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STUDY OF *RICKETTSIA PARKERI* COLONIZATION AND PROLIFERATION IN

AMBLYOMMA MACULATUM (ACARI: IXODIDAE)

by

Khemraj B.C.

A Dissertation
Submitted to the Graduate School
and the Department of Biological Sciences
at The University of Southern Mississippi
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

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May 2017

Published by the Graduate School

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ABSTRACT

STUDY OF *RICKETTSIA PARKERI* COLONIZATION AND PROLIFERATION IN

AMBLYOMMA MACULATUM (ACARI: IXODIDAE)

by Khemraj B.C.

May 2017

Amblyomma maculatum (Gulf coast tick) ticks are prevalent across the
Atlantic to Gulf Coast region of United States. These ticks are recognized vectors
of *Rickettsia parkeri*, a spotted fever group of *Rickettsia* (SFGR) known to cause
American boutonneuse fever associated with fever and eschar rashes localized
to the site of bites. We hypothesized that *Rickettsia parkeri* colonization and
proliferation in the tick vector involve pathogen-symbiont dynamics and tick-
pathogen interactions, which influence rickettsial transmission to the victims after
tick bites. The rickettsial infection is maintained across the tick life cycle for many
generations due to transovarial and transstadial transmission of the pathogen. In
the first part, we hypothesized that dynamic interaction among pathogenic *R.
parkeri* and other tick symbionts inside the tick favors the rickettsial pathogen to
survive, which multiply and infect vertebrates host upon infestation. We
maintained *R. parkeri* infected and uninfected tick colonies in our lab to study
their colonization with I tick. The bacterial loads for *R. parkeri*, *Francisella* like
endosymbionts and "*Candidatus* Midichloria mitochondrii" were estimated in both
tick colonies during different life stages and within various tick organs that are
vital for blood-feeding, reproduction, and disease transmission by using specific
qRT-PCR primers. Our results showed that *R. parkeri* thrives when CMM is

present but displaces FLE along the tick life-cycle. In the second part, we
hypothesized that tick SECIS binding protein (*SBP2*) and selenoprotein P
(*SELENOP*) are essential in selenoprotein biosynthesis, and thereby play a role
in overall tick redox balance and rickettsial colonization. RNAi assays were
employed to specifically silence tick *SBP2* and *SELENOP*. The silencing of *SBP2*
and *SELENOP* impaired synthesis of many known selenoproteins except
selenophosphate synthetase (*SEPHS2*) and selenoprotein O (*SELENOO*).
Neither of the genes impaired tick feeding, but *SBP2* silencing significantly
impacted tick oviposition success and egg hatching. The silencing of *SBP2*
further impaired rickettsial colonization and reduced transovarial transmission,
whereas the *SELENOP* did not show role in tick feeding success or
ovipositioning, but it did impair rickettsial colonization and transovarial
transmission. This study provided new avenues of pathogen-symbiont dynamics
and tick-pathogen interactions within vectors.

ACKNOWLEDGMENTS

DEDICATION

To My Dear Parents

And Beloved Wife

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CHAPTER I - INTRODUCTION

1.1 Ticks

Ticks serve as one of the most successful vectors of a variety of pathogens to humans and animals of any arthropod, second only to the mosquito. They are well adapted for all weather conditions from tropical, temperate and even subarctic habitats, but are found with the greatest density in tropical and subtropical areas. Ticks are classified in the class Arachnida, subclass Acari, order Parasitifomes, and suborder Ixodida with four tick families comprising 878 species. The tick families have been classified as Ixodidae (hard ticks), Argasidae (soft ticks), Nuttalliellidae, and Laelaptidae (Anderson and Magnarelli, 2008). The latter two families are monospecific and are less significant in disease and public health. There are two major families, Argasidae (Soft ticks) and the Ixodidae (hard ticks); they are monophyletic with predicted divergence time 140 million years ago (Late Jurassic) (Black and Piesman, 1994). It was proposed that evolutionarily Ixodidae family developed from the Argasidae family of ticks. The main difference between these two families is in their feeding strategies: Ixodids feed for prolonged periods of time varying from a few days to two weeks, while Argasids typically feed less than an hour and feed multiple times. Within the Ixodidae, ticks have longer barbed mouthparts and use physical mechanisms to stay firmly attached to their hosts are prostriate (*Ixodes*), and ticks with relatively short mouthparts that secrete cement or glue from their salivary glands to firmly anchor to the host are metastriate (*Amblyomma*) (Sonenshine and Roe, 2013). The tick life cycle consists of four

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stages: egg, larva, nymph and adult (male or female). The postembryonic life stages, larva, and nymphs are sexually indistinguishable, but adults are sexually dimorphic as male or female. The overt differences in the immature stages is six-legged larval stage and eight-legged nymphal and adult stage. The adult tick body can be divided into two parts; capitulum (gnathosoma) and the body (idiosoma), which contains legs. The body length of ticks at the unfed stage is 2 mm to 20 mm while the blood-engorged ticks may be 25 to 30 mm and weigh up to 100 times their unfed masses (Fig 1.2) (Anderson and Magnarelli, 2008). The dorsal body of the male tick is covered by scutum whereas only anterior portion is covered in the female. The biological advantage of having small scutum allows female ticks engorge and imbibe more blood due to tapering scutum toward head region with feeding (Fig 1.2)(Anderson and Magnarelli, 2008).

1.1.1 *Amblyomma Maculatum* (Gulf Coast Tick)

The Gulf-Coast tick (*Amblyomma maculatum*) is a hard tick that is distributed across several regions of Central and South American countries bordering the Gulf of Mexico and the Caribbean Sea, with a range larger sized than both *Amblyomma americanum* and *Ixodes scapularis* (Fig 1.1) (Estrada-Pena et al., 2005). Bird migration and animal movement likely contributed to the vast distribution of this tick across the United States (Mukherjee et al., 2014). The adult Gulf coast ticks feed on vertebrate hosts for approximately two weeks, drop off from host. They pre-oviposit for 3-5 days and lays eggs continuously up to 25 days producing about 1,000 eggs/day/female and gravid female dies (Drummond et al., 1970). In the lab, they are managed at 27^0C with 70-98% relative humidity

and a photoperiod of 14/10 light hours. *Amblyomma maculatum* have been

reported to cause tick paralysis (Espinoza-Gomez et al., 2011) and gotch-ear

condition in cattle (Edwards et al., 2011). It is a known vector of *Rickettsia parkeri*

and other bacterial species, *Candidatus Rickettsia andeanae* (Ferrari et al.,

2013) and the protozoan, *Hepatozooan americanum* in dogs (O'Dwyer et al.,

2001). *Ehrlichia chaffensis* were reported to infect (Kocan et al., 2000), but

recently both *Ehrlichia* and *Anaplasma* were considered less likely vectored by *A.*

maculatum (Allerdice et al., 2016).
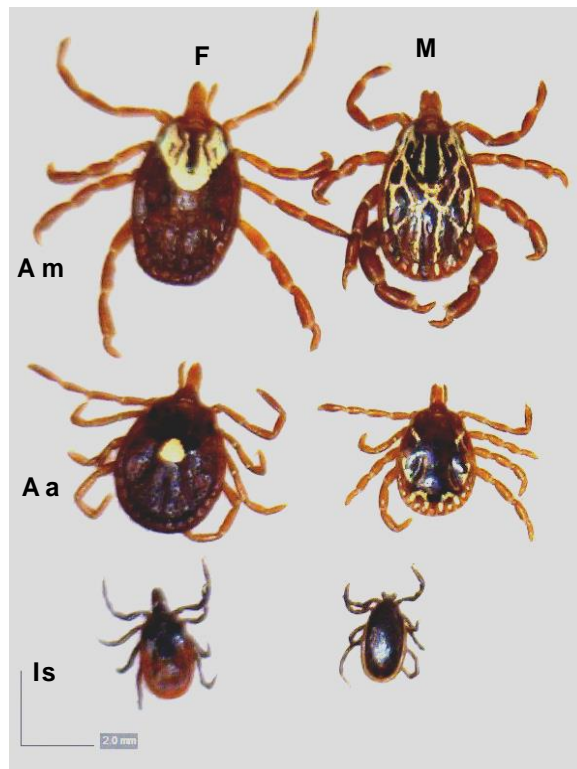
Figure 1.1 Hard Ticks (Ixodidae).

M; male and F; Female, Am; *Amblyomma maculatum*, Aa; *Amblyomma americanum*, Is; *Ixodes scapularis*

1.2 Tick Hematophagy: World's Most Efficient Vampire

1.2.1 Hard Ticks

Ticks are obligatory hematophagous arthropods and require blood meal for growth and development (Fig 1.2). Based on the number of host used, tick life cycle can be single-host, two-host and three- host lifecycle. In *Amblyomma* and *Ixodes,* each post-embryonic life stages: larvae, nymphs, and adults require three different hosts for a blood meal (three-host life cycle). In case of *Rhipicephalus,* larvae attach on a host and the engorgement and molting of larvae and nymphs occurs on the same host, which is called a single-host life cycle. *Hyalomma dromedarii* ticks are two host ticks; they feed on two separate hosts to complete their lifecycle. Soft ticks generally feed on multiple times on multiple hosts. Blood feeding is one of the important attributes of ticks that distinguish between hard ticks (Ixodidae) and soft ticks (Argasidae). In hard ticks, the blood feeding process occurs once in each postembryonic life stage whereas soft ticks feed multiple times in on host. The tick feeding on host can be divided into stage of attachment (1), slow feeding stage (2), fast feeding stages (24-48h before detachment), and repletion or disengagement from the host (4). The tick attaches to the host skin and starts making a blood-pool below the host dermis avoid host responses, it begins feeding slowly (slow feeding stage), then mating induces feeding and rapid engorgement occurs one to two days before detachment (Franta et al., 2010). Based on total the blood meal cycle, duration of the slow and fast feeding stage varies. The fast feeding phase occurs for last 24-48 h before the engorged tick drops off the host and during this period tick

ingests major portion (2/3) of blood meal. During blood feeding, the tick gut pump out water and electrolytes into hemocoel, which is then transferred back to host via saliva, resulting in a concentrated meal (Fig 1.2) (Waladde, 1979).

1.2.2 Soft Ticks

Soft ticks imbibe blood almost immediately after host attachment, and they do not secrete cement or form a new cuticle. The secretion of excess water occurred through their coxal pores. It has been reported that Argasids larval ticks complete feeding within 20 minutes while adults take 35 to 70 minutes (Sonenshine and Roe, 2013). Blood digestion takes place intracellular in the gut cell with the involvement of the cathepsins B, C and D and aspartic endopeptidases (Franta et al., 2010). The blood digestion (haemoglobinolysis) and activities of the cathepsins reach their maximum activities during the rapid engorgement stage (Franta et al., 2010).

1.2.3 Tick Salivary Secretion: Mechanism of Tick Hematophagy

The successful feeding of ticks requires a repertoire of pharmacological proteins, and compounds to evade the host hemostasis consisting of blood coagulation, platelet aggregation, and vasoconstriction. In addition, tissue repair mechanisms induce scar formation, a process that starts onset of vascular injury. The next generation sequencing approach of transcriptomic studies of tick salivary glands yielded a wealth of information about the tick sialome (Anatriello et al., 2010; Francischetti et al., 2011; Karim et al., 2011; Karim and Ribeiro, 2015; Ribeiro et al., 2011). The secreted proteins comprise up to 50% of sialotranscriptome suggested that tick feeding as a complex phenomenon (Karim

and Ribeiro, 2015). The complex blood feeding in tick cannot be impaired by the silencing of one or two genes which may be due to 'sialome switch', a new concept referring the expression of separate sets of tick proteins in different time on host (Karim and Ribeiro, 2015; Kotsyfakis et al., 2015).
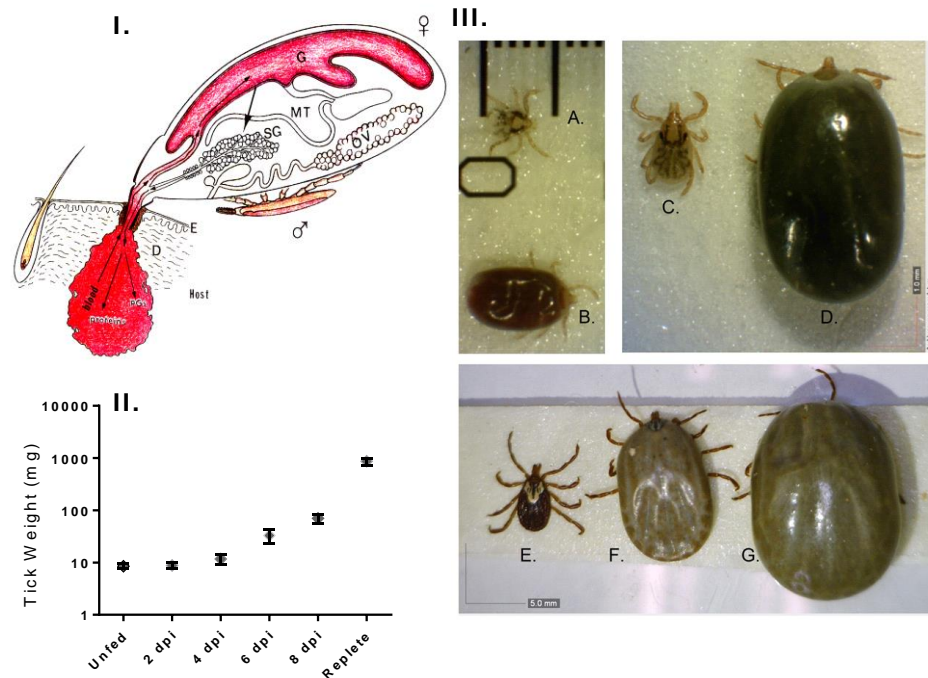
Figure 1.2 Tick Hematophagy.

Cartoon depicting pool feeding mechanism of tick feeding (i), adult female, Amblyomma maculatum tick weight gain across the blood meal cycle (ii), tick life stages; unfed and fed larva (A, B); nymphs (C, D); unfed, partially fed and engorged female (E, F, G).

Ticks face strong host responses from the initiation of attachment to engorgement, and multiple tick factors are released in the saliva that counteract host responses: platelet aggregation and blood coagulations, inflammatory responses and immune responses (Francischetti et al., 2009). The tick antigens that establish strong anti-immune responses in host could be important vaccine

targets for controlling tick attachment and reducing tick-borne pathogens.

Table 1.1

Tick factors known to involved in anti-coagulation of host blood.

Tick	Saliva/SGE/Factor	Effect	Reference
Ornithodorus moubata	Tick anticoagulant peptide and antistatin	Inhibit blood coagulation factor X (fXa)	(Dunwiddie et al., 1991; Schaffer et al., 1991)
Boophilus microplus	microphilin	Inhibits fibrinocoagulation, thrombin-induced platelet aggregation	(Ciprandi et al., 2006)
Ornithodoros savignyi	SGE	Prolonged activated partial thromboplastin time (ATPP) and prothrombin time and inhibited fXa	(Gaspar et al., 1995)
Amblyomma variegatum	Variegin	Thrombin inhibitor	(Koh et al., 2007)
Amblyomma hebraeum	Amblin	Thrombin inhibitor	(Lai et al., 2004)
Ornithodoros savignyi	Savignyrin	Platelet aggregation inhibitor	(Mans et al., 2002)

Ornithodoros savignyi	Savignin	Thrombin inhibitor	(Mans et al., 2002)
Ixodes scapularis	Ixolaris	Antithrombotic and Tissue factor pathway inhibitor	(Monteiro et al., 2005; Nazareth et al., 2006)
Amblyomma americanum	Americanin	Thrombin inhibitor	(Zhu et al., 1997)
Ixodes scapularis	Penthalaris	Tissue factor pathway inhibitor	(Francischetti et al., 2004)
Ixodes scapularis	Salp14 and Salp9ac	fXa inhibitor	(Narasimhan et al., 2002)
Ornithodoros moubata	Serine protease inhibitor	fXa inhibitor	(Waxman et al., 1990)
Ixodes scapularis	Metalloprotease	Fibrinolytic activity	(Francischetti et al., 2003)
Ixodes scapularis	Saliva/Troponin I-like	Inhibitor of endothelial proliferation	(Francischetti et al., 2005; Fukumoto et al., 2006)

1.2.3.2 Platelet Aggregation and Blood Coagulations

Adenosine diphosphate (ADP) and collagen are primary agonists of platelet aggregation, and tick saliva contains apyrase which hydrolyses ADP and

ATP to Adenosine monophosphate (AMP), which restricts platelet aggregation (Mans et al., 1998; Ribeiro et al., 1991). Without platelet activation, phosphatidylserine, a negatively charged phospholipid would stop amplification of coagulation cascade. The end-product of coagulation is thrombin which positively recruits more platelets in tick bite sites. The prevention of platelet aggregation further impairs synthesis of serotonin and thromboxane, impairing vasoconstriction near the bite area, which helps in formation of tick blood pool.

The blood coagulation cascade by tissue factor/FVIIa (extrinsic) and by a cascade of activation leading to activation of factor X. The calcium and other factors (FV and FX) help in formation of activated factor X. Both extrinsic and intrinsic pathways activate factor X that converts prothrombin to thrombin. The challenges of tick are that it requires constant supply of blood overcoming the blood coagulation pathway (Fig 1.2). The anticoagulants discovered include kunitz domain containing proteins, Ixolaris (Francischetti et al., 2004, 2002; Monteiro et al., 2005), and many metalloproteases in tick saliva were reported as fibrinolytic enzymes (Francischetti et al., 2003). Tick created host skin wound remains without healing due to anti-angiogenesis factors in tick saliva (Fukumoto et al., 2006).

1.2.3.3 Inflammatory Responses

Tick reattachment suffers in repeated infestations of *Amblyomma* and *Rhipicephalus* ticks in guinea pigs which was attributed to eosinophils and basophils increased populations in infestation sites (Brown and Askenase, 1982; Brown and Knapp, 1980; Steeves and Allen, 1991). Reinfestation interferences

are not evident in many other vertebrate host probably tick's trick to switch sialome per vertebrate hosts. Ticks were known to possess the anti-inflammatory factor against vertebrate host cytokines and chemokines (Hajnická et al., 2005; Vancová et al., 2007). T-cell response was observed in repeated infestations of ticks on mice (*Ixodes ricinus* nymphs) on mice skin but attachment after repeated infestation was not impaired (Mbow et al., 1994). The tick saliva proteins were reported to suppress the immune response activated as IgG1 and IgG2 in tick susceptible (Holstein) cattle (Kashino et al., 2005).

1.2.3.4 Immune Responses

Tick saliva or salivary glands extracts has ability to suppress the host immune cell populations which were reported in many studies. The *Ixodes scapularis* saliva has been shown to impair cytokine and NO production (Chen et al., 2012), similarly, the *I. ricinus* saliva showed inhibition of maturation, migration and antigen presentation (Skallová et al., 2008). The host reactive oxygen species (ROS), a defense mechanism against tick infestation was reported to be diminished by the tick saliva (Menten-Dedoyart et al., 2012). Tick saliva was reported inhibiting host Th17 immunity, and priming of a mixed Th1/Th2 response (Hannier et al., 2003; Mejri et al., 2002; Rolníková et al., 2003), in addition to suppression of cytotoxicity of natural killer (NK) cells (Kopecký and Kuthejlová, 1998).

The successful tick feeding requires constant blood follow and blood pool maintenance underneath host dermis. The tick prostaglandin E_2 (PGE_2) has been identified as essential in keeping blood pool (Ribeiro, 1989). In one of the

experiments, only 25% ticks were attached and none molted into next

developmental in repeated infestations of *A. maculatum* nymphs (Fig 1.3). The

immune rejections of tick infestation was reported in guinea pigs for *Amblyomma*

americanum and *Rhipicephalus appendiculatus* (Brown et al., 1983, 1982).
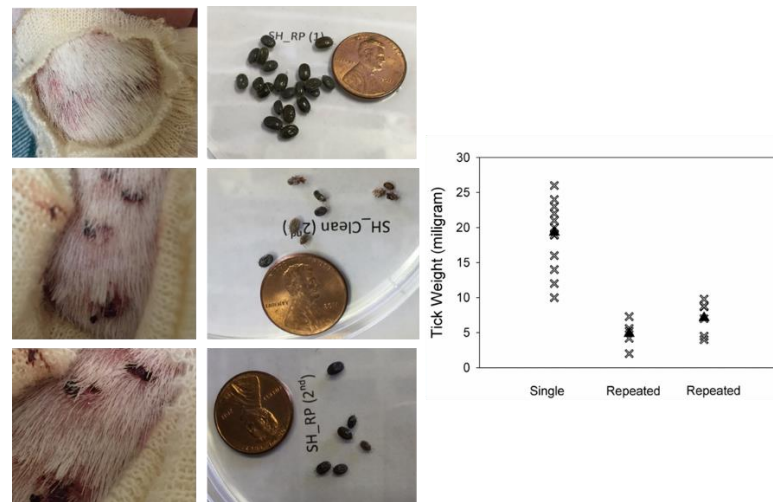
Figure 1.3 Tick Modulation of Host Immunity.

The left panel: First-time tick feeding in guinea pig (top), repeated infestation of naïve nymphs (middle), *R. parkeri* nymphs (bottom) after a month. Right panel: nymphal weights reported from single or repeated infestations.

1.2.4 Tick Sialome Mining and Quest for Vaccine Candidate

All the proteins expressed in the tick salivary glands is collectively referred

as 'sialome'. The next generation sequencing technologies allowed identification

of sialome in many ticks: *Ixodes scapularis* (Valenzuela et al., 2002), *Ixodes*

ricinus (Chmelar et al., 2008) and soft ticks, *Ornithodoros parkeri* and

Ornithodorus coriaceus (Francischetti et al., 2008a, 2008b). The sialo-

transcriptomes of *Amblyomma maculatum* and *A. americanum* ticks provided a

wealth of genomic information and generated knowledge base for new

experiments (Karim et al., 2011; Karim and Ribeiro, 2015). The cysteine protease

inhibitors (cystatins) were reported to impair tick blood feeding by modulating host immunity in *Amblyomma americanum* (Karim et al., 2005) and *Ixodes scapularis* (Kotsyfakis et al., 2007). The tick sialostatin L and sialostatin L2 become important immune modulatory proteins may help in treatment of asthma by inhibiting Th9-derived IL-9 (Horka et al., 2012; Kotsyfakis et al., 2010, 2007). The cystatins from the soft tick, *Ornithodoros* had showed the vaccine potential by reducing tick attachment and engorgement (Salát et al., 2010).

Considering salivary secretions as instrumental in tick feeding success, the tick proteins involved in cellular trafficking were targeted for RNAi assay and reported role in tick feeding success (Browning and Karim, 2013; Villarreal et al., 2013). The genes associated with cellular exocytosis are mediated by SNAREs (Soluble NSF Attachment Protein Receptors) and two molecules aid in the disassembly and recycling of the components of the SNARE complex are α-SNAP and N-ethylmaleimide sensitive fusion protein (NSF) (Zhao et al., 2007). SNAREs are small molecules that are mostly found in the plasma membrane and classified as target (t) or vesicle (v) SNAREs (Fasshauer et al., 1998). The study of SNAREs family of proteins Vti (vesicle transport through interaction with t-SNARE); Vti1a and Vti1b in *Amblyomma maculatum* and *A. americanum* involve in tick hematophagy (Villarreal et al., 2013). Similarly, the proteins involved in disassembly of SNARE complex, SALP25 and NSF upon silencing impaired tick blood-feeding (Browning and Karim, 2013).

The important method in developing anti-tick immunity is harnessing the host immunity by injecting tick antigens. The anti-tick vaccines are developed for

Boophilus microplus ticks using the midgut protein Bm86, which was isolated and purified and used for vaccination study (Willadsen et al., 1989). The integrating of both acaricides and anti-tick vaccination in livestock with Bm86 reported successful in South American countries (de la Fuente et al., 2007). The tick protein Bm86 was further characterized as a glycoprotein, with signal peptide and having repeated six cysteine residues resembling with epidermal growth factor-like protein (Rand et al., 1989). Bm86 has been commercialized in Australia and South American countries where *Boophilus microplus* is the problem with high success rate but this vaccine remained ineffective for *Amblyomma* species (de la Fuente et al., 2007). Some research in anti-tick vaccination used cocktail of tick proteins; vitellin-degrading cysteine endopeptidase (VTDCE) and *Boophilus* yolk pro-cathepsins (BYC) from *R. microplus*, and glutathione S-transferase from *Haemaphysalis longicornis* (GST-Hl) showed the reduction of tick engorgement and oviposition success (Parizi et al., 2012). Serine proteases inhibitors (serpins) from *Hemaphysalis longicornis* was proposed to have anti-tick property (Imamura et al., 2005) and cystatins from *Ixodes scapularis* are on the way to become anti-tick vaccine (Horka et al., 2012; Lieskovská et al., 2015).

1.3 Tick Microbiome

The "sleeping giant" of the biology (Woese, 1998) known as microbiome gained importance across the biology with the advent of next-generation sequencing technology. The human microbiome project (Turnbaugh et al., 2007) and many other microbiome studies provided the previously unknown role of

microbiome playing significant role in host immunity (Lee and Mazmanian, 2010), behavior (Archie and Theis, 2011; Ezenwa et al., 2012) and metabolism (Le Chatelier et al., 2013; Zhu et al., 2011). In vector biology, the microbiome is important with respect to microbial role in metabolism and pathogen transmissions. The study of microbiome has provided significant knowledge in arthropod immunity, pathogen colonization, and transmissions. The pathogen can mediate the overall vectorial capacity interacting with microbiome (Abraham et al., 2017). Microbiome influences the vectorial capacity (Jupatanakul et al., 2014) and vector competences (Weiss and Aksoy, 2011) for many arboviruses and development of pathogen (Dong et al., 2009). The bacterial profiling of the gulf coast tick using next generation technique provided dominance of *Rickettsia* and *Francisella* like endosymbionts (Budachetri et al., 2014). Recently, a RNAseq experiment (unpublished) to determine tick factors differentially expressed with respect to *R. parkeri* revealed unique symbiont *Candidatus* Midichloria mitochondrii (CMM) in infected tick tissues.

1.3.1 *Rickettsia*

The Rickettsial pathogens are classified into order Rickettsiales which includes the family Rickettsiaceae and Anaplasmataceae (Dumler et al., 2001; Raoult and Roux, 1997). The most of the rickettsial diseases are caused by infection with obligate intracellular gram-negative alpha-proteobacteria transmitted by arthropod vectors and affect an estimated one billion people worldwide (Parola et al., 2005; Walker and Ismail, 2008). In United States, five rickettsial agents were reported as public health significant, *Rickettsia rickettsii*

(Niebylski et al., 1999)*, Rickettsia akari* (Krusell et al., 2002)*, Rickettsia felis* (Azad et al., 1992), *Rickettsia parkeri* (Paddock et al., 2004) and *Rickettsia species 364D* (renamed as *Rickettsia philippii*) (Padgett et al., 2016; Shapiro et al., 2010)*.*

In this study, we focused on *Rickettsia parkeri*, a member of Spotted fever group of *Rickettsia* (SFGR), and its association with Gulf coast tick, *Amblyomma maculatum*. After the first human case of *R. parkeri* rickettsiosis in 2004, a tick symbiont *R. parkeri* become a public health problem (Paddock et al., 2004) and many human cases were reported (Straily et al., 2016).

There are few molecular biology and genetic tools discovered to study functional genomics of Rickettsial agents (Burkhardt et al., 2011; Wood et al., 2014). There is dearth of information regarding genetic manipulation of Rickettsial agents and there is huge knowledge gap in expression of Rickettsial gene and functional studies (Welch et al., 2012). *Rickettsia parkeri* infected ticks were reported to maintain infection across the life cycle to multiple generations (Wright et al., 2015) by transovarial and transstadial transmission like that in *Rickettsia conorii* in *Rhipicephalus sanguineus* (Socolovschi et al., 2012); *Dermacentor variabilis* for *Rickettsia montana* and *R. rhipicephali* (Macaluso et al., 2001); *Rickettsia africae* in *Amblyomma variegatum* (Socolovschi et al., 2009), and *Rickettsia rickettsii* in *Dermacentor andersoni* (Burgdorfer, 1963).

1.3.2 *Francisella*

Francisella is gram-negative coccobacilli, a gamma proteobacteria widely recognized because of *Francisella tularensis,* causing fatal disease known to

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infect more than 100 mammalian species. *Francisella tularensis* are found in two

subspecies; type A (tularensis) and type B (holoractica), and the two variants of

type A (A1 and A2) were reported from east and west United States (Farlow et

al., 2005). The arthropod transmission was first reported by Dr. Francis whereas

Francisella tularensis was isolated first time in tick (*Dermacentor andersoni*) by

R. R. parker (Petersen et al., 2009). *Francisella* like endosymbiont (FLE) are

identified in ticks other than *F. tularensis* (Scoles, 2004; Sun et al., 2000) and

reported in *A. maculatum* (Budachetri et al., 2014). There were many genetically

different FLEs were reported in *Dermacentor variabilis* and *D. andersoni*

(Dergousoff and Chilton, 2012) and across tick lifecycle (Liu et al., 2016). It was

hypothesized that pathogenic *Francisella tularensis* transformed into symbiotic

FLE in ticks (Gerhart et al., 2016).

