Tick-pathogen interactions in bovine anaplasmosis

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Interacties tussen teek en pathogeen in anaplasmose bij het rund
(met een samenvatting in het Nederlands)

PROEFSCHRIFT
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For my parents

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1.1 Introduction

Anaplasma marginale is a tick-borne bacterial pathogen that causes bovine anaplasmosis, one of the most important tick-borne diseases of ruminants worldwide. The disease occurs in tropical and subtropical regions of the New World, Europe, Africa, Asia and Australia [1]. Clinical symptoms are most notable in cattle, but other ruminants including buffalo, bison, African antelopes and mule deer can become persistently infected [2].

Bovine anaplasmosis causes large economic losses to the cattle industry. The losses are measured through several parameters: low weight gain, reduction in milk production, abortion, costs of anaplasmosis treatment and mortality. In Latin America the losses were calculated to be approximately $800 million per year and in United States over $300 million [3]. The disease presents a serious constraint to livestock improvement programs in the tropics because *Bos taurus* cattle imported from temperate regions are highly susceptible to anaplasmosis and often do not survive to become part of the planned reproduction programs for genetic improvement [3]. Anaplasmosis is currently classified in List B of the Office International des Epizooties (OIE) Terrestrial Animal Health Code due to its socio-economic importance and significance in terms of restrictions in the international trade of animals and animal products.

Current control measures vary according to geographical region and include the use of acaricides, chemotherapy and vaccination. However, most of these approaches lead primarily to the reduction of clinical symptoms, whereas transmission of the pathogen still occurs. Therefore, development of vaccine formulations capable of preventing transmission would have significant economic impact. This approach would require a better understanding of mechanisms underlying the complex interactions between *A. marginale* and its vector ticks.

In this chapter, an overview of the current knowledge about *A. marginale* biology, importance to animal health and current control measures is presented. Furthermore, current knowledge on *A. marginale*-tick interactions is also presented as a part of a novel integrated approach to *Anaplasma* vaccine development.

1.2 *Anaplasma marginale*

1.2.1 Historical background

The first reports on *A. marginale* appeared as early as in 1894, when Salmon and Smith detected the presence of inclusion bodies in bovine erythrocytes [4]. The first full description came from Sir Arnold Theiler, who observed bacteria in erythrocytes of South African cattle in 1910 and described them as "marginal points" and "peripheral coccus-like bodies" [5]. This was long before he identified these inclusion bodies to be a causative agent of the disease known by the local farmers as gallsickness. Many investigators believed that the small "coccus-like bodies", seen in the red blood cells of affected cattle, was a developmental stage of *Babesia bigemina.*

In 1908 a number of heifers were imported from England and they had been artificially infected with South African isolate of *B. bigemina*, with the aim of strengthening their immunity. In due course, 50 % of these heifers died with symptoms of fever, anaemia, icterus and with "marginal points" in their blood, but without haemoglobinuria, which made Theiler believe that the mortality was due to the different cause. Therefore, he concluded that these "marginal points" were independent pathogenic organisms, which he named *Anaplasma marginale* [6]. The scientific name of the organism is based on its staining characteristics and localization in the host cell, where "Anaplasma" stands for the lack of stained cytoplasm and "marginale" denotes the peripheral location of the organism in the host cell. Theiler subsequently described a subspecies of *A. marginale*, *A. centrale*, for which inclusions were found in the centre of the erythrocytes, which appeared to be less pathogenic for cattle and gave cross protection to *A. marginale* [7]. This discovery was invaluable for the control of anaplasmosis. Theiler did the first isolation of *A. centrale* and since then it has been and it is still used as a live blood vaccine in South Africa, Australia, Israel and several South American countries.

1.2.2 Classification

Since its first description, *A. marginale* has been reclassified several times. In 2001, a new classification of the *Rickettsiales* based on phylogenetic analysis of the 16S rRNA gene and *groESL* operon was proposed [8]. According to this analysis *A. marginale* is now classified as follows:

Phylum: Proteobacteria
Class: Alphaproteobacteria
Order: Rickettsiales

Family: Anaplasmataceae
Genus: *Anaplasma*
Species*: Anaplasma marginale*

The order Rickettsiales, comprises two families Anaplasmataceae and Rickettsiaceae. While organisms within the family Rickettsiaceae are all obligate intracellular bacteria that grow freely within the cytoplasm, Anaplasmataceae are obligate intracellular organisms found exclusively within membrane-bound vacuoles in the vertebrate or tick host cell cytoplasm. Furthermore, almost all organisms in the family Anaplasmataceae multiply in both vertebrates and invertebrates (primarily ticks and nematodes). The phylogenetic analysis consistently supported formation of four different genera within the family Anaplasmataceae: *Anaplasma* (96.1% similarity), *Ehrlichia* (97.7% similarity), *Wolbachia* (95.6% similarity) and *Neorickettsia* (94.9% similarity) [8]. The genus *Anaplasma* (apart from *A. marginale)* includes two more species that infect ruminants: *A. centrale* and *A. ovis*. After the recent reclassification the genus also includes *A. bovis* (formerly *Ehrlichia bovis*), *A. phagocytophilum* (formerly *E. phagocytophila*, *E. equi* and the HGE agent), *A. platys* (formerly *E. platys*) and *Aegyptianella* (genus incertae sedis due to the lack of sequence information).

1.2.3 Pathogenesis

Presently, *A. marginale* is known to infect *in vivo* only mature, circulating erythrocytes of domestic and wild ruminants [9] (Figure 1A). However, recent *in vitro* and *in vivo* studies indicated that *A. marginale* also infects endothelial cells, which may have implications for both pathogenesis and immune mechanisms [10, 11]. *A. marginale* enters erythrocytes by endocytosis and resides in the membrane-bound vacuole, where it divides by binary fission. The membrane vacuole is derived from erythrocyte membrane and contains 4 to 8 organisms [12](Figure 1B). In the acute infection as much as 70% of erythrocytes may become infected. During high rickettsiemias (bacteriemias) multiple infections of individual erythrocytes are common. The incubation period varies with the number of organisms in the infective dose and ranges from 7 to 60 days, with an average of 28 days. *A. marginale* leaves the host cell without disrupting it. Cells that are physically or chemically altered during the course of the disease are recognized by bovine reticulo-endothelial cells and phagocytized, which will result in the development of mild to severe anemia and icterus, without hemoglobinemia and hemoglobinuria. The acute phase of the disease may also include symptoms such as high fever, dramatic weigh loss, abortion, lethargy and often death in animals older than 2 years. Calves less than one year of age

develop a relatively mild form of disease [13]. Importantly, animals that survive acute disease develop a lifelong persistent infection and serve as reservoirs for transmission to new susceptible hosts [14, 15]. Persistence is characterized by sequential rickettsemic cycles, occurring at approximately 5 week intervals, with peaks at 10^6 bacteria /ml of blood followed by a rapid decline when rickettsemia is controlled by a specific immune response [16]. Persistently infected cattle have a lifelong immunity and are resistant to clinical onset of the disease on challenge exposure.

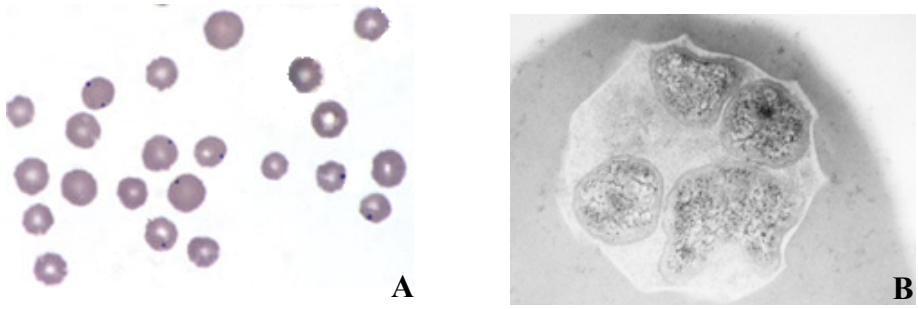

Figure 1. Bovine erythrocytes infected with A. marginale. ***(A)*** *Inclusion bodies located at the periphery of the erythrocyte in a stained blood smear.* ***(B)*** *Electron micrograph of A. marginale inclusion that contains four organisms.*

1.2.4 Transmission

Transmission of *A. marginale* under natural conditions occurs mechanically by blood contaminated fomites and biting flies and biologically by ticks. Moreover, *in utero* transmission during the acute phase of the disease has been described [17, 18] and may contribute to the epidemiology of the disease.

Mechanical transmission of anaplasmosis frequently occurs via blood contaminated needles, dehorning saws, nose tongs, ear tagging devices and castration instruments. Blood sucking dipteras such as horse flies (*Tabanus* spp.), horn flies (*Hematobia irritans*), stable flies (*Stomoxys calcitrans*) and mosquitoes (*Culicidae*) [19-21] also play an important role in mechanical transmission, especially in areas where tick vectors do not occur or in areas where *Rhipicephalus* (*Boophilus*) *microplus*, the cattle tick, does not appear to be a biological vector of *A. marginale* [22]. It is believed that successful mechanical transmission can only be achieved when there is a minimal time lapse (few minutes) between consecutive feedings. However, horse flies were shown to remain mechanically infective for at least 2 hours after they had obtained a partial blood meal from

an acutely infected calf [23]. To date, there is no evidence of multiplication or any developmental cycle in these arthropod vectors. Tick-borne transmission has been shown to be at least two orders of magnitude more efficient than mechanical transmission by horse flies [24].

Table 1. Tick species tested and shown to be vectors of bovine anaplasmosis (*from Kocan et al. 2004 with some modifications).*

Tick Species	Reference
Rhipicephalus (Boophilus) microplus	[30]
R.(Boophilus) decoloratus	[31]
R.(Boophilus) annulatus	[32]
R.(Boophilus) calcaratus	[33]
Dermacentor albipictus	[34]
D. andersoni	[35, 36]
D. hunteri	[37]
D. occidentalis	[35]
D. variabilis	[35, 36]
Hyalomma excavatum	[38]
H. marginatum rufipes	[39]
Ixodes ricinus	[40]
I. scapularis	[41]
Rhipicephalus bursa	[33]
R. evertsi	[39]
R. simus	[31, 39]
R. sanguineus	[38, 42]
Argas persicus	[43]

Biological transmission of *A. marginale* occurs effectively through ticks. Approximately 20 tick species have been incriminated as vectors worldwide [1]. Most of these tick species were shown to transmit *A. marginale* under experimental conditions only, which does not necessarily imply transmission in the field (Table 1). In general, vectors include *Rhipicephalus spp*., *Dermacentor spp*. and *Ixodes spp*., whereas *Amblyomma spp*. do not appear to be able to transmit the pathogen. The one-host ticks *R. microplus* and

R. annulatus are the main vectors in tropical and subtropical areas [25]. In the southern United States, where *R. microplus* was eradicated in the early 1940's by a compulsory accaricide–based program, bovine anaplasmosis continued to be transmitted by *Dermacentor spp*. (*D. variabilis, D. andersoni and D. albipictus*) [26].

Ticks become infected when feeding on an infected host after which extensive and complex multiplication occurs in several tick tissues. Tick transmission can occur from stage to stage (transstadial) or within stage (intrastadial), while transovarial transmission from one tick generation to another does not appear to occur [27]. *D. andersoni*, a three-host tick, has been shown to transmit *A. marginale* both transstadially (nymph to adult) and intrastadially (by adult males). Intrastadial transmission by male ticks is believed to be an important epidemiological mechanism. After initial feeding, adult male ticks feed intermittently and can easily be transferred from one to another host because of social and grooming behavior of cattle. Male ticks become persistently infected and can serve as reservoirs transmitting the pathogen repeatedly when feeding on susceptible animals [28, 29]. Although not described for *Rhipicephalus* species, the developmental cycle of *A. marginale* in these ticks is probably similar and males are likely to play an important role in pathogen transmission.

1.2.5 Life cycle

The life cycle of *A. marginale* in ticks is complex and well coordinated with the tick feeding cycle [28] (Figure 2). Infected erythrocytes are ingested by the ticks with a blood meal and the first sites of infection are gut and Malpighian tubule cells. During the subsequent feeding many other tissues, including salivary glands, become infected from where *A. marginale* can be transmitted to the vertebrate host. At each site of infection two stages of *A. marginale* occur within a membrane bound vacuole in the tick cell cytoplasm. The first form seen within *A. marginale* colonies is the reticulated (vegetative) form, which divides by binary fission and results in formation of large colonies containing hundreds of organisms. The reticulated forms are then transformed into dense forms, which are the infective form and can survive for a short time outside of cells. Cattle become infected when the dense form is transmitted during tick feeding via the salivary glands. Ticks are able to acquire infection after feeding on persistently infected animals with a very low level of rickettsemia. Moreover, once ticks acquire the infection the biological replication of the organism within the ticks makes up for the initial low infective dose [15]. Not all *Anaplasma* strains are equally transmitted by tick. The failure of ticks to become infected

and transmit the infection depends on both early (midgut) and late (salivary glands) barriers [44].

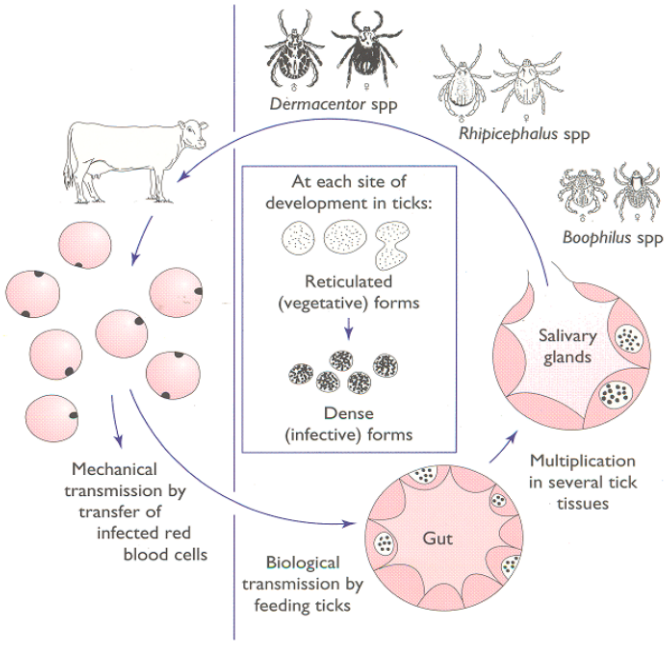

Figure 2. Schematic of the development cycle of A. marginale in cattle and ticks (adapted from the poster in Parasitology today, 1999.).

1.2.6 Geographical distribution

Anaplasmosis occurs in tropical and subtropical areas (~40° N to 32° S) throughout the world including the New World, Europe, Africa, Asia and Australia [1]. In Europe, anaplasmosis is endemic in several Mediterranean countries, including Italy [45], Portugal [46] and Spain [47]. Recently, *A. marginale* was reported in more northern latitudes, such as Austria [48], the Alpine region of Switzerland [49] and Hungary [50]. The distribution of anaplasmosis may however continue to change due to the trend in global warming, which may influence the distribution of the vector ticks [51].

1.3 Control methods

In the past 60 years control measures have not changed much and depending on the geographic location they concern arthropod control, administration of antibiotics and vaccination. Generally, the strategy depends on epidemiology of the disease in the affected region and is influenced by local management practices.

1.3.1 Arthropod control

Arthropod control for anaplasmosis, targeting both ticks and biting flies, is labor intensive and expensive. Environmental pollution and contamination of milk and meat products are becoming an important issue [52]. The problem is further complicated by the fact that repeated use may result in development of acaricide-resistant tick populations [53]. Furthermore, complete prevention of transmission may result in animal populations which are fully susceptible to bovine anaplasmosis or to any other locally occurring tick-borne pathogen, and thus at risk of disease outbreaks if control measures breakdown [54].

Another method successfully used to control tick infestation is based on anti-tick vaccines developed for *Rhipicephalus (Boophilus)* spp. using Bm86 and Bm95 tick gut antigens

1.3.2 Antibiotic therapy

Antibiotic therapy is directed toward prevention of clinical symptoms of anaplasmosis. Several chemotherapeutics have been examined, but only tetracyclines, fluoroquinolones and imidocarb dipropionate have been found to be effective. Antibiotic treatment does not lead to the complete clearance of *A. marginale* and does not prevent persistent infection. Although extensively used in the US, tetracycline therapy is costly and bears the risk of development of resistant *A. marginale* organisms which may limit the usage of antibiotics in the future.

1.3.3 Vaccination

Development of long-term immunity by vaccination has been used extensively for control of bovine anaplasmosis throughout most of the world and it represents the most effective control measure for anaplasmosis. Although several vaccines have been developed, all of them have specific advantages and drawbacks. These vaccines can be divided into two major groups: live and inactivated vaccines. Both types induce protective immunity that reduces morbidity and mortality but they do not prevent cattle to become infected upon

challenge exposure. Immunized animals can develop persistent infection and serve as reservoirs for mechanical and biological transmission.

1.3.3.1 Live vaccines

Immunization of the cattle with attenuated isolates of *A. marginale* or with *A. centrale* is still the method of choice in most parts of the world. Vaccinated cattle develop a persistent infection which induces a life long protection and revaccination is usually not required.

A live strain of *A. centrale* is routinely used for vaccination in Australia [55], South Africa [39], Argentina [56], Uruguay [57], Israel [58], Zimbabwe [59] and Malawi [60] to reduce the impairment of animal health and production losses. The exact mechanisms of protective immunity created by *A. centrale* against various *A. marginale* genotypes are not yet completely understood. It is known that *A. centrale* and *A. marginale* share common immunodominant epitopes [61] and CD^+ T cell epitopes are conserved between the two species [62], which may play a role in protection by *A. centrale*. Protection against challenge is adequate in most of the cases and the use of live *A. centrale* vaccine appears to be justified [63]. However, in widely separated geographic areas, *A. centrale* vaccine does not provide effective cross–protection, as shown in Paraguay and Zimbabwe [59, 64]. The *A. centrale* vaccine is produced from the blood of infected cattle and therefore has major disadvantages, including iatrogenic spread of other blood-borne pathogens.

The infection - and - treatment method was used in the past and it involved inoculation of cattle with *A. marginale*-infected erythrocytes, followed by treatment with low doses of antibiotics [3]. This method required closely monitoring by a veterinarian and timely treatment which makes it impractical.

The attenuation of *A. marginale* by irradiation combined with passage through deer and sheep, has also been reported [65], but its use as a vaccine caused severe reactions in adult cattle [66]. Attempt to attenuate by passage through 65 splenectomised calves and then 35 splenectomised sheep was reported, but reversion to virulence remained a problem [67].

1.3.3.2 Inactivated vaccines

An inactivated vaccine has been developed in the United States and was used effectively until 1999 when it was withdrawn from the market due to company restructuring. The vaccine used *A. marginale* partially purified from infected bovine erythrocytes as an antigen [68, 69]. Inactivated vaccines reduced clinical anaplasmosis and there was no risk of reversion to the virulence, but they were expensive to produce, difficult to standardize

and often not cross-protective in widely separated geographic areas with different endemic *A. marginale* isolates.

1.4 Tick-pathogen interface as a target for new anaplasmosis control strategies

A wide range of arthropods transmitting the disease, persistent infection in both ticks and cattle that serve as a reservoir host, antigenic variation and the presence of a variety of genotypes in nature make the control of bovine anaplasmosis a challenging task.

One of the important goals of future control is the development of a vaccine which can block transmission of *A. marginale*. Factors that mediate the infection and the development of *A. marginale* within the tick are not well defined, but most likely they involve cellular pathways from both the pathogen and the vector. Understanding the molecular details of vector-pathogen interactions may lead to the development of novel vaccine (or therapeutic) strategies that can be rationally designed to target the pathogen life cycle stages within the arthropod host.

The knowledge on molecular interactions between *A. marginale* and the tick is still limited. However, the recent development of a cell culture system for *A. marginale*, novel molecular technologies (such as RNA interference) as well as increasing information on genomics of both the pathogen and the vector, is of a great importance and is expected to rapidly expand the knowledge of the tick-pathogen interface.

1.4.1 Development of a cell culture system

The major impediment to anaplasmosis research has been the lack of an *in vitro* culture system and all research relied therefore on the use of infected cattle as a source of *A. marginale*. Although cell culture systems were investigated using erythrocytes and arthropod cell lines [70], they did not result in continuous propagation of the organism. However, continuous propagation of *A. marginale* in an embryonic tick cell line from *Ixodes ricinus* (IDE8) has been achieved [71]. Colonies of *A. marginale* in this IDE8 cell line are morphologically similar to those found in naturally infected tick cells. The cell culture derived *A. marginale* remained infective for cattle, caused clinical disease in cattle and remained transmissible by ticks [72].

Six major surface proteins (MSPs) present on *A. marginale* derived from bovine erythrocytes were found to be conserved on cell culture-derived organisms. Moreover, the antigenic composition of *A. marginale* remained the same after successive passage in cell

culture or after passage through ticks [73, 74]. Therefore, *A. marginale* tick cell culture showed promise as a source of alternative antigen for use in vaccine development and as a system for basic research. Culture derived *A. marginale* has been used in vaccination trials and immunized cattle were shown to be protected against anaplasmosis after challenge exposure to infected blood or feeding infected ticks [75, 76]. However, protection was partial and clinical signs were not prevented.

A. marginale cell culture system has been adapted for short term growth in 24-well and 96-well plate formats and used for development of competitive ELISA assays [77], drug screening assays [78] and *A. marginale* tick infectivity studies [79, 80].

Tick cell cultures have been used successfully to study vector pathogen interactions [81]. Interestingly, for *A. marginale* most of these studies were performed in ISE6 and IDE8 tick cell lines derived from *Ixodes scapularis*, which is not a natural vector of *A. marginale* [81]. Recently, a Brazilian isolate of *A. marginale* was propagated successfully in BME26 cell line derived from *R. microplus* [82], which provides new opportunities to study the *A. marginale*–tick interface in the cells cultured from a natural vector.

1.4.2 RNA interference (RNAi) as a tool for the characterization of the tick-pathogen interface

RNAi is a nucleic acid–based reverse genetic approach that results in silencing of gene expression and allows for study of the impact of the gene function on metabolic pathways. RNAi was discovered when it was shown that double stranded RNA (dsRNA), when injected into the nematode *Caenorhabditis elegans* silenced the gene of absolute sequence similarity [83]. Small interfering RNAs are the effector molecules of the RNAi pathway, which is initiated by dsRNA and results in a potent sequence specific degradation of cytoplasmatic mRNAs with the same sequence as the dsRNA trigger [84].

RNAi has become widely used in ticks, where the use of alternative methods for genetic manipulations is limited [85]. Little is known about the mechanism of RNAi in ticks. The closest organism in which the mechanism of RNAi was described is *Drosophila melanogaster* [86]. This information was used by Kurscheid et al. to search the existing *R. microplus* EST database and the *I. scapularis* genome reads to identify putative tick RNAi proteins and constructed a putative tick RNAi pathway [87].

Several methods are used for RNAi in ticks, depending on the experimental design or objectives: (1) micro-injection of dsRNA into unfed or fed ticks (2) soaking or incubation of ticks, tick tissues or tick cells with dsRNA (3) feeding of dsRNA to ticks and

(4) virus production of dsRNA. Although most extensively used for the study of the tick gene function, recent studies demonstrated that RNAi constitutes an important tool for the study of the tick-pathogen interface, which will likely contribute to the identification of candidate antigens for use in pathogen transmission blocking tick vaccine [88].

1.4.3 Genomic resources for A. marginale and ticks

Complete genome sequencing and annotation revealed that the surface coat of *A. marginale* is skewed to two families containing immunodominant proteins: the msp1 superfamily and msp2 superfamily [89]. The completion of *A. marginale* genome (St. Maries strain) and genomes of similar tick-borne pathogens has allowed comparative genomics to identify conserved genes and pathways associated with transmission [90].

By contrast, genome information on ticks is still limited mainly due to the unexpectedly large size of the tick genome. So far, only the *I. scapularis* genome is being sequenced (estimated size $2x10^9$ bp) due to its relevance as a principal vector of Lyme borreliosis [91]. The genome size of *R. microplus*, one of the principal vectors of *A. marginale,* is estimated to be $7x10^9$ bp in length, over twice the length of the human genome [92]. Therefore, the obstacle to future sequencing project is obtaining a sufficient funding. However, large scale EST data (>10,000 ESTs) are publically available for *R. microplus* [93].

1.4.4 A. marginale molecules involved in interactions at the tick-pathogen interface

The major surface proteins (MSPs) of tick-borne intracellular bacteria mediate functions necessary for survival, replication and transmission. Therefore, surface expressed proteins are logical candidates for vaccine development targeted to either induce protective immune response or prevent colonization of the tick vector and prevent further transmission. The surface coat of many tick-borne pathogens is remodeled during the transmission between vertebrate and arthropod hosts. This is well studied in *Borrelia burgdorferi*, the causative agent of Lyme disease, which modulates surface protein expression in order to survive in different host tissues. For example, OspA is essential for colonization and survival of this bacteria in the tick midgut [94], while OspC is required to infect the mammalian host and it is up-regulated during the tick feeding in preparation to transmission to a new host [95, 96]. In case of *A. marginale*, such surface alterations are less well defined. Several major surface proteins (MSPs) have been identified and characterized for *A. marginale* [3, 97].

Of all the MSPs, the MSP1 complex has been studied most extensively in *A. marginale*-tick interactions. The MSP1 complex is a heterodimer of two structurally

unrelated polypeptides MSP1a and MSP1b. The MSP1b, encoded by *msp1β1* and *msp1β*, is shown to be adhesin for bovine erythrocytes but not for tick cells [79]. Although MSP1b is encoded by a multigene family, only small variations in protein sequences were observed during the *A. marginale* cycle in ticks and cattle [98]. MSP1a is encoded by a single gene, *msp1α*, which is conserved during the multiplication of the parasite in cattle and ticks [98, 99]. The MSP1a of *A. marginale* geographic strains differs in molecular weight due to a variable number of tandem 23-31 amino acid repeats, and the sequence of MSP1a has been shown to be a stable marker for identification of geographic strains [100, 101]. The analysis of tandemly repeated MSP1a peptides of several geographic isolates of *A. marginale* revealed a complex relationship between the *msp1α* phenotype and the tick transmissibility of the isolate and suggested that both the sequence and conformation of the repetitive region influence the properties of MSP1a [100]. Functionally, MSP1a was shown to be an adhesin for bovine erythrocytes and tick cells, to be involved in infection and transmission by *Dermacentor* spp. ticks and to be involved in immunity to *A. marginale* infection in cattle [79, 102]. MSP1a was found to be differentially regulated in bovine erythrocytes and tick cells. While up-regulated in bovine erythrocytes, MSP1a was found to be down-regulated in tick salivary glands and cultured tick cells [103]. Garcia Garcia et al. demonstrated that MSP1a and MSP1b are glycosylated and suggest that the glycosylation of MSP1a plays a role in the adhesion of *A. marginale* to tick cells [104].

MSP2 is an immunodominant outer membrane protein belonging to MSP2 superfamily of proteins [89]. This superfamily is reported to contain 56 genes, including msp2 (n=8), msp3 (n=8), msp3 reminants (n=2), msp4, opag (n=3), omp-1(n=15), orfX (n=12) and orfY (n=7) [89]. Within the MSP2 superfamily, MSP2 protein is most extensively studied. Analysis of persistent *A. marginale* infection in ticks and cattle revealed that new antigenic variations of MSP2 occur with each rickettsemic cycle [105, 106]. This mechanism of antigenic variations allows *A. marginale* to evade the host immune response and contributes to the maintenance of the persistent infection [16]. In *A. marginale*, there are single expression sites for both *msp2* and *msp3*; however, there are multiple functional *msp2* and *msp3* pseudo genes distributed throughout the chromosome that serve as templates for gene conversion to generate major surface protein 2 (MSP2) and MSP3 surface coat variants. These variants escape the preexisting antibody response and are believed to be critical for the long-term persistence of the organism within the immunocompetent mammalian host [74, 89]. The role of MSP2 in establishing persistent infection in ticks is still unknown, but the protein may be involved in infection of tick cells.

1.4.5 Tick molecules involved in interactions at the tick-pathogen interface

In general, pathogens have evolved together with their vectors and developed an intimate relationship to facilitate survival and transmission. Several studies have shown the importance of tick factors for pathogen infection and life cycle within a tick vector. For example, the tick receptor TROSPA identified in *I. scapularis* guts is required for the colonization of the guts by *B. burgdorferi* [107]. The repression of TROSPA expression by RNA interference, prevented efficient colonization of the vector by the spirochete and subsequently reduced the pathogen transmission. Another study showed that *A. phagocytophilum*, an obligate intracellular bacterium closely related to *A. marginale,* requires a tick protein, Salp 16, for salivary gland colonization [108]. While several Salp proteins were identified that affect tick feeding; only Salp 16 was up-regulated in response to *A. phagocytophilum* infection. Silencing of Salp 16, resulted in reduced infection in tick salivary glands.

Additional studies have been conducted wherein tick proteins have been identified which may contribute to pathogen infection and transmission. Ribeiro and colleagues identified transcripts that were differentially regulated between *B. burgdorferi*-infected and uninfected *I. scapularis* salivary glands [109]. Differential gene expression in response to pathogen infection has also been characterized in *D. variabilis* in response to *Rickettsia montanensis* [110-112] and in salivary glands of female *R. appendiculatus* in response to *Thieleria parva* [113]. However, the function of the affected genes is still largely unknown.

Whereas the presence of tick receptors for tick borne pathogens has been reported [107], the tick receptor for *A. marginale* has not been identified. Moreover, expression of tick genes/ proteins that facilitate the infection and multiplication of this pathogen is still not well described.

1.4.5.1 Tick protective antigens

Recent studies have shown that tick vaccines can also reduce tick vector capacity. It was noticed that vaccination of the cattle with a commercial anti tick vaccine based on the *R. microplus* tick gut antigen Bm86, resulted in reduction of incidence of *B. bigemina* and *A. marginale* [114, 115]. The use of recombinant *R. appendiculatus* P64 putative cement protein (derived form the cement cone that secures tick mouth parts in the host skin), resulted in reduced vector capacity of the tick for infection with tick borne encephalitis virus (TBEV) [116]. These results show a possibility of developing new dual target vaccines that would control both tick infestation and transmission of tick borne pathogens.

Furthermore, the tick protein subolesin was discovered as a tick protective antigen in *I. scapularis* ticks and it was shown to be highly conserved among a broad range of tick species [117]. In a recent study the role of subolesin in the infection and transmission of *A. marginale* was characterized. Subolesin was shown by RNAi gene knock down and immunization trials using the recombinant protein, to protect the host against tick infestations, reduce tick survival and reproduction, and to cause a degeneration of the guts, salivary glands, reproductive tissues and embryos [118-120]. The silencing of subolesin expression also decreased the vector capacity of ticks for *A. marginale* and *A. phagocytophilum* [118]. Further experiments demonstrated that subolesin is differentially expressed in *A. marginale* and *A. phagocytophilum* infected tick cells [118]. Subolesin was differentially expressed in *A. marginale* infected ticks in a tissue specific manner, in which mRNA levels increased in response to *A. marginale* infection in tick salivary gland cells but not in the gut cells. Functional analysis by RNAi demonstrated that *A. marginale* infection levels were reduced in *D. variabilis* salivary glands and IDE8 tick cells after gene knock down. In addition, subolesin was shown to be similar in structure and function to insect and vertebrate akirins which control NK-kB dependent and independent gene expression that impact innate immunity [121]. However, its role in tick innate immunity still remains to be investigated.

1.4.5.2 Tick innate immunity

The tick innate immune system is another factor that influences pathogen development in the tick. Tick-borne pathogens have apparently co-evolved with the tick for their mutual survival because, while pathogens multiply in the ticks, these infections do not appear to influence tick biology. Understanding innate immunity mechanisms of different tick species may help to answer questions as to why some pathogens are transmitted and others not and why some ticks are vector competent and others not.

Antimicrobial peptides are an important component of the tick innate immunity and include lysozymes, cercopins, attacins, defensins, prolin-rich peptides, glycine-rich peptides and others [122]. Among those, defensins are probably the most studied, and are predominantly active against Gram-negative bacteria. Defensins have been identified in variety of ixodid ticks including *D. variabilis* [123], *I. scapularis* [124], *A. americanum* [125], *A. hebraeum* [126] and *R. microplus* [127].

A particular defensin called varisin was identified in *D. variabilis* ticks and its role in immunity to *A. marginale* was studied [128]. Silencing of varisin occurred in tick hemocytes, midguts and salivary glands after RNAi. Varisin knock down did not increase

A. marginale infections as expected, but, on the contrary, reduced infection levels. However, *A. marginale* colonies were morphologically abnormal in varisin silenced ticks, when compared to control group and some ticks had systemic infection with a yeast like microbe. The results suggested that the defensin may not be active against *A. marginale* in a natural tick vector, but rather acts to limit the infection of non-transmissible microorganisms.

1.5 Aims and outline of this thesis

Bovine anaplasmosis presents a major problem to the cattle industry in the tropical and subtropical regions worldwide. The climate change influences distribution of the vector tick species, therefore anaplasmosis may pose a threat to even broader areas in the world. As reviewed in ***Chapter 1***, vaccination may be a promising approach for the control of the disease, but development of the effective vaccine requires better understanding of molecular interactions at the tick-pathogen interface.
The aims of the study described in this thesis are:

- to test the vector competence of *Dermacentor reticulatus* ticks for *A. marginale*,
- to identify and characterize tick genes/proteins that facilitate the infection and multiplication of *A. marginale by* using a functional genomics approach,
- to identify and characterize genes differentially expressed in *R. microplus* salivary glands in response to *A. marginale* infection,
- to characterize the effect of silencing of functionally important genes on *A. marginale* development in ticks,
- to determine the role of tick protective antigen subolesin in *A. marginale* and other pathogen infections in ticks,
- to characterize tick gene expression profiles in response to *A. phagocytophilum* infection and compare tick gene expression responses in *A. marginale* and *A. phagocytophilum* infected tick cells.

In ***Chapter 2*** of this thesis it is described that *D. reticulatus*, a tick species with a wide distributional range within Europe, can indeed act as a competent vector for *A. marginale.* In ***Chapter 3*** it is demonstrated that *A. marginale* infection modifies gene expression in *D. variabilis* ticks and cultured *I. scapularis* cells. A functional genomics approach was used to identify and characterize tick genes/proteins that are differentially regulated in response to *A. marginale* and that facilitate the trafficking of the pathogen from infection of the gut cells through infection and transmission from salivary glands. This study was extended by identifying genes differentially expressed in *R. microplus* male salivary glands (***Chapter 4***). In addition, *R. microplus* derived tick cell line BME26 was used for the first time to study *A. marginale*-tick interactions (***Chapter 4***). Four of the identified differentially expressed genes were further analyzed by gene silencing and the effects of silencing on *A. marginale* development and infection levels in ticks was characterized by means of quantitative PCR and microscopy (***Chapter 5***). The gene knock down affected the

pathogen developmental cycle in *D. variabilis* ticks, suggesting that the four genes are potential candidates for transmission blocking vaccines. In ***Chapter 6*** the expression of tick protective antigen subolesin in several tick species in response to different pathogens (including *A. marginale*) is described. The results suggest possible direct and indirect effects of subolesin knockdown on the level of infection of pathogens in ticks. Finally, the molecular response to infection with different obligate intracellular tick–transmitted bacteria is described in ***Chapter 7***. *A. marginale* and *A. phagocytophilum* were found by microarray and quantitative RT-PCR to elicit different gene expression responses in cultured tick cells. The major findings and conclusions of this thesis are summarized and discussed in ***Chapter 8***.

Overall, the results described in this thesis are expected to contribute to a better understanding of the *A. marginale*-tick interface and to the development of a new generation of pathogen transmission blocking vaccines designed to prevent transmission and reduce exposure of vertebrate hosts to tick-borne pathogens.

1.6 References

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Chapter 2

Experimental transmission of *Anaplasma marginale* by male *Dermacentor reticulatus*

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Abstract

Background
Bovine anaplasmosis has been reported in several European countries, but the vector competency of tick species for *Anaplasma marginale* from these localities has not been determined. Because of the wide distributional range of *Dermacentor reticulatus* within Europe and the major role of *Dermacentor* spp. as a vector of *A. marginale* in the United States, we tested the vector competency of *D. reticulatus* for *A. marginale*.

Results
Male *D. reticulatus* were allowed to feed for 7 days on a calf persistently infected with a Zaria isolate of *A. marginale*, after which they were removed and held off-host for 7 days. The ticks were then allowed to feed a second time for 7 days on a susceptible tick-naïve calf. Infection of calf No. 4291 was detected 20 days post exposure (p.i.) and confirmed by msp4 PCR. Thirty percent of the dissected acquisition fed ticks was infected. In addition, *A. marginale* colonies were detected by light microscopy in the salivary glands of the acquisition fed ticks. Transmission of *A. marginale* to calf No. 9191 was confirmed by examination of Giemsa-stained blood smears and msp4 PCR. Ticks were dissected after transmission feeding and presence of *A. marginale* was confirmed in 18.5% of the dissected ticks.

Conclusions
This study demonstrates that *D. reticulatus* males are competent vectors of *A. marginale*. Further studies are needed to confirm the vector competency of *D. reticulatus* for other *A. marginale* strains from geographic areas in Europe.

Background

Bovine anaplasmosis is one of the most important tick-borne diseases of ruminants worldwide. The disease is caused by infection of cattle with the obligate intraerythrocytic bacteria *Anaplasma marginale* which is classified in the family Anaplasmataceae, order Rickettsiales [1]. The acute phase of the bovine anaplasmosis is characterized by anemia, icterus, weight loss, fever, abortion, decreased milk production and often results in death [2]. Animals surviving the acute phase develop a lifelong persistent infection and can serve as reservoirs for mechanical transmission and biological transmission by ticks [3].

Anaplasmosis is endemic in tropical and sub-tropical regions where the disease constitutes a constraint to the cattle production. In Europe anaplasmosis is endemic in several Mediterranean countries including Italy [4, 5], Portugal [6] and Spain [7], and has occasionally been reported in Austria [8], Switzerland [9] and Hungary [10]. Mechanical transmission of *A. marginale* is effected by blood-contaminated fomites, including hypodermic needles, castration instruments, ear tagging devices, tattooing instruments, and dehorning saws or by blood-contaminated mouthparts of biting flies [11]. Biological transmission is effected by ticks and over 20 species of ticks have been incriminated as vectors worldwide [12]. While the one-host ticks, *Rhipicephalus* (*Boophilus) microplus* and *R. annulatus,* were eradicated from the United States in the early 1940s, they are the main tick vectors in tropical and subtropical areas [13]. Currently, *Dermacentor* spp. (*D. andersoni*, *D. variabilis* and *D. albipictus*) are the major tick vectors of *A. marginale* in the U.S. [14].

A. marginale undergoes a complex developmental cycle in ticks that begins with infection of gut cells from infected erythrocytes ingested with the tick bloodmeal [15, 16]. Development of the final infective stage occurs in salivary glands from where the pathogen is transmitted to cattle. A major means of *A. marginale* transmission appears to be by male *Dermacentor* ticks which become persistently infected. These males are intermittent feeders and can feed and transmit *A. marginale* multiple times as they transfer among cattle, thus effecting intrastadial transmission [15, 16].

