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Discovery and Molecular Characterization of Selenoprotein M in the Salivary Glands of *Amblyomma maculatum*, the Gulf Coast Tick

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DISCOVERY AND MOLECULAR CHARACTERIZATION OF SELENOPROTEIN M IN THE SALIVARY GLANDS OF *AMBLYOMMA MACULATUM*, THE GULF COAST TICK

by

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ABSTRACT

DISCOVERY AND MOLECULAR CHARACTERIZATION OF SELENOPROTEIN M IN THE SALIVARY GLANDS OF *AMBLYOMMA MACULATUM*, THE GULF COAST TICK

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The Gulfcoast ticks transmit disease-causing pathogens to humans and animals. *Rickettsia parkeri* is notable among the pathogens transmitted by *A. maculatum* to humans. Heavy infestations of *A. maculatum* on animal ears cause them to become thickened and curled, a condition commonly called "gotch ear." The tick's multifunctional salivary glands are vital to their biological success and likely also play a critical role in transmission of disease; tick saliva contains a broad array of secretory products that facilitate prolonged tick attachment and feeding; disrupting tick blood feeding or inactivating key tick salivary proteins presents a novel strategy for tick-borne disease prevention. Sequencing of *A. maculatum* salivary gland normalized cDNA library revealed a gene sequence homologous to SelenoproteinM. Trace element Selenium exhibits a variety of functions in the form of Selenoproteins, most importantly, as an antioxidant enzyme. SelenoproteinM is expressed in *A. maculatum* salivary glands in almost all the feeding phases. RNA interference (RNAi) was used to assess the role of this molecule for tick feeding success. Silencing of was demonstrated by reduced transcript in salivary glands removed from partially fed ticks. Disrupting expression of SelenoproteinM by RNAi induced rapid weight gain in engorging female ticks in early

phase of feeding. Since many Selenoproteins are involved in antioxidant activities, we further evaluated the antioxidant capacity of tick tissues treated with SelM-dsRNA. There was a significant reduction in the antioxidant capacity in SelenoproteinM silenced tick tissues.

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CHAPTER I

INTRODUCTION AND BACKGROUND

Tick and Tick Borne Diseases

Ticks (class Arachnida, subclass Acari, suborder Ixodida) are external blood feeding parasites and are capable of transmitting pathogens such as viruses, bacteria and protozoa. Ticks have unique and carefully engineered mouthparts to serve the purpose of attachment and feeding consisting of paired anchoring organs or rostrum, a pair of sharp mandibles that move back and forth on the rostrum. A tick secures its attachment to the host by inserting its chelicerae (cutting mandibles) and hypostome (feeding tube) into the skin. Their bites not only transmit diseases but may cause a severe allergic reaction, various types of toxicosis and tick paralysis. Wounds caused by ticks can be painful and may lead to a secondary infection. A total of 870 species of ticks have been documented so far throughout the world (Wheller and Hayashi, 1998) and have been characterized into two main families: Ixodidae (Hard Ticks) with 683 species and Argasidae (Soft Tick) are restricted to 183 species. In adult argasidae, scutum is absent instead there is a presence of leathery or wrinkled integument; their mouth parts are barely visible. Ixodidae ticks, on other hand, have a shell like covering or scutum on entire dorsal surface of male, which is reduced to the small portion behind capitulum in females thus permitting expansion of female body during blood feeding (AFPMB, 2006).

The Life cycle of a hard tick typically has four developmental stages: egg, the 6-legged larva (seed ticks), 8-legged nymph and an adult (male or female). Larvae and nymphs molt into the next stage by shedding their cuticle after ample blood meal from an appropriate vertebrate host. In case of adults, the fed male tick dies after mating (which

generally occurs on the host). Female ticks engorge themselves by expanding the exoskeleton to repletion and lay eggs to complete the life cycle. In a behaviour known as questing, ticks seek hosts by climbing grass, weeds or bushes waiting to grasp and attach to a suitable host (Okulova, 1978). As the suitable host arrives tick raises its body to an angle, attaches to the host and inserts its mouth parts in the skin epidermis to make a firm grip (Kemp et al., 1982; Sonenshine, 1991). Although hard ticks, take one blood meal per stage, soft ticks can feed several times on the same host. The characteristic comparison between both the families is listed below (see Table 1).

Table 1

Comparison of Two Major Tick Families, Ixodidae and Argasidae (Modified From-Parola, P., Raoult, D., Clin Infect Dis. 2001. 32, 897-928).

Characteristics	Ixodidae (hard ticks)	Argasidae (soft ticks)	References
Capitulum	Anterior, Visible from above	Ventral, not visible from above	Parola and Raoult (2001)
Scutum	Present	Absent	Sonenshine (1991), Hillyard (1996)
Nymphal stages	1	Several	Sonenshine (1991), Hillyard (1996)
Feeding	Several days, only once	Intermittent feeding: (min. to few hours) several times.	Sonenshine (1991), Hillyard (1996)

Table 1 (continued).

Characteristics	Ixodidae (hard ticks)	Argasidae (soft ticks)	References
Weight gain female	High (up to 100 times original unfed weight)	Low (up to 12 times original unfed weight)	Sonenshine (1991),Hillyard (1996
Egg laying events	One	400-500	Parola and Raoult (2001)
Host	1-3 host	Several host	Sonenshine (1991, 1993), Spickett (1996)
Life span	Several months to three years	Long life span up to 10 years	Sonenshine (1991, 1993)

Mouth parts of hard ticks vary in length, *Dermacentor*, *Hemophysalis*, *Rhipicephalus* tick mouth parts they extend to the interface of the dermis and epidermis, but extends well into while in case of *Amblyomma* and *Ixodes* they extend well into the dermis (Kemp et al., 1982; Moorhouse, 1969). Based on their life history and feeding habits, hard ticks are generally classified into "one-host," "two-host" or "three-host" ticks. A one-host tick remains on a single host for feeding and molting and ultimately engorges into adult e.g., *Rhipicephalus annulatus*. In case of two- host tick, such as *Hyalomma marginatum* fed larval ticks molts into nymphs, which then feed on the same host and drops off. After molting into an adult tick, this tick and engorges on a second host. In three -host ticks, ticks at each three life stages feeds on three separate hosts e.g., *Ixodes scapularis*, *Amblyomma maculatum* etc. (AFPMB, 2006).

Ticks were recognized as human and animal parasites for ages but the first clues demonstrating their ability to transmit infectious diseases were made at the beginning of

19th century (Smith and Kilbourne, 1893). Ticks have a wide host range and can feed on multiple hosts, providing them with ample opportunity to acquire and transmit diseases, In addition to this ticks are able to survive without feeding for long intervals of time, They have high reproductive potential, a female tick can lay as much as 60,0000 eggs at a time, All the three life cycle stages feed on blood and are capable carriers of diseases (AFPMB, 2006). Ticks can transmit diseases by transovarial transmission i.e., from infected female to eggs and then to future offspring or by transstadial transmission i.e., passed from one life stage to another during molting. Ticks may transmit more than one pathogen to the host in a single bite (Swanson et al., 2006). They transmit more human diseases than any other arthropod, transmitting a wide variety of pathogens among them are bacteria, Rickettsiae, viruses, spirochetes and protozoan (Parola and Raoult, 2001). The main diseases transmitted by ticks are listed in the below (see Table 2).

Table 2

Common Tick and Tick Borne Diseases (Modified From-Center for Disease Control, 2004)

Tick	Tick-associated Disease(s)	Pathogen associated	Geographic Distribution in U.S.	References
Amblyomma americanum (Lone star tick)	Ehrlichiosis, tularemia, Rocky Mountain spotted fever, tick paralysis	*Ehrlichia chaffeensis, Rickettsia Rickettsii, Ehrlichia ewingii, Francisella tularensis*	Southeast, Midsouth and coastal Northeast	Goddard (2009)
Amblyomma maculatum (Gulf Coast tick)	Heartwater disease, American boutoneusse Fever	*Ehrlichia ruminantium, Rickettsia parkeri*	Gulf Coast to Midsouth and lower Midwest	Uilenberg(1982), Paddock et al.(2004)
Dermacentor variabilis (American dog tick)	Ehrlichiosis, tularemia, Rocky Mountain spotted fever, Q-fever, tick paralysis	*Ehrlichia chaffeensis, Rickettsia Rickettsii, Ehrlichia ewingii, Francisella tularensis, Coxiella burnetii.*	Eastern 2/3 of U.S.; West Coast	Holden et al.(2003)

Table 2 (continued).

Tick	Tick-associated Disease(s)	Pathogen associated	Geographic Distribution in U.S.	References
Dermacentor andersoni (Rocky Mountain wood tick)	Rocky Mountain spotted fever, tularemia, Colarado tick fever, tick paralysis	*Rickettsia Rickettsii, Francisella tularensis, Colorado tick fever virus*	Northwest; Northern Rocky Mountain states, Arizona, New Mexico.	Piesman and Gage (1996) CCHDOE (2001)
Ixodes scapularis (Eastern black-legged tick (deer tick)	Lyme disease, babesiosis, anaplasmosis, tick paralysis	*Borrelia burgdorferi, Babesia microti, Anaplasma phagocytophilum*	States east of and adjacent to Mississippi River; Eastern TX and OK	CDC (1995, 1997), Steere (2001)
Rhipicephalus sanguineus (brown dog tick)	Ehrlichiosis, Rocky Mountain spotted fever, babesiosis, anaplasmosis, hepatozoonosis, haemobartonellosis	*Ehrlichia chaffeensis, Rickettsia Rickettsii, Babesia microti, Anaplasma phagocytophilum*	Throughout U.S	Lord (2001)

Among other diseases is tick paralysis, caused by salivary factors (Edlow and McGillicuddy, 2008). Tick inoculates these toxins into the host upon prolonged attachment which may lead to weakness in the legs and respiratory failure and death (Felz et al., 2000; CDC, 2006). Tick bites may also lead to the production of secondary infection, redness, mild swelling, blister formation, hair loss and in severe cases, necrotic ulcers (McGinley and Smith, 2003).

Amblyomma Species: A. maculatum and A. tuberculatum

Amblyomma is the third largest genus in hard tick family Ixodidae, so far about 130 species of the genus have been identified (Kolonin, 2009); almost half of these occur in the American continent. Several Amblyomma species are crucial from the stand point of public health, because they serve as important vectors for various disease agents' such as Rocky Mountain spotted fever, ehrlichiosis and anaplasmosis (Kolonin, 2009). Adult Amblyomma range from 4-8 mm in size and are often characterized with highly ornate scutum and long

mouth parts and are well established in North America (Keirans and Durden, 1998). My research work focuses around two Amblyomma species: *Amblyomma maculatum*, the gulf coast tick and *Amblyomma tuberculatum*, the gopher tortoise tick.

Amblyomma maculatum, the gulf coast tick was originally known to be distributed across the atlantic to the gulf coast (Bishop and Hixson, 1936). The range of this tick has h expanded, most likely due to transportation of infested livestock (Goddard and Norment, 1983) and migratory birds like cattle ergets, *Bubulcus ibis* (USDA, 2008; Karim et al., 2011 unpublished data). *A. maculatum* has a wide host range; adults are common on large animals both domestic and wild such as sheep's, horses, mules and cattle's (Cooley and Kohls, 1944) deer, feral swine (Strickland et al., 1976; Greiner et al., 1984). Larval and nymphal ticks generally infest grounds inhabiting birds and small mammals (Bishopp and Trembley, 1945). Adult male *A. maculatum* are approximately 4 mm in length, having brown colored shield with silvery white lines that are more or less connected with few isolated white spots. Adult males have a dorsum twice as long as broad with prominent punctures. Their capitulum is long and palpi short. Legs are brownish and heavy. Female AM ticks have 2 mm long shield which is silvery white behind and brown in front rest of the abdomen is dark without hairs. Shield is pentagonal is shape and its lateral lobes are strongly punctured. Legs and capitulum are long, stigma plate is longer than males (Axtell and LeFurgey, 1979) (see Figure 1).

The Gulf Coast tick has great economic and public health importance as it serves as competent vector for various pathogenic agents; it's a confirmed experimental vector of *Ehrlichia ruminantium*, the agent of heart water disease in cattle (Uilenberg, 1982). This disease is prevalent in Africa and causes heavy economic losses, the mortality in infected

cattles is as high as 90% (Uilenberg, 1990). With globalization and the increase in international trade, heartwater presents itself as a significant threat to the livestock and wildlife of United States. U.S. Department of Agriculture estimated that outbreak of *Ehrlichia ruminantium* in the United States may lead to annual losses of $762 million to the livestock industry.