1.3.3 *Candidatus* Midichloria Mitochondrii

In ticks, not so known symbiont, *Candidatus* Midichloria mitochondrii

(CMM) with unique localization in tick cell mitochondria or cytoplasm was

discovered (Sassera et al., 2006). The phylogenetic and statistical studies of *16S*

rRNA sequences of "Midichloria and like organism" proposed a novel family

"Candidatus Midichloriaceae" (Montagna et al., 2013) within order Rickettsiales.

CMM were reported from ticks and many ciliates (Boscaro et al., 2013; Epis et

al., 2008). In a microbiome study, over dominance of CMM created problem in

bacterial profiling (Gofton et al., 2015), and survey on wild collected *Amblyomma*

americanum showed the infection rate of only 0.15% (Williams-Newkirk et al.,

2012) and even not reported in *Amblyomma maculatum* (Budachetri et al., 2014).

The functional role of presence of CMM in ticks and was not known but some research may suggest it can be mere marker of tick bite (Mariconti et al., 2012).

1.3.4 Defensive Symbiosis

The symbiosis between host and microbe can further help host to defend colonization of other bacteria, parasite or parasitoids and even influence predator is known as defensive symbiosis (Clay, 2014) which is different than nutritional symbiosis where bacterial symbionts supplements nutrients to host (Baumann, 2005; Smith et al., 2015).

The bacterial symbionts avoiding the infection of pathogenic bacteria in host environment is considered non-immunological defenses which is achieved by producing antibodies or toxin against other bacteria or priming innate immune signaling pathways (Little and Kraaijeveld, 2004; Parker et al., 2011). The microbial community plays important role in pathogen transmission, vector competence (Burgdorfer et al., 1973; Clay et al., 2008), and tick reproductive fitness (Zhong et al., 2007) and likely has other undiscovered roles in vector ecological and physiological adaptations. The defensive symbiosis was observed in *Wolbachia*-mosquito model where *Wolbachia*, an endosymbiont interferes in colonization or transmission of pathogenic microbes (Teixeira et al., 2008; Werren et al., 2008). Simply, gut microbiota itself can prime the mosquito midgut immunity which resulted into the less infection of *Plasmodium* in *A. gambiae* (Dong et al., 2009). The defensive symbiosis is non-immunological as reported in *Wolbachia*-mediated dengue viral interference (Rances et al., 2013). The pathogen, *Borrelia burgdorferi* interaction with native microbiome in *Ixodes*

results in colonization success in tick gut tissues (Abraham et al., 2017; Narasimhan et al., 2014).

The field studies reported 12-40% of tick infection rate of *Rickettsia parkeri* in *Amblyomma maculatum* (Fig 2.1) (Budachetri et al., 2014). There could be many factors that determine the infection of rickettsial agents in ticks or public and the ecological modeling of the *R. parkeri* infection in ticks, human and animal disease cases may provide significant answers (Eremeeva and Dasch, 2015).

1.4 Tick Pathogen Interfaces

Tick pathogen interfaces determine success of the pathogen inside the tick. The pathogen specificity of the tick such as *Ixodes scapularis* for *Borrelia burgdorferi* (De Silva and Fikrig, 1995), *Dermacentor andersoni* for *Rickettsia rickettsii* (Niebylski et al., 1999) and *Amblyomma americanum* for *Ehrlichia chaffensis* (Long et al., 2003), *Rhipicephalus microplus* for *Babesia bovis* (Howell et al., 2007) depends on tick-pathogen interactions. The success of the pathogen colonization inside the vector defends not only on tick-pathogen interactions but pathogenic and non-pathogenic bacterial interaction or rickettsial interferences in virulence (Price et al., 1954) or transmission from ticks (Telford, 2009). The ability of pathogens to down-regulate the host immune response and to manipulate host cells is important for their establishment inside eukaryotic cells (Rikihisa, 2010; Sahni et al., 2013; Walker et al., 2003). Here, we reviewed tick genes affecting pathogen acquisition, colonization or transmission.

1.4.1 Antioxidants

Antioxidants genes were reported differentially expressed in *Ixodes ricinus* infected with *Borrelia burgdorferi* infections (Rudenko et al., 2005) and *Amblyomma maculatum* infected with *Rickettsia parkeri* (Adamson et al., 2013). Host phagosomes were reported to produce highly reactive oxygen species: HOCl and ·OH that are toxic to ingested bacteria ingested (Rosen et al., 1990). Vector superoxide dismutase determines survival of pathogen against superoxide radicals as reported in mosquito-*Plasmodium* and in tick-*Rickettsia* studies (Crispell et al., 2016; Lanz-Mendoza et al., 2002). The pro-oxidant *Duox* was involved in microbial clearance, redox-dependent modulation of signaling pathways, cross-linking of biomolecules, and discrimination between symbiont and pathogen (Kim and Lee, 2014). Another pro-oxidant, nitric oxide, which is a multifunctional free radical created during the oxidation of L-Arginine to L-Citrulline by the enzyme nitric oxide synthase (Rivero, 2006) kills *Plasmodium* ookinete (Gupta et al., 2009). In *Amblyomma maculatum-Rickettsia parkeri* model, superoxide dismutase (*SODs*) and *Catalase* together with battery of selenogenes quench radicals or catalyzes peroxides during blood feeding and pathogen colonization (Adamson et al., 2013; Crispell et al., 2016).

1.4.2 Innate Immune Signaling Pathways

The arthropod immune responses are mainly dependent on Toll, immune deficiency (Imd), and Janus kinase (JAK)-signaling transducer activator of transcription (STAT) pathways studied mainly in *Drosophila* (Smith and Pal, 2014). In mosquito, JAK-STAT pathway controls early load of *Plasmodium vivax*

(Bahia et al., 2011), Toll pathway control dengue virus infection (Xi et al., 2008) and Imd pathway factors and effectors impact malaria transmission cycle (Garver et al., 2012). The *I. scapularis* 5.3kDa protein activates STAT (signaling transducer activation of transcription) pathway and induced with *Anaplasma phagocytophilum* infection (Liu et al., 2012).

1.4.3 Tick Genes Known to Involve in Pathogen Acquisition

The pathogens from the infectious blood meal acquired by tick-gut differentially regulated tick proteins. There are studies showing tick *subolesin* role in *Anaplasma marginale* acquisition into tick guts (Bensaci et al., 2012; de la Fuente et al., 2010, 2006; Merino et al., 2011). Another tick gene, *α1, 3-fucosyltransferase* which was upregulated with the infection of *A. phagocytophilum* and upon silencing in *I. scapularis* nymphs significantly decreases *A. phagocytophilum* acquisition from the infected mice (Pedra et al., 2010). *Dermacentor variabilis* glutathione S-transferase (*GST*) and *vATPase* (H^+ transporting lysosomal vacuolar proton pump) help in *Anaplasma marginale* acquisition from infectious blood meal (Kocan et al., 2009). Tick immunity genes, *defensin* or antimicrobial peptides impair ability of tick to acquire *Rickettsia montanensis* and *Anaplasma marginale* (Ceraul et al., 2007; Kocan et al., 2008).

1.4.4 Tick Genes Known to Involve in Pathogen Transmission

Tick feeding involves concentration of vertebrate blood and removal of water back to host blood during salivation. The success of tick feeding requires salivary proteins which modulate, blood coagulation, immunomodulation, and pain receptors for a prolonged time (Francischetti et al., 2009). The pathogen

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developed inside tick salivary glands can utilize the immunocompromised tick-host interface or tick factors (proteins) which help pathogen development and transmission during salivation. The *I. scapularis* 5.3kDa protein (gene 15) silencing increased the *A. phagocytophilum* infection of tick salivary glands and transmission to mammalian host (Liu et al., 2012). *Dermacentor variabilis* kunitz protease inhibitor (*DvKPI*) was upregulated across the blood meal and with the *Rickettsia montanensis* infection. After RNAi silencing of *DvKPI,* rickettsial colonization of tick midgut increased by 90% suggesting this molecule can limit *R. montanensis* acquisition by ticks (Ceraul et al., 2008).

1.4.5 Tick Genes Known to Involve in Pathogen Multiplication

The multiplication of the pathogen inside vector is required during transmission to susceptible host or maintaining transovarial transmissions. The pathogen adherence to tick gut tissues, movement of pathogen to hemolymph and migration to salivary glands for saliva assisted transmission were important events of pathogen inside tick. The *Ixodes scapularis* tick TROSPA (Tick receptor of outer surface protein A) plays important role in *Borrelia burgdorferi* adherence to tick gut tissues (Pal et al., 2004). Similarly, tick receptor of BBE31 (TRE31) and *B. burgdorferi* outer surface lipoprotein (BBE31) depletion impaired the spirochete entering inside the hemolymph (Zhang et al., 2011). The *I. scapularis* secretory protein P11 is important in migration of *Anaplasma phagocytophilum* from tick guts to hemolymph or salivary glands (Liu et al., 2011). The silencing of tick selenoprotein M (*SelM*) and glutathione S-transferase

(*GST*) by dsRNA showed reduction of *A. marginale* multiplication in tick salivary glands (Kocan et al., 2009).

1.5 Selenoproteins

Selenoproteins are selenium-containing proteins known for redox function centered around selenium atom in selenocysteine (U) encoded by UGA (Opal codon) (Allmang and Krol, 2006). The alternate coding of UGA to selenocysteine occurs in all three domains of life with mechanism involving specific synthesis machinery involving Sec-tRNA^{sec}, SECIS element in 3'-UTR of selenoprotein mRNAs, SECIS binding protein, selenocysteine-specific elongation factors and selenophosphate synthetase supplying selenium. Though three domains of life contain selenoprotein synthesis machinery, selenoproteome is reduced or absent in some insect species where putative cysteine homologs may replace their functions (Lobanov et al., 2008). There are 25 known human selenoproteins and most studied included are glutathione peroxidases, thioredoxin reductases and iodothyronine deiodinases (Labunskyy et al., 2014). Selenoproteins contain at least one selenocysteine residue whereas selenoprotein P (SELENOP) in human has 10 and Zebrafish selenoprotein P has 17 selenocysteine residues (Kryukov and Gladyshev, 2000). Due to the multiple selenocysteine residues, SELENOP functions as selenium storage and transportation functions in body and the level of which represent the status of selenium micronutrients in body (Schulze et al., 2016).

1.5.1 Selenium as a Trace Mineral

Selenium is the important trace mineral available to body as selenocysteine, incorporated into selenoproteins for biological functions. Selenoproteins are known for their antioxidant property and selenium plays central role in redox reactions. The nature has chosen selenium over sulfur because of (rate advantage and redox advantage) Se, a better nucleophile and react with great ease to reactive oxygen species and Se-O bond readily reduced defying permanent oxidations (Reich and Hondal, 2016). The daily requirement of the selenium for adult human is 55 micrograms readily obtained from plants products and meat products. There are three important diseases due to selenium deficiency, Keshan disease in children (where heart enlargement and poor heart functions) certain region of China (Longo and Loscalzo, 2014), Kashin-Beck disease (arthritis in different bones) (Huang et al., 2013), and myxedematous endemic cretinism (in combination of deficiency of iodine, mental retardation) (Dumont et al., 1994). The selenium toxicity or selenosis was reported to with increased blood selenium level (reaches >1μg/ml) with common complaint of gastrointestinal upset, hair loss, white blotchy nails, garlic breath odor, fatigue, irritability, and mild nerve damage (Goldhaber, 2003).

1.5.2 Biosynthesis of Selenoproteins

Selenocysteine is 21st amino acid represented by UGA codon, which in non-selenoprotein translates as stop codon (Fig 1.4). The alternative coding of UGA to an selenocysteine is unique mechanism involving combined action of tRNA$^{[ser]sec}$, Seryl-tRNA synthetase, phosphoseryl-tRNA$^{[ser]sec}$ kinase,

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selenocysteine synthase, selenophosphate synthetase (Fig 1.4) (Labunskyy et

al., 2014). The mechanism of Sec incorporation required specific SECIS

elements in selenoprotein mRNA at 3'-UTR region. The GA Quartet (SECIS core,

non-Watson crick interactions) in SECIS element is binding site for SECIS

binding protein (SBP2) (Fletcher et al., 2001). SBP2 is determining protein in

selenoprotein synthesis containing three important domains, NH2 terminal

domain, a Sec incorporation domain in the middle of protein and a RNA-binding

domain belonging to L7Ae RNA-binding proteins (Low et al., 2000). The L7Ae

RNA-binding motif found in SBP2 is part of L30 ribosomal protein (rpL30), a

component of large ribosomal (60s) subunit in eukaryotes (Caban and Copeland,

2006). For Sec incorporation, Sec-specific eukaryotic elongation factor (eEFSec)

recruits aminoacylated tRNA [ser] [sec], which in combination of SBP2, rpL30

incorporate selenocysteine (Sec) into nascent protein chain in response to UGA

codon (Tujebajeva et al., 2000). The SBP2 and ribosomal interaction were

considered first step in selenoprotein synthesis in new hypothesis of

selenoprotein biosynthesis (Caban and Copeland, 2006).The selenium donor,

selenophosphate is generated from selenide by selenophosphate synthetase2

(SEPHS2), interestingly during selenium deficiency sulfide instead of selenide

can make cysteine instead of selenocysteine in selenoproteins (Lu et al., 2009).

The excess selenoprotein synthesis is controlled by selenocysteine lyse, that

control over synthesized selenoproteins reduced to alanine and elemental

selenium (Mihara et al., 2000). The overall selenoprotein synthesis can be

regulated by body selenium level (in GPx1, MSRB1, SELENOW, and

SELENOH), eukaryotic translation initiation factor eIF4a3 (selenoprotein

translation during Se deficiency) and nucleolin, a phosphoprotein in nucleolus

which synthesizes *rRNA* (Labunskyy et al., 2014). The nomenclature of

selenoproteins were based on new recommendations of selenoprotein

researcher approved by HUGO gene nomenclature committee

(http://www.genenames.org/cgi-bin/genefamilies/set/890) (Gladyshev et al.,

2016). We hypothesized that tick generate superoxide radicals to defend *R.

parkeri* infections and tick superoxide dismutase, catalase, and selenoproteins

balances altered redox (Fig 1.4).
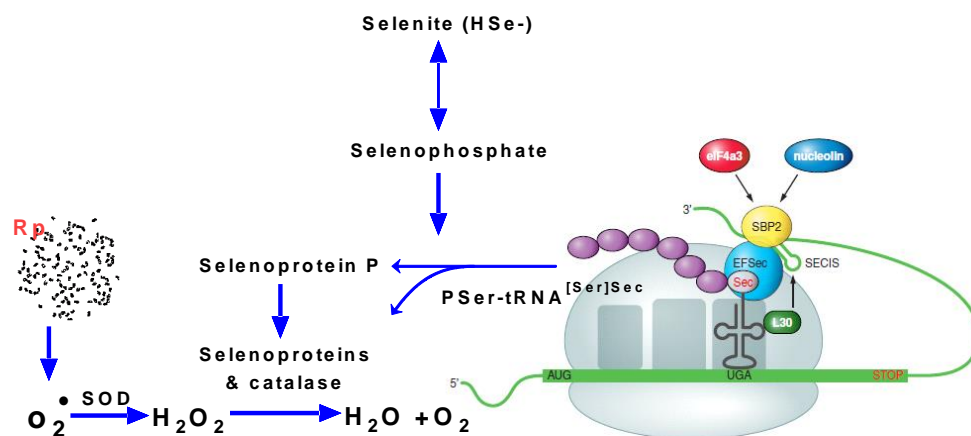
Figure 1.4 Selenoprotein biosynthesis and mechanism of redox hemostasis.

Note: Ticks defend *Rickettsia parkeri* producing superoxide radicals but due to redox imbalance tick superoxide dismutase (*SOD*) expression increases and selenoproteins and catalase in combination reduces organic hydroperoxide and hydroperoxide.

1.5.3 Functionally Characterized Selenoproteins

In human, there are 25 different selenoproteins and most known have

redox properties. The important are glutathione peroxidases, five (Gpx1-4, Gpx6)

of total eight paralogs contain Sec residue whereas three Gpx (Gpx5, 7,8) have Cys (Kryukov et al., 2003). The second most studied selenoproteins were iodothyronine deiodinase (DIO1-3) which reduces prohormone thyroxine into active hormone (Bianco et al., 2002). Other important selenoproteins are thioredoxin reductases (TXNRD, TrxR), they reduce the protein disulphide bonds with help of thioredoxin. Three different TrxR (TXNRD) were present in human, TXNRD1 primary located in cytosol and nucleus bears Sec in penultimate position of –COOH. TXNRD2 is mitochondrial where it reduces mitochondrial thioredoxin. Third thioredoxin reductase (TXNRD3) is thioredoxin/glutathione reductase displaying glutaredoxin activity (Arner and Holmgren, 2000). Other important selenoproteins are methionine-R-sulfoxide reductase (MSRB1), which catalyzes repair of R-enantiomer of oxidized methionine residues in proteins (Kryukov et al., 2002). The 15-kDa selenoprotein (SELENOF or Sel15), SELENOM (SelM), SELENOK (SelK) and SELENOS (SelS) are ER-resident selenoproteins known to regulate protein folding and redox homeostasis in ER (Korotkov et al., 2001). Selenoprotein O (SELENOO) is known to function as mitochondrial kinase apart being antioxidant in mitochondria (Han et al., 2014). Selenoprotein P (SELENOP) is known for multiple Sec-containing proteins and considered important proteins for transport of selenium across the body (Labunskyy et al., 2014). The selenoproteins W, T, H, and V are thioredoxin-like proteins (based on Cys-x-x-Sec motif) belong to Rdx family of selenoproteins known to function as thiol-based oxido-reductase. The computer program developed by Galdyshev, SECISearch (http://genome.unl.edu/SECISearch.html)

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(Kryukov et al., 2003) used in human EST databases helped identification of two new selenoproteins known as selenoprotein T and selenoprotein R (Kryukov et al., 1999).

In *Amblyomma maculatum*, there are 12 known coding sequences from sialotranscriptome project. The selenoprotein K (SELENOK) and selenoprotein M (SELENOM) enhances the antioxidant capacity in ticks (Adamson et al., 2014) and thioredoxin reductase (TXNRD or TrxR) maintains native microbiome in ticks (Budachetri and Karim, 2015). NCBI domain search showed, SELENOK has no specific domain but SELENOM contains Sep15/SelM redox domain which is similar to thioredoxin-like domain suggested to function as thiol-disulphide isomerases in endoplasmic reticulum.

1.6 Rationale of Study

The successful colonization of *Rickettsia parkeri* inside tick vector, *Amblyomma maculatum* is prerequisite for vectorial ability of tick, which is determined by tick-pathogen and pathogen-symbiont interactions inside the tick. Our earlier report showed *Rickettsia parkeri* infection rate of 10-37% in wild caught ticks and presence of *Francisella* like endosymbiont (FLE) (Budachetri et al., 2014). *R. parkeri* is maintained across the tick lifecycle but infection rate remained lower probably *R. parkeri* colonization suffers restriction within tick. The possible restrictions could be from (a) symbionts residing inside tick guts (b) tick defenses comprising anti-oxidants and innate immune pathways. In this study, we explored the phenomenon how *R. parkeri* colonizes inside the tick with focus on the *R. parkeri*-symbiont and tick-pathogen interactions.

FLE and CMM or any other endosymbiont could be influencing rickettsial infections within tick or tick tissues. We hypothesized (displacement hypothesis) that *Rickettsia parkeri* colonizes within the tick vector by displacing the native tick microbiome. We assumed that success of the *R. parkeri* colonization depend on how well rickettsia displaces other symbionts (displacement hypothesis) and make a niche or alternatively, how symbionts defend pathogenic infections (defensive symbiosis) inside tick tissues.

To decipher tick-pathogen interaction paradigm, we aimed to target and disrupt tick-specific molecule (s)/pathways that normally allow the tick to successfully colonize and transmit *Rickettsia parkeri* vertically to the next generation. The studies on tick-pathogen interface with tick antioxidants, superoxide dismutase or catalase supports rickettsial infection inside tick organs by overexpressing during pathogen infections (Crispell et al., 2016; Kumar et al., 2016). Selenoproteins are unique with their biosynthesis and antioxidant property centered on selenium in selenocysteine. Silencing of eukaryotic elongation factor (eEFSec, SEF), which requires during selenoprotein synthesis impaired total antioxidant capacity of tick, *R. parkeri* colonization and significantly altered salivary secretome (Adamson et al., 2013). The selenoprotein P (SELENOP) is known to function as selenium transporter across the body besides being an antioxidant (Mostert, 2000). In this study, we functionally characterize tick SECIS binding protein (SBP2) and a putative selenoprotein P (SELENOP) and observed impact on tick selenoproteome synthesis and *R. parkeri* colonization.

CHAPTER II – DYNAMIC INTERACTIONS AMONG *RICKETTSIA* AND TICK

SYMBIONTS LIMIT PATHOGEN INFECTION IN *AMBLYOMMA MACULATUM*

2.1 Abstract

Amblyomma maculatum (Gulf Coast tick) possesses vectorial capacity to *Rickettsia parkeri*. The rickettsial replication inside the tick vector is prerequisite for vectorial capacity of tick. The ability of pathogen to survive and make niche in tick tissues surrounded by bacterial community is an important aspect in bacterial colonization. *A. maculatum* microbiome comprises of rickettsial agents, R. parkeri and other symbionts like *Francisella* like endosymbiont (FLE) and *Candidatus* Midichloria mitochondrii (CMM). We hypothesized that *R. parkeri* dynamically interacts with tick symbionts and modulates tick host to achieve successful replication, proliferation, and colonization inside tick tissues before transmission. In this study, we investigated dynamic interactions of rickettsial replication with tick symbionts in tick, *A. maculatum*. The *R. parkeri* infected; Rp (+) and non-infected; Rp (-) *A. maculatum* tick colonies were established and maintained for tick-*Rickettsia* interaction studies. The total bacterial load (TBL) and individual bacterial loads for *R. parkeri* load (Rp) along with intracellular symbionts, *Francisella* like endosymbionts (FLE) and *Candidatus* Midichloria mitochondrii (CMM) were quantified across the tick life stages by specific primers in qPCR assays. Quantification of individual bacteria (Rp, FLE, and CMM) across the tick life cycle suggested their ability of transovarial and transstadial transmission in *A. maculatum*. We concluded that *R. parkeri* infection decreased FLE numbers but CMM thrived across the tick lifecycle. Further study of *R. parkeri* and its

associated endosymbionts may yield clues about its replication and transmission to vertebrate hosts.

2.2 Introduction

Ticks are blood-feeding parasites of humans, wild animals, and domestic animals, and are important from a public health perspective because they serve as competent vectors of various disease-causing infectious agents. Many tick-borne pathogens can infect various organs within a tick where they can multiply. Infection of the salivary glands enables tick pathogens to readily infect vertebrate hosts upon tick feeding. The Spotted Fever Group Rickettsial (SFGR) agent, *Rickettsia parkeri*, is maintained in tick populations through transstadial (between life-stage molts) and transovarial (deposition into eggs for next generation pathogen development) transmission (Raoult and Roux, 1997). The Gulf Coast tick, *Amblyomma maculatum*, is an arthropod vector with increasing public health significance because of its role as the primary vector of *R. parkeri* in the USA (Paddock et al., 2004). Rickettsial diseases are caused by obligate intracellular gram-negative bacteria and affect people on all continents except Antarctica (Parola et al., 2005; Walker and Ismail, 2008). In modern times, the rate and ease of global movement creates the risk of transporting ticks and tick-borne diseases, which may have previously been restricted to one region.

A dynamic interaction occurs between tick vectors and their associated disease-causing agents, regarded as a continuous "*bellum omnium contra omes*" or war of all against all (Chmelař et al., 2016). There is unavoidable interaction between pathogens with and obligate tick symbionts during colonization and

transmission. The important roles of non-pathogenic symbionts and disease agent have become evident in mosquitos where *Wolbachia*, affects colonization of malarial agent, yellow fever virus, dengue, and chikungunya virus (Hughes et al., 2014, 2011; Walker et al., 2011). There are few reports of tick symbionts playing role in manipulation of pathogenic bacterial proliferation or transmission (Narasimhan and Fikrig, 2015; Niebylski et al., 1997b). The commonly associated symbionts of hard ticks belong to *Rickettsia*, *Francisella*, *Coxiella*, *Wolbachia*, and *Candidatus* Midichloria genera (Ahantarig et al., 2013). *Francisella* like endosymbiont, which has been detected in many ticks (Burgdorfer et al., 1973; Niebylski et al., 1997a; Sun et al., 2000) is a gamma proteobacterial symbiont found in the ticks and is related to the pathogenic *Francisella tularensis* (Foley and Nieto, 2010). The identification of FLE in different tick organs and specially in ovaries was reported from hard tick, *Haemaphysalis doenitzi* (Liu et al., 2016). Importantly, the recent study claimed the origin of FLE from mammalian pathogen *Francisella tularensis* after transiting to endosymbiotic lifecycle (Gerhart et al., 2016). *Candidatus* Midichloria mitochondrii (CMM) detected in *Ixodes ricinus* has unique intramitochondrial lifestyle (Sassera et al., 2006). Based on phylogenetic and statistical studies of the *16S rRNA* sequences of "Midichloria and like organisms", CMM is proposed to belong to a novel family known as "Candidatus Midichloriaceae"(Montagna et al., 2013), has frequently been reported in various Ixodid ticks (Epis et al., 2008). Though CMM is not abundant in *Amblyomma* ticks but excessive level of CMM in *Ixodes holocyclus* (>95% sequence reads) possess problem in detecting

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pathogenic bacteria and required specific masking primers for CMM (Gofton et al., 2015). Overall, we have shown that *R. parkeri* colonization success is associated with the quantity of CMM present in the tick and the depletion of FLE in the *Amblyomma maculatum*.

2.3 Materials and Methods

2.3.1 Ethics Statement

All the animal experiments were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, USA. The protocols for tick blood feeding on sheep (for adults) and immature ticks (on hamsters) were approved by the institutional Animal Care and Use Committee of the University of Southern Mississippi (protocol # 10042001 and 15101501).

2.3.2 Ticks and Other Animals

Gulf Coast ticks (*A. maculatum*) were maintained at the University of Southern Mississippi according to established methods (Patrick and Hair, 1975). The *R. parkeri*-infected *A. maculatum*; Rp (+) tick and *R. parkeri* uninfected; Rp (-) tick colonies were established and propagated in the laboratory from field collections (Budachetri et al., 2014; Crispell et al., 2016). Adult ticks were kept at room temperature with approximately 90% relative humidity under a photoperiod of 14 h light/10 h dark before infestation on sheep. Ticks were blood fed on sheep and were either allowed to feed to repletion or were removed at 3 to10 days after attachment, depending on the experimental protocol.

2.3.3 Ticks and Tick Tissues Preparations

Eggs, unfed and fed (larva and nymphs) were acquired from the regular Rp (+) and Rp (-) tick colonies. Eggs and unfed larva from three individual ticks (20 milligram) and fed larva (3 pooled) and similarly, unfed nymphs (20 milligram) and fed nymphs were stored in RNAlater. The tick tissues from unfed and partially blood-fed female adult ticks were isolated within 4 h of removal from the animal. The tick tissues from individual adult ticks were dissected in ice-cold M-199 buffer as described by Morgan et al., 1950 and 1955 (Morgan et al., 1955, 1950). After isolation, the tissues were washed gently in the same ice-cold buffer. The tissues were either stored immediately after dissection in RNAlater (Invitrogen, Carlsbad, CA, USA) prior to extracting the mRNA from them, or in protein extraction buffer (0.15 M Tris-HCl, pH 8.0, 0.3M NaCl, 10% glycerol). All the samples were stored at -80°C freezer until further processing.

2.3.4 RNA Isolation and cDNA Synthesis

Extraction of total RNA and cDNA synthesis were conducted as previously described (Crispell et al., 2016). Briefly, the tick tissues stored in RNAlater were used for total RNA extraction using an Illustra™ RNAspin Mini Isolation kit (GE Healthcare, Piscataway, NJ, USA) per manufacturer's instructions. The RNA concentration was determined using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Total RNA (1 µg) was reverse transcribed into cDNA using iScript™ cDNA synthesis kit (Bio-Rad, Inc., Hercules, CA, USA).