The vectorial capacity of tick species for *A. marginale* in Europe has not been well defined. Recent reports of endemicity of anaplasmosis in European countries [10] and of outbreaks in countries previously thought to be free of anaplasmosis, including Switzerland, warranted studies on the role of putative tick vector(s) [17]. The broad distribution range of *D. reticulatus,* which extends from the British isles to Central Asia [18], as well as the expanded geographic distribution of this tick as recently reported in

Germany [19], Hungary [20] and the Netherlands [21], warrants further study of *D. reticulatus* as a vector for *A. marginale* in Europe.

Results

Infection and acquisition feeding

Infection of calf No. 4291 with the *A. marginale* Zaria isolate was detected on day 20 post exposure (PI) when the body temperature increased to 39.9ºC and depression and anorexia were observed. The percent reduction PCV was 50% and the *A. marginale* percent parasitized erythrocytes (PPE) was 6% (Table 1)**.** *A. marginale* infection was subsequently confirmed by *msp4* PCR. After infestations of the calf on the day 34 p.i. with 80 male and 5 female *D. reticulatus* ticks when the PPE was 0.6% (minimum 1000 erythrocytes counted), all female ticks and 66 of the male ticks attached and fed successfully. Based on PCR testing of one salivary gland from each of the 30 male tick halves, the infection percentage was 30%. The presence of *A. marginale* colonies in salivary gland cells was confirmed by light microscopy examination (Figure 1) in the other half of the PCR positive ticks**.**

Table 1**.** Clinical findings of acquisition and transmission feeding of *D. reticulatus* ticks.

Calf number	Number of ticks/feeding (days)	Incubation period (days)[c]	Maximum temperature (˚C)	PCV reduction (%)	Maximum parasitemia (%)
4291[a]	80/7	20	39.9	50	6
9191[b]	30/7	35	39.9	37.5	2

[a] Infected intravenously with A. marginale (Zaria isolate) blood stabilate.[b] Infested with D. reticulatus adult males fed on calf 4291.[c] Number of days to first observation of infected blood cells on a stained blood smears.

Transmission feeding

Male *D. reticulatus* ticks transmitted *A. marginale* Zaria isolate to calf No. 9191. On day 27 p.i. the calf tested PCR positive. Inclusion bodies were detected in erythrocytes on day 35 in Giemsa-stained blood smears and the peak parasitemia was 2%. While minimal clinical symptoms of anaplasmosis were observed, the body temperature increased during the peak of parasitemia up to 39.9ºC, the percent reduction PCV was 37.5 % (Table 1) and the mucosal membranes became pale. Five out of 27 (18.5%) ticks that were attached successfully were PCR positive for *A. marginale.*

Verification of isolate identity

The A. *marginale* isolate genetic identity was confirmed by PCR in samples collected during persistent infection in calf, during replication and development in ticks and after subsequent transmission to the susceptible calf. Subsequent sequencing of the *msp4* and *msp1α* genes also confirmed the isolate identity since the *msp4* and *msp1α* sequences were the same in all tick and cattle samples. The *msp1α* sequence of the Zaria isolate contained two novel repeat forms which were labelled as 54 and 55 following the nomenclature of de la Fuente et al. [22] (Figure 2).

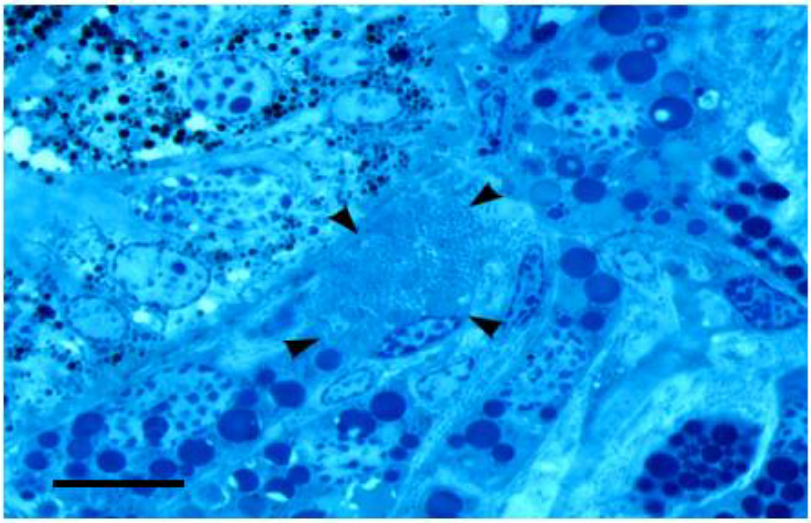

Figure 1. Light micrograph of male D. reticulatus salivary gland cell containing several A. marginale colonies (arrowheads). Bar = 10 μm..

Sequence accession numbers

The GenBank accession numbers for msp1α and msp4 sequences of the Zaria isolate of A. marginale are [GenBank: EU106083] and [GenBank: EU106082] respectively.

Discussion

A. marginale infection in cattle and wild ruminants was reported in several parts of Europe, including the Mediterranean countries of Spain and Portugal [6, 7], Italy [4, 5], and occasionally in France [23], the Alpine region of Switzerland [8, 17] and more recently in

Hungary [10]. However, the epidemiology of anaplasmosis in Europe has not been thoroughly investigated and local vector species were not identified.

(A)

Repeat form	Encoded sequence
A	DDSSSASGQQQESSVSSQSE-ASTSSQLG--
54	LH**********F*TGQ**********
55	T*****GD*H*********GQ**********
F	T******************GQ**********

(B)

A. marginale isolate	Origin	Genbank accession No.	Structure of MSP1a tandem repeats			No. of MSP1a repeats
Zaria	Nigeria	EU106083	54	55	F	3

Figure 2. Sequence and structure of MSP1a tandem repeats in the Zaria isolate of A. marginale. (A) The one letter amino acid code was used to depict the different sequences found in MSP1a repeats. Asterisks indicate identical amino acids and gaps indicate deletions/insertions with respect to the reference repeat A. (B) The structure of the MSP1a repeats region was represented using the repeat forms described in (A). Description of MSP1a repeats was updated after de la Fuente et al. [22].

In the Mediterranean region, several tick species have been incriminated as the potential biological vectors of *A. marginale*. *Hyalomma m. marginatum* and *Rhipicephalus bursa* were found on Iberian red deer in Spain that proved to be infected with *A. marginale* [7] and these ticks were therefore listed as putative tick vectors. Furthermore, *A. marginale* is endemic in Sicily and has been reported elsewhere from Italy, and several tick species have been identified in this area that may be vectors, including *Rhipicephalus turanicus* and *Haemaphysalis punctata* that were collected from *A. marginale* infected cattle [5].

Although anaplasmosis was reported in more northern latitudes, the tick vectors have not been identified. For instance, *A. marginale* was recently reported to be endemic in Hungary and the predominant tick species present on cattle in the study area was *D. reticulatus* [10]. In the United States, several *Dermacentor* spp. (*D. variabilis*, *D. andersoni* and *D. albipictus* are known to be vectors of anaplasmosis[14]. While *D. reticulatus* is known to be a vector of *Babesia canis* [24], *Rickettsia slovaca* [25] and

Coxiella burnetii [26, 27], the vector competency of this tick for *A. marginale* has not been determined until now.

The *A. marginale* strain used in this study originated from Zaria, Nigeria. Although bovine anaplasmosis occurs in large areas of Africa and a few isolates from South Africa have been characterized, this isolate from West Africa had not been reported previously and proved to have unique *msp1a* and *msp4* sequences. The *A. marginale msp4* gene, which is a stable marker for the genetic characterization of strains, does not undergo antigenic variation when cycling between tick and mammalian hosts [28]. MSP1a, encoded by *msp1α*, is involved in the adhesion and transmission of *A. marginale* by ticks and varies in the number and sequence of amino-terminal tandem repeats among geographic strains [22].

Conclusions

In this research we have demonstrated the vector competency of male *D. reticulatus* as an experimental biological vector of *A. marginale* by intrastadial transmission. Further studies are needed to confirm the vectorial role of *D. reticulatus* in Europe by use of *A. marginale* isolates derived from naturally infected cattle and ticks from endemic areas. *Dermacentor* spp. from *A. marginale* endemic regions should be studied, including *D. marginatus* which is commonly found on cattle and wildlife reservoir hosts.

Methods

Experimental animals

Two Holstein-Friesian calves, 8 months of age (No. 4291 and No. 9191), were used. Both animals had no previous exposure to ticks and were confirmed to be *A. marginale* free by examination of Giemsa-stained blood smears and *msp4* PCR. All the ticks feeding and *A. marginale* infection were approved by the Animal Experiments Committee (DEC) of the Faculty of Veterinary Medicine, Utrecht University (DEC No. 0604.0801). Both animals were euthanized at the end of the experiment.

A. marginale isolate

A Nigerian *A. marginale* isolate used for these studies was obtained from a naturally infected bovine from Zaria, Nigeria in 1986. This isolate was subsequently passaged in splenectomized calves, and blood samples were collected at the peak of parasitemia, prepared with 10% DMSO as stabilate and stored in 2 ml aliquots in liquid nitrogen.

Ticks

Adult *D. reticulatus* ticks were collected during October 2006. by dragging vegetation in the area of the Dintelse Gorzen, The Netherlands. The absence of *A. marginale* infection in collected ticks was confirmed in 344 randomly selected ticks by use of an *A. marginale* specific PCR followed by reverse line blot hybridization (RLB) [21]. The ticks were maintained in the laboratory at 20ºC/90% relative humidity. Male ticks, allowed to acquire infection by feeding on an infected calf, were used for these studies because of their putative role in transmission of *A. marginale* [15, 16].

Infection of ticks

For infection of calves, the *A. marginale* blood stabilate was thawed and inoculated intravenously (IV) into the jugular vein of an eight-month old non-splenectomized and tick-naïve Holstein-Friesian calf (No. 4291)**.** Rectal temperature was measured and registered daily and calf was observed for anemia and other signs consistent with anaplasmosis**.** Giemsa-stained blood smears were made and examined daily during the acute stage of the infection and twice weekly during the persistent stage of the infection. The packed cell volume (PCV) was determined using the microhematocrit technique. On day 34 p.i., 5 female and 80 male *D. reticulatus* ticks were placed in cotton patches glued to shaved area on the back of the calf. The ticks were allowed to acquisition feed for 7 days, after which the engorged females were removed and discarded and the fed male ticks were placed in an incubator at 20ºC with 90% relative humidity and a 12:12 h photoperiod for 7 days. This holding period provided time for the development and multiplication of *A. marginale* in tick midguts and other tissues [29]. Thirty male ticks were randomly selected and cut in half with a razor blade separating the right and left sides. The salivary gland from one tick half was dissected for PCR testing, while the other tick half was fixed for light microscopy studies.

Transmission feeding

A second eight-month old, tick-naïve and non-splenectomized Holstein-Friesian calf (No. 9191) was used for the transmission feeding of *D. reticulatus.* A group of 30 acquisition fed male *D. reticulatus* ticks was allowed to feed a second time for 7 days on this calf. After transmission feeding, ticks were removed and the salivary glands from one half of each tick were dissected for subsequent *msp4* PCR testing. Body temperature was recorded daily and calf observed closely for the signs of illness. Blood samples were collected from the calf for determination the percent reduction PCV using the microhematocrit technique and for the

preparation of Giemsa-stained blood smears. DNA was extracted from the blood samples and tested for the presence of *A. marginale* by the *msp4* PCR [22].

Light microscopy studies

For light microscopy studies, a half of each tick was fixed in 2% glutaraldehyde in O.2M sodium cacodylate buffer. The halves were then post fixed in osmium tetraoxide in O.2M sodium cacodylate buffer, dehydrated in graded series of ethanol (70% - 100%) and embedded in epoxy resin. Thick sections (1.0 μm) were cut and stained with Malory's stain for observation with a light microscope. Light micrographs were recorded with Leica DM LB with Spotcam camera system (Oklahoma State University, Stillwater, OK, USA).

Molecular Diagnostics

DNA was extracted from 200μl of blood and from individual tick salivary glands using NucleoSpin DNA extraction kit (Macherey-Nagel, Düren, Germany) following the manufacturer's protocol for the purification of genomic DNA from blood and insects. The DNA was eluted with water and stored at -20˚C. A PCR assay amplifying the *A. marginale msp4* gene was performed on blood samples of the two calves used for tick feeding and *D. reticulatus* salivary gland DNA samples, followed by sequencing as described previously [30] .

The *msp1α* gene was amplified from DNA extracted from the blood of *A. marginale* infected calves and tick salivary glands as described previously [22], but using forward primer **MSP1aATG**: 5′-TGTGTGTGTGTTATGT-3' instead of primer MSP1aP. Amplified and column purified samples were cloned in the pGEM-T vector (Promega) following the manufacturer's protocol and used directly for sequencing (Secugen SL, Madrid, Spain). The resulting *msp1α* and *msp4* gene sequences were compared to sequence data available from GenBank using the BLAST 2.2.15 program [31]. Multiple sequence alignment was performed using the program Align X (Vector NTI Suite V5.5., Invitrogen, North Bethesda, MD USA) with an engine based on the Clustal W algorithm [32].

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Chapter 3

Functional genomic studies of tick cells in response to infection with the cattle pathogen, *Anaplasma marginale*

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Abstract

The coevolution of ticks and the pathogens that they transmit has ensured their mutual survival. In these studies, we used a functional genomics approach to characterize tick genes regulated in response to *Anaplasma marginale* infection. Differentially regulated genes/proteins were identified by suppression-subtractive hybridization and differential in-gel electrophoresis analyses of cultured IDE8 tick cells infected with *A. marginale*. Nine of 17 of these genes were confirmed by real-time RT-PCR to be differentially regulated in ticks and/or IDE8 tick cells in response to *A. marginale* infection. RNA interference was used for functional studies. Six genes, which encode putative selenoprotein W2a, hematopoietic stem/progenitor cells protein-like, proteasome 26S subunit, ferritin, GST, and subolesin control, were found to affect *A. marginale* infection in IDE8 tick cells. Four genes, which encode putative GST, salivary selenoprotein M, vATPase, and ubiquitin, affected *A. marginale* infection in different sites of development in ticks. The results of these studies demonstrated that a molecular mechanism occurs by which tick cell gene expression mediates the *A. marginale* developmental cycle and trafficking through ticks.

Note: Sequence data from this article have been deposited with the GenBank Data Library under Accession Nos. ES429085–ES429112.

Background

Ticks transmit protozoan, rickettsial, and viral pathogens that impact both human and animal health [1,2]. Of these tick-borne pathogens, those belonging to the genus *Anaplasma* (Rickettsiales: Anaplasmataceae) are obligate intracellular organisms found exclusively within parasitophorous vacuoles in the cytoplasm of both vertebrate and tick host cells [3]. The type species, *Anaplasma marginale*, causes the economically important cattle disease anaplasmosis [3]. In the United States, *A. marginale* is vectored by *Dermacentor variabilis, D. andersoni, and D. albipictus* [3,4], while the one-host cattle ticks, *Rhipicephalus (Boophilus) microplus* and *R. annulatus*, are the main vectors in tropical and subtropical regions of the world [3].

The ticks and the pathogens that they transmit have coevolved molecular interactions that ensure their survival [5]. Pathogen multiplication, development, and subsequent transmission to vertebrate hosts are perfectly coordinated with the tick feeding cycle. The life cycle of *A. marginale* in the tick vector is complex and coordinated with tick feeding cycles [6–8]. Bovine erythrocytes infected with *A. marginale* are ingested by ticks in the bloodmeal and the first site of infection in ticks is gut cells, after which many other tick tissues become infected. Infection of salivary glands occurs during a subsequent feeding from where transmission is effected. The first form of *A. marginale* seen within colonies at each site of development is the reticulated (vegetative) form that divides by binary fission and results in formation of large colonies that may contain hundreds of organisms. The reticulated form then transforms into the dense or infective form, which can survive for a short time outside of cells.

At the tick-pathogen interface, *A. marginale* outer membrane proteins important for infection of ticks cells have been identified and partially characterized [5,9]. However, the expression of tick genes/proteins that facilitate *A. marginale* infection and multiplication has not been well described [10].

Herein, we studied molecular tick-pathogen interactions of *A. marginale*. In this study, we used a functional genomics approach to identify and characterize tick genes/proteins that are differentially regulated in response to *A. marginale* infection and that facilitate the trafficking of the pathogen from infection of gut cells through infection and transmission from salivary glands. These results are a fundamental contribution toward the understanding of the *A. marginale*-tick interface and will likely contribute to the development of a new generation of pathogen transmission-blocking vaccines designed to prevent transmission and reduce exposure of vertebrate hosts to tick-borne pathogens.

Results

Differential gene regulation in A. marginale-infected IDE8 tick cells and ticks

Genes differentially regulated in response to *A. marginale* infection were identified by suppression-subtractive hybridization (SSH) in infected and uninfected IDE8 tick cell cultures. The sequence analysis of 455 clones identified from the SSH libraries resulted in 332 (73%) clones without identity to published sequence databases, 86 (19%) clones containing *A. marginale* rRNA sequences, 2 (0.4%) clones with vector sequences, and 35 (7.6%) clones with identity to sequences published previously (Table 1). Of these 35 clones, 27 were upregulated in *A. marginale*-infected cells and 8 were downregulated in infected IDE8 tick cells (Table 1). Gene ontology assignments showed that the differentially regulated genes encoded proteins involved in cellular functions such as cell structure, metabolism, stress, immunity, and enzymatic processes (Table 1). Redundant (not unique) sequences were identified for 3 genes up-regulated in infected tick cells containing sequences identical to lysosomal vacuolar ATPase (vATPase), allatotropin, and heat-shock protein 70 and for 2 genes down-regulated in infected cells encoding putative ferritin and signal sequence receptor δ (Table 1).

Table 1. Genes identified by SSH as differentially regulated in *A. marginale*-infected IDE8 tick cell.

EST	Sequence identity	Up- or down-regulated in infected cells	E value	Short description
1I1B12	*Rhipicephalus appendiculatus* (CD784193)	Up	$1.8\times e^{-42}$	Phenylalanyl-tRNA synthetase β chain (PheRS),similar to Drosophila AY052086
1I1E11	*R. appendiculatus* (TC9)	Up	$2.2\times e^{-12}$	Cytochrome b
1I1H6	*Aedes aegypti* (TC62463)	Up	$4.2\times e^{-9}$	Glutathione S-transferase
1I2G6	*Ixodes pacificus* (AY674238)	Up	$8\times e^{-12}$	Function unknown
1I3A8	*Boophilus microplus* (TC3882)	Up	$6.9\times e^{-23}$	Selenoprotein W2a, weakly similar to AY221261
1I3F5	*Ixodes scapularis* (DN970416)	Up	$2\times e^{-93}$	Ubiquitin

EST	Sequence identity	Up- or down-regulated in infected cells	E value	Short description
1I3H6	*Amblyomma variegatum* (BM290841)	Up	$2.2\times e^{-14}$	Function unknown
1I3H10	*B. microplus* (CK179161)	Up	$1.4\times e^{-28}$	Function unknown, weakly similar to AY058365
1I4C6, 1I4B3, 2I5A3	*A. aegypti* (DQ440373)	Up	$4\times e^{-59}$	vATPase, H+ transporting lysosomal vacuolar proton pump
1I4G12	*Tetraodon nigroviridis* (CR731610)	Up	$3\times e^{-97}$	Function unknown
1I5B9	*I. scapularis* (DQ066115)	Up	$6\times e^{-169}$	Ixodegrin-2A RGD-containing protein
2I1E4	*I. pacificus* (AY674285)	Up	$2\times e^{-17}$	Cytochrome c oxidase polypeptide VIII
2I1F6	*I. scapularis* (DQ066253)	Up	$7\times e^{-104}$	Hematopoietic stem/progenitor cells protein-like
2IP10	*Taeniopygia guttata* (DQ216245)	Up	$3\times e^{-119}$	Ubiquitin C variant 5-like
2I2A7	*Synthetic construct* (AY461597)	Up	$4\times e^{-13}$	Arsenic-like protein
2I2G2	*Ixodes ricinus COII* (AY945419)	Up	$1\times e^{-96}$	Cytochrome c oxidase subunit II
2I3A3	*I. ricinus* (AY333957)	Up	$2\times e^{-137}$	γ -Actin-like protein
2I3A7	*I. pacificus* (AY674286)	Up	$6\times e^{-73}$	NADH-ubiquinone oxidoreductase
2I3A9, 2I3D7	*Spodoptera frugiperda* (AJ508907)	Up	$1\times e^{-48}$	Allatotropin
2I3G1	*Rattus norvegicus* (XM_226439)	Up	$7\times e^{-14}$	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 7
2I3G10	*Haemaphysalis longicornis* (AB020491)	Up	$7\times e^{-10}$	Cathepsin L-like cysteine proteinase B
2I4D3, 2I1C2	*Homo sapiens* (BC057397)	Up	$1\times e^{-46}$	Heat shock protein 70
2I4F6	*I. scapularis* (DQ066085)	Up	$6\times e^{-138}$	Salivary selenoprotein M
U1C8	*Haliclona rubens* (AY226061)	Down	$6\times e^{-49}$	β-Tubulin
U1D2	*I. scapularis* (AY682794)	Down	$9\times e^{-116}$	R2 retrotransposon reverse transcriptase-like
UP8, U3H10	*I. scapularis* (AY277906)	Down	0.0	Ferritin
U2A8, U3H12	*I. scapularis* (DQ066337)	Down	$8\times e^{-104}$	Signal sequence receptor δ
U2D4	*Gadus morhua* (AY281321)	Down	$2\times e^{-21}$	Sec61 γ subunit
U3A10	*Homo sapiens CW-1* (U56255)	Down	$5\times e^{-166}$	Mouse Tctex-1 (t complex sterility protein) homolog

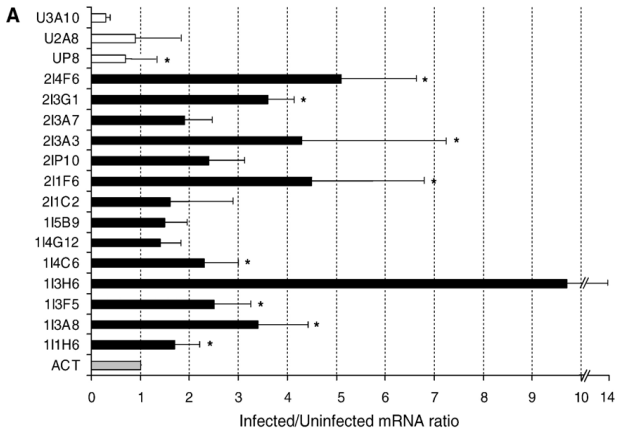
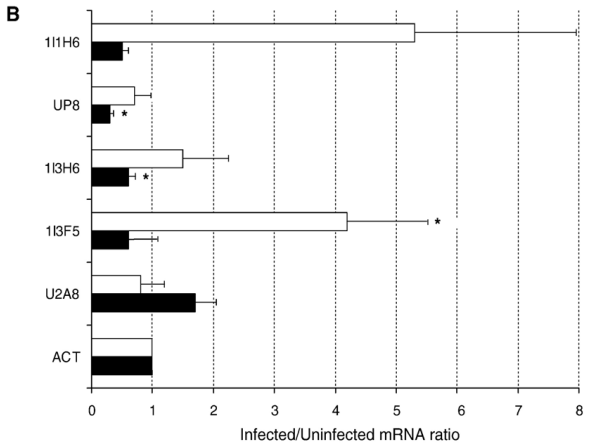
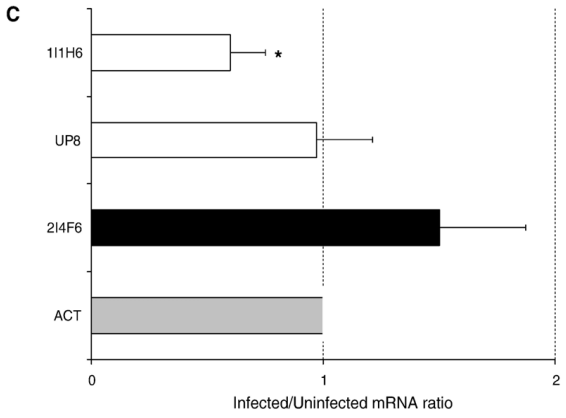


Fig. 1. Differential mRNA expression in A. marginale-infected IDE8 tick cells and ticks. Real-time RT-PCR was done on uninfected and infected (A) IDE8 cells (three independent cultures each; genes up-regulated in infected (black bars) and uninfected (white bars) cells are shown),



(B) male D. variabilis guts (black bars) and salivary glands (white bars) (three groups of 10 ticks each), and



(C) pooled male R. microplus salivary glands (two independent experiments; genes up-regulated in infected (black bars) and uninfected (white bars) ticks are shown). Bars represent average +SD mRNA ratios. mRNA levels were normalized against tick β-actin (ACT) using the comparative C_t method. mRNA levels were compared between infected and uninfected ticks and IDE8 tick cells by Student's t test ($p \leq 0.05$).*

While real-time RT-PCR analysis was attempted on all candidate differentially regulated genes with identity to sequence databases (Table 1), conditions were established for only 17/29 (59%) of the unique IDE8 tick cell sequences. The results of real-time RT-PCR were similar to the SSH analysis and confirmed by statistical analysis differential expression of 9 genes in *A. marginale*-infected IDE8 tick cells (Fig. 1A). These genes encoded putative glutathione S-transferase (GST) (1I1H6), selenoprotein W2a (1I3A8), polyubiquitin (1I3F5), vATPase (1I4C6), hematopoietic stem/progenitor cells proteinlike (2I1F6), γ-actin-like protein (2I3A3), proteasome 26S subunit (2I3G1), salivary selenoprotein M (2I4F6), and ferritin (UP8) (Fig. 1A). For the other 8 genes, mRNA levels were not significantly different between infected and uninfected tick cells (Fig. 1A).

To expand these differential gene expression results to tick species that are natural vectors of *A. marginale*, primers designed for IDE8 tick cell sequences were tested with RNA from uninfected and *A. marginale*-infected *D. variabilis* and *R. microplus*. We anticipated that many of the primers designed using the IDE8 tick cell sequences would not work with other tick species in RT-PCR studies. Therefore, homologues were identified for some of the differentially regulated genes in *D. variabilis* (GST (1I1H6) and ferritin (UP8)) and *R. microplus* (GST (1I1H6), ferritin (UP8), and selenoprotein M (2I4F6)) sequence databases and their expression was tested in uninfected and *A. marginale*-infected ticks. In *D. variabilis*, positive RT-PCR results were obtained for five genes encoding putative GST (1I1H6), ferritin (UP8), protein of unknown function (1I3H6), ubiquitin (1I3F5), and signal sequence receptor δ (U2A8), which revealed tissue-specific differences between gut and salivary gland expression levels (Fig. 1B). As expected, tick-to-tick variations in mRNA levels affected the statistical significance in some samples. Nevertheless, statistically significant differences were observed for ferritin (UP8) and protein of unknown function (1I3H6) mRNA levels in guts and for ubiquitin (1I3F5) in the salivary glands, which corroborated the differential expression of these genes in *A. marginale*-infected *D. variabilis* (Fig. 1B). While GST (1I1H6) and selenoprotein M (2I4F6) were differentially expressed in *R. microplus* salivary glands, only GST (1I1H6) mRNA levels were significantly higher in uninfected ticks (Fig. 1C).

Proteomics analysis of A. marginale-infected and uninfected IDE8 tick cells

The proteome of IDE8 tick cells was compared between uninfected and *A. marginale*-infected cells by differential in-gel electrophoresis (DIGE) (Fig. 2). Protein spots with greater than twofold change between infected and uninfected cells were subjected to matrix-assisted laser desorption/ionization time-offlight (MALDI-TOF) mass spectrometry

(MS)/MS analysis (Table 2). Of the 17 proteins analyzed, 7 (41%) were from *A. marginale*, 7 (41%) could not be identified, and 3 (18%) were of tick origin and had homology to sequence databases (Table 2). Of the differentially regulated tick proteins, only 1, homologous to translation elongation factor 1γ, was up-regulated in infected cells. Proteins homologous to GST and a putative high-mobility group-like protein were down-regulated in infected tick cells.

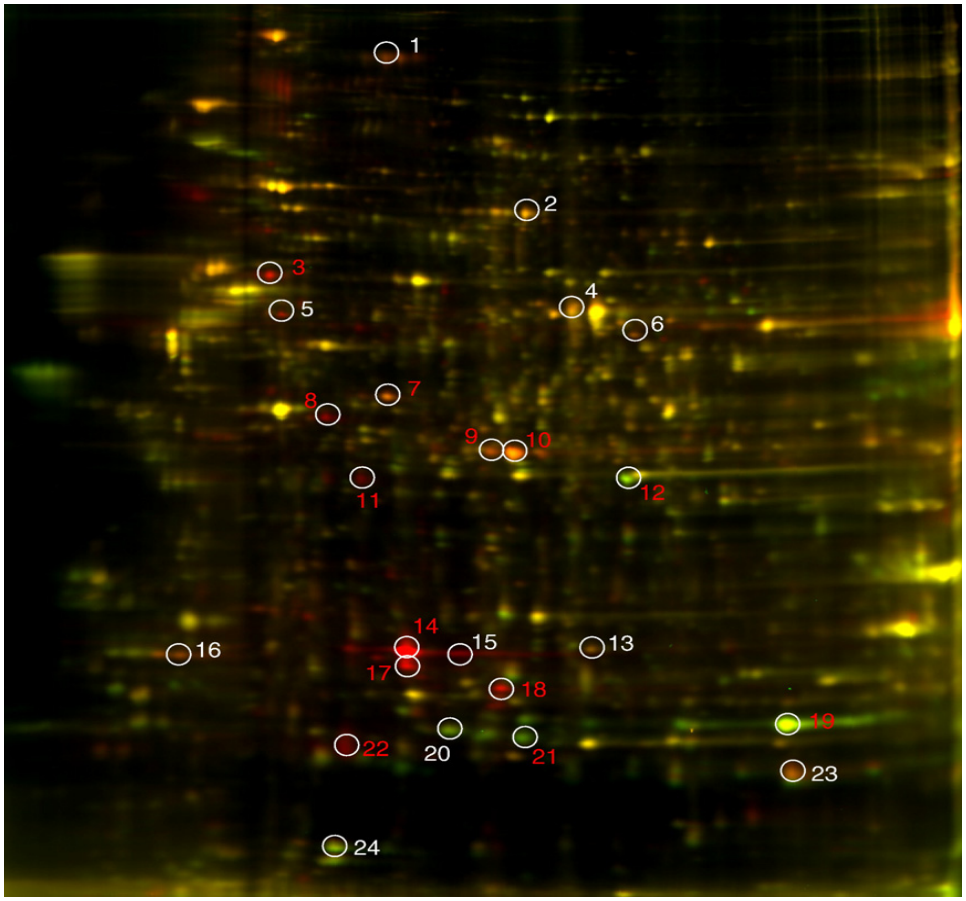


Fig. 2. A representative 2D map of DIGE analysis of proteins in uninfected and A. marginale-infected IDE8 tick cells. Overlaid images of Cy5- and Cy3-labeled proteins from infected (red) and uninfected (green) cells, respectively. Differentially regulated proteins, including those analyzed using a MALDI-TOF/TOF mass spectrometer, are numbered (Table 2).

Table 2. Differentially regulated proteins between *A. marginale*-infected and uninfected IDE8 tick cells identified by 2D-DIGE and MALDI-TOF/TOF mass spectrometry.

Spot number[a]	Fold change (I/C) [b]	Protein ID [c]	Up- or down regulated in infected cells [b]	Accession number [d]	Matched peptides	Sequence coverage (%)	Ion score	Ion score CI (%)
1	2.13	No ID	Up					
2	1.50	ND	Up					
3	14.42	*A. marginale* 60-kDa heat-shock protein	Up	27901660	2	5	97	100
4	1.50	ND	Up					
5	4.65	No ID	Up					
6	1.83	ND	Up					
7	2.35	*Bombyx mori* translation elongation factor 1γ	Up	12328431	1	3	44	76.64
8	7.28	*A. marginale* translation elongation factor Tu	Up	56417005	4	15	173	100
9	2.27	No ID	Up					
10		No ID	Up					
11	4.21	*A. marginale* major surface protein 2	Up	56417171	2	6	78	99.99
12	−2.10	No ID	Down					
13	1.83	ND	Up					
14	239.54	*A. marginale* major surface protein 4	Up	56404202	6	26	413	100
15	33.26	No ID	Up					
16	2.36	No ID	Up					
17	21.08	*A. marginale* major surface protein 4	Up	56404202	8	36	565	100
18	11.33	*A. marginale* major surface protein 4	Up	56404192	2	9	137	100
19	2.16	*Haemaphysalis longicornis* glutathione S-transferase	Down	34539115	4	20	227	100
20	1.78	ND	Down					
21	−2.25	*Dermacentor variabilis* putative HMG-like protein	Down	29825377	3	16	93	100
22	6.03	*A. marginale* transcriptional regulator	Up	23168766	6	44	503	100
23	1.45	ND	Up					
24	−1.52	ND	Down					

[a] *Spot numbers refer to the 2D gel proteins of interest that were analyzed by mass spectrometry (Fig. 2).*

[b] *Determined with respect to A. marginale-infected cells. Positive and negative values correspond to up- and down-regulated proteins in infected tick cells, respectively.*

[c] *Abbreviations: No ID, no matching protein was found; ND, not done because only spots with fold change N2 were analyzed; I, infected tick cells; C, control tick cells.*

[d] *Protein name and accession number are listed according to the NCBI nr database.*

Functional role of differentially regulated genes in ticks and IDE8 tick cells infected with A. marginale

The nine genes that were corroborated by real-time RT-PCR to be differentially regulated in *A. marginale*-infected IDE8 tick cells (Fig. 1A), which included one encoding a putative GST protein that was also identified by proteomics, were selected for functional analysis. RNA interference (RNAi) was used to evaluate the effect of silencing differentially regulated genes on *A. marginale* infection and multiplication in *D. variabilis* ticks. After RNAi, mRNA levels were reduced ($p<0.05$) after transmission feeding (TF) for GST

(1I1H6) (100±0.0 and 100±0.4% silencing in guts and salivary glands, respectively) and for salivary selenoprotein M (2I4F6) (100±0.0 and 74± 25% silencing in guts and salivary glands, respectively). For other genes, either silencing of expression was not statistically significant compared with control ticks or real-time RT-PCR conditions could not be established for *D. variabilis*. Although RNAi was demonstrated in ticks with dsRNA from heterologous tick species [11], the sequence identity may not have been sufficient for efficient RNAi in *D. variabilis* with some of the IDE8 tick cells-derived dsRNAs.

The effect of RNAi of selected genes on *D. variabilis* tick attachment and mortality was determined and analyzed statistically. Tick attachment was affected ($\alpha<0.01$) in ticks injected with dsRNAs of putative GST (1I1H6; 50% attachment) and selenoprotein W2a (1I3A8; 55% attachment) compared to the saline-injected controls (97% attachment). Tick mortality was significantly higher ($\alpha<0.01$) after acquisition feeding (AF) for ticks injected with dsRNAs of putative ubiquitin (1I3F5; 74% mortality) and γ-actin-like protein (2I3A3; 53% mortality) compared to saline-injected controls (9% tick mortality).

The effect of gene knockdown by RNAi was observed on different stages of *A. marginale* infection of *D. variabilis* ticks (Table 3). Silencing the expression of genes encoding putative ubiquitin (1I3F5), vATPase (1I4C6), selenoprotein M (2I4F6), and GST (1I1H6) resulted in statistically significant differences in *A. marginale* infection levels compared to saline-injected controls (Table 3). In ticks in which the expression of putative GST was silenced, *A. marginale* infection was inhibited both in tick guts after AF and in salivary glands after TF. However, those pathogens that infected the ticks after AF were able to multiply in the guts during TF. When putative vATPase expression was silenced, *A. marginale* infection was inhibited in tick guts after AF but the pathogens were still able to infect and multiply in the salivary glands after TF. The RNAi of salivary selenoprotein M expression resulted in the inhibition of pathogen infection and/or multiplication in tick salivary glands after TF. Targeting the expression of the putative ubiquitin also inhibited *A. marginale* gut infection but the effect during TF could not be analyzed due to the high tick mortality. As reported previously [10], subolesin RNAi affected the infection of tick salivary glands after TF. As commonly reported, tick-to-tick variations in the *A. marginale* infection levels in guts and salivary glands were found (Table 3).

RNAi was done on cultured IDE8 tick cells infected with *A. marginale* to characterize the effect of differentially regulated genes on the multiplication of *A. marginale*. The effect of RNAi on *A. marginale* infection of IDE8 tick cells was not analyzed because a tick cell-free inoculation of cells could not be produced and infection by

bovine erythrocytes, requiring several weeks to establish infections, would not provide consistent culture conditions [12]. Silencing of gene expression was demonstrated for 7 of the 10 sequences analyzed (Table 4). Of them, two genes encoding putative selenoprotein W2a (1I3A8) and hematopoietic stem/progenitor cells protein-like (2I1F6) produced significantly higher *A. marginale* infections as determined by major surface protein 4 (*msp4*) mRNA levels with respect to the controls after RNAi (Table 4). The knockdown of genes encoding putative proteasome 26S subunit (2I3G1), ferritin (UP8), GST (1I1H6), and subolesin (positive control) resulted in significantly lower *A. marginale msp4* mRNA levels, and RNAi silencing of putative vATPase (1I4C6) did not affect *msp4* mRNA levels in treated cells (Table 4).

Table 3. *A. marginale* infection levels in *D. variabilis* male guts and salivary glands after RNAi.

Experimental group	**Infection levels in the guts after AF (*A. marginale*/ tick±SE)**	**Infection levels in the guts after TF (*A. marginale*/ tick±SE)**	**Infection levels in the salivary glands after TF (*A. marginale*/ tick±SE)**
1I1H6 (GST)	25±15*	99,060±68,462	2±0*
1I3A8	364,245±439,264	1854±164	571±473
1I3F5 (ubiquitin)	2±0*	ND	ND
1I4C6 (vATPase)	81±5*	795±227	247±205
2I1F6	718±481	313,957±73,403	12,846±5,880
2I3A3	88,204±70,610	2392±904	39±2
2I3G1	556±106	20,877±3387	21±13
2I4F6 (salivary selenoprotein M)	389,095±282,048	1451±443	2±0*
UP8	5360±1059	1891±342	30±35
TGST (GST)	182±82*	19,201±1967	2±0*
Subolesin	814±122	1517±1025	2±0*
Saline control	40,579±6993	28,252±27,788	287±144

The A. marginale infection levels were analyzed in D. variabilis male ticks after RNAi. Salivary glands and/or guts were dissected from five ticks after AF and TF and analyzed by quantitative msp4 PCR to determine A. marginale infection levels. Infection levels in tick guts and salivary glands were compared between dsRNA- and saline-injected ticks by Student's t test. The putative function of tick genes targeted by RNAi is indicated in parentheses for experimental groups with statistically significant differences.

ND, not determined because ticks did not survive RNAi.