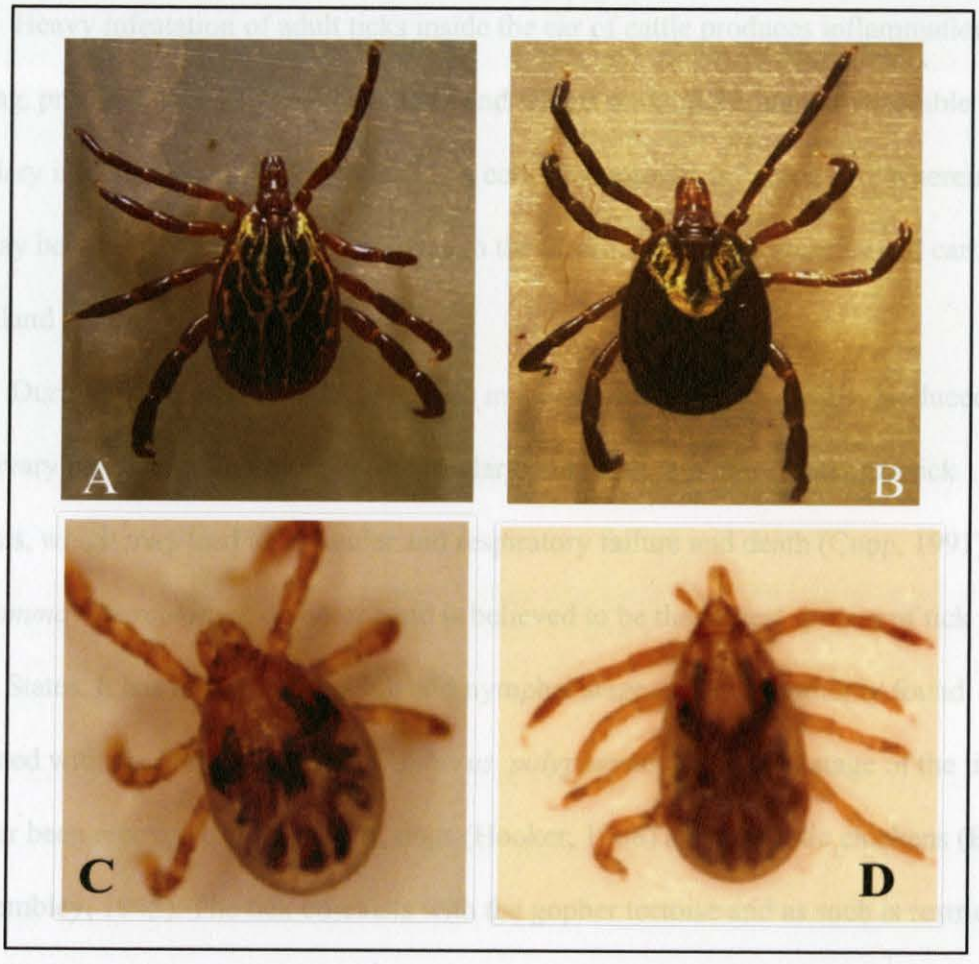

Figure 1. Amblyomma maculatum ticks, various life stages. A: Adult male *A. maculatum* tick; B: Adult female *A. maculatum* tick; C: *A. maculatum* larva; D: *A. maculatum* nymph

The Gulf Coast tick is now recognized as the primary vector of *Rickettsia parkeri,* a spotted fever group rickettsia (Paddock et al., 2004). *R. parkeri* was first identified in the Gulf Coast tick in 1937 (Parker et al., 1939) the first confirmed human case reported in

2004 (Paddock et al., 2004) and there has been increase in the number of cases (Finley et al., 2006; Whitman et al., 2007). *R. parkeri* infection presents clinically 2-10 days after tick bite; these symptoms may differ from RMSF in form of an eschar that resembles a sore or pimple at the site of infection. Other symptoms include fever, fatigue, headache, muscle pain, and generalized rash (Sumner et al. 2007; Paddock et al., 2004).

Heavy infestation of adult ticks inside the ear of cattle produces inflammation, swelling, production of yellowish exudates and edema making the animal venerable for secondary infection and it may also lead to a condition known as "gotch ear" wherein the ears may become curled and deformed due to the destruction of ear muscles and cartilage (Strickland et al., 1976).

During blood feeding, AM ticks may inoculate the host with a toxin produced from the salivary glands, leading to a neuromuscular condition commonly known as tick paralysis, which may lead to muscular and respiratory failure and death (Cupp, 1991). *Amblyomma tuberculatum*, on other hand is believed to be the largest species of tick in United States. It has host-specific adult and nymphal stages and is commonly found associated with the Gopher tortoise, *Gopherus polyphemus*. The larval stage of the tick has however been recovered from rabbits, dogs (Hooker, 1908) and domestic chickens (Bishop and Trembley, 1945). The tick co-exists with the gopher tortoise and as such is restricted to parts of Florida, Alabama, South Carolina, Mississippi and Georgia.

Figure 2. *Amblyomma tuberculatum*, the Gopher tortoise tick. A: Picture of an adult male *A. tuberculatum* tick. B: Engorged *A. tuberculatum* Female. C: Adult *A. tuberculatum* Tick attached to the shell of Gopher Tortoise. D: Adult *A. tuberculatum* attached to the limbs of Gopher tortoise.

Since July 7, 1987, *Gopherus polyphemus* has been listed as "Threatened" across its range, the main cause being habitat destruction, predation and diseases, the tortoise is quite susceptible to upper respiratory tract disease (URTD) caused by *Mycoplasma* sp (Blake, 2007). Because of the threatened status of the Gopher tortoise, there is growing interest in their diseases. Ticks may play an important role in determining the status of diseases in the tortoise serving as their vector. They may also help the disease to persist in the host by compromising the host defense system as long as they feed.

Importance of tick Salivary Glands

The biological success of ticks depends upon the multifunctional, morphologically complex salivary glands (Sauer et al., 1995). The general organization of salivary gland is similar in most Ixodid ticks. All hard ticks generally have a pair of large grape- like salivary glands that are connected to the mouth parts. There are three types of acini in salivary agranular glands: acini I and granular acini II and III (Binnington and Stone, 1981).

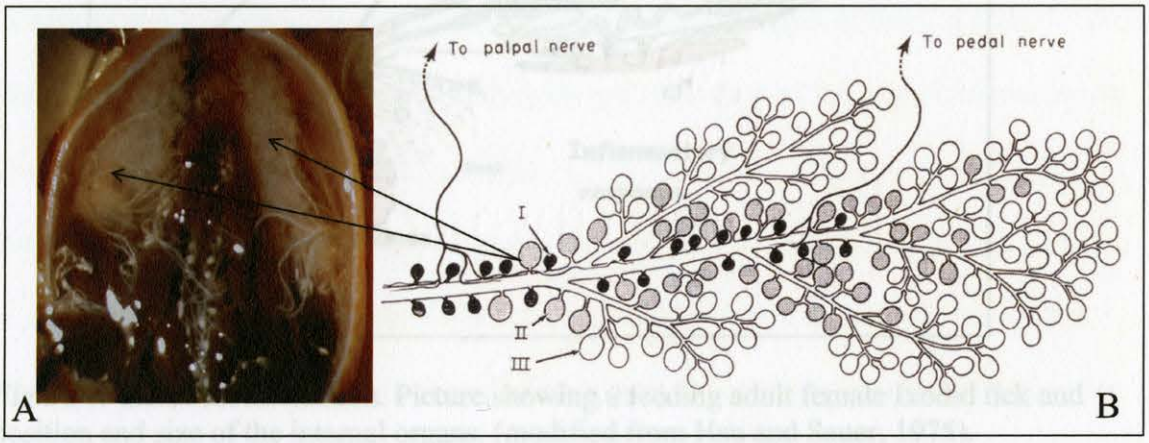

Figure 3. A dissected adult *A. maculatum* tick, A: Showing pairs of salivary gland in white and B: Internal morphology of salivary glands showing various types of Acini (modified from Binnington, K. C., 1978. Int. J. Parasitology. 8, 97-115).

The type-I acini are directly attached to the main duct, while the remaining two acini containing the secretory granules are distributed across the branches of intralobular ducts. Type-III acini are the most prevalent. Type-I acini are osmoregulatory in function they help the tick to remain hydrated during months between the feeding cycles (McMullen et al., 1976; Rudolph and Knulle 1974, 1978) and may also play an important role in feeding, though they don't undergo substantial change in the size number or appearance of cells (Baker et al., 1984). Type-II and III acini have various granular cells; they may be involved in fluid and electrolyte transport and salivary secretion during the fast feeding

phase of the tick. They may also be involved in producing a variety of cement like secretory proteins for synthesizing the attachment cone, anticoagulants, immuno-modulatory compounds and vasodilators to maintain blood flow to the attachment site. (see Figure 4).

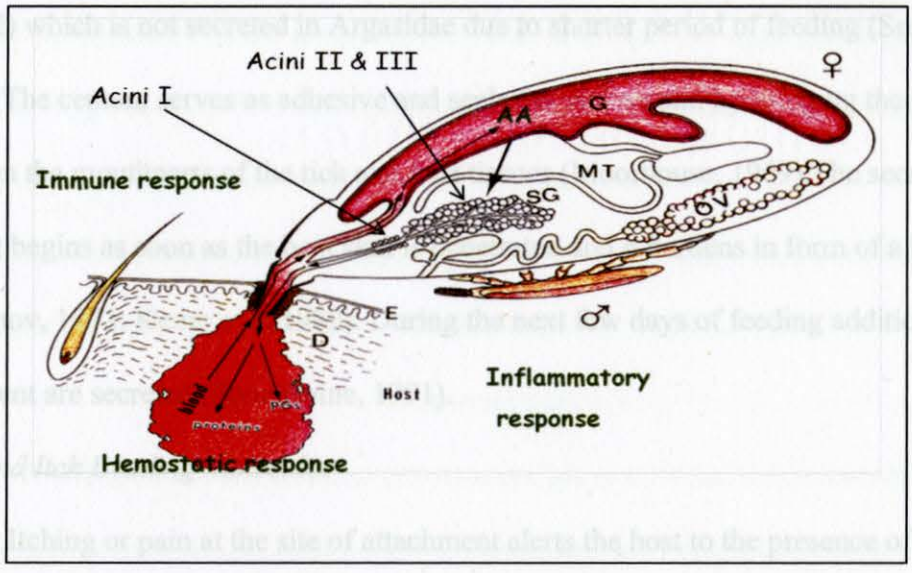

Figure 4. Tick-host interaction. Picture showing a feeding adult female Ixodid tick and position and size of the internal organs. (modified from Hsu and Sauer, 1975).

Hard ticks must feed on the host for extended period of time, thereby providing the host immune system with sufficient time to mount a hemostatic response at the feeding site (Ribeiro et al., 1985). Salivary glands of ticks are miniscular labs. In non-feeding ticks these glands produce hygroscopic saliva that help absorb water vapor from the atmosphere and keep the tick hydrated (Bowman and Sauer, 2004). Blood feeding-ticks have to face various challenges as part of host defense, to overcome this innate and acquired immune response, ticks secrete saliva which contain a cocktail of biologically active molecules acting as immune suppressors and modulators preventing hemostasis, inflammation, clotting\, vasoconstriction, even pain and itching (Ribeiro and Francischetti, 2003). A

detailed description of various salivary compounds secreted by ticks during attachment and feeding is as follows:

Cement

Attachment cement is secreted by the salivary glands of Ixodid ticks (Kemp et al.1982) which is not secreted in Argasidae due to shorter period of feeding (Sonenshine, 1991). The cement serves as adhesive and seals the bite wound by bridging the gap between the mouthparts of the tick and host tissues (Moorhouse, 1969).The secretion of the cement begins as soon as the host skin is penetrated and it hardens in form of a tube or cone (Balashov, 1972; Kemp et al.1982). During the next few days of feeding additional layers of cement are secreted (Sonenshine, 1991).

Pain and Itch Evading Molecules

Itching or pain at the site of attachment alerts the host to the presence of the tick resulting in a response to remove the tick. Bradykinin are an important class of molecules that mediate pain (Clark, 1979) and itch (Alexander, 1986). Serine protease inhibitors produced in Ixodid tick saliva are effective inhibitors of Bradykinin action (Tanaka, 1999). One carboxypeptidase in saliva degrades active bradykinin. Histamine and serotonin binding proteins counteracts itch sensation (Alexander, 1986) and innate and acquired immune response (Paine et al., 1983), these proteins possesses affinity for histamine and serotonin thus rendering them ineffective (Paesen et al., 1999).

Tick Anticoagulants and Inhibitors of Platelet Aggregation

For blood-feeding ectoparasites, blood coagulation is a major obstacle in prolonged feeding. Ticks secrete various inhibitors of factor Xa and thrombin (Francischetti et al., 2002) that prevent the conversion of fibrinogen into fibrin and thereby block coagulation

(Mann, 1999). Platelet aggregation in soft ticks is inhibited by apyrase activity, in hard ticks metalloproteases serve the purpose by degrading extracellular matrix (Ravanti and Kahari 2000).

Other Important Biomolecules

Prostaglandins E2 act as vasodilators and inhibit platelet aggregation (Dickinson et al., 1976; Higgs et al., 1976); they help the engorging ticks by increasing blood flow to the feeding site. Calreticulins are highly conserved molecules that are known to be involved in interfering with complement activation, regulating adhesion and works as molecular chaperones (Coppolino and Dedhar, 1998; Kovacs et al., 1998).

RNA Interference

Gene regulation is a complex process, and plays a crucial role in maintaining the identity and integrity of cell during developmental and adult stage. The discovery of RNA mediated gene regulation has opened new avenues for identifying of gene function and producing knockdown mutants. RNA interference or small RNA mediated gene silencing has become a powerful tool in molecular biology since its discovery in 1998 where sequence-specific gene silencing was observed in the nematode *Caenorhabditis elegans* (Fire et al., 1998). Remarkably, this effect was determined to be systemic and heritable. Fire et al., performed RNAi by injecting dsRNA into the gonads and body cavity of the worm and examining the phenotypic changes in the next generation. They subsequently determined there were other ways to introduce dsRNA such feeding the worms with bacteria expressing the dsRNA (Timmons and Fire, 1998) and soaking the worms in the solution of dsRNA (Tabara et al., 1998). RNA mediated gene silencing has thus been

frequently used to analyze the functional aspect of genes in various plant and animal species (Kennerdell and Carthew, 1998; Cogoni and Macino, 2000; Nakano et al., 2000).

RNA mediated gene regulation is triggered by small non-coding RNA molecules that initiate suppression or down regulation of target/unwanted mRNA and also degrades aberrant RNA (viral origin, transgene etc.). Micro RNA (miRNAs) (Bartel, 2004) and small interfering RNA (siRNAs) (Hamilton and Baulcombe, 1999) are two well-known classes of small RNA that are involved in gene regulation.

