

IDENTIFICATION AND CHARACTERIZATION OF
MACROPHAGE MIGRATION INHIBITORY FACTOR
FROM THE AMERICAN DOG TICK, *DERMACENTOR*
VARIABILIS (SAY)

By

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TABLE OF CONTENTS

Chapter	Page
I. INTRODUCTION.....	1
II. REVIEW OF LITERATURE.....	5
Ticks and their diversity.....	5
Public health significance of Ixodid ticks.....	6
Tick-borne diseases.....	6
The American dog tick, <i>Dermacentor variabilis</i>	7
Tick salivary glands.....	8
Tick salivary gland physiology.....	9
Control and mechanism of salivation.....	11
Salivary gland degeneration.....	12
Gene expression in Ixodid tick salivary glands.....	13
Tick midgut.....	15
Blood meal digestion.....	15
Tick midgut ultrastructure and digestion process.....	16
Macrophage migration inhibitory factor (MIF).....	18
Structure and function of MIF.....	19
MIF in humans.....	20
MIF expression in human diseases.....	22
The role of MIF in human immunity.....	23
MIF and glucocorticoids.....	24
MIF in parasitic nematodes.....	25
MIF in Ixodid ticks.....	26
Functions of tick MIF.....	27
Expression and localization of MIF in ticks.....	28
III. MATERIALS AND METHODS.....	29
Ticks.....	29
Tick infestation.....	29
Tick dissections.....	30
Double stranded RNA synthesis.....	30
RNA isolation.....	31
Protein isolation.....	32
Primers.....	32
Reverse transcriptase PCR.....	33

Chapter	Page
Agarose gel electrophoresis and DNA sequencing.....	33
Cloning, transformation, cell growth and plasmid isolation.....	33
Rapid amplification of cDNA ends (RACE)	34
Reverse transcriptase-relative quantitative PCR.....	35
Polyacrylamide gel electrophoresis (PAGE) and Western blot analysis	36
Database searching and sequence retrieval for MIF in arthropods and nematodes	37
Multiple sequence analysis and phylogenetic analysis.....	37
Identifying a homology model for MIF	38
Structure conservation analysis using the homology model.....	38
 IV. RESULTS AND DISCUSSION.....	 39
Identification of the presence of MIF in <i>Dermacentor variabilis</i> tissues.....	39
Elucidation of full length cDNA sequence	40
Expression of MIF gene in midgut and salivary glands during 0-96 hrs of feeding.....	41
Analysis of MIF protein in midgut and salivary glands during 0-96 hrs of feeding.....	44
The effect of MIF on tick blood meal acquisition	45
Multiple sequence alignment and phylogenetic analysis.....	47
Identifying a homology model for MIF	49
Structure conservation analysis using homology model.....	49
 V. SUMMARY AND CONCLUSION.....	 51
 REFERENCES	 75

LIST OF TABLES

Table	Page
1. Primers used for RT-PCR, RT-qPCR and RACE experiments.....	56

LIST OF FIGURES

Figure	Page
1. Control mechanism of Ixodid tick female salivary gland secretion	57
2. Structures of parasitic and mammalian macrophage migration inhibitory factor (MIF)	58
3. Identification of the presence of MIF gene from 0-96 hrs fed <i>Dermacentor variabilis</i> female tick tissues.....	59
4. <i>Dermacentor variabilis</i> MIF nucleotide sequence and putative amino acid sequence	60
5. Change in MIF gene expression in <i>Dermacentor variabilis</i> midgut tissues during 0-96 hr feeding interval.....	61
6. Change in MIF gene expression in <i>Dermacentor variabilis</i> salivary gland tissues during 0-96 hr feeding interval	62
7. Detection of midgut proteins from <i>D. variabilis</i> females during feeding intervals from 0-96 hrs in a polyacrylamide gel	63
8. Detection of salivary gland proteins from <i>D. variabilis</i> females during feeding intervals from 0-96 hrs in a polyacrylamide gel	64
9. Effect of MIF on blood meal acquisition in <i>Dermacentor variabilis</i>	65
10. RT-qPCR analysis of MIF gene expression for dsRNA injected female <i>D. variabilis</i> midgut tissue.....	66
11. RT-qPCR analysis of MIF gene expression for dsRNA injected female <i>D. variabilis</i> salivary gland tissue.....	67
12. Multiple sequence alignment of tick, nematode and insect MIFs	68
13. Phylogenetic analysis of MIF from ticks, nematodes and insects	70
14. Sequence alignment between <i>D. variabilis</i> MIF (Target) and its homology model identified using automated modeling mode at SwissModel	72
15. A schematic presentation of the results from the Consurf analysis to find the conserved amino acids in the homology model for <i>D. variabilis</i> MIF	73

CHAPTER I

INTRODUCTION

Ticks are second only to mosquitoes as life threatening disease vectors of animals and humans (Sonenshine, 1991). There are three major tick families, the Ixodidae, the Argasidae and the Nuttalliellidae. The highest species diversity is observed in the family Ixodidae, commonly known as hard ticks due to their sclerotized dorsal scutum. In contrast soft ticks have a flexible leathery cuticle. Family Nuttalliellidae is found only in Africa and has only a single tick species (Amsden et al, 2005). The hard ticks are the most diverse group with over 700 different species and 13 genera (Amsden et al, 2005). Ticks are obligatory blood feeders and an Ixodid female tick can uptake a blood meal more than 100 times of initial body weight (Sauer et al., 2005).

Ixodid ticks can transmit pathogens that cause mild to severe illnesses in humans and animals. Some tick-borne diseases can even be fatal (Taege, 2000, Jongejan et al., 2004 and Magnarelli, 2009). The tick's ability to ingest blood from several hosts in each consecutive life stage aids pathogen transmission between different hosts (Amsden et al, 2005 and Fritz, 2009). Ixodid ticks in the genera *Amblyomma*, *Ixodes*, *Dermacentor* and *Rhipicephalus* are identified as the most common vectors of tick-borne pathogens in the United States (Amsden et al, 2005).

Dermacentor variabilis is a three-host tick that feeds on a wide range of hosts including humans, cattle, dogs, horses, deer and many other wild and domestic mammals (Comer, 1991). A three-host tick feeds on three different hosts at each life stage, i.e. larvae, nymph and adult, to complete their life cycle. *D. variabilis* is the primary vector of Rocky Mountain spotted fever (RMSF) and bovine anaplasmosis in North America (Kocan et al., 1981, Sonenshine, 1993, Lankester et al., 2007 and Torres, 2007). It has also been shown to transmit *Cytauxzoon felis* and *Ehrlichia canis* (Blouin et al., 1984, Johnson et al., 1998). Both *Ehrlichia chaffeensis* and *Ehrlichia ewingii* have been identified in *D. variabilis* but transmission has not been tested (Steiert et al., 2002).

More than 3400 different putative salivary gland proteins belonging to at least 32 different gene families have been identified from different tick species (Francischetti et al., 2010). A number of these salivary gland proteins have been shown to be secreted while others contain putative signal sequence that suggest a secreted protein. There have been many studies done on tick salivary gland physiology and biochemistry to identify and understand the functions of salivary secretions in tick feeding (Sauer, 1977, Needham and Teel., 1986, Shipley et al., 1993, Sauer et al., 1995, Bowman et al., 1995, Zhu et al., 1997, Bowman et al., 1997, Sauer et al., 2000, Bowman et al., 2001, Bowman et al., 2004 and Bowman et al., 2008, Šimo et al., 2009a, Šimo et al., 2009b).

Tick blood feeding is facilitated by two main organs in the tick, the salivary gland and the midgut. The midgut ultrastructure has been studied in different tick species at different life stages to identify the cell types involved in blood meal digestion and to study the ultrastructure changes during blood meal digestion as described in detail in Chapter II (Raikhel, 1978, Jaworski et al., 1983, Williams et al., 1985, Agbede, 1986,

Agbede et al., 1987 and Tarnowski et al., 1989, Ageyi et al., 1992, Ageyi et al., 1995). The information at the molecular level is very limited. Recently a high throughput transcriptome study has identified a total of 82 transcripts that were involved in blood meal digestion (Anderson et al., 2008).

Macrophage migration inhibitory factor (MIF) was among one of the first cytokines to be identified about 40 years ago (Calandra et al., 2003). It was named for its activity in inhibiting random macrophage migration *in vitro* (Bucala et al., 2003). MIF homologs has been identified in many organisms including mammals, nematodes, arthropods, jawed/jawless fish, plants, cyanobacteria and parasites (Pastrana et al., 1998, Jaworski et al., 2001, Sato et al., 2003, Calandra et al., 2003, Jin et al., 2007, Cordery et al., 2007 and Ito et al., 2008).

One of the first midgut proteins characterized has been developed for an anti-tick vaccine (Willadsen et al., 1989). Tick MIF was first identified from a midgut cDNA library from partially fed Lone star ticks, *Amblyomma americanum* (Jaworski et al., 2001). Two other tick MIF sequences are available for *Haemaphysalis longicornis* and *D. variabilis* midgut transcripts, with the latter being the study overlapping the research reported here (Umemiya et al., 2007 and Anderson et al., 2008). The protein is 116 amino acids long and the recombinant tick MIF is approximately 12 kDa in size (Jaworski et al., 2001 and Umemiya et al., 2007). Our lab has also identified the presence of MIF in *Ixodes scapularis* using high-throughput bioinformatics tools from the VectorBase (<http://www.vectorbase.org>). Possible biological functions for tick MIF, as suggested by Jaworski et al., (2001) are to increase inflammation at the feeding site facilitating blood

meal uptake and to inhibit the macrophage migration toward the mouth parts as the tick feeds or within tick midgut after feeding.

Previously our group characterized *A. americanum* MIF expression, localization and gene expression in midgut, salivary gland, ovaries and carcass tissues of adult female lone star ticks fed from 0-9 days feeding intervals (Bowen et al., 2010). We have also shown that immunization of rabbits with tick MIF specific antibodies lengthens the feeding intervals in *A. americanum* (Jaworski et al., 2009).