2.3.5 Quantification of Total Bacterial Load

The bacterial load in each tick tissue was estimated as described previously (Budachetri and Karim, 2015; Narasimhan et al., 2014). Briefly, 25ng of cDNA from the tick tissues, 200 nM *16sRNA* gene primers, and iTaq™ Universal SYBR® Green Supermix (Bio-Rad Inc., Hercules, CA, USA) in a 25-µl volume reaction was used with the following thermocycler parameters: 94°C for 5 min followed by 35 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 30 s. Standard curves were used to estimate the copy numbers of each gene. The bacterial copy numbers were normalized against *A. maculatum* tick *GAPDH*.

Table 2.1

The oligonucleotides used in this study.

Gene	GenBank ID	Forward Primer (5'-3')	Reverse Primer (5'-3')	Size (bp)
GyrB	AM159536	CTTGAGAGCAGAAC CACCTA	CAAGCTCTGCCG AAATATCTT	146
rOmpB	AF123717	CAAATGTTGCAGTT CCTCTAAATG	AAAACAAACCGTT AAAACTACCG	96
16S rRNA		AGAGTTTGATCCTG GCTCAG	CATGCTGCCTCC CGTAGGAGT	
FRAN*16 S rRNA*		CAACATTCTGGACC GAT	TGCGGGACTTAA CCCAACAT	373
GAPDH	JO842341	CACCCATCACAAAC ATGGGTGCAT	TTTCAGGAAATGA AGCCTGCCAGC	175

2.3.6 Quantification of *Francisella* Like Endosymbiont (FLE) in Tick Tissues

The *Francisella* like endosymbiont from *A. maculatum* tick tissues were quantified according to published method with slight modification (Dergousoff and Chilton, 2012). *16S rRNA* primers specific to FLE and tick *GAPDH* primers were

used to amplify tick FLE and tick *GAPDH* in thermal cycler and PCR products were purified and sequenced and verified prior to further assays (Table 2.1). Two standard curves for quantification of tick FLE and *GAPDH* genes were determined based on serially diluted PCR products. The serial dilutions of 10^8 to 10^1 copies of each gene were amplified in the thermal cyclic conditions and obtained cycle threshold (Ct) values for known dilutions were used to determine standard curve which was used to calculate copies of each genes. The 25µL of qRT-PCR reactions consisted of 125 nM of each primer, iTaq™ Universal SYBR® Green Supermix (Bio-Rad Inc., Hercules, CA, USA) and the serially diluted PCR products prepared for each standard curve. The reaction mixtures were subjected to thermal cycle parameters of 95°C for 5min followed by 29 cycles of 95°C for 30s, 52°C for 30s and 72°C for 30s with final extension of 72°C for 5 min in CFX96 Real-Time System (Bio-Rad Inc. Hercules, CA, USA). Using the same reaction conditions and thermal cycle parameters, 25 ng *A. maculatum* cDNA for tissues were amplified and the standard curves produced were used to estimate the copy numbers of each gene in each sample. The FLEs copy numbers were normalized against the *A. maculatum GAPDH* gene. As with the other qRT-PCR reactions, all the samples were run in triplicate.

2.3.7 Quantification of the *Candidatus* Midichloria Mitochondrii (CMM) in Tick Tissues

We followed the protocol described previously for quantification of *Candidatus* Midichloria mitochondrii (Sassera et al., 2006). CMM specific *GyrB* gene and tick *GAPDH* were amplified from tick *A. maculatum* using tick primers

(Table 2.1). The amplified PCR products were serially diluted 10-fold (10^8 to 10^1

copies) and used to generate a standard curve. The qRT-PCR reactions

consisted of 400 nM of each primer, iTaq™ Universal SYBR® Green Supermix

(Bio-Rad Inc., Hercules, CA, USA) and the serially diluted PCR products

prepared for each standard curve. The reaction mixture was subjected to thermal

cycling at 95°C for 2 min, 40 cycles at 95°C for 15 s and at 60°C for 30 s, and

melt curve from 55°C to 95°C with increasing increments of 0.5°C per cycle in a

CFX96 Real-Time System (Bio-Rad Inc., Hercules, CA, USA). The standard

curves generated were used to calculate the copy number of CMM and the tick

GAPDH gene. Using the same reaction conditions and thermal cycle parameters,

25 ng *A. maculatum* cDNA tissues were amplified and the standard curves

produced were used to estimate the copy numbers of each gene in each sample.

The CMM copy numbers were normalized against the *A. maculatum GAPDH*

gene. As with the other qRT-PCR reactions, all the samples were run in triplicate.

2.3.8 Quantification of *Rickettsia Parkeri* Load in Tick Tissues

Rickettsia parkeri load was estimated by quantifying rickettsial load per

tick *GAPDH* gene copy numbers estimated by using two standard curves for

rickettsial outer membrane protein B (*rompB*) and tick *GAPDH* (Jiang et al.,

2012). The specific primers for *R. parkeri ompB* and tick *GAPDH* (Table 2.1)

were used for amplification of each gene. The amplified PCR products of known

concentrations were used to make standard curves using iTaq™ Universal

SYBR® Green Supermix (Bio-Rad Inc., Hercules, CA, USA), 250nM of each

primer concentrations in qRT-PCR using thermal cyclic parameters of 10 minutes

at 95 °C, followed by 35 cycles of 15 seconds at 95 °C, 30 seconds at 60 °C, and 30 seconds at 72 °C using CFX96 Real-Time System (Bio-Rad Inc., Hercules, CA, USA).

2.3.9 Data Analysis

All data are expressed as mean ± SEM unless otherwise mentioned. Statistical significance between the two experimental groups or their respective controls was determined by t-test (P value, 0.05) using Graph Pad (Graph pad Prism 6.05, La Jolla, CA, USA).

2.4 Results

2.4.1 *Rickettsia Parkeri* Quantification Across the Tick Lifecycle

The study of *R. parkeri* colonization inside tick tissues started with identification of field infected tick tissues (Fig 2.1). The yearly survey of field collected *A. maculatum* from 2011 to 2016 showed the infection rate of 12-37% in tested ticks except on year 2015 where pooled ticks were tested (3 per group) (Fig 2.1). Presence of *Rickettsia parkeri* infected and non-infected ticks in field provide opportunity to develop infected and non-infected colonies in lab. In lab-cultured tick colonies, we observed that *R. parkeri* increases across the developmental stages from eggs to adults (Fig 2.2). The infected eggs hatched into infected larva, with blood meal, Rp significantly increases (>3.5 fold) in fed larva (t=4.12, df=7, p=0.0041). Similarly, Rp transstadially transmitted to nymphal stages, where blood meal significantly increases (>2 fold) the Rp load (t=3.08, df=10, p=0.01). The Rp transstadially maintained to adult ticks (Fig 2.2). In adult

ticks, the blood meal negatively impacted Rp load (Fig 2.2). The decreased Rp

load was further represented in tick midgut and salivary glands (Fig 2.2).
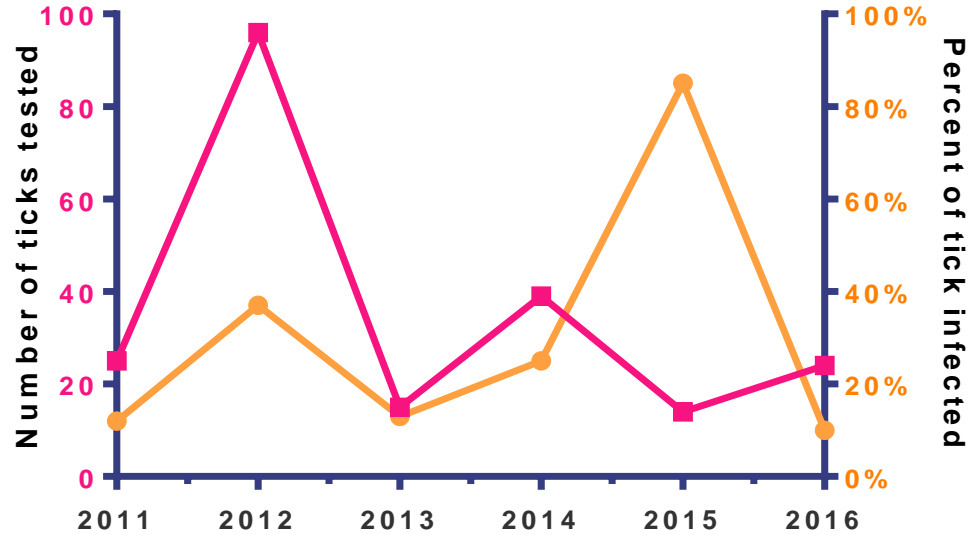
Figure 2.1 Dynamics of *Rickettsia parkeri* in South Mississippi.
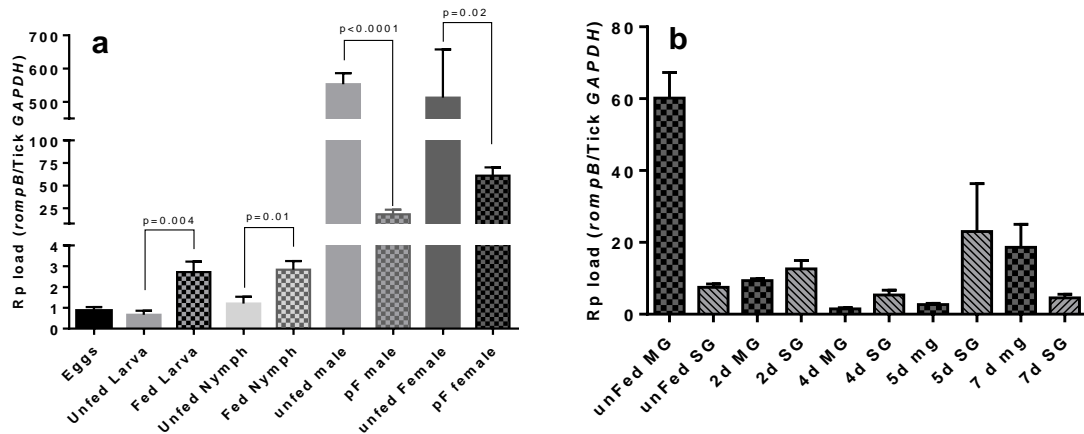
Figure 2.2 *Rickettsia parkeri* (Rp load) across the tick life stages encompassing transovarial and transstadial maintenance in *Amblyomma maculatum.*

2.4.2 Symbionts Across the Blood Meal

FLE decreased rapidly like the overall bacterial load changes in both tick midgut and salivary gland tissues across the blood meal (Budachetri and Karim, 2015) (Fig 2.3) whereas the level of the CMM remained constant in the midgut across the tick blood meal but decreasing rapidly following blood meal in salivary glands (Fig 2.3).
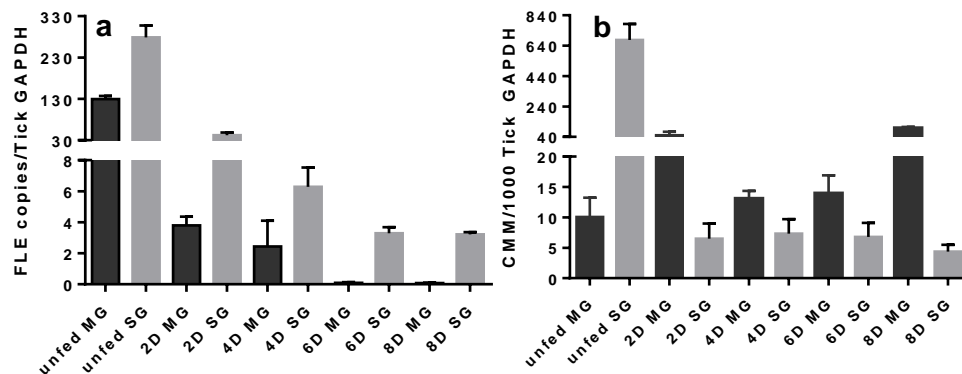
Figure 2.3 The FLE and CMM load across the blood meal in naïve tick tissues.

FLE load (A) and CMM load (B) in midguts (MG) and salivary glands (SG) in different time points in tick tissues. D; days post infestation.

2.4.3 Total Bacterial Load (BL) Across the Tick Lifecycle

In partially fed tick tissues, total bacterial load increased in Rp (+) ticks (100 fold in gut tissues) but only 1.2-fold in salivary glands compared to Rp (-) tick (Fig 2.4). The increases in bacterial loads were observed with rickettsial infections in embryonic and immature tick stages as well (Fig 2.4). The bacterial load increases 40-fold in Rp (+) eggs, from 104 to 4232. The bacterial load in Rp (+) unfed larva was lower than in Rp-unfed larva possibly because of lower Rp load in unfed larva but with the blood meal Rp load increases in fed larva making

total bacterial load 6-fold higher compared to Rp (-) ticks (Fig 2.5). The bacterial

loads in unfed and fed nymphal tissues were higher in Rp+ticks with 3.5 and 1.5-

fold higher than Rp (-) ticks respectively (Fig 2.5).
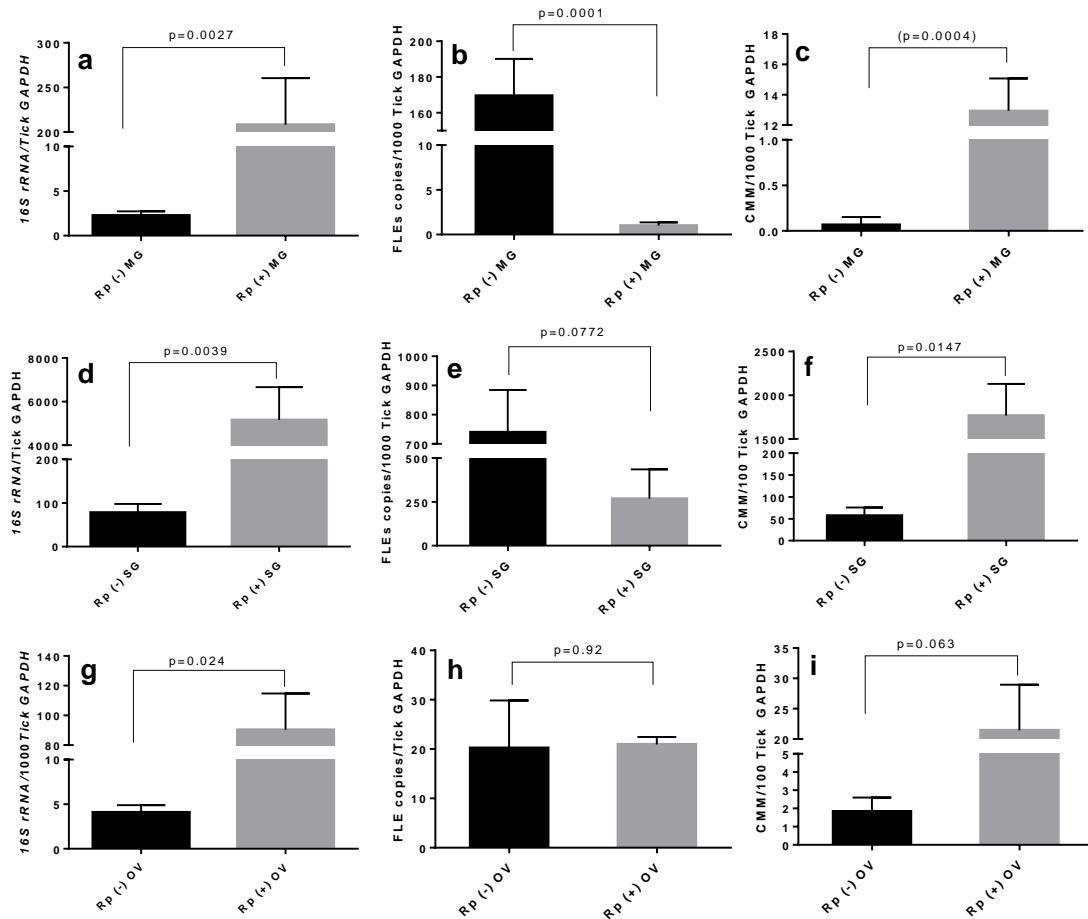
Figure 2.4 *Rickettsia parkeri* and symbionts dynamics in partially fed *Amblyomma maculatum*

Total bacterial load, FLE and CMM copies/tick *GAPDH* were measured by qPCR in tick guts tissues (a, b, c); salivary

glands (d, e, f) and ovaries (g, h, i) in Rp+ticks (black bar) and Rp-free ticks (gray bar). Rp; *Rickettsia parkeri*, OV; ovarian

tissues; Mg; midguts, Sg; Salivary glands.

2.4.4 *Francisella* Like Endosymbiont (FLE) Across the Tick Lifecycle

Except in ovaries, female adult tick midguts and salivary glands showed

decreased levels of FLE in Rp (+) ticks compared to Rp (-) ticks (Fig 2.4, 2.5). In

midgut tissues, it depressed to 1 from 169 copies/*GAPDH* in Rp-ticks (t=10.31,

df=8, p<0.0001), whereas in salivary glands it decreased from 739 to 267

copies/tick *GAPDH* (t=2.13, df=6, p=0.077) (Fig 2.4). The FLE load remained

unchanged in tick ovarian tissues with Rp infections (t=0.10, df=2, p=0.93). Like

in ovarian tissues, we did not observe alteration of FLE in eggs with Rp infections

(t=0.43, df=6, p=068) (Fig 2.5). Although FLE load was similarly unaffected in

unfed larval tissues (t=0.89, df =6, p=0.41), and the FLE load increased greatly in

Rp (+) fed larva (t=1.95, df=6, p=0.099) (Fig 2.5). The FLE depleted in Rp (+)

unfed nymphal tissues compared to Rp (-) unfed nymphal tissues but this was

not significant (unfed: t=0.66, df =10, p=0.53; fed: t=1.944, df =10, p=0.08) (Fig

2.5).

2.4.5 *Candidatus* Midichloria Mitochondrii (CMM) in Tick Lifecycle

The CMM were more than 180-fold higher in Rp (+) midgut (t=5.49, df=9,

p=0.0004), >30-fold higher in Rp (+) Salivary glands (t=3.22, df=7, p=0.015) and

>6 fold higher in ovarian tissues (t=2.59, df=4, p=0.06) compared to Rp (-)

tissues (Fig 2.4). It was increased fourfold in Rp (+) eggs (t=1.55, df=4, p=0.195),

20-fold in Rp+fed larva (t=5.29, df=6, p=0.0019) compared to Rp (-) tick eggs and

fed larva respectively. Similarly, CMM load was observed to be 30-fold higher in

Rp (+) unfed nymphal (t=10.38, df=10, p<0.0001) and >100 fold higher in fed

nymphs (t=16.68, df=8, p<0.0001) with respect to Rp (-) tick nymphs (Fig 2.5).

CMM load reduced to one third in unfed larva again with Rp infection (Fig 2.5).
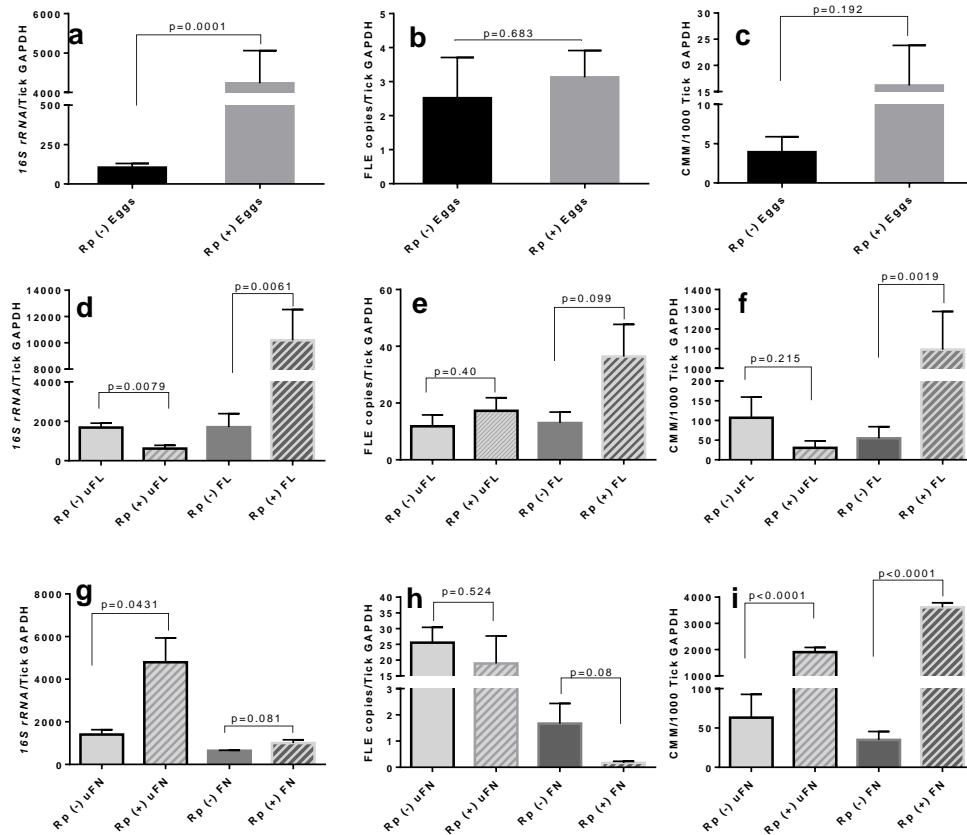
Figure 2.5 *Rickettsia parkeri* and symbionts dynamics in immature ticks.

The total bacterial load, FLE and CMM copies/Tick *GAPDH* in *R. parkeri* infected (Rp+ticks) and uninfected (Rp-free ticks) in embryonic and immature tick stages. In eggs (a, b, c); in larva (d, e, f) and in nymphal ticks (g, h, i). uFL; unfed larva, FL; fed larva, uFN; unfed nymph, FN; Fed nymphs.

2.5 Discussions

Pathogen replication inside tick tissues is an important aspect of the overall vector competence of ticks. We studied the dynamics of *Rickettsia parkeri* and two symbionts, *Francisella* like endosymbiont (FLE) and *Candidatus* Midichloria mitochondrii (CMM) across the tick life cycles. In *A. maculatum*, the most notable bacteria besides *R. parkeri* was *Francisella* like endosymbiont (FLE) (Budachetri et al., 2014) and recently *Candidatus* Midichloria mitochondrii

(CMM) was observed in RNAseq experiment. The replication of *R. parkeri* inside its tick vector, *A. maculatum* is the prerequisites for successful transmission to a vertebrate host or to the next life stage by transovarial transmission. This study investigated possible interactions between pathogenic *R. parkeri* with non-pathogenic symbionts inside *A. maculatum*.

In ticks, a blood meal can adversely affect bacterial replication because of the blood-induced oxidative stress (Budachetri and Karim, 2015); however *R. parkeri* defies oxidative stress and multiplies despite the blood meal in larva and nymphs after blood meal (Fig 2.2). The *Borrelia burgdorferi* in *Ixodes scapularis* multiplies with blood meal in tick tissues (Piesman et al., 2001). Both studies lack detail about how many bacteria a tick inoculates during feeding, and consider only the bacterial load in ticks, particularly in the unfed and fed stages and at particular time points in the tick tissues. The total bacterial load in Rp (+) ticks compared to Rp (-) ticks is higher possibly due to the rickettsial load in Rp (+) ticks increasing instead of native bacteria (Fig 2.4, 2.5). In both Rp (+) ticks and Rp (-) ticks, the total bacterial load decreases after the blood meal similar to earlier observation in naïve ticks (Budachetri and Karim, 2015).

Nevertheless, our results confirm the transovarial and transstadial nature of *R. parkeri*, along with FLE and CMM in *A. maculatum*, as reported earlier (Baldridge et al., 2009; Sassera et al., 2006; Wright et al., 2015). The FLE and CMM symbionts, whose distinct function has not been determined yet, could be an important source of nutrient limitation in ticks (Rio et al., 2016). The dynamics of interaction between microbes inside tick that could be utilized for transmission

blocking strategies for human pathogen of tick origin. Microbial symbionts play crucial roles in arthropod physiology and pathogen colonization and innovative use of these symbionts could offer a novel method for controlling vector-borne disease transmission (Sassera et al., 2013).

This study showed the CMM is intricately related to Rp and multiples with Rp infections across the tick life cycle (Fig 2.3, 2.4). Though the CMM diminishes with blood meal in Rp (-) ticks with blood meal (Fig 2.3), it always thrived with Rp infections. CMM had been reported from many arthropods including tick as having unique intra-mitochondrial life which overwhelmingly dominates bacterial profiles in *Ixodes holocylcus* (Gofton et al., 2015; Richard et al., 2009; Sassera et al., 2006). Interestingly, overwhelming CMM loads have been found in *I. holocyclus* and these microbes are known to flourish in the presence of a blood meal in *I. ricinus* (Sassera et al., 2008) but was not observed in *A. maculatum* (Budachetri et al., 2014)*. Rather, CMM in *A. maculatum* can be viewed as a successful colonizing partner of *R. parkeri*. Based on the sero-positivity to *Candidatus* Midichloria mitochondrii in human parasitized by *Ixodes ricinus* tick, pathogenicity status of CMM has been proposed (Mariconti et al., 2012).

FLE level depleted in *R. parkeri* infected ticks suggesting inverse relationship between them but with exception in ovaries and eggs (Fig 2.3), indicating possible collaboration between these two bacteria during transovarial transmission. The genus *Francisella* belongs to gram-negative coccobacilli and reported from tick not limited to ovaries (Scoles, 2004).The refractoriness of the human pathogen transmission from arthropod vector has been linked to mosquito

symbiont, *Wolbachia* (Mousson et al., 2012). Interestingly, mosquito symbiont, *Wolbachia* when infected to *Aedes aegypti* (which naturally lacks) impairs arboviral development and transmissions (Jupatanakul et al., 2014). The inverse relationship between human pathogen and arthropod symbiont had been reported in *Glossina* flies, where absence of the *Wiggleswrothia* symbionts leads to higher colonization of *Trypanosome* parasites (Pais et al., 2008). But, *Glossina* itself help proliferating *Wiggleswrothia* by secreting peptidoglycan recognition protein to prevent induction of tsetse's immune deficiency (Imd) pathway suggesting important role in fly reproduction (Wang and Aksoy, 2012). The importance of the microbiome in mosquito or tick survival or reproduction and pathogen infection had been reported but identification of particular bacteria is important milestone for killing vectors of human diseases using their own gut microbes (Beier et al., 1994; Zhong et al., 2007).There are reports gut bacteria *Serratia* sp or *Entobacteria* sp. from field-collected mosquitoes can impair sporogonic development of malarial parasite in insectary mosquito (Cirimotich et al., 2011; Gonzalez-Ceron et al., 2003).

There is more study needed to but our results provided that FLE or CMM could be an important symbiont like *Wolbachia* or *Chromobacterium* (Csp_P) in mosquito in interfering in pathogen colonization (Hughes et al., 2014; Ramirez et al., 2014). The bacteria residing inside tick or any arthropods as mutualistic, parasitic or commensal collectively referred as endosymbiosis. In this study, it was not determined that host response to *R. parkeri* further altered the symbionts or it is not known that symbiont displacement is immunological or non-

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immunological. The understanding of intricate dynamics of tick endosymbiosis and alteration or breakdown of symbiosis could generate more options for control of vector by impairing host physiology or vector competences.

2.6 Conclusions

The successful colonization of *Rickettsia parkeri* inside its competent vector, *A. maculatum* is associated with unique ability of *R. parkeri* to dynamically interact with tick symbionts. This study opens more research questions to confirm if pathogen-symbiont dynamics is due to tick response to pathogenic *R. parkeri* which influenced symbionts. Further utilization of FLE or CMM in relation to impairing *R. parkeri* colonization inside tick guts tissues would yield important discovery.

CHAPTER III –SECIS BINDING PROTEIN 2 (SBP2) AND PUTATIVE

SELENOPROTEIN P (SELENOP) ARE REQUISITE FOR TICK FECUNDITY

AND *RICKETTSIA PARKERI* COLONIZATION

3.1 Abstract

Ticks are hematophagous ectoparasitic arthropods that are the second

deadliest vector of disease after mosquitoes. *Amblyomma maculatum* is the

known vector of *Rickettsia parkeri*, an infectious bacterial pathogen. In this study,

we hypothesized that tick SECIS binding protein 2 (*SBP2*) and putative tick

selenoprotein P (*SELENOP*, *SelP*) are requisite for selenoproteome synthesis

and rickettsial colonization inside ticks. To assess role in physiology and

pathogen colonization, RNAi assay was used to selectively knockdown tick *SBP2*

and *SELENOP* genes in *Rickettsia parkeri* infected ticks. The rickettsial

colonization and displacement of tick symbionts was assessed using qPCR

assay in both *SBP2* and *SELENOP* knockdown ticks. Both tick genes were

significantly depleted in *R. parkeri* infected tick tissues (Rp (+) tick) using RNAi

assay. The knockdown of the *SBP2* gene negatively impacted transcriptional

activity most of selenoproteins tested including *SELENOP*. Whereas tick

selenophosphate synthetase (*SEPHS2*, *SPS*) and *SELENOO* expressions were

increased with *SBP2* knockdown. Whereas *SELENOP* knockdown showed

upregulation selenophosphate synthetase (*SEPHS2*), *SBP2* and *Cu/Zn-SOD* in

tick gut tissues and *SELENOO* and *TrxR (TXNRD2)* were upregulated but

Catalase was depleted in salivary glands. Both tick *SBP2* and putative

SELENOP knockdown did not affect tick engorgement but *SBP2,* not *SELENOP*

silencing significantly impaired ovipositioning as estimated by egg mass conversion ratio. Moreover, the transovarial transmission of the *R. parkeri* to tick eggs and hatching of eggs were significantly impaired by *SBP2* knockdown. We conclude that the novel tick selenoprotein synthesis gene, *SBP2,* and putative tick *SELENOP* were functionally related to synthesis of selenoproteins and rickettsial colonization in tick.