MicroRNAs

MicroRNAs are evolutionary conserved endogenous RNA molecules averaging 19-23 nucleotides long and are found in most eukaryotic cells (plants and animals). They were recognized as gene regulators in the early 2000's (Bartel, 2009). Most miRNA are synthesized in the nucleus from intergenic regions that are in the antisense orientation. They contain their own promoters and regulatory units. (Lau et al., 2001; Lee et al., 2004; Lagos et al., 2001; Lee and Ambros, 2001). They are transcribed by RNA Polymerase II into ~ 1000 nucleotide long pri-miRNA that are single-stranded and have a characteristic hair-pin structure, pri-miRNA are processed into pre-miRNA (70-100) nucleotide long by specific RNase III endonuclease "Drosha" with help of RNA- binding protein "Pasha."

Theses pre-miRNAs are then transported into cytoplasm via Exportin-5. Once in the cytoplasm, pre-miRNAs are cleaved by RNase III "Dicer" and are converted into ds miRNAs 19-23 nucleotide long (Lee et al., 2004; Cai et al., 2004). Once the guide strand of the ds miRNA is recognized, it is assembled into a ribonucleoprotein (RNP) complex. RISC (RNA induced silencing complex) other strand is degraded. RISC is a ribonucleoprotein complex that has an active component in form of endonuclease

Argonaute protein. The guide strand directs RISC to complementary mRNA binds to this mRNA and is degraded by Argonaute. In animal, miRNAs bind to multiple, partially complementary sites. The fate of the target mRNA and degree of gene expression is determined by the extent of base pairing to miRNA. The mRNA can either be translationally repressed (partial) or cleaved (degraded completely) (Schwarz and Zamore, 2002; Krol et al., 2004; Khvorova et al., 2003; Schwarz et al., 2003; Lin et al., 2005; Okamura et al., 2008).

Gene Regulation effect: In plants the miRNA must have near perfect homology to their target mRNA in order for gene suppression, the miRNA-containing RISCs function as endonucleases, causing the degradation of mRNA (Rhoades et al., 2002). In animals the complementarity of miRNA and its target mRNA is usually restricted to the 3′ region of the target site i.e. (nucleotides 2-8) of the miRNA (Lewis et al., 2003; Lai, 2004; Brennecke et al., 2005; Lewis et al., 2005). As a result, binding of the miRISC to the target mRNA blocks its translation into protein, rather than catalyzing its cleavage (Olsen and Ambros, 1999). In plants and yeast miRNAs are believed to be involved in repression of transcription by processing chromatin methylation.

Small Interfering RNAs

siRNA are dsRNA molecules 21-23 nucleotides in length, siRNA can be endogenous or exogenous in origin. Endogenous siRNA are produced *in vivo* from perfectly base paired dsRNA molecules derived from two complementary RNA strands. It can also originate from repetitive elements within genome (rasiRNA). Exogenous siRNAs are produced from dsRNA introduced into the cell by lab manipulation or viral infection. In the cytoplasm, these ds RNA are processed into a 21-23 nucleotide long ds siRNA

containing 2-nucleotide 3' overhangs with 5' phosphate and 3' hydroxyl termini at each end by member of RNase III family of ribonuclease protein "Dicer." These ds siRNA are separated into ss siRNA and the guide strand is incorporated into the RISC complex (other strand is degraded). The RISC complex base pairs with region of complementary mRNA, inducing its degradation thus preventing translation (Bernstein et al., 2001; Siomi et al., 2009; Zamore et al., 2000; Vermeulen et al., 2005; Castanotto et al., 2009; Qiu et al., 2005; Ahlquist, 2002).

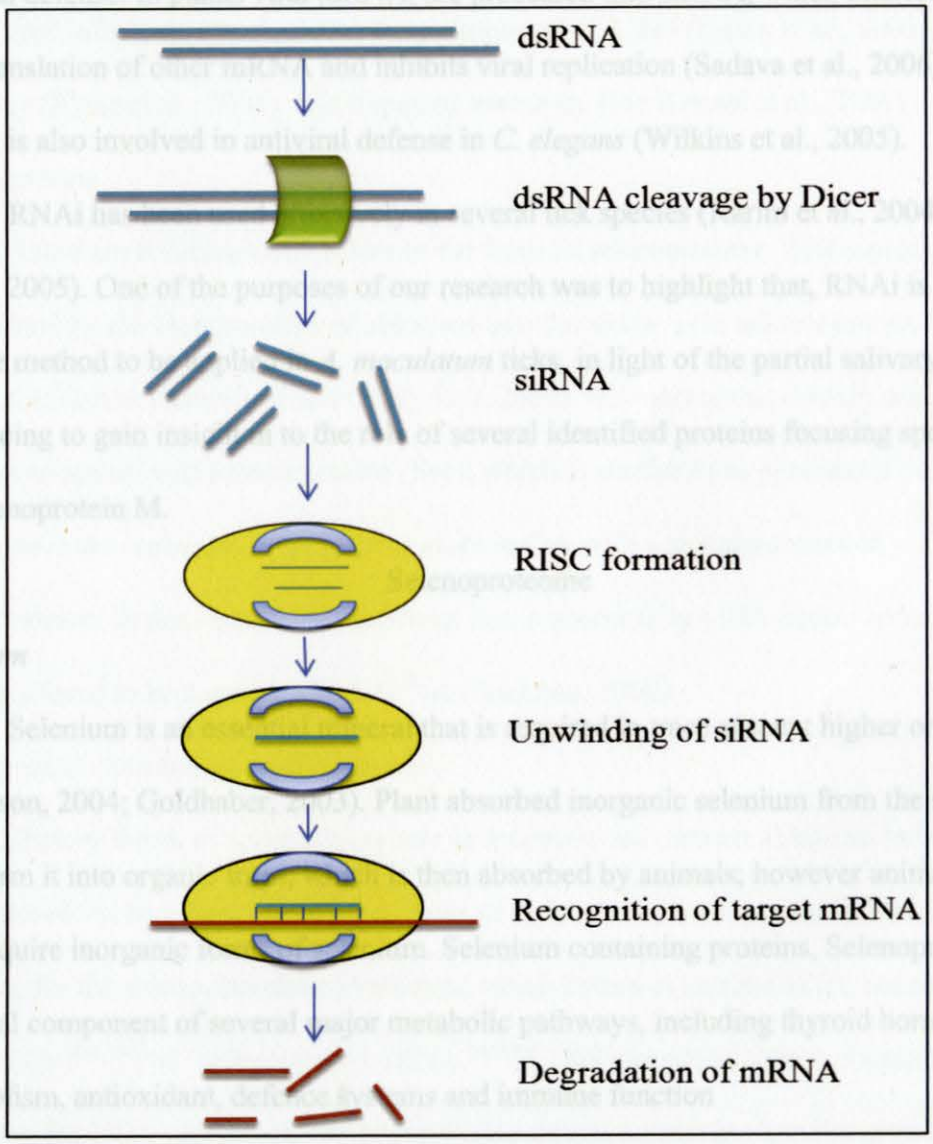

Figure 5. Mechanism of RNA interference.

siRNAs have become an important tool in gene knockdown studies, siRNA of exogenous or endogenously origin could be incorporated into RISC to induce sequence-specific degradation of target RNAs. This has developed a powerful tool to "knock-down" gene expression by introducing synthetic dsRNA to the medium, adding viral vectors that express siRNAs into cells or generating transgenic animals that synthesize siRNAs. Evidence supports the fact that in certain plant and animal species, siRNA is involved in antiviral defense. In plants viral ssRNA, are processed into siRNA, which interferes with translation of other mRNA and inhibits viral replication (Sadava et al., 2006). siRNA is also involved in antiviral defense in *C. elegans* (Wilkins et al., 2005).

RNAi has been used effectively in several tick species (Karim et al., 2004, 2004a, 2004b, 2005). One of the purposes of our research was to highlight that, RNAi is easy and reliable method to be applied in *A. maculatum* ticks, in light of the partial salivary gland sequencing to gain insight in to the role of several identified proteins focusing specifically on Selenoprotein M.

Selenoproteome

Selenium

Selenium is an essential mineral that is required in trace amount higher organisms (Thomson, 2004; Goldhaber, 2003). Plant absorbed inorganic selenium from the soil and transform it into organic form, which is then absorbed by animals; however animals can also acquire inorganic forms of selenium. Selenium containing proteins, Selenoproteins are essential component of several major metabolic pathways, including thyroid hormone metabolism, antioxidant, defence systems and immune function

(Holben and Smith, 1999). The decline in blood selenium concentration is involved in potential public health implications, particularly in relation to the chronic disease such as cancer and cardiovascular disease (Comb and Comb, 1999). Selenium deficiency has been known to play its part in various diseases such as Keshan disease, a cardiomyopathy affecting women and children in restricted regions of China that have low selenium levels in soil, also prevalent in China is Kashin-Beck disease, a deforming osteoarthritis (Coopinger and Diamond, 2001). Selenium deficiency among humans may cause, increase risk of viral infections (Beck, 2001) the progression of AIDS (Baum et al., 2001), male infertility (Flohe et al., 2001), and impaired immunity (Mc Kenzei et al., 2001).

Selenocysteine

Selenium is biologically active in the form of selenoproteins. Selenoproteins are synthesized by the incorporation of selenium into the amino acid selenocysteine during protein translation (Hartsfield and Gladyshev, 2002). Selenoproteins contain selenium in the form of amino acid selenocysteine (Sec), which is similar to aa cysteine (Cys), the difference is the replacement of sulphur atom in Cys with a selenium atom in selenocysteine. In this cotranslational event Sec is encoded by UGA codon and as such Sec was considered to be a nonstandard 21 st aa (Stadman, 1990).

Selenoprotein Metabolism in Eukaryotes

Dietary forms of selenium organic or inorganic are converted into an intermediate form selenide to be utilized for the synthesis of Selenoproteins. Selenide is used as a substrate for the selenophosphate synthetase which in turn is utilized in the conversion of seryl tRNA $^{(ser)\,sec}$ to selenocysteyl tRNA $^{(ser)\,sec}$. Selenocysteyl tRNA contains the anticodon for UGA and directs the incorporation of selenocysteine into the growing

polypeptide chain with the help of unique SECIS elements that are located at the 3′ UTR's of mRNA. So far 25 types of selenium containing proteins (selenoproteins) have been identified in eukaryotes (Kryukov et al., 2003).

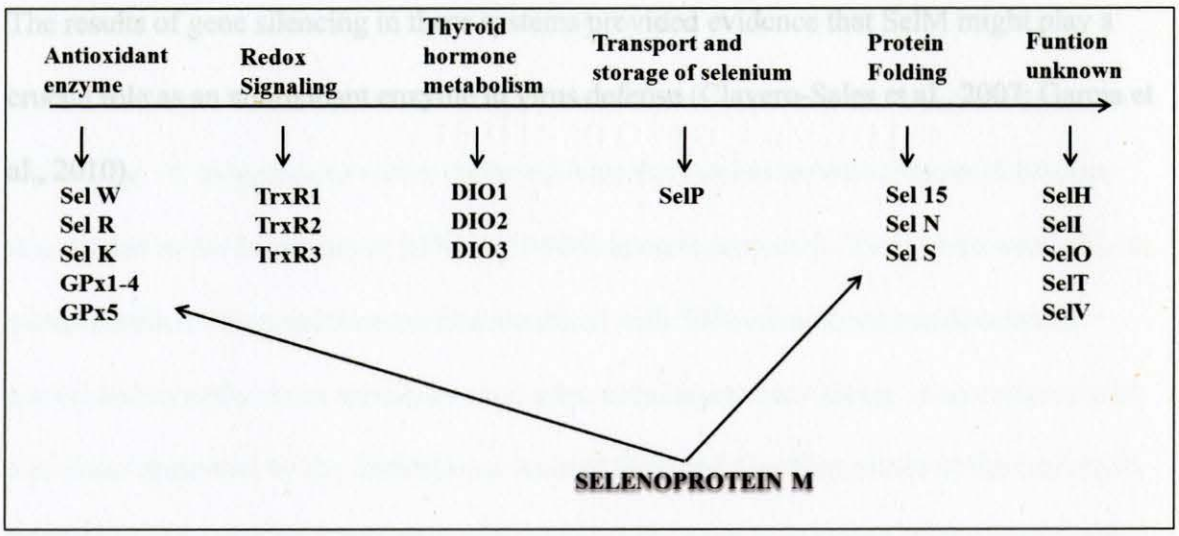

Figure 6. Role of various Selenoproteins so far identified in Eukaryotes.

A Salivary gland cDNA library from *A. maculatum* revealed the sequence of putative Selenoprotein M (SelM), this is a 105 aa sequence that was expressed in almost all the tick tissues and feeding stages. SelM is known to be involved in redox activities in eukaryotes (Ferguson et al., 2006). Hwang *et al, 2005* suggested that SelM may protect against Alzheimer's disease also suggested by him was the fact that the overexpression of the SelM protein in transgenic rats differentially regulated the activity of other antioxidant enzymes in various tissues (Hwang et al., 2008). In Drosophila SelM known to be involved in salivary gland development, survival and fertility (Martin-Romero et al., 2001; Kwon et al., 2003).

In ticks Selenoprotein expression has been identified in response to *B. burgdorferi* infection (Ribeiro et al., 2006). The IDE8 cell lines in tick were observed overexpressing SelM in response to *Anaplasma marginale* infection (de la Fuente et al., 2007). In

Dermacentor variabilis, SelM was overexpressed in midguts and salivary glands of male ticks infected with *A. marginale* (Kocan et al., 2009). Similarly in white shrimp, SelM expression was induced after infection with White spot syndrome virus (WSSV) infection. The results of gene silencing in these systems provided evidence that SelM might play a crucial role as an antioxidant enzyme in virus defense (Clavero-Sales et al., 2007; Garcia et al., 2010).