The specific objectives in this study were to:

1. Identify a MIF homolog from American dog tick *D. variabilis* and complete the full-length cDNA sequence
2. Analyze MIF gene expression in female midgut and salivary gland tissues from 0-96 hrs at 0, 8, 24, 48, 72, 96 hrs time intervals of feeding
3. Analyze the MIF protein in female midgut and salivary gland tissues from 0-96 hrs at 0, 8, 24, 48, 72, 96 hrs time intervals of feeding
4. Study the effect of MIF on blood meal acquisition in adult females using RNA interference
5. Compare phylogenetic relationships between tick, nematode and insect MIFs

CHAPTER II

REVIEW OF LITERATURE

Ticks and their diversity

Ticks are very important vectors of human and animal pathogens. They are second only to mosquitoes as vectors of life threatening human and animal diseases (Sonenshine, 1991). Ticks belong to the class Arachnida, subclass Acari, order Parasitiformes and suborder Ixodida. The suborder Ixodida has three families, the Ixodidae, the Argasidae and the Nuttalliellidae (Sonenshine, 1991). Family Ixodidae is composed of approximately 700 species and 13 genera. The family Argasidae is composed of approximately 180 species and 5 genera. The family Nuttalliellidae is found only in Africa and has only a single tick species (Amsden et al, 2005). Hard ticks belong to the family Ixodidae and are the largest and most important group as vectors of human and animal pathogens. Ticks have four life stages: egg, larva, nymph and adult. All of the motile life stages in the Ixodid ticks attach to the host and feed for days, whereas the Argasid ticks feed for minutes up to an hour. Most Argasid ticks uptake blood, 5-10 times their initial body weight while the Ixodid female ticks can ingest more than 100 times of the initial body weight (Sauer et al., 1995). Ticks in the family Ixodidae show either one-host, two-host or three-host life cycles and it is a species specific feature to which life cycle they belong (Sonenshine, 1991).

Public health significance of Ixodid ticks

Ticks have a significant impact on animals and humans in the northern hemisphere (Jongejan et al., 2004). They are medically important ectoparasites living in diversified habitats from the tropics to subarctic areas (Magnarelli, 2009). They can transmit an array of pathogens (bacteria, viruses and parasites) causing mild to severe illnesses in humans and these tick-borne diseases can even be fatal (Taeye, 2000, Dennis et al., 2005 and Magnarelli, 2009). Tick blood meal acquisition from several hosts consecutively facilitates the blood-borne pathogen transmission between different hosts (Amsden et al, 2005 and Fritz, 2009). The risk of tick-borne disease is limited by the coexistence of the microbial pathogen, competent vector tick, a reservoir host and a susceptible host (Fritz, 2009). Some tick species have the potential to hold multiple pathogens causing co-infections in vertebrate host (Swanson, 2006). Early treatment of tick-borne bacterial or protozoan infections is more effective compared to treating the later stages of the diseases (Magnarelli, 2009).

Tick-borne diseases

In the United States, ticks in the genera *Amblyomma*, *Ixodes*, *Dermacentor* and *Rhiphicephalus* are identified as common vectors of bacterial, rickettsial, spirochetal and protozoan pathogens (Amsden et al, 2005). Major tick-borne diseases include Rocky Mountain spotted fever, Human Monocytic Ehrlichiosis, Human Granulocytic Anaplasmosis, Tularemia, Lyme borreliosis, Bovine Anaplasmosis, tick-borne encephalitis and Babesiosis (Fritz, 2009; Rim et al., 2007; Amsden et al, 2005; Jongejan et al., 2004; Gayle et al., 2001; Taeye, 2000 and Golightly et al., 1999). Many of these

diseases are considered emerging tick-borne diseases (Beugnet et al., 2009). Lyme borreliosis has continued to increase at a higher rate due to socioeconomic changes increasing the risk of tick exposure while RMSF has shown cyclic fluctuations of the incidence over decades (Torres, 2007 and Piesman et al., 2008). Patterns of seasonal activity for host seeking can vary greatly among different tick species depending on the climatic and environmental conditions (Dennis et al., 2005). Both sexes and people of all ages are equally susceptible to tick-borne diseases. Infection and disease rates may vary due to personal behaviors, residence, activities and host immunity (Dennis et al., 2005). Clinical recognition and treatment of patients with tick-borne diseases is very challenging since very few clinical or laboratory diagnostics are available (Goodman, 2005). Amasden et al., 2005 suggests that it is critical to treat the patients in early onset for tick-borne diseases as it will significantly lower incidence of disease and mortality (Amasden et al., 2005). Disease control measures include different approaches for avoiding tick bites, using strategies for suppression of host-seeking ticks and avoiding disease following a bite via vaccination or antibiotic treatments (Piesman et al., 2008). According to Piesman et al., 2008, academic research on tick-borne diseases must be brought into the real world to develop efficient preventive measures. It is necessary to have ready access to information about the personal risk of tick exposure and how to ensure proper actions to lessen the risk of tick bite and pathogen exposure (Piesman et al., 2008).

The American dog tick, *Dermacentor variabilis*

Dermacentor variabilis is a three-host tick that feeds on a wide range of hosts including humans, cattle, dogs, horses, deer and many other wild and domestic mammals

(Comer, 1991). *D. variabilis* is abundant in the eastern US from Florida to New England and from the Atlantic seaboard to the Mississippi basin. Discontinuous populations are also found in southeastern Canada, midwestern and western US (McEnroe, 1974 and 1975). *D. variabilis* populations in southern and middle parts of its range have a characteristic one year life cycle whereas the populations in the northern part of its range have a two year life cycle (Garvie et al., 1978, Campbell, 1979 and Sonenshine 1993). *D. variabilis* is the primary vector of Rocky Mountain spotted fever (RMSF) in North America (Sonenshine, 1993 and Torres, 2007). It also transmits *Anaplasma marginale* causing bovine anaplasmosis in cattle (Kocan et al., 1981 and Lankester et al., 2007). *D. variabilis* has shown to transmit *Cytauxzoon felis*, the protozoan parasite of cytauxzoonosis in domestic cats (Blouin et al., 1984). In addition to these three pathogens *D. variabilis* is also capable of transmitting *Ehrlichia canis* (Johnson et al., 1998). Several *Ehrlichia spp* including *Ehrlichia chaffeensis*, *Ehrlichia ewingii* have been identified in *D. variabilis* but transmission has not been tested (Steiert et al., 2002).

Due to its importance as a vector of human and animal diseases, the American dog tick, *D. variabilis* has been extensively studied for the tick biology at molecular and ecological perspectives.

Tick salivary glands

The salivary glands are the largest glands in the tick body (Sonenshine, 1991). They consist of grape-like clusters of acini (alveoli). Female Ixodid ticks have three major types of alveoli and males have four types (Kaufman, 1989 and Sauer et al., 1995). Type I alveoli are agranular, do not change during tick feeding and are believed to

function in atmospheric water absorption by unfed ticks (Sonenshine, 1991 and Sauer et al., 1995). Granular alveoli contain dense, large granules in their cytoplasm and are classified as Type II, III and IV (Fawcett et al., 1986 and Sonenshine, 1991). Type II and III are morphologically similar but contain different granular cell types. Type III is the most abundant alveoli type and both Type II and III undergo remarkable transformation during tick feeding. During the slow feeding period salivary glands undergo extensive changes (Kaufman, 1989). The changes include enlargement of the nuclei and cytoplasm of certain granular cells, an increase in the mass of alveoli and proliferation of plasma membrane and mitochondria (Sauer et al., 1995). These transformations are not observed in every granular cell type of the alveoli. Male Ixodid ticks that do not feed on a host have type I and IV alveoli only. Type IV alveoli are male specific and contain only one granular cell type (Sauer et al., 1995).

Tick salivary gland physiology

Acarine salivary glands are essential to several major tick feeding processes as well as nonfeeding activities. Early studies on tick salivary glands have identified the essential role of salivary glands for the blood feeding process. These included secretion of the cement substances, cytolysins, anticoagulants, enzymes and inhibitors, histamine agonists and antagonists, prostaglandins, antihemostatic factors, immune-modulating compounds and toxic components (Sauer, 1977 and Sauer et al., 1995). Recent transcriptome studies have identified thousands of hard tick proteins expressed in salivary glands and the function of the majority of these proteins are still not understood. From different tick species, approximately 3400 different putative salivary gland proteins,

belonging to about 32 different gene families, have been identified (Francischetti et al., 2010). Tick saliva has shown to possess hemolytic activity due to phospholipase A₂ (PLA₂) (Zhu et al., 1997). A specific thrombin inhibitor, americanin, which inhibits the activity of high potent platelet aggregation activator thrombin, has been characterized from the lone star tick (Zhu et al., 1997). Ticks also use the salivary glands for fluid elimination during blood meal concentration by returning excess water and ions to the host after the ingestion (Sauer, 1977 and Sauer et al., 1995). Ticks can eliminate about 70% of the fluid and ion content of the blood meal into the host by salivating into the feeding site (Bowman et al., 2004). Several studies revealed two other functions of salivary glands not related to feeding. These included salivary secretions by males during copulation and the use of salivary glands for water uptake from unsaturated air while they are not feeding (Sauer, 1977 and Needham and Teel., 1986). Ticks survive prolonged periods without a blood meal by maintaining water balance (reviewed by Bowman et al., 2004). The most common route of pathogen transmission appears to be via the salivary glands (Sauer et al., 1995 and Bowman et al., 1997). Anti-platelet aggregatory, anticoagulatory and anti-vasoconstrictory factors in tick saliva thwart the host's haemostatic mechanisms to facilitate a continuous blood flow to the feeding lesion. Salivary components also suppress the immune and inflammatory response of the host facilitating the ticks to attach the host prolong. This process permits and enhances the tick-borne pathogen transmission and establishment (Bowman et al., 1997). For some viral pathogens, there is a less cell specificity within tick salivary glands whereas for some protozoan pathogens there is high cell specificity (reviewed by Bowman et al.,

2008). Understanding the specific interactions between pathogens and salivary glands is likely to identify novel blocking targets for pathogen transmission.

