Study of *Rickettsia parkeri* Colonization and Proliferation in the Tick Host *Amblyomma maculatum* (Acari: Ixodidae)

Khemraj Budachetri

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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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THE UNIVERSITY OF SOUTHERN MISSISSIPPI
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

I would like to acknowledge my mentor Dr. Shahid Karim for his guidance and help during entire period of my graduate education. I am indebted to him for believing and trusting, creating curiosity in science and motivating in times of difficulties. The advice and support from academic advisors Dr. Mohamed O. Elasri, Dr. Alex S. Flynt, Dr. Fengwei Bai and Dr. Gregory Dasch was valuable in shaping this dissertation.

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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
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°C 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, *Hepatozoon 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).](image)

*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
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.

<table>
<thead>
<tr>
<th>Tick</th>
<th>Saliva/SGE/Factor</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ornithodorus moubata</em></td>
<td>Tick anticoagulant peptide and antistatin</td>
<td>Inhibit blood coagulation factor X (fXa)</td>
<td>(Dunwiddie et al., 1991; Schaffer et al., 1991)</td>
</tr>
<tr>
<td><em>Boophilus microplus</em></td>
<td>microphilin</td>
<td>Inhibits fibrinocoagulation, thrombin-induced platelet aggregation</td>
<td>(Ciprandi et al., 2006)</td>
</tr>
<tr>
<td><em>Ornithodoros savignyi</em></td>
<td>SGE</td>
<td>Prolonged activated partial thromboplastin time (ATPP) and prothrombin time and inhibited fXa</td>
<td>(Gaspar et al., 1995)</td>
</tr>
<tr>
<td><em>Amblyomma variegatum</em></td>
<td>Variegin</td>
<td>Thrombin inhibitor</td>
<td>(Koh et al., 2007)</td>
</tr>
<tr>
<td><em>Amblyomma hebraeum</em></td>
<td>Amblin</td>
<td>Thrombin inhibitor</td>
<td>(Lai et al., 2004)</td>
</tr>
<tr>
<td><em>Ornithodoros savignyi</em></td>
<td>Savignyrin</td>
<td>Platelet aggregation inhibitor</td>
<td>(Mans et al., 2002)</td>
</tr>
<tr>
<td>Species</td>
<td>Saliva Component</td>
<td>Function</td>
<td>Reference</td>
</tr>
<tr>
<td>-------------------------</td>
<td>------------------</td>
<td>------------------------------------------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td><em>Ornithodoros savignyi</em></td>
<td>Savignin</td>
<td>Thrombin inhibitor</td>
<td>(Mans et al., 2002)</td>
</tr>
<tr>
<td><em>Ixodes scapularis</em></td>
<td>Ixolaris</td>
<td>Antithrombotic and Tissue factor pathway inhibitor</td>
<td>(Monteiro et al., 2005; Nazareth et al., 2006)</td>
</tr>
<tr>
<td><em>Amblyomma americanum</em></td>
<td>Americanin</td>
<td>Thrombin inhibitor</td>
<td>(Zhu et al., 1997)</td>
</tr>
<tr>
<td><em>Ixodes scapularis</em></td>
<td>Penthalaris</td>
<td>Tissue factor pathway inhibitor</td>
<td>(Francischetti et al., 2004)</td>
</tr>
<tr>
<td><em>Ixodes scapularis</em></td>
<td>Salp14 and Salp9ac</td>
<td>fXa inhibitor</td>
<td>(Narasimhan et al., 2002)</td>
</tr>
<tr>
<td><em>Ornithodoros moubata</em></td>
<td>Serine protease inhibitor</td>
<td>fXa inhibitor</td>
<td>(Waxman et al., 1990)</td>
</tr>
<tr>
<td><em>Ixodes scapularis</em></td>
<td>Metalloprotease</td>
<td>Fibrinolytic activity</td>
<td>(Francischetti et al., 2003)</td>
</tr>
<tr>
<td><em>Ixodes scapularis</em></td>
<td>Saliva/Troponin I-like</td>
<td>Inhibitor of endothelial proliferation</td>
<td>(Francischetti et al., 2005; Fukumoto et al., 2006)</td>
</tr>
</tbody>
</table>

**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 E2 (PGE2) 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 development 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 africæ* 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
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 depends 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
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,
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 (Tujetbajeva 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 elf4a3 (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/gene families/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.](image)

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)
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
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 to 10 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.