CHAPTER II

METHODS AND MATERIALS

Sample Preparation

Ticks

Pathogen-free adult female *A. maculatum* ticks were obtained from Oklahoma State University. *A. tuberculatum* were collected from the Gopher tortoise. Theses ticks were maintained in the laboratory at 23°C and >90% relative humidity. Ticks were washed with sterile distilled water and then surface sterilized with 70% ethanol prior to dissection. Larval and nymphal ticks were harvested after detachment from sheep, in accordance with a protocol approved by the Institutional Animal Care and Use Committee at the University of Southern Mississippi. Engorged larva and nymphs were maintained at 23°C and >90% relative humidity to allow them to molt into the adult stage.

Salivary Gland Dissections

Salivary glands were separated from the rest of the organs. Tick salivary glands were dissected in ice-cold 100 mM 3-(N-morpholino)-propanesulfonic acid (MOPS) buffer (pH 6.8). The dorsal cuticle was removed with a razor blade and the salivary glands (SG) were separated from the rest of the tissues (midgut (MG), reproductive system (RS) and synganglion (SG) with fine-tipped tweezer. All tissues used in gene expression studies were washed in MOPS buffer and preserved in 200 µl of RNA later (Ambion, USA) in separate RNase-free microfuge tubes (Ambion, USA). RNase free microfuge tubes were stored at −20°C until use.

Total RNA and mRNA Extraction

Total RNA was extracted from dissected tissue samples isolated from adult ticks, unfed and partially 4–5 days fed ticks, using the RNAspin Mini RNA Isolation Kit and QuickPerp Micro mRNA Purification Kit (GE Healthcare) according to the manufacturer's protocol. Briefly for total RNA extraction following were the major steps: tissues samples were lysed/ homogenized in 350 µl Lysis Solution and 3.5 µl β-Mercaptoethanol with a pestle until no tissue pieces were visible then the lysate was passed 4-5 times through a 27 gauge needle fitted to a syringe. Following the protocol 350 µl Ethanol (70%) was added to the lysate and was mixed by vortexing, the binding process involves binding negatively charged RNA to the silica membrane so that all the other contaminates are separated. This is followed by washing after centrifugation to remove the residual proteins and salt. DNaseI enzyme is applied to the RNA bound membrane and it is incubated at room temperature for 15 min to digest any residual amount of DNA. Finally total RNA was eluted in 50-100 µl RNase-free water, elution helps in separating the RNA from the silica surface with the help of elution buffer.

Similarly mRNA extraction was done following the protocol specified by the manufacturer tissues were homogenized adding 0.4 ml of Extraction Buffer. Cells were lysed with 0.8 ml of Elution Buffer using a vortex. The homogenate was transferred to Oligo(dT)-Cellulose pellet and was gently mix by inversion for 3 min manually. mRNA bound to the Oligo(dT) was washed several times with high and low salt buffers to remove contaminating proteins, nucleic acids and carbohydrates. mRNA bound to the resin bed was eluted using 70 ul of prewarmed (65°C) Elution Buffer.

The quantity of the RNA was quantified by both Nanodrop and Bioanalyzer. Concentrations were measured by pipetting 1.0 µl of the eluted RNA solution to the measurement pedestal, with UV @ 260 nm, ratio A260:A280 provided us with information regarding the purity of RNA. Samples with purity ratio between 1.8 to 2.2 were selected for further experiments. In addition to the Nanodrop Agilent Bioanalyzes was used to determine the quality of RNA. Electrodes of the analyser were cleaned with 350 µL RNAseZAP and 350 µl RNAse-free water. RNA Ladder and RNA samples (1µ in RNase-free vials) were heat denature it for 2 min at 70°C. A new RNA 6000 Nano chip was placed on the chip priming station, 9.0 µl of gel-dye mix was added to the well-marked G with help of a plunger, 5 µl of RNA 6000 Nano marker was added in all 12 sample wells and in the well-marked with ladder sign. 1 µl of prepared ladder and samples were added in each of the corresponding wells the sample chip was vortexed at 2400 rpm and the run was started.

Preparation of c DNA Library

Non-Normalized Library

Complementary DNA was synthesized from 3 µg of Poly A+RNA using the SMART cDNA library construction kit (Clontech) following the manufacturer's instructions. Briefly 3µl of mRNA sample, 1µl of SMART IV Oligonucleotide, 1µl of CDS III/3 polymerase chain reaction (PCR) Primer were incubated at 72°C for 2 min, then at 0°C for 2 min immediately. 5×First-Strand buffer 2µl, 1µl of dithiothreitol (DTT) (20 mmol /L), 1µl of dNTP mix (10 mmol/ L)and 1µl of SMARTScribe MMLV reverse transcriptase (200 U/µl) were added and then incubated at 42°C for 1 h. PCR was performed according to the manufacturer's instructions. The PCR mixture consisted of 2µl of first-strand cDNA, 10 µl of advantage 2 PCR buffer, 2µl of 50×dNTP mix, 2µl of 5'

PCR primer, 2 µl of CDS III/3'PCR primer, 2µl of 50×advantage 2 polymerase mix and 80 µl of deionized H2O. The amplification program was as follow: 95°C for 20 sec, followed by 24 cycles of 94°C for 30 sec, 68°C for 6 min, PCR products 5 µl were analysed on 1.2% agarose/ethidium bromide (EtBr) gel. The PCR products of the double stranded (ds) cDNA was purified and stored at -20°C. Double-stranded cDNA 50 µl was then treated with 2 µl of proteinase K (20 µg/µl) at 45°C for 20 min followed by phenol:chloroform:isoamyl alcohol separation it was then digested with 100 µl of Sfi I restriction enzyme at 50°C for 2 h, fractionated by CHROMA SPIN-400 column (Clontech) to collect larger than 400 bp cDNA. Approximately, 250 ng of cDNA was ligated into 100 ng of Sfi I digested pDNR-LIB vector (Clontech) and electroporated into *Escherichia coli* J DH10b cells using the Bio-RAD Gene Pulser. To calculate the titer of the library, the library was plated directly on selective medium (containing 30 µg/ml of chloramphenicol) and incubated at 37°C overnight. Randomly 16 clones were selected and amplified by PCR according to the following program:94°C for 5 min; 94°C for 1 min, 56°C for 1 min, 72°C for 1 min for 29 cycles; 72°C for 10 min.

cDNA Library Normalization

The expressed genes in eukaryotic cells generate mRNA varying from a few copies to over 200,000. The random sequencing of ESTs from the cDNA library will have greater probability of selecting abundant transcripts to be sequenced and leading to inefficient detection of rare transcripts. Normalization is multi-step process performed using the sequencing to increase the random sequencing efficiency and the detection of rare genes

The Trimmer direct kit (Evrogen) was used in conjunction with the Creator SMART kit to reduce the numbers of abundant transcripts thus normalizing our cDNA

library. The first step is hybridization that denatures cDNA at high temperature (98°C) and then allows to renature at lower temperature (68°C). The hybridization reaction mixture was precipitated with the following reagents: 6µl of Duplex-specific nuclease (DSN), 4µl of 800 ng DNA template, 4µl of 4×hybridization buffer. Sterile water was added to a total volume of 16µl, mixed and incubated in a thermal cycler at 98°C for 2 min, and then 68°C for 5 h. Denaturation produces single stranded cDNA fragments of both the rare and abundant transcripts. As the temperature is lowered, the ss cDNA starts to reassociate. In a given amount of time, the abundant transcript anneal more rapidly and in greater number as compared to rare transcript.

The second step is use of enzyme Duplex Specific Nuclease (DSN), which has a strong affinity for cleaving ds DNA as compared to ssDNA. This helps in removing most of the ds DNA that belong to abundant transcripts though some might be rare ds DNA. Two sterile tubes were prepared 15 min before the end of the hybridization procedure. In the first tube, 1µl of DSN storage buffer and 1µl of DSN solution (in storage buffer) were added which was labeled as 1/2 DSN enzyme. In the second tube, 3µl of DSNstorage buffer and 1µl of DSN solution was added and labeled as 1/4 DSN. Then the two tubes were placed on ice, and 5µl of the preheated DSN master buffer, respectively were added, incubated at 68°C for 25min after this step 10 µl of DSN stop solutions was added, incubated at 68°C for 5min.

The cDNA fragments are then amplified with the PCR cycling of 95°C for 1 min, cycling 12-13 at 95°C for 15 s; 66°C for 20 s; 72°C for 3 min and the normalization efficiency is checked on agarose gel by using marker gene of known abundance.

Sequencing

Library plates were outsourced for sequencing to USDA, Poplarville. Details of the protocol followed and equipment used are not provided here, however, the basic principle behind the technique is summarized. This technique utilizes 2',3'-dideoxynucleotide triphosphates (ddNTPs), molecules that differ from deoxynucleotides by the having a hydrogen atom attached to the 3' carbon rather than an hydroxyl group. These molecules terminate DNA chain elongation because they cannot form a phosphodiester bond with the next deoxynucleotide. The ssDNA to be sequenced serves as a template strand for *in vitro* DNA synthesis; a synthetic 5′-end-labeled oligodeoxynucleotide is used as the primer. When a small amount of a specific dideoxy NTPs (ddNTPs) is included along with the four deoxy NTPs normally required in the reaction mixture for DNA polymerase, the products are the series of chains that are specifically terminated at the dideoxy residue. Thus four separate reactions, each containing a different ddNTP,can be run, and their products displayed on a High-resolution polyacrylamide gel.

Sequence Analysis

Vector Trimming- Vector contamination was removed from all the sequences using NCBI (National Center for Biotechnology Information's) Vec Screen (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html). Sequence homology search was done using BLASTX (Basic Local Alignment Search Tool: Translated query versus protein database) of the NCBI (www.ncbi.nlm.nih.gov/BLAST/). Sequence selection was done on the basis of e-values and homology all the selected sequences were translated into protein using the SIB (Swiss institute of Bioinformatics) Expasy Translation tool (www.expasy.org). Sequence alignment- Multiple sequence alignment was done using

CLUSTALW (www.ebi.ac.uk/Tools/msa/clustalw2/) of EMBL-EBI (European Molecular Biology Laboratory). Gene ontologies of some sequences were derived using SIB (Swiss institute of Bioinformatics) AmiGO (amigo.geneontology.org). Mega software was used to construct phylogenetic trees for the sequences (www.megasoftware.net).

In Vivo Gene Silencing

Synthesis of dsRNA

Synthesis of dsRNA was done according to the protocol mentioned in the BLOCK iT RNA TOPO transcription kit (Invitrogen, USA). PCR reaction 50 µl was set up, 100 ng DNA Template, Gene specific PCR Primers (SelM and GFP) (10 µM each) 1 µl, 25µl PCR supermix (Invitrogen) and Sterile water add to a final volume of 49.5 µl using PCR program of 94°C for 1 minute followed by 34 cycles each of 94°C for 1 minute, 61°C for 1 minute and 72°C for 1 minute followed by a 7 minute extension at 72°C. For the TOPO linking reaction, PCR product 1 µl, Salt Solution 1 µl, Sterile water 3 µl BLOCK-iT™ T7-TOPO® Linker 1 µl were mixed together for a total volume 6 µl. Reaction mix was incubated for 15 minutes at 37°C.

The TOPO linking reaction produces sense and antisense linear DNA template when used in a PCR reaction with gene specific and T7 PCR primers, 10X PCR BLOCK-iT™ T7 Primer (75 ng/µl) 1 µl, 1 µl Gene-specific forward/Reverse primer (10 µM), TOPO Linking reaction 1 µl, PCR supermix 25µl made upto total volume of 50 µl. Using the BLOCK iT RNA TOPO transcription kit (Invitrogen, USA), the sense and antisense linear DNA template are used to generate sense and antisense transcripts. For each sample above (sense and antisense DNA template), following components were added 75 mM NTPs 8 µl, DNA template 10 µl, 10X Transcription Buffer 4 µl, BLOCK-iT™ T7

Enzyme Mix 6 µl, Total volume 40 µl, the reaction were incubated at 37°C for 2 hours afterwards 2 µl of DNase I to each reaction. Incubated for 15 minutes at 37°C. These two transcripts are purified and ligated together in water (reached to the boiling point) that is allowed to cool to room temperature for 1-1.5 hours. dsRNA synthesized is kept in small aliquot and analyze by agarose gel electrophoresis to check the quality of your dsRNA. The remaining dsRNA is stored at -80°C.

Microinjection of dsRNA in Ticks

Pathogen-free unfed female *Amblyomma maculatum* (AM) ticks were divided into three groups (A, B and C) of 60 female ticks. *In vivo* gene silencing of selected genes was done according to method described earlier by (Karim et al., 2005; 2008). The ticks were securely fastened on a tape, the ticks in Group A were injected with 1.5µl of dsRNA for SelM (500ng/µl) and Group B with dsRNA for GFP (500ng/µl), respectively, using a microinjecting syringe (Hamilton, Reno, NV, USA) via the foramen between the capituli and the scutum, Group C served as untreated control. A lesion generally formed at the site of injection, the ticks were carefully observed for any mortality due to injection injury. The ticks were incubated overnight at 37°C to make sure that their wounds healed. Before infestation on the sheep, 40 males ticks were added in each group induce successful feeding.

Sheep Infestation

According to protocols approved by the Institutional Care and Use Committee, The University of Southern Mississippi, Microinjected ticks of each group were infested on the three cells isolated by stockinet. Sheep were observed daily post infestation in order to observe attachment, fatality, feeding pattern, engorgement and phenotypic change. Partially

fed ticks were collected from each group after 7 days of feeding, the ticks were weighed and dissected, salivary and midguts tissues were collected from each group. The final collection of ticks was done on day 12, these ticks were used either for the purpose of saliva collection, or to determine the egg production and reproductive efficiency in SelM silenced and non-silenced ticks.