Control and mechanism of salivation

Salivation is under neural regulation and direct innervations of salivary nerves are supported by induction of salivation with the neurotransmitter dopamine (Sauer et al., 1995). Action of dopamine in the regulation of saliva secretion is through a G protein-coupled receptor which eventually activates the adenylate cyclase and forms cyclic AMP (cAMP) (Sauer et al., 1995). cAMP-dependent protein kinase, which is involved in protein phosphorylation, has also been identified in *A. americanum* salivary glands, but the specific functions of phosphoproteins in salivary gland fluid secretion have not yet been identified (Sauer et al., 2000). There are several other receptors have been identified other than G protein-coupled receptors that are also involved in controlling salivary gland function. Some of these are for dopamine antagonists such as spiperone, pimozide and haloperidol while others are for neurotransmitter inhibitors such as γ -aminobutyric acid (GABA) and ergot alkaloids. All these potentiate the activity of dopamine on saliva secretion except the ergot alkaloid receptor. Ergot alkaloid receptor inhibits stimulating the salivation but does not reduce the salivary glands response to dopamine (Sauer et al., 1995). Sauer et al. (2000) suggested that water transport in tick salivary gland may occur via transmembrane aquaporin following dopamine initiation of cAMP production, protein phosphorylation and eventual fluid secretion (Sauer et al., 2000). Dopamine also acts as a stimulant for the opening of voltage-dependent Ca^{2+} channels in salivary gland (Bowman et al., 1995). Influx of Ca^{2+} to the cell activates the cyclic phospholipase A₂ (cPLA₂)

which increases the levels of free arachidonic acid in salivary glands (Bowman et al., 1995). Arachidonic acid constitutes of about 9% of the total fatty acids in tick salivary glands and during feeding salivary gland arachidonic acid content increases rapidly (Shipley et al., 1993). Arachidonic acid is the source for prostaglandin 2-series (PGE₂) biosynthesis in tick salivary glands via cyclooxygenase (COX) pathway and assumed to be a dietary source for ticks since they lack the ability to synthesize arachidonic acid *in vivo* (Bowman et al., 1995). PGE₂ may be secreted with saliva or it can interact with PGE₂ receptor in salivary glands and stimulate an increase of inositol triphosphate (IP₃) mobilizing intracellular Ca²⁺ to stimulate secretion of salivary gland proteins (Sauer et al., 2000 and Bowman et al., 2001). A recent study on tick nervous system have identified four types of peptidergic neurons in the synganglion, each innervating different parts of the salivary gland, producing distinct neuropeptides and have a highly characteristic anatomy (Šimo et al., 2009a). They hypothesize that each of these neuronal types controls the activity of specific secretory cells or duct contraction of a feeding tick. Two novel neuropeptides, myoinhibitory peptide (MIP) and SIFamide have been identified from *I. scapularis* and their proposed functions might be antagonistic with MIP showing an inhibitory function and SIFamide showing a stimulatory function (Šimo et al., 2009b). The overall action of dopamine in secretory regulation in salivary glands is summarized in the Figure 1 (reproduced from Sauer et al., 2000).

Salivary gland degeneration

After the immature ticks or the mated females are fed to repletion, the cells in the salivary gland lose the secretion capability followed by autolysis and salivary gland degeneration (Sonenshine, 1991). Kaufman (1986) suggested that the salivary gland

degeneration following the blood meal uptake is controlled by a humoral factor “tick salivary gland degeneration factor” (TSGDF) which is identified as an 20-hydroxyecdysone (Kaufman, 1986). Within 3-4 days after repletion, ticks lose over 95% of their fluid secretion capability. Even partially fed small ticks (0.18-0.29 g in weight) lose about 75% of fluid secretory competence within 4-5 days after removal from the host (Kaufman, 1986). Severing opisthosomal nerves can inhibit the salivary gland degeneration and this is believed to be due to the lack of abdominal stretching for the secretion of TSGDF. Salivary gland degeneration is also triggered by copulation. It has been suggested that a male factor accelerates this process and is a protein or a peptide (Sauer et al., 1995). Salivary gland degeneration in replete females is unlikely to be reversed whereas partially fed small ticks can restore the salivary glands by reattaching to a host later (Kaufman, 1986).

Gene expression in Ixodid tick salivary glands

During blood feeding female tick salivary glands undergo growth, differentiation and development and there is also a sequential buildup and reduction of materials in specific salivary gland cells (McSwain et al., 1982). Some proteins appear to be synthesized feeding ticks in response to tick attachment and feeding whereas the others are abundant in partially fed ticks suggesting that they secreted or converted to other substances during feeding (McSwain et al., 1982). McSwain et al., (1982) identified 31 different proteins between unfed and fed female *A. americanum*. Mating or feeding to repletion acts as stimuli to the amount of protein secreted, but not the differential protein synthesis (McSwain et al., 1982). The changes in proteins and salivary gland cytology are

also accompanied by a significant increase of RNA synthesis at the onset of feeding (Oaks et al., 1991). A differential gene expression between the slow feeding and the fast feeding stages in adult *A. americanum* females has been observed. At least eight genes were found to express during the fast feeding stage producing proteins ranged from 8- 129 kDa (Oaks et al., 1991). Protein and RNA profiles of the male salivary glands can vary both qualitatively and quantitatively (Bior et al., 2002). Saliva quantity limits the analysis of salivary protein using traditional protein chemistry and abundant host proteins can cause problems in characterizing salivary gland proteins using novel proteomics approaches (Madden et al., 2002). Recent expressed sequence tags (ESTs) analyses, microarray analyses and transcriptome studies provided a significant insight into differential gene expression in the salivary glands of different tick species (Valenzeula et al., 2002, Nene et al., 2004, Santos et al., 2004, Francischetti et al., 2005, Alcorn-Chaidez et al., 2007, Aljamali et al., 2009 and Aljamali et al., 2009). Aljamali et al., (2009) found that gene expression in female lone star ticks was dramatically higher at the beginning of the feeding and towards the end of feeding. They proposed that genes up-regulated at the onset of the feeding may be involved in survival on the host and the genes that are up-regulated towards the end of feeding are transport-associated genes involved in organ degeneration (Aljamali et al., 2009).

Tick midgut

Tick midgut is the digestive organ of the tick and the largest organ in the tick body. The midgut consists of a ventriculus, numerous blind diverticula (caeca) and the rectal tube (Coons et al., 1986). The blood meal undergoes hemolysis in the midgut lumen and it is thought that digestion is completely intracellular (Balashov, 1972). The uptake of the blood meal occurs through receptor-mediated endocytosis (Tarnowski et al., 1989).

Blood meal digestion

The blood meal digestion consists of three phases. During the first phase the ingested cells undergo rapid agglutination and hemolysis and the slower second phase, which may last up to several weeks to several months, assimilates the hemoglobin mass into gut cells. During the third phase, in adult unfed ticks, semi-digested hemoglobin is consumed slowly (Jaworski et al., 1983). The process of digestion in mated females is also differentiated into three distinct phases. First phase is initiated by feeding and is a continuous digestion occurs during slow engorgement. The second phase, reduced digestion occur during the rapid engorgement, is initiated by mating. Third phase of the digestion is a continuous digestion initiated by detachment from the host and occurs throughout the post-feeding period (Tarnowski et al., 1989).

Tick midgut ultrastructure and digestion process

Raikhel (1978) identified two types of digestive cells in unfed and feeding nymphs of *Hyalomma asiaticum*, whose activity varied depending on the physiological stage of the tick (Raikhel, 1978). Jaworski et al., 1983 observed the changes in midgut ultrastructure with age and observed decrease in the size of siderosomes (iron containing electron-dense particles) suggesting that hemoglobin is digested within these particles (Jaworski et al., 1983). Ultrastructural changes have also been observed in the amount of lipids, siderosomes myelinosiderosomes (hemosiderin with myelin figures) in different aged ticks (Williams et al., 1985). Scanning electron microscopy has revealed the presence of microvilli in Ixodid tick midgut epithelium increasing the surface area and hence the efficiency of membrane transport. In ticks these microvilli are supposed to be involved in receptor mediated endocytosis (Agbede, 1986). Agbede and Kemp, 1987 suggested that basophilic cells (digestive cells) in the gut involve in active transport of ions to create an osmotic gradient to draw water from the lumen and digestive cells across the gut wall into the hemolymph (Agbede et al., 1987 and Tarnowski et al., 1989). In unfed ticks the midgut epithelium is lined by stem cells and empty digest cells filled with hematin. Upon the attachment to the host the stem cells differentiate and hematin is lost from the midgut epithelium and digestive activity starts from the anterior end of the midgut (Ageyi et al., 1995).

The midgut epithelial cells (stem cells) of unfed ixodod ticks acts as the precursor cells of replacement, digestive and vitellogenic cells in the mated female midgut (Tarnowski et al., 1989). These digestive cells are found in all three phases of the blood meal digestion. The digestive cells filled up with residual bodies, rupture and slough or

the whole cell slough into the lumen only during the first continuous digestion phase and the replacement cells are found only during this phase. At the end of the oviposition the digestive cells are filled with residual bodies. Presumed vitellogenic cells were found during both the reduced digestion phase as well as during the second continuous digestion phase (Tarnowski et al., 1989).

The midgut is one of many other organs involved in the synthesis and/or processing of vitellogenin and there is a feeding signal associated with the initiation of synthesis and/or processing (Rosell et al., 1992). After detaching from the host, concentrations of protein and hemoglobin decrease to about 50% of the initial concentration whereas hematin increases by about two-fold (Tarnowski et al., 1989). The secretory activity in the midgut is continuous throughout the tick's life. The lysosomal enzyme containing cell number varies with the phase of feeding period and the secretory cells are loaded and present before the blood meal reaches the midgut (Agyei et al., 1991). An accumulation of RNA in the midgut epithelia at the time of attachment and prior to the arrival of blood meal suggested that the midgut epithelial cells act as secretory cells (Agyei et al., 1992). Acid phosphatase containing vesicles are elongated or tubular in shape and involved in blood meal digestion and identified as tubular lysosomes. They share morphological and cytochemical characteristics with similar structures in mammals (Gough et al., 1995). The presence of enzymes such as peroxidase and alkaline phosphatases indicate that midgut digest cells are multifunctional and have both secretory and digestive activities (Agyei et al., 1992). In addition to these two functions, the midgut epithelium can also serve as a sink to bind the by-products of digestion and facilitate excretion (Agyei et al., 1992).

It has been observed that the midgut epithelium ultrastructure of *Haemaphysalis longicornis* nymphs also changes as the feeding progresses. During the unfed stage, it functions as a nutrition reserve; and during feeding stage, it serves as a digestive organ (Koh et al., 1991).