** $p \leq 0.05$.*

Table 4. *A. marginale msp4* mRNA levels in IDE8 tick cells after RNAi.

Experimental group	**Silencing of gene expression (%)±SD**	**Change in *A. marginale msp4* mRNA levels (average fold with respect to control cells±SD)**
1I3A8 (selenoprotein W2a)	84±2*	4.4±0.3**
1I3F5	0±0	NS
1I4C6	93±4*	NS
2I1F6 (hematopoietic stem/ progenitor cells protein-like)	55±26*	7.6±1.7*
2I3A3	0±0	NS
2I3G1 (proteasome (prosome, macropain) 26S subunit, non-ATPase, 7)	77±21*	0.6±0.05*
2I4F6	0±0	NS
UP8 (ferritin)	93±2*	0.6±0.03*
1I1H6/TGST (GST)	70±29*	0.3±0.16*
Subolesin	61±26*	0.5±0.06*

The A. marginale msp4 mRNA levels were analyzed in IDE8 tick cells after RNAi. Total RNA was extracted from infected tick cells after RNAi and analyzed by quantitative RT-PCR to determine gene expression silencing and A. marginale msp4 mRNA levels with respect to control cells treated with unrelated Rs86 dsRNA. Tick genes and A. marginale msp4 mRNA levels were compared between test and control cells by Student's t test. The putative function of tick genes targeted by RNAi is indicated in parentheses for experimental groups with statistically significant differences. NS, not significantly different.

$^{*}p \leq 0.05$. $^{**}p \leq 0.01$

Summary of results of differential regulation and functional analyses in IDE8 tick cells and ticks in response to A. marginale infection

In the study reported herein, we characterized the tick cell response to infection with *A. marginale* at the mRNA and protein levels and used RNAi to study the function of these molecules during *A. marginale* infection and multiplication in ticks and cultured IDE8 tick cells. Of the 472 molecules (455 from SSH and 17 from DIGE analyses) that were identified as candidates for differential regulation in *A. marginale*-infected IDE8 cells, 38 (representing 31 unique sequences) had identity/ homology to nucleotide and protein sequence databases. Of these, 17 were analyzed by real-time RT-PCR and 9 were confirmed to be differentially regulated in response to *A. marginale* infection of IDE8 cells by statistical analysis of mRNA levels in infected and uninfected cells. Some of these genes were also differentially regulated in tissues of *D. variabilis* and *R. microplus* ticks infected with *A. marginale*. The sequences of the 9 genes that were confirmed to be differentially regulated in response to *A. marginale* infection of IDE8 tick cells were then used for functional analysis by RNAi in *D. variabilis* and IDE8 tick cells. Four genes (encoding

putative GST, salivary selenoprotein M, vATPase, and ubiquitin) had significantly lower *A. marginale* infection levels after RNAi in *D. variabilis* guts and/or salivary glands. Six genes (encoding putative selenoprotein W2a, hematopoietic stem/ progenitor cells protein-like, proteasome 26S subunit, ferritin, GST, and subolesin control) affected *A. marginale* multiplication in IDE8 tick cells after RNAi.

Discussion

Studies of the molecular events that mediate interactions between hosts and *A. marginale* have been the focus of recent investigations [3,5,9]. However, most of this research has focused on the vertebrate host-pathogen interactions rather than those of the tick and pathogen. While outer membrane proteins of *A. marginale* that are involved in interactions with tick cells have been partially characterized [5,9], tick-pathogen coevolution is most likely highly influenced by genetic traits of the vector. Recently, tick proteins have been characterized that play a role in the infection and transmission of *Borrelia burgdorferi* [13–15] and *A. phagocytophilum* and/or *A. marginale* [10,16]. Furthermore, genetic factors have been associated with intraspecific variation in vector competence for a variety of vector-borne pathogens, including *A. marginale* [17,18]. These results illustrate the complexity of tick-pathogen coevolution relationships and suggest that genetic loci of both the vector and the pathogen are required for infection and transmission of pathogens by ticks [5].

Few studies have characterized gene expression in ticks in response to pathogen infection. Differential gene expression has been characterized in *Ixodes ricinus* and *I. scapularis* infected with *B. burgdorferi* [19,20], in *D. variabilis* in response to *Rickettsia montanensis* infection [21–23], and in salivary glands of female *Rhipicephalus appendiculatus* infected with *Theileria parva* [24]. These studies have suggested that pathogens modify the expression of tick genes involved in the establishment of infection and cellular defense mechanisms. However, the functions of these genes during tick-pathogen interactions are largely unknown.

In the study reported herein, we used RNAi for functional studies of differentially regulated tick genes. RNAi is currently the most efficient method for the genetic manipulation of gene expression in ticks [11]. The results of RNAi suggested that the genes identified as differentially regulated in *A. marginale*-infected IDE8 tick cells may perform different functions during the infection, trafficking, and multiplication of the pathogen in ticks (Fig. 3) and provided additional evidence for the distinct role that guts and salivary glands play on *Anaplasma* infection and transmission by ticks [25]. The putative GST,

vATPase, and ubiquitin may be involved in the initial *A. marginale* infection of tick gut cells. Results of the RNAi experiments also suggested that GST and salivary selenoprotein M expression may be involved in trafficking and/or infection and multiplication of the pathogen in tick salivary glands. Furthermore, RNAi experiments in IDE8 tick cells provided evidence that putative selenoprotein W2a and hematopoietic stem/progenitor cells protein-like may participate in the cellular response to limit pathogen infection, while proteasome 26S subunit, ferritin, and GST may enhance *A. marginale* multiplication in tick cells.

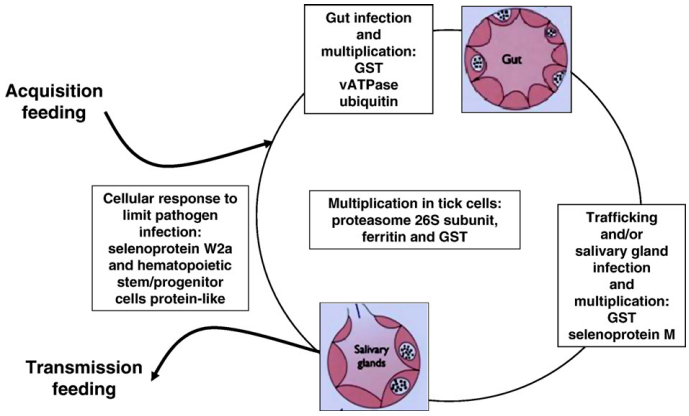


Fig. 3. Genes differentially regulated in A. marginale-infected ticks and IDE8 tick cells may have different functions during the infection, trafficking, and multiplication of the pathogen in ticks. A schematic representation of the A. marginale life cycle in ticks and the putative roles of genes identified in this study is presented.

Some of the tick genes identified as differentially regulated in response to *A. marginale*, such as selenoproteins, GST, vATPase, and ferritin, have been reported to be regulated by tick blood feeding or infection with other pathogens [19–23,26,27]. However, other genes such as ubiquitin, proteasome 26S subunit, and hematopoietic stem/progenitor cells protein-like constitute new findings of tick genes differentially regulated in response to pathogen infection.

The effects of differentially regulated genes on *A. marginale* infection and multiplication in ticks were different from that produced by subolesin, which has pleiotropic effects on gut, salivary gland, and reproductive tissues that affect tick feeding, development, and reproduction [10,28,29]. Except for the putative ubiquitin and GST, which affected tick survival and attachment, respectively, RNAi of differentially regulated genes did not produce notable effects on tick tissues and feeding. These results suggest that

it may be possible to combine tick proteins that are functionally important for the tick and the pathogen to produce vaccines for the control of tick infestations and the transmission of tick-borne pathogens [30].

For some genes studied herein, the results of mRNA expression analysis were similar between *A. marginale*-infected IDE8 tick cells and ticks. These results confirm the utility of IDE8 tick cell culture, in part, as a model to study *Anaplasma*-tick interactions [12]. However, tissue-specific differences in gene expression between guts and salivary glands and between ticks and IDE8 tick cells also demonstrated that results in cultured IDE8 tick cells should be validated in infected and uninfected tick species that are natural vectors of the pathogen.

In summary, we have used a functional genomics approach to study *A. marginale*-tick interactions. The multifaceted approach of using genomics, proteomics, and real-time RT-PCR with RNAi functional analysis has allowed the identification and characterization of tick genes that are involved in cellular responses to limit pathogen multiplication or are essential for *A. marginale* infection and multiplication in the tick vector. Interestingly, some of these genes had different expression patterns in tick guts and salivary glands and affected the *A. marginale* life cycle at different sites in the tick, thus supporting our hypothesis that *A. marginale* trafficking through ticks and subsequent transmission is mediated by tick cell gene expression. Experiments will be required to define further the role of these genes during the *A. marginale* life cycle in ticks. The results reported in this study expand our understanding of the role of tick gene expression in pathogen development and will likely contribute to the development of a new generation of pathogen transmissionblocking vaccines designed to prevent or minimize infections in vertebrate hosts.

Materials and methods

Experimental design

We first identified tick genes and proteins that are differentially regulated by *A. marginale* in *I. scapularis* IDE8 cells by SSH and DIGE coupled with MALDI-TOF/TOF MS/MS analysis, respectively. The partial sequences of SSH clones and DIGE proteins were used to search for sequence identity/homology to nucleotide and protein databases. Oligonucleotide primers were then designed to validate differential expression by real-time RT-PCR in uninfected and *A. marginale*-infected IDE8 tick cells and *D. variabilis* ticks. The differential expression of some genes was also studied by real-time RT-PCR in uninfected

and *A. marginale*-infected *R. microplus.* The genes that proved to have statistically significant differences in the mRNA levels between uninfected and *A. marginale*-infected IDE8 tick cells were then selected for functional analysis by RNAi. RNAi was used to characterize the function of selected genes in the infection and/or multiplication of *A. marginale* in *D. variabilis* and IDE8 tick cells.

Uninfected and A. marginale-infected ticks

D. variabilis male ticks were obtained from the laboratory colony maintained at the Oklahoma State University, Tick Rearing Facility. Off-host ticks were maintained in a 12-h light:12-h dark photoperiod at 22–25 °C and 95% relative humidity. To obtain infected *D. variabilis*, male ticks were fed for 1 week on a splenectomized calf with ascending *A. marginale* parasitemia that was experimentally infected with the Virginia isolate of *A. marginale*. The ticks were then removed and maintained off-host for 4 days and then fed for an additional week on an uninfected calf. Uninfected ticks were fed in a similar way on the normal uninfected calf only. *R. microplus* male ticks (Mozambique strain) were reared in cattle at the Utrecht Center for Tick-borne Diseases, Utrecht University. Ticks were infected by feeding on a calf experimentally infected with the Texas isolate. Uninfected ticks were fed in a similar way on an uninfected calf. The infection of ticks with *A. marginale* was corroborated by *msp4* PCR [31]. Animals were housed with the approval and supervision of the respective Institutional Animal Care and Use Committees.

Uninfected and A. marginale-infected tick cells

The tick cell line IDE8 (ATCC CRL 11973), derived from *I. scapularis* embryos, was cultured as described previously [32]. Briefly, tick cells were maintained at 31 °C in L15B medium, pH 7.2, supplemented with 5% heatinactivated fetal bovine serum (Sigma, St. Louis, MO, USA), 10% tryptose phosphate broth (Difco, Detroit, MI, USA), and 0.1% lipoprotein concentrate (ICN Biomedicals, Inc., Costa Mesa, CA, USA), and the culture medium was replaced weekly. IDE8 tick cells were inoculated with the Virginia isolate of *A. marginale* and monitored by stained smears and with phase-contrast microscopy [32]. The IDE8 tick cell monolayers in T-25 flasks were infected with 1 ml (1:5 dilution) terminal infected cell cultures, in which approximately 100% of the cells were infected. Flasks were washed two times with PBS and harvested 3 days postinfection (dpi), when approximately 40% of the cells were infected. Companion cell cultures were terminal at 7 dpi. Uninfected cells were cultured in the same way but with the addition of 1 ml of

culture medium instead of infected cells. Cells were centrifuged at 10,000 g for 3 min and cell pellets were frozen in liquid N until used for RNA extraction. The infection of tick cells with *A. marginale* was corroborated by *msp4* PCR [31].

Suppression–subtractive hybridization

Total RNA was isolated from three uninfected and three *A. marginale*-infected IDE8 cell cultures at 3 dpi using TriReagent (Sigma) according to the manufacturer's instructions. RNA quality was checked by gel electrophoresis to verify the integrity of RNA preparations. Pools of 21 μg RNA were made from uninfected (Ns=3; 7 μg RNA/each) and infected (N=3; 7 μg RNA/each) tick cells. SSH was performed at Evrogen JCS (Moscow, Russia) as described previously [33]. Tester and driver RNAs were subtracted in both directions to construct two SSH libraries enriched for differentially regulated cDNAs in uninfected (reverse-subtracted) and infected (forward-subtracted) tick cells. Approximately 100 clones from each library were randomly picked and subjected to differential hybridization with subtracted and nonsubtracted probes using the PCR-select differential screening kit (Clontech, Palo Alto, CA, USA), which resulted in N95% candidate differentially regulated cDNAs. Then, 400 clones from the forward-subtracted library and 100 clones from the reversesubtracted library were sequenced from one end of the pAL-16 vector with vector-specific primers.

Sequence analysis and database search

Partial sequences were determined for 455 of the 500 sequenced SSH library clones. Multiple sequence alignment was performed using the program AlignX (Vector NTI Suite version 8.0, InforMax; Invitrogen, Carlsbad, CA, USA) to exclude vector sequences and to identify redundant (not unique) sequences. Searches for sequence similarity were performed with the BLASTN program (http://www.ncbi.nlm.nih.gov/BLAST) against the nonredundant sequence database (nr) and databases of tick-specific sequences (http://www.vectorbase.org/index.php; http://www.tigr.org/). Protein ontology was determined using the protein reference database (http://www.proteinlounge. com).

Proteomics analysis

Approximately 10^6 uninfected and *A. marginale*-infected cells at 3 dpi were pooled from three independent cultures prepared as described above and used to perform proteomics analysis at Applied Biomics (Hayward, CA, USA; http://www.appliedbiomics.com) by the DIGE technique [34] according to the manufacturer's protocol. Briefly, cell samples were

lysed in 2D lysis buffer containing 30 mM Tris-HCl, pH 8.8, 7 M urea, 2 M thiourea, and 4% Chaps electrophoresis reagent (Sigma) and sonicated for 5 s using VirSonic 100 (VirTis, Gardiner, NY, USA) at power level 4. After vigorous shaking at room temperature for 30 min, cell lysates were cleared by high-speed centrifugation for 30 min at 4 °C. Supernatants were then transferred to fresh Eppendorf tubes and protein concentration was adjusted for each sample to 5 mg/ml with the lysis buffer. Infected and uninfected samples were labeled with Cy5 and Cy3, respectively (Amersham Biosciences, Inc., Piscataway, NJ, USA). An internal pool was generated by combining equal amounts of lysates from each cell sample and this pool was labeled with Cy2 dye and was included in all gel runs to aid cross-gel statistical analysis. After second-dimension SDS-PAGE, the gel was scanned using the Typhoon Trio scanner (Amersham Biosciences) and images were analyzed using ImageQuant software (Amersham Biosciences). The resulting gel was then visualized by Sypro Ruby staining. Statistics and quantification of protein expression were carried out in DeCyder-differential in-gel analysis software (Amersham Biosciences). Protein spots with greater than twofold change between infected and uninfected cells were excised from the gel using an Ettan spot picker (Amersham Biosciences) and digested with trypsin and protein mass spectra were obtained by MS and MS/MS analysis using a MALDI-TOF/TOF mass spectrometer (ABI-4700; Applied Biosystems, Inc., Foster City, CA, USA; ACHC matrix) [34,35]. For sequence identification, peptide mass fingerprinting was performed with the Mascot search engine (Matrix Science, Boston, MA, USA) and NCBI and SwissProt protein databases were searched (two variable modifications, carbamidomethyl and oxidation, one missed cleavage; precursor tolerance, 100 ppm; MS/MS tolerance, 0.3D). A match was considered successful when de novo sequences were derived from high-quality mass spectra and thepeptide score was >95%.

Real-time RT-PCR analysis

The same RNA samples prepared as described above for SSH from three *A. marginale*-infected and three uninfected IDE8 tick cell cultures were used for real-time RT-PCR analysis. Total RNA was extracted as described above from uninfected and *A. marginale*-infected male *D. variabilis* guts and salivary glands (three groups of 10 ticks each) and from pooled uninfected and *A. marginale*-infected male *R. microplus* salivary glands for real-time RT-PCR analysis. Two primers were synthesized based on the sequences determined for candidate differentially regulated genes in IDE8 tick cells and used for real-time RT-PCR analysis of mRNA levels in uninfected and *A. marginale*-infected IDE8 tick

cells and male *D. variabilis* ticks. Identical sequences were mined for some genes in *D. variabilis* (GST and ferritin) and R. (Boophilus) microplus (GST, ferritin, and selenoprotein) sequence databases and used to synthesize primers for real-time RT-PCR analysis of mRNA levels in uninfected and *A. marginale*-infected male tick tissues. Real-time RT-PCR was done using the QuantiTec Sybr Green RTPCR kit (Qiagen, Valencia, CA, USA) and a Bio-Rad iQ5 thermal cycler (Hercules, CA, USA) following the manufacturer's recommendations and using the oligonucleotide primers and PCR conditions described in Table 5. mRNA levels were normalized against tick β-actin using the comparative Ct method. mRNA levels were compared between infected and uninfected IDE8 tick cells and ticks by Student's *t* test (p=0.05).

RNA interference in ticks

Oligonucleotide primers homologous to pAL-16 SSH vector plasmid sequences and containing T7 promoters for in vitro transcription and synthesis of dsRNA (PAL5T7, 5′-TAATACGACTCACTATAGGGTACTGGCGGCCGCGGGAATTCGAT, and PAL3T7, 5′-TAATACGACTCACTATAGGGTACTGCCGCGAATTCACTAGTGAT) were synthesized to amplify selected tick cDNAs. For GST dsRNA synthesis, oligonucleotides were designed to be homologous to reported tick sequences (GST5T7, 5′-TAATACGACTCACTATAGGGTACTACTACATCGATGGGGACGT, and GST3T7, 5′-TAATACGACTCACTATAGGGTACTAYKTYYYKGAGCAGCAGG). *D. variabilis* subolesin dsRNA was synthesized as described previously [10,28]. PCR and dsRNA synthesis reactions were performed as described previously [10,28], using the Access RT-PCR system (Promega, Madison, WI, USA) and the Megascript RNAi kit (Ambion, Austin, TX, USA). The dsRNAwas purified and quantified by spectrometry.

Male *D. variabilis* ticks were injected with approximately 0.4 μl of dsRNA (5×10^{10}–5×10^{11} molecules per microliter) in the lower right quadrant of the ventral surface of the exoskeleton of the tick [10,28]. The injections were done on 20 ticks per group using a Hamilton syringe with a 1-in., 33-gauge needle. Control ticks were injected with *D. variabilis* subolesin dsRNA (positive control) or injection buffer (10 mM Tris-HCl, pH 7, 1 mM EDTA) alone (saline negative control). We have previously demonstrated that there is no difference between using an unrelated dsRNA or injection buffer alone for negative control ticks in tick RNAi experiments [28]. The ticks were held in a humidity chamber for 1 day after which they were allowed to feed for 7 days on a splenectomized calf that was experimentally infected with the Virginia isolate of *A. marginale* (rickettsemia during tick feeding, 4.8–35.9% infected erythrocytes). Unattached ticks were removed 2

days after infestation. All ticks were removed after 7 days of feeding and held in a humidity chamber for 4 days to allow ticks to digest the bloodmeal completely to detect only those pathogens that had infected gut cells. Guts were then dissected from 5 ticks and stored in RNAlater (Ambion) for extraction of DNA and RNA to determine the *A. marginale* infection levels by msp4 quantitative PCR [31] and to confirm gene knockdown by RT-PCR using sequence-specific primers (Table 5) as described above. The remaining ticks were allowed to feed for 7 days on a sheep to promote development of *A. marginale* in tick salivary glands. Salivary glands and guts were dissected from 5 ticks and processed for RNA and DNA studies as described above for the guts. Animals were cared for in accordance with standards specified in the Guide for the Care and Use of Laboratory Animals. mRNA levels and *A. marginale* infection in tick guts and salivary glands were compared between dsRNA- and saline-injected ticks by Student's t test (p=0.05). Tick attachment was evaluated as the ratio of attached ticks 48 h after infestation on the calf to the total number of ticks. Tick mortality was evaluated as the ratio of dead ticks after AF on the calf or TF on the sheep to the total number of fed ticks. Tick attachment and mortality were compared between dsRNA- and saline-injected ticks by $\chi 2$ test as implemented in Mstat 4.01 (α=0.01).

RNA interference in tick cells

A flask of IDE8 tick cells with a terminal infection of *A. marginale* (Virginia isolate) was disrupted and used to infect two T-25 flasks of uninfected tick cell monolayers. The cells were collected 2 dpi and used to seed two 24-well plates at 6×105 cells/well. Cells were cultured for 24 h to allow attachment and incubated with the *I. scapularis* dsRNAs described above for RNAi in ticks. The unrelated Rs86 dsRNA [36] was used as negative control. Each well received a mix of 10 µl dsRNA (5×10^{9}–5×10^{10} molecules per microliter) and 90 µl L15B medium and incubated for 24 h. Two wells were used for each treatment. After 24 h, 1 ml of L15B medium was added to each well. Medium was replaced after 24 h. Cells were harvested at 4 days posttreatment, centrifuged, washed once with PBS, and used to purify RNA as described above. *A. marginale msp4* mRNA levels were determined by real-time RT-PCR as described above using oligonucleotide primers MSP4A.m5, 5′-GACGTGCTGCACACAGATTT, and MSP4A.m3, 5′-CTCATCAAATAGCCCGTGGT. Gene expression silencing was confirmed by RT-PCR using sequence-specific primers (Table 5) as described above.

Table 5. RT-PCR oligonucleotide primers and conditions for the characterization of the expression profiles of differentially regulated tick genes.

Gene description [a]	Upstream/downstream primer sequences (5′-3′)	PCR annealing conditions
I. scapularis sequences		
1I1H6	GGTACATGGAATCCGACTGC GTCCCCTTTTGCTTCGACTT	54 °C, 30 s
	ACTACATCGATGGGGACGT AYKTYYYKGAGCAGCAGG	45 °C, 30 s
1I3A8	GACGCAAAACTTCCTTCGAG GCACTTTCCAAGAGCCTGAC	54 °C, 30 s
1I3F5	GCTTTCACGTTTTCGATGGT GGCAAAGATCCAAGACAAGG	50 °C, 30 s
1I3H6	GCCTAGGGAGGACGTCGTAG ACGTGGAACACATCGAGTCA	50 °C, 30 s
1I4C6 G	AATGCGAGACACTGGAGGAC AATCCAGGAATGTTGCCAA	50 °C, 30 s
1I4G12	GACGGACCTTGTCCGACTAC ATTCCCTCCTTGTCCTGGAT	53 °C, 30 s
1I5B9	CGTCCCCTTCTGTGGAATTA TCATCGTTGTTCTGGTCTCG	53 °C, 30 s
2I1C2	GAGACCATCAAGTGGCTGGA CTTGGTGATGATGGGGTTG	53 °C, 30 s
2I1F6	CAACCCCAAGATCGTCAACT ACGCGTCCTTACGTTTCACT	53 °C, 30 s
2IP10	TCTTGCCGGTCAGAGTCTTT GAAGGCGAAAATTCAGGACA	53 °C, 30 s
2I3A3	TAAAACCCCTTTCCCCACTT GCACTCGAACCTAGCAAACC	53 °C, 30 s
2I3A7	TCGACTCTGTTCAGGAGGAAG GGTCCAAATGGCAGAGCAT	53 °C, 30 s
2I3G1	AGGAAGTGCACGATGATGG GGTTGGTTATCCTCTGGGAGA	54 °C, 30 s
2I4F6	CTTTCTTGCCGTGCTTCTTT GCTCAACTTCCTCGTCGTTC	53 °C, 30 s
UP8	CCTCCCTCGCTAACCTCTCT ATCGTCACGGTCGAAGTAGC	54 °C, 30 s
U2A8	GCTCATCGTCGCCAACAT GAGTTCCTCCGTCCAGCTC	54 °C, 30 s
U3A10	GCCTATGGCCTTTCTCCTTT ATCTGACTTCGGTGCCATTC	53 °C, 30 s
β-Actin (AF426178)	GAGAAGATGACCCAGATCA GTTGCCGATGGTGATCACC	50 °C, 30 s
D. variabilis sequences		
	ATTGGTGCAGGACCATTCTC	57 °C, 30 s
GST (DQ224235)	GGCATATCTGCACGACAGAA	
Ferritin (AY277904)	GAGCGTGAACATGCTGAGAA CAGCTGAGCGTCATTGTGAT	57 °C, 30 s
R. microplus sequences		
GST (AF077609)	GGCTGAACGAGAAGACCAAG CAGGGTTGTAGCACAGACGA	55 °C, 30 s
Selenoprotein (TC3882)	CGTGACTGGCACAGTAGGAC GAGCAAATGTCCAACGAGGT	55 °C, 30 s
Ferritin (AY277902)	CTCAGCCCCGTCAGAACTAC CTCCTCATCGCTGCTCTTCT	55 °C, 30 s
β-Actin (AY255624)	CACGGTATCGTCACCAACTG TGAT	55 °C, 30 s

[a] *IDs for I. scapularis ESTs are described in Table 1. GenBank accessionnumbers are shown in parentheses for R. microplus and D. variabilis sequences.*

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Chapter 4

Differential expression of genes in salivary glands of male *Rhipicephalus* (*Boophilus*) *microplus* in response to infection with *Anaplasma marginale*

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Submitted for publication.

Abstract

Background

Bovine anaplasmosis, caused by the rickettsial tick-borne pathogen *Anaplasma marginale* (Rickettsiales: Anaplasmataceae), is vectored by *Rhipicephalus* (*Boophilus*) *microplus* in many tropical and subtropical regions of the world. *A. marginale* undergoes a complex developmental cycle in ticks which results in infection of salivary glands from where the pathogen is transmitted to cattle. In previous studies, we reported modification of gene expression in *Dermacentor variabilis* and cultured *Ixodes scapularis* tick cells in response to infection with *A. marginale*. In these studies, we extended these findings by use of a functional genomics approach to identify genes differentially expressed in *R. microplus* male salivary glands in response to *A. marginale* infection. Additionally, a *R. microplus*-derived cell line, BME26, was used for the first time to also study tick cell gene expression in response to *A. marginale* infection

Results

Suppression subtractive hybridization libraries were constructed from infected and uninfected ticks and used to identify genes differentially expressed in male *R. microplus* salivary glands infected with *A. marginale*. A total of 279 ESTs were identified as candidate differentially expressed genes. Of these, five genes encoding for putative histamine-binding protein (22Hbp), von Willebrand factor (94Will), flagelliform silk protein (100Silk), Kunitz-like protease inhibitor precursor (108Kunz) and proline-rich protein BstNI subfamily 3 precursor (7BstNI3) were confirmed by real-time RT-PCR to be down-regulated in tick salivary glands infected with *A. marginale*. The impact of selected tick genes on *A. marginale* infections in tick salivary glands and BME26 cells was characterized by RNA interference. Silencing of the gene encoding for putative flagelliform silk protein (100Silk) resulted in reduced *A. marginale* infection in both tick salivary glands and cultured BME26 cells, while silencing of the gene encoding for subolesin (4D8) significantly reduced infection only in cultured BME26 cells. The knockdown of the gene encoding for putative metallothionein (93Meth), significantly up-regulated in infected cultured BME26 cells, resulted in higher *A. marginale* infection levels in tick cells.

Conclusions

Characterization of differential gene expression in salivary glands of *R. microplus* in response to *A. marginale* infection expands our understanding of the molecular mechanisms at the tick-pathogen interface. Functional studies suggested that differentially expressed

genes encoding for subolesin, putative von Willebrand factor and flagelliform silk protein could play a role in *A. marginale* infection and multiplication in ticks, while metallothioneins may play a role in defense against bacterial infections. These tick genes found to be functionally relevant for tick-pathogen interactions will likely be candidates for development of vaccines designed for control of both ticks and tick-borne pathogens.

Note: Gene sequences deposited in the GenBank Data Library, Accession numbers: GO496166-G0496262

Background

Bovine anaplasmosis, caused by the obligate intracellular rickettsial pathogen, *Anaplasma marginale* (Rickettsiales: Anaplasmataceae), is characterized in cattle by anemia, fever, weight loss and reduced milk production [1]. Transmission of *A. marginal*e occurs mechanically by biting flies and blood-contaminated fomites, while ticks are biological vectors [2]. Approximately 20 tick species have been incriminated worldwide as vectors of *A. marginale* [2]. Of these tick species, the southern cattle tick, *Rhipicephalus* (*Boophilus*) *microplus,* found in tropical and subtropical regions of the world, is considered to be the most economically important ectoparasite of livestock [3]. *R. microplus* vectors several pathogens and *A. marginale* is among the most important ones, causing notable economic loss in milk and beef production [4].

The developmental cycle of *A. marginale* was described in *Dermacentor* ticks, and male ticks were shown to become persistently infected with *A. marginale* and able to transmit infection repeatedly when transferred among cattle [5]. The *A. marginal*e development, multiplication in the tick and transmission to the vertebrate host are coordinated with tick feeding. Within *Dermacentor* ticks, *A. marginale* undergoes a complex developmental cycle that begins with the infection of the gut cells. After a second tick feeding, many other tick tissues become infected, including the salivary glands from where the *A. marginale* is transmitted to cattle [5, 6]. Although the developmental cycle of *A. marginale* has not been described for *Rhipicephalus* (*Boophilus*) spp., the developmental cycle in *R. microplus* is most likely similar and males may also play an important role in pathogen transmission [7].

Molecular interactions at the tick-pathogen interface ensure survival and development of both the pathogen and vector. While recent studies on several pathogens have demonstrated that tick gene expression is modified in response to pathogen infection [8-10], information on the function of the differentially expressed genes is limited [11]. RNA interference (RNAi) has been shown to be a useful tool for the characterization of the function of genes involved in tick-host-pathogen interactions and the transmission of tick-

borne pathogens and for screening for tick protective antigens [12]. Recently, genes differentially expressed in cultured IDE8 tick cells in response to *A. marginale* infection were identified and their impact on pathogen infection in *D. variabilis* ticks was characterized by RNAi during the pathogen developmental cycle [11].

Tick cell lines have been used successfully to study vector-pathogen interactions [13]. However, these studies were conducted in the IDE8 and ISE6 tick cell lines derived from *Ixodes scapularis* embryos which is not a natural vector of *A. marginale* [13]. Recently, a Brazilian isolate of *A. marginale* was propagated successfully in the BME26 cell line derived from *R. microplus* [14] which provided the opportunity to study the *A. marginale*-tick interface in the cells cultured from a natural tick vector.

The objective of this study was to identify *R. microplus* genes differentially expressed in male salivary glands in response to infection with *A. marginale* by using suppression subtractive hybridization (SSH) approach and to characterize the function of those genes by RNAi. SSH enables identification of low-abundant rare transcripts through the comparison of two cDNA populations by selective amplification of the genes expressed in one population but not in the other [15, 16]. The results of these SSH studies were validated by real-time RT-PCR in *R. microplus* ticks and cultured BME26 tick cells for selected genes. Finally, functional analyses were conducted on selected genes by RNAi in both *R. microplus* male ticks and cultured BME26 cells to determine the putative role of these genes in *A. marginale*-tick interactions.

Results

Identification of differentially expressed genes in R. microplus male salivary glands

Two SSH libraries, forward and reverse, were constructed to identify genes up- and down-regulated in *R. microplus* male salivary glands in response to *A. marginale* infection. From each library, 288 randomly selected clones were identified and sequenced. After removing vector sequences and eliminating EST clones with poor sequence quality, datasets of 128 and 151 ESTs from forward and reverse subtracted libraries were obtained, respectively, and used for bioinformatics analyses. Clustering and assembly of ESTs from forward subtracted library (up-regulated in infected cells) resulted in 43 unique transcripts of which 10 were derived from two or more ESTs (consensus sequences) and 33 were derived from a single EST (singletons). Assembly of the ESTs in the reverse subtracted library (down-

regulated in infected cells) yielded 56 unique sequences (24 consensus sequences and 32 singletons). Automated annotation was then used to search public domain protein databases for putative functions (Table 1). Gene ontology assignments demonstrated that up-regulated genes encoded for proteins with putative functions of binding (21%), structural molecules (11%), catalytic/enzymatic activity (6%), DNA/RNA metabolism (4%), and 58% had no known function (Figure 1A). Putative functions assigned to down-regulated genes included binding (20%), structural molecules (20%), catalytic/enzymatic activity (7%), transport (5%) and 48% had no known function (Figure 1B).

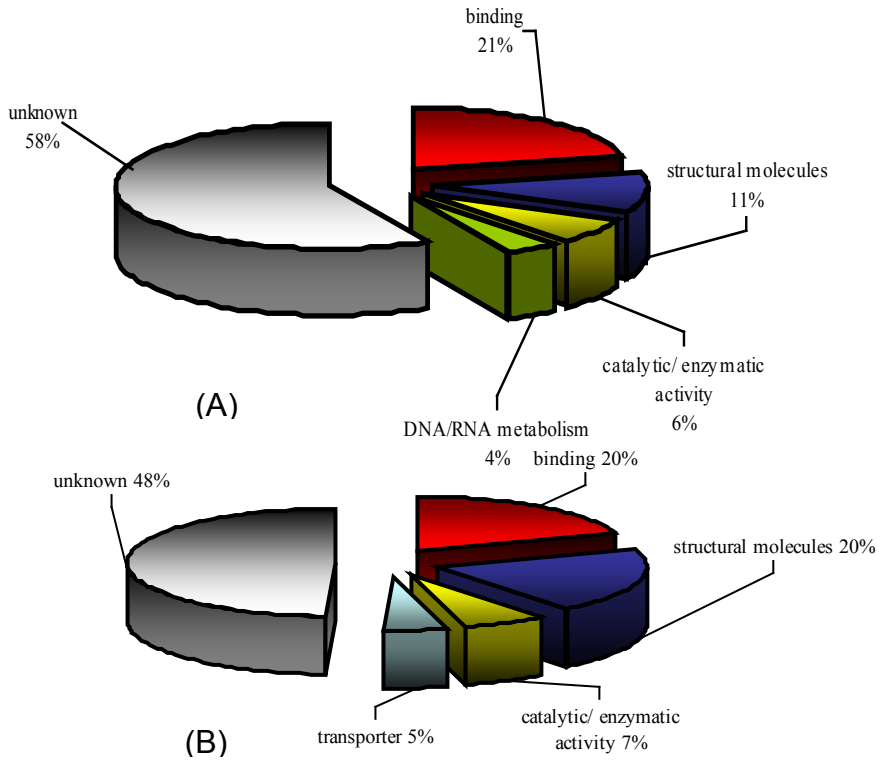

Figure 1. Gene ontology assignments of ESTs differentially expressed in R. microplus male salivary glands in response to A. marginale infection. (A) Genes up-regulated in infected salivary glands. (B) Genes down-regulated in infected salivary glands.

Differential gene expression in A. marginale-infected R. microplus male salivary glands and cultured BME26 cells

Fourteen candidate genes with putative functions in tick-pathogen interactions were selected for validation of SSH results by real-time RT-PCR (Table 2). Real-time RT-PCR analyses were done on the same pooled RNA samples used for SSH. Statistically significant differences in expression were obtained for 5 genes (Figure 2). Similar to the SSH results, genes encoding for putative female-specific histamine-binding protein (22Hbp), flagelliform silk protein (100Silk), Kunitz-like protease inhibitor precursor (108Kunz), and proline-rich protein BstNI subfamily 3 precursor (7BstNI3) were significantly down-regulated in infected tick salivary glands. The gene encoding for the putative von Willebrand factor (94Will), identified to be up-regulated by SSH, was shown by real-time RT-PCR to be significantly down-regulated in the infected tick salivary glands. For the other 9 genes, mRNA levels were not significantly different between infected and uninfected ticks. Subolesin (4D8), used as a positive control, was down-regulated in *A. marginale*-infected tick salivary glands.

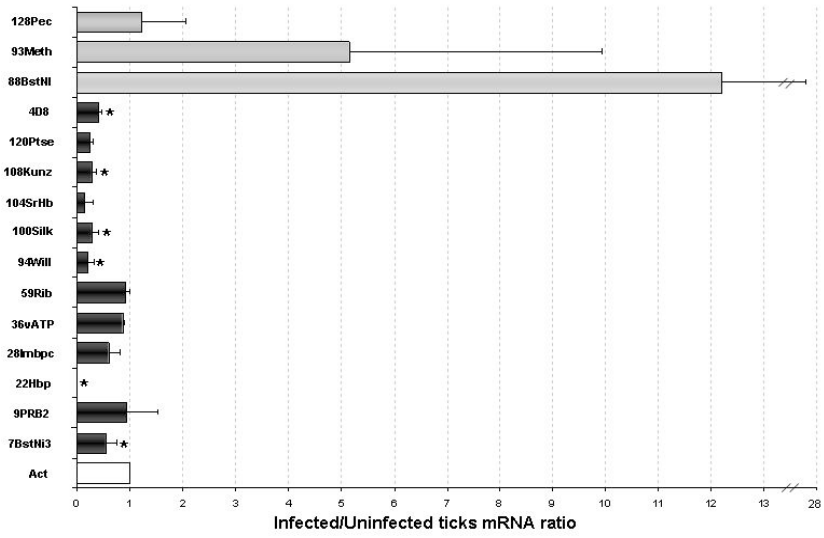

*Figure 2. Differential gene expression in A. marginale-infected R. microplus male salivary glands. Real-time RT-PCR was done on uninfected and infected pooled salivary glands (two independent experiments). Genes up-regulated (white bars) and down-regulated (black bars) in infected salivary glands are shown. Bars represent average + SD mRNA ratios. The mRNA levels were normalized against tick β-actin using the comparative Ct method. The mRNA levels were compared between infected and uninfected tick salivary glands by Students's t test (*p≤0.05). Gene IDs are described in Table 2.*

To evaluate the use of cultured BME26 cells for studying *A. marginale*-tick interactions, the same primers were used for real-time RT-PCR analysis of uninfected and *A. marginale*-infected BME26 tick cells. Twelve of the 14 selected genes were amplified from BME26 cultured cells. Gene expression profiles were studied for each gene at 6, 24 and 72 hours post-infection (hpi) and compared with uninfected cells collected at the same time points (Figure 3). Genes encoding for putative vacuolar H+-ATPase (36vATP) and ribosomal protein S29 (59Rib) were significantly up-regulated at 6 hpi, while putative Kunitz-like protease inhibitor precursor (108Kunz), metallothionein (93Meth) and von Willebrand factor (94Will) were significantly up-regulated 24 and 72 hpi. The mRNA levels for the rest of the genes were not statistically different between infected and uninfected BME26 cells. The subolesin (4D8) control was significantly downregulated by 24 hpi. All the tick sequence-derived primers were tested against bovine RNA by RT-PCR. Amplicons were not obtained for any of the primer pair tested (data not shown).