Transcriptional Expression in SelM Silenced and Non-Silenced Ticks

Total RNA was isolated from SG and MG tissues of both the groups using illustra RNAspin Mini RNA isolation kit (GE healthcare, UK) according to manufacturer protocol. Concentration and purity of isolated RNA was determined using nanodrop. Total RNA was normalized to 100ng/µl and was reverse transcribed using M-MLV (Moloney Murine Leukemia virus) reverse transcriptase according to the protocol given by Invitrogen as follows, 1 µl oligo (dT) 12-18 (500 µg/ml), 1µg total RNA, 1 µl 10 mM dNTP Mix, were made upto 12µl with Sterile, distilled water the reaction was incubated at 65°C for 5 min and quick chill on ice. Further 4 µl 5X First-Strand Buffer, 2 µl 0.1 M DTT, 1 µl RNaseOUT™ Recombinant Ribonuclease Inhibitor (40 units/µl) were mixed the contents of the tube gently and incubate at 37°C for 2 min. M-MLV RT 1 µl (200 units) was added and the reaction was incubated at 50 min at 37°C followed heating at 70°C for 15 min. In order to remove RNA complementary to the cDNA, 1 µl of *E. coli* RNase H was added to the reaction mix and it was incubate at 37°C for 20 min. cDNA was PCR-amplified using gene-specific primers for SelM and PDI. All amplifications were performed using a PCR program of 75°C for 3 minutes, 94°C for 2 minutes, 22 cycles of 94°C for 1 minute, 49°C for 1 minute and 72°C for 80 seconds, followed by 10 minutes at 72°C. PCR products were analyzed on a 2% agarose gel (Bio-Rad) stained with ethidium bromide.

Antioxidant Assay

Tick Saliva Harvest

For saliva collection, ticks were allowed to feed on the sheep for 5–7 days. Near-replete ticks were removed from the cell and immobilized, a fine capillary tube was fitted over their mouthparts, and 2–5 µl of 5% Dopamine in methanol was injected into their dorsa. Saliva was collected over a period of 1–2 h pooled and stored at -70°C until used.

Total Antioxidant Capacity Assay

Antioxidant protects the cell from damages caused by reactive oxygen species by slowing or preventing the process of oxidation of reactive oxygen species by slowing or preventing the process of oxidation. Reactive oxygen species lead to oxidative stress those results in development of many diseases such as Parkinson's disease, diabetes, rheumatoid arthritis and neurodegeneration. Total antioxidant assay is based upon the reduction of Cu^{2+} to Cu^{+}. The resulting Cu^{+} forms a colored complex with a dye reagent the color intensity at 570nm is proportional to TAC (Total antioxidant capacity) in the sample (Quantichrome Antioxidant Assay kit DTAC-100). Before using the samples for TAC assay all samples were normalized for protein concentration using Biorad protein assay kit. Sample preparation for both protein estimation and TAC assay was done by sonicating tissues from the salivary glands and midguts in 10 mM PBS (phosphate buffer saline). The homogenate were centrifuged to separate soluble fractions at 14000 rpm for 5 mins at 4°C, the supernatant was used for assay. Bradford assay was done according to the Biorad microassay protocol. Trolox was used as a standard; five dilutions of standard were prepared (0.1µg/µl-3µg/µl). The Samples and standard were added to the plate, 100µl of dye reagent solution (200µl of dye concentrate and 800µl of dH_20) was added to each of

the wells. Plate was incubated at RT for 5 mins. Absorbance was calculated at 630nm. Protein concentrations were normalized for TAC assay. For TAC the assay standards were diluted in dH_20 to final concentrations of 10-1200µM. 20µl of standards and samples were transferred into separate wells of a clear 96 well plate. The working reagent was prepared by adding 100µl of reagent A to 8µl of reagent B. 100µl of working reagent was added to each sample well. Plates were incubated at RT for 10 min. Optical Density was calculated at 630nm.

Statistical Analyses

All statistical analyses and graphing were done with Microsoft Exel software. Reported results are mean values±SD.

CHAPTER III

RESULTS AND DISCUSSION

cDNA Library from the Salivary Glands of Ticks

Total RNA was extracted from the salivary glands of adult AM and AT ticks and the quality of the RNA was determined. The Bioanalyzer provides a more sensitive qualitative analysis from less RNA as compared to other traditional methods. The gel picture below (see Figure 7) shows distinct bands and negligible degradation products between the bands, suggesting high quality RNA. As determined by the Nanodrop the concentration of the total RNA extracted in all the samples was between 1-3 µg/µl. The ratio of A_{260}/A_{280} to the total RNA was well within the range between 2.0 and 2.2, indicating the total RNA isolated was suitable for cDNA library construction.

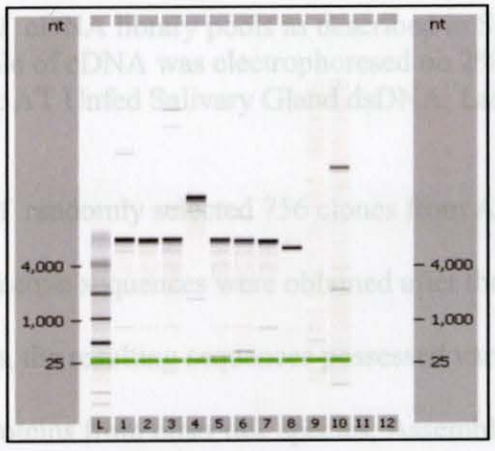

Figure 7. Bioanalyzer results showing total RNA recovery from the salivary gland of Adult female AM and AT. Lane L: RNA ladder; Lane 1:AM Unfed Salivary Gland; Lane 2: AM Partially Fed Salivary Gland; Lane 3: AT Unfed Salivary Gland; Lane 4: AT Partially Fed Salivary Gland.

The cDNA was synthesized from 3 µg of total RNA using the SMART cDNA library construction kit (Clontech) was subjected to reverse transcription for synthesis of the first and double-strand cDNAs (see Figure 8). Smear of ds cDNA obtained was more than 300bp, Comparison of the intensity of the banding pattern of ds cDNA product to the

1 kb DNA size marker showed that there were abundant transcripts in our AM library. The clone evaluation of random colonies revealed cDNA inserts length ranged from 500-1000bp.

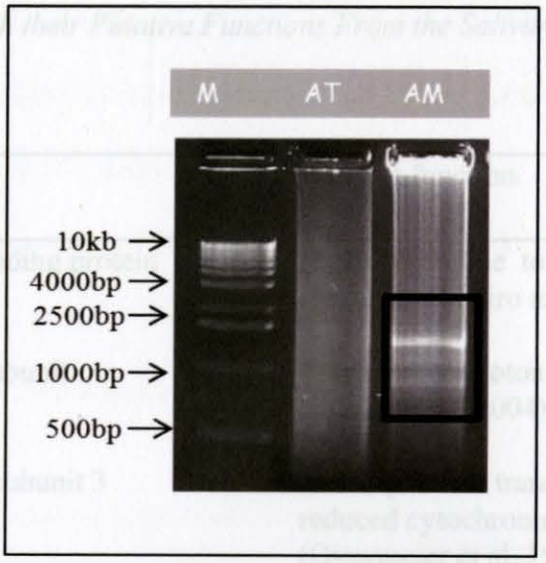

Figure 8. Gel analysis of cDNA library pools as described in SMART cDNA construction kit. 4µl sample of cDNA was electrophoresed on 2% Agarose gel. Lane M: 1 kb DNA ladder; Lane AT: AT Unfed Salivary Gland dsDNA; Lane AM: AM Unfed Salivary Gland dsDNA.

EST sequencing of randomly selected 756 clones from AT and 210 clones from AM library was done. Effective sequences were obtained after the removal of the vector and low-quality sequences, the resulting sequences possessed various degrees of homology to previously described proteins from other tick species; Assembly of the expressed sequence tags from AT yielded 48 contigs and 140 singletons, based on the blast similarity 50% of the assembled contigs and 60% of assembled singletons showed similarity to known proteins by the NCBI BLASTX program and appeared to be coding for functional predicted proteins, among the protein sequences obtained from *A. tuberculatum* were several putative proteins with histamine-binding domains, several protease inhibitors of distinct classes, metalloproteases, several peptide families of unknown function,

antimicrobial peptides, several housekeeping genes and death associated proteins, some of them are listed below (see Tables 3).

Table 3

Selected Proteins with their Putative Functions From the Salivary Gland Library of A.tuberculatum

Protein	Putative function
Putative histamine binding protein	Binds histamine to prevent allergic response (Ribeiro and Walker, 1994)
ATP synthase, beta subunit	Transport of protons across a membrane (Zhang et al., 2004)
Cytochrome oxidase subunit 3	It catalyzes the transfer of electrons from reduced cytochrome c to molecular oxygen (Ostermeier et al.,1996)
NADH dehydrogenase 1 alpha subcomplex (B275)	Essential component of complex I of the respiratory chain, which transfers electrons (Brandt, 2006)
Serotonin and histamine binding protein	Binds serotonin and histamine in the cells (Ribeiro and Walker, 1994 and Mans, 2005)
Microplusin	Tick antimicrobial peptide (Esteves et al., 2009)
Death associated protein	Role in apoptosis (Yang et al.,1997)

This work provides information about the diversity of transcripts expressed in the salivary glands of *A. tuberculatum*, describing putative sequences that may be responsible for various biological activities, analysis of these genes and their products has the potential to reveal novel functions and processes associated with tick physiology. These gene products may constitute potential targets for designing tick control strategies. Similarly the

analysis of 210 clones from *A. maculatum* library provided 22 contigs and 101 sigletons, , based on the blast similarity 50% of the assembled contigs and 78% of assembled singletons showed similarity to known proteins by the NCBI BLASTX program and appeared to be coding for functional predicted proteins such as histamine binding proteins, there were several protease inhibitors, proteins with Kunitz domains, several peptide Selenoproteins, families of unknown function, metalloproteases, several housekeeping genes and metalloproteins some of them are listed below (see Tables 4).

Table 4

Selected Proteins with their Putative Functions from the Salivary Gland Library of A.maculatum

Protein	Putative function
Protein disulfide isomerase	Catalyzes the formation and breakage of disulfide bonds between cysteine residues within proteins as they fold (Wilkinson and Gilbert, 2004)
Cytochrome C Oxidase	Respiratory electron transport (Ostermeier et al.,1996)
Putative Kunitz-BPTI protein	Serine proteinase inhibitors and potassium channel blockers (Laskowski,1986)
Histamine Binding Protein	Binds histamine suppress inflammation during blood feeding (Ribeiro and Walker, 1994)
Salivary lipocalin	Inhibiting various hemostatic or inflammatory systems, prostaglandin synthesis (Andersen et al., 2005)
Selenoprotein M	Binds Selenium to perform variety of roles (Kryukov et al., 1999)