In a transcriptome study of the American dog tick, *D. variabilis* midgut, a total of 82 transcripts belonging to 11 functional categories, were identified as putative proteins that are directly involved in blood meal digestion (Anderson et al., 2008).

Macrophage Migration Inhibitory Factor (MIF)

Macrophage migration inhibitory factor (MIF) was among one of the first cytokines to be identified about 40 years ago (Calandra et al., 2003). This molecule has been identified as a component in the host antimicrobial response system and promotes pro-inflammatory functions of immune cells. Cytokines are essential molecules to initiate the host inflammatory response and to coordinate cellular and humoral responses. These responses eventually eradicate or suppress the invasive pathogen (Calandra et al., 2003). It was named for its activity in inhibiting random macrophage migration *in vitro* (Bucala et al., 2003).

MIF is different from other cytokines by two major characteristics. That is MIF signaling is occurring through a cell surface receptor and that it catalyzes chemical reactions through an enzymatic active site, both of which are not observed with other cytokines (Lolis et al., 2009). Presence of human MIF homolog has been confirmed in many organisms including mammals, nematodes, arthropods, jawed/jawless fish, plants, cyanobacteria and parasites but not found in the insects from order Diptera (Pastrana et

al., 1998, Jaworski et al., 2001, Sato et al., 2003, Calandra et al., 2003, Jin et al., 2007, Cordery et al., 2007 and Ito et al., 2008 and). Cross species conservation of MIF protein suggests that it has an important biological function.

Structure and function of MIF

Crystal structure for Ixodid tick MIF is not available. Structural information is available for MIFs from rat, nematodes, parasites, human and amphibians (Suzuki et al., 1996, Sun et al., 1996, Sugimoto et al., 1996, Cho et al., 2007, Orita et al., 2001, Shao et al., 2010, Dobson et al., 2009, Kamir et al., 2008, Richardson et al., 2009 and Suzuki et al., 2004). The Protein Data Bank lists about 45 different crystal structures for MIF from various organisms and all of these proteins exist as trimers (<http://www.rcsb.org/pdb/home/home.do>). Each monomer consists of two $\beta/\alpha/\beta$ motifs, in which two parallel β sheets are in the N-terminal and other two parallel β sheets are in C-terminal opposing the direction of N-terminal sheets. Two α helices are found in the same side of the β sheets (Suzuki et al., 1996). Three monomers aggregate to form a tightly packed trimer structure with extensive hydrogen bonds reflecting the physiological structure for this protein (Suzuki et al., 1996). The trimer forms an inner core lined by four β strands of each monomer and the two α helices of each monomer are located external to the β strands (Zang et al., 2002). This structure is more or less conserved among all the crystal structures of MIF known to date. MIF shares a common subunit topology with *E. coli* 5-carboxymethyl-2-hydroxymuconate isomerase (CHMI) although the sequence similarity is very limited (Suzuki et al., 1996). MIF is known to enzymatically catalyze the keto enol tautomerization of small aromatic molecules such as

dopachrome and phenylpyruvate (Zang et al., 2002). The N-terminal proline (Pro-2) has been identified as an essential residue for tautomerase activity in MIF in both human and *Brugia malayi* and it acts as the base catalyst for the tautomerization (Zang et al., 2002). The tautomerase active site is composed of amino acid residues 1, 33-34 and 64-66 (Dobson et al., 2009). Certain parasites have two forms of MIF protein and only a single form shows the tautomerase activity and the loss of function is due to the different conformation of amino acid residues in the three dimensional protein structure (Richardson et al., 2009). Many MIF sequences also have a conserved oxidoreductase domain containing two cysteine residues at 56th and 59th positions in the amino acid sequence (Suzuki et al., 2004). Although MIF shows both tautomerization and oxidoreductase activities, their physiological significance is not understood (Dobson et al., 2009). Some of the parasitic MIFs have been found to bind to the cell surface human MIF receptor CD74 but the specific surface contact between the receptor and MIF is unknown (Cho et al., 2007, Kamir et al., 2008 and Dobson et al., 2009). The ribosomal protein S19 (RPS19) has also been identified to interact with MIF and low doses of RPS19 strongly inhibit MIF-CD74 interactions suggesting that RPS19 to be an extracellular negative regulator of MIF (Filip et al., 2009).

MIF in humans

MIF expression has been identified in T cells, monocytes, macrophages, blood dendritic cells, B cells, neutrophils, eosinophils, mast cells and basophils. MIF expression is notably observed in tissues that have a direct contact with host's natural environment such as, the lung, skin epithelial lining, gastrointestinal and gastrourinary tracts. Several

tissues in the endocrine system, especially organs involved in stress response such as hypothalamus, pituitary gland and adrenal gland, have shown a high level of MIF expression (Calandra et al., 2003). Unlike many other cytokines, MIF is constitutively expressed and stored as intracellular pools (Calandra et al., 2003). MIF activates extracellular signal-regulated kinase 1/2 (ERK1/ERK2) members of the mitogen-activated protein kinases (MAPKs) (Calandra et al., 2003). MIF binds to the extracellular domain of CD74, the cell-surface form of the MHC class-II-associated invariant chain. CD74 is required for the downstream activities such as MIF-induced activation of the ERK1/2 MAPK cascade, cell proliferation and PGE₂ production (Leng et al., 2003). Adhesion of cells to fibronectin in mouse fibroblasts induces the secretion of MIF inducing ERK/MAPK activation (Liao et al., 2003). MIF also promotes the recognition of lipopolysaccharides (LPS) and Gram-negative bacteria by cells of the innate immune system by up-regulating the expression of Toll-like receptor 4 (TLR4). It has been shown that MIF has a synergistic effect with low concentrations of LPS (Kudrin et al., 2006). This enhances the production of inflammatory cytokines such as TNF- α and initiates the host defense response (Roger et al., 2001). The high expression of MIF in primary tumors and different tumor cell lines associates MIF as a negative regulator of p53-mediated growth arrest and apoptosis (Calandra et al., 2003). MIF physically interacts with the p53 tumor suppressor and this negative regulation of p53 is essentially linked to the cysteine residue 81 (Jung et al., 2008).

MIF expression in human diseases

MIF has been identified as a critical mediator of immune and inflammatory diseases since it has broad regulatory properties. They include septic shock, rheumatoid arthritis, delayed-type hypersensitivity, inflammatory lung diseases and cancer (Lue et al., 2002). Elevated expression of MIF has been identified in alveolar endothelium and infiltrating macrophages in lung tissues from patients suffering from acute respiratory distress syndrome (ARDS). MIF was found to induce the TNF- α and lead to alveolar inflammation in ARDS (Lai et al., 2003). Increased expression of MIF enhances the invasive capacity of the tumor cells (Hagemann et al., 2005). High level of MIF expression has been also detected in the basal and supra-basal keratinocytes of scleroderma affected patients with diffuse cutaneous systemic sclerosis (dcSSc) (Wu et al., 2006). Neuroblastomas also have an elevated expression of MIF and it is suggested that MIF may initiate a pathway to suppress T cell immunity leading to partly suppress the antitumor immunity (Zhou et al., 2008). High levels of MIF in breast cancer tissue induce angiogenesis leading to breast cancer (Xu et al., 2008). The number of MIF expressed cells in the mucosa of colorectal carcinoma (CRC) patients is 20-40 times higher than in normal tissue and this increase is only observed in the diseased tissue (He et al., 2009). Overexpression of MIF in ovarian cancer contributes to the immune evasion of ovarian carcinoma by transcriptionally down-regulating NK cell receptor group 2D (NKG2D) (Krockenberger et al., 2008). Knocking down of MIF using RNAi or anti-MIF antibodies has shown to reduce the risk of disease (Hagemann et al., 2005 and He et al., 2009). A well-developed case for therapeutic antagonism is available for rheumatoid arthritis using anti-MIF antibodies or genetic MIF deficiency (Morand, 2005). Similar

studies have also been done using MIF-deficient mice in models of atheroma, colitis and multiple sclerosis (Morand, 2005). Administration of anti-MIF antibodies has shown beneficial effect in autoimmune diabetes, autoimmune myocarditis, experimental allergic neuritis and atherosclerosis (Cvetkovic et al., 2006). In addition to anti-MIF antibodies, chemical inhibitors and plant-derived inhibitors such as *N*-acetyl-*p*-benzoquinone imine (NAPQI), curcumin and caffeic acid have been identified as potent inhibitors of MIF (Cvetkovic et al., 2006).

The role of MIF in human immunity

MIF induces the phagocytosis of foreign particles by macrophages (Onodera et al., 1997). MIF has been identified as a critical mediator of LPS mediated endotoxemia and Gram-negative septic shock (Bernhagen et al., 1998). MIF promotes the recognition of LPS and Gram-negative bacteria by cells of the innate immune system by up-regulating the expression of Toll-like receptor 4 (TLR4). This enhances the production of inflammatory cytokines such as TNF- α and initiates the host defense response (Roger et al., 2001). Responses to the bacterial infections have been useful to study MIF as a novel therapeutic target in patients with septic shock (Froidevaux et al., 2001 and Calandra et al., 2003). Administration of recombinant MIF can reduce the severity of parasitic bacterial and viral infections (Calandra, 2003 and Calandra et al., 2003). Transcription factors specificity protein 1 (Sp 1) and cAMP response element binding protein (CREB) are two main positive regulators of constitutive human MIF expression (Roger et al., 2007). Immune cells rapidly release MIF when they are exposed to microbial products or to pro-inflammatory cytokines and during antigen-specific activation and it is an essential component of host inflammatory responses (Calandra et al., 2003). MIF is incompatible

with other cytokines in its pro-inflammatory nature in that it is induced by glucocorticoids rather than inhibited by glucocorticoids hormones (Calandra et al., 1995).