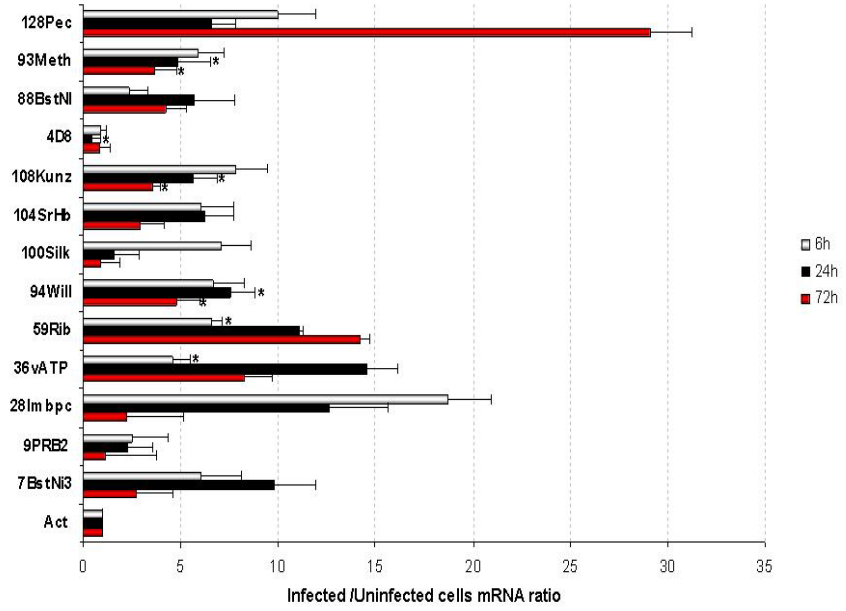

*Figure 3. Differential gene expression in A. marginale-infected cultured BME26 tick cells. Real-time RT-PCR was done on uninfected and infected BME26 cells collected at 6, 24 and 72 hpi (four independent cultures each). Bars represent average + SD mRNA ratios. The mRNA levels were normalized against tick β-actin using the comparative Ct method. The mRNA levels were compared between infected and uninfected tick cells by Students's t test (*p≤0.05). Gene IDs are described in Table 2.*

Functional roles of genes differentially expressed in R. microplus ticks and cultured BME26 cells in response to infection with A. marginale

The five genes corroborated by real-time RT-PCR to be differentially expressed in *A. marginale* infected tick salivary glands, female-specific histamine-binding protein (22Hbp), flagelliform silk protein (100Silk), Kunitz-like protease inhibitor precursor (108Kunz), proline-rich protein BstNI subfamily 3 precursor (7BstNI3) and von Willebrand factor (94Will) (Figure 2), were selected for functional analyses in ticks. The effect of gene knockdown on *A. marginale* infection and multiplication in *R. microplus* male tick salivary glands was evaluated by RNAi. The mRNA levels after RNAi were reduced for putative von Willebrand factor (94Will), flagelliform silk protein (100Silk) and subolesin (4D8), while silencing of the other genes did not result in statistically significant differences from the control (Table 3).

Table 3. *A. marginale* infection levels in tick salivary glands after RNAi and tick mortality rates in dsRNA-injected *R. microplus* males.

Experimental group	Gene expression silencing (%) ± SD[a]	*A. marginale* infection levels (% change with respect to controls)[b]	Mortality rate[c]
94Will	85.6±16.9	29.4±0.6 (-63%)	80 %**
100Silk	89.8±9.4	17.0±1.1 (-79%)*	74.3 %**
Subolesin (4D8)	82.7±12.7	32.0±1.6 (-60%)	68.6 %**
Control	--	80.0±1.9	57.1 %

*[a]Total RNA was extracted from infected tick salivary glands after RNAi and analyzed by real-time RT-PCR to determine gene expression silencing with respect to control ticks injected with the GIII dsRNA. [b]The A. marginale infection levels were analyzed by msp4 PCR, expressed as msp4 copies per tick ± SD and compared between test and control ticks injected with the GIII dsRNA by Student's t test (*p<0.05). [c]Tick mortality was evaluated as the ratio of dead male ticks 7 days after dsRNA injection to the total number of the attached male ticks, and was compared between test and control ticks injected with the GIII dsRNA by χ^2-test (**α < 0.025).*

The effect of RNAi of selected genes on male *B. microplus* mortality was determined. Tick mortality was significantly higher in groups injected with dsRNA for von Willebrand factor (94Will), flagelliform silk protein (100Silk) and subolesin when compared with the unrelated dsRNA-injected control ticks (Table 3). Despite the fact that individual variation in gene expression affected the statistical significance of results, silencing of the genes encoding for putative von Willebrand factor (94Will), flagelliform

silk protein (100Silk) and subolesin (4D8) resulted in 63%, 79% and 60% decrease in *A. marginale* infection levels in *R. microplus* male salivary glands, respectively (Table 3). Characterization of the effect of gene knockdown on *A. marginale* infections in cultured BME26 tick cells was attempted for all the genes which were shown to be differentially expressed in tick cells and/or tick salivary glands. However, mRNA levels were reduced only for putative ribosomal protein S29 (59Rib), metallothionein (93Meth), flagelliform silk protein (100Silk) and subolesin (4D8) genes (Table 4). Of these genes, two genes encoding for putative flagelliform silk protein (100Silk) and subolesin resulted in 12% and 17% reduction of *A. marginale* infection levels, respectively, when compared with controls (Table 4). The knockdown of the gene encoding for putative metallothionein (93Meth) resulted in higher *A. marginale* infection levels in tick cells (Table 4).

Table 4. *A. marginale* infection levels in cultured BME26 tick cells after RNAi.

Experimental group	**Silencing of gene expression (%) ± SD[a]**	***A. marginale* infection levels (% change with respect to controls)[b]**
59Rib	71.7±31.1	8.6±0.5 (-3%)
93Meth	65.0±14.4	18.0±0.0 (+102%)*
100Silk	99.5±0.3	7.8±0.6 (-12%)*
Subolesin (4D8)	88.1±7.1	7.4±0.3 (-17%)*
Control	--	8.9±1.4

*[a]Total RNA was extracted from infected tick cells after RNAi and analyzed by real-time RT-PCR to determine gene expression silencing with respect to control cells treated with buffer only. [b]The A. marginale infection levels were analyzed by msp4 PCR, expressed as msp4 DNA (ng) ± SD and compared between test and control ticks by Student's t test (*p<0.05).*

Discussion

In the present study we identified *R. microplus* male salivary gland genes differentially expressed in response to *A. marginale* infection by use of SSH and real-time RT-PCR. Development and multiplication of *A. marginale* in salivary gland cells involves molecular interactions between pathogen- and tick-derived molecules. Salivary gland, the tissue of interest in this study, is a critical site in the developmental cycle from where the pathogen is transmitted to cattle. Recently, tick salivary gland proteins were shown to play a role in the infection and transmission of *Borrelia burgdorferi* [17, 18], *A. phagocytophilum* [19] and *A. marginale* [20]. *A. marginale* membrane surface proteins involved in tick salivary gland colonization have been identified and partially characterized [21, 22]. Understanding the

molecular mechanisms of *A. marginale*-tick interactions for *R. microplus*, one of the most important vectors of *A. marginale* worldwide, is fundamental toward development of novel control measures [23].

Some of the genes identified by SSH, including those genes encoding for putative tick cement proteins, female specific histamine binding protein, IgG binding protein C, salivary gland-associated protein 64P, flagelliform silk protein and von Willebrand factor, were identified previously in different tick species and appear to be involved in tick feeding or pathogen infection [10, 24-26]. However, most differentially-expressed genes identified in this study have not been reported previously. Some cellular functions affected by *A. marginale* infection of *R. microplus,* such as cell structure and enzymatic processes, were reported previously in infected tick IDE8 cultured cells [11]. The discrepancy observed for some studied genes between SSH and real-time RT-PCR results may reflect differences between both methods for identifying differentially expressed genes or the presence of multiple sequences targeted during RT-PCR reactions that affect the results of mRNA quantification for some genes.

In a recent study, genes differentially expressed in cultured IDE8 tick cells in response to *A. marginale* infection were identified and functional studies conducted in *D. variabilis* suggested that these genes may play different roles during pathogen infection, development and trafficking from midguts to salivary glands [11]. Some of the genes identified by de la Fuente et al. [11] such as gluthathione S-tranferase, selenoprotein M and ferritin were also shown to be differentially expressed in *R. microplus* salivary glands in response to *A. marginale* infection. However, these genes were absent from the current EST dataset which could be due to differences in the system used for EST discovery (cultured IDE8 tick cells versus *R. microplus* salivary glands) and/or other factors such as tick species and/or *A. marginale* strain and infection levels.

While tick cell lines have been used successfully in *A. marginale* functional genomics studies [11], this is the first report of the use of the BME26 tick cell line derived originally from a natural vector of *A. marginale* for functional studies of tick-pathogen interactions. Since these studies were conducted on ticks and tick cells of the same species, most of the genes identified in tick salivary glands were also amplified from cultured BME26 tick cells. However, expression profiles of selected genes observed in cultured BME26 cells was not identical to that found in tick salivary glands. For example, the expression of the putative von Willebrand factor (94Will) was down-regulated in tick salivary glands but up-regulated in cultured BME26 tick cells infected with *A. marginale*.

These differences may have resulted from tissue-specific regulation of gene expression or because we only observed early stages of infection in the cultured BME26 tick cells (6-72 hpi). These studies demonstrated the utility of BME26 cells for the screening of tick genes expressed in response to *A. marginale* infection. However, as was reported previously [11], results of studies using cultured tick cells must be validated in naturally infected ticks. Interestingly, expression of putative vacuolar H+-ATPase (36vATP) was significantly up-regulated in *A. marginale*-infected cultured BME26 cells, as reported for previous gene expression studies of cultured IDE8 cells in response to *A. marginale* infection [11].

RNAi was used in this study to assign the effect of selected gene knockdown on *A. marginale* infection and multiplication in ticks. Although statistically significant for flagelliform silk protein (100Silk) only, results of RNAi experiments suggested that putative von Willebrand factor (94Will), flagelliform silk protein (100Silk) and subolesin could play a role in pathogen infection of *R. microplus* male salivary glands. RNAi experiments in cultured BME26 tick cells provided further evidence that flagelliform silk protein (100Silk) and subolesin may play a role in *A. marginale* infection and/or multiplication in tick cells and suggested that metallothionein (93Meth) may be involved tick defense against pathogen infection.

The flagelliform silk protein was identified previously in tick and orb weaving spider salivary glands but its function was not linked to pathogen infection [27-29]. Mulenga et al. [30] demonstrated that the flagelliform silk protein may be involved in tick attachment. In previous studies of *I. ricinus* after *B. burgdorferi* infection, the von Willebrand factor was isolated from tick salivary glands and shown to be up-regulated but its possible role in infection was not studied [10]. A von Willebrand factor-like motif is present in the major hemelipoglycoprotein found in ixodid ticks and this protein has been shown to play a role as a heme-sequestering factor during tick feeding [31]. Therefore, silencing of these genes may affect tick feeding, mortality and development of *A. marginale* in salivary glands. However, as shown previously for subolesin [20], gene expression studies in cultured BME26 tick cells have provided evidence that that the flagelliform silk protein may play a role in the infection of ticks with *A. marginale*.

The results for gene expression and silencing of subolesin in *R. microplus* male salivary glands and cultured BME26 cells infected with *A. marginale* reported herein are in agreement with previous studies in which subolesin knockdown reduced *A. marginale* infection in *D. variabilis* and cultured IDE8 cells [11, 20]. Subolesin, discovered as a tick protective antigen in *I. scapularis,* has been shown to be conserved in many tick species [32, 33]. Subolesin was shown by both RNAi gene knockdown and immunization trials

using the recombinant protein to protect vertebrate hosts against tick infestations, reduce tick survival and reproduction, and cause degeneration of gut, salivary gland, reproductive tissues and embryos [32-38]. Targeting of subolesin by RNAi or vaccination also decreased tick vector capacity for *A. marginale* and *A. phagocytophilum* [20]. In addition, subolesin was shown to function in the control of gene expression in ticks [39, 40] and to be differentially expressed in *Anaplasma*-infected ticks and cultures tick cells [11, 41]. However, subolesin expression in *R. microplus* tick salivary glands and cultured BME26 cells was different to previous reports showing up-regulation in *A. marginale*-infected *D. variabilis* salivary glands and cultured IDE8 cells [41]. These differences could be due to tick species-specific differences in gene regulation or to other factors such as pathogen strain and infection levels. Nonetheless, these results expanded our knowledge on the role of subolesin in tick-*Anaplasma* interactions.

Metallothioneins are a family of low molecular weight proteins with a high affinity for divalent metals that function in cell detoxification, apoptosis, stress response and immunity [42-44]. Metallothioneins control the cellular zinc ion levels, which are known to be important in the immune system, and their expression has been associated with protective response against pathogens [45-49]. The results suggested a role for tick metallothioneins in defense against bacterial infections. Interestingly, selenoproteins that regulate the levels of another important trace mineral in the organism were suggested to participate in the cellular response to limit *A. marginale* infection in tick cells [11].

As described herein, RNAi has proven to be an important tool for the characterization of the function of genes involved in tick-host-pathogen interactions and the transmission of tick-borne pathogens and for screening for tick protective antigens [11, 12, 20, 50]. Although dsRNA sequences used in this study do not contain any significant overlap with other known *R. microplus* genes, the possibility of off-target gene silencing effects cannot be excluded due to the limited amount of sequence data available. Availability of the complete *R. microplus* genome sequence data will facilitate screening for potential off-target effects. These can subsequently be minimized by avoiding the use of dsRNAs or siRNAs containing sequences which are present in multiple genes.

In our study *R. microplus* male salivary gland genes differentially expressed in response to *A. marginale* infection were identified by using SSH approach. Recently a *R. microplus* microarray (NimbleGen) has been developed and used for the analysis of acaricide- inducible genes in *R. microplus* [51]. Microarray chip hybridization could be an

alternative approach for identifying *R. microplus* differentially expressed genes in response to *A. marginale* infection.

Conclusions

In this study, *A. marginale* infection of *R. microplus* was shown to modulate in male salivary glands the expression of genes encoding for putative proteins involved in binding, catalytic/enzymatic activity, transport, DNA/RNA metabolism and structural molecules. Five genes encoding for putative histamine-binding protein (22Hbp), von Willebrand factor (94Will), flagelliform silk protein (100Silk), Kunitz-like protease inhibitor precursor (108Kunz) and proline-rich protein BstNI subfamily 3 precursor (7BstNI3) were confirmed by real-time RT-PCR to be down-regulated in tick salivary glands infected with *A. marginale*. Functional studies suggested that differentially expressed genes encoding for subolesin, putative von Willebrand factor and flagelliform silk protein could play a role in *A. marginale* infection and multiplication in ticks, while metallothioneins may play a role in defense against bacterial infections. Additionally, for the first time RNAi in cultured BME26 tick cells was used to study *A. marginale*-tick molecular interactions and suggested that subolesin and flagelliform silk protein may be required by *A. marginale* for infection and multiplication in these cultured cells. Collectively these data are important toward understanding the molecular mechanisms involved in *R. microplus*-*A. marginale* interactions.

Methods

Experiment design

A suppression subtractive hybridization (SSH) approach was used to identify genes differentially expressed in *R. microplus* male salivary gland genes in response to *A. marginale* infection. Sequences obtained by SSH were used to search for homology/identity to nucleotide and protein databases. Real-time RT-PCR was used to validate differential expression of selected genes in uninfected and *A. marginale*-infected *R. microplus* salivary glands. Differential expression patterns of selected genes were also studied in cultured BME26 cells at 6, 24 and 72 hpi by real-time RT-PCR. The genes that proved to have significantly different mRNA levels between uninfected and *A. marginale*-infected ticks or cultured BME26 tick cells were then selected for functional studies. RNAi was used to characterize the function of selected genes in *A. marginale* infection *in vivo* in *R. microplus* male ticks and *in vitro* in cultured BME26 tick cells.

Rhipicephalus microplus ticks

The *R. microplus* ticks used for construction of the SSH libraries originated from Mozambique and were provided by ClinVet International (Pty), Bloemfontein, South Africa. The *R. microplus* (Susceptible, CENAPA, Mexico strain) ticks used for the RNAi experiments were obtained from laboratory colonies maintained at the University of Tamaulipas, Mexico. Originally, these tick strains were collected from infested cattle in Tapalpa, Jalisco, Mexico. The ticks were maintained on cattle at the tick rearing facilities at the Utrecht Center for Tick-Borne Diseases, Utrecht University or the University of Tamaulipas. Larvae were kept off-host in an incubator at 20 ºC with 95% relative humidity and 12 hours light: 12 hours dark photoperiod. Cattle were cared for in both The Netherlands and Mexico in accordance with standards specified in the Guide for Care and Use of Laboratory Animals.

Tick cell line

The tick cell line BME26 was derived originally from embryos of *R. microplus* following the protocol established by Esteves et al. [52]. The cells were maintained in L-15B300 medium [53] supplemented with 5% heat-inactivated FBS (Gibco/Invitrogen, NY, USA), 10% TPB (Difco, Detroid, MI, USA), 0,1% bovine lipoprotein (ICN, Irvine, CA), 100 units ml^{-1} penicillin (Gibco/Invitrogen) and 100 μg ml^{-1} streptomycin (Gibco/Invitrogen) at pH 7.2. Cultures were grown at 34°C in 25 cm^2 plastic flasks (Nalge Nunc Int. Rochester, NY) containing 5 ml of the complete medium, which was changed weekly. Monolayers were subcultured when they reached a density of approximately 10^7 cells/ ml and approximately $8x10^5$ cells/ml were transferred to the new flask.

A. marginale isolates

The *A. marginale* isolate used for infection of *R. microplus* ticks and construction of SSH libraries was obtained from an infected calf in Texas, USA, in 1977. The isolate was subsequently passaged in splenectomized calves and blood samples were collected at the peak parasitemia (40%), prepared as a stabilates with 10 % DMSO and stored in 2 ml aliquots in liquid nitrogen. A Brazilian strain of *A. marginale* with an inclusion appendage (UFMG1) [54], which was recently established and propagated in IDE8 tick cells, was used to infect BME26 cells [14].

Uninfected and A. marginale-infected R. microplus male ticks for SSH libraries construction

To obtain *A. marginale*-infected *R. microplus* male ticks, eight month-old non-splenectomized and tick-naive Holstein-Friesian calf (No.4280) was infected intravenously with the Texas isolate of *A. marginale*. *R. microplus* larvae were then fed on the calf with ascending parasitemia. Approximately 200 partially fed male ticks were collected after 21 days of feeding and the presence of *A. marginale* infection in salivary glands was confirmed in 20 individually dissected tick salivary glands by *msp4* PCR [55]. Salivary glands from the remaining ticks were dissected in cold PBS, pooled and immediately stored in TriReagent (Sigma-Aldrich, Zwijndrecht, The Netherlands) at -80^{o}C. Uninfected ticks were fed in a similar way on an uninfected calf and the male tick salivary glands were dissected and stored as described previously. Cattle and tick feeding studies were conducted in accordance with approval of the Animal Experiments Committee (DEC) of the Faculty of Veterinary Medicine, Utrecht University (DEC No. 0604.0801).

Uninfected and A. marginale-infected cultured BME26 tick cells

The tick cell line BME26 was cultured as described above. Approximately 4x10^{6} cells from the passage 72 were plated in 24-well plates (Nunc) and maintained in *Anaplasma* medium [56] at 34ºC for 3 days. The cells were infected with the 15 day-old culture of *A. marginale* in BME26 cells. Five milliliters of this suspension were transferred to a plastic tube. The tube was immersed in liquid nitrogen for 5 min for cell disruption and rickettsia releasing, followed by thawing in a water bath at 37ºC. Approximately 500 µl of infected cell suspension was inoculated into each well onto uninfected BME26 monolayer. The plate was maintained at 34°C and cells harvested at 6, 24 and 72 hpi from 4 wells for each time point. Uninfected control cells (n = 4 wells) were cultured in the same way but with the addition of 500 µl of *Anaplasma* medium only and the cells were harvested at the same time points. Cells were centrifuged at 800 g for 10 min and RNA/DNA was isolated using Trizol reagent (Gibco/Invitrogen), as recommended by the manufacturer.The infection of the tick cells by *A. marginale* was corroborated by *msp4* PCR [55].

Suppression-subtractive hybridization

Total RNA was isolated from uninfected and *A. marginale*-infected tick salivary glands using Tri reagent (Sigma-Aldrich) following the manufacturer's protocol. RNA quality was checked by gel electrophoreses to confirm integrity of RNA preparations. Pools of 2 µg of total RNA were made from uninfected and *A. marginale*-infected salivary glands. The

cDNA synthesis was done using the Super SMART PCR cDNA synthesis kit (Clontech-Takara, Saint-Germain-en-Laye, France), a method for producing high quality cDNA from a low amount of starting material. The cDNA was then directly used for PCR select subtraction (Clontech-Takara) based on a technique previously described by Diatchenko at al. [15, 16] and SSH libraries were constructed according to manufacturer's instructions. The double stranded cDNA from both groups (infected and uninfected salivary glands) was *Rsa*I digested. Part of the digested cDNA was ligated with Adapter 1 and part with the Adapter 2R, and the rest was saved for use as a driver in preparation for hybridization. The forward subtracted library was made by hybridizing adapter ligated cDNA from *A. marginale*-infected tick salivary glands as the tester in the presence of uninfected tick salivary gland CDNA as the driver. This forward reaction library was designed to produce clones that are overexpressed or up-regulated in infected salivary glands. The reverse library was made in the same way but in this case the adapter ligated cDNA from uninfected tick salivary glands was used as the tester and infected salivary gland cDNA as the driver. The reverse reaction library was designed to produce clones underexpressed or down-regulated in infected salivary glands. In either case the driver cDNA was added in excess during each hybridization to remove common cDNAs by hybrid selection and leaving over expressed and novel tester cDNA to be recovered and cloned. Differentially expressed cDNAs were PCR amplified with Advantage PCR polymerase mix (Clontech-Takara), cloned using pGEM Easy T/A cloning kit (Promega, Madison, WI, USA), transformed into JM109 and plated on LB with ampicillin, X-gal and IPTG. Approximately 300 clones were randomly picked up from each plated library in 96- well plates containing LB medium supplemented with ampicillin and grown overnight. Plasmids were purified using Wizard SV 96 Plasmid DNA purification system (Promega). Plasmid inserts were PCR amplified and PCR products were fully sequenced in an accredited service laboratory (BaseClear, Leiden, The Netherlands) using vector specific primers.

Sequence analysis and database search

Partial sequences were obtained for 279 out of 576 randomly selected sequenced SSH library clones (288 from each forward- and reverse-subtracted libraries). The cDNA Annotation System software (CAS; Bioinformatics and Scientific IT Program (BSIP), Office of Technology Information Systems (OTIS), National Institute of Allergy and Infectious Diseases (NIAID), Bethesda, MD, USA) (http://exon.niaid.nih.gov) was used for automated sequence clean up, contig assembly, Blast analysis [57] against multiple

sequence databases (nonredundant sequence database and databases of tick-specific sequences http://www.vectorbase.org/index.php and BmGI2; http://compbio.dfci.harvard.edu/), identification and locating of signal peptide cleavage sites and gene ontology (GO) assignments. Genes for further analyses were annotated by manual curation.

Table 5. Real-time RT-PCR oligonucleotide primers and conditions.

EST	Upstream/downstream primer sequences (5'-3')	PCR annealing conditions
7BstNI3	AAACTGGGGAATCCAAAAGG	55ºC/30s
	GGGGTTTGGGATAGGGTTC	
9PRB2	AACGACCGCCCAAAAATAAC	55ºC/30s
	AATTTGTTCCGGTTTTGTTCC	
22Hbp	GGAGGTTACGAACTATGGGC	55ºC/30s
	ATGAGTTGGCAGTGCCTTAG	
28ImbpC	CGGTACCATGATGCACTTTG	55ºC/30s
	TGATGGCGTCCCTAGTTACC	
36vATP	GAAGGCTTCGAACAGAGTCG	55ºC/30s
	CTCAATTCTGGTGGCCAAG	
59Rib	CCAGCAAGCGAGATTGTGTA	55ºC/30s
	GCGTACTGTCTGAAGCAACG	
88BstNI	GTTTGGGGGCCTTAAGAAAA	55ºC/30s
	TTTTTCCCAAAAGGTTCTCC	
93Meth	CTGAACTGAACGCATCATGG	55ºC/30s
	GCACAACATTTTGCAGATGG	
94Will	TCATTGACGAAGAAGCGATC	55ºC/30s
	TACAAGTCGCCCTGACACC	
100Silk	TGAACCAGAGGGACCAACTC	55ºC/30s
	GTCTTGGACTCGGCAGTAGC	
104SrHb	CGAACCCGAATGGATTATG	55ºC/30s
	TTCAAACATGAAGCGACAGC	
108Kunz	ATGGAACTGTTCGGTTTTGC	55ºC/30s
	ATCCGCCGTAAATGAAGTTC	
120Ptse	GCGCGACCTCTTTGTTAAAC	55ºC/30s
	CGAATACGCACAGAAGGTGAC	
128Pec	AGGCCCAATTCTGATCTTTC	55ºC/30s
	CAAAGCTCAAACGTGTGGTG	
Subolesin	GAGACCAGCCCCTGTTCA	54ºC/30s
(4D8)	CTGTTCTGCGAGTTTGGTAGATAG	
Beta-actin	GACATCAAGGAGAAGCT(TC)TGC	55ºC/30s
	CGTTGCCGATGGTGAT(GC)	

Real-time RT- PCR analysis

The same RNA samples prepared before for SSH from uninfected and *A. marginale*-infected tick salivary glands were used for real-time RT-PCR. Total RNA was extracted as described above from uninfected and *A. marginale*-infected cultured BME26 tick cells from quadruplicate cultures at 6, 24 and 72 hpi and used for real-time RT-PCR. Oligonucleotide primers were synthesized based on the sequences determined for candidate differentially expressed genes (Table 5) and used in 25 µl RT-PCR reactions performed using the iScript one step RT-PCR kit with SYBR green (Bio-Rad, Hercules, CA,USA) and a Bio-Rad iQ5 thermal cycler following the manufacturer's protocol. The mRNA levels were normalized against tick β-actin (Genbank accession number AY255624) using the comparative Ct method [58]. The mRNA levels were compared between infected and uninfected tick salivary glands and tick cells by Student's *t* test (p=0.05). Total RNA was isolated from bovine blood and RT-PCR reactions were performed using the oligonucleotide primers and conditions described in Table 5. PCR products were electrophoretically separated using 1.5 % agarose gel stained with ethidium bromide for visualization.

RNA interference in ticks

Oligonucleotide primers containing T7 promoter sequences at the 5'-end were synthesized for *in vitro* transcription of dsRNA using the Acess RT-PCR system (Promega, Madison, WI, USA) and the Megascript RNAi kit (Ambion, Austin, TX, USA) following manufacturer's instructions. The dsRNA was purified and quantified by spectophotometry. Newly molted uninfected *R. microplus* males, were injected with approximately 0.3 µl of dsRNA (5 x 10^{10} molecules/µl) in the lower right quadrant of the ventral surface of the exoskeleton of the tick [20]. Ticks (35/group) were injected using a Hamilton syringe with a 1 inch, 33 gauge needle. Control ticks were injected with *R. microplus* subolesin dsRNA (positive control) or the unrelated GIII dsRNA (negative control). The GIII sequence was identified in *R. microplus* and did not affect tick feeding, mortality and oviposition after RNAi (unpublished results). Ticks were held in a humidity chamber for 3-5 hours and mortality was recorded before the living ticks were allowed to feed in seven separate patches (five test genes and two controls), each one for a different group, glued on the back of a calf naturally infected with *A. marginale* in Tamaulipas, Mexico (approximately 4% rickettsemia during tick feeding). Ten females were placed in each patch simultaneously with injected males. Unattached ticks were removed 2 days after infestation and attached ticks were allowed to feed for 7 days. All the males were collected and salivary glands were

dissected from individual ticks from each group. The salivary glands were stored in RNAlater (Qiagen) until used for DNA and RNA extraction to determine *A. marginale* infection levels by quantitative *msp4* PCR [55] and to confirm gene knockdown by real-time RT-PCR. The mRNA levels of the target gene and the *A. marginale* infection were compared between test and control ticks by Student's *t*-test (p=0.05). Tick mortality was evaluated as the ratio of the dead male ticks 7 days after dsRNA injection to the total number of attached male ticks feeding on the animal and was compared between test and control groups by χ^2-test (α=0.025).

RNA interference in cultured BME26 tick cells

Approximately $5x10^5$ BME26 cells/well were placed in 24-well plates (Nunc). The dsRNA prepared in the way described above for each of the 8 target genes was added to the culture wells (n = 4 for each treatment). Subolesin dsRNA was used as a positive control and control wells received elution buffer only. Each of the treated wells received 10 µl of dsRNA ($5x10^{10}$ molecules per microliter) and 190 µl of L15B *Anaplasma* medium and was incubated for 24 h. After this period, additional 300 µl of medium were added to each well. After 48 h cells were infected as described above. Three days post infection the cells were harvested from the plate, centrifuged at 800 x g for 10 min and used to extract RNA and DNA with TriReagent (Sigma). *A. marginale* infection levels were determined by *msp4* PCR [55]. Gene expression silencing was confirmed by real-time RT-PCR using sequence-specific primers (Table 3) as described above.

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Supplementary tables

Table 1. Genes identified by SSH as differentially expressed in *A. marginale*-infected *R. microplus* male salivary glands.

GenBank Acc. No.	Best match to non-repetitive protein database and the *R. microplus* BmGI2 database	E-value	Molecular function
Up-regulated in infected salivary glands			
GO496166	metallothionein - *Oscillatoria brevis* (BAC76027.1); b_microplus|TC23383	$1^{x}e^{+00}$	binding
GO496167	Von Willebrand factor - *Ixodes ricinus* (AAQ01562.1)	$2^{x}e^{-04}$	binding
GO496168	salivary proline-rich protein - *Homo sapiens* (CAA30394.2)	$1^{x}e^{-14}$	binding
GO496169	proline-rich protein BstNI subfamily 1 isoform 1 precursor - *Homo sapiens* (NP_005030.2)	$4^{x}e^{-25}$	binding
GO496170	hydroxyproline-rich glycoprotein DZ-HRGP - *Volvox carteri f. nagariensis* (CAB62280.1)	$4^{x}e^{-23}$	binding
GO496171	hypothetical protein isoform 2 - *Pan troglodytes* (XP_001151718.1)	$2^{x}e^{-25}$	binding
GO496172	putative glycine-rich protein - *Arabidopsis thaliana* (CAA22153.1); b_microplus|U92761	$2^{x}e^{-46}$	binding
GO496173	vacuolar ATP synthase subunit G-like protein - *Nasonia vitripennis* (XP_001600097.1); b_microplus|TC24424	$1^{x}e^{-19}$	binding
GO496174	predicted protein - *Coprinopsis cinerea okayama* (EAU88308.1); b_microplus|TC17118	$6^{x}e^{-03}$	binding
GO496175	shematrin-4 - *Pinctada fucata* (BAE93436.1); b_microplus|CK184579	$2^{x}e^{-28}$	structural molecule activity
GO496176	putative cement protein RIM36 - *Rhipicephalus appendiculatus* (AAK98794.1); b_microplus|U92790	$1^{x}e^{-130}$	structural molecule activity
GO496177	hypothetical protein PGUG_00176 - *Pichia guilliermondii* ATCC 6260 (XP_001486799.1)	$2^{x}e^{-04}$	structural molecule activity
GO496178	oxidoreductase domain protein - *Enterobacter sp.* 638 (YP_001175795.1); b_microplus|TC17531	$3^{x}e^{+0}$	catalytic activity
GO496179	cytochrome c oxidase subunit III - *Rhipicephalus sanguineus* (NP_008515.1); b_microplus|TC17087	$5^{x}e^{-38}$	catalytic activity
GO496180	AC002304_31 F14J16.10 - *Arabidopsis thaliana* (AAF79338.1); b_microplus|TC22130	$3^{x}e^{-01}$	catalytic activity
GO496181	enhancer of Polycomb CG7776-PA, isoform A – *Apis mellifera* (XP_397232.1)	$6^{x}e^{-79}$	transcription regulatory activity
GO496182	hypothetical protein OsI_035683 - *Oryza sativa* (EAY81724.1); b_microplus|TC22921	$6^{x}e^{-04}$	transcription regulatory activity
GO496183	Predicted gene, EG381818 - *Mus musculus* (AAH69935.1); b_microplus|CV449693	$1^{x}e^{-12}$	molecular function unknown
GO496184	putative secreted protein - *Ixodes scapularis* (AAY66614.1); b_microplus|CV440197	$3^{x}e^{-21}$	molecular function unknown
GO496185	hypothetical protein DDBDRAFT_0190925 - *Dictyostelium discoideum* AX4 (XP_646663.1); b_microplus|TC22779	$1.3^{x}e^{-01}$	molecular function unknown
GO496186	hypothetical protein MAL7P1.142 - *Plasmodium falciparum* 3D7 (XP_001349158.1); b_microplus|TC24862	$2^{x}e^{-20}$	molecular function unknown
GO496187	putative secreted protein - *Ixodes scapularis* (AAY66614.1); b_microplus|CV440198	$4^{x}e^{-03}$	molecular function unknown

GO496188	hypothetical protein - *Arabidopsis thaliana* (AAC17072.1)	$3^{x}e^{-01}$	molecular function unknown
GO496189	microneme-rhoptry antigen - *Theileria annulata* strain Ankara (XP_953099.1)	$5^{x}e^{-05}$	molecular function unknown
GO496190	putative secreted protein - *Ixodes scapularis* (AAY66614.1)	$7^{x}e^{-03}$	molecular function unknown
GO496191	hypothetical protein - *Thermobia domestica* (CAM36311.1)	$8^{x}e^{-06}$	molecular function unknown
GO496192	Putative lipoprotein - *Burkholderia xenovorans* LB400 (YP_560200.1); b_microplus|TC19098	$6^{x}e^{-16}$	molecular function unknown
GO496193	hypothetical protein Kpol_464p3 - *Vanderwaltozyma polyspora* DSM 70294 (XP_001642230.1); b_microplus|CV440198	$3^{x}e^{-01}$	molecular function unknown
GO496194	hypothetical protein - *Thermobia domestica* (CAM36311.1); b_microplus|TC20527	$1^{x}e^{-05}$	molecular function unknown
GO496195	unnamed protein product - *Tetraodon nigroviridis* (CAG11595.1) b_microplus|TC16479	$7.5^{x}e^{-01}$	molecular function unknown
GO496196	hypothetical protein - *Magnetospirillum gryphiswaldense* MSR-1 (CAM73970.1)	$2.3^{x}e^{-02}$	molecular function unknown
GO496197	putative secreted salivary gland peptide - *Ixodes scapularis* (AAV80791.1); b_microplus|TC21841	$2^{x}e^{-34}$	molecular function unknown
GO496198	AGAP009763-PA - *Anopheles gambiae* str. PEST (EAA14477.4); b_microplus|TC21159	$2.7^{x}e^{-02}$	molecular function unknown
GO496199	MGC79481 protein - *Xenopus tropicalis* (NP_001005003.1)	$1.7^{x}e^{-01}$	molecular function unknown
GO496200	hypothetical protein - *Equus caballus* (XP_001494787.1); b_microplus|CK172681	$9^{x}e^{-03}$	molecular function unknown
GO496201	salivary mucin with chitin-binding domain - *Argas monolakensis* (ABI52758.1)	$1^{x}e^{-07}$	molecular function unknown
GO496202	A Chain A, Structure Of Iodinated Cbm25 Amylase - *Bacillus halodurans* (82408236); b_microplus|TC21438	$6.2^{x}e^{-01}$	molecular function unknown
GO496203	hypothetical protein - *Pan troglodytes* (XP_001151054.1); b_microplus|CK178776	$1.7^{x}e^{-01}$	molecular function unknown
GO496204	predicted protein - *Aspergillus terreus* NIH2624 (XP_001213801.1)	$1.8^{x}e^{-01}$	molecular function unknown
GO496205	conserved hypothetical protein - *Beggiatoa sp.* SS (ZP_01998230.1)	$6^{x}e^{-12}$	molecular function unknown
GO496206	salivary secreted basic tail protein - *Ornithodoros parkeri* (ABR23390.1); b_microplus|TC19863	$2^{x}e^{-10}$	molecular function unknown
Down-regulated in infected salivary glands			
GO496207	Female-specific histamine-binding protein 1 precursor - *Rhipicephalus appendiculatus* (O77420); b_microplus|TC18188	$1^{x}e^{-23}$	binding
GO496208	immunoglobulin G binding protein C - *Rhipicephalus appendiculatus* (AAB68803.1); b_microplus|TC20577	$1^{x}e^{-81}$	binding
GO496209	serotonin and histamine binding protein - *Dermacentor reticulatus* (AAL56644.1)	$5^{x}e^{-12}$	binding
GO496210	Kunitz-like protease inhibitor precursor - *Ancylostoma caninum* (AAN10061.1); b_microplus|TC20102	$6^{x}e^{-22}$	binding
GO496211	Hypothetical proteinT20B6.3 - *Caenorhabditis elegans* (NP_497637.1); b_microplus|TC23476	$1^{x}e^{-10}$	binding
GO496212	GR-RBP3 (glycine-rich RNA-binding protein 3) - *Arabidopsis thaliana* (NP_200911.1); b_microplus|TC20164	$1^{x}e^{-07}$	binding
GO496213	proline-rich protein BstNI subfamily 3 precursor - *Homo sapiens* (NP_006240.4)	$2^{x}e^{-20}$	binding