Selenoproteins were among the abundant class of transcripts in *A. maculatum* cDNA library. Among several identified Selenoproteins were Protein disulphide isomerase (PDI) and SelenoproteinM (SelM). These discoveries thus provided us with the blueprint to uncover additional selenoprotein mRNA and understand the underlying mechanism for Se regulation in these ticks. Various bioinformatics approaches were used to curate, annotate and analyse the Selenoprotein sequences. The multiple sequence alignment of Protein disulphide isomerase shows homology with other tick species (see Figure 9).

```
A.maculatum       GGHVCSWGYAKHIVLLAAFVSVSL-GSDVLDYSGSDFDDRIKEHDTALVEFFAPWCGHCK 59
A.variegatum      ---------MKHIVLLAVFVSASL-ASDVLDYSGSDFEDRIKEHDTALVEFFAPWCGHCK 50
H.longicornis     ---------MKQIILLAAFVSAVL-GSDVLDYSGSDFDDRIREHDTALVEFFAPWCGHCK 50
I.scapularis      ---------MRWFPLLAVLIPAAL-ASDVLDYS-ADFDTKIQDHDAALVEFFAPWCGHCK 49
B.floridae        --------MFSWLSLLVVCALARVNADDVLDYSGDDFSDRIGEHDVALVEFFAPWCGHCK 52
                            : **..   . : ..****** **. :* :**.**************

A.maculatum       RLAPEYEKAATTLKGNDPPVPLVKVDCTSESGGKDTCSKYGVSGYPTLKIFKGGEFSSEY 119
A.variegatum      RLAPEYEKAATTLKSNDPPVPLVKVDCTSDSG-KETCSKYGVSGYPTLKIFKGGEFSSEY 109
H.longicornis     RLAPEYEKAATALKDNDPPVPLVKVDCTSETGGKDTCQKHGVSGYPTLKIFKGGEFSSEY 110
I.scapularis      RLAPEYEKAATELKTNDPPVPLIKVDCTSDGG-KDTCSKHGVSGYPTLKIFRGGEFSADY 108
B.floridae        RLAPEYEKAATVLKDNDPPVALVKVDCTSESGGKDTCSKFGVSGYPTLKIFRGGEFSSEY 112
                  ********** ** *****.*:******: * *:**.*.**********:*****::*

A.maculatum       NGPREAGGIVKH-RSQVGPSSKECTSAEELAKLLEKDEVVIVGFFENKDVDLHEHFP--- 175
A.variegatum      NGPREAGGIVKHMRSQVGPSSKECTSAEELAKLLEKDEVVIVGFFESKDVDLHEHFLKVA 169
H.longicornis     NGPREFSGIVKHMRSQVGPASKECTSAEELEKLLSKDEVVIVGFFENKDVALHEHFLKVA 170
I.scapularis      NGPREAGGIVKYMKAQVGPSSKELLSVAEVEKYLSKDDVVIFGFFESKDASLHENFLKVA 168
B.floridae        QGPREQNGIVSFMRKQVGPSAKPVLDKDAMEKFIGNSEPSVVGFFAE-DSDLKKAFLKTA 171
                  :**** .***.. : ****::*   .   : * : :.:  :.*** . *  *:: *

A.maculatum       -------------QGLL---------------------------------------- 179
A.variegatum      DKQRESWVFGHTFNKDLLKKYGHTN-KVVLFRPKLLKSKFEESEVAYDGAADKAALEKFL 228
H.longicornis     DKQRESWVFGHTFNKDLLKKHGHTN-KVVLFRPSVLKNKFEENEAVYEGAADKNELEKFL 229
I.scapularis      DKQREAWTFGHSFDKDVLKKYGYKN-QVVLFRPKILKNKFEESFAVYSGSDDKTELETFI 227
B.floridae        DNNRDDYRFAYTEARDVIDKYGYQDDAVVLFYPPRLHNKFEEKQLVYEGKSSENKIKTWL 231
                            :.::

A.maculatum       ------------------------------------------------------------
A.variegatum      KQNYHGLVGHRTQDNYNQFETPLLVAYFDVDYTKNAKGTNYWRNRILKVAQNYKG---KL 285
H.longicornis     KENYHGLVGHRTQDNYNQFQAPLLVAYYDVDYTKNAKGTNYWRNRVLKVAQKFKG---KL 286
I.scapularis      KENYHGLVGHRTQDNYNMFQAPLLVAYYDVDYTKNAKGTNYWRNRILKVAQNYKG---KL 284
B.floridae        KDNVLGLCGHMTDGNADKFKKPLVVAYYDVDYVKNAKGSNYWRNRVLKVATKLKEEGKEV 291

A.maculatum       ------------------------------------------------------------
A.variegatum      NFAISNKDSFAAEMDDYGLS-SHGNKPVVAVRNANNEKFRMTNEFSVENLEKFLEEYTAG 344
H.longicornis     NFAISNKESFAAEMDDYGLS-SHGNKPVVAIRNAQSEKFRMTDEFSVESLEKFLNDYVAG 345
I.scapularis      NFAVSNKDSFAAEMDDYGVT-VKANKPAIAVRNSENEKFRMTNDFSVENLEKFLEEYLAG 343
B.floridae        YFAIAARGDFYGQLSEFGLDSSSSDKPVVAARDTSDDKFIMTDEFSVDNLEKFVRDFLDG 351
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Figure 9. ClustalW analysis of putative *A.maculatum* Protein disulfide isomerase, showing its multiple alignment with the protein disulphide originating from other tick species such as *A.variegatum, I.scapularis, H.longicornis* where "*": identical. ":": conserved substitutions ".": semi-conserved substitution.

AM Protein disulfide isomerase belongs to the thioredoxin- like superfamily and has a single conserved domain (Marchler-Bauer et al., 2011). ClustalW analysis showed closely related to *Amblyomma variegatum* showing 86% homology, 82% with *Hemophysalis longicornis*. Similar analysis done with AM SelenoproteinM revealed that among ticks its related closely to *Ixodes scapularis.* The phylogenetic analysis (see Figure 10) showed its relatedness to other arthropods like Red Flour Beetles (*Tribolium Castaneum*), Jewel Wasp (*Nasonia vitripennis*) and Red Pacific white shrimp (*Litopenaeus vannamei).*

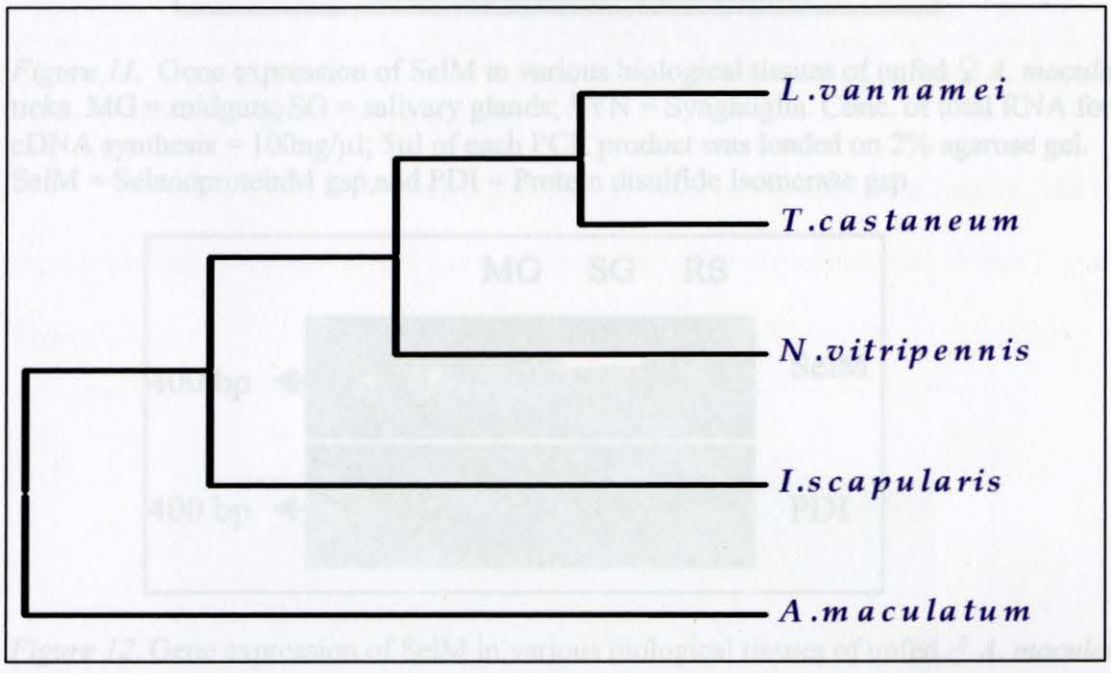

Figure 10. Phylogenetic analysis showing the relatedness of *A.maculatum* selenoprotein M, *Ixodes sacpularis* and other arthropods, *Litopenaeus vannamei, Nasonia vitripennis and Tribolium Castaneum.*

Transcriptional Expression of putative Selenoprotein M

SelM gene expression in various feeding stages and tissues of the gulf coast tick was determined using RT-PCR and gene specific primers (gsp). In AM females, (see Figure 11) SelM is expressed in midgut (MG), salivary glands (SG) and synganglion

(SYN). In AM males the gene was expressed in midgut (MG), salivary glands (SG) and reproductive system (RS). The expression of SelM is comparatively higher in salivary glands as compared to other tissues both in female and male ticks.

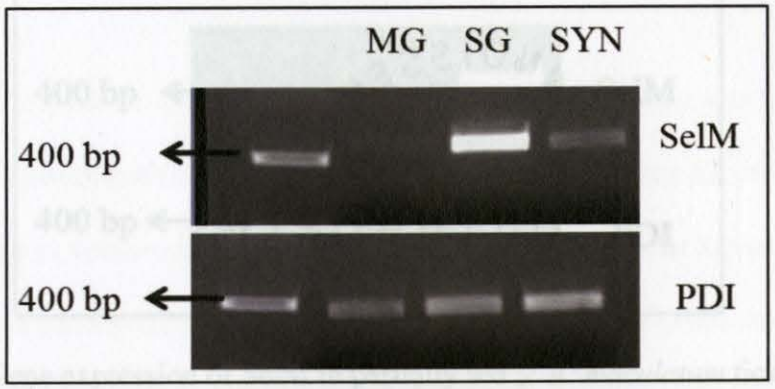

Figure 11. Gene expression of SelM in various biological tissues of unfed ♀ *A. maculatum* ticks. MG = midguts; SG = salivary glands; SYN = Synganglia. Conc. of total RNA for cDNA synthesis = 100ng/µl; 5µl of each PCR product was loaded on 2% agarose gel. SelM = SelenoproteinM gsp and PDI = Protein disulfide Isomerase gsp

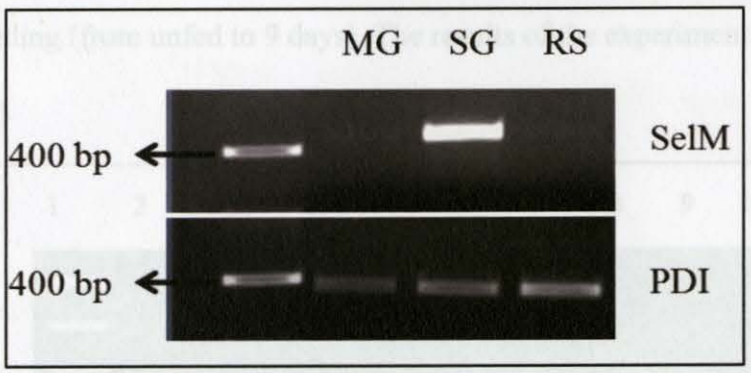

Figure 12. Gene expression of SelM in various biological tissues of unfed ♂ *A. maculatum* ticks. MG = midguts; SG = salivary glands; RS = reproductive system; Conc. of total RNA for cDNA synthesis = 100ng/µl; 5µl of each PCR product was loaded on 2% agarose gel. SelM = SelenoproteinM gsp and PDI = Protein disulfide isomerase gsp.

In male SelM showed some expression in the reproductive systems and was thus targeted by us to determine its role in fertility of male ticks (results discussed latter in the thesis). We observed the expression of the gene in partially fed females, it was slightly

higher in both midguts and salivary gland tissues (see Figure 13) indicating that feeding up regulates the expression of SelM in the above tissues.

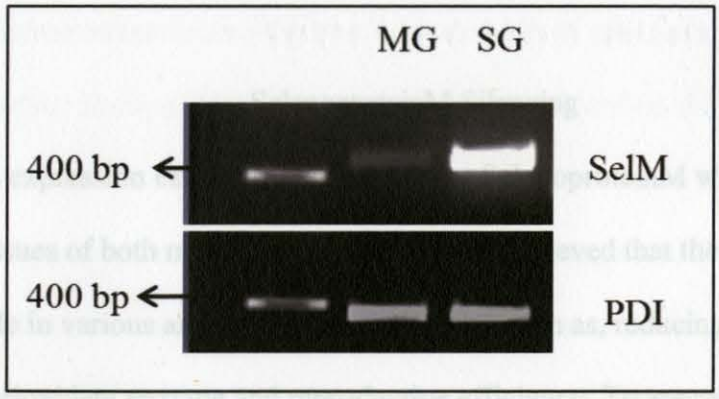

Figure 13. Gene expression of SelM in partially fed ♀ *A. maculatum* ticks. MG = midguts; SG = salivary glands; Conc. of total RNA for cDNA synthesis = 100ng/µl; 5µl of each PCR product was loaded on 2% agarose gel. SelM = SelenoproteinM gsp and PDI = Protein disulphide isomerase gsp.

We investigated the global expression of SelM in the salivary glands of AM ticks during feeding (from unfed to 9 days). The results of the experiment are shown in (see Figure 14).

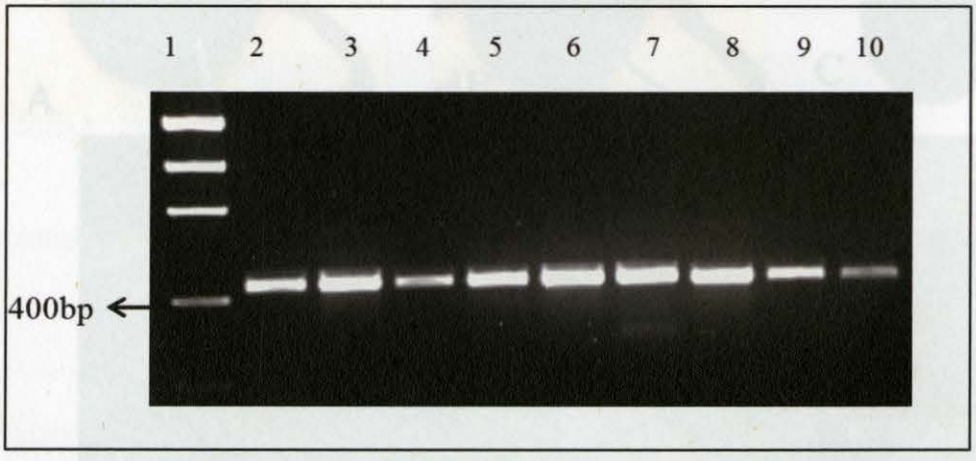

Figure 14. Transcriptional expression of SelM in salivary glands of female *A. maculatum* ticks. Lane 1: DNA ladder, Lane 2: SelM expression in unfed ticks; Lane 3: SelM expression in 24 hrs fed ticks; Lane 4: SelM expression in 48 hrs fed ticks; Lane 5: SelM expression in 72 hrs fed ticks; Lane 6: SelM expression in 96 hrs fed ticks; Lane 7: SelM expression in 120 hrs fed ticks; Lane 8: SelM expression in 6day fed ticks; Lane 9: SelM expression in 7day fed ticks; Lane 10: SelM expression in 9day fed ticks