MIF and Glucocorticoids

Glucocorticoids are anti-inflammatory molecules which have a regulatory effect on the host immune responses (Flaster et al., 2007). The inhibition of the inflammation is performed by affecting several signaling pathways. Glucocorticoids exert their effect by reducing the production of pro-inflammatory cytokines, prostaglandins, reactive oxygen and nitrogen species, which are inflammatory mediators. They also reduce the migration of leukocytes to the inflammation site by inhibition of adhesion molecules and directing leukocytes for apoptosis (Flaster et al., 2007). These anti-inflammatory properties have enabled glucocorticoids to be used in treatments for various inflammatory and autoimmune diseases (Molle et al., 2005). However, the use of glucocorticoids induces significant side effects such as osteoporosis, diabetes and hypertension. Therefore, the use of glucocorticoids is limited (Molle et al., 2005). Calandra et al. (1995) has reported that low concentrations of glucocorticoids induce production of MIF that acts to override glucocorticoids-mediated inhibition of cytokine secretion by lipopolysaccharide-stimulated monocytes (Calandra et al., 1995). They postulated that MIF might counter-regulate the anti-inflammatory activities of glucocorticoids and concluded that both act together to regulate inflammation and immunity. Several pathways to explain interactions between glucocorticoids and MIF have been proposed and the regulation of glucocorticoid-sensitivity by MIF occurs via modulation of the MAPK and phospholipase A2 (PLA2) activation, hydrocortisone-induced increases in cytosolic I κ B α expression and

activation of ERK MAPK pathways (Fingerle-Rowson et al., 2003 and Flaster et al., 2007). Its role has also been described as a concentration dependent responsiveness to the glucocorticoids (Kudrin et al., 2006). It has been suggested that MIF antagonism of glucocorticoids could supplement steroids providing the first definitive steroid-sparing therapy (Aeberli et al., 2006).

MIF in parasitic nematodes

Homologs of human macrophage migration inhibitory factor have been identified in many parasitic and some free living nematodes. To date, there are 35 different nematode MIF sequences identified from 28 different organisms (Vermeire et al., 2008). A MIF homolog in nematodes was identified from a *B. malayi* infective-stage larva with a 42% amino acid sequence identity to human and murine MIF (Pastrana et al., 1998). Localization of the protein revealed that it is expressed in the hypodermis/lateral chord, the uterine wall and larvae developing in utero. In a macrophage migration assay, recombinant *B. malayi* MIF and human MIF inhibited the macrophage migration equally. Recombinant *B. malayi* MIF was shown to have significantly lower level of tautomerase activity compared to mammalian MIFs. The level of MIF expression in *B. malayi* adults and microfilariae (Mf) stages is approximately twice compared to larvae 3 (L3) and larvae 4 (L4) stages (Pastrana et al., 1998). *B. malayi* MIF has been identified as a member of the immune evasion cytokines found in filarial nematodes (Maizels et al., 2001). Four distinct MIF homologues have been identified from the free living nematode *Caenorhabditis elegans*; *Ce-mif-1*, *Ce-mif-2*, *Ce-mif-3* and *Ce-mif-4*; each of the genes residing on a different chromosome with a distinct genomic organization varying from

three to five exons with the exception of *Ce-mif-1* (Marson et al., 2001). The level of amino acid sequence identity among *C. elegans* MIFs ranged between 15-30% and between 22-35% to mammalian MIFs. *Ce-mif-1, 2 and 4* encode for 117, 120 and 121 amino acids respectively whereas *Ce-mif-3* encodes 146 amino acids producing a larger MIF protein of 16.4 kDa (Marson et al., 2001). *Ce-mif-1, Ce-mif-2 and Ce-mif-3* genes are transcribed by eggs, major larval stages (L1-L4) and adults. When the *C. elegans* larvae enters a reversible developmental arrest called dauer larvae, *Ce-mif-1* and *Ce-mif-3* transcription levels increases over 100-fold suggesting a potential role of MIF protein in homeostasis mechanisms under stressed conditions (Marson et al., 2001). MIF homologs identified so far in other parasitic nematodes represent four major clades of the phylum Nematoda (Vermeire et al., 2008). Amino acid sequence alignments of the 35 MIF proteins differentiate the proteins into two different types, MIF-1 and MIF-2, based on the homology to *Ce-mif-1* or *Ce-mif-2*. The MIF-1 type proteins share an amino acid sequence identity between 18%-51% with *Ce-mif-1* whereas MIF-2 type proteins share 28%-65% sequence identity with *Ce-mif-2* (Vermeire et al., 2008). MIF proteins characterized from different parasitic nematodes have exhibited activities similar to that of mammalian MIF (Falcone et al., 2001; Pastrana et al., 1998; Tan et al., 2001; Zang et al., 2002 and Wu et al., 2003).

MIF in Ixodid ticks

Homologs of human macrophage migration inhibitory factor (MIF) have been characterized from ticks *A. americanum* and *H. longicornis* (Jaworski et al., 2001 and Umemiya et al., 2007). A putative MIF homolog was also identified from the midgut

transcriptome of *D. variabilis* (Anderson et al., 2008). Lone star tick MIF was identified from a midgut cDNA library of 3-day fed female ticks and the expression was detected in salivary gland and midgut using antiserum to a tick-MIF specific antibody (Jaworski et al., 2001). The tick origin of MIF was confirmed by sequencing the gene from tick genomic DNA. The open reading frame for cDNA clone was 348 nucleotides and encoded a peptide of 116 amino acids. The *A. americanum* MIF gene consisted of three exons of 108, 173 and 67 bases intervened by two large introns of 647 and 1382 bases from 5'-3' end (Jaworski et al., 2001). In a macrophage migration assay, recombinant tick MIF and recombinant human MIF showed equal activity in inhibiting the migration of macrophages (Jaworski et al., 2001). *H. longicornis* MIF was also characterized from ESTs constructed from cDNA libraries produced from midgut tissues of partially fed tissues. The deduced amino acid sequences in *A. americanum* and *H. longicornis* MIF proteins shared a 77% sequence similarity (Umemiya et al., 2007).

Functions of tick MIF

Possible biological functions for tick MIF, as suggested by Jaworski et al., (2001) are to increase inflammation at the feeding site facilitating blood meal uptake and to inhibit the macrophage migration toward the mouth parts as the tick feeds or within tick midgut after feeding. Umemiya et al. (2007) suggested that tick MIF may involve in the proliferation and differentiation of cells in the tick body. Lengthening of the Lone star tick feeding intervals following the tick MIF peptide injection to the host indirectly support that MIF is secreted into the host with tick saliva during feeding (Jaworski et al., 2009).

Expression and localization of MIF in ticks

An increase in the expression levels of MIF has been observed in the salivary glands, midgut and integument tissues of *H. longicornis* fed adults compared to unfed adult tissues. The increase in the expression of *H. longicornis* MIF in salivary glands and midgut tissues was also observed in an immunoblot analysis using polyclonal antibodies against recombinant *H. longicornis* MIF (Umemiya et al., 2007). An immunohistochemical assay in partially fed *H. longicornis* adult ticks showed a strong binding of antibodies in the cytoplasm of midgut cells and epidermal cells and a slightly weaker binding to salivary gland cells. Immunization of New Zealand white rabbits with tick MIF specific peptide have shown to lengthen the *A. americanum* feeding intervals by almost one day, suggesting that tick MIF is neutralized in the feeding lesion by circulating anti-tick MIF antibodies (Jaworski et al., 2009). In Lone star ticks, highest MIF expression was observed in midgut epithelial tissue of 5 days fed females and the expression is decreased near the end of feeding (Bowen et al., 2010). Localization of MIF using specific antibody has confirmed the abundance of protein in midgut tissues at 5 days of feeding. Tick MIF gene silencing using RNA interference in *A. americanum* adult females has not shown any impact on gross physiological feeding parameters (Bowen et al., 2010).

CHAPTER III

MATERIALS AND METHODS

Ticks

Adult *Dermacentor variabilis* ticks were purchased from the National Tick Research and Education Facility at Oklahoma State University. They were maintained at 96% relative humidity with saturated K₂SO₄ solution and 12:12 (light: dark) photoperiod prior to use. Standard tick rearing procedures were utilized for the production of American dog ticks on sheep and rabbit hosts (Patrick and Hair, 1975).

Tick Infestation

Ticks were placed in stockinette cells that were glued to the backs of shorn sheep. Tick infestations were done in groups of 30 tick pairs per cell. Female ticks were collected at feeding intervals of 0, 8, 24, 48, 72 and 96 hrs after they are attached to the host.

For RNAi studies, ticks were placed on sheep so at least one dsRNA or buffer-only group was located on either side of the sheep. Partially fed female ticks were collected after 72 hrs after attached to the host and remaining females were allowed to replete. All the ticks were allowed to reach final engorgement were individually weighed after collection on a Mettler-Toledo AT-20 analytical balance (Mettler-Toledo, Columbus, OH).

To verify whether tick RNA was contaminated with sheep blood RNA, unfed and partially fed tick midgut RNA were checked for the presence of sheep glyceraldehyde-6-phosphate dehydrogenase using specific primer set (Bowen et al., 2010).

Tick dissections

Harvested ticks were dissected to obtain salivary gland (SG) and midgut (MG) tissues. Tissues from each feeding interval were pooled and collected into 500µl Tri-Reagent (Molecular Research Center, Cincinnati, OH). Collected tissues were frozen in liquid nitrogen and stored at -80⁰C.

Double stranded RNA synthesis

Double-stranded RNA (dsRNA) for *D. variabilis* was synthesized using modified methods based on those developed by de la Fuente et al. (2006). Briefly, *D. variabilis* RNA was amplified using T7MIFxQ primer set using Ambion Ag-Path ID™ One-Step RT-PCR protocol (Ambion Inc. Austin, TX). Ribomax™ Large Scale RNA Production System- T7 (Promega corporation, Madison, WI) was used to synthesize RNA with T7 promoters. Next, Megascript® RNAi Kit (Ambion Inc. Austin, TX) was used for transcription and purification. dsRNA was quantified using a Nanodrop ND-1000 (Thermo Scientific, Wilmington, DE) and stored at -80⁰C. The dsRNA was later thawed on ice and standardized to 1µg/µl prior to administer into ticks. As a positive control, subolesin dsRNA was administered into female ticks (de la Fuente et al., 2006).