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank ID</th>
<th>Forward Primer (5’-3’)</th>
<th>Reverse Primer (5’-3’)</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GyrB</td>
<td>AM159536</td>
<td>CTTGAGAGCAGAACACCTA</td>
<td>CAAGCTCTGCGAAATATCTT</td>
<td>146</td>
</tr>
<tr>
<td>rOmpB</td>
<td>AF123717</td>
<td>CAAATGTTGCAGTTCTCTAAATG</td>
<td>AAAACAAAACCGTTAAAACCCG</td>
<td>96</td>
</tr>
<tr>
<td>16S rRNA</td>
<td></td>
<td>AGAGTTTGATCCTGGCTCAG</td>
<td>CATGCTGCCTCCGTAGGAGT</td>
<td></td>
</tr>
<tr>
<td>FRAN 16S rRNA</td>
<td></td>
<td>CAACATTCTGGACCGAT</td>
<td>TCGGGGACTTAAACACAT</td>
<td>373</td>
</tr>
<tr>
<td>GAPDH</td>
<td>JO842341</td>
<td>CAACCATACCAAAACATGGGTGCAT</td>
<td>TTCAGGAAATGAGCCTGCCAGC</td>
<td>175</td>
</tr>
</tbody>
</table>

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 \textit{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 \textit{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 \textit{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 \textit{A. maculatum GAPDH} gene. As with the other qRT-PCR reactions, all the samples were run in triplicate.

\textbf{2.3.7 Quantification of the \textit{Candidatus Midichloria Mitochondrii} (CMM) in Tick Tissues}

We followed the protocol described previously for quantification of \textit{Candidatus Midichloria} mitochondrii (Sassera et al., 2006). CMM specific \textit{GyrB} gene and tick \textit{GAPDH} were amplified from tick \textit{A. maculatum} using tick primers
The amplified PCR products were serially diluted 10-fold ($10^6$ 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/\textit{GAPDH} in Rp-ticks \((t=10.31, \ df=8, \ p<0.0001)\), whereas in salivary glands it decreased from 739 to 267 copies/tick \textit{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 \((t=0.43, \ df=6, \ p=0.068)\) (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).

\textbf{2.4.5 \textit{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 *Wigglesworthia* symbionts leads to higher colonization of *Trypanosome* parasites (Pais et al., 2008). But, *Glossina* itself help proliferating *Wigglesworthia* 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-
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<sup>(sec)</sup> 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 (*SELENO*M) 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
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.

<table>
<thead>
<tr>
<th>mRNA ID</th>
<th>Forward (5’-3’)</th>
<th>Reverse (5’-3’)</th>
<th>size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SELENO P or SelP</strong></td>
<td>GGA GGC TCT TAC ACA TGT TCT C</td>
<td>CAC GTT CGA AGC TCC AGT ATC</td>
<td>85</td>
</tr>
<tr>
<td><strong>SELENO P or SelP</strong> (dsRNA)</td>
<td>CCTGCAGTTCTA GTGGTCA</td>
<td>AAGCTCCAGTATC ATGCAGGG</td>
<td>403</td>
</tr>
<tr>
<td><strong>SBP2</strong></td>
<td>CTA CTG CGA CCA TGT CCT AAC</td>
<td>GTC CTT CTG GTA AAG TCG ATC C</td>
<td>97</td>
</tr>
<tr>
<td><strong>SBP2</strong> (dsRNA)</td>
<td>ATGACCGTGAAGA CGTTGCT</td>
<td>GTCCTCAGAGTCG GACTCCT</td>
<td>567</td>
</tr>
<tr>
<td><strong>SEPHS2 or SPS</strong></td>
<td>GCA AAC TGA CGG AGA AGG A</td>
<td>TGC CAC AGC CAC CAA TAA</td>
<td>141</td>
</tr>
</tbody>
</table>
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.
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.,

58
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
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) residues 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.
Note: The evolutionary history was inferred by Maximum likelihood method based on JTT matrix-based model (Jones et al., 1992) in MEGA6 software (Tamura et al., 2013).

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
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

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

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 (SEP2S), 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 \textit{SBP2} drastically reduced \textit{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 \textit{SBP2} silencing suggested the critical role of \textit{SBP2} in the synthesis of selenoproteins. The reduced selenoprotein synthesis machinery probably increased the activity of selenophosphate synthetase (\textit{SEPHS2}) for increased recycling of selenium in tick hemolymph (Fig 3.6). An important selenoprotein, \textit{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 \textit{SELENOK} and \textit{TrxR (TXNRD2)} were reduced or remain unchanged but Selenoprotein N (\textit{SELENON}), Selenoprotein T (\textit{SELENOT}) and \textit{MSRB1 (SelX)} were significantly downregulated with \textit{SBP2} silencing (Fig 3.6). The \textit{MSRB1} or \textit{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 (\textit{SELENON}) function in ticks was not known yet but in human \textit{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 (\textit{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.,
The tick superoxide dismutase \((Cu/Zn\text{-SOD})\) 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 \(Cu/Zn\text{-SOD}\) but depletion of \(Catalase\) showed superoxides 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 \(Cu/Zn\text{-SOD}\) 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
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.
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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 EXPIRATION DATE: September 30, 2015

Jodie M. Jawor, Ph.D.
IACUC Chair

Inclusions – see ‘Procedures’

19 February 2013
Date
APPENDIX B - IACUC Approval Letter

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 Salolome
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 EXPIRATION DATE: September 30, 2018

Date: 10/01/15

Frank Moore, PhD
IACUC Chair
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