3.2 Introduction

The eukaryotic selenoproteins mRNA form stem-loop structure at 3'-untranslated regions known as selenocysteine insertion sequence (SECIS). This stem-loop structure helps in recoding of UGA stop codon as selenocysteine (Sec). The machinery of Sec incorporation requires SECIS binding protein (*SBP2*) that recognizes SECIS element, this interaction of protein and RNA along with elongation factor (eEFSec), specific Sec-tRNA[(sec)] are the important factors involved in selenoprotein synthesis (Labunskyy et al., 2014). The SBP2 protein is unique in selenoprotein synthesis that bind to SECIS core and allows binding of ribosomal protein (rpL30) which stimulate in recoding of UGA. The *SBP2* sequence has conserved region (L7Ae family) of RNA binding proteins (binds to *28S rRNA* ribosomal proteins) near C-terminus, which is site for SECIS binding as well (Copeland et al., 2001). SBP2 selectively and with high affinity binds to SECIS elements, and this SBP2: SECIS binding highly correlated with ability of SECIS to compete for recoding of UGA (Bubenik et al., 2015). The mutation of SBP2 impairs the binding to SECIS elements and severely halts selenoproteome synthesis (Schoenmakers et al., 2010).

There are 25 selenoproteins known in human containing at least one selenocysteine (U) to 10 (in SELENOP) and play antioxidant role (Labunskyy et al., 2014). The redox property of selenoproteins depend in selenium atom in selenocysteine residue. The selenium's inability to form many forms of pi bonds, ease of reducing organic and inorganic hydrogen peroxides without oxidative inactivation make selenoproteins as unique antioxidants (Reich and Hondal, 2016). All the function of selenium is collectively performed by selenoproteins and level of selenium affects the selenoprotein biosynthesis, in severe selenium deficiency sulphur can take selenium's position. The health importance of selenium were reported with strengthening of immune functions, reducing viral infections, critical risk factor for cardiovascular diseases and also known to reduce chances of getting cancer (Rayman, 2000). Selenium has been associated with reducing oxidative stress or inflammatory conditions and enhancing fertility. It's been reported that the reduced serum selenium level corresponds to loss of CD4 T cells in HIV-1 infections (Look et al., 1997). Further, selenium supplementation was reported in reducing the chances of miscarriage (Barrington et al., 1997; Hidiroglou, 1979) and even inhibit proliferation of cancerous cells (Sun et al., 2013).

The glutathione peroxidase (GPx4), a selenoprotein protects the developing sperm cells from oxidative damages and become structural protein in mitochondrial capsule of sperm midpiece (Ursini et al., 1999). The selenoprotein P (SELENOP) is multiple selenocysteine (Sec, U) containing selenoprotein and most of selenium (>50% of total in body) in human plasma is associated with this

protein. Plasma selenoprotein P is the indicator of selenium level in body and considered to be storage and transporter of selenium across the body (Richardson, 2005).

Amblyomma maculatum vectors Rickettsia parkeri, a pathogen which cause spotted fever similar to Rocky Mountain Spotted Fever (RMSF) with distinct clinical features of mild fever, eschar-associated rashes not observed in RMSF (Paddock et al., 2008). Amblyomma selenoproteins were discovered in massive transcriptome project from tick salivary glands (Karim et al., 2011) and previous work suggested antioxidant role for selenocysteine elongation factor, selenoprotein M (SELENOM) and SELENOK (Adamson et al., 2014, 2013). In this study, we have targeted important selenoprotein synthesis machinery protein, SECIS binding protein 2 (SBP2) and putative tick selenoprotein P (SELENOP) and characterized their roles in tick physiology and pathogen colonization and transovarial transmissions.

3.3 Methods

3.3.1 Tick and Tick Tissues

The Gulf coast tick (A. maculatum) adults, collected from Sand Hill wildlife refuge, Gautier, Mississippi were blood fed and allowed to lay eggs. The screening of R. parkeri at egg stages allowed us opportunity to segregate R. parkeri infected; Rp (+) and uninfected; Rp (-) ticks. The transovarial and transstadial maintenance of R. parkeri across the tick life cycle helped us the establishment of tick colonies with or with R. parkeri infection. The tick colonies were maintained at the University of Southern Mississippi according to

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established methods (Patrick and Hair, 1975). Ticks were kept at room temperature with approximately 90% relative humidity under a photoperiod of 14 h light/10 h dark before infestation on sheep. Ticks were blood fed on sheep and was either allowed to replete or removed between 3-10 days from host, depending on the experimental protocol. The protocols for tick blood feeding on sheep (for adults) and immature ticks (on hamsters) were approved by the institutional Animal Care and Use Committee of the University of Southern Mississippi (protocol #10042001 & 15101501).

3.3.2 Bioinformatics Analyses

The coding sequences for SECIES binding protein 2 (*SBP2*) and selenoprotein P (*SELENOP*) were obtained from *A. maculatum* sialotranscriptome (Karim et al., 2011). Both nucleotide sequences were conceptually translated into its amino acid sequence and aligned using ClustalX2 multiple sequence alignment with the protein sequences from other species. In case of SBP2, *Homo sapiens*, human (Q96T21), *Equus caballus,* horse (F6V110), *Danio rerio,* Zebrafish (F1R3N8) and *Ixodes scapularis,* black legged tick (B7Q610) sequences obtained from uniprot (www.uniprot.org) (Larkin et al., 2007; Thompson et al., 2002) and graphically represented in Jalview after multiple sequence alignment with tick SBP2 (Jones et al., 1992). The phylogenetic tree was built using MEGA6 software (Tamura et al., 2013) using additional sequences for SBP2 from *Oryctolagus cuniculus,* rabbit (G1T4E3), *Rattus norvegicus*, rat (Q9QX72), *Mus musculus*, house mouse (NP_083555.1), *Clupea harengus*, atlantic herring (XP_012670383.1), *Poeciliopsis prolifica*,

blackstripe livebearer (A0A0S7GUM1), *Ixodes scapularis*, blacklegged tick (ISCW021662 or B7Q610) for phylogenetic insights.

Amblyomma maculatum SELENOP were aligned with that from *Ixodes scapularis* (B7Q2F3), *Ixodes ricinus* (V5GWR4), *Rhipicephalus appendiculatus* (A0A131YE48), *Homo sapiens* (P49908), *Rattus norvegicus* (P25236), *Mus musculus* (P70274), two sequences from *Danio rerio* (Q98SV1 and Q98SV0) and visualized in Jalview and MEGA6 software was used to get phylogenetic history (Tamura et al., 2013).

3.3.3 Tick Tissues Isolations

The unfed and partially blood-fed female adult ticks were dissected within four hours of after removal from the sheep. Tick midgut and salivary gland tissues were dissected ice-cold M-199 buffer (Morgan et al., 1955, 1950). After removal, midguts and salivary glands were washed gently in the same ice-cold buffer. The tick tissues were either stored immediately after dissection in RNAlater (Invitrogen, Carlsbad, CA, USA) prior to extracting mRNA or protein extraction buffer.

3.3.4 RNA Preparation, cDNA Synthesis and qRT-PCR

The tick tissues stored in RNAlater were used for total RNA extraction using an Illustra™ RNAspin Mini Isolation kit (GE Healthcare, Piscataway, NJ, USA) per manufacturer's instructions. The total RNA concentration was determined using Nano drop spectrophotometer. Total RNA (1 µg) was reverse transcribed into cDNA using iScript™ cDNA synthesis kit (Bio-Rad, Inc., Hercules, CA, USA). Gene-specific primers were designed to amplify the cDNA

from *A. maculatum* tissues (Table 3.1). Transcriptional gene expression study

was normalized using glyceraldehyde 3-phosphate dehydrogenase (*GAPDH).* All

genes used in this study were first amplified using gene-specific primers (Table

3.1) and their sequences were confirmed by sequencing prior to either dsRNA

synthesis or gene expression studies. iTaq™ Universal SYBR® Green Supermix

(Bio-Rad Inc., Hercules, CA, USA) and 25ng of cDNA and 150nM of gene-

specific primers were used for each reaction mixture. The qRT-PCR reaction

mixtures were subjected to 10 minutes at 95 °C, followed by 35 cycles of 15

seconds at 95 °C, 30 seconds at 60 °C, and 30 seconds at 72 °C using CFX96

Real-Time System (Bio-Rad Inc., Hercules, CA, USA).

Table 3.1

The Oligonucleotides Used in this Study.

	mRNA ID	Forward (5'-3')	Reverse (5'-3')	size (bp)
SELENO P or SelP		GGA GGC TCT TAC ACA TGT TCT C	CAC GTT CGA AGC TCC AGT ATC	85
SELENO P or SelP (dsRNA)		CCTGCAGTTCCTA GTGGTCA	AAGCTCCAGTATC ATGCGGG	403
SBP2		CTA CTG CGA CCA TGT CCT AAC	GTC CTT CTG GTA AAG TCG ATC C	97
SBP2 (dsRNA)		ATGACCGTGAAGA CGTTGCT	GTCCTCAGAGTCG GACTCCT	567
SEPHS2 or SPS		GCA AAC TGA CGG AGA AGG A	TGC CAC AGC CAC CAA TAA	141

rOmpB	AF123717	CAAATGTTGCAGTT CCTCTAAATG	AAAACAAACCGTT AAAACTACCG	96
EEFSec or SEF	KC989559	TGGCTCCAGAAAT GCTGCTCATTG	ACGCCTTTGCGAC TCTTCTCCTTA	157
SELENO K or SelK	JO843326	AGTTCCAGCAGGT CATCAGTGTCA	TCCAGGAATAGGG CAGTCCATTGT	132
SELENO M or SelM	JO842653	GCTGTACGATGAG AATGGAGAG	AGCCAGGTGCTCA AACAA	94
SELENO N or SelN	KC989560	TTAGTTTGGACACT GTGGACGGGT	AGGCTTCTCTAAC AACGGCACTCA	150
SELENO O or SelO	KC989561	AAGCTCGGCCTTG TGAAGAGAGAA	TACAGCACGACAA GAGCTTGGACA	190
SELENO S or SelS	JO842687	AGAACAAGTGCAC CACAACAGCAG	ATTTCTTGCATCCT TCGACGTGCC	107
SELENO T or SelT	KC989562	TCTTTGTGTGTGGA GCCATCGAGA	ACCACACCCGCAC GTCATTAAAGT	81
MSRB1 or SelX	JO845128	ACCACTCTCCTTG GCCATCATTCA	TGCACTTCCCACA GTACACCTTGA	108
TrxR or TXNRD2	JO843723	TGTGACTACACCA ACGTGCCTACA	AGTAGCCTGCATC CGTTCCTCTTT	175
GAPDH	JO842341	CACCCATCACAAA CATGGGTGCAT	TTTCAGGAAATGA AGCCTGCCAGC	175

3.3.5 RNAi Assay: Silencing of Tick *SBP2* and *SELENOP*

Synthesis of double-stranded RNA (dsRNA) (for *SBP2* and *SELENOP*) and tick manipulations were performed according to the methods described previously (Crispell et al., 2016). Briefly, the PCR product, purified using PCR purification kit (Qiagen, Valencia, CA, USA), was used in a second set of PCR

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using the same primers (Table 3.1), but with the addition of flanking T7 binding site sequence (GAATTAATACGACTCACTATAGGG) that allow the binding of RNA polymerase and the generation of dsRNA. After confirming the T7-flanked *SBP2* and *SELENOP* gene sequence, the secondary PCR product was invitor transcribed using a T7 Quick High Yield RNA synthesis kit (New England Bio labs, Ipswich, MA, USA) by incubating the PCR product with T7 polymerase overnight at 37°C. The resulting dsRNAs were purified by ethanol precipitation and their concentrations were measured spectrophotometrically using a Nanodrop (Thermo Fisher Scientific, Wilmington, DE, USA). The product was visualized by gel electrophoresis using a 2% agarose gel. The dsSBP2 and dsSELENOP were diluted to make working concentration of 1µg/µL. The same protocol was used to synthesize dsRNA-LacZ to be used as an irrelevant dsRNA control. The 20-25 unfed adult female ticks (*R. parkeri* infected) for each group for *SBP2*, *SELENOP* and *LacZ* were microinjected with 1µl dsRNA using a 27-guage needle. Ticks were kept overnight at 37°C to alleviate needle trauma and promote survival. Surviving ticks were infested on a sheep and in the presence of male ticks. For sample collection, total of 12 partially fed experimental, control ticks were removed on day 5 and 7 post tick infestation, with the remaining ticks allowed to remain attached, and blood feed until repletion. The feeding success of the individual ticks was evaluated by recording the attachment duration, repletion weight and the ability to oviposition (Karim et al., 2012). The period of oviposition in ticks completes by 25-30 days and after completion of oviposition, eggs were collected from each control and treatment groups (dsLacZ, dsSBP2

and dsSELENOP) ticks and stored in RNAlater and placed in -80°C before RNA extraction. Partially fed ticks removed from each group were dissected to obtain their midguts and salivary gland tissues.

3.3.6 Quantification of Total Bacterial, *Rickettsia parkeri*, FLE and CMM Loads

The *R. parkeri* load was determined quantifying rickettsial outer membrane protein B (*ompB*) copies per tick *GAPDH* copies using qRT-PCR assay as described earlier (Budachetri et al., 2014; Jiang et al., 2012). The bacterial load in each tick tissue was estimated as described previously (Budachetri and Karim, 2015; Narasimhan et al., 2014). Similarly, slight modification of published protocol was used to estimate *Francisella* like endosymbionts (Dergousoff and Chilton, 2012) and *Candidatus* Midichloria mitochondrii (Sassera et al., 2006). We made standard curves for each genes for quantifying total bacterial load, Rp load, CMM and FLE using published method and similarly, tick *GAPDH* standard curves were prepared corresponding to each quantification protocols. The copy numbers were calculated using the respective standard curves and loads were represented per tick *GAPDH* copies.

3.3.7 Lipid Peroxidation Assay

The lipid peroxidation assay kit (Sigma-Aldrich, St. Louis, MO, USA) measures malondialdehyde (MDA) level formed after peroxidation of polyunsaturated lipids by reactive oxygen species which provides oxidative stress level in tissues. In this kit, lipid peroxidation is determined by estimation of reaction product of MDA with thiobarbituric acid (TBA) and measured

colorimetrically at wavelength of 532nm which is proportional to MDA level

present in the sample. All the procedures were followed according to

manufactures' recommendations.

3.3.8 Data Analysis

All data are expressed as mean ± SEM unless otherwise mentioned.

Statistical significance between the two experimental groups or their respective

controls was determined by the Mann-Whitney rank sum test or t-test (P value,

0.05). Comparative differences amongst multiple experimental groups were

determined by analysis of variance with statically significant P values of <0.05

(Graph pad Prism6.05, La Jolla, CA). Transcriptional expression levels were

determined by Bio-Rad software (Bio-Rad CFX MANAGER v.3.1), and

expression values were considered significantly if P value, 0.05, when compared

to control.

3.4 Results

3.4.1 Bioinformatics Analyses

The sequence for SECIS binding protein 2 (*SBP2*) from *Amblyomma*

maculatum was aligned with sequences from *Homo sapiens*, human (Q96T21),

Equus caballus, horse (F6V110), *Danio rerio*, Zebrafish (F1R3N8) and *Ixodes*

scapularis, Blacklegged tick (B7Q610) (Fig 3.1). The *A. maculatum* SECIS

binding protein showed 27-28% sequence identities to sequences from

vertebrates, *E. caballus*, and *D. rerio* but 42-45% with that of prostriate tick

species, *I. scapularis* and *I. ricinus* (V5IFJ7). The gray highlight (96 amino acid

residues) represents the conserved domain between vertebrates and tick SBP2
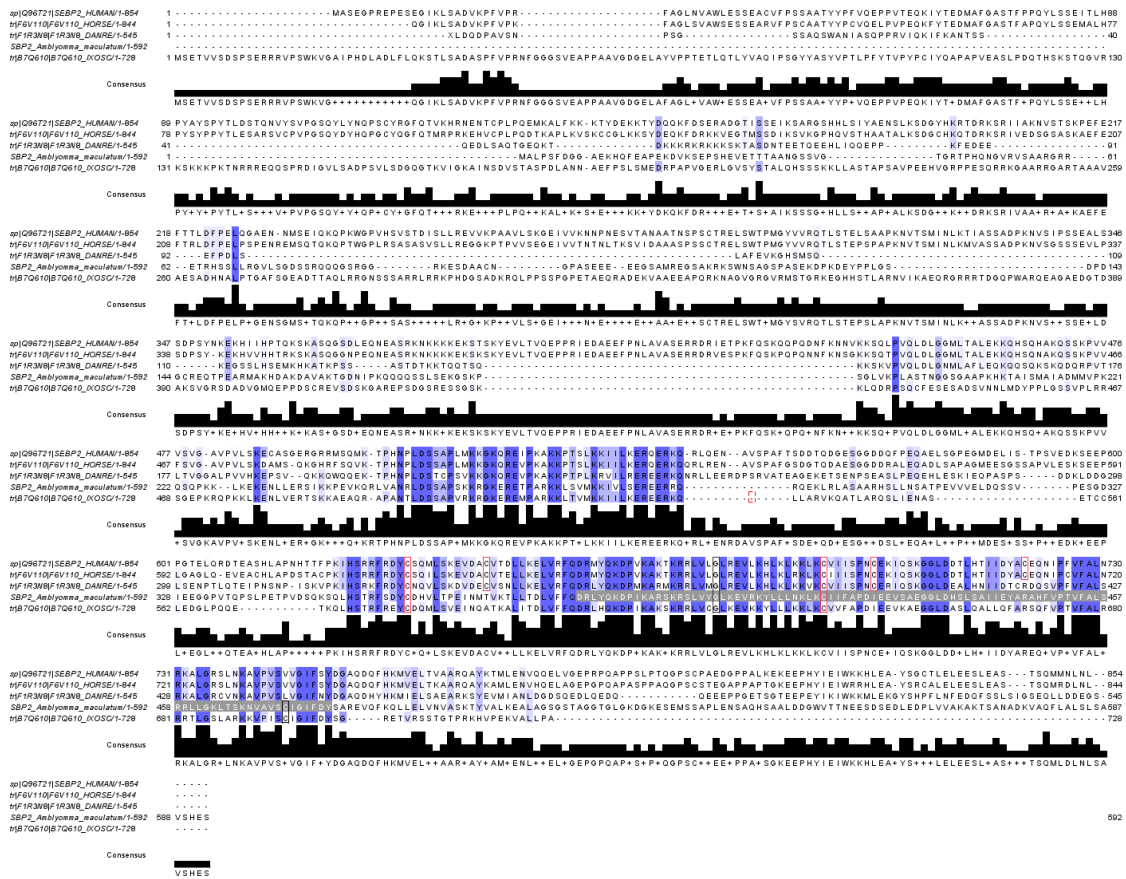
Figure 3.1 Multiple sequence alignment of SECIS binding protein 2(SBP2) amino acids sequences from different taxa.

The regions outlined by red box are conserved region between listed species. The sequences of SBP2 from *Amblyomma maculatum* were aligned with sequences from *Homo sapiens*, human (Q96T21), *Equus caballus*, horse (F6V110), *Danio rerio*, Zebrafish (F1R3N8) and *Ixodes scapularis*, Blacklegged tick (B7Q610).

(Fig 3.1). The amino acid identities in conserved domains showed *A. maculatum* shared 54-56% identities with vertebrates but about 73.96% identities with *I. scapularis*. Cysteine residues are conserved and represented in box with red border are important to SECIS region binding capacity of selenoprotein mRNAs (Bubenik et al., 2015). NCBI CDD databases search showed the binding domain from ribosomal protein 7Ae/L30e/S12e/Gadd45 family and RNA binding domain in overlapped region shaded gray in alignment (Fig 3.1) (Marchler-Bauer et al.,

2015). The SBP2 domain structure and ribosomal binding studies showed the SBP2 interaction with ribosome occurs via *28S rRNA*. They showed RNA binding as prerequisite for Sec insertion, and importance of Glycine residue in RNA binding ability (boxed in black in Fig 3.1) the region lies between amino acid 517-777 in rat SBP2 (Copeland et al., 2001).

The evolutionary relationship between SBP2 amino acid sequences from vertebrates, ticks, mosquitoes, flies and worms was derived from phylogenetic tree prepared using MEGA6 software (Tamura et al., 2013) (Fig 3.2). The SECIS binding protein 2 (SBP2) sequences from vertebrates: human, horse, zebrafish, rabbit, rat, house mouse, Atlantic herring, Black stripe livebearer, Chicken, and Ticks: Black-legged tick and *Ixodes ricinus* were used with *Amblyomma maculatum* SBP2 (Karim et al., 2011). Similarly, SBP2 sequences from *Plasmodium gaboni*, *P. reichenowi*, and *P. falciparum*; flies: *Drosophila melanogaster*, *D. simulans*, and *Glossina morsitans.* And, similarly SBP2 sequences from parasitic worms: *Echinococcus granulosus*, *Ascaris lumbricoides* and *Enterbious vermicularis* along with model organism *Caenorhabditis elegans* (Fig 3.2) were used to deduce phylogenetic relationships. The SBP2 sequences from each taxonomic class made their own clades (Fig 3.2).
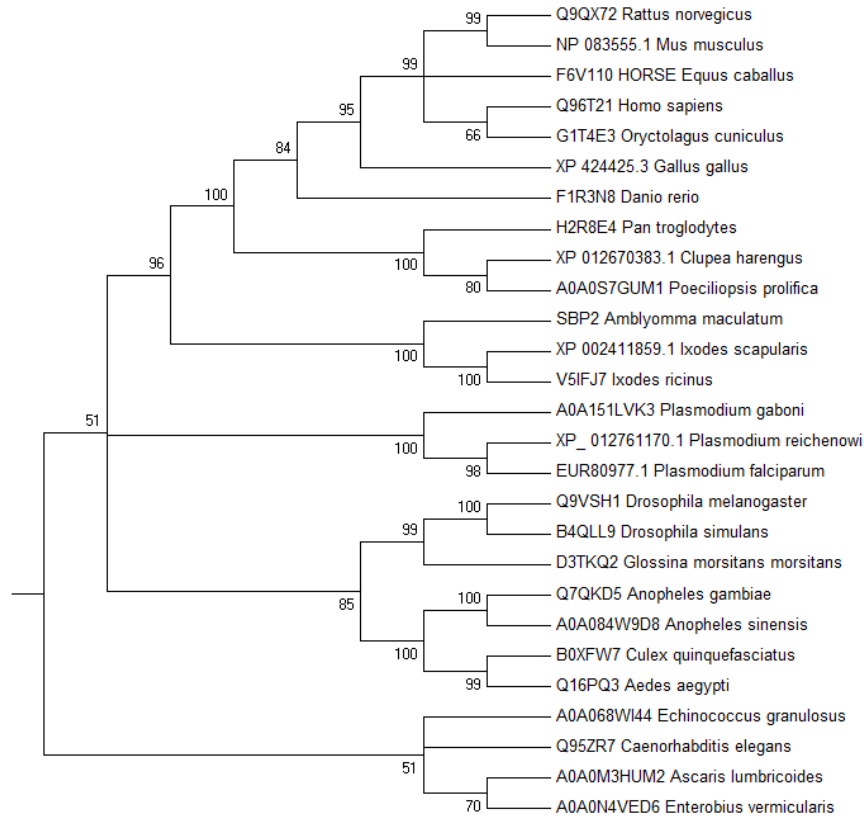
Figure 3.2 Evolutionary relationships of taxa based on SECIS binding protein (SBP2) using neighbor-joining method.

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 1000 replicates (Joseph Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The analysis involved 27 amino acid sequences. All the position with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There was a total of 132 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013). SBP2 sequences with accession number followed by species in tree.

Putative selenoprotein P (SELENOP) in *Amblyomma maculatum* showed 27-28% identities with vertebrate selenoprotein P (SELENOP) from rat, mouse, and humans but less than both SELENOPs from zebrafish (23 % identities) (Fig 3.3) which is very low identities compared to 72% identities between human and

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mouse or rat. Though there is 92% sequence identities between *Ixodes scapularis* and *I. ricinus* SELENOPs but *Amblyomma* SELENOP shared only 36% sequence identities with both *Ixodes* SELENOPs. The *Rhipicephalus appendiculatus* SELENOP sequence shared 67% with *Amblyomma* SELENOP but only 36-37% with that of *Ixodes scapularis* and *I. ricinus* (Fig 3.3). In the Fig 3.3, sequences in green border represent signal peptide suggesting of secretory nature of the SELENOP sequences only from vertebrates. The red border was made to highlight selenocysteine (U) resides which are selenium-containing amino acid specific to selenoproteins. In the N-terminal side, up to poly histidine (region of sequences represented by black borders, many histidine residues) only one selenocysteine residue was observed (Fig 3.3). The number of selenocysteine residues along the vertebrates SELENOP varies from 10-17. The rat, mouse and human selenoprotein P contains 10 selenocysteine residues (one in N-terminal and 9 at C-terminus) whereas zebrafish (SELENOPa) contains 17 selenocysteine residues (one in N-terminal and 16 at C-terminus) (Fig 3.3). The position of selenocysteine residues in rat and mouse differs from that of human (Fig 3.3). The tick selenoprotein P (SELENOP) were unique in not having signal peptide sequences and did not match with N-terminal selenocysteine residue of SELENOP from vertebrate (Fig 3.3). NCBI CDD databases search showed the putative domain in SELENOP (SelP_N) from *Amblyomma maculatum*. The zebrafish SELENOPb contains only one selenocysteine (U) at N-terminus (Fig 3.3).
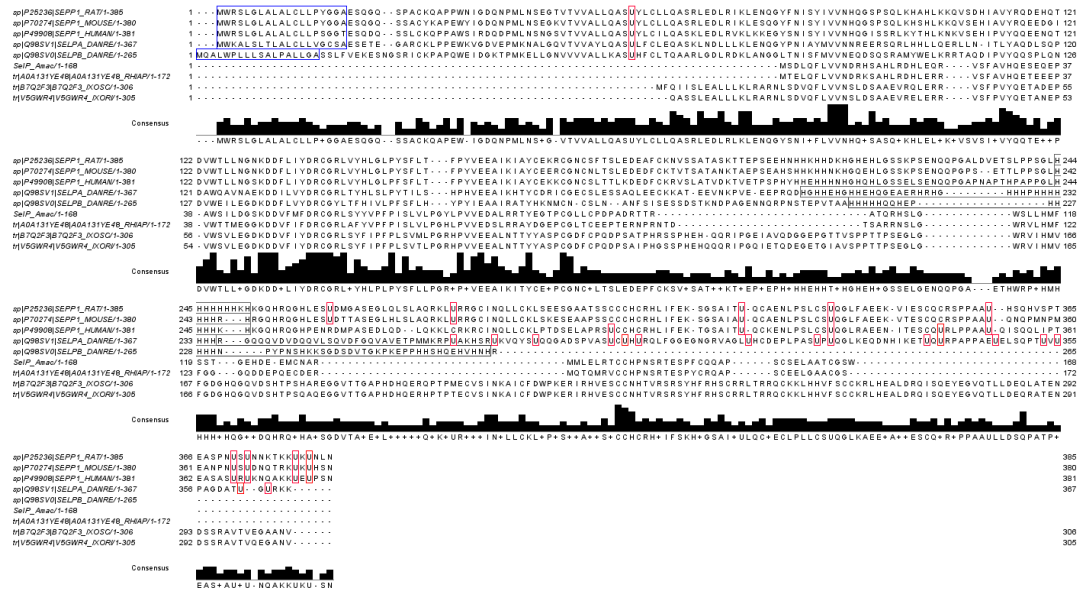
Figure 3.3 Multiple sequence alignment for selenoprotein P (SELENOP).