GO496214	PRB2_HUMAN Basic salivary proline-rich protein - *Homo sapiens* (P02812)	$6^{x}e^{-24}$	binding
GO496215	plus agglutinin - *Chlamydomonas incerta* (AAX33674.1); b_microplus|TC15758	$2^{x}e^{-15}$	binding
GO496216	minus agglutinin - *Chlamydomonas incerta* (AAW51128.1); b_microplus|TC19098	$4^{x}e^{-05}$	binding
GO496217	keratin associated protein 19-3 - *Homo sapiens* (EAX09897.1); b_microplus|TC21957	$7^{x}e^{-01}$	structural molecule activity
GO496218	CG6004-PB - *Drosophila melanogaster* (NP_648504.2); b_microplus|CV452616	$9^{x}e^{-06}$	structural molecule activity
GO496219	flagelliform silk protein - *Nephila madagascariensis* (AAF36091.1); b_microplus|TC23771	$1^{x}e^{-22}$	structural molecule activity
GO496220	RS29_IXOSC 40S ribosomal protein S29 ribosomal protein S29 - *Ixodes scapularis* (Q4PM47)	$3^{x}e^{-28}$	structural molecule activity
GO496221	Basic proline-rich protein - *Sus scrofa* (AAK61382.1)	$1^{x}e^{-21}$	structural molecule activity
GO496222	cement-like antigen – *Haemaphysalis longicornis* (BAF35848.1); b_microplus|TC1909	$2^{x}e^{-07}$	structural molecule activity
GO496223	Putative cement protein RIM36 - *Rhipicephalus appendiculatus* (AAK98794.1); b_microplus|TC17863	$1^{x}e^{-139}$	structural molecule activity
GO496224	Putative cement protein RIM36 - *Rhipicephalus appendiculatus* (AAK98794.1)	$1^{x}e^{-145}$	structural molecule activity
GO496225	Putative cement protein RIM36 - *Rhipicephalus appendiculatus* (AAK98794.1)	$1^{x}e^{-151}$	structural molecule activity
GO496226	salivary gland-associated protein 64P - *Rhipicephalus appendiculatus* (AAM09648.1); b_microplus|TC18640	$9^{x}e^{-48}$	structural molecule activity
GO496227	putative cement protein RIM36 - *Rhipicephalus appendiculatus* (AAK98794.1) ; b_microplus|TC24800	$2^{x}e^{-39}$	structural molecule activity
GO496228	PRB1L precursor protein - *Homo sapiens* (AAB27289.1)	$4^{x}e^{-13}$	catalytic activity
GO496229	dipeptidyl peptidase - *Nasonia vitripennis* (XP_001601820.1); b_microplus|TC20123	$5^{x}e^{-07}$	catalytic activity
GO496230	matrix metalloproteinase 1 (interstitial collagenase) - *Xenopus laevis* (NP_001080518.1)	$7^{x}e^{-01}$	catalytic activity
GO496231	Salivary gland metalloprotease - *Boophilus microplus* (AAZ39660.1); b_microplus|TC18456	$1^{x}e^{-50}$	catalytic activity
GO496232	26kDa protease - *Sarcophaga peregrine* (BAA22400.1) b_microplus|TC17022	$1^{x}e^{-17}$	catalytic activity
GO496233	invertase/pectin methylesterase inhibitor family protein - *Arabidopsis thaliana* (NP_176463.2); b_microplus|TC16199	$1^{x}e^{-01}$	enzyme regulatory activity
GO496234	homeobox B1 - *Mus musculus* (NP_032292.2); b_microplus|TC17118	$1^{x}e^{-01}$	transporter activity
GO496235	mannose dehydratase, NAD(P)-binding - *Frankia aln* (YP_712286.1); b_microplus|TC22512	$3^{x}e^{-04}$	transporter activity
GO496236	surface-erythrocyte phosphoprotein - *Babesia rossi* (CAD10043.1) ; b_microplus|TC23771	$5^{x}e^{-01}$	transporter activity
GO496237	conserved hypothetical protein - *Trichomonas vaginalis* G3 (XP_001318272.1); b_microplus|TC21871	$4^{x}e^{-08}$	molecular function unknown
GO496238	hypothetical protein FRAAL1158 - *Frankia alni* ACN14a (YP_712532.1); b_microplus|TC21414	$1^{x}e^{-04}$	molecular function unknown

GO496239	hypothetical protein EhV364 - *Emiliania huxleyi* virus 86 (YP_294122.1)	$9^{*}e^{-23}$	molecular function unknown
GO496240	cell surface SD repeat antigen precursor, putative - *Streptococcus sanguinis* SK36 (YP_001035905.1); b_microplus|TC16475	$3^{*}e^{-06}$	molecular function unknown
GO496241	hypothetical protein -*Strongylocentrotus purpuratus* (XP_783923.2) b_microplus|CK176028	$5^{*}e^{-06}$	molecular function unknown
GO496242	SJCHGC03140 protein - *Schistosoma japonicum* (AAX26495.2)	$4.7^{*}e^{-01}$	molecular function unknown
GO496243	hypothetical protein; putative membrane protein - *Frankia alni* ACN14a (YP_712532.1); b_microplus|TC17022	$1.1^{*}e^{+00}$	molecular function unknown
GO496244	SET domain containing 1B -*Homo sapiens* (XP_946855.2); b_microplus|TC20104	$1.5^{*}e^{-01}$	molecular function unknown
GO496245	hypothetical protein GLP_609_40294_39134 -*Giardia lamblia* (XP_770752.1); b_microplus|TC19098	$9.3^{*}e^{-01}$	molecular function unknown
GO496246	hCG1793893 - *Homo sapiens* (EAX07174.1)	$5^{*}e^{-24}$	molecular function unknown
GO496247	hypothetical protein -*Cryptococcus neoformans var. neoformans* JEC21 (XP_569355.1); b_microplus|TC21386	$3.3^{*}e^{+00}$	molecular function unknown
GO496248	hypothetical protein TTHERM_00849310 -*Tetrahymena thermophila* SB210 (XP_001019233.1)	$5.5^{*}e^{-01}$	molecular function unknown
GO496249	unnamed protein product -*Candida glabrata* (XP_448999.1); b_microplus|TC21702	$4^{*}e^{-03}$	molecular function unknown
GO496250	hypothetical protein OsJ_010161 - *Oryza sativa* (EAZ26678.1); b_microplus|TC16663	$3^{*}e^{-12}$	molecular function unknown
GO496251	hypothetical protein - *Rattus norvegicus* (XP_001054782.1); b_microplus|TC17460	$2^{*}e^{-09}$	molecular function unknown
GO496252	hypothetical protein - *Monodelphis domestica* (XP_001376188.1)	$2^{*}e^{-05}$	molecular function unknown
GO496253	PRB3 protein - *Homo sapiens* (AAH96211.1); b_microplus|TC20839	$4^{*}e^{-06}$	molecular function unknown
GO496254	predicted protein - *Nematostella vectensis* (XP_001638632.1)	$2^{*}e^{-01}$	molecular function unknown
GO496255	unnamed protein product - *Tetraodon nigroviridis* (CAF94182.1)	$1.5^{*}e^{+00}$	molecular function unknown
GO496256	predicted protein - *Sclerotinia sclerotiorum* (XP_001596482.1)	$2.5^{*}e^{+00}$	molecular function unknown
GO496257	Period gamma - *Apis cerana* (BAD06463.1)	$1.4^{*}e^{+00}$	molecular function unknown
GO496258	hypothetical protein - *Haemaphysalis longicornis* (BAE02553.1); b_microplus|TC21071	$1^{*}e^{-10}$	molecular function unknown
GO496259	hypothetical protein 3 - *Microplitis demolitor bracovirus* (YP_239367.1); b_microplus|TC16665	$1^{*}e^{-03}$	molecular function unknown
GO496260	20/24 kDa immunodominant saliva protein - *Rhipicephalus appendiculatus* (AAO60049.1); b_microplus|CV444142	$7^{*}e^{-08}$	molecular function unknown
GO496261	unnamed protein product - *Homo sapiens* (BAC86958.1); b_microplus|TC15758	$1.4^{*}e^{-02}$	molecular function unknown
GO496262	hypothetical protein PC104124.00.0-*Plasmodium chabaudi chabaudi* (XP_743423.1)	$3^{*}e^{-01}$	molecular function unknown

Table 2. Differentially expressed genes selected based on their putative role during *A. marginale* infection to validate SSH results by real-time RT-PCR.

EST	Sequence identity	Up- or Down-regulated in infected salivary glands	Short description
7BstNI3	*Homo sapiens* (NP_006240.4)	down	proline-rich protein BstNI subfamily 3 precursor
9PRB2	*Homo sapiens* (P02812)	down	PRB2_HUMAN Basic salivary proline-rich protein
22Hbp	*Rhipicephalus appendiculatus* (077420)	down	female-specific histamine-binding protein 1 precursor
28ImbpC	*R. appendiculatus* (AAB68803.1)	down	immunoglobulin G binding protein C
59Rib	*Ixodes scapularis* (Q4PM47)	down	ribosomal protein S29 mRNA
100Silk	*Nephila madagascariensis* (AAF36091.1)	down	flagelliform silk protein
104SrHb	*Dermacentor reticulatus* (AAL566441)	down	serotonin and histamine binding protein
108Kunz	*Ancylostoma caninum* (AAN10061.1)	down	Kunitz-like protease inhibitor precursor
120Ptse	*Sarcophaga peregrine* (BAA22400.1)	down	26kDa protease
128PecIn	*Arabidopsis thaliana* (NP_176463.2)	down	invertase/pectin methylesterase inhibitor family protein
36vATP	*Aedes aegypti* (XP_001600097.1)	up	vacuolar H+-ATPase V1 sector subunit
88BstNI	*Homo sapiens* (NP_005030.2)	up	proline-rich protein BstNI subfamily 1 isoform 1
93Meth	*Oscillatoria brevis* (BAC76027.1)	up	metallothionein
94Will	*Ixodes ricinus* (AAQ01562.1)	up	von Willebrand factor mRNA

Chapter 5

Silencing of genes involved in *Anaplasma marginale*-tick interactions affects the pathogen developmental cycle in *Dermacentor variabilis*

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Abstract

Background

The cattle pathogen, *Anaplasma marginale*, undergoes a developmental cycle in ticks that begins in gut cells. Transmission to cattle occurs from salivary glands during a second tick feeding. At each site of development two forms of *A. marginale* (reticulated and dense) occur within a parasitophorous vacuole in the host cell cytoplasm. However, the role of tick genes in pathogen development is unknown. Four genes, found in previous studies to be differentially expressed in *Dermacentor variabilis* ticks in response to infection with *A. marginale,* were silenced by RNA interference (RNAi) to determine the effect of silencing on the *A. marginale* developmental cycle. These four genes encoded for putative glutathione S-transferase (GST), salivary selenoprotein M (SelM), H+ transporting lysosomal vacuolar proton pump (vATPase) and subolesin.

Results

The impact of gene knockdown on *A. marginale* tick infections, both after acquiring infection and after a second transmission feeding, was determined and studied by light microscopy. Silencing of these genes had a different impact on *A. marginale* development in different tick tissues by affecting infection levels, the densities of colonies containing reticulated or dense forms and tissue morphology. Salivary gland infections were not seen in any of the gene-silenced ticks, raising the question of whether these ticks were able to transmit the pathogen.

Conclusions

The results of this RNAi and light microscopic analyses of tick tissues infected with *A. marginale* after the silencing of genes functionally important for pathogen development suggest a role for these molecules during pathogen life cycle in ticks.

Background

Ticks transmit pathogens that impact both human and animal health [1]. Of these tick-borne pathogens, those belonging to the genus *Anaplasma* (Rickettsiales: Anaplasmataceae) are obligate intracellular organisms found exclusively within parasitophorous vacuoles in the cytoplasm of both vertebrate and tick host cells [2]. The type species, *A. marginale*, causes the economically important cattle disease bovine anaplasmosis [2]. In the United States, *A. marginale* is vectored by *Dermacentor variabilis, D. andersoni*, and *D. albipictus* [2,3].

The life cycle of *A. marginale* in the tick vector is complex and coordinated with tick feeding cycle [4-6]. Bovine erythrocytes infected with *A. marginale* are ingested by ticks in the bloodmeal and the first site of infection in ticks is gut and Malpighian tubule cells. *A. marginale* then infects and develops in salivary glands, the site of transmission to the vertebrate host. Gut muscle and fat body cells may also become infected with *A. marginale* during tick feeding.

Two stages of *A. marginale* occur within a parasitophorous vacuole in the tick cell cytoplasm. The first form of *A. marginale* seen within colonies is the reticulated (vegetative) form (RF) that divides by binary fission and results in formation of large colonies that may contain hundreds of organisms. The RFs then transform into the dense form (DF) which can survive for a short time outside of cells and is the infective form. This developmental cycle occurs at every site of *A. marginale* development in ticks.

The evolution of ticks and the pathogens that they transmit have co-evolved molecular interactions that mediate their development and survival [7], and these interactions involve genetic traits of both the tick and the pathogen. Recently, a functional genomics approach was used to discover genes/proteins that are differentially expressed in tick cells in response to infection with *A. marginale* [7]. In these studies, 4 genes found to be downregulated after RNA interference (RNAi) affected *A. marginale* infection levels in *D. variabilis* guts and/or salivary glands. These four genes encoded for putative glutathione S transferase (GST), salivary selenoprotein M (SelM), H+ transporting lysosomal vacuolar proton pump (vATPase) and subolesin. The results of these experiments further confirmed that a molecular mechanism occurs by which tick cell gene expression mediates the *A. marginale* developmental cycle and trafficking through ticks [7].

In this study, we characterized the effect of silencing GST, SelM, vATPase and subolesin genes by RNAi on *A. marginale* development and infection levels in *D. variabilis* by quantitative PCR and light microscopy. The analysis was conducted in ticks after acquisition feeding (AF) and transmission feeding (TF) to characterize the effect on gene

expression during pathogen trafficking from guts during AF to salivary glands and other tissues after TF [4,5]. The results demonstrated that gene knockdown reduced the number of RF- and DF-containing colonies in various tick tissues with implications for pathogen replication, development and transmission in ticks, and suggested that these genes may be good targets for development of a new generation of pathogen transmission-blocking vaccines for control of bovine anaplasmosis directed toward reducing exposure of vertebrate hosts to *A. marginale.*

Results

Confirmation of RNAi of tick genes and A. marginale infection levels in ticks

The effect of RNAi on GST, SelM and subolesin gene silencing was confirmed in ticks after AF and TF (Table 1).

Table 1. Expression silencing of selected genes in *D. variabilis* male guts and salivary glands after RNAi.

Experimental group	Silencing in guts after AF (%± SD)	Silencing in guts after TF (% ± SD)	Silencing in salivary glands after TF (% ± SD)
GST	81.2.6±12.4*	100±0.0*	100±0.4*
vATPse	ND	ND	ND
SelM	74.1±17.3*	100±0.0*	74.0±25.2*
Subolesin	90.0±21.4*	99.7±0.6*	99.4 ± 0.0*

*The silencing of gene expression was analyzed in D. variabilis male ticks after RNAi. Salivary glands and/or guts were dissected from 5 ticks after AF and TF and mRNA levels were analyzed by real time RT-PCR. Percent reduction in transcript levels relative to that in tissues from control ticks were averaged over five replicate samples and expressed as average ± SD. Significance was determined by comparing mRNA levels between dsRNA and saline injected control ticks by Student's t-test (*P≤0.05). Abbreviation: ND, not done because RT-PCR conditions could not be established.*

Silencing the expression of genes encoding for putative GST, vATPase, SelM and subolesin resulted in statistically significant differences in the *A. marginale* infection levels in guts and/or salivary glands when compared to saline-injected controls (Table 2). In ticks in which the expression of putative GST was silenced, *A. marginale* infection was inhibited both in tick guts after AF and in salivary glands after TF. When putative vATPase dsRNA was injected, *A. marginale* infection was inhibited in tick guts after AF but the pathogens were still able to infect and multiply in the salivary glands after TF. The RNAi of salivary

SelM expression resulted in the inhibition of pathogen infection and/or multiplication in tick salivary glands after TF. As reported previously [8], subolesin RNAi affected *A. marginale* infection of tick salivary glands after TF. In all cases, infection levels were not affected in guts after TF (Table 2).

Table 2. *A. marginale* infection levels in *D. variabilis* male guts and salivary glands after RNAi of selected tick genes.

Experimental group	Infection levels in guts after AF (*A. marginale*/tick ± SE)	Infection levels in guts after TF (*A. marginale*/tick ± SE)	Infection levels in salivary glands after TF (*A. marginale*/tick ± SE)
GST	5 ± 15*	99,060 ± 68462	2 ± 0*
vATPse	81 ± 5*	795 ± 227	247 ± 205
SelM	389,095 ± 282048	1,451 ± 443	2 ± 0*
Subolesin	814 ± 122	1,517 ± 1025	2 ± 0*
Saline control	40,579 ± 6993	28,252 ± 27788	287 ± 144

*The A. marginale infection levels were analyzed in D. variabilis male ticks after RNAi. Salivary glands and/or guts were dissected from 5 ticks after AF and TF and DNA was used for quantitative msp4 PCR to determine A. marginale infection levels. Infection levels in tick guts and salivary glands were expressed as average ± SE and compared between dsRNA and saline injected ticks by Student's t-test (*P≤0.05).*

Light microscopy analysis of A. marginale colonies in tick tissues

Colonies of *A. marginale* containing RFs or DFs were easily recognizable with light microscopy (Fig. 1) The main site of *A. marginale* infection in ticks after AF was in the gut and Malpighian tubule cells (Figs. 1 and 2), while after TF colonies were also seen in gut muscle, salivary gland acinar and fat body cells (Fig. 2). Quantitative analysis of *A. marginale* colonies in dsRNA-injected ticks demonstrated that gene knockdown by RNAi significantly reduced the density of RFs in the gut of subolesin dsRNA-injected ticks after both AF and TF when compared to controls (Table 3). The density of DFs after AF was significantly lower in guts of ticks injected with GST dsRNA and in guts of ticks injected with subolesin dsRNA after TF (Table 3). In contrast, the density of RFs was significantly higher in guts of ticks injected with SelM dsRNA after AF and TF and the density of DFs was significantly higher in vATPase dsRNA-injected ticks after TF (Table 3). In all silenced ticks, *A. marginale* colonies were not seen in salivary glands after AF or TF; infection of salivary glands was seen only in the control ticks after TF (Table 3).

Overall, the qualitative analysis of *A. marginale* in *D. variabilis* silenced ticks resulted in the reduction of colonies in gut muscle, Malpighian tubule and fat body cells

after TF as compared to the controls (Table 4). Only subolesin dsRNA-injected ticks showed a reduction of *A. marginale* colonies in the malpighian tubules after AF (Table 4). Interestingly, an increase in the number of fat body colonies was seen in GST dsRNA injected ticks after TF, suggesting that the silencing of this gene enhanced infection of fat body cells and thus represented a shift in the *A. marginale* tick developmental cycle (Table 4).

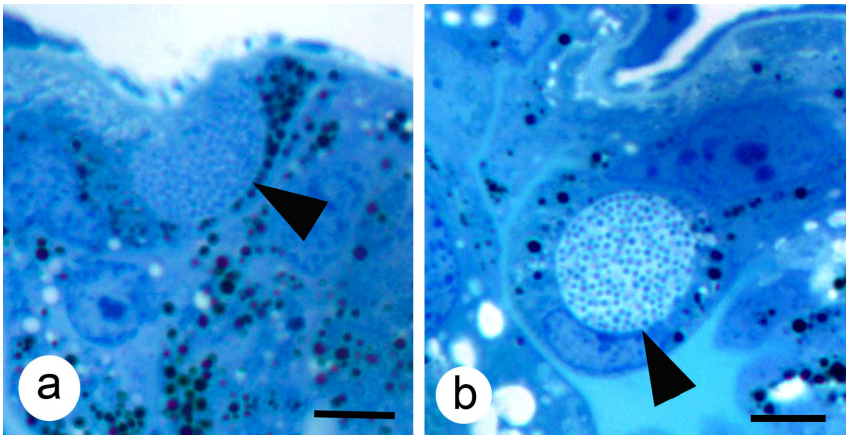

Figure 1. Light photomicrographs of colonies of A. marginale in the gut of male D. variabilis. (a) A colony containing reticulated forms (arrow) of A. marginale and (b) a gut cell containing a colony with dense forms (arrow). Mallory's stain, Bar = 5 µm..

Compared with the controls, differences in tissue degeneration were observed in the salivary glands, testis and/or guts of vATPase and subolesin dsRNA-injected ticks after AF and TF (Table 4; Fig. 3). In ticks with SelM knockdown, fat body degeneration was observed after TF (Table 4; Fig 3).

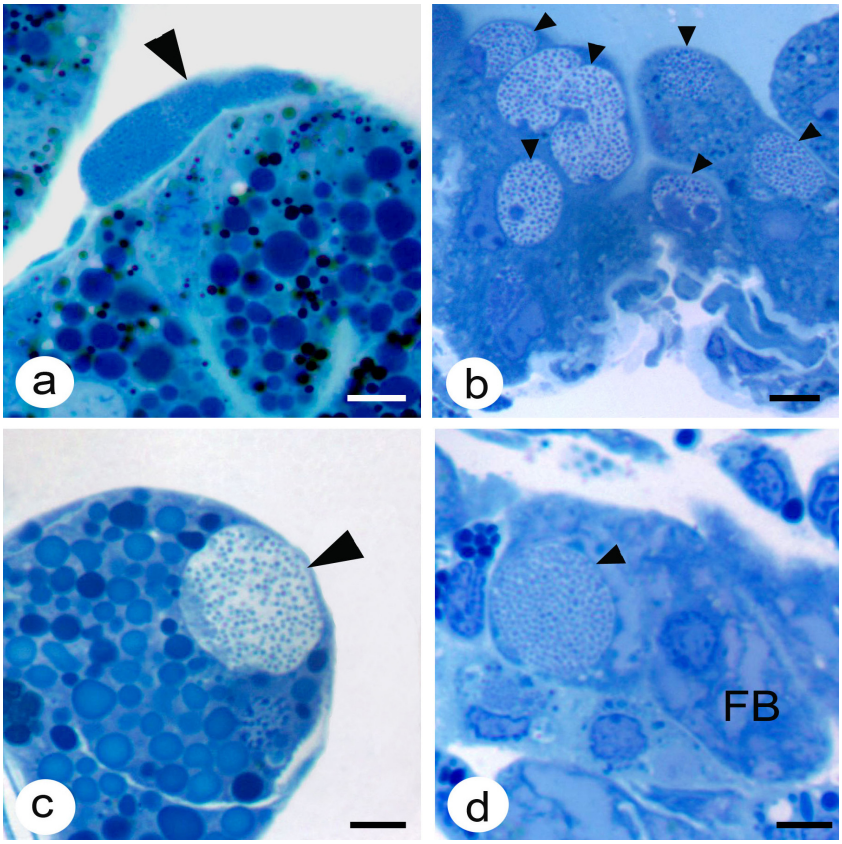

Figure 2. Light photomicrographs of colonies of A. marginale in various tissues of male D. variabilis. (a) A colony of A. marginale in a gut muscle cell (large arrow); (b) several colonies (small arrows) in Malpighian tubules cells; (c) a colony in a salivary gland cell (large arrow) and (d) a colony of A. marginale (small arrow) in a fat body cell. Mallory's stain, bar = 5 μm.

Discussion

Previous reports documented differential gene expression in *A. marginale*-infected tick guts and salivary glands and cultured tick cells [7]. The expression of GST, SelM, vATPase and subolesin was upregulated in *D. variabilis* and/or IDE8 tick cells in response to infection with *A. marginale* [7, 9]. Conversely, functional analysis of these genes by RNAi demonstrated that *A. marginale* infection levels in *D. variabilis* guts and/or salivary glands were reduced after gene knockdown [7]. However, these experiments did not provide

evidence of how these genes affected the developmental cycle of *A. marginale* in ticks, which was the objective of the experiments reported herein.

The results reported in this study further confirm that GST, SelM, vATPase and subolesin are overexpressed in response to infection of ticks with *A. marginale* to increase infection/multiplication rate [7]. In general, the number of *A. marginale* colonies was lower in most tissues in gene knockdown ticks when compared to controls. Notably, colonies were not seen by light microscopy in salivary glands of the gene silenced ticks, suggesting that transmission may be diminished or prevented. The results of the light microscopy analysis further suggested that the proteins encoded by these genes have different impacts on the development of *A. marginale* in ticks. GST may be important for the development of DF in guts of AF ticks. Subolesin was also essential for the multiplication of the pathogen in gut cells both after AF and TF as gene knockdown resulted in significantly lower RF-containing colonies. The increase in the densities of RFs in SelM dsRNAinjected ticks after AF and TF and DFs in the gut of vATPase dsRNA-injected ticks after TF provided interesting results suggesting that gene silencing affected the development ofthe pathogen. SelM knockdown in tick guts after AF and TF resulted in higher densities of colonies containing RFs, and thus appeared to inhibit development of *A. marginale* to the dense or infective forms. However, densities of colonies containing DFs were not significantly different from the controls in guts after AF or TF.

In most cases, the results of *A. marginale* infection levels determined by *msp4* PCR were similar to light microscopy findings of RF- and DF-containing colonies in guts and salivary glands. However, some incongruence was observed between both types of analysis. In all cases except for the number of RF-containing colonies in the gut of SelM knockdown ticks after AF and TF, the *msp4* PCR results showed higher infection levels than those predicted by light microscopy analysis when compared to controls. The detection of higher infection levels by PCR may be explained either by the PCR amplification of DNA from organisms not forming colonies or resulted from the sampling observed in a single cross section of the tick halves. Also, PCR did not differentiate between tissues that may be dissected together while light microscopy analysis allowed for examination of individual tick tissues. In ticks with SelM knockdown, light microscopy analysis showed an increase in *A. marginale* RF but not DF-containing colonies, which may have also influenced the results obtained by both methods.

Table 3. Quantitative analysis of *A. marginale* colony densities in *D. variabilis* guts and salivary glands after gene knockdown by RNAi.

Tissue/colonies containing RF or DF	Tick genes silenced by RNAi				
	GST	**SelM**	**vATPase**	**Subolesin**	**Control**
Ticks collected after AF					
Gut/RF	0.27 ± 0.24	0.85 ± 0.31*	0.62 ± 0.57	0.00 ± 0.00*	0.28 ± 0.20
Gut/DF	0.07 ± 0.01*	0.26 ± 0.26	0.15 ± 0.12	0.17 ± 0.06	0.18 ± 0.13
Salivary glands/RF	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Salivary glands/DF	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Ticks collected after TF					
Gut/RF	1.00 ± 0.72	1.43 ± 1.29*	0.63 ± 0.47	0.04 ± 0.01*	0.75 ± 0.59
Gut/DF	0.29 ± 0.23	0.53 ± 0.48	2.62 ± 2.31*	0.04 ± 0.03*	0.32 ± 0.25
Salivary glands/RF	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*	0.003 ± 0.001
Salivary glands/DF	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*	0.01 ± 0.01

*The density of A. marginale reticulated forms (RF) and dense forms (DF) containing colonies (average ± SD) was calculated for tick gut and salivary gland (sg) sections after acquisition feeding (AF) and transmission feeding (TF) and compared between dsRNA-injected and control ticks by Student's t-test with unequal variance (*P< 0.05). The values were underlined when gene knockdown resulted in higher RF or DF when compared to controls.*

The mechanism by which these proteins affect *A. marginale* developmental cycle in ticks is still unknown. However, information on the function of these proteins can be incorporated into discussion of their role in *A. marginale* infection/multiplication. Selenoproteins are selenocysteine (Sec)-containing proteins that are involved in a variety of cellular processes such as oxidant metabolism [10]. In humans, SelM is expressed in many tissues and is localized in the endoplasmic reticulum [11]. In ticks, Ribeiro et al. [12] identified selenoproteins in salivary glands of *I. scapularis* after blood feeding or *B. burgdorferi* infection. However, little is known about the function of these proteins in ticks. In other arthropods such as *Drosophila*, selenoproteins have been implicated in survival, salivary gland development and fertility [13,14]. SelM was overexpressed in IDE8 tick cells infected with *A. marginale* and a selenoprotein gene was overexpressed in *A. marginale*infected *R. microplus* ticks [7]. SelM was also overexpressed in the gill of white shrimp (*Litopenaeus vannamei*) infected with the white spot syndrome virus [15]. Taken

together, these results suggest that selenoproteins may function to reduce the oxidative stress caused by pathogen infection in ticks. However, as shown herein, SelM may have other functions in ticks, perhaps related to salivary gland development, that explain why reduction in its expression prevents *A. marginale* from infection and/or multiplication in salivary glands after TF. The increase noted in the colony densities containing RFs in SelM silenced ticks both after AF and TF, suggests that expression of this gene directly impacts the *A. marginale* developmental cycle.

Table 4. Qualitative analysis of *A. marginale* colonies in gut muscle, Malpighian tubule and fat body and tissue degeneration in *D. variabilis* after gene knockdown by RNAi.

Collection time/tissue	**Genes silenced by RNAi**				
	GST	**SelM**	**vATPase**	**Subolesin**	**Control**
AF/GM	-	-	-	-	-
AF/MT	++	++	++	(-)	++
AF/FB	-	-	-	-	-
TF/GM	(++)	+++	(++)	(-)	+++
TF/MT	(++)	(++)	(++)	(-)	+++
TF/FB	+++	++	++	(+)	++
AF tissue degeneration	None	None	Testis and SG	Guts and SG	None
TF tissue degeneration	None	FB	Testis and SG	Guts and SG	None

The number of A. marginale colonies was evaluated for tick gut muscle (GM), Malpighian tubule (MT) and fat body (FB) sections after acquisition feeding (AF) and transmission feeding (TF). Scale: – (colonies not found), + (very rare; colonies found in < 10% sections), ++ (rare; colonies found in 10–39% sections), +++ (abundant; colonies found in > 40% sections). Tissue degeneration was evaluated for tick guts, salivary glands (sg), testis and fb after AF and TF. The findings were parenthesized or underlined when gene knockdown resulted in lower or higher colony counts, respectively when compared to controls.

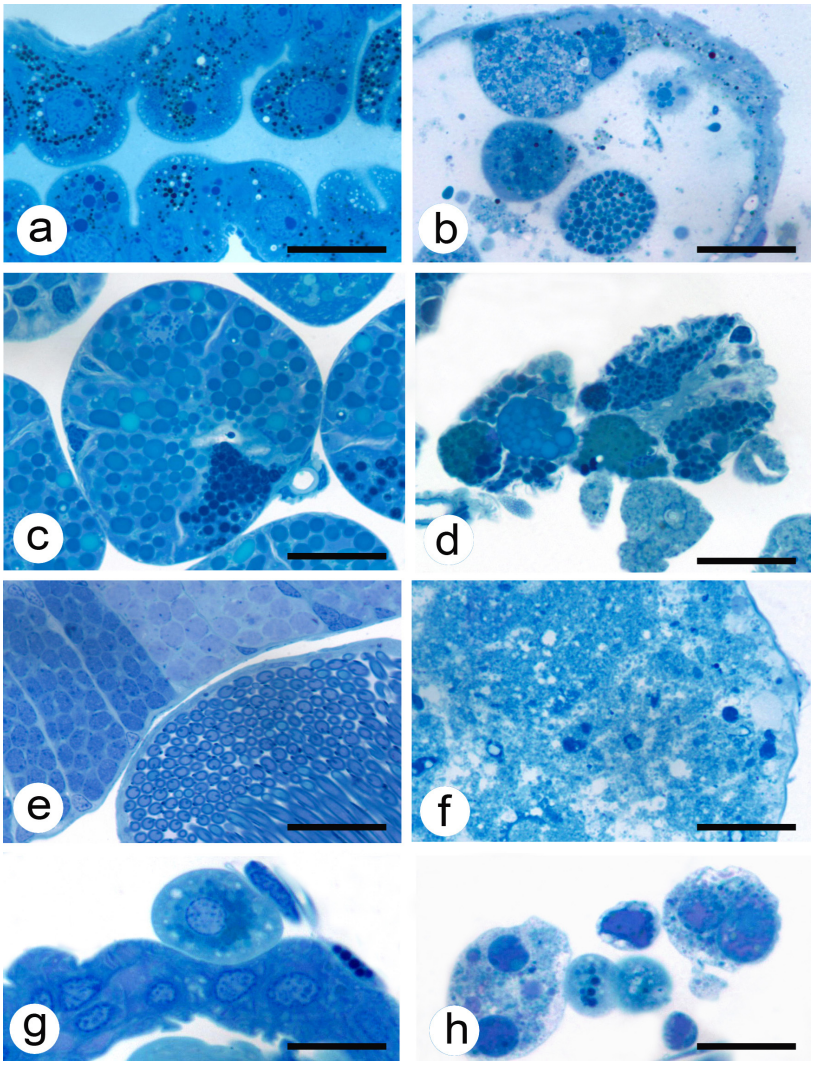

Figure 3. Light micrographs of tick tissues from saline- and dsRNA-injected ticks. Saline injected ticks had normal gut (a), salivary gland (c), spermatogonia and prospermatids (e) and (g) fat body tissues. Tissue degeneration was observed in guts (b), salivary glands (d), spermatogonia and prospermatids (f) and/or fat body cells (h) in ticks injected with subolesin, vATPase, GST, or SelM dsRNA. Mallory's stain, bar = 10 μm.

GST belongs to a gene family that functions in the detoxification of xenobiotic compounds and metabolites produced by cell oxidative stress [16-18]. GSTs have been found to be overexpressed in both infected [19-20] and uninfected ticks [21]. In human cells infected with *A. phagocytophilum* or *R. rickettsii*, GST genes were down-regulated [22-23]. GST was overexpressed both in IDE8 tick cells and *D. variabilis* salivary glands in response to infection with *A. marginale* [7]. However, congruent with proteomics results, real-time RTPCR analysis of GST expression in *D. variabilis* guts and *R. microplus* ticks revealed that mRNA levels were higher in uninfected ticks [7]. These results suggest that ticks have multiple GST genes with different tissue-specific expression patterns that could play different roles during *A. marginale* infection [21]. As in other arthropods [24-27], GSTs may be involved in tick innate immunity by protecting cells from oxidative stress as a result of bacterial infection [18]. Additionally, GST may function as a stress response protein during blood feeding in ticks [19-21]. As determined by RNAi combined with PCR and light microscopy analysis of *A. marginale*, GST appears to be required for pathogen infection of *D. variabilis* guts and salivary glands and IDE8 cells, thus suggesting that the pathogen benefits from GST function, perhaps by diminishing the deleterious effect that cell oxidative stress metabolites may have on bacterial multiplication and development [7, 28]. Most interesting in this study was the notable increase of *A. marginale* infection in fat body cells in the GST silenced ticks, which represents a change in the *A. marginale* developmental cycle.

vATPase is a multisubunit enzyme that mediates acidification of eukaryotic intracellular organelles which has been associated with the cytoskeleton and clathrin-coated vesicles that facilitate receptor-mediated endocytosis required for rickettsial infection [6, 29-30]. Functional vATPase was shown to be required for the normal function of the Golgi complex, endoplasmic reticulum, vacuoles and endocytotic and exocytotic vesicles [29]. vATPase was also implicated in immunity [31]. Genetic knockout of vATPase subunits resulted in lethal phenotypes in yeast, *Neurospora*, *Drosophila* and mice [29]. The vATPase knockdown in *Drosophila* and human cells reduced influenza virus replication [32]. In ticks, vATPase has been implicated in salivary fluid secretion in *Amblyomma americanum* [33]. The results in *A. marginale*-infected tick cells were similar to those in *D. variabilis* ticks infected with *R. montanensis* in which vATPase mRNA levels were increased [7, 34], as well as studies in which human HL-60 cells were infected with *A. phagocytophilum* [23]. Furthermore, RNAi of vATPase expression reduced *A. marginale* infection of *D. variabilis* gut cells but not pathogen multiplication in IDE8 cells [7]. These

results together with those reported herein suggest that vATPase may be functionally important for *A. marginale* development in ticks by affecting pathogen infection of guts and salivary glands. Additionally, vATPase knockdown resulted in testis and salivary gland degeneration, suggesting a role for this molecule in the function of these organs.

The tick subolesin was recently discovered as a tick protective antigen in *Ixodes scapularis* [35]. Subolesin was shown by both RNAi gene knockdown and immunization trials using the recombinant protein to protect hosts against tick infestations, reduce tick survival and reproduction, and cause degeneration of gut, salivary gland, reproductive tissues and embryos [36-41]. Subolesin was shown to function in the control of gene expression in ticks through the interaction with other regulatory proteins [7, 42, 43]. These studies demonstrated a role of subolesin in the control of multiple cellular pathways by exerting a regulatory function on global gene expression in ticks. Subolesin was also shown to be differentially expressed in *Anaplasma*-infected ticks and cultured tick cells [7, 42]. The targeting of tick subolesin by RNAi or immunization was also resulted in decreased vector capacity of ticks for *A. marginale* and *A. phagocytophilum*, respectively [8]. Consistent with these results, in the experiments reported herein subolesin knockdown resulted in gut and salivary gland degeneration and affected the development of both DFs and RFs in the gut and the movement to and infection of salivary glands. These results provide additional evidence of the role of subolesin during *A. marginale* developmental cycle in ticks. RNAi has become an important tool for the study of gene expression and function in ticks [44]. However, little is known about the process of RNAi in ticks [45]. In a recent study, we analyzed the possible off-target effects after tick subolesin RNAi and found that it is a highly specific process [42]. However, these studies have not been performed for other tick genes. Therefore, the possibility of off-target effects may exist particularly for multigene families such as those including SelM and GST. Nevertheless, it is likely that off-target effects, if present would affect the expression of other members of the gene family that are relevant for the results presented and discussed herein.

Conclusions

The results of this RNAi and light microscopic analyses of tick tissues infected with *A. marginale* after the silencing of genes functionally important for pathogen development support previous findings [7] and suggest a role for these molecules during pathogen life cycle in ticks. The decrease in the number of DF-containing colonies suggests an effect of these genes in pathogen development from RFs to infective DFs with a possible decrease in

pathogen transmission by ticks. The decrease in the number of RF-containing colonies suggests an effect of these genes on pathogen infection and replication in ticks. These results suggested that *A. marginale* may increase the expression of SelM and GST to reduce the oxidative stress caused by pathogen infection and thus increase pathogen multiplication in tick cells. The vATPase may be involved in pathogen infection of tick guts and salivary gland cells by facilitating pathogen infection by receptor-mediated endocytosis. For tick subolesin, the results presented herein provide further support for its role in different molecular pathways including those required for *A. marginale* infection and multiplication in ticks. Salivary gland infections were not seen in any of the gene-silenced ticks, raising the question of whether these ticks were able to transmit the pathogen. Finally, the results of these studies suggest that GST, SelM, vATPase and subolesin may be candidate antigens for use in the development of transmission-blocking vaccines for control bovine anaplasmosis.

Methods

Ticks

Dermacentor variabilis male ticks were obtained from the laboratory colony maintained at the Oklahoma State University, Tick Rearing Facility. Larvae and nymphs were fed on rabbits and adults were fed on sheep. Off-host ticks were maintained in a 12hr light: 12 hr dark photoperiod at 22-25 °C and 95% relative humidity. To obtain infected *D. variabilis*, male ticks were allowed to AF for one week on a splenectomized calf experimentally infected with the Virginia isolate of *A. marginale* when the parasitemia was ascending. The ticks were then removed and maintained off-host for 4 days and then allowed to TF for an additional week on an uninfected calf. Uninfected ticks were fed in a similar way on the uninfected control calf. Animals were housed with the approval and supervision of the Oklahoma State University, Institutional Animal Care and Use Committee.