It was observed that the gene is expressing highly in all the feeding stages, the expression was seen higher in 5-8 days of feeding which were the fast feeding stages of the tick.

SelenoproteinM Silencing

Gene expression experiments showed that SelenoproteinM was highly expressed in various tissues of both male and female ticks, we believed that the protein plays an important role in various aspects of tick physiology such as, reducing oxidative stress serving as antioxidant enzyme and reproductive efficiency. To assess the role of SelenoproteinM in *A .maculatum* we planned gene silencing or RNAi experiment.

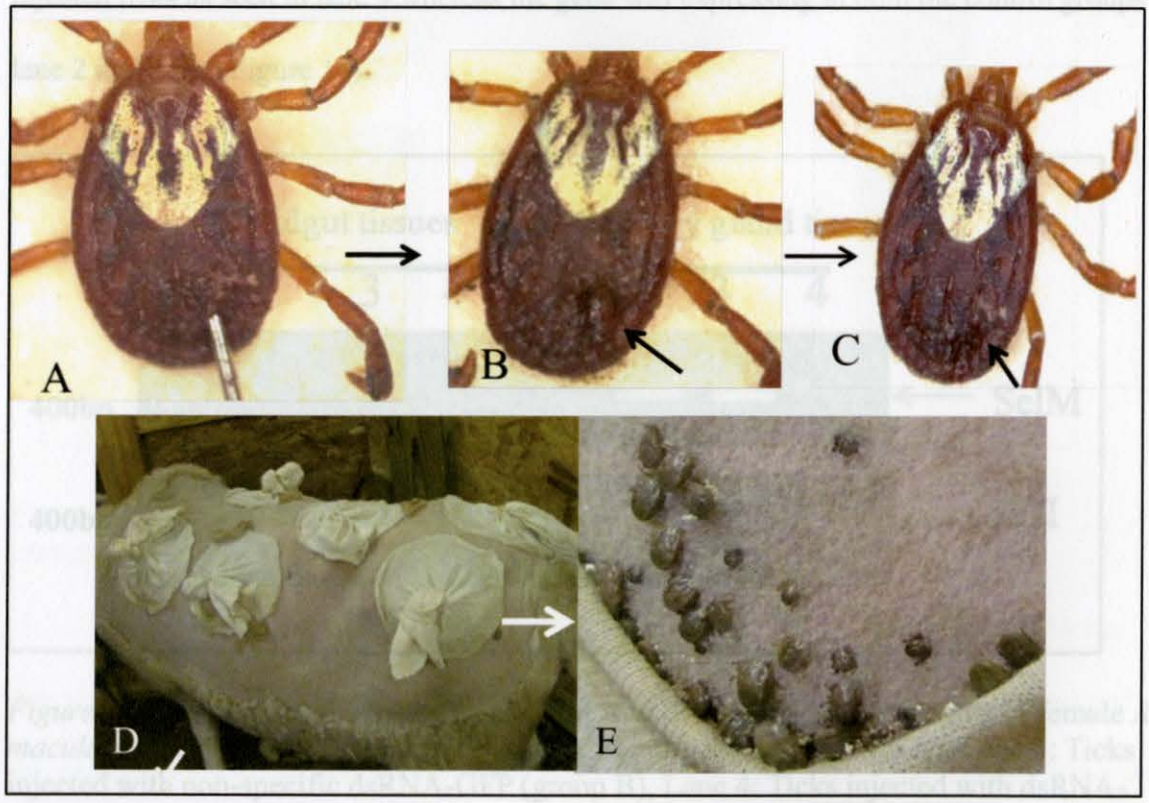

Figure 15. Microinjection of dsRNA in *A. maculatum* ticks. A: dsRNA-SelM is injected in adult female ticks with help of a fine syringe. B: A wound appears at the site of microinjection. C: The wound is sealed after the ticks are incubated overnight at 37°C. D: The ticks are infested on the sheep on separate cells according the experimental plan. E: Ticks are feeding and increasing in size in one of the cell.

dsRNA was injected in ticks using a microinjecting. A lesion is formed at the site of injection. These ticks were incubated overnight at 37°C to make sure that their wounds are healed. Before infestation on the host 40 male ticks of same species was added in each group induce feeding. Ticks of each group were infested on the three cells isolated by stockinet. Sheep was observed daily post infestation in order to observe attachment, fatality, feeding pattern, engorgement and phenotypic change (see Figure 15).

Post RNAi, we checked the transcriptional expression of SelM in order to determine the success of the RNAi procedure in form of gene knockdown, RT-PCR revealed the silencing of SelM in both Midguts and salivary glands of ds RNA SelM injected ticks as seen in lane 4 whereas the gene was expressing in both the control groups lane 2 and 3 (see Figure 16).

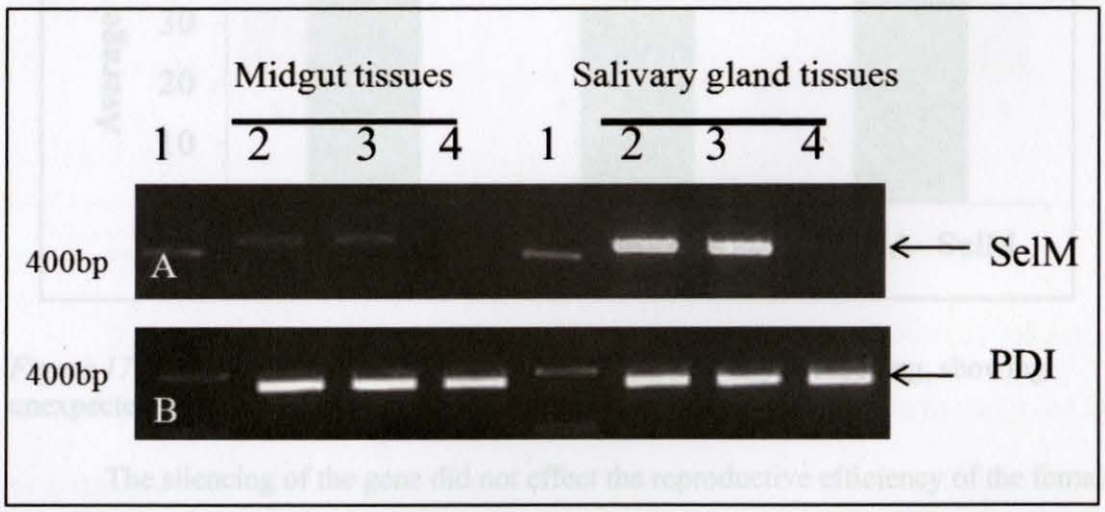

Figure16. Transcriptional expression of SelM in salivary glands and midguts of female *A. maculatum* ticks. Lane 1: DNA ladder, Lane 2: Untreated ticks (group A), Lane 3: Ticks injected with non-specific dsRNA-GFP (group B), Lane 4: Ticks injected with dsRNA-SelM (group C). Panel A represents amplification with SelM (SelenoproteinM gsp); Panel B represents amplification with PDI (Protein disulpfide Isomerase gsp).

We selected various phenotypic criteria to determine the effects silencing of SelenoproteinM on adult AM ticks. The effect gene silencing on the tick attachment,

feeding and reproductive efficiency was considered. We observed that during the early phase of feeding, the silencing of SelenoproteinM caused rapid weight gain in ticks (see Figure 17). ds RNA-SelM injected groups attained an average weight of ~66 mg compared to an average weight of ~33-37mg in control groups.

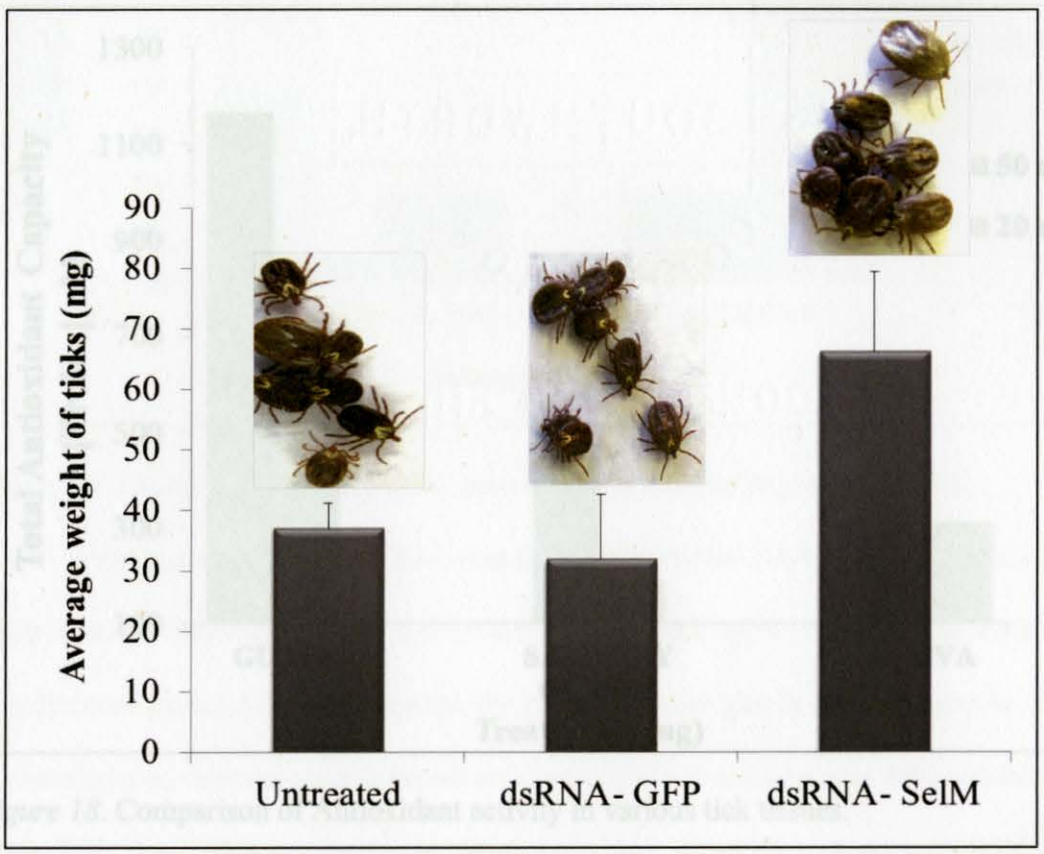

Figure 17. Comparison of average weight of tick after 7 days of feeding, showing unexpected gain of weight in dsRNA SelM injected ticks.

The silencing of the gene did not effect the reproductive efficiency of the female tick as after engorging, all the surviving females in the three groups were laying eggs and the egg masses were almost similar in the egg laying females of the three groups. We also checked the effect of silencing on the reproductive efficiency of male ticks, there was no evidence of any significant effect on male fertility.

Further we determined antioxidant in various tissues of AM ticks. The comparison of TAC (Total antioxidant capacity) in various tissues of the ticks showed that the antioxidant capacity is highest in the midgut tissues and AM saliva also showed significantly higher capacity when compared to salivary glands (see Figure.18).

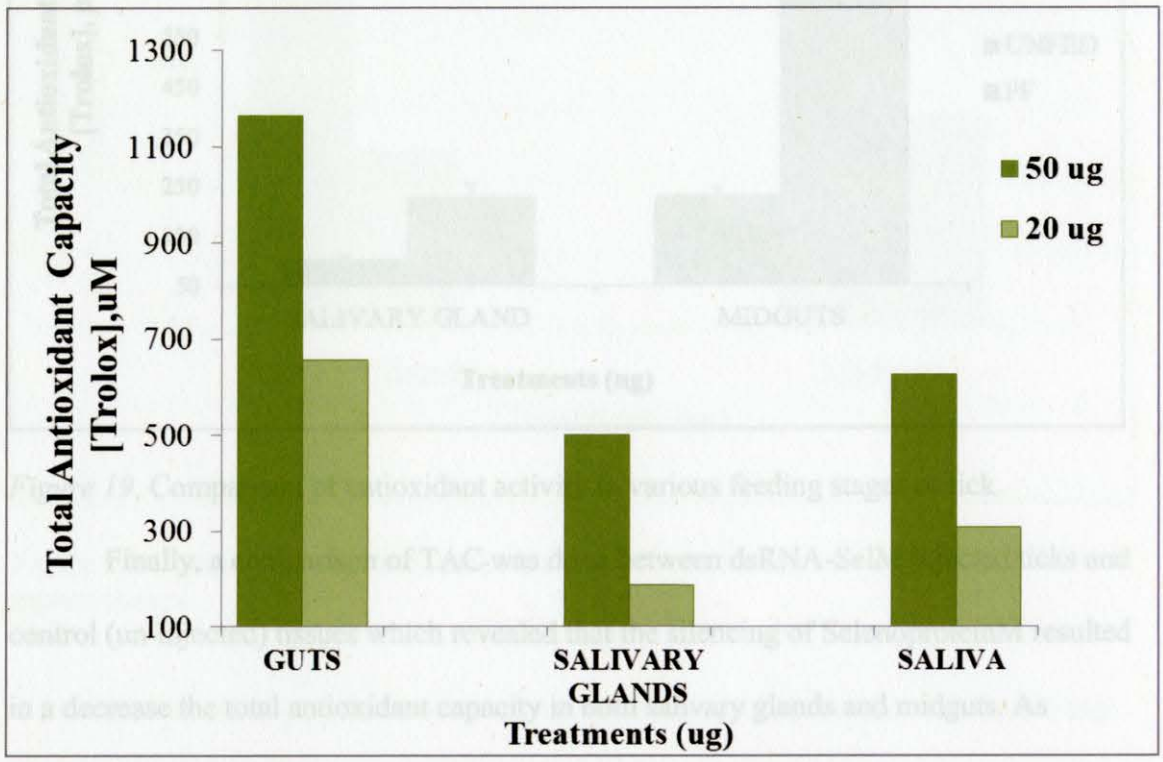

Figure 18. Comparison of Antioxidant activity in various tick tissues.

Similarly a comparison of antioxidant activity in tissues from partially fed and unfed ticks revealed that in both SG and MG the activity was higher in partially fed ticks as compared to the unfed state. There was a 60% increase in TCA activity in partially fed SG and 68% increase in partially fed MG (see Figure 19).

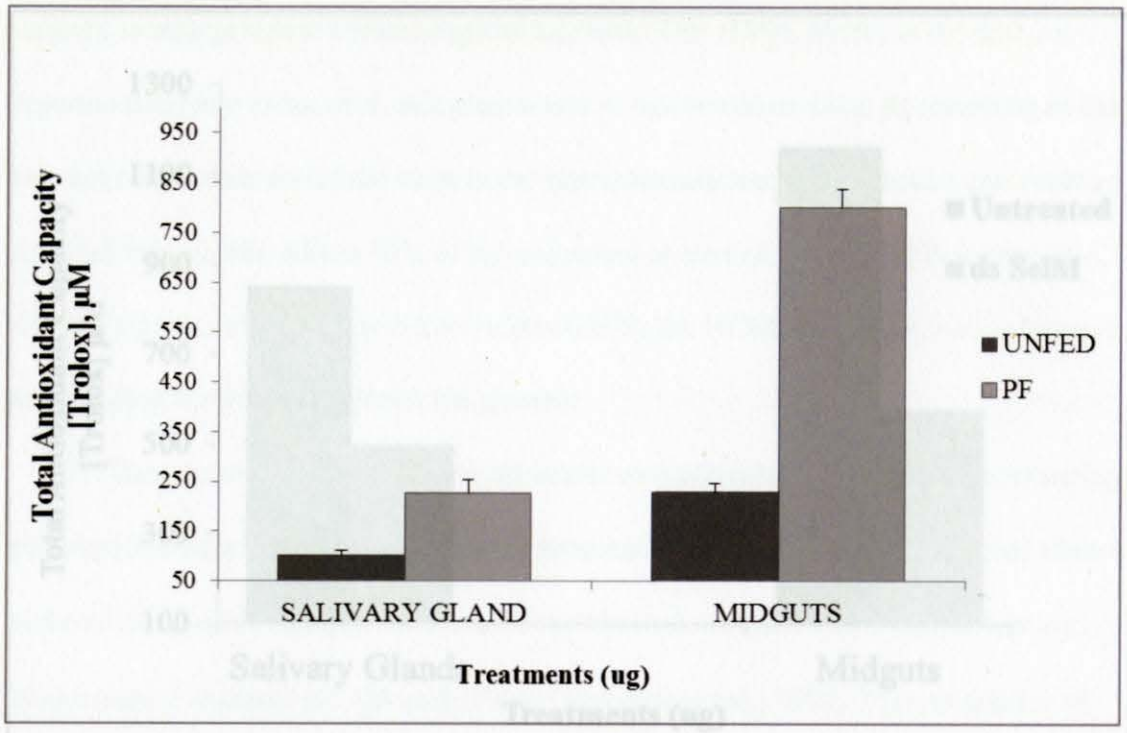

Figure 19. Comparison of antioxidant activity in various feeding stages of tick.

Finally, a comparison of TAC was done between dsRNA-SelM injected ticks and control (un-injected) tissues which revealed that the silencing of SelenoproteinM resulted in a decrease the total antioxidant capacity in both salivary glands and midguts. As compared to un-injected control the antioxidant activity in midguts was 49% reduced in ds RNA-SelM injected ticks whereas in salivary glands it was 45% reduced in ds RNA-SelM injected ticks (see Figure 20).

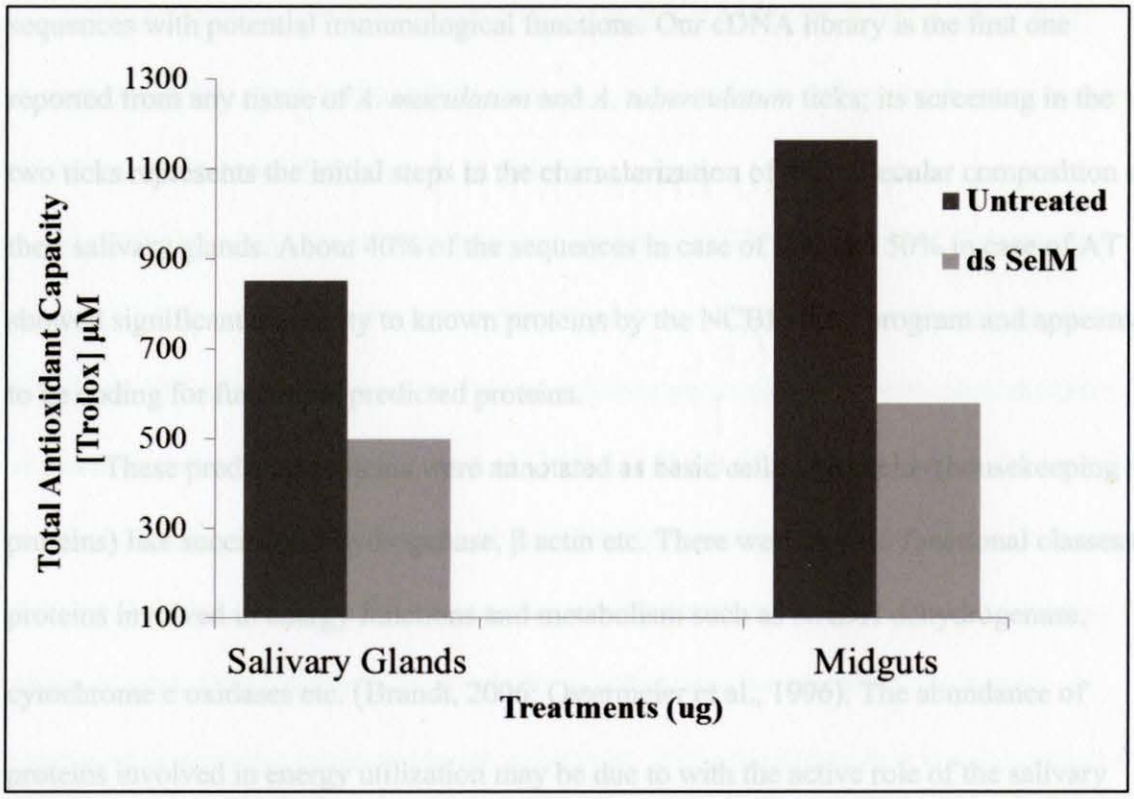

Figure 20. Comparison of antioxidant capacity between un-injected and dsRNA-SelM treated tissues.

Discussion

In absence of genomic sequences for most tick species, Expressed sequence tags (EST's) sequencing has proven to be a valuable tool for novel gene discovery. NCBI's database provides EST's for tick species such as *Ixodes scapularis* (Hill and Wikel, 2005), *Dermacentor variabilis* (Alarcon-Chaidez et al., 2007), *Boophilus microplus* (Guerrereo et al, 2005) *Amblyomma americanum* (Hill and Gutierrez, 2005) and many more. Salivary glands of various tick species have been studied for over 40 years describing the importance of these glands in the tick attachment, feeding and pathogen transmission. There has been a tremendous growth in understanding tick salivary gland physiology with the advancement of molecular biology techniques. A total of 756 ESTs from *A. tuberculatum* and 210 from *A. maculatum* were analysed. There were several important

sequences with potential immunological functions. Our cDNA library is the first one reported from any tissue of *A. maculatum* and *A. tuberculatum* ticks; its screening in the two ticks represents the initial steps in the characterization of the molecular composition of their salivary glands. About 40% of the sequences in case of AM and 50% in case of AT showed significant similarity to known proteins by the NCBI blastx program and appeared to be coding for functional predicted proteins.

These predicted proteins were annotated as basic cellular proteins (housekeeping proteins) like succinate dehydrogenase, β actin etc. There were several functional classes of proteins involved in energy functions and metabolism such as NADH dehydrogenase, cytochrome c oxidases etc. (Brandt, 2006; Ostermeier et al., 1996). The abundance of proteins involved in energy utilization may be due to with the active role of the salivary glands in osmoregulation and blood feeding (see introduction). Putative anticoagulants and immunosuppressive protein like lipocalins, histamine and serotonin binding proteins were identified both in *A. maculatum* and *A. tuberculatum*. Lipocalin proteins interfere with platelet aggregation, blood coagulation, activation of the complement system, completion of the blood meal, and inflammation (Benoit et al., 2008) similarly histamine and serotonin are released by the host in response to tissue damage, increasing the permeability of blood vessels allowing wound repair (see Introduction), presence of lipocalins and binding proteins for both histamine and serotonin suggests with the necessity for these ticks to overcome a strong innate immune response while feeding on the host for prolonged periods. The Presence of serine protease inhibitors, like Kuntz BPTI is important factors which disrupt defensive host processes, serine protease inhibitor gene family is involved in the regulation of several physiological functions such as the blood clotting cascade, clot