Note: The putative selenoprotein P (SELENOP) sequences from *Amblyomma maculatum* were aligned with SELENOP sequences from *Rattus norvegicus*, rat (P25236), *Mus musculus*, mouse (P70274), *Danio rerio*, Zebrafish (Q98SV1, Q98SV0), *Ixodes scapularis*, Black-legged tick (B7Q2F3), *Ixodes ricinus*, castor bean tick (V5GWR4) and *Rhipicephalus appendiculatus*, Brown ear tick (A0A131YE48).

The phylogenetical history among SELENOP were obtained using MEGA6 software after changing all the selenocysteine to cysteine (U to C) showed the marked differences between SELENOP from vertebrate to that of ticks and SELENOPb from zebrafish made separate clade as well (Fig 3.4).
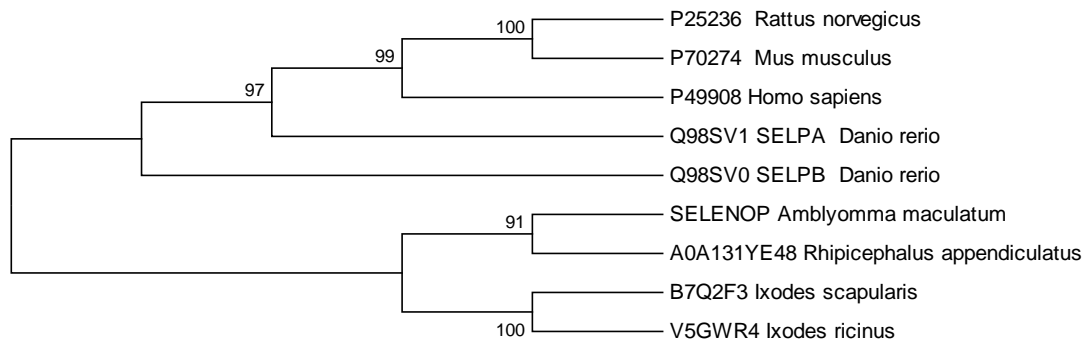
Figure 3.4 Molecular phylogenetic analysis of SELENOP by Maximum likelihood method.

3.4.2 *SBP2, SELENOP* and *SEPHS2* Expressions with Blood Meal and Pathogen Infections

The transcriptional expressions of SECIS binding protein (*SBP2*), *SELENOP* and *SEPHS2* were estimated in unfed, slow feeding stage (day 4) and fast feeding stage (day 8) of *Amblyomma maculatum* blood meal. *SBP2* transcriptional activity depleted with blood meal in both tick tissues, except in gut tissues at 8 dpi (days post infestations), which is like transcriptional level at unfed stage (Fig 3.5a). The selenoprotein P (*SELENOP*) and selenophosphate synthetase (*SEPHS2*) transcriptional activities were observed to see relative importance of selenoprotein synthesis machinery in relation to selenium transportation (*SELENOP*) and as a selenium donor (*SEPHS2*). Both, *SELENOP* and *SEPHS2* transcripts diminished with blood meal at slow and fast feeding stages (Fig 3.5 b, c) whereas in gut tissues *SELENOP* up-regulated to >3 folds at slow feeding stage but diminishes later (Fig 3.3b). Like *SBP2*, transcriptional activity of *SEPHS2* in gut tissues was up-regulated >40 fold in fast feeding stage (Fig 3.5c).

The transcriptional expression of *SBP2*, *SELENOP* and *SEPHS2* were estimated in *R. parkeri* infected tick tissues to assess the tick response to pathogen infection (Fig 3.5d). The tick tissues were first evaluated for the infection of *R. parkeri* in tick tissues and transcriptional expressions were estimated in uninfected and infected tick tissues. In both tissues tested, *SBP2*

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expressions were significantly upregulated with *R. parkeri* infections; 2-fold in gut tissues (p<0.005) whereas fivefold in salivary glands (P<0.005). Similarly, rickettsial infection increases the expression of *SELENOP* in both tissues (P<0.05) whereas *SEPHS2* was up-regulated only in gut tissues (Fig 3.5d).
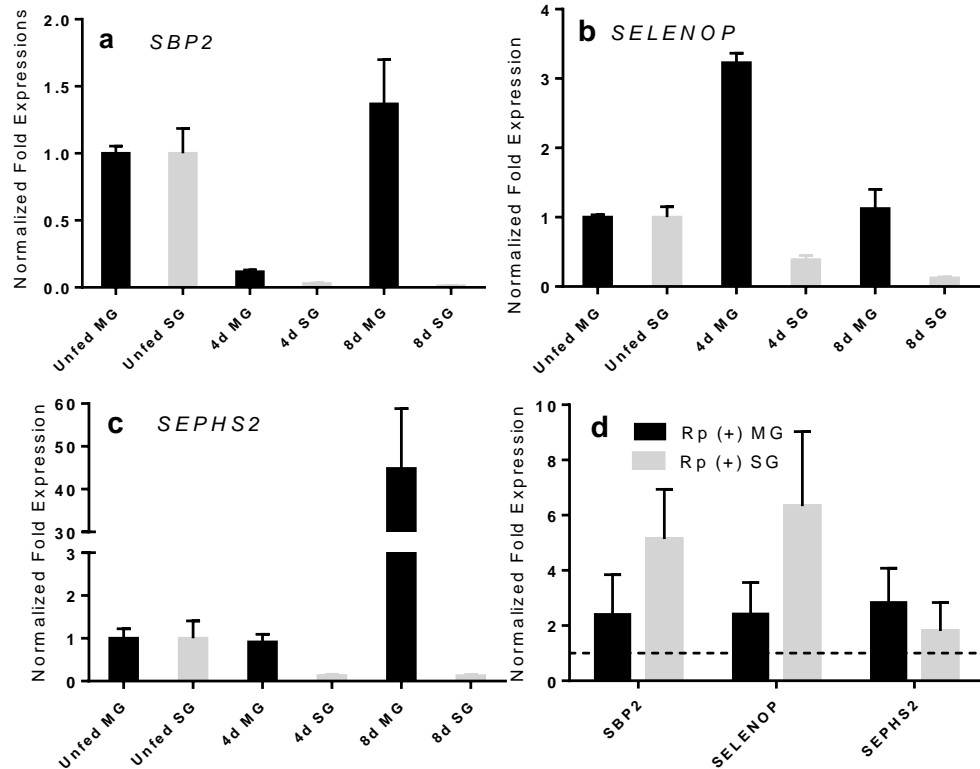
Figure 3.5 Transcriptional gene expressions of *Amblyomma maculatum* SECIS binding protein (*SBP2*), selenoprotein P (*SELENOP*) and selenophosphate synthetase (*SEPHS2*) with blood meal and *Rickettsia parkeri* infection.

Transcriptional gene expressions for (a) *SBP2*, (b) *SELENOP* and (c) *SEPHS2* were estimated at unfed, slow feeding ticks (4dpi) and fast feeding ticks (8dpi) in female *A. maculatum*. The expression at unfed was made 1 for reference. (d) Tick *SBP2*, *SELENOP* and *SEPHS2* were upregulated in *R. parkeri* infected tick midgut and salivary glands tissues. The transcription activities were normalized with tick *GAPDH*.

3.4.3 Tick *SBP2* and *SELENOP* Silencing in Vivo

We have significantly knocked down *SBP2* transcripts with 93% in midguts and 90% in salivary glands in Rp (+) ticks (Fig 3.6). The impact of knockdown of tick *SBP2* in selenoprotein synthesis machinery genes and selenoproteins were assessed using transcriptional gene expressions. The transcriptional expressions of *SELENOP*, *SELENOS*, *SELENOK*, *TXNRD2*, *SELENON*, *SELENOT*, *SELENOX* were depleted with silencing of tick *SBP2* but *SEPHS2* and *SELENOO* were significantly upregulated whereas, transcriptional expressions of *eEFSec*, *SELENOM* were remained unaffected (Fig 3.6). Though catalase did not alter its expression but superoxide dismutase (*Cu/Zn-SOD*) significantly upregulated with *SBP2* knockdowns (Fig 3.6).

In separate experiment, RNAi assay for silencing of *SELENOP* showed the 96% knockdown in tick midgut tissues and about 97% knockdown in tick salivary glands (Fig 3.6). We observed significant upregulation ($P<0.05$) of selenophosphate synthetase (*SEPHS2*), SECIS binding protein 2 (*SBP2*), and *Cu/Zn-SOD* in gut tissues with *SELENOP* silencing (Fig 3.6). Whereas, Selenoprotein O (*SELENOO*) and thioredoxin reductase (*TXNRD2*) were significantly upregulated in tick salivary glands with *SELENOP* silencing (Fig 3.6). The Selenoprotein S (*SELENOS*) in both tick tissues and *MRSB1* (*SelX)* in midguts and *Catalase* in salivary glands were significantly depleted ($p<0.05$) with *SELENOP* knockdown (Fig 3.6).
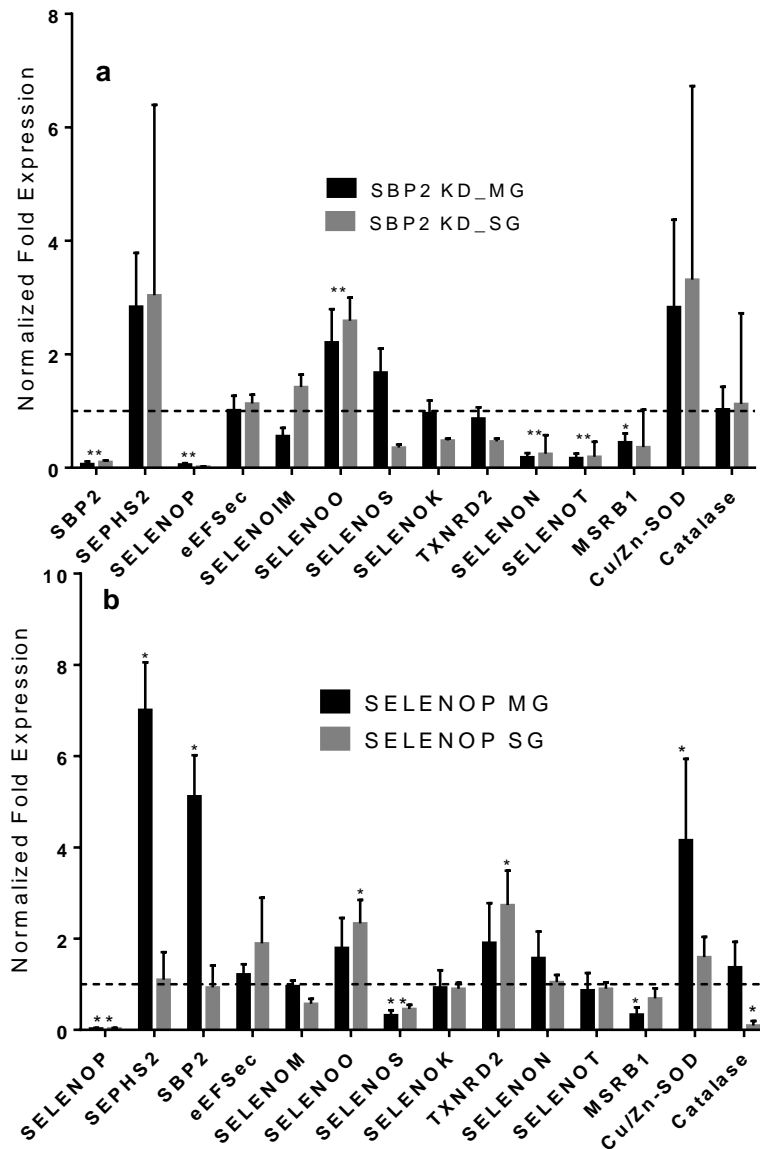
Figure 3.6 Knockdown of the SECIS binding protein 2 (*SBP2*) (a) and *SELENOP* (b) in Rp+ticks.

The transcriptional expressions of the selenogenes were assessed in *SBP2* and *SELENOP* knockdown tick tissues. The

qRT-PCR was used to determine transcriptional expression of tick selenoproteins using tick *GAPDH* as reference gene.

The expressions of the all the target genes in control samples were made 1.

The lipid peroxidation was estimated to assess the level of oxidative stress

in tick with presence of *R. parkeri* and with silencing of tick *SBP2* and *SELENOP*

genes (Fig 3.7). *R. parkeri* increase the lipid peroxidation (t=1.89, df =2) without statistical significance and contrary to our expectation we did not observe lipid peroxidation with silencing of tick *SBP2* (p=0.09) and *SELENOP* (p=0.35) (Fig 3.7).
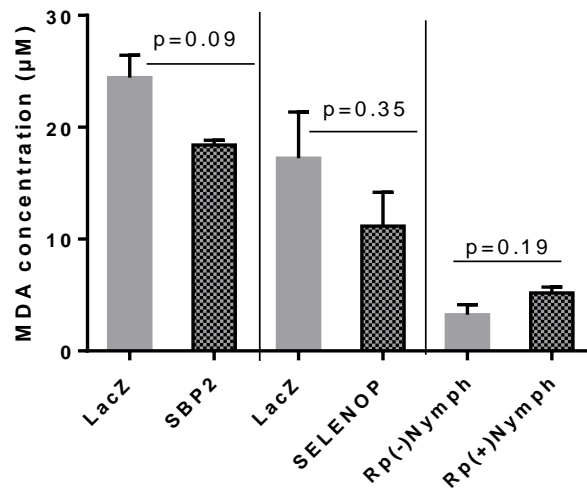
Figure 3.7 Lipid peroxidation measurement in ticks.

The lipid peroxidation was measured for estimation of oxidative stress in tick *SBP2* and *SELENOP* knockdowns and with or without *Rickettsia parkeri* infected nymphal ticks. Rp (-) Nymph, *R. parkeri* free nymphs; Rp (+) Nymph, *R. parkeri* infected nymphs.

3.4.4 Impact of *SBP2* and *SELENOP* Knockdown in the Tick Phenotype

We assessed the tick engorgement weights, tick egg laying (egg mass) and egg conversion ratios (the ratio of eggs mass per engorged tick weights) with the silencing of tick *SBP2* and *SELENOP* (Table 3.2). We observed that the engorged weight did not significantly reduced with *SBP2* knockdowns (t=0.198, df=18, p=0.85), but egg conversion ratio was significantly impaired with the knockdowns (t=5.72, df=8, p=0.0004) (Fig 3.8a-e) and eggs did not hatch to complete tick lifecycle.

In *SELENOP* knockdown assay, though small sample size for tick engorgement estimation, we observed no difference between control and *SELENOP* knockdown in tick engorgement and egg conversion (Table 3.2).

Table 3.2

Phenotype data for dsLacZ (irrelevant control) and dsSBP2 injected *Amblyomma maculatum* ticks.

Group	Infection status	No. of ticks	Engorged tick weight (Range)	Egg mass (Range)	Egg conversion ratio (Range)
dsLacZ	Rp+Ticks	25	769±46 (435-1090)	325±106 (38-497)	0.55±0.04 (0.44-0.67)
dsSBP2	Rp+Ticks	25	754±38 (632-904)	83±56 (22-253	0.11±0.08 (0.034-0.345)
dsSELENOP	Rp+Ticks	15	539 (504-574)	0-312	0.54

The weights are in milligrams. Egg conversion, the ratio of total tick eggs weight at the completion of egg laying to gravid female tick weight.

3.4.5 *SBP2* and *SELENOP* Silencing Impact on *Rickettsia-symbionts* Dynamics

The *R. parkeri* load (Rp load) slightly increased but not significant in gut tissues (p=0.25) whereas Rp load decreases in salivary glands (p=0.6) with *SBP2* knockdowns (Fig 3.7a). Similarly, silencing of tick *SELENOP* did not affect the Rp load in gut tissues (t=1.76, df=10, p=0.10) but Rp load decreased in salivary glands (t=1.19, df=10, p=0.25) (Fig 3.8a).
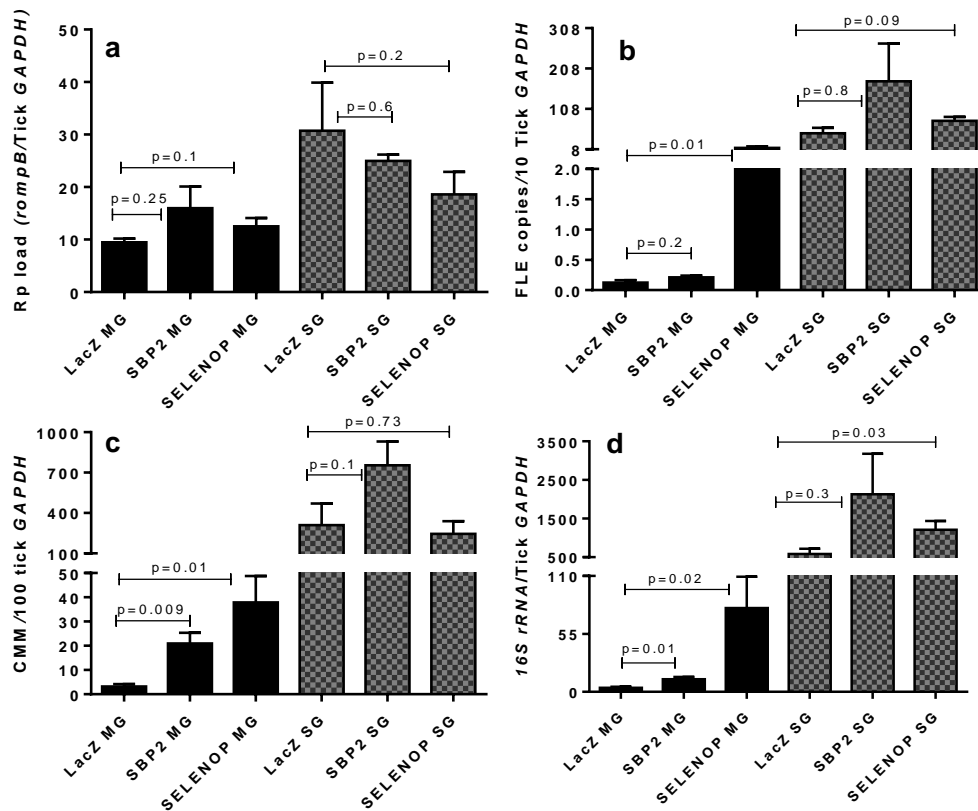
Figure 3.8 The *Rickettsia parkeri* and symbionts in tick *SBP2* and *SELENOP* knockdown tissues.

(a) *R. parkeri* load in tick midguts (MG) and salivary glands (SG). (b) FLE load in dsRNA-injected tick tissues. (c) CMM load in dsRNA-injected tick tissues. (d) Total bacterial load in dsRNA-injected tick tissues after 5dpi of dsRNA injection.

The bacterial symbionts were further quantified in tick *SBP2* and

SELENOP knockdown tissues. The FLE load remained unchanged in gut tissues

(p=0.2) but its load was increased (p=0.09) in salivary glands (Fig 3.8b) with

SBP2 silencing. The *SELENOP* knockdowns showed significant increased FLE

load in gut tissues (t=3.29, df=6, p=0.01) and slightly increased in salivary glands

(t=1.83, df =10, p=0.09) (Fig 3.8b). The silencing of *SBP2* significantly increased

CMM load in gut tissues (p=0.009), but slightly increased in salivary glands

(p=0.1) compared to control tissues (Fig 3.8c). CMM load was also increased in

SELENOP silenced tick gut tissues (t=3.015, df=10, p=0.01), but remained

unchanged in salivary glands (t=0.349, df=10, p=0.73) (Fig 3.8c). In tick guts

tissues, with silencing of *SBP2*, total bacterial load significantly increased

(p=0.01) but slightly increased in salivary glands (p=0.3) (Fig 3.8d). Whereas,

silencing of *SELENOP* significantly increased microbiota load in gut tissues

(t=2.54, df=10, p=0.029) and salivary glands (t=2.36, df=10, p=0.039) (Fig 3.8d).

3.4.6 Tick Fecundity and Transovarial Transmission of *Rickettsia parkeri*

The transovarial transmission of *R. parkeri* was estimated in tick eggs

collected after completion of tick oviposition (25 days after start of oviposition) in

both control and *SBP2* and *SELENOP* silenced groups. We observed that *R.

parkeri* in *SBP2* silenced tick eggs showed significant depletion of *R. parkeri*

compared to dsLacZ injected tick eggs (t=2.90, df=5, p=0.033) (Fig 3.9). The tick

SELENOP did not affect egg conversion ratio (Table 3.2) but reduced

transovarial transmission of *R. parkeri* (p=0.78) (Fig 3.9f).
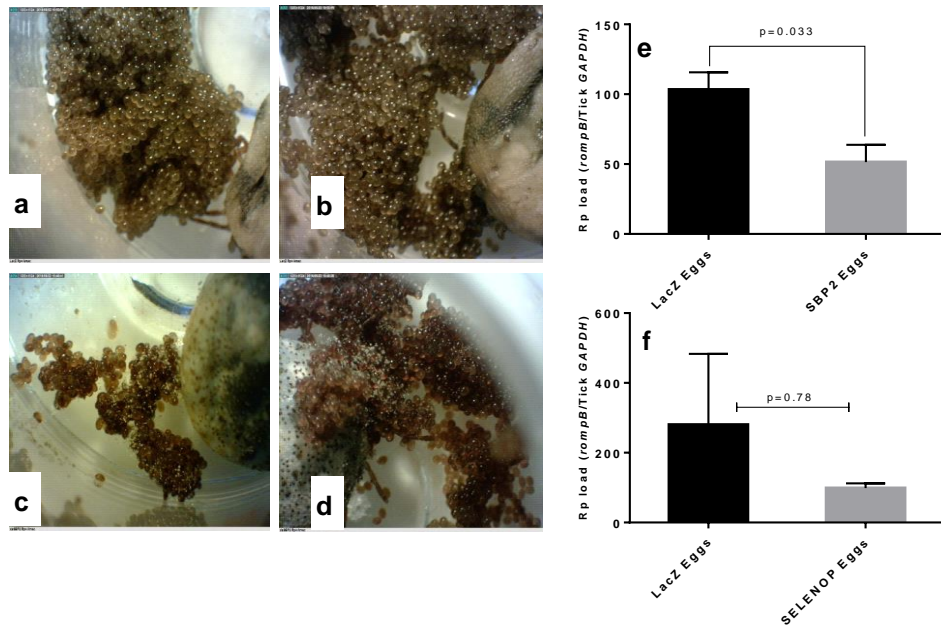
Figure 3.9 The impact of *SBP2* silencing tick fecundity and transovarial transmission impact of *Rickettsia parkeri* with *SBP2* and *SELENOP* silencing.

Eggs from the control (dsLacZ-injected) (a, b) and dsSBP2 injected (c, d) ticks. (e, f) *R. parkeri* load in tick eggs with silencing of *SBP2* and *SELENOP*.

3.5 Discussions

In this study, we have characterized functional role of tick SECIS binding protein 2 (*SBP2*) and tick selenoprotein P (*SELENOP*) in tick physiology and pathogen colonization. *Amblyomma SBP2* is the important factor in selenoprotein biosynthesis; it determines incorporation of selenium in protein as a selenocysteine. Ticks *SBP2* binds to 3' UTR region of selenoprotein mRNA which has specific stem-loop structure, like in case of phospholipid hydroperoxide glutathione peroxidase, direct cross-linking and competition experiments showed *SBP2* binds between UTR at 82 to 104 nucleotides (Copeland and Driscoll, 1999). Selenocysteine insertion sequence (SECIS), in

prokaryotes lies immediately downstream of in-frame opal codon (UGA)

(Hüttenhofer et al., 1996) which determine incorporation of Sec during translation

whereas its position in eukaryotic mRNA is variable between 500 to 5300

nucleotides far from in-frame opal codon (UGA) therefore the incorporation of

Sec determined by 3' UTR as it does for other translations (Sachs et al., 1991).

The SECIS binding site in SBP2 proteins is highlighted region in Fig 3.1.

Importantly, this highlighted region, conserved across the invertebrates and

vertebrates (Fig 3.2), is the binding site for ribosomal protein, rpL30 too, the

important step in selenoprotein biosynthesis. The evolutionary link of SBP2

across the taxa showed the separate clade for tick species which is different than

other arthropods or invertebrates (Fig 3.2).

The important genes in tick selenoprotein synthesis are selenium donor

selenophosphate synthetase (*SEPHS2)*, selenium transporter (*SELENOP)* and

SECIS binding protein (*SBP2)* including selenocysteine elongation factor

(eEFSec, SEF) and specific sec-tRNA[sec] (Kryukov et al., 2003). The tick

selenoprotein P (*SELENOP*) is unique and no studies were performed to

decipher functional significance yet. *SELENOP* is the only protein containing

multiple selenocysteine residues, 17 in zebrafish SELENOPa whereas 10 in

SELENOP from rat, mouse, and human (Fig 3.4). From bioinformatics analyses,

we showed that tick *SELENOP* is unique compared to that of vertebrate or even

zebrafish SELENOPb (Fig 3.3). Though further studies are required to identify

the position of the selenocysteine in tick *SELENOP*, it's intriguing that tick

SELENOP does not contain signal peptide and did not show selenocysteine in

multiple sequence alignment as expected compared to other sequences (Fig 3.4). The question still arises if there is a separate SELENOP gene or any other protein functionally equivalent to vertebrate SELENOP or tick SELENOP plays different role than that of Selenoprotein P.

The temporal transcription activity of *Amblyomma SBP2* showed blood meal negatively influences the mRNA abundances for *SBP2* except at 8 dpi in gut tissues (Fig 3.5). Temporal transcriptional expressions of the *SELENOP* showed cyclical: it increased and then decreases in gut tissues while gradually decreases in salivary glands (Fig 3.5) whereas *SEPHS2* gradually increases with blood meal in gut tissues but decrease gradually in salivary glands tissues (Fig 3.5). The transcriptional expressions of *SELENOP*, *SBP2,* and *SEPHS2* at day 8 post infestation co-occur with fast feeding stage of tick suggesting need for a maximum activity (Horn et al., 2009). *SEPHS2* plays role in synthesizing selenophosphate, a selenium donor from ATP and selenide (Veres et al., 1994). Whereas, *SELENOP* helps in transportation of selenium to different tissues, even in selenium storage and recycling in brain (Richardson, 2005). The optimal nutritional intake range for selenium (Se) in individual is determined by maximal expression of *SELENOP* (Hoeflich et al., 2010) and serum *SELENOP* expression is related to bone turnover and bone mineral density (Hoeg et al., 2012).

The upregulation of *SBP2, SEPHS2,* and *SELENOP* with *R. parkeri* infection (Fig 3.5) suggested the strong evidence for role selenium incorporation machinery in pathogen colonization, which probably by the antioxidant defense

required to neutralize superoxide formation during rickettsial infections (Santucci et al., 1992).

The knockdown of tick *SBP2* drastically reduced *SELENOP* in addition of reduction of expression levels of other selenoproteins (Fig 3.6). The transcriptional expressions of most of the selenoproteins remained depleted with *SBP2* silencing suggested the critical role of *SBP2* in the synthesis of selenoproteins. The reduced selenoprotein synthesis machinery probably increased the activity of selenophosphate synthetase (*SEPHS2*) for increased recycling of selenium in tick hemolymph (Fig 3.6). An important selenoprotein, *SELENOO,* an antioxidant as well as putative mitochondrial kinase property remained highly expressed probably compensating the reduced oxidative balance in mitochondria (Han et al., 2014). The *SELENOK* and *TrxR (TXNRD2)* were reduced or remain unchanged but Selenoprotein N (*SELENON*), Selenoprotein T (*SELENOT*) and *MSRB1* (*SelX*) were significantly downregulated with *SBP2* silencing (Fig 3.6). The *MSRB1 or SelX* contains domain of methionine sulfoxide reductase which reduces methionine-R-sulfoxide formed during oxidation of methionine residues in protein (Kim and Gladyshev, 2004). Selenoprotein N (*SELENON*) function in ticks was not known yet but in human SELENON deficiency causes the neuromuscular disorder, generalized muscle atrophy, and muscle weakness, but no function has been described in ticks (Castets et al., 2012). Selenoprotein T (*SELENOT*) has domain related to Rdx family, this protein binds UDP-glucose glucosyltransferase (UGTR) and known to facilitate insulin secretion induced by neuropeptide (Prevost et al.,

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2013). The tick superoxide dismutase (*Cu/Zn-SOD*) plays role in detoxifying superoxide radicals formed as normal physiology or that induced against *R. parkeri* infection (Crispell et al., 2016) probably compensated the *SBP2* depletion. This may be reason why we did not observe increased lipid peroxidation (Fig 3.7).