RNA interference in ticks and sample collection

The GST (Genbank accession number DQ224235), SelM (ES429105), vATPase (ES429091) and subolesin (AY652657) dsRNA synthesis was done using the Access RT-PCR system (Promega, Madison, WI, USA) and the Megascript RNAi kit (Ambion, Austin, TX, USA) as reported previously [7-8,38]. For RNAi, male *D. variabilis* ticks were injected with approximately 0.4 μl of dsRNA ($5x10^{10}$-$5x10^{11}$ molecules per μl) in the lower right

quadrant of the ventral surface of the exoskeleton. The injections were done on 50 ticks per group using a Hamilton syringe with a 1 inch, 33 gauge needle. Control ticks were injected with injection buffer (10 mM Tris-HCl, pH 7, 1 mM EDTA) alone. The ticks were held in a humidity chamber for 1 day after which they were allowed to AF and acquire infection for 7 days on a splenectomized calf that was experimentally infected with the Virginia isolate of *A. marginale* (rickettsemia during tick feeding ranged from 4.8% to 35.9% infected erythrocytes). Unattached ticks were removed two days after infestation. All ticks were removed after AF and held in a humidity chamber for four days to allow ticks to digest the bloodmeal, thus allowing for detection of only those pathogens that had infected gut cells. For studies on AF fed ticks, 10 ticks per group were cut in half, fixed and processed for light microscopy analysis. From an additional 5 ticks from each group midguts were dissected and the DNA and RNA were extracted and used to determine the *A. marginale* infection levels by *msp4* quantitative PCR and to confirm gene expression silencing by RT-PCR. The remaining ticks were allowed to TF for 7 days on an uninfected calf to promote development of *A. marginale* in tick salivary glands. After TF, 10 ticks per group were cut in half, fixed and processed for light microscopy analysis. In addition, salivary glands and guts were dissected from 5 ticks from each group for extraction of DNA and RNA and used for determination of the *A. marginale* infection levels by *msp4* quantitative PCR and to confirm gene expression silencing by RT-PCR.

Confirmation of gene silencing and determination of A. marginale infection levels

Guts collected after AF and guts and salivary glands collected after TF were placed in RNAlater (Ambion) for extraction of DNA and RNA as previously reported [7]. *A. marginale* infection levels in ticks were determined by *msp4* quantitative PCR as described by de la Fuente et al. [46]. Tick gut and salivary glands infections in dsRNA and saline injected ticks were compared by Student's *t*-test (P=0.05). Gene expression knockdown was confirmed by determination of mRNA expression levels by real-time RT-PCR as described by de la Fuente et al. [7]. The mRNA levels were normalized against tick β–actin using the comparative Ct method and significance of gene silencing was determined by comparison of mRNA levels in dsRNA and saline injected ticks by Student's *t*-test (*P≤0.05).

Light microscopy and data analysis

For the microscopic studies, ticks were cut in half with a razor blade, separating the right and left sides, and the tick halves were fixed in 2% glutaraldehyde in 0.1 M sodium

cacodylate buffer (pH 7.2). The tick halves were then dehydrated in a graded series of ethanol, washed and embedded in epoxy resin after Kocan et al. [47]. Thick sections (1.0 μm) were cut with an ultramicrotome, stained with Mallory's stain [48] and examined using a light microscope. A calibrated grid (each square, 0.07 mm2) was used to estimate the area of the gut examined in each section. The number of RF- and DF-containing colonies in the gut of each section was recorded. For the salivary glands, total number of salivary acini was tabulated in each tick section and the number of RF- and DF-containing colonies was recorded. The densities of RF- and DF-containing colonies in guts and salivary glands were determined as the number of colonies per gut mm2 or salivary gland acini in each section and compared between dsRNA-injected and control ticks by Student's t-test with unequal variance ($P<0.05$). *A. marginale* colonies seen in other tick tissues (gut muscle, Malpighian tubule and fat body cells) were also counted and tabulated for evaluation of the qualitative role of these tissue infections in the *A. marginale* tick developmental cycle in silenced and control ticks.

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Chapter 6

Subolesin expression in response to pathogen infection in ticks

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Abstract

Background

Ticks (Acari: Ixodidae) are vectors of pathogens worldwide that cause diseases in humans and animals. Ticks and pathogens have co-evolved molecular mechanisms that contribute to their mutual development and survival. Subolesin was discovered as a tick protective antigen and was subsequently shown to be similar in structure and function to akirins, an evolutionarily conserved group of proteins in insects and vertebrates that controls NF-kB-dependent and independent expression of innate immune response genes. The objective of this study was to investigate subolesin expression in several tick species infected with a variety of pathogens and to determine the effect of subolesin gene knockdown on pathogen infection. In the first experiment, subolesin expression was characterized in ticks experimentally infected with the cattle pathogen, *Anaplasma marginale*. Subolesin expression was then characterized in questing or feeding adult ticks confirmed to be infected with *Anaplasma*, *Ehrlichia*, *Rickettsia*, *Babesia* or *Theileria* spp. Finally, the effect of subolesin knockdown by RNA interference (RNAi) on tick infection was analyzed in *Dermacentor variabilis* males exposed to various pathogens by capillary feeding (CF).

Results

Subolesin expression increased with pathogen infection in the salivary glands but not in the guts of tick vector species infected with *A. marginale*. When analyzed in whole ticks, subolesin expression varied between tick species and in response to different pathogens. As reported previously, subolesin knockdown in *D. variabilis* infected with *A. marginale* and other tick-borne pathogens resulted in lower infection levels, while infection with *Francisella tularensis* increased in ticks after RNAi. When non-tick-borne pathogens were fed to ticks by CF, subolesin RNAi did not affect or resulted in lower infection levels in ticks. However, subolesin expression was upregulated in *D. variabilis* exposed to *Escherichia coli*, suggesting that although this pathogen may induce subolesin expression in ticks, silencing of this molecule reduced bacterial multiplication by a presently unknown mechanism.

Conclusions

Subolesin expression in infected ticks suggested that subolesin may be functionally important for tick innate immunity to pathogens, as has been reported for the akirins. However, subolesin expression and consequently subolesin-mediated innate immunity varied with the pathogen and tick tissue. Subolesin may plays a role in tick innate immunity in the salivary glands by limiting pathogen infection levels, but activates innate immunity

only for some pathogen in the guts and other tissues. In addition, these results provided additional support for the role of subolesin in other molecular pathways including those required for tissue development and function and for pathogen infection and multiplication in ticks. Consequently, RNAi experiments demonstrated that subolesin knockdown in ticks may affect pathogen infection directly by reducing tick innate immunity that results in higher infection levels and indirectly by affecting tissue structure and function and the expression of genes that interfere with pathogen infection and multiplication. The impact of the direct or indirect effects of subolesin knockdown on pathogen infection may depend on several factors including specific tick-pathogen molecular interactions, pathogen life cycle in the tick and unknown mechanisms affected by subolesin function in the control of global gene expression in ticks.

Background

Ticks transmit pathogens of the genera *Anaplasma*, *Ehrlichia*, *Rickettsia*, *Babesia* and *Theileria* that impact both human and animal health [1-3]. Of these tick-borne pathogens, *Anaplasma marginale* causes the economically important cattle disease, bovine anaplasmosis [2]. Worldwide, *A. marginale* is vectored by tick species of the genera *Dermacentor* and *Rhipicephalus* [1-3]. The developmental cycle of *A. marginale*, which is presently the most completely characterized rickettsial cycle in ticks, is complex and coordinated with tick feeding cycle [4-6]. Ticks become infected with *A. marginale* when they ingest infected bovine erythrocytes in the bloodmeal, and the first sites of infection are in gut and Malpighian tubule cells. After a second tick feeding, *A. marginale* infects and develops in salivary glands, the site of transmission to the vertebrate host.

The ticks and the pathogens that they transmit have co-evolved molecular interactions involving genetic traits of both the tick and the pathogen that mediate their development and survival [7]. Recent studies have shown that pathogen infection modifies the expression of subolesin and other tick genes [7-11]. Tick subolesin was discovered as a tick protective antigen in *Ixodes scapularis* [12]. Subolesin was shown by RNAi gene knockdown and immunization trials using the recombinant protein to protect hosts against tick infestations, reduce tick survival and reproduction, cause degeneration of guts, salivary glands, reproductive tissues and embryos and to decrease the vector capacity of ticks for *A. marginale* and *A. phagocytophilum* [8, 13-18]. In addition, subolesin was shown to be similar in structure and function to insect and vertebrate akirins which control NF-kB-dependent and independent gene expression that impact innate immunity [19-22]. Based on

the proposed function for tick subolesin, this molecule would be involved in the initial host innate immune response to pathogen infection. However, subolesin expression and its role in tick innate immunity to pathogen infection have not been reported.

The objective of this study was to investigate subolesin expression in several tick species infected with a variety of pathogens and to determine the effect of subolesin gene knockdown on pathogen infection.

Results

Expression of subolesin in tick vectors experimentally infected with A. marginale

Subolesin expression was analyzed in the tick vector species, *D. variabilis*, *D. andersoni*, *D. reticulatus*, *R. sanguineus, R. microplus* and *R. annulatus* experimentally infected with *A. marginale*. Characterization of subolesin expression in guts and salivary glands was done in *D. variabilis*, *D. andersoni* and *R. sanguineus*. Differences in subolesin expression were observed between guts and salivary glands when correlated with *A. marginale* infection in *D. variabilis*, *D. andersoni* and *R. sanguineus* (Figs. 1A-F). While subolesin expression in salivary glands correlated positively with pathogen infection in all three tick species (correlation coefficient, R^2=0.7, 0.6 and 0.9, for *D. variabilis*, *D. andersoni* and *R. sanguineus*, respectively; Figs. 1A-C), a correlation was not found in guts (R^2=0.1, 0.3 and 0.2, respectively; Figs. 1D-F). Interestingly, as shown by differences in the linear correlation slope, the increase in pathogen infection levels resulted in larger variations in subolesin expression in *R. sanguineus* (Fig. 1C) as compared to *Dermacentor spp*. (Figs. 1A and 1B).

When subolesin expression was analyzed in whole ticks, differences were observed in response to *A. marginale* infection between tick species, but in all cases subolesin levels remained unchanged (4 of 6 species analyzed) or were significantly lower in infected ticks than in the uninfected controls (2 of 6 species analyzed) (Fig. 2). However, notable tick-to-tick variation in subolesin expression was also observed (Fig. 2).

Subolesin expression in questing and feeding adult ticks naturally infected with Anaplasma, Ehrlichia, Rickettsia, Babesia or Theileria species

To characterize subolesin expression in ticks naturally infected with different pathogens, questing and feeding adult ticks were collected and analyzed for pathogen infection. The ticks were found to be infected with various pathogens: *R. sanguineus* and *D. marginatus* were infected with *Rickettsia conorii*; *R. bursa* was infected with *Theileria annulata*;

Hyalomma lusitanicum was infected with *Babesia bigemina*; *Hyalomma marginatum marginatum* was infected with *Theileria buffeli*; *R. sanguineus* was infected with *Ehrlichia canis*; and *R. turanicus* and *R. bursa* were infected with *A. ovis* (Table 1).

Table 1. Adult ticks naturally infected with *Anaplasma*, *Ehrlichia*, *Rickettsia*, *Babesia* or *Theileria* species.

Tick species (N)	Sex	Collection	Pathogen infection
R. sanguineus (3)	female	questing	*R. conorii*
D. marginatus (3)	female	questing	*R. conorii*
R. bursa (9)	female	sheep	*T. annulata*
H. lusitanicum (5)	male	questing	*B. bigemina*
H. m. marginatum (8)	male	cattle	*T. buffeli*
R. sanguineus (2)	female	dog	*E. canis*
R. turanicus (2)	female	sheep	*A. ovis*
R. bursa (3)	female	sheep	*A. ovis*

Questing and feeding adult ticks were collected in Sicilian farms and analyzed for pathogen infection by PCR or RLB. To define pathogen species infecting ticks, PCR and sequence analysis of cloned amplicons were performed for Anaplasma, Ehrlichia and Rickettsia spp. For Theileria and Babesia spp., RLB results were confirmed at the species level. For analysis of subolesin expression, sex and collection-matching uninfected controls were used. Uninfected ticks were negative for all pathogens analyzed.

Subolesin mRNA levels were analyzed in infected ticks and in sex and collection-matched uninfected controls. Under natural infection conditions, differences in subolesin expression were observed between tick species in response to different pathogens (Figs. 3A-D). However, similar to ticks experimentally infected with *A. marginale*, subolesin levels remained unchanged or were lower in infected ticks as compared with uninfected controls (Figs. 3A-D) with the exception of *H. lusitanicum* infected with *B. bigemina* (Fig. 3B). Tick-to-tick variations in subolesin expression were also observed as shown previously in ticks experimentally infected with *A. marginale* (Figs. 3A-D). When analysis was conducted in the same tick species infected with different pathogens, *R. sanguineus* infected with *R. conorii* or *E. canis* (Figs. 3A and 3D) and *R. bursa* infected with *T. annulata* or *A. ovis* (Figs. 3B and 3C), subolesin expression levels did not differ with the pathogen and were similar between infected and uninfected ticks. `

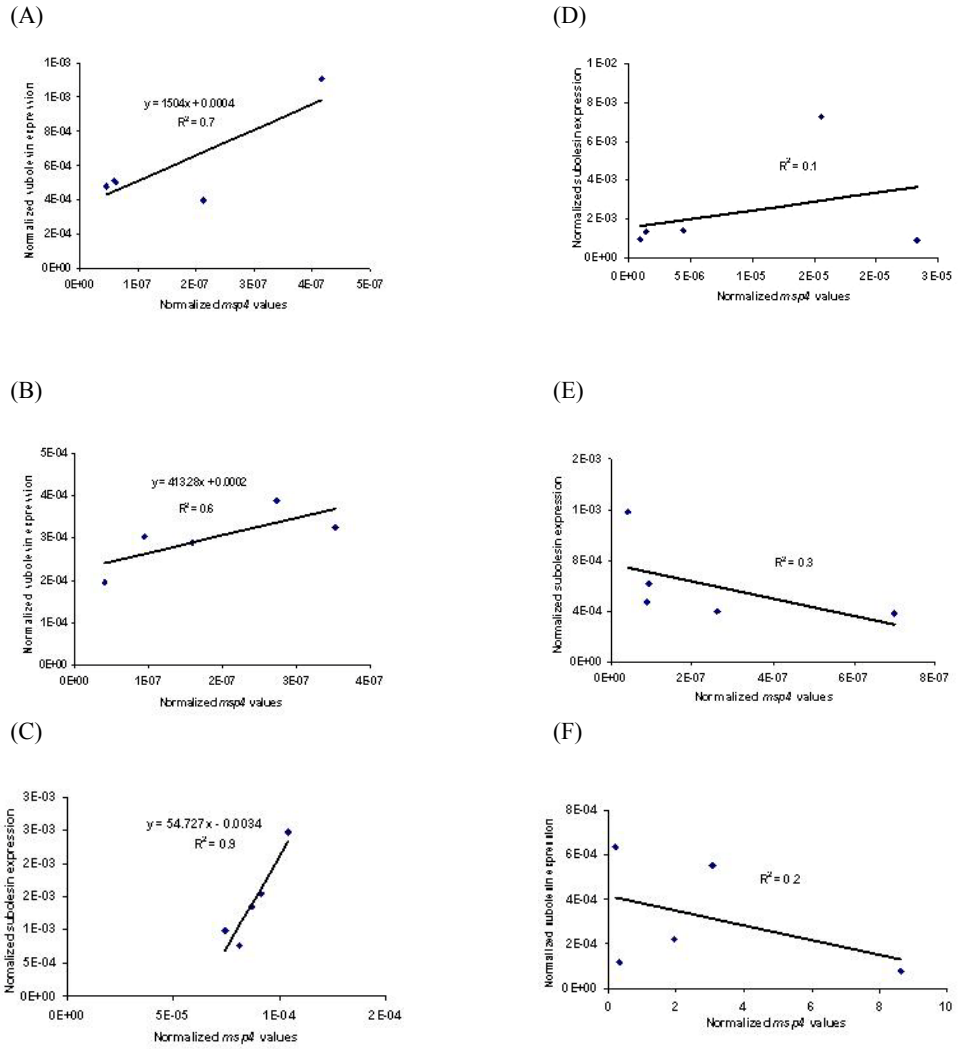

Figure 1. Correlation between subolesin expression and A. marginale infection levels in D. variabilis male guts and salivary glands. RNA was extracted from guts collected after acquisition feeding (D-E) and salivary glands collected after transmission feeding (A-C) in 5 pools of 10 ticks each of D. variabilis (A and D), D. andersoni (B and E) and R. sanguineus (C and F) male ticks experimentally infected with A. marginale. Subolesin and msp4 mRNA levels were analyzed by real-time RT-PCR and normalized against tick 16S rRNA using the comparative Ct method [9, 32]. Regression analyses were conducted in Microsoft Excel to compare normalized A. marginale msp4 and subolesin mRNA levels.

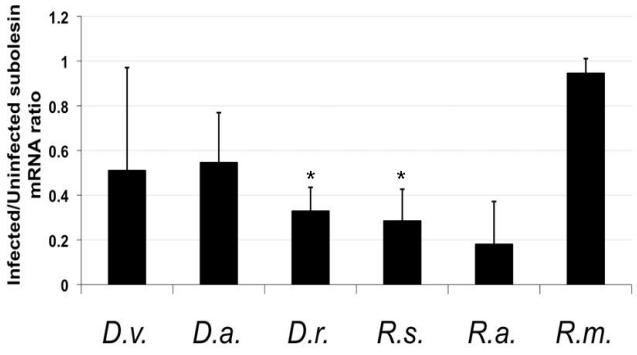

*Figure 2. Subolesin expression in tick vector species experimentally infected with A. marginale. Subolesin expression was characterized in D. variabilis (D.v.), D. andersoni (D.a.), D. reticulatus (D.r.), R. sanguineus (R.s.), R. annulatus (R.a.) and R. microplus (R.m.) whole ticks after transmission feeding (5 pools of 10 ticks each). Subolesin mRNA levels were analyzed by real-time RT-PCR and normalized against tick 16S rRNA using the comparative Ct method [9, 32]. The graph depicts the infected to uninfected subolesin mRNA ratio (±SD) calculated by dividing normalized subolesin mRNA levels in infected ticks by the average of the normalized subolesin mRNA level in uninfected control ticks (N=20). Normalized subolesin mRNA levels were compared between infected and uninfected ticks by Student's t-Test (*P<0.05).*

Effect of subolesin knockdown on the tick response to pathogen infection

The results of subolesin expression studies in response to pathogen infection suggested a role for this molecule in tick innate immunity, at least in salivary glands and in whole ticks in response to some pathogens.

To define the role of subolesin in tick innate immunity, the effect of subolesin gene knockdown was analyzed in *D. variabilis* males capillary fed Gram-positive and Gram-negative bacteria and the yeast, *Pichia pastoris* (Table 2). The results demonstrated that subolesin knockdown after RNAi was effective with an average of 55-99% gene silencing (Table 2). The effect of subolesin knockdown on tick infection levels varied among pathogens (Table 2). While *Francisella tularensis* infection levels were higher in subolesin-silenced ticks when compared to controls, the *A. marginale*, *A. phagocytophilum*, *Ehrlichia canis* and *Escherichia coli* levels were lower. *Bacillus subtilis* and *P. pastoris* infection levels were not significantly different between subolesin-silenced and control ticks.

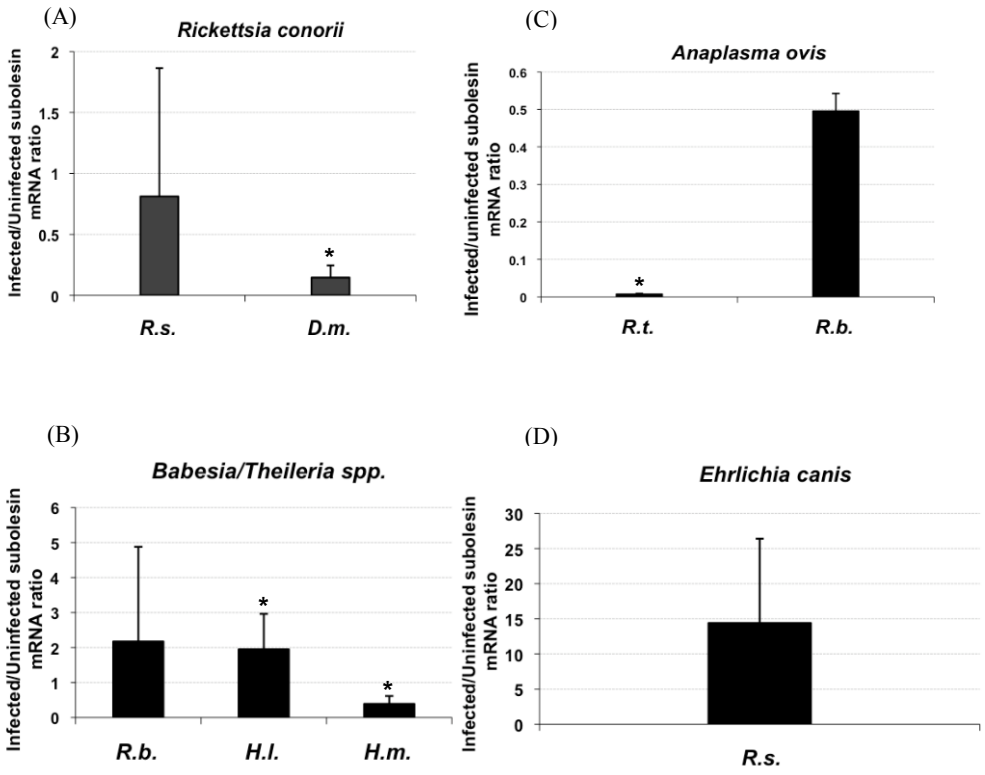

*Figure 3. Subolesin expression in questing or feeding adult ticks naturally infected with different pathogens. Subolesin expression was characterized in R. sanguineus (R.s.) and D. marginatus (D.m.) infected with R. conorii (A), R. bursa (R.b.), H. lusitanicum (H.l.) and H. m. marginatum (H.m.) infected with T. annulata, B. bigemina and T. buffeli, respectively (B), R. turanicus and R. bursa infected with A. ovis (C) and R. sanguineus infected with E. canis (D). In all cases, sex and collection-matching groups of uninfected tick samples were analyzed for comparison. Subolesin mRNA levels were analyzed by real-time RT-PCR and normalized against tick 16S rRNA using the comparative Ct method [9, 32]. The graph depicts the infected to uninfected subolesin mRNA ratio (±SD) calculated by dividing normalized subolesin mRNA levels in infected ticks by the average of the normalized subolesin mRNA level in uninfected control ticks. Normalized subolesin mRNA levels were compared between infected and uninfected ticks by Student's t-Test (*P<0.05).*

Table 2. Experimental conditions and results of *D. variabilis* subolesin RNAi and CF with different pathogens.

Pathogen (isolate/strain)	Inoculum	CF tickmeal	Subolesin expression silencing (%)[a]	Tick infection ratio (Subolesin/ Rs86)[b]
A. marginale (Oklahoma, OK [33])	4.3% (OK), 3.3% (FL) and 7.4% (Bison) infected erythrocytes	Blood from splenectomized calves experimentally infected with isolate stabilates	89±17*	0.85±0.09*
A. marginale (Okeechobee, FL [33])			55±32*	0.83±0.10*
A. marginale (Bison) [33]			86±17*	0.95±0.10*
A. phagocytophilum (NY18) [34]	50% infected cells	ISE6 cultured tick cells in L15B with 10% FBS	92±14*	0.91±0.09*
F. tularensis (Live Vaccine Strain LVS; ATCC 29684)	10^7 CFU/ml	DMEM with 10% FBS	99±2*	1.74±0.86*
E. canis (Ebony)	2% infected cells	DH82 cultured dog cells in DMEM with 10% FBS	94±11*	0.89±0.16*
E. coli (JM109; Promega)	10^7 CFU/ml	DMEM with 10% FBS	97±3*	0.92±0.07*
B. subtilis (culture 125-1 kindly supplied by H. Evers)	10^7 CFU/ml	DMEM with 10% FBS	71±21*	0.65±0.58
P. pastoris (X33; Invitrogen)	10^6 CFU/ml	YPD	80±16*	0.60±0.31

*[a]Subolesin mRNA levels were determined by real-time RT-PCR and normalized against tick 16S rRNA using the comparative Ct method. Percent subolesin expression silencing was calculated in subolesin dsRNA-injected ticks with respect to control ticks injected with the unrelated Rs86 dsRNA and expressed as average±SD. Subolesin normalized Ct values were compared between subolesin dsRNA and control Rs86 dsRNA injected ticks by Student's t-test (*P<0.05). [b]Infection levels were determined by real-time PCR using pathogen-specific gene sequences and normalizing against tick 16S rRNA using the comparative Ct method. Tick infection ratio was calculated as subolesin dsRNA to average control Rs86 dsRNA injected ticks normalized Ct values and expressed as average±SD. Pathogen-specific gene normalized Ct values were compared between subolesin dsRNA and control Rs86 dsRNA injected ticks by Student's t-test (*P<0.05). Abbreviations: CF, capillary feeding; CFU, colony forming units; L15B, modification of Leibovitz's L15 medium containing additional glucose, amino acids, vitamins and trace minerals (Sigma-Aldrich, St Louis, MO, USA); FBS, fetal bovine serum (Sigma); DMEM, Dulbecco's Modified Eagle Medium (Gibco, Invitrogen, Carlsbad, CA, USA); YPD, Yeast Extract Peptone Dextrose medium (10 g/l yeast extract, 20 g/l peptone, 20 g/l glucose) (Sigma).*

To characterize the effect of pathogen infection by CF on subolesin expression, subolesin mRNA levels were compared between ticks injected with control dsRNA and

then fed pathogen-infected or plain media by CF (Fig. 4). The results demonstrated that, with the exception of the *E. coli*-fed ticks, subolesin levels remained unchanged or were lower in infected ticks. However, when subolesin expression was analyzed in individual ticks, some ticks in groups infected with *E. coli*, *E. canis*, *A. marginale* (Bison), *P. pastoris* and *A. phagocytophilum* had subolesin mRNA levels higher than the controls (Fig. 5). This result explained the tick-to-tick variation observed in previous experiments with experimentally and naturally infected ticks and suggested that other factors affected subolesin expression independent of infection levels because subolesin expression only correlated positively with pathogen infection levels in *F. tularensis*-infected ticks (Fig. 4).

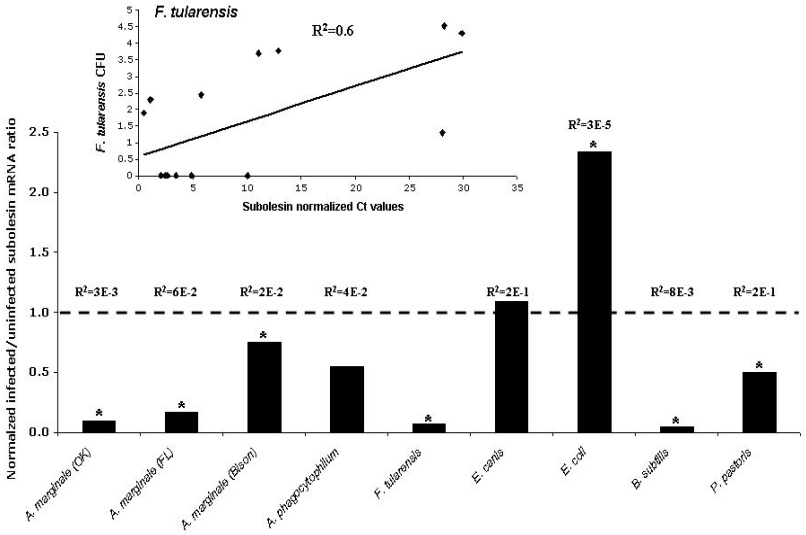

*Figure 4. Subolesin expression in D. variabilis male ticks infected with different pathogens by capillary feeding (CF). Subolesin expression levels were compared between ticks injected with control Rs86 dsRNA and then fed pathogen-infected or plain media by CF (N=27-29). Whole individual ticks were dissected and used for DNA/RNA extraction to determine pathogen infection levels by real-time PCR and subolesin mRNA levels by real-time RT-PCR after normalization against tick 16S rRNA using the comparative Ct method [9, 32]. The graph depicts the infected to uninfected subolesin mRNA ratio (±SD) calculated by dividing normalized subolesin mRNA level in infected ticks by the average of the normalized subolesin mRNA level in uninfected control ticks. Normalized subolesin mRNA levels were compared between infected and uninfected ticks by Student's t-Test (*P<0.05). Regression analyses were conducted in Microsoft Excel to compare normalized pathogen infection levels and subolesin mRNA levels. Regression coefficients are shown for all groups. The correlation graph is shown in the insert for F. tularensis, the only group in which a positive correlation was found between subolesin expression and pathogen infection levels.*

Discussion

Differential expression of subolesin in *Anaplasma*-infected tick guts and salivary glands and cultured tick cells was reported previously [7, 9, 10]. In these experiments, subolesin expression was significantly upregulated in *D. variabilis* salivary glands and IDE8 tick cells but not in *D. variabilis* guts and *R. microplus* salivary glands in response to infection with *A. marginale* [9]. In contrast to *A. marginale*, subolesin expression in *A. phagocytophilum*-infected *I. scapularis* nymphs was significantly downregulated and remained unchanged in infected ISE6 cultured tick cells [9]. The differences in subolesin expression between *A. marginale* and *A. phagocytophilum* infected cultured tick cells were also demonstrated recently for other genes [11]. In addition, functional analysis by subolesin RNAi demonstrated that *A. marginale* infection levels were reduced in *D. variabilis* salivary glands and IDE8 tick cells after gene knockdown [8-10]. Subolesin knockdown affected *A. marginale* development in *D. variabilis* by affecting rickettsial development and infection levels in different tissues [10]. Interestingly, salivary gland infections were not observed in these subolesin-silenced ticks, raising the question of whether they would have been able to transmit *A. marginale* [10]. Additionally, the function of subolesin was recently suggested to be similar to insect and vertebrate akirins in the control of NF-kB-dependent and independent gene expression in ticks [20, 21]. These results suggested that subolesin expression would likely be affected by pathogen infection and to have a role on tick innate immunity, a hypothesis that was tested in the experiments reported herein.

Results reported herein (Table 1) further confirmed subolesin upregulation in salivary glands of *A. marginale*-infected ticks. However, *A. marginale* infection did not affect subolesin expression in the gut of infected ticks. When subolesin expression was analyzed in whole ticks infected with various pathogens, expression levels remained generally unchanged or were lower in infected ticks. This result suggested that while subolesin expression may be upregulated in salivary glands, expression may not be affected or even decreased in other tissues of infected ticks. The overall effect of pathogen infection on subolesin expression in whole ticks may be different from that observed in isolated tissues and reinforces the role that different tissues play in pathogen infection and multiplication in ticks [10, 23]. These results were similar to those obtained in *I. scapularis* nymphs infected with *A. phagocytophilum* [9] but demonstrated differences in tick response to other pathogens, as illustrated by subolesin upregulation in *H. lusitanicum* infected with *B. bigemina*. However, the results with naturally infected ticks should be taken with caution

due to the small number of ticks analyzed. Furthermore, although naturally infected ticks were analyzed for the presence of the most prevalent tick-borne pathogens in Sicily [30], the infection with other pathogens not considered in these studies could affect subolesin expression levels in ticks. However, it is likely that these pathogens would be present in both groups of ticks considered in the study, "infected" and "uninfected", therefore rendering no differences in subolesin mRNA levels.

As demonstrated herein, subolesin may play a role in tick innate immunity in salivary glands but not in the gut. Since the gut is the first tick barrier to pathogen infection [2], subolesin may not be involved in protecting ticks against *A. marginale* infection because of mechanisms that have co-evolved between the pathogen and the tick vector to support pathogen transmission while insuring tick survival [24]. However, subolesin may function in the salivary glands to limit pathogen infection to levels that are not detrimental for ticks. The differential role of subolesin in tick gut and salivary gland cells was further demonstrated by RNAi experiments [7, 9]. Gene knockdown may not affect *A. marginale* infection levels in tick guts because subolesin may not be involved in innate immunity in this tissue. The decrease in *A. marginale* infection levels in the salivary glands of subolesin-silenced *D. variabilis* may not be related to innate immune response but may be due to suppression of other genes regulated by subolesin and required for pathogen infection and multiplication [7, 19, 20] and/or involved in salivary gland function [10, 15]. This effect is most likely not relevant in the gut perhaps because gene silencing was shown to occur 3 days after injection of subolesin dsRNA [15], when gut cells may have already become infected with *A. marginale*.

Recently, we studied the role of *D. variabilis* defensin, varisin, in tick innate immunity to *A. marginale* [25]. Silencing of varisin occurred in tick hemocytes, midguts and salivary glands after RNAi. Varisin knockdown did not increase *A. marginale* infections, which actually were significantly reduced in the varisin-silenced ticks. However, *A. marginale* colonies were morphologically abnormal in varisin-silenced ticks when compared with the controls and some ticks had systemic infections with a yeast-like microbe that may have resulted from varisin RNAi. These results suggested that tick-pathogen interactions may have evolved in natural tick vector species to prevent innate immunity mechanisms against the vectored pathogen and to limit infection with non-tick vectored microorganisms.

The effect of subolesin knockdown in ticks on infection with tick-borne and non-tick-borne organisms was tested in *D. variabilis* using RNAi and CF. In these experiments, infection levels of the tick-borne pathogens, *A. marginale*, *A. phagocytophilum* and *E. canis*

were lower in subolesin-silenced ticks. In contrast, *F. tularensis* infections were higher after subolesin RNAi and CF. These results could be explained in several ways: (1) as previously discussed, tick-pathogen interactions may have evolved in the natural tick vector species to prevent innate immunity against tick-borne pathogens; (2) the life cycle of pathogens in ticks varies and may be accompanied by different impacts of subolesin expression; (3) although *D. variabilis* has been demonstrated to be a vector for *F. tularensis* [26], as shown herein, subolesin function in innate immunity could differ among pathogens; (4) subolesin control of gene expression in ticks may include the expression of genes crucial for tissue structure and function and pathogen infection and multiplication [8-10, 19, 20]; (5) as suggested in CF experiments, unknown factors may affect tick subolesin expression in response to pathogen infection that could result in individual differences in tick capacity to mount an effective subolesin-mediated innate immunity independent of pathogen infection levels; and (6) pathogen infection by CF may differ from natural conditions and thus change the role of subolesin in pathogen infection and development. For example, *A. marginale* infection and multiplication in capillary fed ticks occurred only in the gut [27], thus differing from the natural life cycle.

For non-tick-borne pathogens, *E. coli* and *P. pastoris*, RNAi experiments suggested that subolesin did not have an effect on infection, at least after CF. However, subolesin expression was upregulated in *D. variabilis* exposed to *E. coli*, suggesting that although this pathogen may induce subolesin expression in ticks, silencing of this molecule reduced bacterial multiplication by a presently unknown mechanism. As discussed previously, tick-to-tick variations in subolesin expression in response to pathogen infection occurred for *E. coli* and *P. pastoris*, again suggesting that subolesin may play a role in tick innate immunity against these pathogens but this effect could be affected by unknown individual factors.

Previously, Goto et al. [21] demonstrated that akirin or relish knockdown in flies resulted in lower survival rates after *Agrobacterium tumefasciens* infection when compared to controls. The experiments conducted in ticks were not designed to study the effect of subolesin knockdown on infected tick survival. However, in agreement with lower infection levels in ticks after subolesin silencing, we did not observe an increase in tick mortality after experimental infection with *A. marginale* and other pathogens by CF. The discrepancy between the results in flies and ticks after akirin/subolesin knockdown and pathogen infection could be explained by tick-pathogen co-evolved interactions which are not present in *A. tumefasciens*-infected flies, the limitations of CF to mimic natural tick

feeding and infection conditions, differences in the function of subolesin when compared to that of akirin in insects or to a combination of these factors.

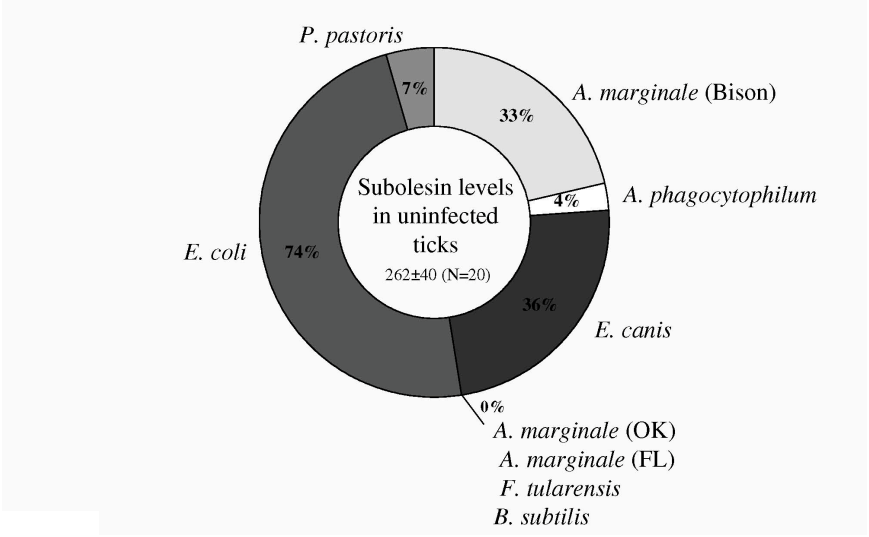

Figure 5. Tick-to-tick variations in subolesin expression in response to pathogen infection. The graph depicts the percent of infected male D. variabilis ticks that showed normalized subolesin mRNA levels higher than the average expression level in uninfected ticks. In all experiments, 27-29 infected ticks were analyzed. For experimental details see figure 4 legend.

Conclusions

The studies demonstrated that subolesin expression varies with pathogen infection in tick salivary glands and in the guts in response to some pathogens, thus suggesting a role of subolesin in tick innate immunity. A hypothetical function for subolesin-mediated innate immune response in ticks is depicted in Figure 6. Subolesin may play a role in tick innate immunity in the salivary glands to by limiting pathogen infection levels but may activate innate immunity to some pathogens in the guts and other tissues. Furthermore, these results provided additional evidence for the role of subolesin in different molecular pathways including those required for tick physiology and pathogen infection and multiplication in ticks. Consequently, subolesin knockdown in ticks may affect pathogen infection directly by reducing tick innate immune responses that result in higher infection levels and indirectly by affecting tissue structure and function and the expression of genes that

interfere with pathogen infection and multiplication. The prevalence of direct or indirect effects of subolesin knockdown on pathogen infection would depend on several factors including the evolution of tick-pathogen interactions, pathogen life cycle in ticks and unknown mechanisms conditioned by subolesin function in the control of global gene expression in ticks.

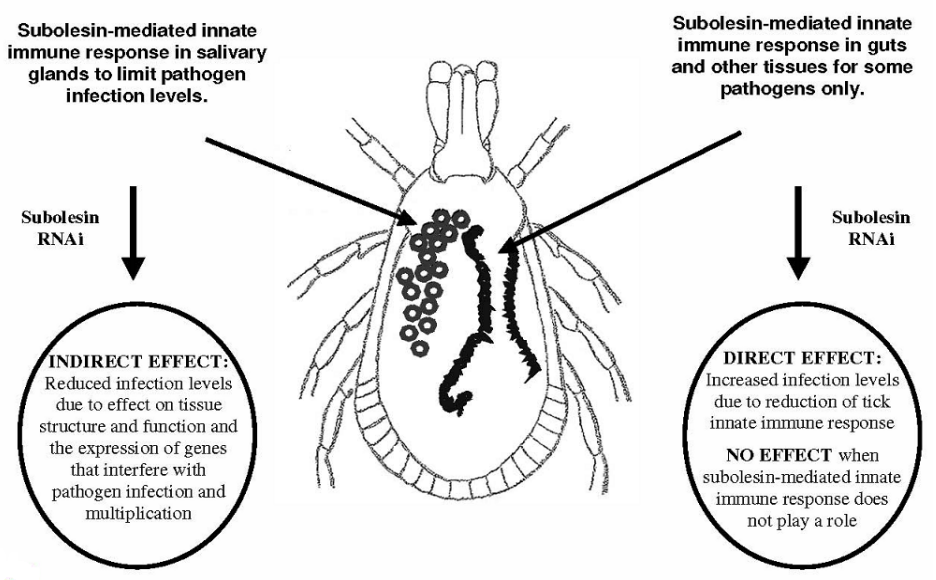

Figure 6. Hypothetical function for subolesin-mediated innate immune response in ticks. It may be possible that subolesin plays a role in tick innate immunity in the salivary glands to limit pathogen infection levels but in the guts for some pathogens only. Consequently, subolesin knockdown in ticks may affect pathogen infection directly by reducing tick innate immune responses that result in higher infection levels and indirectly by affecting tissue structure and function and the expression of genes that interfere with pathogen infection and multiplication.