Three experimental groups of 60 female ticks were used: dsRNA (MIF)-injected, dsRNA (Subolesin)-injected and buffer-only injected. Ticks were secured on masking

tape adhered to a glass plate, positioning ventral side up. A Hamilton 10 μ l syringe (Hamilton Company, Reno, NV) was used to inject ~ 1.0 μ l of dsRNA into each tick, delivering ~ 1x 10⁸ molecules of dsRNA per tick. After injection, ticks were transferred to a fresh container and allowed to recover overnight in a humidity chamber before the sheep was infested.

RNA isolation

Total RNA was extracted from tissues homogenized in Tri-Reagent using manufacturer's RNA isolation protocol. Briefly, the tissue was ground in Tri-Reagent and incubated at room temperature. The RNA was separated into the aqueous layer by adding chloroform and shaking vigorously. RNA was then precipitated using iso-propanol. The RNA pellet was washed with 75% ethanol and the final RNA pellet was reconstituted in nuclease free water (Ambion, Austin, TX) and stored at -80⁰C. RNA concentration was estimated using NanoDrop ND-1000 (Thermo Scientific, Wilmington, DE) and using the estimated RNA concentrations, working solutions were standardized to 10ng/ μ l.

For rapid amplification of cDNA ends (RACE), female ticks were pooled from each feeding interval and lyophilized with liquid nitrogen and macerated to a fine powder. The powder was re-suspended in 500 μ l Tri-Reagent for RNA isolation. Working RNA solutions were standardized to 250ng/ μ l.

Protein Isolation

Protein extractions were performed using tissues homogenized in Tri-Reagent using manufacturer's protein isolation protocol. Briefly, after the aqueous layer was removed for RNA isolation, the remaining interface and organic layer was precipitated with 100% ethanol, mixed by inversion and centrifuged. The resulting phenol-ethanol supernatant was used for the protein isolation. The protein was precipitated by adding 3 volumes of acetone and the pellet was washed 3 times with guanidine hydrochloride/ethanol/glycerol mix. A final wash was done with 95% ethanol/2.5% glycerol mix. The pellet was dried at room temperature and the final protein pellet was reconstituted in 1X PBS with 1% SDS and stored at -20⁰C. Protein concentration was estimated using NanoDrop ND-1000 and working solutions were standardized to 1µg/µl.

Primers

Reverse transcriptase PCR (RT-PCR) and reverse transcriptase-relative quantitative PCR (RT-qPCR) were performed using MIF1xQ primers which yielded a 180bp product (Bowen et al., 2010). Tick 16S rRNA was used as the control for the RT-PCR whereas human 18S rRNA was used as the internal control to normalize the gene expression in RT-qPCR experiments. MIF1xQ primers were generated using *Amblyomma americanum* MIF sequence (Jaworski et al., 2001, Bowen et al., 2010) and the primers were designed to cross an intron to prevent genomic DNA amplification (Fernando et al., 2006). For dsRNA synthesis, T7MIF1xQ primers were used for the initial amplification of *D. variabilis* RNA. Gene Specific Primers (GSPs) for rapid amplification of cDNA ends (RACE) were designed using the initial sequence obtained

for *D. variabilis* MIF. All the primers were generated by Integrated DNA Technologies (IDT) (IDT DNA, Coralville, IA) and were used at the concentration of 10 μ M (Table 1). Amplifications were performed using the PTC-100TM thermocycler (MJ Research Inc.).

Reverse transcriptase PCR

Using the previously established methods of Bowen et al. (2010), reverse transcriptase PCR (RT-PCR) was performed using the Ambion Ag-Path IDTM One-Step RT-PCR protocol. Amplification conditions were 50⁰C for 4 min, 95⁰C for 15 min, 35 cycles of 94⁰C for 1 min, 52⁰C for 1 min and 68⁰C for 1min followed by 72⁰C for 10 min then stored at 4⁰C.

Agarose gel purification and DNA sequencing

The PCR products were excised from the agarose gel and gel purified using GENECLAN[®] II kit (MP Biomedicals, Solon, OH) using manufacturer's protocol. Concentration of the purified product was estimated by agarose gel electrophoresis together with Low DNA MassTM Ladder (Invitrogen Corporation, Carlsbad, CA). DNA sequencing was performed by the Biochemistry Core Facility, Oklahoma State University using ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA) and specific primers used for amplifications or T7 primers in the vector.

Cloning, transformation, cell growth and plasmid isolation

Gel purified product was cloned into a pGEM[®]-T Vector System (Promega Corporation, Madison, WI), transformed into *E.coli* JM109 Competent cells and plated on 1.5% agar in Luria-Bertoli media with 0.2 μ g/ μ l ampicillin, 100 μ l of 0.1M Isopropyl

β -D-1-thiogalactopyranoside (IPTG) and 1 mg of bromo-chloro-indolyl-galactopyranoside (BCIG/X-Gal) then incubated overnight. Plates were screened for the presence of blue or white colonies and a single white colony was transferred to 50 ml of Luria broth with 0.2 μ g/ μ l ampicillin then incubated overnight. Eppendorf Fast Plasmid[®] Mini Prep kit (Eppendorf North America, USA) was used to plasmid extraction from bacteria according to manufacturer's guidelines. Plasmid isolation was confirmed by analyzing them on a 1.5% agarose gel with 0.1% ethidium bromide. NanoDrop ND-1000 was used to estimate the plasmid yield.

Rapid amplification of cDNA ends (RACE)

Gene specific primers (GSP 1 and 2) for *D. variabilis* MIF were made using the initial sequence obtained from RT-PCR. Marathon[®] cDNA Amplification Kit and Advantage[™] 2 Polymerase Mix (Clontech Laboratories Inc. Mountain View, CA) were used for the RACE reactions using manufacturer's guidelines. First stranded synthesis was performed using 1 μ g of total RNA at 42⁰C for 1 hr immediately followed by the second strand synthesis according to manufacturer's guidelines. Adaptor ligation was performed at 16⁰C overnight and the adaptor ligated cDNA was diluted 1:50 with Tricine-EDTA buffer, to be used in 3' and 5' RACE reactions, and stored at -20⁰C. 5' and 3' RACE reactions were performed at optimized conditions using adaptor ligated cDNA, GSP primers and adaptor primers. Amplification conditions for 5' RACE were 94⁰C for 30 s, 35 cycles of 94⁰C for 5 s, 70⁰C for 2 min then 4⁰C and 3' RACE amplification conditions were, 94⁰C for 30 s, 35 cycles of 94⁰C for 5 s, 68⁰C for 2 min then 4⁰C. Amplification product from each reaction were analyzed on 1.5% agarose gels

with 0.1% ethidium bromide, recovered from the gel, cloned into a pGEM[®]-T Vector System, transformed into *E.coli* JM109 Competent cells and plate on 1.5% agar in Luria-Bertoli media with 0.2µg/µl ampicilin then incubated overnight. Plates were screened for the presence of white colonies and a single colony was transferred to 50 ml of Luria broth with 0.2µg/µl ampicillin then incubated overnight. Eppendorf Fast Plasmid[®] Mini Prep kit was used to plasmid extraction from bacteria. Insert was sequenced with T7 primers using ABI 3730 DNA Analyzer. The full length cDNA sequence for *D. variabilis* MIF was deduced by performing sequence alignments of 3' and 5' RACE sequences with known tick MIF sequences individually and manually editing them using BioEdit Version 7.0.9.0 sequence editing software (Hall, 1999).

Reverse transcriptase- relative quantitative PCR

Reverse transcriptase–relative quantitative PCR (RT-qPCR) was performed using a modified AgPath-ID[™] One-Step RT-PCR kit (Ambion). The AgPath 2X RT-PCR buffer was replaced with FastStart Universal SYBR Green Master (ROX) (Roche Diagnostics, Mannheim, Germany). The primers were diluted to 10 µM working stock and 5ng of RNA was used per reaction. Ag-Path ID protocols were followed using appropriate reagent volumes for a 25µl final volume. Samples were mixed in 96-well plates and the reaction was performed in an Applied Biosystems 7500 Real Time PCR System (Applied Biosystems, Foster City, CA). Amplification conditions used were 50⁰C for 2 min, 95⁰C for 15 min, 40 cycles of 95⁰C for 15 s and 58⁰C for 1 min. The default dissociation step was added following the 40 cycles. Ct values were exported into Microsoft Excel and gene expression levels were normalized against H18S rRNA controls. At least Ct values from four replicates were averaged and the averaged Ct

values were used for downstream delta Ct calculations. The change in delta Ct values was calculated relative to 0 hrs fed tissue or buffer-injected tick tissues, i.e. 0 hrs fed tissues or buffer-injected tissues were considered as references for estimating the fold difference in gene expression (Fernando et al., 2006).

Polyacrylamide gel electrophoresis (PAGE) and Western blot analysis

The standardized protein samples were analyzed on Ready Gel[®] Tris- Tricine Gel, 10-20% precast polyacrylamide gels (BioRad Laboratories, Hercules, CA). The protein sample was mixed with Tricine Sample Buffer containing 2-Mercaptoethanol, boiled for 5 min at 100⁰C and any insoluble material was sedimented by centrifugation. Electrophoresis was done at 100 V for 90 min and the same sample set was simultaneously electrophorated in another gel. Following the electrophoresis, one gel was stained in 0.1% Coomassie brilliant blue for 2 hrs, destained with destaining solution for 2 hours and stored in the storage solution. The other gel was transferred to a nitrocellulose membrane at 100 V for 45 min. The nitrocellulose membrane containing proteins was incubated in the blocking solution containing 2% horse serum followed by an antibody (1:500) specific to tick MIF peptide (amino acids- CLSPKENKKHSAVLFEHIEKTL, Jaworski et al., 2001, 2009) synthesized by conjugating the peptide to bovine serum albumin and subsequent immunization of rabbits (Affinity BioReagents, Rockford, IL), then with anti mouse/ anti rabbit IgG (1:100). VECTASTAIN[®] ABC kit with peroxidase-based detection system (Vector Laboratories, Burlingame, CA) was used as the detection system. Color development was achieved using 4-chloronaphthol with methanol and H₂O₂.