The unique selenoprotein P (*SELENOP*) with depletion showed increased expressions of *SEPHS2* probably for utilizing excess selenium unused with less biosynthesis of selenoproteins. The upregulation of the *SBP2* in gut tissues upon *SELENOP* depletion suggested need for biosynthesis of selenoproteins. Due to thioredoxin-like function of *SELENOP* (based on Sec-X-X-C domain), the *TrxR* (TXNRD2) upregulated probably compensated depletion of *SELENOP* (Mostert, 2000). The upregulation of *Cu/Zn-SOD* but depletion of *Catalase* showed super-oxides radicals being generated with depletion of *SelP* (Burk et al., 2003). The lipid peroxidation assay did not show increased peroxidation with silencing of putative tick *SELENOP* probably due to compensation from *Cu/Zn-SOD* or *Catalase* (Fig 3.7).The significantly depleted *SELENOS* with silencing of *SELENOP* probably created endoplasmic reticulum stress (ER-stress) impacting protein folding functions (Shchedrina et al., 2010). The transcriptional expressions of *SELENOM*, *SELENOK*, *SELENON* or *SELENOT* and *eEFsec* did not alters with *SELENOP* silencing showing less impact on the biosynthesis of these selenoproteins but increased expressions of selenophosphate (*SEPHS2*) and *SBP2* upregulation in tick gut tissues showed the probably high demand of selenoprotein synthesis (Fig 3.6).

The silencing of *SBP2* or *SELENOP* depleted *R. parkeri* loads in salivary glands but remained unchanged in gut tissues (Fig 3.8) correlated to their upregulated expressions in salivary glands but not in gut tissues with *R. parkeri* infection (Fig 3.5). The antioxidant functions of *SBP2* probably playing role in depletion of rickettsial load (Copeland and Driscoll, 1999). Similarly, *SELENOP* provides protection against oxidative damage and lipid peroxidation (Burk et al., 2003). The reduced rickettsial colonization inside tick salivary glands means reduced transmission to vertebrate host though we did not estimated *R. parkeri* before or after the day 5 post infestation. The *SBP2* known to function as an anti-apoptotic (Papp et al., 2010) and *SELENOP* functions as an anti-peroxidation property provide redox balance to cellular environment (Rock and Moos, 2010).

We tested the rickettsia-symbiont interaction hypothesis by quantifying FLE, CMM and total bacterial loads in *SBP2* and *SELENOP* depleted ticks. The FLE loads were increased with silencing of both tick genes whereas CMM loads were increased in all but *SELENOP* silenced salivary glands where *R. parkeri* load was depleted. With the depletion of the *SBP2* and *SELENOP*, tick tissues showed the increased bacterial load due to increased FLE and CMM loads (Fig 3.8). The depletion of two genes *SBP2* and *SELENOP* were significantly upheld by tick superoxide dismutase which probably inhibited the lipid peroxidation allowing the symbionts to proliferate.

The reduced rickettsial load inside the tick organs probably could be the reason for less transmission to eggs stage of tick through transovarial transmission (Fig 3.9). The oxidative stress with the *SBP2* knockdown probably

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impaired tick fecundity success but the role of selenium in reproductive fitness might be another reason for reduced eggs conversion ratio (Kumar et al., 2016; Tinggi, 2008). We hypothesized that silencing of selenoprotein synthesis machinery gene, *SBP2,* and *SELENOP* impacted the tick oviposition due to altered selenium level.

3.6 Conclusions

Our results support the tick *SBP2* plays role in tick fecundity and rickettsial colonization whereas *SELENOP* impacted on rickettsial colonization and transovarial transmissions. This study opened possibility of tick selenoproteome in tick redox homeostasis, tick physiology and exploring tick's ability to harbor pathogenic microbes.

CHAPTER IV –CONCLUSIONS

We started field collection of *Amblyomma maculatum*, a gulf coast tick,

and quantified *Rickettsia parkeri* in Mississippi for five years (2011 to 2016) and

estimated infection less than 40% out of total ticks tested. In nature, ticks avoid

the rickettsial infections which implies *R. parkeri* is a less symbiont and more

pathogen to *A. maculatum.* But, rickettsial infections faithfully transfer across the

tick lifecycle once infected at least for few generations but the possibility of

disappearance of infections from tick after many generations probably occurs in

nature. This possibility may be due to interactions among the rickettsia with

symbionts or tick defense may overpower pathogen infections. We showed the

dynamic interactions among FLE, CMM and *R. parkeri* within tick tissues and

across all tick stages. The bacterial symbiont, FLE probably displaces rickettsial

infections completely in long run or tick may lose CMM, which may make *R.

parkeri* unable to colonize in the tick tissues. The future studies on how

symbionts like FLE and CMM defend *R. parkeri* infections would yield important

findings about defensive symbiosis in the ticks.

The tick selenoproteome is unexplored area and they are generally

regarded to perform antioxidant functions. The selenium-containing proteins,

selenoproteins, perform redox reaction more efficiently and resist deactivation by

peroxides or oxidants unlike to their cysteine homologs. The redox center in

selenoprotein is selenium which has advantages of being easily reacting to

oxygen species and readily getting reduced. In this study, we studied tick-

pathogen interactions focusing in tick selenoproteome synthesis machinery,

SECIS binding protein 2 (*SBP2*) and unique tick Selenoprotein P (*SELENOP*) in tick physiology and rickettsial colonization success. The functional characterization of *SBP2* showed its importance in selenoprotein biosynthesis, tick fecundity, and pathogen colonization or transovarial transmissions. The *SELENOP* plays role in pathogen colonization and transovarial transmissions and tick redox balance. This study provides importance of selenoproteome in tick physiology and pathogen colonization and provides important future guide to tick-pathogen interaction studies as well as pathogen-symbiont dynamics.

APPENDIX A - IACUC Approval letter

THE UNIVERSITY OF SOUTHERN MISSISSIPPI

Institutional Animal Care and Use Committee

118 College Drive #5147
Hattiesburg, MS 39406-0001
Phone: 601.266.4063
Fax: 601.266.4377

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE NOTICE OF COMMITTEE ACTION

The proposal noted below was reviewed and approved by The University of Southern Mississippi Institutional Animal Care and Use Committee (IACUC) in accordance with regulations by the United States Department of Agriculture and the Public Health Service Office of Laboratory Animal Welfare. The project expiration date is noted below. If for some reason the project is not completed by the end of the three year approval period, your protocol must be reactivated (a new protocol must be submitted and approved) before further work involving the use of animals can be done.

Any significant changes (see attached) should be brought to the attention of the committee at the earliest possible time. If you should have any questions, please contact me.

PROTOCOL NUMBER:	**10042001**
PROJECT TITLE:	**Tick Sialome**
PROPOSED PROJECT DATES:	**February 2013 – September 2015**
PROJECT TYPE:	**Renewal**
PRINCIPAL INVESTIGATOR(S):	**Shahid Karim**
DEPARTMENT:	**Biological Sciences**
FUNDING AGENCY/SPONSOR:	**NIH NIAID, DOS NAS, AHA**
IACUC COMMITTEE ACTION:	**Full Committee Approval**
PROTOCOL EXPIRATON DATE:	September 30, 2015

19 February 2013
Date

Jodie M. Jawor, Ph.D.
IACUC Chair

Inclusions – see 'Procedures'

THE UNIVERSITY OF
SOUTHERN MISSISSIPPI.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

118 College Drive #5116 | Hattiesburg, MS 39406-0001
Phone: 601.266.6791 | Fax: 601.266.4377 | iacuc@usm.edu | www.usm.edu/iacuc

NOTICE OF COMMITTEE ACTION

The proposal noted below was reviewed and approved by The University of Southern Mississippi Institutional Animal Care and Use Committee (IACUC) in accordance with regulations by the United States Department of Agriculture and the Public Health Service Office of Laboratory Animal Welfare. The project expiration date is noted below. If for some reason the project is not completed by the end of the approval period, your protocol must be reactivated (a new protocol must be submitted and approved) before further work involving the use of animals can be done.

Any significant changes should be brought to the attention of the committee at the earliest possible time. If you should have any questions, please contact me.

PROTOCOL NUMBER:	15101501 (Replaces 10042001)
PROJECT TITLE:	Tick Sialome
PROPOSED PROJECT DATES:	10/2015 - 09/2018
PROJECT TYPE:	Renewal
PRINCIPAL INVESTIGATOR(S):	Shahid Karim
DEPARTMENT:	Biological Sciences
FUNDING AGENCY/SPONSOR:	N/A
IACUC COMMITTEE ACTION:	Full Committee Approval
PROTOCOL EXPIRATON DATE:	September 30, 2018

_____   _____ 10/01/2015 _____
Frank Moore, PhD   Date
IACUC Chair

REFERENCES

Abraham, N.M., Liu, L., Jutras, B.L., Yadav, A.K., Narasimhan, S.,
Gopalakrishnan, V., Ansari, J.M., Jefferson, K.K., Cava, F., Jacobs-
Wagner, C., Fikrig, E., 2017. Pathogen-mediated manipulation of
arthropod microbiota to promote infection. Proc. Natl. Acad. Sci. U. S. A.
201613422. doi:10.1073/pnas.1613422114

Adamson, S., Browning, R., Singh, P., Nobles, S., Villarreal, A., Karim, S., 2014.
Transcriptional activation of antioxidants may compensate for
selenoprotein deficiencies in *Amblyomma maculatum* (Acari: Ixodidae)
injected with selK- or selM-dsRNA. Insect Mol. Biol. 23, 497–510.
doi:10.1111/imb.12098

Adamson, S.W., Browning, R.E., Budachetri, K., Ribeiro, J.M.C., Karim, S., 2013.
Knockdown of Selenocysteine-Specific Elongation Factor in *Amblyomma
maculatum* Alters the Pathogen Burden of Rickettsia parkeri with
Epigenetic Control by the Sin3 Histone Deacetylase Corepressor
Complex. PLoS One 8, e82012. doi:10.1371/journal.pone.0082012

Ahantarig, A., Trinachartvanit, W., Baimai, V., Grubhoffer, L., 2013. Hard ticks
and their bacterial endosymbionts (or would be pathogens). Folia
Microbiol. (Praha). 58, 419–28. doi:10.1007/s12223-013-0222-1

Allerdice, M.E.J., Hecht, J.A., Karpathy, S.E., Paddock, C.D., 2017. Evaluation of
Gulf Coast Ticks (Acari: Ixodidae) for *Ehrlichia* and *Anaplasma* Species. J.
Med. Entomol.54 :481-484. doi:10.1093/jme/tjw176

Allmang, C., Krol, A., 2006. Selenoprotein synthesis: UGA does not end

the story. Biochimie 88, 1561–1571. doi:10.1016/j.biochi.2006.04.015

Anatriello, E., Ribeiro, J.M.C., de Miranda-Santos, I.K.F., Brandao, L.G., Anderson, J.M., Valenzuela, J.G., Maruyama, S.R., Silva, J.S., Ferreira, B.R., 2010. An insight into the sialotranscriptome of the brown dog tick, *Rhipicephalus sanguineus*. BMC Genomics 11. doi:Artn 450Doi 10.1186/1471-2164-11-450

Anderson, J.F., Magnarelli, L.A., 2008. Biology of ticks. Infect Dis Clin North Am 22, 195–215, v. doi:S0891-5520(07)00123-7 [pii]10.1016/j.idc.2007.12.006

Archie, E.A., Theis, K.R., 2011. Animal behaviour meets microbial ecology. Anim. Behav. 82, 425–436. doi:10.1016/j.anbehav.2011.05.029

Arner, E.S., Holmgren, A., 2000. Physiological functions of thioredoxin and thioredoxin reductase. Eur J Biochem 267, 6102–6109. doi:ejb1701 [pii]

Azad, A.F., Sacci Jr., J.B., Nelson, W.M., Dasch, G.A., Schmidtmann, E.T., Carl, M., 1992. Genetic characterization and transovarial transmission of a typhus-like rickettsia found in cat fleas. Proc Natl Acad Sci U S A 89, 43–46.

Bahia, A.C., Kubota, M.S., Tempone, A.J., Araújo, H.R.C., Guedes, B.A.M., Orfanó, A.S., Tadei, W.P., Ríos-Velásquez, C.M., Han, Y.S., Secundino, N.F.C., Barillas-Mury, C., Pimenta, P.F.P., Traub-Csekö, Y.M., 2011. The JAK-STAT pathway controls *Plasmodium vivax* load in early stages of Anopheles aquasalis infection. PLoS Negl. Trop. Dis. 5, e1317. doi:10.1371/journal.pntd.0001317

Baldridge, G.D., Scoles, G.A., Burkhardt, N.Y., Schloeder, B., Kurtti, T.J., Munderloh, U.G., 2009. Transovarial transmission of *Francisella*-like endosymbionts and *Anaplasma phagocytophilum* variants in *Dermacentor albipictus* (Acari: Ixodidae). J. Med. Entomol. 46, 625–32.

Barrington, J.W., Taylor, M., Smith, S., Bowen, P., Simpkins, J., 1997. Selenium and recurrent miscarriage. J. Obstet. Gynaecol. (Lahore). 17, 199–200. doi:10.1080/01443619750113870

Baumann, P., 2005. Biolgy of bacteriocyte-associated enodsymbionts of plant sap-sucking insects. Annu. Rev. Microbiol. 59, 155–189. doi:10.1146/annurev.micro.59.030804.121041

Beier, M.S., Pumpuni, C.B., Beier, J.C., Davis, J.R., 1994. Effects of para-aminobenzoic acid, insulin, and gentamicin on *Plasmodium falciparum* development in anopheline mosquitoes (Diptera: Culicidae). J. Med. Entomol. 31, 561–5.

Bensaci, M., Bhattacharya, D., Clark, R., Hu, L.T., 2012. Oral vaccination with vaccinia virus expressing the tick antigen subolesin inhibits tick feeding and transmission of *Borrelia burgdorferi*. Vaccine 30, 6040–6. doi:10.1016/j.vaccine.2012.07.053

Bianco, A.C., Salvatore, D., Gereben, B., Berry, M.J., Larsen, P.R., 2002. Biochemistry, Cellular and Molecular Biology, and Physiological Roles of the Iodothyronine Selenodeiodinases. Endocr. Rev. 23, 38–89. doi:10.1210/edrv.23.1.0455

Black, W.C. th, Piesman, J., 1994. Phylogeny of hard- and soft-tick taxa (Acari:

Ixodida) based on mitochondrial *16S rDNA* sequences. Proc Natl Acad Sci U S A 91, 10034–10038.

Boscaro, V., Petroni, G., Ristori, A., Verni, F., Vannini, C., 2013. "*Candidatus* Defluviella procrastinata" and "*Candidatus* Cyrtobacter zanobii", Two Novel Ciliate Endosymbionts Belonging to the "Midichloria Clade." Microb. Ecol. 65, 302–310. doi:10.1007/s00248-012-0170-3

Brown, S.J., Askenase, P.W., 1982. Blood eosinophil and basophil responses in guinea pigs parasitized by *Amblyomma americanum* ticks. Am. J. Trop. Med. Hyg. 31, 593–8.

Brown, S.J., Galli, S.J., Gleich, G.J., Askenase, P.W., 1982. Ablation of immunity to *Amblyomma americanum* by anti-basophil serum: cooperation between basophils and eosinophils in expression of immunity to ectoparasites (ticks) in guinea pigs. J. Immunol. 129, 790–6.

Brown, S.J., Knapp, F.W., 1980. *Amblyomma americanum*: sequential histological analysis of adult feeding sites on guinea pigs. Exp Parasitol 49, 303–318.

Brown, S.J., Worms, M.J., Askenase, P.W., 1983. *Rhipicephalus appendiculatus*: Larval feeding sites in guinea pigs actively sensitized and receiving immune serum. Exp. Parasitol. 55, 111–120. doi:10.1016/0014-4894(83)90004-8

Browning, R., Karim, S., 2013. RNA interference-mediated depletion of N-ethylmaleimide Sensitive Fusion Protein and Synaptosomal Associated Protein of 25 kDa results in the inhibition of blood feeding of the Gulf

Coast tick, *Amblyomma maculatum*. Insect Mol Biol.
doi:10.1111/imb.12017

Bubenik, J.L., Miniard, A.C., Driscoll, D.M., 2015. Characterization of the UGA-recoding and SECIS-binding activities of SECIS-binding protein 2.
http://dx.doi.org/10.1080/15476286.2014.996472.

Budachetri, K., Browning, R.E., Adamson, S.W., Dowd, S.E., Chao, C.-C., Ching, W.-M., Karim, S., 2014. An Insight Into the Microbiome of the *Amblyomma maculatum* (Acari: Ixodidae). J. Med. Entomol. 51, 119–129.
doi:10.1603/ME12223

Budachetri, K., Karim, S., 2015. An insight into the functional role of thioredoxin reductase, a selenoprotein, in maintaining normal native microbiota in the Gulf Coast tick (*Amblyomma maculatum*). Insect Mol. Biol. 24, 570–581.
doi:10.1111/imb.12184

Burgdorfer, W., 1963. Investigation of "transovarial transmission" of *Rickettsia rickettsii* in the wood tick, *Dermacentor andersoni*. Exp Parasitol 14, 152–159. doi:http://dx.doi.org/10.1016/0014-4894(63)90019-5,

Burgdorfer, W., Brinton, L.P., Hughes, L.E., 1973. Isolation and characterization of symbiotes from the Rocky Mountain wood tick, *Dermacentor andersoni*. J Invertebr Pathol 22, 424–434.

Burk, R.F., Hill, K.E., Motley, A.K., 2003. Selenoprotein metabolism and function: evidence for more than one function for selenoprotein P. J. Nutr. 133, 1517S–20S.

Burkhardt, N.Y., Baldridge, G.D., Williamson, P.C., Billingsley, P.M., Heu, C.C.,

Felsheim, R.F., Kurtti, T.J., Munderloh, U.G., 2011. Development of shuttle vectors for transformation of diverse *Rickettsia* species. PLoS One 6, e29511. doi:10.1371/journal.pone.0029511

Caban, K., Copeland, P.R., 2006. Size matters: a view of selenocysteine incorporation from the ribosome. Cell. Mol. Life Sci. 63, 73–81. doi:10.1007/s00018-005-5402-y

Castets, P., Lescure, A., Guicheney, P., Allamand, V., 2012. Selenoprotein N in skeletal muscle: from diseases to function. J. Mol. Med. 90, 1095–1107. doi:10.1007/s00109-012-0896-x

Ceraul, S.M., Dreher-Lesnick, S.M., Gillespie, J.J., Rahman, M.S., Azad, A.F., 2007. New tick defensin isoform and antimicrobial gene expression in response to *Rickettsia montanensis* challenge. Infect. Immun. 75, 1973–83. doi:10.1128/IAI.01815-06

Ceraul, S.M., Dreher-Lesnick, S.M., Mulenga, A., Rahman, M.S., Azad, A.F., 2008. Functional characterization and novel rickettsiostatic effects of a Kunitz-type serine protease inhibitor from the tick *Dermacentor variabilis*. Infect. Immun. 76, 5429–35. doi:10.1128/IAI.00866-08

Chen, G., Severo, M.S., Sohail, M., Sakhon, O.S., Wikel, S.K., Kotsyfakis, M., Pedra, J.H.F., 2012. *Ixodes scapularis* saliva mitigates inflammatory cytokine secretion during *Anaplasma phagocytophilum* stimulation of immune cells. Parasit. Vectors 5, 229. doi:10.1186/1756-3305-5-229

Chmelar, J., Anderson, J.M., Mu, J., Jochim, R.C., Valenzuela, J.G., Kopecký, J., 2008. Insight into the sialome of the castor bean tick, *Ixodes ricinus*. BMC

Genomics 9, 233. doi:10.1186/1471-2164-9-233

Chmelař, J., Kotál, J., Kopecký, J., Pedra, J.H.F., Kotsyfakis, M., 2016. All For One and One For All on the Tick-Host Battlefield. Trends Parasitol. doi:10.1016/j.pt.2016.01.004

Ciprandi, A., de Oliveira, S.K., Masuda, A., Horn, F., Termignoni, C., 2006. *Boophilus microplus*: its saliva contains microphilin, a small thrombin inhibitor. Exp. Parasitol. 114, 40–6. doi:10.1016/j.exppara.2006.02.010

Cirimotich, C.M., Dong, Y., Clayton, A.M., Sandiford, S.L., Souza-Neto, J.A., Mulenga, M., Dimopoulos, G., 2011. Natural Microbe-Mediated Refractoriness to *Plasmodium* Infection in *Anopheles gambiae*. Science (80- 332), 855 LP-858.

Clay, K., 2014. Defensive symbiosis: a microbial perspective. Funct. Ecol. 28, 293–298. doi:10.1111/1365-2435.12258

Clay, K., Klyachko, O., Grindle, N., Civitello, D., Oleske, D., Fuqua, C., 2008. Microbial communities and interactions in the lone star tick, *Amblyomma americanum*. Mol Ecol 17, 4371–4381.

Copeland, P.R., Driscoll, D.M., 1999. Purification, redox sensitivity, and RNA binding properties of SECIS-binding protein 2, a protein involved in selenoprotein biosynthesis. J. Biol. Chem. 274, 25447–54.

Copeland, P.R., Stepanik, V.A., Driscoll, D.M., 2001. Insight into Mammalian Selenocysteine Insertion: Domain Structure and Ribosome Binding Properties of Sec Insertion Sequence Binding Protein 2. Mol. Cell. Biol. 21, 1491–1498. doi:10.1128/MCB.21.5.1491-1498.2001

Crispell, G., Budachetri, K., Karim, S., 2016. *Rickettsia parkeri* colonization in

    Amblyomma maculatum: the role of superoxide dismutases. Parasit.

    Vectors 9, 291. doi:10.1186/s13071-016-1579-1

de la Fuente, J., Almazán, C., Blouin, E.F., Naranjo, V., Kocan, K.M., 2006.

    Reduction of tick infections with *Anaplasma marginale* and *A.*

    phagocytophilum by targeting the tick protective antigen subolesin.

    Parasitol. Res. 100, 85–91. doi:10.1007/s00436-006-0244-6

de la Fuente, J., Almazán, C., Canales, M., Pérez de la Lastra, J.M., Kocan,

    K.M., Willadsen, P., 2007. A ten-year review of commercial vaccine

    performance for control of tick infestations on cattle. Anim. Health Res.

    Rev. 8, 23–8. doi:10.1017/S1466252307001193

de la Fuente, J., Kocan, K.M., Blouin, E.F., Zivkovic, Z., Naranjo, V., Almazán,

    C., Esteves, E., Jongejan, F., Daffre, S., Mangold, A.J., 2010. Functional

    genomics and evolution of tick–*Anaplasma* interactions and vaccine

    development. Vet. Parasitol. 167, 175–186.

    doi:10.1016/j.vetpar.2009.09.019

De Silva, A.M., Fikrig, E., 1995. Growth and migration of *Borrelia burgdorferi* in

    Ixodes ticks during blood feeding. Am J Trop Med Hyg 53, 397–404.

Dergousoff, S.J., Chilton, N.B., 2012. Association of different genetic types of

    Francisella-like organisms with the rocky mountain wood tick

    (*Dermacentor andersoni*) and the American dog tick (*Dermacentor*

    variabilis) in localities near their northern distributional limits. Appl.

    Environ. Microbiol. 78, 965–71. doi:10.1128/AEM.05762-11

Dong, Y., Manfredini, F., Dimopoulos, G., 2009. Implication of the mosquito
midgut microbiota in the defense against malaria parasites. PLoS Pathog.
5, e1000423. doi:10.1371/journal.ppat.1000423

Drummond, R.O., Whetstone, T.M., R. O. Drummond, Wheatstone, T.M., 1970.
Oviposition of the Gulf Coast Tick. J. Econ. Entomol. 63, 1547–1551.

Dumler, J.S., Barbet, A.F., Bekker, C.P., Dasch, G.A., Palmer, G.H., Ray, S.C.,
Rikihisa, Y., Rurangirwa, F.R., 2001. Reorganization of genera in the
families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales:
unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with
Ehrlichia and *Ehrlichia* with *Neorickettsia*, descriptions of six new species
combi. Int J Syst Evol Microbiol 51, 2145–2165. doi:10.1099/00207713-
51-6-2145

Dumont, J.E., Corvilain, B., Contempre, B., 1994. The biochemistry of endemic
cretinism: roles of iodine and selenium deficiency and goitrogens. Mol.
Cell. Endocrinol. 100, 163–166. doi:10.1016/0303-7207(94)90297-6

Dunwiddie, C.T., Smith, D.E., Nutt, E.M., Vlasuk, G.P., 1991. Anticoagulant
effects of the selective factor XA inhibitors tick anticoagulant peptide and
antistasin in the APTT assay are determined by the relative rate of
prothrombinase inhibition. Thromb. Res. 64, 787–94.

Edwards, K.T., Varela-Stokes, A.S., Paddock, C.D., Goddard, J., 2011. Gotch
ear in a goat: a case report. Vector Borne Zoonotic Dis. 11, 1217–9.
doi:10.1089/vbz.2010.0204

Epis, S., Sassera, D., Beninati, T., Lo, N., Beati, L., Piesman, J., Rinaldi, L.,

McCoy, K.D., Torina, A., Sacchi, L., Clementi, E., Genchi, M., Magnino, S., Bandi, C., 2008. *Midichloria mitochondrii* is widespread in hard ticks (Ixodidae) and resides in the mitochondria of phylogenetically diverse species. Parasitology 135, 485–94. doi:10.1017/S0031182007004052

Eremeeva, M.E., Dasch, G.A., 2015. Challenges posed by tick-borne rickettsiae: eco-epidemiology and public health implications. Front. public Heal. 3, 55. doi:10.3389/fpubh.2015.00055

Espinoza-Gomez, F., Newton-Sanchez, O., Flores-Cazares, G., De la Cruz-Ruiz, M., Melnikov, V., Austria-Tejeda, J., Rojas-Larios, F., 2011. Tick paralysis caused by *Amblyomma maculatum* on the Mexican Pacific Coast. Vector Borne Zoonotic Dis 11, 945–946. doi:10.1089/vbz.2010.0154

Estrada-Pena, A., Venzal, J.M., Mangold, A.J., Cafrune, M.M., Guglielmone, A.A., Estrada-Peña, A., Venzal, J.M., Mangold, A.J., Cafrune, M.M., Guglielmone, A.A., 2005. The *Amblyomma maculatum* Koch, 1844 (Acari: Ixodidae: Amblyomminae) tick group: diagnostic characters, description of the larva of *A. parvitarsum* Neumann, 1901, *16S rDNA* sequences, distribution and hosts. Syst Parasitol 60, 99–112. doi:10.1007/s11230-004-1382-9

Ezenwa, V.O., Gerardo, N.M., Inouye, D.W., Medina, M., Xavier, J.B., 2012. Microbiology. Animal behavior and the microbiome. Science (80-.). 338, 198–199. doi:10.1126/science.1227412

Farlow, J., Wagner, D.M., Dukerich, M., Stanley, M., Chu, M., Kubota, K., Petersen, J., Keim, P., 2005. *Francisella tularensis* in the United States.

Emerg. Infect. Dis. 11, 1835–1841. doi:10.3201/eid1112.050728

Fasshauer, D., Sutton, R.B., Brunger, A.T., Jahn, R., 1998. Conserved structural features of the synaptic fusion complex: SNARE proteins reclassified as Q- and R-SNAREs. Proc. Natl. Acad. Sci. U. S. A. 95, 15781–6.

Ferrari, F.A.G., Goddard, J., Moraru, G.M., Smith, W.E.C., Varela-Stokes, A.S., 2013. Isolation of "*Candidatus* Rickettsia andeanae" (Rickettsiales: Rickettsiaceae) in embryonic cells of naturally infected *Amblyomma maculatum* (Ixodida: Ixodidae). J. Med. Entomol. 50, 1118–25.

Fletcher, J.E., Copeland, P.R., Driscoll, D.M., Krol, A., 2001. The selenocysteine incorporation machinery: interactions between the SECIS RNA and the SECIS-binding protein SBP2. RNA 7, 1442–53.

Foley, J.E., Nieto, N.C., 2010. Tularemia. Vet. Microbiol. 140, 332–338. doi:10.1016/j.vetmic.2009.07.017

Francischetti, I.M.B., Anderson, J.M., Manoukis, N., Pham, V.M., Ribeiro, J.M.C., 2011. An insight into the sialotranscriptome and proteome of the coarse bontlegged tick, *Hyalomma marginatum* rufipes. J. Proteomics 74, 2892–2908. doi:10.1016/j.jprot.2011.07.015

Francischetti, I.M.B., Mans, B.J., Meng, Z., Gudderra, N., Veenstra, T.D., Pham, V.M., Ribeiro, J.M.C., 2008a. An insight into the sialome of the soft tick, *Ornithodorus parkeri*. Insect Biochem. Mol. Biol. 38, 1–21. doi:10.1016/j.ibmb.2007.09.009

Francischetti, I.M.B., Mather, T.N., Ribeiro, J.M.C., 2005. Tick saliva is a potent inhibitor of endothelial cell proliferation and angiogenesis. Thromb.

Haemost. 94, 167–74. doi:10.1160/TH04-09-0566

Francischetti, I.M.B., Mather, T.N., Ribeiro, J.M.C., 2004. Penthalaris, a novel

recombinant five-Kunitz tissue factor pathway inhibitor (TFPI) from the

salivary gland of the tick vector of Lyme disease, *Ixodes scapularis*.