Methods

Ticks

D. variabilis, *D. andersoni* and *R. sanguineus* male ticks were obtained from the Tick Rearing Facility, Department of Entomology and Plant Pathology, Oklahoma State University. Larvae and nymphs were fed on rabbits and adults were fed on sheep. The *R. annulatus* (Mercedes strain, Texas, USA) and *R. microplus* (Mozambique strain) ticks were obtained from laboratory colonies maintained on cattle at the University of Tamaulipas and the Utrecht Centre for Tick-Borne Diseases, University of Utrecht, The Netherlands,

respectively. *D. reticulatus* ticks were also obtained from a laboratory colony at the tick rearing facility at the University of Utrecht. Larvae and nymphs were fed on rabbits and adults were fed on calves. Off-host ticks were maintained in a 12hr light: 12 hr dark photoperiod at 22-25 °C and 95% relative humidity. Animals were cared for in accordance with standards specified in the Guide for Care and Use of Laboratory Animals of each institution.

To obtain *A. marginale*-infected ticks, *D. variabilis*, *D. andersoni* and *R. sanguineus,* male ticks were allowed to acquisition feed (AF) for one week, during an ascending parasitemia, on a splenectomized calf experimentally-infected with the Virginia isolate of *A. marginale*. The ticks were then removed and maintained off-host for 4 days and then allowed to transmission feed (TF) for an additional week on an uninfected calf. *R. annulatus* larvae were allowed to feed on a calf naturally-infected with *A. marginale* in Tamaulipas, Mexico (approximately 4% rickettsemia during tick feeding) and collected as adults after 21 days of feeding. *R. microplus* larvae and *D. reticulatus* adult male ticks were allowed to feed on an intact calf experimentally infected with the Nigeria isolate of *A. marginale. R. microplus* males were collected after 21 days of feeding. *D. reticulatus* ticks were allowed to AF for 7 days, removed and maintained 5 days off-host and then allowed to TF for an additional week on the same infected calf. Uninfected ticks were allowed to feed in the same way on uninfected calves to serve as controls. Infection of ticks with *A. marginale* was determined by *msp4* PCR [28]. Cattle were maintained according to approved protocols and under the supervision of the respective Institutional Animal Care and Use Committees.

Questing and feeding adult ticks were collected on 27 farms located in different Sicilian regions (Palermo, Enna, Messina, Siracusa and Trapani). A total of 678 ticks were collected and analyzed for this study. Of them, 29 were questing ticks and 649 were collected from cattle, sheep, goats or dogs. Ticks were identified using morphological keys for the Italian Ixodidae [29]. The ticks were incubated for three days in the laboratory prior to dissection and RNA/DNA extraction.

Identification of pathogen infection in naturally infected ticks

DNA was extracted from individual whole tick samples using TriReagent (Sigma, St. Louis, MO, USA) following manufacturers recommendations. The DNA was resuspended in sterile distilled water and stored at -20ºC until used. For the initial screening, PCR analyses for *Anaplasma*, *Ehrlichia* and *Rickettsia* spp. were performed as described previously [30] with 1 µl (0.1-10 ng) DNA using 10 pmol of each primer and the Ready-

To-Go PCR beads (Amersham, Piscataway, NJ, USA). Reactions were performed in an automated DNA thermal cycler for 35 cycles. PCR products were electrophoresed on 1% agarose gels to check the size of amplified fragments by comparison to a DNA molecular weight marker (1 Kb DNA Ladder, Promega). Control reactions were done without the addition of DNA to the reaction to rule out contaminations during PCR. Reverse line blot (RLB) was used for detection of *Babesia*/*Theileria* spp. as described previously [31]. Uninfected ticks were confirmed to be negative for all pathogens analyzed.

To identify and confirm pathogens in ticks, PCR and sequence analysis of cloned amplicons were performed for *Anaplasma*, *Ehrlichia* and *Rickettsia* spp. Amplified fragments were resin purified (Promega), cloned into pGEM-T vector (Promega) and sequenced in an accredited service laboratory (BaseClear, Leiden, The Netherlands) using vector specific primers. The BLAST tool was used to search the NCBI databases in order to identify sequences reported previously with identity to sequences obtained herein. Gene sequences were deposited in the GenBank with accession numbers GQ857075-GQ857078.

Gene expression analysis by real-time RT-PCR in experimentally and naturally infected ticks

Total RNA was extracted using TriReagent (Sigma) following manufacturers recommendations. In *D. variabilis, D. andersoni* and *R. sanguineus* male ticks experimentally infected with *A. marginale*, RNA was extracted from guts collected after AF and salivary glands collected after TF in 5 pools of 10 ticks each. *A. marginale* infection in tick guts and salivary glands was characterized by *msp4* real-time RT-PCR as described previously [7]. Subolesin expression was characterized by real-time RT-PCR using species-specific oligonucleotide primers (Table 3) as described previously [9]. Subolesin levels were characterized in guts and salivary glands of *D. variabilis*, *D. andersoni* and *R. sanguineus* and in whole ticks experimentally infected with *A. marginale* after TF (5 pools of 10 ticks each) and in individual whole ticks naturally-infected with different pathogens. In all cases, matching groups of uninfected tick samples were analyzed concurrently for comparison. Real-time RT-PCR was done using the QuantiTec SYBR Green RT-PCR kit (Qiagen, Valencia, CA, USA) and a Bio-Rad iQ5 thermal cycler (Hercules, CA, USA) following manufacturer's recommendations. mRNA levels were normalized against tick 16S rRNA using the comparative Ct method [9, 32]. Normalized subolesin mRNA levels were compared between infected and uninfected ticks by Student's t-Test (P=0.05). Regression analyses were conducted in Microsoft Excel to compare normalized *A. marginale msp4* and subolesin mRNA levels in the guts and salivary glands

of *D. variabilis*, *D. andersoni* and *R. sanguineus* male ticks experimentally infected with *A. marginale*.

Tick RNA interference and capillary feeding

D. variabilis subolesin dsRNA and unrelated control Rs86 dsRNA were synthesized as described previously [8, 15, 32], using the Access RT-PCR system (Promega, Madison, WI, USA) and the Megascript RNAi kit (Ambion, Austin, TX, USA). The dsRNA was purified and quantified by spectrometry. Male *D. variabilis* ticks were injected with approximately 0.4 µl of dsRNA ($5x10^{10}$-$5x10^{11}$ molecules per µl) in the lower right quadrant of the ventral surface of the exoskeleton of ticks [8, 15]. The injections were done on 30 ticks per group using a Hamilton syringe with a 1-inch, 33 gauge needle. The ticks were held in a humidity chamber for 1 day after which they were allowed to feed for 3 days on a sheep prior to CF. Ticks were removed from the sheep and immobilized for CF [27]. Fifty-µl volume capillary tubes were placed over the capitulum of the ticks to feed them with the pathogen containing tick meal (Table 2). CF was done for 3 days with daily changes of capillary tubes containing fresh tick meal. Whole individual ticks were then dissected and used for DNA/RNA extraction to determine pathogen infection levels by real-time PCR and subolesin mRNA levels by real-time RT-PCR using pathogen-specific gene sequences (Table 3) and subolesin primers, respectively, as described above. Subolesin and pathogen-specific gene normalized Ct values were compared between subolesin dsRNA and control Rs86 dsRNA injected ticks by Student's *t*-test (P=0.05). For *F. tularensis*, dissected tick tissues were homogenized, centrifuged and supernatants plated to count pathogen colony forming units (CFU) per tick and to compare CFU between subolesin dsRNA and control Rs86 dsRNA injected ticks by Student's *t*-test (P=0.05). Regression analyses were conducted in Microsoft Excel to compare normalized pathogen infection levels and subolesin mRNA levels.

Table 3. Oligonucleotide primers and PCR conditions for the characterization of subolesin and pathogen-specific gene expression.

Gene description[a]	Upstream/downstream primer sequences (5´-3´)	PCR annealing conditions
D. variabilis	CCAGCCTCTGTTCACCTTTC	54ºC,
subolesin [9]	CCGCTTCTGAATTTGGTCAT	30 sec
R. microplus	CACAGTCCGAGTGGCAGAT	55ºC,
subolesin [9]	GATGCACTGGTGACGAGAGA	30 sec
A. marginale	GGGAGCTCCTATGAATTACAGAGAATTGTTTAC	60ºC,
msp4 [28]	CCGGATCCTTAGCTGAACAGGAATCTTGC	1 min
A. phagocytophilum	GACGTGCTGCACACAGATTT	54ºC,
msp4 [9]	CTCATCAAATAGCCCGTGGT	1 min
E. canis	GTGGCAGACGGGTGAGTAAT	57 ºC,
16S (M73221)	GCTGATCGTCCTCTCAGACC	30 sec
B. subtilis dal	AATTGAAAGGGACCGACATC-	59 ºC,
[35]	TTAATGGTTTCGAGCCTTCC	30 sec
E. coli dxs [36]	CGAGAAACTGGCGATCCTTA	60ºC,
	CTTCATCAAGCGGTTTCACA	30 sec
P. pastoris CTA 1	CCTGAAGGACGCCAATATGT	57 ºC,
(AB472085)	GCTTTCCAGCCTCTTCATTG	30 sec
Tick 16S rRNA [9]	GACAAGAAGACCCTA	42 ºC,
	ATCCAACATCGAGGT	30 sec

[a]*When published, references are shown for oligonucleotide sequences. When designed for this study, GenBank accession numbers are shown in parenthesis.*

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Chapter 7

Anaplasma phagocytophilum and *A. marginale* elicit different gene expression responses in cultured tick cells

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Abstract

The genus *Anaplasma* (Rickettsiales: Anaplasmataceae) includes obligate tick-transmitted intracellular organisms, *A. phagocytophilum* and *A. marginale* that multiply in both vertebrate and tick host cells. Recently, we showed that *A. marginale* affects the expression of tick genes that are involved in tick survival and pathogen infection and multiplication. However, the gene expression profile in *A. phagocytophilum*-infected tick cells is currently poorly characterized. The objectives of this study were to characterize tick gene expression profile in *Ixodes scapularis* ticks and cultured ISE6 cells in response to infection with *A. phagocypthilum* and to compare tick gene expression responses in *A. phagocytophilum* and *A. marginale* infected tick cells by microarray and real-time RT-PCR analyses. The results of these studies demonstrated modulation of tick gene expression by *A. phagocytophilum* and provided evidence of different gene expression responses in tick cells infected with *A. phagocytophilum* and *A. marginale*. These differences in *Anaplasma*-tick interactions may reflect differences in pathogen life cycle in the tick cells.

Introduction

Ticks transmit pathogens that greatly impact both human and animal health [1]. The genus *Anaplasma* (Rickettsiales: Anaplasmataceae) includes obligate tick-transmitted intracellular organisms found exclusively within membrane-bound inclusions or vacuoles in the cytoplasm of both vertebrate and tick host cells [2, 3]. *A. marginale* infects cattle and wild ruminants and causes bovine anaplasmosis [2]. *A. phagocytophilum* infects humans and wild and domesticated animals [2, 4, 5] and is the causative agent of human, equine and canine granulocytic anaplasmosis and tick-borne fever of ruminants [5, 6]. In the United States, *A. phagocytophilum* is transmitted by *Ixodes scapularis* and *I. pacificus* [2, 4].

The ticks and the pathogens they transmit have evolved molecular interactions that affect their survival and life cycle [3]. The *A. phagocytophilum* outer membrane proteins that are involved in interactions with tick cells have been identified and partially characterized [7, 8]. Recently, we identified and characterized tick molecules that are involved in *A. marginale*-tick interactions, demonstrating that *A. marginale* affects the expression of tick genes essential for tick survival and pathogen infection and multiplication [9]. However, tick molecules that are affected by and participate in *A. phagocytophilum* infection and multiplication are currently poorly characterized [10, 11].

The objectives of this study were the characterization of tick gene expression profiles in *I. scapularis* ticks and cultured tick cells in response to infection with *A. phagocytophilum* and to compare tick gene expression responses in *A. phagocytophilum* and *A. marginale* infected cultured tick cells by microarray and real-time RT-PCR analyses. The results reported herein demonstrated modulation of tick gene expression by *A. phagocytophilum* and identified differentially expressed genes that are relevant for the understanding of basic biological questions of *A. phagocytophilum* life cycle in *I. scapularis*. The results also provided evidence of differences in tick gene expression in response to infection with *A. phagocytophilum* or *A. marginale*

Materials and Methods

Uninfected and Anaplasma-infected ticks and tick cells

The *I. scapularis* nymphs uninfected and infected with *A. phagocytophilum* (Gaillard and Dawson strains) were obtained from a laboratory colony reared at the Centers for Disease Control and Prevention, Atlanta, GA, USA. Tick larvae were fed on uninfected or infected mice, collected after feeding and allowed to molt to nymphs. Animals were housed with the approval and supervision of the Institutional Animal Care and Use Committee.

The tick cell line ISE6, derived from *I. scapularis* embryos (provided by U.G. Munderloh, University of Minnesota, USA) was cultured in L15B medium as described previously for IDE8 cells [12], but the osmotic pressure was lowered by the addition of one-fourth sterile water by volume after Munderloh et al. [13]. The ISE6 cells were first inoculated with *A. phagocytophilum* (NY18 isolate)-infected HL-60 cells and maintained according to Munderloh et al [13] until infection was established and routinely passaged. Infected ISE6 cells were frozen in liquid nitrogen and served as inoculum for uninfected cells. The ISE6 cells were initially infected with *A. marginale* (Virginia isolate) from a frozen inoculum of infected bovine erythrocytes. Following infection and routine passage, infected ISE6 cells were frozen in liquid nitrogen and served to infect uninfected ISE6 cells. For the current study each inoculum of infected cells was thawed and centrifuged and the pellet was resuspended in culture medium and put on the ISE6 cells. When the infection reached approximately 80% of the tick cells, the monolayer was passaged onto uninfected ISE6 monolayers and maintained in L15B medium as described above. Monolayers of infected ISE6 cells were collected at different time points as described above. Uninfected cells were cultured in the same way but adding 1 ml of culture medium instead of infected cells. Collected cells were centrifuged at 10,000 x g for 3 min and cell pellets were frozen in liquid N until used for RNA extraction.

The infection of ticks and tick cells with *A. phagocytophilum* or *A. marginale* was corroborated by major surface protein 4 (*msp4*) PCR [14, 15].

Microarray analysis

Infected tick ISE6 cells were sampled at 6 days post-infection (dpi) with approximately 70% infected cells (separate cell cultures grown under similar conditions had >90% cells infected at 8 dpi). Uninfected cells were sampled at the same time point as infected cells to account for culture time effects. Total RNA was extracted from three *A. marginale*-infected, three *A. phagocytophilum*-infected and three uninfected ISE6 cell cultures using the RNeasy Mini Kit (Qiagen) including the on-column DNA digestion with the RNase-free DNase set following manufacturer's instructions. RNA quality was checked by gel electrophoresis to verify the integrity of RNA preparations. Total RNA (5 µg) were labeled using the 3DNA Array900 kit with Alexa Fluor dyes (Genisphere, Hatfield, PA, USA), Superscript II (Invitrogen, Carlsbad, CA, USA), the supplied formamide-based hybridization buffer and 24x60mm LifterSlips (Erie Scientific, Portmouth, NH, USA) according to the manufacturer's (Genisphere) instructions. The microarray was constructed

with 768 random *I. scapularis* sequences enriched for genes differentially expressed after subolesin knockdown as previously described [16] (NCBI Gene Expression Omnibus (GEO) platform accession number GPL6394 and series number GSE10222). Eight pools of 12 clones each from an unsubtracted *I. scapularis* cDNA library and subolesin cDNA were also arrayed and used to validate normalization. Hybridization signals were measured using a ScanArray Express (PerkinElmer, Boston, MA, USA) and the images were processed using GenePix Pro version 4.0 (Axon, Union City, CA, USA). Ratios were calculated as *Anaplasma*-infected cells versus uninfected control cells. Pre-processing of data was accomplished using R-project statistical environment (http://www.r-project.org) and Bioconductor (http://www.bioconductor.org) and the LIMMA package as previously described [17]. This included: (1) removal of data points where signal was less than the background plus two standard deviations in both channels, (2) removal of data points where signal was less than 200 RFU in both channels, (3) removal of poor quality spots flagged during image processing, (4) removal of spots with less than 50% valid biological and technical replicates, (5) log transformation of the background subtracted mean signal ratios and (6) normalization using global Lowess intensity-dependent normalization. Normalized ratio values obtained for each probe were averaged across 3 biological replicates and four technical replicates and significant differences were defined as P-value $\leq$ 0.05 and displaying an expression fold change greater than 2-fold in either *A. phagocytophilum* or *A. marginale* infected cells.

Sequence analysis and database search

Partial sequences were determined for cDNA sequences identified as differentially expressed in the microarray analysis. Multiple sequence alignment was performed using the program AlignX (Vector NTI Suite V 8.0, InforMax, Invitrogen, Carisbad, CA, USA) to exclude vector sequences and to identify redundant (not unique) sequences. Searches for sequence similarity were performed with the BLASTX program (http://www.ncbi.-nlm.nih.gov/BLAST) against the non-redundant sequence database (nr) and databases of tick specific sequences (http://www.vectorbase.org/index.php; http://compbio.dfci.harvard.-edu/tgi/).

Table 1. RT-PCR oligonucleotide primers and conditions for the characterization of the expression profiles of differentially expressed tick genes.

Gene ID[a]	Upstream/downstream primer sequences	PCR annealing temperature
1I1H6	GGTACATGGAATCCGACTGC GTCCCCTTTTGCTTCGACTT	54 ºC
1I3A8	GACGCAAAACTTCCTTCGAG GCACTTTCCAAGAGCCTGAC	54 ºC
1I3F5	GCTTTCACGTTTTCGATGGT GGCAAAGATCCAAGACAAGG	50 ºC
1I3H6	GCCTAGGGAGGACGTCGTAG ACGTGGAACACATCGAGTCA	50 ºC
1I4C6	AATGCGAGACACTGGAGGAC AATCCAGGAATGTTGCCAAG	50 ºC
1I4G12	GACGGACCTTGTCCGACTAC ATTCCCTCCTTGTCCTGGAT	53 ºC
1I5B9	CGTCCCCTTCTGTGGAATTA TCATCGTTGTTCTGGTCTCG	53 ºC
2I1C2	GAGACCATCAAGTGGCTGGA CTTGGTGATGATGGGGTTG	53 ºC
2I1F6	CAACCCCAAGATCGTCAACT ACGCGTCCTTACGTTTCACT	53 ºC
2IP10	TCTTGCCGGTCAGAGTCTTT GAAGGCGAAAATTCAGGACA	53 ºC
2I3A3	TAAAACCCCTTTCCCCACTT GCACTCGAACCTAGCAAACC	53 ºC
2I3A7	TCGACTCTGTTCAGGAGGAAG GGTCCAAATGGCAGAGCAT	53 ºC
2I3G1	AGGAAGTGCACGATGATGG GGTTGGTTATCCTCTGGGAGA	54 ºC
2I4F6	CTTTCTTGCCGTGCTTCTTT GCTCAACTTCCTCGTCGTTC	53 ºC
UP8	CCTCCCTCGCTAACCTCTCT ATCGTCACGGTCGAAGTAGC	54 ºC
U2A8	GCTCATCGTCGCCAACAT GAGTTCCTCCGTCCAGCTC	54 ºC
C2E6	GTAAAGCCCGCTCTCAAGAA CATTCGGGTTTGTCCACAG	53 ºC
C3B2	GAGTAGTGCCCGTCTTCGAC AGGTGATGCTGCCCTTGTAG	53 ºC
C4G3	AACTGCCTTGGAGTTGCAGT CTTGTGTCCCAGGTGGAAGT	53 ºC
C4B10	GTTCTTCTAACGGCCACTGC AGTCTTTGGTGCAAGCGAGT	53 ºC
R1E12	ATGTGAAGCTGAGGCCAAAC GGAATTCGATTAGCGTGGTC	53 ºC
R4G5	CCTTCCCTGCAATGTCAAAT CACAAGTGGGCAATCAACAC	53 ºC
Beta actin (AF426178)	GAGAAGATGACCCAGATCA GTTGCCGATGGTGATCACC	50ºC
16S rRNA (L34293)	GACAAGAAGACCCTA ATCCAACATCGAGGT	42ºC

[a]*IDs for I. scapularis genes are described herein and in de la Fuente et al. [9]*

Table 2. Microarray analysis of gene expression profile in *A. marginale* and *A. phagocytophilum* infected and uninfected tick ISE6 cells.

Probe ID[a]	Description[b]	*A. marginale* infection vs. control		*A. phagocytophilum* infection vs. control	
		Fold change[c]	SD[d]	Fold change[c]	SD[d]
C4A10	No homolog found	6.249	0.000	2.208	0.294
R1A6	No homolog found	2.539	0.162	1.049	0.346
C3C5	[Genbank:L22271] internal transcribed spacer 1 (*Ixodes dammini*)	2.406	1.211	1.383	0.000
C4A8	No homolog found	2.239	0.165	1.188	0.442
R3A7	No homolog found	2.209	0.805	-1.384	0.562
C4G3	[Genbank:AAY66629] putative secreted salivary protein (*Ixodes scapularis*)	2.167	0.326	1.037	0.614
C2E6	[Genbank:ABB89211] troponin I protein (*Rhipicephalus haemaphysaloides*)	2.040	0.400	-1.068	0.442
C3C3	No homolog found	1.916	0.559	3.422	1.037
C4A1	No homolog found	1.857	0.000	2.121	0.517
R2A12	No homolog found	1.199	0.232	-2.219	0.450
C3A7	No homolog found	1.135	0.282	-3.028	0.141
C3D9	[Genbank:XP_791420] hypothetical protein (*Strongylocentrotus purpuratus*)	1.076	0.335	4.875	2.069
C3B2	[Genbank:BAE53722] aspartic protease (*Haemaphysalis longicornis*)	-1.311	0.330	-6.986	0.379
R2G1	No homolog found	-1.497	0.495	-2.086	0.826
R2D6	No homolog found	-1.538	0.309	-2.440	0.563
C3C11	No homolog found	-2.053	0.401	-2.477	0.488
C4D12	No homolog found	-2.066	0.547	-1.022	0.533
R4G5	[Genbank:AAP84098] ML domain-containing protein (*Ixodes ricinus*)	-2.066	0.161	1.020	0.266
C4C9	[Genbank: AAH56007] H13-prov protein (*Xenopus laevis*)	-2.070	0.270	1.081	0.689
C4G11	[Genbank:EAA09467] ENSANGP00000010016 (*Anopheles gambiae*)	-2.093	0.550	-1.193	0.598
C4G9	No homolog found	-2.095	0.402	-1.598	0.888
R3F5	[Genbank:AAY66764] putative secreted salivary protein (*Ixodes scapularis*)	-2.118	0.310	-1.037	0.320
R3G4	No homolog found	-2.292	0.259	1.090	0.415
R1F3	No homolog found	-2.339	0.570	-1.344	0.855
R1E12	[Genbank:NP_001119682] ribosomal protein L32 (*Acyrthosiphon pisum*)	-2.379	0.000	2.488	0.000
C3F10	[Genbank:AAM93633] putative secreted protein (*Ixodes scapularis*)	-2.386	0.545	-1.647	0.315
R3D4	No homolog found	-2.529	1.046	-2.377	0.518
C4D2	No homolog found	-2.702	0.860	1.043	0.972
R3F4	No homolog found	-2.928	0.298	1.174	0.396
C1H10	No homolog found	-3.341	0.307	-1.057	0.000
C4A4	No homolog found	-3.678	1.181	-1.430	0.331
C4E12	[Genbank:AAY66942] ribosomal protein S17 (*Ixodes scapularis*)	-3.964	0.822	1.430	0.993
C4B10	[Genbank:AAQ01562] von Willebrand factor (*Ixodes ricinus*)	-4.422	0.000	2.413	0.000

[a]Probe ID (library plate and well) identifies sample (clone) in stock plates. [b]Description of the probe based on top (best) BLASTX alignment. [c]Fold change is the fold change of Lowess intensity-dependent normalized log2 ratio of valid background-corrected means averaged between valid replicates. Only entries displaying an expression change greater than 2-fold and P<0.05 in either A. phagocytophilum or A. marginale infected cells are shown. Positive and negative values correspond to genes upregulated and downregulated in infected cells, respectively. [d]SD is the standard deviation determined from the normalized average log2 ratio but determined on data from valid spots only.

Real-time reverse transcription (RT)-PCR analysis

Total RNA was extracted from uninfected and *A. phagocytophilum*-infected and *A. marginale*-infected ISE6 cells (three cultures each) and *A. phagocytophilum*-infected *I. scapularis* whole unfed nymphs (three groups of uninfected ticks, three groups of ticks infected with the Gaillard strain, and three groups of ticks infected with the Dawson strain with 10 nymphs each) using the RNeasy Mini Kit (Qiagen) including the on-column DNA digestion with the RNase-free DNase set following manufacturer's instructions. Infected tick cells were sampled at a single time point at 3 dpi with approximately 40% infected cells (companion cultures were terminal at 8 dpi) or at 2, 5 and 8 dpi. When included in the analysis, uninfected cells were sampled at the same time point as infected cells to account for culture time effects. Two primers were synthesized based on the sequences determined for candidate differentially expressed genes for real-time RT-PCR analysis. Real-time RT-PCR was done using the QuantiTec SYBR Green RT-PCR kit (Qiagen, Valencia, CA, USA) and a Bio-Rad iQ5 thermal cycler (Hercules, CA, USA) following manufacturer's recommendations. Reactions were done for 40 cycles and 30 sec annealing using oligonucleotide primers and annealing temperatures described in Table 1. Negative controls included reactions without RNA. mRNA levels were normalized against tick β–actin or 16S rRNA using the comparative Ct method and compared between infected and uninfected tick cells and ticks or between *A. phagocytophilum* and *A. marginale* infected tick cells by Student's *t*-Test (P=0.05).

The *A. phagocytophilum* and *A. marginale* infection levels were evaluated in tick ISE6 cells at 2, 5 and 8 dpi by real-time PCR of *msp4* and normalizing against tick 16S rDNA sequences using the QuantiTec SYBR Green PCR kit (Qiagen) in an iQ5 thermal cycler (Bio-Rad) as described above. Known amounts of the full length *A. phagocytophilum* and *A. marginale msp4* PCR product were used to construct a standard for quantitation of pathogens per cell.

Nucleotide sequence accession numbers

The nucleotide sequences of the ESTs reported in this paper have been deposited in the GenBank database under accession numbers FL685631-FL685658.

Results

A. phagocytophilum modulates gene expression in infected I. scapularis nymphs and tick ISE6 cells

The infection with *A. marginale* has been shown to modulate tick gene expression [9]. However, the effect of *A. phagocytophilum* infection on tick gene expression is unknown. Here, two experimental approaches were used to characterize gene expression profiles in tick cells infected with *A. phagocytophilum*. In the first approach, tick gene expression was characterized by microarray analysis of RNA from infected and uninfected tick ISE6 cells. In the second approach, genes identified as differentially expressed in tick IDE8 cells and ticks infected with *A. marginale* were used to characterize the effect of *A. phagocytophilum* on tick gene expression in infected *I. scapularis* nymphs and tick ISE6 cells by real-time RT-PCR.

The microarray analysis showed in *A. phagocytophilum*-infected tick ISE6 cells the upregulation of genes C4B10 with homology to von Willebrand factor, R1E12 with homology to ribosomal protein L32, C4A10, C3C3, C4A1 and C3D9 with unknown function and the downregulation of genes C3B2 with homology to an aspartic protease and R2A12, C3A7, R2G1, R2D6, C3C11 and R3D4 with unknown function (Table 2). The expression of other genes with homology to troponin I (C2E6), putative secreted salivary protein (C4G3) and ML domain-containing protein (R4G5) did not change after infection of tick ISE6 cells with *A. phagocytophilum* (Table 2).

The mRNA levels of selected genes differentially expressed in *A. phagocytophilum*-infected tick ISE6 cells were evaluated by real-time RT-PCR in infected and uninfected cells (Figure 1). Similar to microarray hybridization results, the analysis of mRNA levels by real-time RT-PCR showed significant upregulation of C4B10 (von Willebrand factor) and R1E12 (ribosomal protein L32) and downregulation of C3B2 (aspartic protease) in tick ISE6 cells infected with *A. phagocytophilum* (Figure 1). The mRNA levels of genes identified previously as differentially expressed in tick IDE8 cells and ticks infected with *A. marginale* [9] were also evaluated by real-time RT-PCR in infected and uninfected tick ISE6 cells. The mRNA levels of genes differentially expressed in *A. marginale*-infected ISE6 cells were similar to those reported previously in infected IDE8 cells [9] and data not shown. The results in *A. phagocytophilum*-infected ISE6 cells showed that pathogen infection significantly upregulated the expression of U2A8 (signal sequence receptor delta), 1I5B9 (ixodegrin-2A RGD containing protein), 1I4G12 (unknown

function) and downregulated the expression of 2I3A7 (NADH-ubiquinoe oxidoreductase) and 1I1H6 (glutathione S-transferase (GST)) in tick ISE6 cells (Figure 1).

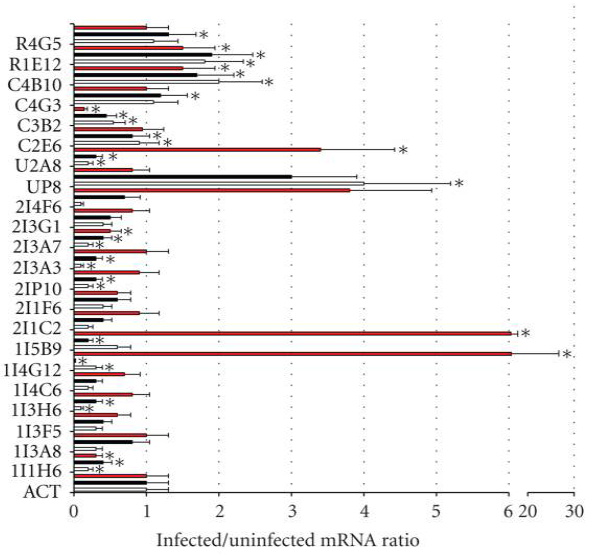

*Figure 1. Differential gene expression in A. phagocytophilum-infected I. scapularis ticks and cultured tick ISE6 cells. Real-time RT-PCR was done on uninfected and infected I. scapularis nymphs (three groups each of uninfected ticks, infected ticks (Gaillard strain; black bars) and infected ticks (Dawson strain; white bars) with 10 nymphs each) and uninfected and NY18 isolate-infected tick ISE6 cells (three independent cultures each; red bars). Bars represent the ratio between infected normalized Ct values/uninfected average normalized Ct values (+SD). The mRNA levels were normalized against tick β-actin (ACT) and compared between infected and uninfected ticks and tick cells by Student's t-test (*P ≤0.05).*

The expression of selected genes was also analyzed in *I. scapularis* nymphs infected with two different *A. phagocytophilum* strains (Figure 1). In *I. scapularis* nymphs, the expression of C4G3 (putative secreted salivary protein), C4B10 (von Willebrand factor), R1E12 (ribosomal protein L32) and R4G5 (ML domain-containing protein) was significantly upregulated and the expression of U2A8 (signal sequence receptor delta), UP8 (ferritin), 2I3A7 (NADH-ubiquinoe oxidoreductase), 2I3A3 (gamma actin-like protein), 2IP10 (ubiquitin C variant 5-like), 1I5B9 (ixodegrin-2A RGD containing protein), 1I1H6 (GST), C2E6 (troponin I), C3B2 (aspartic protease) and 1I4G12 and 1I3H6 with unknown function was significantly downregulated. Interestingly, the mRNA levels were similar in *I.*

scapularis nymphs infected with two different strains of *A. phagocytophilum* but differed from those obtained in infected ISE6 cells for some genes such as U2A8, 1I5B9, 1I4G12, C2E6, C4G3 and R4G5 (Figure 1).

Differential gene expression in A. phagocytophilum-infected I. scapularis nymphs and ISE6 cells differed from that observed after A. marginale infection

The results reported herein showed that gene expression profiles were different for *A. phagocytophilum* and *A. marginale* infected tick cells (Figures 2 and 3). The microarray analysis in infected and uninfected tick ISE6 cells showed that the expression of genes with homology to internal transcribed spacer 1 (probe C3C5), putative secreted salivary protein (C4G3), troponin I (C2E6), aspartic protease (C3B2), ML domain-containing protein (R4G5), H13-prov protein (C4C9), ribosomal protein L32 (R1E12), putative secreted protein (C3F10), ribosomal protein S17 (C4E12), von Willebrand factor (C4B10) and sequences with unknown function (R1A6, C4A8, R3A7, R2A12, C3A7, C3D9, R2D6, C3C11, R3G4, R1F3, C4D2, R3F4, C1H10, C4A4) was different between *A. phagocytophilum* and *A. marginale*-infected cells (Figure 2). The expression of other genes changed in a similar way after infection of tick ISE6 cells with *A. marginale* or *A. phagocytophilum* (C4A10, C3C3, C4A1, R2G1, C4D12, C4G11, C4G9, R3F5, R3D4; Figure 2).

By real-time RT-PCR, the mRNA levels of U2A8 (signal sequence receptor delta), 2I3G1 (proteasome 26S subunit, non-ATPase), 2I3A3 (gamma actin-like protein), 2I1F6 (hematopoietic stem/progenitor cells protein-like), 1I5B9 (ixodegrin-2A RGD containing protein), 1I4G12 (unknown function), 1I3H6 (unknown function), 1I3F5 (ubiquitin) and 1I1H6 (GST) were significantly different between *A. phagocytophilum* and *A. marginale* infected ISE6 cells collected at 3 dpi with approximately 40% infected cells (Figure 3). Except for U2A8, 1I5B9, 1I4G12, C2E6, C4G3 and R4G5 which had different mRNA levels in *A. phagocytophilum*-infected nymphs and tick ISE6 cells (Figure 1), the mRNA levels of the studied genes were also different between *A. phagocytophilum*-infected *I. scapularis* nymphs and *A. marginale*-infected tick cells (data not shown).

Because the kinetics of differentially expressed genes may vary with *Anaplasma* infection levels, the expression of selected genes was compared by real-time RT-PCR in *A. phagocytophilum* and *A. marginale* infected ISE6 cells collected at 2, 5 and 8 dpi (Figure 4(a)). The results showed time-dependent variation in the mRNA ratios of studied genes between *A. phagocytophilum* and *A. marginale* infected cells (Figure 4(b)). However, significant differences were observed between *A. phagocytophilum* and *A. marginale*

infection at all time points, thus suggesting that differences in gene expression profiles elicited by these pathogens are present throughout the infection cycle in tick ISE6 cells.

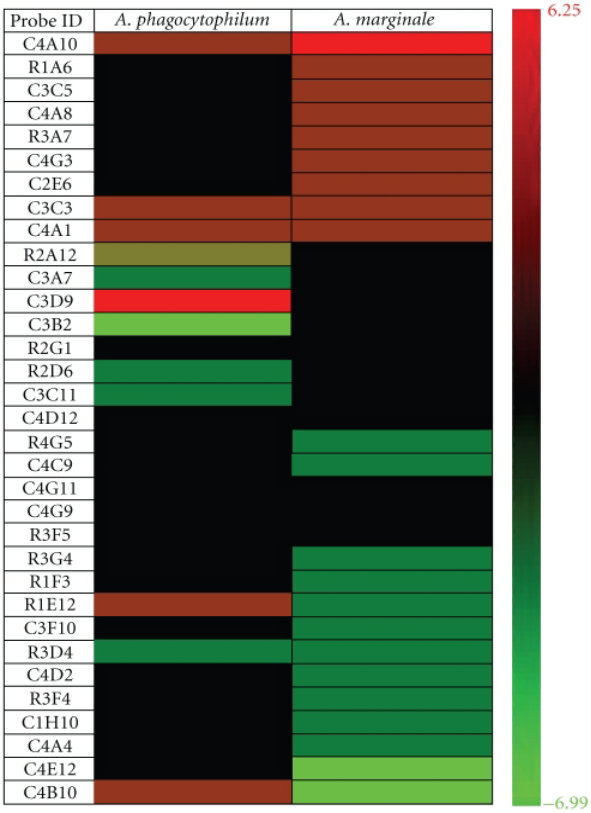

Figure 2. Effect of A. phagocytophilum and A. marginale infection on tick ISE6 cells gene expression. Total RNA was extracted from three A. marginale- infected, three A. phagocytophilum-infected, and three uninfected ISE6 cell cultures. The expression fold change was determined by microarray hybridization at 6 days postinfection (dpi) (approximately 70% infected cells; companion cultures were terminal at 8 dpi). Uninfected cells were sampled at the same time point as infected cells to account for culture time effects. Ratios were calculated as Anaplasma-infected cells versus uninfected control cells. Normalized ratio values obtained for each probe were averaged across 3 biological replicates and four technical replicates and only entries displaying a significant ($P<0.05$) expression fold change >2 in either A. phagocytophilum- or A. marginale-infected cells are shown. Clone ID (library plate and well) are shown. The graph was constructed with the HCE software (http://www.-cs.umd.edu/hcil/hce/hce3.html).