resolution, the inflammatory response and complement activation (Mulega et al., 2003; Armstrong, 2008). Protein disulphide isomerase is involved in energy production, oocyte development and viability (Liao et al., 2008).

Selenoproteins are present in almost all forms of life (i.e., bacteria, archaea, and eukarya), 30 selenoproteins have been identified among these lineages (Castellano, 2005; Kryukov, 2003). Selenoproteins such as glutathione peroxidases, thioredoxin reductases, and iodothyronine deiodinases have well-defined roles and are involved in oxidoreductions, redox signaling, antioxidant defence, thyroid hormone metabolism, and immune responses (Lu and Holmgren, 2009). SelM expression was analyzed in various tissues and feeding stages of adult Gulf Cost ticks by RT-PCR, in unfed ticks the transcript abundantly expressed in salivary glands, moderate in midguts, while the expression in synganglion and reproductive system are lower. In feeding ticks, the expression of selenoproteinM was higher as compared to unfed ticks (both in salivary glands and midguts) which may be due to higher enzymatic activity in these tissues during blood meal intake. In fast feeding stages of tick increased expression of SelM was observed which may responsible for alleviating high toxicity of reactive oxygen species produced during fats feeding due to reduction in actin cytoskeleton (Gourlay and Ayscough 2005).

RNAi was used successfully to suppress the expression of SelM in *Amblyomma maculatum*. Earlier also the technique was applied to selectively knock-down genes in tick salivary glands both *invivo* and isolated ones, to access their roles. The studies on using dsRNA induced silencing were done initially on the secreted salivary component a histamine binding protein (Aljamali et al., 2003) and synaptobrevin homologue (Karim et al., 2004). The silencing of SelM caused rapid weight gain in early phase feeding; this was

quite a surprising result, the only possible justification for this result could likely be the bioavailability of selenide, silencing of SelM may have resulted in the increased sequestration of some free selenide that was easily available for incorporation into other selenoproteins. Thus, with this expansion of the selenide pool, selenoproteins that utilize selenium in various other metabolic pathways may result in weight gain. However, additional information is needed to understand the regulation of selenoproteins.

In our study, we also evaluated the role of SelM as an antioxidant enzyme, our comparison of the antioxidant capacity in partially fed midgut, salivary glands and saliva showed that the capacity is highest in guts (despite of the low expression of SelM) we could reason this with the fact that tick guts are lined with an enzyme-secreting tissue and serve as the main site of digestion and absorption. In Ixodid ticks, the volume of blood ingested by a female can be as much as one hundred times its initial weight, for the purpose of Heme digestion which could be a toxic molecule due to its ability to generate reactive oxygen species, there may be other protective antioxidants active in the midguts (Souza et al., 1997).

In white shrimp, SelM expression was induced after White spot syndrome virus (WSSV) infection and the results of gene silencing provide evidence that SelM might play crucial role as an antioxidant enzyme (Clavero-Sales et al., 2007; Garcia et al., 2010). Similarly in the experiments we conducted it was observed that the antioxidant activity in dsRNA- SelM injected tissues was lower compared to controls, indicating that SelM is an important component of the antioxidant response in Gulf coast ticks. Many biochemical studies involving individual selenoproteins, including their targeted removal using RNAi technology, have also elucidated our understanding of the functions and characteristics of

selenoproteins in various other organisms, the functions of number of selenoproteins are unknown in ticks. We would like develop models to understand the functions, interactions and interplay of other tick selenoproteins.

To summarize this work initiates the molecular characterization of a novel set of genes in *A. maculatum* and *A. tuberculatum*. By analyzing the two cDNA libraries, several transcripts were identified. These transcripts may be of particularly important in understanding tick physiology. Further experiments will evaluate mechanisms by which these genes contribute to the blood feeding and pathogen transmission. Increased sequence information should provide insight and better understanding of salivary gland gene expression and function can contribute to the development of effective tick control strategies, reducing the impact of using chemical control strategies for tick treatment. Ticks harbor many microorganisms, both symbiotic and pathogenic; the Gulf coast tick can transmit pathogens of agricultural or medical impact. Availability of EST data in conjunction with ongoing high throughput sequencing projects for *Amblyomma maculatum* will provide evidences to discern the molecular basis of host-vector -pathogen interactions. Sequence information will enhance the ability to identify candidates for RNA interference (RNAi) studies along with insight on the regulation of various biological processes in the tick.

APPENDIX A

SUMMARY OF PRIMERS USED IN VARIOUS EXPERIMENTS

Target gene	Forward primer (5'-3')	Reverse primer (5'-3')	Tm (◦C)
M 13	GTAAAACGACGGCCAGT	CAGGAAACAGCTATGACC	51
PDI	TCCCTTGGAAGCGATGTT CTCGAT	CAACTTGCGACCGCATGT GTTTGA	60.6
SelM	CGCAGCATTGTAGCTGTG CTTCTT	GTGGCATTCACAAGTCGC CCTTAT	59.7
GFP	GGGCTGCAGATGAGTAA AGGAGAAGAACTTT	GG A AGC T TACCC ATGG A ACAGGTA	60

APPENDIX B

IACUC APPROVAL FORM

The University of Southern Mississippi

Institutional Animal Care and Use Committee

118 College Drive #5147
Hattiesburg, MS 39406-0001
Tel: 601.266.6820
Fax: 601.266.5509
www.usm.edu/spa/policies/animals

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: **10040901**
PROJECT TITLE: **Tick Spitome: Developing a Vaccine for the Tick Paralysis**
PROPOSED PROJECT DATES: **09/30/2009 – 09/29/2011**
PROJECT TYPE: **New Project**
PRINCIPAL INVESTIGATOR(S): **Shahid Karim, Ph.D.**
COLLEGE/DIVISION: **College of Science & Technology**
DEPARTMENT: **Biological Sciences**
FUNDING AGENCY/SPONSOR: **National Institutes of Health/USDA AFRI**
IACUC COMMITTEE ACTION: **Full Committee Review**
PROTOCOL EXPIRATION DATE: **09/30/2012**

Robert C. Bateman, Jr., Ph.D.
IACUC Chair

4-19-2010
Date

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