Thromb. Haemost. 91, 886–98. doi:10.1267/THRO04050886

Francischetti, I.M.B., Meng, Z., Mans, B.J., Gudderra, N., Hall, M., Veenstra,

T.D., Pham, V.M., Kotsyfakis, M., Ribeiro, J.M.C., 2008b. An insight into

the salivary transcriptome and proteome of the soft tick and vector of

epizootic bovine abortion, *Ornithodoros coriaceus*. J. Proteomics 71, 493–

512. doi:10.1016/j.jprot.2008.07.006

Francischetti, I.M.B., Sa-Nunes, A., Mans, B.J., Santos, I.M., Ribeiro, J.M.C.,

2009. The role of saliva in tick feeding. Front. Biosci. (Landmark Ed. 14,

2051–88.

Francischetti, I.M.B., Valenzuela, J.G., Andersen, J.F., Mather, T.N., Ribeiro,

J.M.C., 2002. Ixolaris, a novel recombinant tissue factor pathway inhibitor

(TFPI) from the salivary gland of the tick, *Ixodes scapularis*: identification

of factor X and factor Xa as scaffolds for the inhibition of factor VIIa/tissue

factor complex. Blood 99, 3602–12. doi:10.1182/blood-2001-12-0237

Francischetti, I.M.B.B., Mather, T.N., Ribeiro, J.M.C.C., 2003. Cloning of a

salivary gland metalloprotease and characterization of gelatinase and

fibrin(ogen)lytic activities in the saliva of the Lyme disease tick vector

Ixodes scapularis. Biochem. Biophys. Res. Commun. 305, 869–75.

doi:10.1016/S0006-291X(03)00857-X

Franta, Z, Frantova, H., Konvickova, J., Horn, M., Sojka, D., Mares, M.,
Kopacek, P., 2010. Dynamics of digestive proteolytic system during blood
feeding of the hard tick *Ixodes ricinus*. Parasit Vectors 3, 119.
doi:10.1186/1756-3305-3-1191756-3305-3-119 [pii]

Fukumoto, S., Sakaguchi, T., You, M., Xuan, X., Fujisaki, K., 2006. Tick troponin
I-like molecule is a potent inhibitor for angiogenesis. Microvasc. Res. 71,
218–21. doi:10.1016/j.mvr.2006.02.003

Garver, L.S., Bahia, A.C., Das, S., Souza-Neto, J.A., Shiao, J., Dong, Y.,
Dimopoulos, G., 2012. *Anopheles* Imd pathway factors and effectors in
infection intensity-dependent anti-*Plasmodium* action. PLoS Pathog. 8,
e1002737. doi:10.1371/journal.ppat.1002737

Gaspar, A.R., Crause, J.C., Neitz, A.W., 1995. Identification of anticoagulant
activities in the salivary glands of the soft tick, *Ornithodoros savignyi*. Exp.
Appl. Acarol. 19, 117–27.

Gerhart, J.G., Moses, A.S., Raghavan, R., 2016. A *Francisella*-like endosymbiont
in the Gulf Coast tick evolved from a mammalian pathogen. Sci. Rep. 6,
33670. doi:10.1038/srep33670

Gladyshev, V.N., Arnér, E.S., Berry, M.J., Brigelius-Flohé, R., Bruford, E.A.,
Burk, R.F., Carlson, B.A., Castellano, S., Chavatte, L., Conrad, M.,
Copeland, P.R., Diamond, A.M., Driscoll, D.M., Ferreiro, A., Flohé, L.,
Green, F.R., Guigó, R., Handy, D.E., Hatfield, D.L., Hesketh, J.,
Hoffmann, P.R., Holmgren, A., Hondal, R.J., Howard, M.T., Huang, K.,
Kim, H.-Y., Kim, I.Y., Köhrle, J., Krol, A., Kryukov, G. V., Lee, B.J., Lee,

B.C., Lei, X.G., Liu, Q., Lescure, A., Lobanov, A. V., Loscalzo, J.,

Maiorino, M., Mariotti, M., Sandeep Prabhu, K., Rayman, M.P., Rozovsky,

S., Salinas, G., Schmidt, E.E., Schomburg, L., Schweizer, U., Simonović,

M., Sunde, R.A., Tsuji, P.A., Tweedie, S., Ursini, F., Whanger, P.D.,

Zhang, Y., 2016. Selenoprotein Gene Nomenclature. J. Biol. Chem. 291,

24036–24040. doi:10.1074/jbc.M116.756155

Gofton, A.W., Oskam, C.L., Lo, N., Beninati, T., Wei, H., McCarl, V., Murray,

D.C., Paparini, A., Greay, T.L., Holmes, A.J., Bunce, M., Ryan, U., Irwin,

P., 2015. Inhibition of the endosymbiont "*Candidatus* Midichloria

mitochondrii" during *16S rRNA* gene profiling reveals potential pathogens

in Ixodes ticks from Australia. Parasit. Vectors 8, 345.

doi:10.1186/s13071-015-0958-3

Goldhaber, S.B., 2003. Trace element risk assessment: essentiality vs. toxicity.

Regul. Toxicol. Pharmacol. 38, 232–42.

Gonzalez-Ceron, L., Santillan, F., Rodriguez, M.H., Mendez, D., Hernandez-

Avila, J.E., 2003. Bacteria in Midguts of Field-Collected *Anopheles*

albimanus Block *Plasmodium vivax* Sporogonic Development. J. Med.

Entomol. 40.

Gupta, L., Molina-Cruz, A., Kumar, S., Rodrigues, J., Dixit, R., Zamora, R.E.,

Barillas-Mury, C., 2009. The STAT pathway mediates late-phase immunity

against *Plasmodium* in the mosquito *Anopheles gambiae*. Cell Host

Microbe 5, 498–507. doi:10.1016/j.chom.2009.04.003

Hajnická, V., Vancová, I., Kocáková, P., Slovák, M., Gasperík, J., Sláviková, M.,

Hails, R.S., Labuda, M., Nuttall, P.A., 2005. Manipulation of host cytokine network by ticks: a potential gateway for pathogen transmission. Parasitology 130, 333–42.

Han, S.-J., Lee, B.C.B.B., Yim, S.H., 2014. Characterization of mammalian selenoprotein o: a redox-active mitochondrial protein. PLoS One 9, e95518. doi:10.1371/journal.pone.0095518

Hannier, S., Liversidge, J., Sternberg, J.M., Bowman, A.S., 2003. *Ixodes ricinus* tick salivary gland extract inhibits IL-10 secretion and CD69 expression by mitogen-stimulated murine splenocytes and induces hyporesponsiveness in B lymphocytes. Parasite Immunol. 25, 27–37.

Hidiroglou, M., 1979. Trace Element Deficiencies and Fertility in Ruminants: A Review. J. Dairy Sci. 62, 1195–1206. doi:10.3168/jds.S0022-0302(79)83400-1

Hoeflich, J., Hollenbach, B., Behrends, T., Hoeg, A., Stosnach, H., Schomburg, L., 2010. The choice of biomarkers determines the selenium status in young German vegans and vegetarians. Br. J. Nutr. 104, 1601–1604. doi:10.1017/S0007114510002618

Hoeg, A., Gogakos, A., Murphy, E., Mueller, S., Köhrle, J., Reid, D.M., Glüer, C.C., Felsenberg, D., Roux, C., Eastell, R., Schomburg, L., Williams, G.R., 2012. Bone turnover and bone mineral density are independently related to selenium status in healthy euthyroid postmenopausal women. J. Clin. Endocrinol. Metab. 97, 4061–70. doi:10.1210/jc.2012-2121

Horka, H., Staudt, V., Klein, M., Taube, C., Reuter, S., Dehzad, N., Andersen,

J.F., Kopecky, J., Schild, H., Kotsyfakis, M., Hoffmann, M., Gerlitzki, B., Stassen, M., Bopp, T., Schmitt, E., 2012. The Tick Salivary Protein Sialostatin L Inhibits the Th9-Derived Production of the Asthma-Promoting Cytokine IL-9 and Is Effective in the Prevention of Experimental Asthma. J. Immunol. 188, 2669–2676. doi:DOI 10.4049/jimmunol.1100529

Horn, M., Nussbaumerova, M., Sanda, M., Kovarova, Z., Srba, J., Franta, Z., Sojka, D., Bogyo, M., Caffrey, C.R., Kopacek, P., Mares, M., 2009. Hemoglobin digestion in blood-feeding ticks: mapping a multipeptidase pathway by functional proteomics. Chem Biol 16, 1053–1063. doi:10.1016/j.chembiol.2009.09.009S1074-5521(09)00291-9 [pii]

Howell, J.M., Ueti, M.W., Palmer, G.H., Scoles, G.A., Knowles, D.P., 2007. Transovarial transmission efficiency of *Babesia bovis* tick stages acquired by *Rhipicephalus* (*Boophilus*) *microplus* during acute infection. J. Clin. Microbiol. 45, 426–31. doi:10.1128/JCM.01757-06

Huang, L., Shi, Y., Lu, F., Zheng, H., Liu, X., Gong, B., Yang, J., Lin, Y., Cheng, J., Ma, S., Lin, H., Yang, Z., 2013. Association Study of Polymorphisms in Selenoprotein Genes and Kashin–Beck Disease and Serum Selenium/Iodine Concentration in a Tibetan Population. PLoS One 8, e71411. doi:10.1371/journal.pone.0071411

Hughes, G.L., Koga, R., Xue, P., Fukatsu, T., Rasgon, J.L., 2011. Wolbachia infections are virulent and inhibit the human malaria parasite *Plasmodium falciparum* in *Anopheles gambiae*. PLoS Pathog 7, e1002043. doi:10.1371/journal.ppat.1002043

Hughes, G.L., Rivero, A., Rasgon, J.L., 2014. Wolbachia Can Enhance

    Plasmodium Infection in Mosquitoes: Implications for Malaria Control?

    PLoS Pathog. 10, e1004182. doi:10.1371/journal.ppat.1004182

Hüttenhofer, A., Westhof, E., Böck, A., 1996. Solution structure of mRNA hairpins

    promoting selenocysteine incorporation in *Escherichia coli* and their base-

    specific interaction with special elongation factor SELB. RNA 2, 354–66.

Imamura, S., da Silva Vaz Junior, I., Sugino, M., Ohashi, K., Onuma, M., 2005. A

    serine protease inhibitor (serpin) from *Haemaphysalis longicornis* as an

    anti-tick vaccine. Vaccine 23, 1301–1311.

    doi:10.1016/j.vaccine.2004.08.041

Jiang, J., Stromdahl, E.Y., Richards, A.L., 2012. Detection of *Rickettsia parkeri*

    and *Candidatus* Rickettsia andeanae in *Amblyomma maculatum* Gulf

    Coast ticks collected from humans in the United States. Vector Borne

    Zoonotic Dis. 12, 175–82. doi:10.1089/vbz.2011.0614

Jones, D.T., Taylor, W.R., Thornton, J.M., 1992. The rapid generation of

    mutation data matrices from protein sequences. Bioinformatics 8, 275–

    282. doi:10.1093/bioinformatics/8.3.275

Joseph Felsenstein, 1985. Confidence Limits on Phylogenies: An Approach

    Using the Bootstrap. Evolution (N. Y). 39, 783–91.

Jupatanakul, N., Sim, S., Dimopoulos, G., 2014. The insect microbiome

    modulates vector competence for arboviruses. Viruses 6, 4294–313.

    doi:10.3390/v6114294

Karim, S., Adamson, S.W., Simpson, S.J., Casas, J., 2012. RNA Interference in

Ticks: A Functional Genomics Tool for the Study of Physiology, in: Jockusch, E. (Ed.), Advances in Insect Physiology Small RNAs: Their Diversity, Roles and Practical Uses. Academic Press, San Diego, CA, pp. 119–154.

Karim, S., Miller, N.J., Valenzuela, J., Sauer, J.R., Mather, T.N., 2005. RNAi-mediated gene silencing to assess the role of synaptobrevin and cystatin in tick blood feeding. Biochem. Biophys. Res. Commun. 334, 1336–1342. doi:10.1016/j.bbrc.2005.07.036

Karim, S., Ribeiro, J.M.C., 2015. An Insight into the Sialome of the Lone Star Tick, *Amblyomma americanum*, with a Glimpse on Its Time Dependent Gene Expression. PLoS One 10, e0131292. doi:10.1371/journal.pone.0131292

Karim, S., Singh, P., Ribeiro, J.M.C., 2011. A deep insight into the sialotranscriptome of the gulf coast tick, *Amblyomma maculatum*. PLoS One 6, e28525. doi:10.1371/journal.pone.0028525

Kashino, S.S., Resende, J., Sacco, A.M., Rocha, C., Proenca, L., Carvalho, W.A., Firmino, A.A., Queiroz, R., Benavides, M., Gershwin, L.J., De Miranda Santos, I.K., 2005. *Boophilus microplus*: the pattern of bovine immunoglobulin isotype responses to high and low tick infestations. Exp Parasitol 110, 12–21. doi:10.1016/j.exppara.2005.01.006

Kim, H.-Y., Gladyshev, V.N., 2004. Methionine sulfoxide reduction in mammals: characterization of methionine-R-sulfoxide reductases. Mol. Biol. Cell 15, 1055–64. doi:10.1091/mbc.E03-08-0629

Kim, S.-H., Lee, W.-J., 2014. Role of DUOX in gut inflammation: lessons from *Drosophila* model of gut-microbiota interactions. Front. Cell. Infect. Microbiol. 3, 116. doi:10.3389/fcimb.2013.00116

Kocan, A.A., Ewing, S.A., Stallknecht, D., Murphy, G.L., Little, S., Whitworth, L.C., Barker, R.W., 2000. Attempted transmission of *Ehrlichia chaffeensis* among white-tailed deer by *Amblyomma maculatum*. J. Wildl. Dis. 36, 592–4. doi:10.7589/0090-3558-36.3.592

Kocan, K.M., de la Fuente, J., Manzano-Roman, R., Naranjo, V., Hynes, W.L., Sonenshine, D.E., 2008. Silencing expression of the defensin, varisin, in male *Dermacentor variabilis* by RNA interference results in reduced *Anaplasma marginale* infections. Exp. Appl. Acarol. 46, 17–28. doi:10.1007/s10493-008-9159-5

Kocan, K.M., Zivkovic, Z., Blouin, E.F., Naranjo, V., Almazán, C., Mitra, R., de la Fuente, J., 2009. Silencing of genes involved in *Anaplasma marginale*-tick interactions affects the pathogen developmental cycle in *Dermacentor variabilis*. BMC Dev. Biol. 9, 42. doi:10.1186/1471-213X-9-42

Koh, C.Y., Kazimirova, M., Trimnell, A., Takac, P., Labuda, M., Nuttall, P.A., Kini, R.M., 2007. Variegin, a novel fast and tight binding thrombin inhibitor from the tropical bont tick. J. Biol. Chem. 282, 29101–13. doi:10.1074/jbc.M705600200

Kopecký, J., Kuthejlová, M., 1998. Suppressive effect of *Ixodes ricinus* salivary gland extract on mechanisms of natural immunity in vitro. Parasite Immunol. 20, 169–74.

Korotkov, K. V., Kumaraswamy, E., Zhou, Y., Hatfield, D.L., Gladyshev, V.N., 2001. Association between the 15-kDa Selenoprotein and UDP-glucose:Glycoprotein Glucosyltransferase in the Endoplasmic Reticulum of Mammalian Cells. J. Biol. Chem. 276, 15330–15336. doi:10.1074/jbc.M009861200

Kotsyfakis, M., Horka, H., Salat, J., Andersen, J.F., 2010. The crystal structures of two salivary cystatins from the tick *Ixodes scapularis* and the effect of these inhibitors on the establishment of *Borrelia burgdorferi* infection in a murine model. Mol Microbiol 77, 456–470. doi:MMI7220 [pii]10.1111/j.1365-2958.2010.07220.x

Kotsyfakis, M., Karim, S., Andersen, J.F., Mather, T.N., Ribeiro, J.M.C., 2007. Selective cysteine protease inhibition contributes to blood-feeding success of the tick *Ixodes scapularis*. J. Biol. Chem. 282, 29256–29263. doi:10.1074/jbc.M703143200

Kotsyfakis, M., Schwarz, A., Erhart, J., Ribeiro, J.M.C., 2015. Tissue- and time-dependent transcription in *Ixodes ricinus* salivary glands and midguts when blood feeding on the vertebrate host. Sci. Rep. 5, 9103. doi:10.1038/srep09103

Krusell, A., Comer, J.A., Sexton, D.J., 2002. Rickettsialpox in North Carolina: a case report. Emerg Infect Dis 8, 727–728.

Kryukov, G. V., Castellano, S., Novoselov, S. V, Lobanov, A. V, Zehtab, O., Guigó, R., Gladyshev, V.N., 2003. Characterization of mammalian selenoproteomes. Science 300, 1439–43. doi:10.1126/science.1083516

Kryukov, G. V., Gladyshev, V.N., 2000. Selenium metabolism in zebrafish: multiplicity of selenoprotein genes and expression of a protein containing 17 selenocysteine residues. Genes to Cells 5, 1049–1060. doi:10.1046/j.1365-2443.2000.00392.x

Kryukov, G. V, Kryukov, V.M., Gladyshev, V.N., 1999. New mammalian selenocysteine-containing proteins identified with an algorithm that searches for selenocysteine insertion sequence elements. J. Biol. Chem. 274, 33888–97.

Kryukov, G. V, Kumar, R.A., Koc, A., Sun, Z., Gladyshev, V.N., 2002. Selenoprotein R is a zinc-containing stereo-specific methionine sulfoxide reductase. Proc. Natl. Acad. Sci. U. S. A. 99, 4245–50. doi:10.1073/pnas.072603099

Kumar, D., Budachetri, K., Meyers, V.C., Karim, S., 2016. Assessment of tick antioxidant responses to exogenous oxidative stressors and insight into the role of catalase in the reproductive fitness of the Gulf Coast tick, *Amblyomma maculatum*. Insect Mol. Biol. 25, 283–94. doi:10.1111/imb.12218

Labunskyy, V.M., Hatfield, D.L., Gladyshev, V.N., 2014. Selenoproteins: molecular pathways and physiological roles. Physiol. Rev. 94, 739–77. doi:10.1152/physrev.00039.2013

Lai, R., Takeuchi, H., Jonczy, J., Rees, H.H., Turner, P.C., 2004. A thrombin inhibitor from the ixodid tick, *Amblyomma hebraeum*. Gene 342, 243–9. doi:10.1016/j.gene.2004.07.012

Lanz-Mendoza, H., Hernandez-Martinez, S., Ku-Lopez, M., del Carmen
  Rodriguez, M., Herrera-Ortiz, A., Rodriguez, M.H., 2002. Superoxide
  Anion in *Anopheles albimanus* Hemolymph and Midgut Is Toxic to
  Plasmodium berghei Ookinetes. J. Parasitol. 88, 702.
  doi:10.2307/3285346

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A.,
  McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson,
  J.D., Gibson, T.J., Higgins, D.G., 2007. Clustal W and Clustal X version
  2.0. Bioinformatics 23, 2947–2948. doi:10.1093/bioinformatics/btm404

Le Chatelier, E., Nielsen, T., Qin, J., Prifti, E., Hildebrand, F., Falony, G.,
  Almeida, M., Arumugam, M., Batto, J.-M., Kennedy, S., Leonard, P., Li, J.,
  Burgdorf, K., Grarup, N., Jorgensen, T., Brandslund, I., Nielsen, H.B.,
  Juncker, A.S., Bertalan, M., Levenez, F., Pons, N., Rasmussen, S.,
  Sunagawa, S., Tap, J., Tims, S., Zoetendal, E.G., Brunak, S., Clement, K.,
  Dore, J., Kleerebezem, M., Kristiansen, K., Renault, P., Sicheritz-Ponten,
  T., de Vos, W.M., Zucker, J.-D., Raes, J., Hansen, T., consortium, M.,
  Bork, P., Wang, J., Ehrlich, S.D., Pedersen, O., 2013. Richness of human
  gut microbiome correlates with metabolic markers. Nature 500, 541–546.

Lee, Y.K., Mazmanian, S.K., 2010. Has the microbiota played a critical role in the
  evolution of the adaptive immune system? Science 330, 1768–73.
  doi:10.1126/science.1195568

Lieskovská, J., Páleníková, J., Širmarová, J., Elsterová, J., Kotsyfakis, M.,
  Campos Chagas, A., Calvo, E., Růžek, D., Kopecký, J., 2015. Tick

salivary cystatin sialostatin L2 suppresses IFN responses in mouse
dendritic cells. Parasite Immunol. 37, 70–78. doi:10.1111/pim.12162

Little, T.J., Kraaijeveld, A.R., 2004. Ecological and evolutionary implications of
immunological priming in invertebrates. Trends Ecol. Evol. 19, 58–60.
doi:10.1016/j.tree.2003.11.011

Liu, J.-N., Yu, Z.-J., Liu, L.-M., Li, N.-X., Wang, R.-R., Zhang, C.-M., Liu, J.-Z.,
2016. Identification, Distribution and Population Dynamics of Francisella-
like Endosymbiont in *Haemaphysalis doenitzi* (Acari: Ixodidae). Sci. Rep.
6, 35178. doi:10.1038/srep35178

Liu, L., Dai, J., Zhao, Y.O., Narasimhan, S., Yang, Y., Zhang, L., Fikrig, E., 2012.
Ixodes scapularis JAK-STAT pathway regulates tick antimicrobial
peptides, thereby controlling the agent of human granulocytic
anaplasmosis. J. Infect. Dis. 206, 1233–41. doi:10.1093/infdis/jis484

Liu, L., Narasimhan, S., Dai, J., Zhang, L., Cheng, G., Fikrig, E., 2011. Ixodes
scapularis salivary gland protein P11 facilitates migration of *Anaplasma
phagocytophilum* from the tick gut to salivary glands. EMBO Rep. 12,
1196–203. doi:10.1038/embor.2011.177

Lobanov, A. V, Hatfield, D.L., Gladyshev, V.N., 2008. Selenoproteinless animals:
selenophosphate synthetase SPS1 functions in a pathway unrelated to
selenocysteine biosynthesis. Protein Sci. 17, 176–82.
doi:10.1110/ps.073261508

Long, S.W., Zhang, X., Zhang, J., Ruble, R.P., Teel, P., Yu, X.-J., 2003.
Evaluation of transovarial transmission and transmissibility of *Ehrlichia*

chaffeensis (Rickettsiales: Anaplasmataceae) in *Amblyomma americanum* (Acari: Ixodidae). J. Med. Entomol. 40, 1000–4.

Longo, D.L., Loscalzo, J., 2014. Keshan Disease, Selenium Deficiency, and the Selenoproteome. N. Engl. J. Med. 370, 1756–1760. doi:10.1056/NEJMcibr1402199

Look, M.P., Rockstroh, J.K., Rao, G.S., Kreuzer, K.A., Spengler, U., Sauerbruch, T., 1997. Serum selenium versus lymphocyte subsets and markers of disease progression and inflammatory response in human immunodeficiency virus-1 infection. Biol. Trace Elem. Res. 56, 31–41. doi:10.1007/BF02778982

Low, S.C., Grundner-Culemann, E., Harney, J.W., Berry, M.J., 2000. SECIS-SBP2 interactions dictate selenocysteine incorporation efficiency and selenoprotein hierarchy. EMBO J. 19, 6882–90. doi:10.1093/emboj/19.24.6882

Lu, J., Zhong, L., Lonn, M.E., Burk, R.F., Hill, K.E., Holmgren, A., 2009. Penultimate selenocysteine residue replaced by cysteine in thioredoxin reductase from selenium-deficient rat liver. FASEB J. 23, 2394–2402. doi:10.1096/fj.08-127662

Macaluso, K.R., Sonenshine, D.E., Ceraul, S.M., Azad, A.F., 2001. Infection and transovarial transmission of rickettsiae in *Dermacentor variabilis* ticks acquired by artificial feeding. Vector Borne Zoonotic Dis 1, 45–53.

Mans, B.J., Gaspar, A.R., Louw, A.I., Neitz, A.W., 1998. Apyrase activity and platelet aggregation inhibitors in the tick *Ornithodoros savignyi* (Acari:

Argasidae). Exp. Appl. Acarol. 22, 353–66.

Mans, B.J., Louw, A.I., Neitz, A.W.H., 2002. Savignygrin, a platelet aggregation inhibitor from the soft tick *Ornithodoros savignyi*, presents the RGD integrin recognition motif on the Kunitz-BPTI fold. J. Biol. Chem. 277, 21371–8. doi:10.1074/jbc.M112060200

Marchler-Bauer, A., Derbyshire, M.K., Gonzales, N.R., Lu, S., Chitsaz, F., Geer, L.Y., Geer, R.C., He, J., Gwadz, M., Hurwitz, D.I., Lanczycki, C.J., Lu, F., Marchler, G.H., Song, J.S., Thanki, N., Wang, Z., Yamashita, R.A., Zhang, D., Zheng, C., Bryant, S.H., 2015. CDD: NCBI's conserved domain database. Nucleic Acids Res. 43, D222-6. doi:10.1093/nar/gku1221

Mariconti, M., Epis, S., Gaibani, P., Dalla Valle, C., Sassera, D., Tomao, P., Fabbi, M., Castelli, F., Marone, P., Sambri, V., Bazzocchi, C., Bandi, C., 2012. Humans parasitized by the hard tick *Ixodes ricinus* are seropositive to *Midichloria mitochondrii* : is *Midichloria* a novel pathogen, or just a marker of tick bite? Pathog. Glob. Health 106, 391–396. doi:10.1179/2047773212Y.0000000050

Mbow, M.L., Rutti, B., Brossard, M., 1994. Infiltration of CD4+ CD8+ T cells, and expression of ICAM-1, Ia antigens, IL-1 alpha and TNF-alpha in the skin lesion of BALB/c mice undergoing repeated infestations with nymphal Ixodes ricinus ticks. Immunology 82, 596–602.

Mejri, N., Rutti, B., Brossard, M., 2002. Immunosuppressive effects of *Ixodes ricinus* tick saliva or salivary gland extracts on innate and acquired immune response of BALB/c mice. Parasitol. Res. 88, 192–7.

Menten-Dedoyart, C., Faccinetto, C., Golovchenko, M., Dupiereux, I., Van

    Lerberghe, P.-B., Dubois, S., Desmet, C., Elmoualij, B., Baron, F.,

    Rudenko, N., Oury, C., Heinen, E., Couvreur, B., 2012. Neutrophil

    extracellular traps entrap and kill *Borrelia burgdorferi* sensu stricto

    spirochetes and are not affected by *Ixodes ricinus* tick saliva. J. Immunol.

    189, 5393–401. doi:10.4049/jimmunol.1103771

Merino, O., Almazán, C., Canales, M., Villar, M., Moreno-Cid, J.A., Galindo, R.C.,

    de la Fuente, J., 2011. Targeting the tick protective antigen subolesin

    reduces vector infestations and pathogen infection by *Anaplasma*

    marginale and *Babesia bigemina*. Vaccine 29, 8575–9.

    doi:10.1016/j.vaccine.2011.09.023

Mihara, H., Kurihara, T., Watanabe, T., Yoshimura, T., Esaki, N., 2000. cDNA

    cloning, purification, and characterization of mouse liver selenocysteine

    lyase. Candidate for selenium delivery protein in selenoprotein synthesis.

    J. Biol. Chem. 275, 6195–200. doi:10.1074/JBC.275.9.6195

Montagna, M., Sassera, D., Epis, S., Bazzocchi, C., Vannini, C., Lo, N., Sacchi,

    L., Fukatsu, T., Petroni, G., Bandi, C., 2013. "Candidatus Midichloriaceae"

    fam. nov. (Rickettsiales), an ecologically widespread clade of intracellular

    alphaproteobacteria. Appl. Environ. Microbiol. 79, 3241–8.

    doi:10.1128/AEM.03971-12

Monteiro, R.Q., Rezaie, A.R., Ribeiro, J.M.C., Francischetti, I.M.B., 2005.

    Ixolaris: a factor Xa heparin-binding exosite inhibitor. Biochem. J. 387,

    871–7. doi:10.1042/BJ20041738

Morgan, J.F., Campbell, M.E., Morton, H.J., 1955. The nutrition of animal tissues cultivated in vitro. I. A survey of natural materials as supplements to synthetic medium 199. J. Natl. Cancer Inst. 16, 557–67.

Morgan, J.F., Morton, H.J., Parker, R.C., 1950. Nutrition of animal cells in tissue culture; initial studies on a synthetic medium. Proc. Soc. Exp. Biol. Med. 73, 1–8.