Database searching and sequence retrieval for MIF in arthropods and nematodes

A PSI-BLAST search was performed at National Center for Biotechnology Information (NCBI) database for arthropods, using *A. americanum* MIF as a query (Jaworski et al., 2001). From the resulting sequences, accession numbers of the sequences with more than 35% sequence identity were taken. After accession numbers were obtained, corresponding sequences were obtained from the GenBank. Similarly, amino acid sequences in nematode worms were found by performing PSI-BLAST using *A. americanum* MIF as q query. All these sequences were renamed using the first letter of the genus name followed by the first three letters of the species name. When there are more than one sequence is available for a species, they were designated by numbers of alphabetical letters. The amino acid sequence for *Ixodes scapularis* MIF was obtained from the VectorBase (<http://www.vectorbase.org>) by performing a tblastn using *A. americanum* MIF as the query sequence (Zee et al., 2007).

Multiple sequence alignment and Phylogenetic analysis

Multiple sequence alignment and phylogenetic analysis of MIF were performed using ClustalW2 program (<http://www.ebi.ac.uk/Tools/clustalw2/>) (Labarga et al., 2007). The BLOSUM matrix was used for the alignment with all default parameters.

Phylogenetic tree was constructed using the program Phylogeny.fr Version 2 (Dereeper et al., 2008). The multiple sequence alignment file was manually edited using BioEdit Version 7.0.9.0 sequence editing software (Hall, 1999). The phylogeny.fr used MUSCLE to align the sequences (Edgar, 2004), and Gblocks to curate the appropriate algorithm (Castresana, 2000). Phylogenetic tree was constructed using maximum

likelihood implemented in the PhyML program (Guindon and Gascuel, 2003, Anisimova and Gascuel 2006) using 500 bootstrap replications. Trees were visualized using TreeDyn (Chevenet et al., 2006).

Identifying a homology model for MIF

To identify a closer MIF homolog, a homology modeling was performed at the SwissModel (<http://swissmodel.expasy.org/>) server using *D. variabilis* MIF putative amino acid sequence as the query sequence. SwissModel Automatic Modeling mode was used to obtain the MIF homolog (Peitsch, 1995, Guex, and Peitsch, 1997, Schwede et al., 2003, Arnold et al., 2006, Kiefer et al., 2009).

Structure conservation analysis using the homology model

The resulting model from the SwissModel analysis was used to perform a structure conservation analysis at ConSurf Server (<http://consurf.tau.ac.il/>) (Glaser et al., 2003, Landau et al., 2005). The analysis was performed using the Bayesian method. The MSA was built using ClustalW and the homologs were collected from the SWISS-PROT database. Number of PSI-BLAST iterations was set to 1 at an e-value cut off of 0.001.

CHAPTER IV

RESULTS AND DISCUSSION

Identification of the presence of MIF in Dermacentor variabilis tissues

In ticks, MIF was first identified from the lone star tick, *Amblyomma americanum* (Jaworski et al., 2001). To date, including results from this study, MIF has been identified from three hard tick species *A. americanum*, *Haemaphysalis longicornis* and *Dermacentor variabilis* (Jaworski et al., 2001, Umemiya et al., 2007 and Anderson et al., 2008). In addition, we have identified expressed sequence tags (ESTs) representing *Ixodes scapularis* MIF from the genome database in VectorBase (<http://www.vectorbase.org>) and the identified *I. scapularis* MIF has been verified in our lab (Jaworski, unpublished).

In this study *D. variabilis* MIF was detected in both salivary gland and midgut tissues of unfed and partially fed adult female ticks using RT-PCR and DNA sequencing. Amplification of *D. variabilis* RNA using MIF1xQ primers generated a single product approximately about 180bp. Sequencing the *D. variabilis* MIF product was followed by analysis against the NCBI database using BLAST tool, verifying that this product was at least 80% similar to the existing tick MIF sequences. The amplified product was analyzed on a 1.5% agarose gel containing 0.1% ethidium bromide and is shown in Figure 3. Amplification of sheep glyceraldehyde-6-phosphate dehydrogenase was also done to

verify that tick RNA contamination with sheep blood RNA did not generate any product in our experimental protocol. This result suggested that sheep blood RNA was quickly degraded in tick midgut tissue.

Elucidation of full length cDNA sequence

The full length cDNA for tick MIF is about 450 bp and the putative protein is about 116 amino acids long (Jaworski et al., 2001 and Umemiya et al., 2007). Rapid Amplification of cDNA Ends (RACE) was used to obtain the full length cDNA sequence for *D. variabilis* MIF. Amplification of adaptor-ligated cDNA using gene specific primers (GSP) and adaptor primers generated a single product for 3'- and 5'- ends of the gene separately. Sequencing of the product followed by analysis against the NCBI database verified that the sequences were homologous to other tick MIF genes. The full length cDNA sequence for *D. variabilis* MIF was deduced by performing sequence alignments of 3' and 5' RACE sequences with known tick MIF sequences individually, and then manually editing them using BioEdit Version 7.0.9.0 sequence editing software (Hall, 1999). The deduced full length cDNA was 555 bp in length, with an open reading frame of 348 bp, producing a 116 amino acids long MIF peptide. The 5' untranslated region (5'UTR) is 102 bp in length and there were subtle differences in the nucleotide sequence at 5'UTR region compared to the *D. variabilis* MIF homolog identified from midgut transcriptome (Anderson et al., 2008). They were observed within the first 60 nucleotides in the 5' UTR and were A-T (1) substitution, T-C (4) substitution, G-A (1) substitution and G-C (2) substitutions to the sequence published by Anderson et al. (2008). The 3' UTR is 105 bp long and is identical to the MIF homolog identified from

midgut transcriptome. The full length cDNA sequence for *D. variabilis* MIF and encoding amino acid sequence is shown in Figure 4. In the putative amino acid sequence, neither *D. variabilis* MIF nor any other tick MIFs contain the 59th cysteine residue. Therefore, the signature CXXC domain observed in many other MIF peptides (Suzuki et al., 2004) is missing in the tick sequence. It is possible that the tick MIF may not have the conserved oxidoreductase activity like observed with MIF from other species. In human MIF, oxidoreductase activity is well conserved with CXXC domain. Cys 56 mutants retained the reduction activity up to 50% for insulin as the substrate and 68% for 2-hydroxyethyl disulphide (HED), whereas Cys 59 mutants abolished these activities (Dobson et al., 2009).

Expression of MIF gene in midgut and salivary glands during 0-96 hrs of feeding

The RT-qPCR analysis was performed to evaluate MIF expression in midgut and salivary gland tissues at 0, 8, 24, 48, 72 and 96 hr feeding intervals. The expression of MIF was calculated relative to the 0 hr fed (unfed) tick tissues and presented as a fold difference in gene expression as described in Fernando et al. (2006). In the midgut tissues, the fold difference in gene expression ranged from 1.91 through 34.14 compared to the control (Figure 5). During the first 48 hr period, there was an uninterrupted up-regulation in the gene expression. At 72 hrs the MIF expression was down-regulated compared to the 24 and 48 hr feeding intervals but higher than that of control and 8 hr feeding intervals. Highest expression was observed at the 96 hr feeding interval. Bowen et al., (2010) reported an up-regulation of MIF expression in *A. americanum* midgut tissue during first 5 days of feeding and a down-regulation by the 9th day of feeding. An

up-regulation of MIF in partially fed *H. longicornis* compared to the unfed ticks has also been reported (Umehiya et al., 2007). The results from the current study also suggest the MIF expression is up-regulated during 0-96 hr feeding period and the highest level of MIF expression in *D. variabilis* is also observed in the midgut tissue. The up-regulation of MIF in midgut tissue may function to evade the effect of host macrophages imbibed with the blood meal. There is very limited information available about the blood meal digestion in ticks at molecular level. Anderson et al. (2008) reported identification of 82 transcripts from the tick midgut transcriptome and the functions of these proteins are not known. The exact role of MIF in the midgut is still not known; therefore we cannot attribute an exact function or speculate as to why MIF is down-regulated at 72 hrs of feeding. Studying basic physiology of blood meal acquisition and digestion at molecular level will help in identifying the important genes, involvement and function of these gene products within the midgut.

The expression of MIF in *D. variabilis* salivary gland is less compared to the expression in the midgut tissue. This result was consistent with the MIF expression in the lone star tick, *A. americanum* where the expression of MIF in salivary gland was lower compared to the midgut tissue (Bowen et al., 2010). Figure 6 shows the fold difference in MIF expression during 0-96 hr feeding intervals in salivary gland tissue. The fold differences for MIF gene expression ranged from 3.79 to 12.78 compared to the 0 hrs fed (unfed) control tissue. The MIF gene was consistently up-regulated at each feeding interval. Bowen et al. (2010) observed an up-regulation of MIF within 2 days of feeding and then the expression started to lower by the day 5 in *A. americanum* salivary glands. Umehiya et al. (2007) also observed an up-regulation of MIF in partially fed *H.*

longicornis compared to the unfed ticks. In this study, the feeding intervals only up to 96 hrs (4 days) was compared and did not observe a decline of the gene expression during that period. We hypothesize the up-regulation of MIF expression in salivary gland during the early feeding intervals may play a vital role in increasing the blood meal acquisition by increasing the inflammation at the feeding site of the host. The presence of tick MIF in saliva has not been shown, but the lengthening of *A. americanum* feeding interval following the tick MIF peptide injection to the host indirectly support that MIF is secreted into the host with tick saliva during feeding (Jaworski et al., 2009). In parasitic nematodes, MIF has been identified as an immune evasion cytokine and was shown to play a role in homeostasis mechanisms during stressed conditions (Maizels et al., 2001 and Marson et al., 2001). For example, tick attachment to the mammalian host could be considered a stressful condition for the tick due to pressure of the mammalian immune response to reject the feeding tick. The up-regulation of MIF expression and secretion of the protein into the feeding lesion by the tick salivary glands may aid in masking the tick from some host immune responses. This may also facilitate pathogen transmission from the tick to the host. In addition, while ticks are generally thought to secrete anti-inflammatory factors, it is more likely that a “trade-off” of anti-inflammatory and pro-inflammatory factors is necessary to facilitate blood feeding for extended periods of time (5-21 days). So far more than 3400 putative salivary gland proteins have been identified from ticks belonging to 32 different gene families and the function of most of these proteins are not known (Francischetti et al., 2010). Many of these 3400 putative salivary gland proteins are likely to be secreted during tick feeding. Further, MIF along with other

proteins may have a synergetic, dose-dependent response to overcome the mammalian immune response against tick feeding.