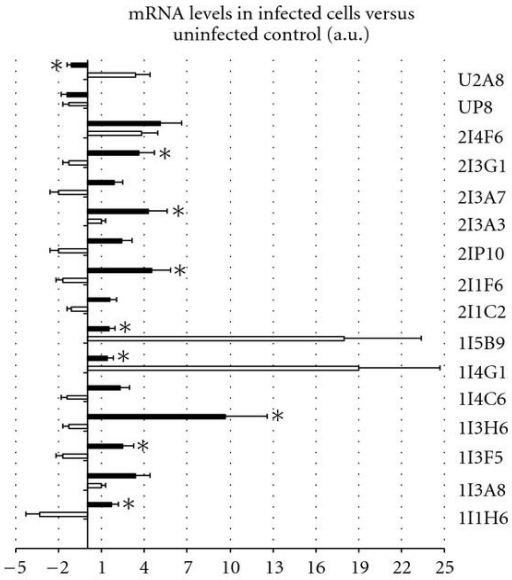

*Figure 3. Effect of A. phagocytophilum and A. marginale infection on tick ISE6 cells gene expression. The mRNA levels were compared between A. phagocytophilum white bars) and A. marginale (black bars) infected tick ISE6 cells by real-time RT-PCR. Bars represent the ratio between infected normalized Ct values and uninfected average normalized Ct values (+SD). The mRNA levels were normalized against tick 16S rRNA and compared between A. phagocytophilum- and A. marginale-infected tick cells by Student's t-test (*P≤0.05). Positive and negative values denote upregulation and downregulation, respectively, with respect to uninfected controls.*

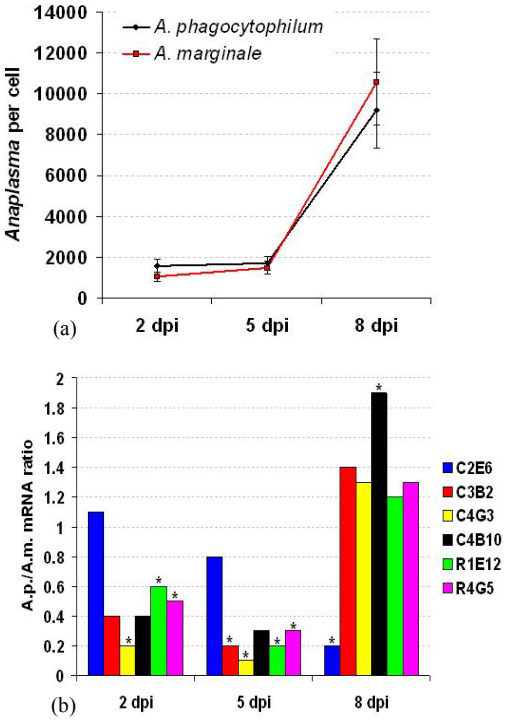

Figure 4. Comparison between differential gene expression in A. phagocytophilum- and A. marginale-infected tick ISE6 cells at different time points after infection. Studies were done on A. phagocytophilum (A.p.) and A. marginale (A.m.) infected tick ISE6 cells (two independent cultures each) at 2, 5, and 8 days postinfection (dpi) with approximately 30%–40%, 60%–70%, and >90% infected cells, respectively. ***(a)*** *The A.p. and A.m. infection levels were evaluated by real-time PCR of msp4 and normalized against tick 16S rDNA. Known amounts of the full length A.p. and A.m. msp4 PCR product were used to construct a standard curve for quantitation of pathogens per cell. Data represent average ± SD.* ***(b)*** *The mRNA levels of selected genes were evaluated by real-time RT-PCR and normalized against tick 16S rRNA. Bars represent the ratio between average Ct values in A.p.-infected cells/average Ct values in A.m.-infected cells. The mRNA levels were compared between A.p.- and A.m.-infected tick cells by Student's t-test (*P ≤0.05). Identical mRNA levels in A. phagocytophilum and A. marginale infected cells equal one.*

Discussion

We have shown previously that *A. marginale* modulates gene expression in infected ticks and tick cells [9]. In the experiment reported herein we hypothesized that *A. marginale* and *A. phagocytophilum* may elicit similar gene expression responses in infected cultured tick cells. To test this hypothesis, gene expression profiles were compared between *A. phagocytophilum* and *A. marginale* in infected tick ISE6 cells. The results showed that *A. phagocytophilum* modulates gene expression in infected *I. scapularis* nymphs and cultured tick ISE6 cells but with different gene expression profiles when compared with *A. marginale*. These results suggested that *A. marginale* and *A. phagocytophilum* produce different differential gene expression profiles in infected tick cells. These differences in *Anaplasma*-tick interactions may reflect differences in pathogen developmental cycle in the tick cells. Alternatively, differences in gene expression profiles between *A. phagocytophilum* and *A. marginale* infected tick ISE6 cells may be due to the fact that *I. scapularis* is not a natural vector of *A. marginale*. However, *I. scapularis* cultured cells have shown to provide functionally relevant data for the study of tick-*A. marginale* interactions [9]. The differences in gene expression between *A. marginale* and *A. phagocytophilum* infected tick cells could be attributed to non-specific responses to the presence of bacterial components that differ between the two *Anaplasma* spp. While this explanation is potentially possible, it is more likely that gene expression profiles resulted from *Anaplasma* intracellular infection because at least for some genes differential expression persisted until 8 dpi, when > 90% cells were infected. Taken together the results reported here consistently provided differences in gene expression profiles between *A. phagocytophilum* and *A. marginale* infected tick cells. Importantly, sampling time points during *Anaplasma* infection of tick ISE6 cells may be important to characterize the expression of particular genes. Although not addressed in this study, these differences may be also present during tick feeding and development.

The genes differentially expressed in *I. scapularis* nymphs and tick ISE6 cells infected with *A. phagocytophilum* included some genes such as GST and ferritin shown previously to affect *A. marginale* infection and/or multiplication in ticks and/or tick cells [9]. However, while GST and ferritin were upregulated and downregulated after *A. marginale* infection, respectively, they were regulated in the opposite direction in *A. phagocytophilum* infected ticks and tick cells. GST, ferritin and aspartic protease (C3B2), also found to be differentially expressed in *A. phagocytophilum*-infected ISE6 cells have

been reported to be regulated by tick feeding or infection with other pathogens [12-18]. Other genes differentially expressed after *A. phagocytophilum* infection such as U2A8 (signal sequence receptor delta), 1I5B9 (ixodegrin-2A RGD containing protein), 2I3A7 (NADH-ubiquinone oxidoreductase), 2IP10 (ubiquitin C variant 5-like), 2I3A3 (gamma actin-like protein), C4B10 (von Willebrand factor), C2E6 (troponin I) and R1E12 (ribosomal protein L32) constitute new findings and may be involved in infection and/or multiplication of the pathogen in ticks or are part of tick cell immune response to moderate infection levels. As shown previously for *A. marginale* [9], RNA interference experiments may help to characterize the function of differentially expressed genes during *A. phagocytophilum* infection of ticks and tick ISE6 cells.

The expression of selected genes was analyzed in *I. scapularis* ISE6 cells and nymphs infected with *A. phagocytophilum*. These experiments allowed us to compare the results of gene expression in vitro and in vivo. The nymphs were selected for analysis because this stage plays an important role during pathogen transmission to humans [5]. The ISE6 cell line was obtained from embryos of *I. scapularis*, one of the natural vectors of *A. phagocytophilum* [19]. However, this cell line is heterogeneous in the cell types represented [19], which may have different susceptibility and response to pathogen infection. Although cultured tick cells have been shown to be a good model for the study of tick-*Anaplasma* interactions [19-21], these results demonstrated that differences may exist between *I. scapularis* cultured tick cells and nymphs in the mRNA levels of certain genes, at least under the experimental conditions used herein. These differences may account for differences in gene expression between infected ISE6 cells and tick whole nymph tissues and/or due to differences in the infection levels in both systems. An additional source of potential differences in gene expression between *I. scapularis* ISE6 cells and nymph tissues could be attributed to the fact that different cells types may be infected in both systems resulting in different responses to infection. Finally, although less likely, these differences may be related to differences between the NY18 isolate used to infect ISE6 cells and the Gaillard and Dawson strains used to infect *I. scapularis* nymphs.

Recent studies have characterized *A. marginale* and *A. phagocytophilum* proteins that are involved in interactions with tick cells [7, 8, 22]. However, tick-*Anaplasma* co-evolution also involves genetic traits of the vector as demonstrated recently in studies on the role of tick proteins in the infection and transmission of *A. marginale* [9, 11] and *A. phagocytophilum* [10,11]. Furthermore, genetic factors have been associated with intraspecific variation in vector competence for a variety of vector-borne pathogens, including *A. phagocytophilum* [23] and *A. marginale* [24, 25].

Conclusions

In summary, we have characterized the gene expression profile in *A. phagocytophilum*-infected *I. scapularis* nymphs and cultured ISE6 cells. Interestingly, differential gene expression seems to differ between *A. marginale* and *A. phagocytophilum* infected cultured tick cells. Future experiments would provide detailed information on the role of these genes during *A. phagocytophilum* life cycle in ticks. These results provide fundamental information toward understanding tick-*Anaplasma* interactions and may lead to formulations of new interventions for the prevention of the transmission of tick-borne pathogens.

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Chapter 8

Summarizing discussion

Contents

8.1 Introduction

Despite the importance of bovine anaplasmosis for livestock production no satisfactory control methods are available. The most efficient way to prevent disease is by vaccination. The development of effective and safe vaccines requires better knowledge on the tick-host-pathogen interactions. Studies of the molecular events that mediate interactions between hosts and *Anaplasma marginale* have been the focus of recent investigations. However, most of this research has focused on the vertebrate host-pathogen interactions rather than those between the tick and the pathogen. In this dissertation, a series of experiments were designed to uncover new vectors and some of the mechanisms involved in *A. marginale*-tick interactions.

8.2 New experimental vector of *A. marginale*

Although *A. marginale* infection in cattle and wild ruminants has been reported in several parts of Europe, the epidemiology of anaplasmosis has not been thoroughly investigated and local vector tick species have not been identified. Recent outbreaks of anaplasmosis in the more northern latitudes, in countries previously thought to be free of anaplasmosis [1-3], warranted studies on the role of putative vector ticks. The broad distribution range of *D. reticulatus*, as well as recently reported increase in distribution in Germany, Hungary and the Netherlands, prompted to study this tick as a vector for *A. marginale* in Europe [4-6]. We have demonstrated that male *Dermacentor reticulatus* ticks are competent as experimental biological vectors of *A. marginale* by intrastadial transmission (***Chapter 2***). The transmission of *A. marginale* occurred when ticks were transferred from infected to a naïve calf; *A. marginale* colonies were detected by light microscopy in the tick salivary glands. The *A. marginale* strain used in this study originated from Zaria, Nigeria. *A. marginale* strains are shown to vary geographically in genotype, morphology and infectivity for ticks. Therefore, further studies are needed to confirm the vectorial capacity of *D. reticulatus* in Europe using of *A. marginale* isolates derived from naturally infected cattle and ticks from endemic areas.

8.3 Functional genomics studies of tick cells in response to infection with *A. marginale*

Ticks and the pathogens that they transmit have co-evolved complex relationships to ensure their mutual survival. Although, recent studies have demonstrated the presence of receptors

for particular tick borne pathogens [7, 8], a tick receptor for *A. marginale* remains unknown. In ***Chapter 3*****,** a functional genomics approach was used to identify and characterize tick genes/proteins differentially expressed in response to *A. marginale* infection. The global tick cell response to *A. marginale* infection was characterized by suppression subtractive hybridization (SSH) in IDE8 cultured tick cells. *A. marginale* infection affected many molecular functions such as protein binding and metabolism, enzymatic/catalytic activities, structural molecules, transporter activity, DNA/RNA metabolism and stress whereas most of the molecules affected are of unknown function. In addition, the proteome of IDE8 cells was compared between uninfected and *A. marginale* infected cells by differential in-gel electrophoresis analysis.

Some of the tick genes identified as differentially regulated in response to *A. marginale,* such as selenoproteins, GST, vATPase, and ferritin, have been reported to be regulated by tick blood feeding or infection with other pathogens [9-14]. However, other genes such as ubiquitin, proteasome 26S subunit, and hematopoietic stem/progenitor cells protein-like constitute new findings of tick genes differentially regulated in response to pathogen infection. The results of RNAi suggest that the genes identified as differentially regulated in *A. marginale*-infected IDE8 tick cells may perform different functions during the infection, trafficking, and multiplication of the pathogen in ticks and provide additional evidence for the distinct role that guts and salivary glands play on *Anaplasma* infection and transmission by ticks. The putative GST, vATPase, and ubiquitin may be involved in the initial *A. marginale* infection of tick gut cells. Results of the RNAi experiments also suggest that GST and salivary selenoprotein M expression may be involved in trafficking and/or infection and multiplication of the pathogen in tick salivary glands. Furthermore, RNAi experiments in IDE8 tick cells provide evidence that putative selenoprotein W2a and hematopoietic stem/progenitor cells protein-like may participate in the cellular response to limit pathogen infection, while proteasome 26S subunit, ferritin, and GST may enhance *A. marginale* multiplication in tick cells. Except for the putative ubiquitin and GST, which affected tick survival and attachment, respectively, RNAi of differentially regulated genes did not produce notable effects on tick tissues and feeding.

The effect of silencing GST, SelM, vATPase and subolesin on *A. marginale* development and infection levels in *D. variabilis* ticks both after acquiring infection and after transmission feeding was characterized by quantitative PCR and light microscopy (***Chapter 5***). The results support previous findings and suggest a role for these molecules during pathogen life cycle in tick. In general, the number of *A. marginale* colonies was lower in most tissues in gene knockdown ticks when compared to controls. Notably,

colonies were not seen by light microscopy in salivary glands of the gene silenced ticks, suggesting that transmission may be diminished or prevented. The results suggest that *A. marginale* may increase the expression of GST and SelM to reduce the oxidative stress caused by pathogen infection and in that way increase pathogen multiplication. The vATPse may be involved in pathogen infection of tick guts and salivary glands by facilitating pathogen infection through the receptor-mediated endocytosis. Tick subolesin, was confirmed to play a role in different molecular pathways including those involved in infection and multiplication of the pathogen in the tick. Collectively, the data suggest that tested genes may be candidate genes for transmission blocking vaccines to control bovine anaplasmosis.

Salivary glands are the critical site in the developmental cycle of *A. marginale,* from where the pathogen is directly transmitted to the bovine host. Recent studies have shown that tick salivary gland proteins play an important role in infection and transmission of tick-borne pathogens [7, 8, 15, 16]. The tick cell response to infection with *A. marginale* was studied in *R. microplus* male salivary glands as described in ***Chapter 4*** of this thesis. A similar approach was used for the identification and functional studies of differentially expressed genes. Some of the genes identified in this study, such as those encoding putative tick cement proteins, female specific histamine binding protein, IgG binding protein C, salivary gland-associated protein 64P, flagelliform silk protein and von Willebrand factor, were identified previously in different tick species and appear to be involved in tick feeding or pathogen infection [11, 17-19]. However, most of the genes identified to be differentially expressed in *R. microplus* salivary glands are novel (not previously identified) genes. Interestingly, genes identified in ***Chapter 3***, such as GST, selenoprotein M and ferritin genes were also found to be differentially expressed in *R. microplus* salivary glands in response to *A. marginale* infection. However, these genes were absent from the EST dataset obtained from *R. microplus* salivary glands, which could be due to differences in the system used for EST discovery (cultured IDE8 tick cells versus *R. microplus* salivary glands) and/or other factors such as the tick species and/or *A. marginale* strain and infection levels. Five genes encoding for putative histamine-binding protein (22Hbp), von Willebrand factor (94Will), flagelliform silk protein (100Silk), Kunitz-like protease inhibitor precursor (108Kunz) and proline-rich protein BstNI subfamily 3 precursor (7BstNI3) were confirmed by real-time RT-PCR to be down-regulated in tick salivary glands infected with *A. marginale*. Functional studies suggest that differentially expressed genes encoding for subolesin, putative von Willebrand factor and flagelliform silk protein could play a role in *A. marginale* infection and multiplication in tick salivary glands.

Cultured tick cells have provided a valuable tool for the study of tick-pathogen interactions [20]. Most of these studies have been done using *I. scapularis* cell lines (IDE8 or ISE6). In ***Chapter 4*** of this thesis, BME26 tick cell line derived originally from *R. microplus*, a natural vector of *A. marginale,* was used for the first time for functional studies on tick-pathogen interactions. Since these studies were conducted on ticks and tick cells of the same species, most of the genes identified in tick salivary glands were also amplified from cultured BME26 tick cells. RNAi experiments in cultured BME26 tick cells provided further evidence that flagelliform silk protein (100Silk) and subolesin may play a role in *A. marginale* infection and/or multiplication in tick cells and suggest that metallothionein (93Meth) may be involved in defense against pathogen infection in ticks.

8.4 Subolesin and *A. marginale*–tick interactions

The tick subolesin was recently discovered as a tick protective antigen in *I. scapularis* [21]. Subolesin was shown by both RNAi gene knockdown and immunization trials using the recombinant protein to protect hosts against tick infestations, reduce tick survival and reproduction, and cause degeneration of gut, salivary gland, reproductive tissues and embryos [22-26]. The targeting of tick subolesin by RNAi or immunization also resulted in decreased vectorial capacity of ticks for *A. marginale* and *A. phagocytophilum* [16]. Consistent with these results, in the experiments reported in ***Chapter 5***, subolesin knockdown resulted in gut and salivary gland degeneration and affected the development of both dense and reticulated forms of *A. marginale* in the gut and the movement to and infection of salivary glands. Subolesin was recently found to be similar to insect and vertebrate akirins in the control of NF-kB-dependent and independent gene expression in ticks [27]. These results suggest that subolesin expression would likely be affected by pathogen infection and to have a role in tick innate immunity, a hypothesis that was tested in the experiments reported in ***Chapter 6***. Subolesin expression levels were investigated in several tick species infected with variety of pathogens, including *A. marginale*. Furthermore, the effect of subolesin knockdown on pathogen infection was also studied.

The subolesin expression varies with pathogen infection in tick salivary glands and in the gut in response to some pathogens. In *A. marginale*-infected ticks subolesin was up-regulated in infected salivary glands. However, *A. marginale* infection did not affect subolesin expression in the gut of infected ticks. This suggests that subolesin may play a role in tick innate immunity in salivary glands but not in the gut. Since the gut is the first tick barrier to pathogen infection, subolesin may not be involved in protecting ticks against

A. marginale infection because of mechanisms that have co-evolved between the pathogen and the tick vector to support pathogen transmission while ensuring tick survival. However, subolesin may function in the salivary glands to limit pathogen infection to levels that are not detrimental for ticks. Similar results were obtained for *I. scapularis* nymphs infected with *A. phagocytophilum*, but differences were observed in the tick response to other pathogens, as illustrated in *Hyalomma lusitanicum* ticks infected with *Babesia bigemina*.

The effect of subolesin knockdown in ticks on infection with tick-borne and non-tick-borne organisms was tested in *D. variabilis* using RNAi and capillary feeding (CF). In these experiments, infection levels of the tick-borne pathogens, *A. marginale*, *A. phagocytophilum* and *hrlichia canis* were lower in subolesin-silenced ticks. In contrast, *Francisella tularensis* infections were higher after subolesin RNAi and CF. For non-tick-borne pathogens, *Escherichia coli* and *Pichia pastoris*, RNAi experiments suggested that subolesin did not have an effect on infection, at least after CF. However, subolesin expression was up-regulated in *D. variabilis* exposed to *E. coli*, suggesting that although this pathogen may induce subolesin expression in ticks, silencing of this molecule reduced bacterial multiplication by a presently unknown mechanism.

These studies demonstrate that subolesin may play a role in tick innate immunity in the salivary glands by limiting pathogen infection levels but may activate innate immunity to some pathogens in the guts and other tissues. Furthermore, these results provide additional evidence for the role of subolesin in different molecular pathways, including those required for tick physiology and pathogen infection and multiplication in ticks. Consequently, subolesin knockdown in ticks may affect pathogen infection directly by reducing the innate immune response, thus resulting in higher infection levels and indirectly by affecting gene expression that interferes with tissue physiology and pathogen infection and multiplication. The direct or indirect effects of subolesin knockdown on pathogen infection would depend on several factors including the evolution of tick-pathogen molecular interactions, pathogen tick life cycle and other presently unknown mechanisms mediated by subolesin function in the control of global gene expression in ticks.

8.5 Tick molecules involved in interactions with *A. phagocytophilum*

A. phagocytophilum is another member of the genus Anaplasmataceae, that infects humans and wild and domesticated animals [28]. Tick molecules that are affected by

A. phagocytophilum infection and multiplication are not well characterized. The results of our study (***Chapter 7***) demonstrate modulation of tick gene expression by *A. phagocytophilum*. We hypothesized that *A. marginale* and *A. phagocytophilum* may elicit similar gene expression responses in infected cultured tick cells. To test this hypothesis, gene expression profiles were compared between *A. phagocytophilum* and *A. marginale* in infected tick ISE6 cells by microarray and real-time RT-PCR analyses. The results showed that *A. phagocytophilum* modulates gene expression in infected *I. scapularis* nymphs and cultured tick ISE6 cells, but with different gene expression profiles when compared with *A. marginale*. These differences in *Anaplasma*-tick interactions may reflect differences in pathogen developmental cycle in tick cells. Alternatively, differences in gene expression profiles between *A. phagocytophilum*- and *A. marginale*-infected tick ISE6 cells may be due to the fact that *I. scapularis* is not a natural vector of *A. marginale*. Sampling time points during *Anaplasma* infection of tick ISE6 cells may be important to further characterize the expression of particular genes.

The genes differentially expressed in *I. scapularis* nymphs and tick ISE6 cells infected with *A. phagocytophilum* included some genes such as GST and ferritin shown previously to affect *A. marginale* infection and/or multiplication in ticks and/or tick cells (***Chapter 3***). However, while GST and ferritin were up-regulated and down-regulated after *A. marginale* infection, respectively, they were regulated in the opposite direction in *A. phagocytophilum*-infected ticks and tick cells. RNAi experiments are required to further characterize the function of differentially expressed genes during *A. phagocytophilum* infection of ticks and tick ISE6 cells.

8.6 Concluding remarks

Tick transmission studies and phylogenetic analysis have provided evidence of a complex tick-*Anaplasma* co–evolutionary relationship and suggested that genetic traits from both the vector and the pathogen are required for successful infection and transmission of the pathogen by ticks. For *A. marginale*, MSPs and other proteins are involved in interactions with vertebrate and invertebrate host cells. The results shown in this thesis suggest that tick molecules play an important role in tick-pathogen interactions as well. The studies demonstrate that molecular mechanisms exist by which tick cell gene expression mediates the *A. marginale* development and trafficking through ticks. *A. marginale* infection affects several tick cell pathways and differential gene expression varies between *A. marginale* and *A. phagocytophilum* infected ticks. The data obtained for the tick protein subolesin provide additional evidence of its role during *A. marginale* developmental cycle in ticks and show that it may play a role in tick innate immunity to pathogens. Collectively, these data will be important for providing the basis for new strategies for development of effective dual-action vaccines that will control both tick infestation and transmission of *A. marginale*.

8.7 Future perspectives

The knowledge on molecular interactions at the *A. marginale*-tick interface is still limited. Different approaches could be used for the molecular dissection of the complex tick-pathogen relationships. In the studies described in this thesis differentially expressed genes were identified by using suppression-subtractive hybridization approach [29]. Recently, a *R. microplus* microarray (NimbleGen) has been developed and used for the analysis of acaricide-inducible genes in *R. microplus* [30]. Therefore, microarray chip hybridization could be used in the future as an alternative approach for identifying differentially expressed genes in response to *A. marginale* infection.

More comprehensive understanding of tick pathogen interface requires characterization of the function of identified genes. For the short time that it has been available, RNA interference (RNAi) was shown to be a valuable tool for studying tick gene function and the functional characterization of genes involved in tick-host-pathogen interactions [31]. Although RNAi seems to be very sequence-specific in ticks, the possibility of off-target gene silencing effects cannot be excluded due to the limited amount of tick sequence data available. Availability of the complete tick genome sequence data will facilitate screening for potential off-target effects.

The absence of suitable laboratory animal model as well as the lack of long term in-vitro culture system, have placed severe constraints on the progress of anaplasmosis research. The first continuous in vitro culture system for *A. marginale* was established in tick derived cell IDE8. Since then, *A. marginale* has been successfully passaged in other tick cell lines including a natural vector tick cell line (BME26). Cultured tick cells have provided a valuable tool for study of *A. marginale*-tick interactions. However, tissue-specific differences in gene expression between gut and salivary glands and between ticks and IDE8/BME26 tick cells were observed in our experiments, indicating that results of gene expression studies *in vitro* should be validated in infected and uninfected vector tick species and in different tick tissues.

Although bovine anaplasmosis presents a major problem in tropical and subtropical areas, recent reports of outbreaks in more northern latitudes indicate that the distributional range is likely to increase in the future. The knowledge of potential vector tick species from new areas is therefore necessary. In this thesis, *D. reticulatus* ticks were shown to be able to transmit *A. marginale* under experimental conditions. Considering the distribution and abundance of *D. reticulatus* in Europe, this tick species may play an important role in transmission of bovine anaplasmosis. The recent finding of increase in *D. reticulatus* populations in The Netherlands indicates that in cases of an anaplasmosis outbreak, it could play an important role in the epidemiology of the disease. Future studies are required that will confirm the role of this tick species in transmission of anaplasmosis in endemic areas. Moreover, other tick species should be also studied, including *D. marginatus* which is also common on cattle and wildlife reservoir hosts.

The research described here represents the first step in discovering complex interactions that are likely to be the result of millions of years long co–evolution between ticks and *A. marginale*. Rapidly increasing genomics information from the sequencing of tick genomes together with the development of novel molecular technologies that are suitable for tick research will certainly provide valuable novel insight in tick-pathogen interactions. The knowledge gained will be important for the design of the effective vaccines to control tick infestations and pathogen transmission. However, it is likely that vaccines for the control of bovine anaplasmosis will contain a combination of both pathogen and tick–derived antigens.

8.8 References

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Summary

Anaplasma marginale, an obligate intracellular gram negative rickettsia, is the causative agent of bovine anaplasmosis, a tick- borne disease of cattle. The disease occurs in tropical and subtropical regions of the world and causes economically important losses to the cattle industry. Despite its importance no satisfactory control methods are available. The development of new vaccine formulations capable of preventing transmission would have significant economic impact. This approach would require a better understanding of mechanisms underlying the complex interactions between *A. marginale* and its vector ticks. Recent studies have provided evidence that *Anaplasma* infection and transmission is mediated by molecular mechanisms involving both tick cell and pathogen genes. The research presented in this thesis has delineated new molecular interactions that occur at the tick–pathogen interface by identification and characterization tick genes/proteins that facilitate infection and multiplication of *A. marginale*.

During the course of the experiments described in this thesis, a new tick species is tested for the vector competence of *A. marginale*. It was found that *Dermacentor reticulatus* male ticks are competent biological vectors of *A. marginale* by intrastadial transmission (***Chapter 2***). *D. reticulatus* ticks are commonly found in the Northern part of Europe and may play a role in epidemiology of anaplasmosis in these regions. Further studies are needed to confirm the vector competency of this tick species for other *A. marginale* strains from endemic areas in Europe.

The *A. marginale* development in the ticks is mediated by tick cell gene expression. In ***Chapter 3*****,** a functional genomics approach was used to identify and characterize genes/proteins in tick cells differentially expressed in response to *A. marginale* infection. The results suggested that gluthathion S-transferase (GST), H^+ transporting lysosomal vacuolar proton pump (vATPase) and ubiquitin may be in initial infection of the tick gut cells, while GST and selenoprotein M (SelM) may be involved in trafficking and/ or infection of the pathogen in tick salivary glands. Furthermore, the experiments in IDE8 tick cell line suggested that selenoprotein W2a and hematopoietic stem/progenitor cell protein-like may play a role in limiting pathogen infection, while proteasome 26S subunit, ferritin and GST may enhance multiplication.

Similar approach was used to identify genes differentially expressed in *Rhipicephalus* (*Boophilus*) *microplus* male salivary glands in response to *A. marginale* infection (***Chapter 4***). *Anaplasma* infection in *R. microplus* was shown to modulate the expression of the salivary glands genes with various functions. Functional studies suggested

that differentially expressed genes encoding for subolesin, putative von Willebrand factor and flagelliform silk protein could play a role in *A. marginale* infection and multiplication in ticks, while metallothioneins may play a role in defence against bacterial infections. In addition, for the first time a *R. microplus* derived cell line, BME26, was used to study *A. marginale*-tick molecular interactions and the results supported the finding that subolesin and flagelliform silk protein may be required for infection and multiplication in tick cells.

Four genes, found to be differentially expressed (***Chapter 3***), were silenced by RNAi and the effect of silencing on the *A. marginale* development in ticks was studied, both after acquiring infection and after a second transmission feeding, by light microscopy and quantitative PCR (***Chapter 5***). The genes studied encoded for putative GST, SelM a, vATPase and subolesin. Silencing of these genes had a different impact on *A. marginale* development in different tick tissues by affecting infection levels, the densities of colonies containing reticulated or dense forms and tissue morphology. These results suggested that *A. marginale* may increase the expression of SelM and GST to reduce oxidative stress by pathogen infection and in that way increase multiplication in the tick cells. The vATPase may be involved in pathogen infection of tick gut and salivary gland cells by facilitating pathogen infection. For the tick subolesin, results showed that it affects multiple molecular pathways required for *A. marginale* infection and multiplication in ticks. Interestingly, salivary gland infections were not seen in any of the gene silenced ticks raising question weather these ticks were able to transmit the pathogen

The genes identified (***Chapters 3, 4, 5***) may be candidates for the transmission blocking vaccines for control of bovine anaplasmosis. However, experiments will be required to further determine role of identified genes.

Tick subolesin was recently discovered as a tick protective antigen in *I. scapularis.* Subolesin was shown to protect hosts against tick infestations, reduce tick survival and reproduction, cause degeneration of gut, salivary gland, reproductive tissues and decrease vector capacity of ticks for *A. marginale* and *A. phagocytophilum*. Subolesin was recently found to be similar to insect and vertebrate akirins in the control of NF-kB-dependent and independent gene expression in tick. These results suggested that subolesin expression would likely be affected by pathogen infection and to have a role on tick innate immunity, a hypothesis that was tested in the experiments reported in the ***Chapter 6***.

It was found that subolesin expression varies with pathogen infection in tick salivary glands and in the guts in response to some pathogens, thus suggesting a role of subolesin in tick innate immunity. Subolesin may play a role in tick innate immunity in the salivary glands by limiting pathogen infection levels but may activate innate immunity

to some pathogens in the gut and other tissues. Furthermore, these results provided additional evidence for the role of subolesin in different molecular pathways, including those required for tick physiology and pathogen infection and multiplication in ticks. Consequently, subolesin knockdown in ticks may affect pathogen infection directly by reducing the innate immune response, thus resulting in higher infection levels and indirectly by affecting gene expression that interferes with tissue physiology and pathogen infection and multiplication.

A. phagocytophilum is another member of the genus *Anaplasma*, which infects humans and wild and domestic animals. Tick molecules that are affected by *A. phagocytophilum* infection and multiplication are not well characterized. Our study demonstrated modulation of tick gene expression by *A. phagocytophilum* (***Chapter 7***). The gene expression profiles were characterized in *A. phagocytophilum* infected ISE6 cells and *I. scapularis* nymphs. Interestingly, the analyses showed that *A. marginale* and *A. phagocytophilum* elicit different tick cell gene responses in infected cultured tick cells. These differences in *Anaplasma*-tick interactions may reflect differences in pathogen developmental cycle in the tick cells. Alternatively, differences in gene expression profiles between *A. phagocytophilum*- and *A. marginale*-infected tick ISE6 cells may be due to the fact that *I. scapularis* is not a natural vector of *A. marginale*.

The work described in this thesis adds significantly to better understanding of mechanisms underlying *Anaplasma*-tick interactions and will contribute to the development of a new generation transmission-blocking vaccines designed to prevent transmission and reduce exposure of vertebrate hosts to tick –borne pathogens.

Samenvatting

Anaplasma marginale, een obligaat intra-cellulair Gram-negatieve rickettsia, veroorzaakt anaplasmose, een door teken overgedragen ziekte bij het rund. De ziekte komt voor in tropische en sub-tropische gebieden en veroorzaakt belangrijke economische schade aan de rundveehouderij. Desondanks zijn er geen afdoende bestrijdingsmethoden beschikbaar. De ontwikkeling van nieuwe vaccins die de overdracht door teken zouden kunnen blokkeren, zijn dus economisch interessant. Deze benadering vereist een beter begrip van de mechanismen die ten grondslag liggen aan de complexe interacties tussen *A. marginale* en vector teken. Recente studies hebben aangetoond dat er bij *Anaplasma* infecties ook genen van de teek betrokken zijn. In het onderzoek, zoals in dit proefschrift beschreven, zijn nieuwe moleculaire interacties opgespoord die plaats vinden tussen *A. marginale* en vector teken, waarbij een aantal genen in teken zijn geïdentificeerd die de infectie met *A. marginale* lijken te faciliteren.

Eén van de experimenten beschreven in dit proefschrift betrof het uittesten van de vector competentie van *Dermacentor reticulatus* teken voor *A. marginale*. Het bleek dat mannelijke *D. reticulatus* teken is staat zijn om *A. marginale* intrastadieel over te brengen bij het rund (***hoofdstuk 2***). *D. reticulatus* komt voor in Noord Europa, waar deze soort een rol als vector zou kunnen spelen. Nader onderzoek is nodig om de vector competentie van deze soort te bepalen voor *A. marginale* stammen die voorkomen in endemische gebieden elders in Europa.

In ***hoofdstuk 3*** is een functionele genoom analyse toegepast om genen in teken te identificeren en te karakteriseren die differentieel tot expressie komen na een infectie met *A. marginale*. De resultaten wezen erop dat glutathion S-transferase (GST), vATPase en ubiquitine betrokken zijn bij de invasie van *A. marginale* in darmcellen van de teek, terwijl GST en selenoprotein M (SelM) mogelijk betrokken zijn bij de invasie van *A. marginale* in de speekselklier van de teek. Bovendien bleek uit experimenten *in vitro* in de teken cellijn IDE8 dat selenoprotein W2a en hematopoietic stem/progenitor cell protein-like, de infectie met *A. marginale* afremmen, terwijl proteasome 26S subunit, ferritine en GST de ontwikkeling van *A. marginale* juist stimuleren.

Dezelfde benadering werd gebruikt voor de identificatie van genen in de speekselklieren van mannelijke *Rhipicephalus (Boophilus) microplus* teken na een infectie met *A. marginale* (***hoofdstuk 4***). Een functionele genoom analyse had tot resultaat dat de genen die coderen voor subolesin, von Willebrand factor en flagelliform silk protein

mogelijk een rol spelen bij de vermeerdering van *A. marginale* in teken, terwijl metallothioneins wellicht een rol spelen bij de afweer tegen bacteriële infecties in teken. Ook werd er voor het eerst in een teken cellijn afkomstig van *R. microplus* (BME26) gevonden dat subolesin en flagelliform silk protein nodig zijn voor de vermeerdering van *A. marginale* in teken cellen.

Vervolgens werden vier genen, GST, SelM, vATPase en subolesin, uitgeschakeld in teken door middel van RNA interferentie, waarna de ontwikkeling van *A. marginale* in deze teken werd bestudeerd met licht microscopie en kwantitatieve PCR (***hoofdstuk 5***). De conclusie die uit de resultaten kon worden getrokken was dat *A. marginale* kan leiden tot een hogere expressie van SelM en GST waardoor mogelijk de oxidatieve stress door de infectie wordt verlaagd en zodoende de vermeerdering van *A. marginale* toeneemt. Verder zou vATPase betrokken zijn bij de invasie van *A. marginale* in darm en speekselklieren van de teek. Tenslotte bleek dat subolesin verschillende moleculaire pathways beïnvloedt die van belang zijn voor de ontwikkeling van *A. marginale* in teken. Het is interessant om te vermelden dat er géén *A. marginale* infecties werden aangetroffen in speekselklieren van de teken waarin genen waren uitgeschakeld, hetgeen de vraag oproept of deze teken nog wel in staat waren om *A. marginale* over te dragen.

De genen zoals in geïdentificeerd in ***hoofdstuk 3, 4***, en ***5*** zijn mogelijke kandidaten voor vaccins bedoeld om de transmissie van *A. marginale* te kunnen blokkeren. Verder onderzoek is nodig om de mogelijke rol van deze genen voor vaccin ontwikkeling nader te bepalen.

Subolesin is recent geïdentificeerd in *Ixodes scapularis* teken en met succes gebruikt als anti-teken vaccin. Bovendien is aangetoond dat subolesin de vector capaciteit voor *A. marginale* en ook voor *Anaplasma phagocytophilum* verlaagd. Verder is ook gevonden dat subolesin vergelijkbaar is met akirinen in insecten en vertebraten voor wat betreft de regulering van NF-κB-afhankelijke en onafhankelijke gen expressie in teken. Dit suggereert dat subolesin een rol speelt in de natuurlijke afweer in teken tegen pathogenen, een hypothese die in ***hoofdstuk 6*** werd getest.

Het bleek dat subolesin expressie werd beinvloedt door de aanwezigheid van bepaalde pathogenen in darm en speekselklieren van de teek. Subolesin leek de infectie in de speekselklieren te beperken, maar daarentegen te stimuleren in de darm en andere weefsels. De resultaten wezen erop dat subolesin een rol speelt in verschillende moleculaire pathways. Het uitschakelen van subolesin in teken zou het infectie nivo direct kunnen beïnvloeden door een verlaging van de natuurlijke immuniteit in teken tegen pathogenen te

bewerkstelligen. Indirect zou een veranderde gen expressie kunnen resulteren in veranderingen in weefsel fysiologie en daardoor de infectie met *A. marginale*.

Tenslotte is de functionele genoom analyse ook toegepast op *A. phagocytophilum,* een *Anaplasma* soort die zowel bij de mens als bij wilde en gedomesticeerde dieren voorkomt **(*hoofdstuk 7*)**. Er werd gekeken naar de expressie profielen van genen in de teken cellijn ISE6 en in *I. scapularis* geinfecteerd met *A. phagocytophilum* of met *A. marginale*. Het bleek dat *A. phagocytophilum* en *A. marginale* verschillende gen expressie patronen opwekten in teken cellijnen. Deze verschillen zijn wellicht veroorzaakt door verschillen in ontwikkelingscyclus van beide *Anaplasma* soorten *in vitro*. Het is ook mogelijk dat deze verschillen te maken hebben met het feit dat *I .scapularis*, waar de teken cellijn van afkomstig is, wel vector is van *A. phagocytophilum* maar niet van *A. marginale*.

Het onderzoek zoals beschreven in dit proefschrift draagt bij aan een beter begrip van de moleculaire interacties tussen *Anaplasma* infecties en vector teken, hetgeen kan leiden tot de ontwikkeling van een nieuwe generatie vaccins die in staat zijn om de transmissie door teken te blokkeren en om zodoende het risico op *Anaplasma* infecties bij het rund te reduceren.

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Zorica

Curriculum Vitae

Zorica Živković was born in Zaječar (Serbia) on the 30th of May 1979, but she lived in Bor for the first 19 years of her life. She graduated from Gymnasium (natural and mathematical scientific orientation) in 1998 and started her studies at the Faculty of Veterinary Medicine in Belgrade, Serbia. After obtaining her Doctor of Veterinary Medicine diploma she carried out clinical work at the small animal practice in Belgrade. In October 2005 she became a PhD student at the Utrecht Centre for Tick-borne Diseases working on tick-pathogen interactions in bovine anaplasmosis. During her PhD (2005-2010) she was a visiting researcher at the Center for Veterinary Health Sciences (Oklahoma State University, USA), Instituto de Investigación en Recursos Cinegéticos IREC (Spain) and Facultad de Medicina Veterinaria y Zootecnia (Universidad Autónoma de Tamaulipas, Mexico). The results obtained in this period are written down in this thesis.

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