Mostert, V., 2000. Selenoprotein P: Properties, Functions, and Regulation. Arch. Biochem. Biophys. 376, 433–438. doi:10.1006/abbi.2000.1735

Mousson, L., Zouache, K., Arias-Goeta, C., Raquin, V., Mavingui, P., Failloux, A.-B., 2012. The Native *Wolbachia* Symbionts Limit Transmission of Dengue Virus in *Aedes albopictus*. PLoS Negl. Trop. Dis. 6, e1989. doi:10.1371/journal.pntd.0001989

Mukherjee, N., Beati, L., Sellers, M., Burton, L., Adamson, S., Robbins, R.G., Moore, F., Karim, S., 2014. Importation of exotic ticks and tick-borne spotted fever group rickettsiae into the United States by migrating songbirds. Ticks Tick Borne Dis 5, 127–134. doi:10.1016/j.ttbdis.2013.09.009

Narasimhan, S., Fikrig, E., 2015. Tick microbiome: the force within. Trends Parasitol 31, 315–323. doi:10.1016/j.pt.2015.03.010

Narasimhan, S., Koski, R.A., Beaulieu, B., Anderson, J.F., Ramamoorthi, N., Kantor, F., Cappello, M., Fikrig, E., 2002. A novel family of anticoagulants from the saliva of *Ixodes scapularis*. Insect Mol. Biol. 11, 641–50.

Narasimhan, S., Rajeevan, N., Liu, L., Zhao, Y.O., Heisig, J., Pan, J., Eppler-

Epstein, R., Deponte, K., Fish, D., Fikrig, E., 2014. Gut microbiota of the tick vector *Ixodes scapularis* modulate colonization of the lyme disease spirochete. Cell Host Microbe 15, 58–71. doi:10.1016/j.chom.2013.12.001

Nazareth, R.A., Tomaz, L.S., Ortiz-Costa, S., Atella, G.C., Ribeiro, J.M.C., Francischetti, I.M.B., Monteiro, R.Q., 2006. Antithrombotic properties of Ixolaris, a potent inhibitor of the extrinsic pathway of the coagulation cascade. Thromb. Haemost. 96, 7–13. doi:10.1160/TH06-02-0105

Niebylski, M.L., Peacock, M.G., Fischer, E.R., Porcella, S.F., Schwan, T.G., 1997a. Characterization of an endosymbiont infecting wood ticks, *Dermacentor andersoni*, as a member of the genus Francisella. Appl Env. Microbiol 63, 3933–3940.

Niebylski, M.L., Peacock, M.G., Schwan, T.G., 1999. Lethal effect of *Rickettsia rickettsii* on its tick vector (*Dermacentor andersoni*). Appl Env. Microbiol 65, 773–778.

Niebylski, M.L., Schrumpf, M.E., Burgdorfer, W., Fischer, E.R., Gage, K.L., Schwan, T.G., 1997b. *Rickettsia peacockii* sp. nov., a new species infecting wood ticks, *Dermacentor andersoni*, in western Montana. Int J Syst Bacteriol 47, 446–452.

O'Dwyer, L.H., Massard, C.L., Pereira de Souza, J.C., 2001. *Hepatozoon canis* infection associated with dog ticks of rural areas of Rio de Janeiro State, Brazil. Vet Parasitol 94, 143–150. doi:10.1016/S0304-4017(00)00378-2

Paddock, C.D., Finley, R.W., Wright, C.S., Robinson, H.N., Schrodt, B.J., Lane, C.C., Ekenna, O., Blass, M.A., Tamminga, C.L., Ohl, C.A., McLellan, S.L.,

Goddard, J., Holman, R.C., Openshaw, J.J., Sumner, J.W., Zaki, S.R., Eremeeva, M.E., 2008. *Rickettsia parkeri* rickettsiosis and its clinical distinction from Rocky Mountain spotted fever. Clin Infect Dis 47, 1188–1196. doi:10.1086/592254

Paddock, C.D., Sumner, J.W., Comer, J.A., Zaki, S.R., Goldsmith, C.S., Goddard, J., McLellan, S.L., Tamminga, C.L., Ohl, C.A., 2004. *Rickettsia parkeri*: a newly recognized cause of spotted fever rickettsiosis in the United States. Clin Infect Dis 38, 805–811. doi:10.1086/381894

Padgett, K.A., Bonilla, D., Eremeeva, M.E., Glaser, C., Lane, R.S., Porse, C.C., Castro, M.B., Messenger, S., Espinosa, A., Hacker, J., Kjemtrup, A., Ryan, B., Scott, J.J., Hu, R., Yoshimizu, M.H., Dasch, G.A., Kramer, V., 2016. The Eco-epidemiology of Pacific Coast Tick Fever in California. PLoS Negl. Trop. Dis. 10, e0005020. doi:10.1371/journal.pntd.0005020

Pais, R., Lohs, C., Wu, Y., Wang, J., Aksoy, S., 2008. The obligate mutualist *Wigglesworthia glossinidia* influences reproduction, digestion, and immunity processes of its host, the tsetse fly. Appl. Environ. Microbiol. 74, 5965–74. doi:10.1128/AEM.00741-08

Pal, U., Li, X., Wang, T., Montgomery, R.R., Ramamoorthi, N., Desilva, A.M., Bao, F., Yang, X., Pypaert, M., Pradhan, D., Kantor, F.S., Telford, S., Anderson, J.F., Fikrig, E., 2004. TROSPA, an *Ixodes scapularis* receptor for *Borrelia burgdorferi*. Cell 119, 457–68. doi:10.1016/j.cell.2004.10.027

Papp, L. V., Lu, J., Bolderson, E., Boucher, D., Singh, R., Holmgren, A., Khanna, K.K., 2010. SECIS-Binding Protein 2 Promotes Cell Survival by Protecting

Against Oxidative Stress. Antioxid. Redox Signal. 12, 797–808. doi:10.1089/ars.2009.2913

Parizi, L.F., Reck, J., Oldiges, D.P., Guizzo, M.G., Seixas, A., Logullo, C., de Oliveira, P.L., Termignoni, C., Martins, J.R., da Silva Vaz, I., 2012. Multi-antigenic vaccine against the cattle tick *Rhipicephalus* (*Boophilus*) *microplus*: A field evaluation. Vaccine 30, 6912–6917. doi:10.1016/j.vaccine.2012.08.078

Parker, B.J., Barribeau, S.M., Laughton, A.M., de Roode, J.C., Gerardo, N.M., 2011. Non-immunological defense in an evolutionary framework. Trends Ecol. Evol. 26, 242–8. doi:10.1016/j.tree.2011.02.005

Parola, P., Paddock, C.D., Raoult, D., 2005. Tick-borne rickettsioses around the world: emerging diseases challenging old concepts. Clin Microbiol Rev 18, 719–756. doi:18/4/719 [pii]10.1128/CMR.18.4.719-756.2005

Patrick, C.D., Hair, J.A., 1975. Laboratory rearing procedures and equipment for multi-host ticks (Acarina: Ixodidae). J Med Entomol 12, 389–390.

Pedra, J.H.F., Narasimhan, S., Rendić, D., DePonte, K., Bell-Sakyi, L., Wilson, I.B.H., Fikrig, E., 2010. Fucosylation enhances colonization of ticks by *Anaplasma phagocytophilum*. Cell. Microbiol. 12, 1222–34. doi:10.1111/j.1462-5822.2010.01464.x

Petersen, J.M., Mead, P.S., Schriefer, M.E., 2009. *Francisella tularensis*: an arthropod-borne pathogen. Vet. Res. 40, 7. doi:10.1051/vetres:2008045

Piesman, J., Schneider, B.S., Zeidner, N.S., 2001. Use of quantitative PCR to measure density of *Borrelia burgdorferi* in the midgut and salivary glands

of feeding tick vectors. J. Clin. Microbiol. 39, 4145–8.

doi:10.1128/JCM.39.11.4145-4148.2001

Prevost, G., Arabo, A., Jian, L., Quelennec, E., Cartier, D., Hassan, S., Falluel-Morel, A., Tanguy, Y., Gargani, S., Lihrmann, I., Kerr-Conte, J., Lefebvre, H., Pattou, F., Anouar, Y., 2013. The PACAP-Regulated Gene Selenoprotein T Is Abundantly Expressed in Mouse and Human β-Cells and Its Targeted Inactivation Impairs Glucose Tolerance. Endocrinology 154, 3796–3806. doi:10.1210/en.2013-1167

Price, W.H., Johnson, J.W., Emerson, H., Preston, C.E., 1954. Rickettsial-Interference Phenomenon: A New Protective Mechanism. Science (80-.). 120, 599.

Ramirez, J.L., Short, S.M., Bahia, A.C., Saraiva, R.G., Dong, Y., Kang, S., Tripathi, A., Mlambo, G., Dimopoulos, G., 2014. *Chromobacterium* Csp_P Reduces Malaria and Dengue Infection in Vector Mosquitoes and Has Entomopathogenic and In Vitro Anti-pathogen Activities. PLoS Pathog. 10, e1004398. doi:10.1371/journal.ppat.1004398

Rances, E., Johnson, T.K., Popovici, J., Iturbe-Ormaetxe, I., Zakir, T., Warr, C.G., O'Neill, S.L., 2013. The Toll and Imd Pathways Are Not Required for *Wolbachia*-Mediated Dengue Virus Interference. J. Virol. 87, 11945–11949. doi:10.1128/JVI.01522-13

Rand, K.N., Moore, T., Sriskantha, A., Spring, K., Tellam, R., Willadsen, P., Cobon, G.S., 1989. Cloning and expression of a protective antigen from the cattle tick *Boophilus microplus*. Proc. Natl. Acad. Sci. U. S. A. 86,

112

9657–61.

Raoult, D., Roux, V., 1997. Rickettsioses as paradigms of new or emerging infectious diseases. Clin Microbiol Rev 10, 694–719.

Rayman, M.P., 2000. The importance of selenium to human health. Lancet 356, 233–241. doi:10.1016/S0140-6736(00)02490-9

Reich, H.J., Hondal, R.J., 2016. Why Nature Chose Selenium. ACS Chem. Biol. 11, 821–41. doi:10.1021/acschembio.6b00031

Ribeiro, J.M., 1989. Role of saliva in tick/host interactions. Exp. Appl. Acarol. 7, 15–20.

Ribeiro, J.M., Endris, T.M., Endris, R., 1991. Saliva of the soft tick, *Ornithodoros moubata*, contains anti-platelet and apyrase activities. Comp. Biochem. Physiol. A. Comp. Physiol. 100, 109–12.

Ribeiro, J.M.C., Anderson, J.M., Manoukis, N.C., Meng, Z.J., Francischetti, I.M.B., 2011. A further insight into the sialome of the tropical bont tick, *Amblyomma variegatum*. BMC Genomics 12, 136. doi:10.1186/1471-2164-12-136

Richard, S., Seng, P., Parola, P., Raoult, D., Davoust, B., Brouqui, P., 2009. Detection of a new bacterium related to "*Candidatus* Midichloria mitochondrii" in bed bugs. Clin. Microbiol. Infect. 15, 84–85. doi:10.1111/j.1469-0691.2008.02244.x

Richardson, D.R., 2005. More roles for selenoprotein P: local selenium storage and recycling protein in the brain. Biochem. J. 386, e5-7. doi:10.1042/BJ20050149

Rikihisa, Y., 2010. *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis*: subversive manipulators of host cells. Nat. Rev. Microbiol. 8, 328–339. doi:10.1038/nrmicro2318

Rio, R.V.M., Attardo, G.M., Weiss, B.L., 2016. Grandeur Alliances: Symbiont Metabolic Integration and Obligate Arthropod Hematophagy. Trends Parasitol. doi:10.1016/j.pt.2016.05.002

Rivero, A., 2006. Nitric oxide: an antiparasitic molecule of invertebrates. Trends Parasitol. 22, 219–25. doi:10.1016/j.pt.2006.02.014

Rock, C., Moos, P.J., 2010. Selenoprotein P protects cells from lipid hydroperoxides generated by 15-LOX-1. Prostaglandins, Leukot. Essent. Fat. Acids 83, 203–210. doi:10.1016/j.plefa.2010.08.006

Rolníková, T., Kazimírová, M., Buc, M., 2003. Modulation of human lymphocyte proliferation by salivary gland extracts of ixodid ticks (Acari: Ixodidae): effect of feeding stage and sex. Folia Parasitol. (Praha). 50, 305–12.

Rosen, H., Orman, J., Rakita, R.M., Michel, B.R., VanDevanter, D.R., 1990. Loss of DNA-membrane interactions and cessation of DNA synthesis in myeloperoxidase-treated *Escherichia coli*. Proc Natl Acad Sci U S A 87, 10048–10052.

Rudenko, N., Golovchenko, M., Edwards, M.J., Grubhoffer, L., 2005. Differential Expression of *Ixodes ricinus* Tick Genes Induced by Blood Feeding or *Borrelia burgdorferi* Infection. J. Med. Entomol. 42, 36–41. doi:10.1093/jmedent/42.1.36

Sachs, A.B., Sarnow, P., Hentze, M.W., 1991. Starting at the Beginning, Middle,

and End: Translation Initiation in Eukaryotes. Cell 89, 831–838. doi:10.1016/S0092-8674(00)80268-8

Sahni, S.K., Narra, H.P., Sahni, A., Walker, D.H., 2013. Recent molecular insights into rickettsial pathogenesis and immunity. Future Microbiol. 8, 1265–88. doi:10.2217/fmb.13.102

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–25.

Salát, J., Paesen, G.C., Rezácová, P., Kotsyfakis, M., Kovárová, Z., Sanda, M., Majtán, J., Grunclová, L., Horká, H., Andersen, J.F., Brynda, J., Horn, M., Nunn, M.A., Kopácek, P., Kopecký, J., Mares, M., 2010. Crystal structure and functional characterization of an immunomodulatory salivary cystatin from the soft tick *Ornithodoros moubata*. Biochem J 429, 103–112. doi: 10.1042/BJ20100280

Santucci, L.A., Gutierrez, P.L., Silverman, D.J., 1992. *Rickettsia rickettsii* induces superoxide radical and superoxide dismutase in human endothelial cells. Infect. Immun. 60, 5113–8.

Sassera, D., Beninati, T., Bandi, C., Bouman, E.A.P., Sacchi, L., Fabbi, M., Lo, N., 2006. "*Candidatus* Midichloria mitochondrii", an endosymbiont of the tick Ixodes ricinus with a unique intramitochondrial lifestyle. Int J Syst Evol Microbiol 56, 2535–2540. doi:10.1099/ijs.0.64386-0

Sassera, D., Epis, S., Pajoro, M., Bandi, C., 2013. Microbial symbiosis and the control of vector-borne pathogens in tsetse flies, human lice, and triatomine bugs. Pathog. Glob. Health 107, 285–92.

doi:10.1179/2047773213Y.0000000109

Sassera, D., Lo, N., Bouman, E.A.P., Epis, S., Mortarino, M., Bandi, C., 2008. "Candidatus Midichloria" endosymbionts bloom after the blood meal of the host, the hard tick *Ixodes ricinus*. Appl. Environ. Microbiol. 74, 6138–40. doi:10.1128/AEM.00248-08

Schaffer, L.W., Davidson, J.T., Vlasuk, G.P., Siegl, P.K., 1991. Antithrombotic efficacy of recombinant tick anticoagulant peptide. A potent inhibitor of coagulation factor Xa in a primate model of arterial thrombosis. Circulation 84, 1741–8.

Schoenmakers, E., Agostini, M., Mitchell, C., Schoenmakers, N., Papp, L., Rajanayagam, O., Padidela, R., Ceron-Gutierrez, L., Doffinger, R., Prevosto, C., Luan, J., Montano, S., Lu, J., Castanet, M., Clemons, N., Groeneveld, M., Castets, P., Karbaschi, M., Aitken, S., Dixon, A., Williams, J., Campi, I., Blount, M., Burton, H., Muntoni, F., Donovan, D., Dean, A., Warren, A., Brierley, C., Baguley, D., Guicheney, P., Fitzgerald, R., Coles, A., Gaston, H., Todd, P., Holmgren, A., Khanna, K.K., Cooke, M., Semple, R., Halsall, D., Wareham, N., Schwabe, J., Grasso, L., Beck-Peccoz, P., Ogunko, A., Dattani, M., Gurnell, M., Chatterjee, K., 2010. Mutations in the selenocysteine insertion sequence–binding protein 2 gene lead to a multisystem selenoprotein deficiency disorder in humans. J. Clin. Invest. 120, 4220–4235. doi:10.1172/JCI43653

Schulze, K.J., Cole, R.N., Chaerkady, R., Wu, L.S.F., Nonyane, B.A.S., Lee, S.E., Yager, J.D., Groopman, J.D., Christian, P., West, Jr, K.P., 2016.

Plasma Selenium Protein P Isoform 1 (SEPP1): A Predictor of Selenium

Status in Nepalese Children Detected by Plasma Proteomics. Int. J.

Vitam. Nutr. Res. 1–10. doi:10.1024/0300-9831/a000256

Scoles, G.A., 2004. Phylogenetic analysis of the *Francisella*-like endosymbionts

of *Dermacentor* ticks. J Med Entomol 41, 277–286.

Shapiro, M.R., Fritz, C.L., Tait, K., Paddock, C.D., Nicholson, W.L., Abramowicz,

K.F., Karpathy, S.E., Dasch, G.A., Sumner, J.W., Adem, P. V, Scott, J.J.,

Padgett, K.A., Zaki, S.R., Eremeeva, M.E., 2010. Rickettsia 364D: a newly

recognized cause of eschar-associated illness in California. Clin Infect Dis

50, 541–548. doi:10.1086/649926

Shchedrina, V.A., Zhang, Y., Labunskyy, V.M., Hatfield, D.L., Gladyshev, V.N.,

2010. Structure-function relations, physiological roles, and evolution of

mammalian ER-resident selenoproteins. Antioxid. Redox Signal. 12, 839–

49. doi:10.1089/ars.2009.2865

Skallová, A., Iezzi, G., Ampenberger, F., Kopf, M., Kopecky, J., 2008. Tick saliva

inhibits dendritic cell migration, maturation, and function while promoting

development of Th2 responses. J. Immunol. 180, 6186–92.

Smith, A.A., Pal, U., 2014. Immunity-related genes in *Ixodes scapularis*--

perspectives from genome information. Front. Cell. Infect. Microbiol. 4,

116. doi:10.3389/fcimb.2014.00116

Smith, T.A., Driscoll, T., Gillespie, J.J., Raghavan, R., 2015. A *Coxiella*-like

Endosymbiont is a potential vitamin source for the Lone Star Tick.

Genome Biol Evol. doi:10.1093/gbe/evv016

Socolovschi, C., Gaudart, J., Bitam, I., Huynh, T.P., Raoult, D., Parola, P., 2012.
Why are there so few *Rickettsia conorii conorii*-infected *Rhipicephalus sanguineus* ticks in the wild? PLoS Negl Trop Dis 6, e1697.
doi:10.1371/journal.pntd.0001697

Socolovschi, C., Huynh, T.P., Davoust, B., Gomez, J., Raoult, D., Parola, P., 2009. Transovarial and trans-stadial transmission of *Rickettsiae africae* in *Amblyomma variegatum* ticks. Clin Microbiol Infect 15 Suppl 2, 317–318.
doi:10.1111/j.1469-0691.2008.02278.x

Sonenshine, D.E., Roe, R.M., 2013. Biology of Ticks, Second. ed. Oxford University Press, New York, NY.

Steeves, E.B., Allen, J.R., 1991. Tick resistance in mast cell-deficient mice: histological studies. Int J Parasitol 21, 265–268. doi:0020-7519(91)90020-8

Straily, A., Feldpausch, A., Ulbrich, C., Schell, K., Casillas, S., Zaki, S.R., Denison, A.M., Condit, M., Gabel, J., Paddock, C.D., 2016. *Notes from the Field* : *Rickettsia parkeri* Rickettsiosis — Georgia, 2012–2014. MMWR. Morb. Mortal. Wkly. Rep. 65, 718–719. doi:10.15585/mmwr.mm6528a3

Sun, L.-H., Li, J.-G., Zhao, H., Shi, J., Huang, J.-Q., Wang, K.-N., Xia, X.-J., Li, L., Lei, X.G., 2013. Porcine serum can be biofortified with selenium to inhibit proliferation of three types of human cancer cells. J. Nutr. 143, 1115–22. doi:10.3945/jn.113.177410

Sun, L. V, Scoles, G.A., Fish, D., O'Neill, S.L., 2000. *Francisella*-like endosymbionts of ticks. J Invertebr Pathol 76, 301–303.

doi:10.1006/jipa.2000.4983

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6:
Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol 30,
2725–2729. doi:10.1093/molbev/mst197mst197

Teixeira, L., Ferreira, Á., Ashburner, M., 2008. The Bacterial Symbiont Wolbachia
Induces Resistance to RNA Viral Infections in *Drosophila melanogaster*.
PLoS Biol. 6, e1000002. doi:10.1371/journal.pbio.1000002

Telford, S.R., 2009. Status of the "East Side Hypothesis" (Transovarial
Interference) 25 Years Later. Ann. N. Y. Acad. Sci. 1166, 144–150.
doi:10.1111/j.1749-6632.2009.04522.x

Thompson, J.D., Gibson, T.J., Higgins, D.G., 2002. Multiple sequence alignment
using ClustalW and ClustalX. Curr Protoc Bioinforma. Chapter 2, Unit 2 3.
doi:10.1002/0471250953.bi0203s00

Tinggi, U., 2008. Selenium: its role as antioxidant in human health. Environ.
Health Prev. Med. 13, 102–8. doi:10.1007/s12199-007-0019-4

Tujebajeva, R.M., Copeland, P.R., Xu, X.M., Carlson, B.A., Harney, J.W.,
Driscoll, D.M., Hatfield, D.L., Berry, M.J., 2000. Decoding apparatus for
eukaryotic selenocysteine insertion. EMBO Rep. 1, 158–63.
doi:10.1038/sj.embor.embor604

Turnbaugh, P.J., Ley, R.E., Hamady, M., Fraser-Liggett, C.M., Knight, R.,
Gordon, J.I., 2007. The human microbiome project. Nature 449, 804–10.
doi:10.1038/nature06244

Ursini, F., Heim, S., Kiess, M., Maiorino, M., Roveri, A., Wissing, J., 1999. Dual

function of the selenoprotein PHGPx during sperm maturation. Science
285, 1393–6. doi:10.1126/science.285.5432.1393

Valenzuela, J.G., Francischetti, I.M.B., Pham, V.M., Garfield, M.K., Mather, T.N.,
Ribeiro, J.M.C., 2002. Exploring the sialome of the tick *Ixodes scapularis*.
J. Exp. Biol. 205, 2843–64.

Vancová, I., Slovák, M., Hajnická, V., Labuda, M., Simo, L., Peterková, K., Hails,
R.S., Nuttall, P.A., 2007. Differential anti-chemokine activity of
Amblyomma variegatum adult ticks during blood-feeding. Parasite
Immunol. 29, 169–77. doi:10.1111/j.1365-3024.2006.00931.x

Veres, Z., Kim, I.Y., Scholz, T.D., Stadtman, T.C., 1994. Selenophosphate
synthetase. Enzyme properties and catalytic reaction. J. Biol. Chem. 269,
10597–603.

Villarreal, A.M., Adamson, S.W., Browning, R.E., Budachetri, K., Sajid, M.S.,
Karim, S., 2013. Molecular characterization and functional significance of
the Vti family of SNARE proteins in tick salivary glands. Insect Biochem.
Mol. Biol. 43, 483–493.

Waladde, S.M., 1979. Feeding electrograms and fluid uptake measurements of
cattle tick *Boophilus microplus* attached on artificial membranes. Int. J.
Parasitol 9, 89–95.

Walker, D.H., Ismail, N., 2008. Emerging and re-emerging rickettsioses:
endothelial cell infection and early disease events. Nat Rev Microbiol 6,
375–386. doi:nrmicro1866 [pii]10.1038/nrmicro1866

Walker, D.H., Valbuena, G.A., Olano, J.P., 2003. Pathogenic mechanisms of

diseases caused by Rickettsia. Ann N Y Acad Sci 990, 1–11.

Walker, T., Johnson, P.H., Moreira, L.A., Iturbe-Ormaetxe, I., Frentiu, F.D., McMeniman, C.J., Leong, Y.S., Dong, Y., Axford, J., Kriesner, P., Lloyd, A.L., Ritchie, S.A., O'Neill, S.L., Hoffmann, A.A., 2011. The wMel Wolbachia strain blocks dengue and invades caged *Aedes aegypti* populations. Nature 476, 450–3. doi:10.1038/nature10355

Wang, J., Aksoy, S., 2012. PGRP-LB is a maternally transmitted immune milk protein that influences symbiosis and parasitism in tsetse's offspring. Proc. Natl. Acad. Sci. U. S. A. 109, 10552–7. doi:10.1073/pnas.1116431109

Waxman, L., Smith, D.E., Arcuri, K.E., Vlasuk, G.P., 1990. Tick anticoagulant peptide (TAP) is a novel inhibitor of blood coagulation factor Xa. Science 248, 593–6.

Weiss, B., Aksoy, S., 2011. Microbiome influences on insect host vector competence. Trends Parasitol 27, 514–522. doi:10.1016/j.pt.2011.05.001S1471-4922(11)00083-3 [pii]

Welch, M.D., Reed, S.C.O., Lamason, R.L., Serio, A.W., 2012. Expression of an epitope-tagged virulence protein in *Rickettsia parkeri* using transposon insertion. PLoS One 7, e37310. doi:10.1371/journal.pone.0037310

Werren, J.H., Baldo, L., Clark, M.E., 2008. Wolbachia: master manipulators of invertebrate biology. Nat. Rev. Microbiol. 6, 741–51. doi:10.1038/nrmicro1969

Willadsen, P., Riding, G.A., McKenna, R. V, Kemp, D.H., Tellam, R.L., Nielsen, J.N., Lahnstein, J., Cobon, G.S., Gough, J.M., 1989. Immunologic control

of a parasitic arthropod. Identification of a protective antigen from
Boophilus microplus. J. Immunol. 143, 1346–51.

Williams-Newkirk, A.J., Rowe, L.A., Mixson-Hayden, T.R., Dasch, G.A., 2012.
Presence, genetic variability, and potential significance of "*Candidatus*
Midichloria mitochondrii" in the lone star tick *Amblyomma americanum*.
Exp. Appl. Acarol. 58, 291–300. doi:10.1007/s10493-012-9582-5

Woese, C.R., 1998. Default taxonomy: Ernst Mayr's view of the microbial world.
Proc. Natl. Acad. Sci. U. S. A. 95, 11043–6.
doi:10.1073/PNAS.95.19.11043

Wood, D.O., Wood, R.R., Tucker, A.M., 2014. Genetic systems for studying
obligate intracellular pathogens: an update. Curr. Opin. Microbiol. 17, 11–
6. doi:10.1016/j.mib.2013.10.006

Wright, C.L., Gaff, H.D., Sonenshine, D.E., Hynes, W.L., 2015. Experimental
vertical transmission of *Rickettsia parkeri* in the Gulf Coast tick,
Amblyomma maculatum. Ticks Tick. Borne. Dis. 6, 568–73.
doi:10.1016/j.ttbdis.2015.04.011

Xi, Z., Ramirez, J.L., Dimopoulos, G., 2008. The *Aedes aegypti* toll pathway
controls dengue virus infection. PLoS Pathog. 4, e1000098.
doi:10.1371/journal.ppat.1000098

Zhang, L., Zhang, Y., Adusumilli, S., Liu, L., Narasimhan, S., Dai, J., Zhao, Y.O.,
Fikrig, E., 2011. Molecular interactions that enable movement of the Lyme
disease agent from the tick gut into the hemolymph. PLoS Pathog. 7,
e1002079. doi:10.1371/journal.ppat.1002079

Zhao, C., Slevin, J.T., Whiteheart, S.W., 2007. Cellular functions of NSF: not just

    SNAPs and SNAREs. FEBS Lett. 581, 2140–9.

    doi:10.1016/j.febslet.2007.03.032

Zhong, J., Jasinskas, A., Barbour, A.G., 2007. Antibiotic treatment of the tick

    vector *Amblyomma americanum* reduced reproductive fitness. PLoS One

    2, e405. doi:10.1371/journal.pone.0000405

Zhu, K., Bowman, A.S., Brigham, D.L., Essenberg, R.C., Dillwith, J.W., Sauer,

    J.R., 1997. Isolation and characterization of americanin, a specific inhibitor

    of thrombin, from the salivary glands of the lone star tick *Amblyomma*

    americanum (L.). Exp. Parasitol. 87, 30–8. doi:10.1006/expr.1997.4175

Zhu, L., Wu, Q., Dai, J., Zhang, S., Wei, F., 2011. Evidence of cellulose

    metabolism by the giant panda gut microbiome. Proc. Natl. Acad. Sci. U.

    S. A. 108, 17714–9. doi:10.1073/pnas.1017956108