It has been suggested for many years that vaccination strategies using tick-derived factors will be important for generating vaccines against tick feeding and tick-borne diseases (Valenzuela, 2004). A recent study on tick immune proteins has identified several up-regulated immune proteins from the *D. variabilis* in response to microbial pathogen infections (Jaworski et al., In press). It is important to understand the function of these proteins and the relationship of these proteins to MIF during tick feeding for the development of vaccines against tick feeding.

Analysis of MIF protein in midgut and salivary glands during 0-96 hrs of feeding

The presence of MIF protein in midgut and salivary gland tissues was confirmed using SDS-polyacrylamide gel analysis followed by Western blot using tick MIF specific antibody (Figures 7 and 8 shows the detection of MIF in polyacrylamide gels and in the immunoblots for midgut and salivary glands respectively). There was an increase in MIF gene expression during 0-96 hrs feeding intervals in both midgut and salivary gland tissues. In contrast, the level of protein changes in the opposite direction in both tissues. The level of MIF protein in both salivary glands and midgut declined as the feeding progressed. Highest level of protein was observed at 0 hrs for midgut and salivary gland tissues. The rate of decline was higher in salivary glands compared to the midgut. At 96 hrs, the level of protein in salivary glands is barely detectable whereas in the midgut tissues, it is still at an easily detectable level. The size of the protein is about 35 kDa in our gels suggesting that it may present as a trimer like other MIF proteins found in human

and parasitic nematodes. The trimer structure of the protein is expected to reduce to monomers after the treatment with 2-Mercaptoethanol, but for unknown reasons *D. variabilis* MIF was detected at 30- 35 kDa range in repetitive experiments. It will be necessary to have more structural information to find out how and why the protein withstands the reducing conditions. Also, it may be that the protein isolation method using Tri-reagent induces this artifact.

MIF gene expression is elevated in midgut and salivary gland tissues likely due to the increased necessity for its function (Figures 5 and 6). The elevated expression of MIF indicates MIF protein production in midgut and salivary gland. The decline of the protein in the immunoblot suggests that the produced protein is not stored in these tissues and is possibly secreted into the feeding lesion or to the midgut lumen. The feeding lesion is the interface of active tick-host interaction and the common route for pathogen transmission (Sauer et al., 1995 and Bowman et al., 1997). MIF produced in the salivary glands may be continuously secreted into the feeding lesion to evade the host immune responses and to facilitate the blood meal uptake. This may also enhance the pathogen transmission from tick salivary glands to the host via feeding lesion by masking host immune responses against tick feeding. It is also possible that MIF produced in the midgut epithelium may be secreted into the lumen to prevent the migration of host macrophages towards the midgut epithelium (Jaworski et al., 2001).

The effect of MIF on tick blood meal acquisition

To observe the effect of MIF in blood meal acquisition of the female *D. variabilis*, an RNAi mediated gene silencing approach was used. The average female

body weight after engorgement was used as the physiological parameter to gauge blood meal uptake. Figure 9 shows the average body weights of ticks after treatment with buffer, dsRNA for MIF and dsRNA for Subolesin. There was no significant difference between the body weights between the buffer-injected females and dsRNA for MIF-injected females. In the positive control (subolesin dsRNA-injected group) average engorged tick body weights were significantly lower compared to the other two groups.

Gene silencing was confirmed by RT-qPCR. Figures 10 and 11 show the data analysis from RT-qPCR for midgut and salivary glands respectively. The analysis clearly shows a down-regulation of MIF expression in the MIF dsRNA-injected tissues. The down-regulation is about 90% compared to the buffer-injected controls for both midgut and salivary gland. These findings suggest that the dsRNA injection successfully suppresses the MIF expression in midgut and salivary glands. Bowen et al. (2010) showed the dsRNA mediated gene silencing of MIF in *A. americanum* in both midgut and salivary gland tissues, but did not observe a significant difference in blood meal uptake by adult female ticks (Bowen et al., 2010). The results from my study are consistent with Bowen et al., (2010) suggesting that silencing of MIF in salivary gland or midgut has no affect in the blood meal acquisition. In addition, while not statistically significant, we observed MIF silenced ticks fed faster and engorged slightly more. These results also suggest that the tick might compensate the gene silencing by producing other proteins during feeding. To test this hypothesis, it will be necessary to identify the differences in protein profiles between MIF silenced and non-silenced ticks.

Interestingly, we observed a down-regulation of MIF expression in the subolesin dsRNA-injected tick tissues. MIF expression is down-regulated about 50% compared to

the buffer-injected controls (Figures 10 and 11). Subolesin has been identified as a potential tick protective antigen from *I. scapularis* and found to be conserved among Ixodid tick species (Almazan et al., 2003). The suggested function of this protein is modulation of tick blood ingestion and reproduction (de la Fuente et al., 2006). Subolesin has also been identified as an ortholog of insect and vertebrate akirins, which was proposed to function as transcription factors in both *Drosophila* and mice (Galindo et al., 2008). These proteins involve in NF- κ B dependent and independent signal transduction and innate immune responses. In humans, MIF has been identified to activate extracellular signal-regulated kinase 1/2 (ERK1/ERK2) members of the mitogen-activated protein kinases (MAPKs) and also to promote the recognition of lipopolisaccharides (LPS) and Gram-negative bacteria by cells of the innate immune system by up-regulating the expression of Toll-like receptor 4 (TLR4) (Calandra et al., 2003 and Kudrin et al., 2006). Our results suggest that any interaction between MIF and subolesin may be an indication of MIF's involvement in signal transduction and immune responses in tick tissues.

Multiple sequence alignment and Phylogenetic analysis

Using data mining, 37 amino acid sequences for MIF were identified from ticks, nematodes and insects. The multiple sequence alignment for these 37 amino acid sequences is shown in Figure 12. It shows the high conservation of MIF amino acid sequence across the species. Amino acid residues, Pro 1, 3, 55; Thr 7, 112; Asn 8, 72; Ile 64, 96; Gly 65, 110, Ser 63 and Leu 87 are found to be the ones with very high level of conservation across the species. Any two tick MIF sequences show more than 78%

sequence similarity between them. Two *Tribolium castaneum* MIF sequences are 84% similar to each other whereas two *Acyrtosiphon pisum* sequences are the least similar showing only 40% sequence similarity between Apis_MIFA and B. All the other arthropod sequences show approximately 50% sequence similarity to tick MIF sequences. Nematode MIF sequences have at least 80% sequence similarity among them and they are at least 25% dissimilar to the tick MIF sequences.

Within the multiple sequence alignment, highlighted in blue is the CXXA pattern observed in ticks. Ticks and some other nematodes do not have the oxidoreductase domain (CXXC) (Suzuki et al., 2004) observed with many other MIF proteins. This suggests that the ticks and some nematodes may not have the oxidoreductase activity. Crystallization data for the tick MIF protein and site-directed mutagenesis assays could be useful in finding active sites for tick MIF.

Another unique amino acid sequence found in tick MIF sequence is highlighted in grey in the Figure 12. The CLSPKENKKHSAVLFEHIEKTL of *A. americanum* (Jaworski et al., 2001 and Jaworski et al., 2009) sequence shares 73-91% sequence similarity with other tick MIF sequence and the amino acid sequence similarity for nematode and insect MIF is well below 50% through this part of the MIF protein. Antibodies to this peptide have been used to detect MIF in tick tissues from *A. americanum* (Jaworski et al., 2001 Jaworski et al., 2009 and Bowen et al., 2010), *D. variabilis* (this study) and *R. sanguineus* (Jaworski unpublished).

A phylogenetic tree generated using 37 MIF amino acid sequence is shown in Figure 13. Interestingly, tick MIFs cluster together with some nematode MIF sequences and not with the insect MIF sequences suggesting that tick MIFs are closely related to the

parasitic nematode MIF rather than insect MIFs. A high level of sequence conservation and phylogenetic proximity of MIF in ticks and nematodes is likely to be a result of selective pressure from parasitism during their evolution. It appears that parasite-specific MIFs may be essential to parasitism.

Identifying a homology model for MIF

The automated modeling mode at SwissModel (<http://swissmodel.expasy.org/>) identified one protein from the protein data bank as the homology model for *D. variabilis* MIF. The protein identified was Macrophage Migration Inhibitory Factor from *Trichinella spiralis* (PDB ID: 1hfoE) (Tan et al., 2001). This has 48.673% sequence identity to the query sequence with an E-value of 5.40e-43. The sequence alignment between the query sequence and the 1hfoE is shown in Figure 14. The α -helices β -sheets in the 3-dimensional protein are indicated as 'h' and 's' respectively. The phylogenetic tree (Figure 13) also suggests that *D. variabilis* MIF and *T. spiralis* MIF are closely related. They are in two closest clades arising from the same node suggesting their close evolutionary proximity.

Structure conservation analysis using the homology model

The schematic presentation of the conservation analysis is shown in Figure 15. The resulted homology model (*T. spiralis* MIF) has 9 highly conserved amino acids according the analysis at ConSurf server. Pro 1, Gly 65/110, Thr 7/112, Ser 63, Asn 8/72 and Ile 96 are recognized as the most conserved amino acid residues. Interestingly, all these highly conserved amino acid residues derived from the model are conserved in *D.*

variabilis and other tick MIF sequences. They are also among the highly conserved amino acid residues for nematodes and insects (Figure 12). This further suggests that tick MIF may also exist as trimer showing a more or less similar structure to the *T. spiralis* MIF.

CHAPTER V

SUMMARY AND CONCLUSIONS

Ticks as blood feeding ectoparasites have caused health problems in both animals and humans. It is important to establish multi-target vaccine strategies to control ticks and the tick-borne pathogens they transmit. The success of ticks as blood feeders is supported by many factors and one of the most important factors is evasion of host-immune responses. The proteins and other components secreted by the salivary glands play a major role in mediating the host-immune responses facilitating the blood meal uptake. This process also permits the transmission of tick-borne pathogens to the vertebrate host via the feeding lesion. Out of more than 3400 putative tick salivary proteins, functional data is not available for the majority; and it is unlikely, that the functional studies could be performed for each of these proteins. It is very important to categorize proteins into different groups with similar functions, and identify orthologs from other tick, parasite and insect species. Current information on blood meal digestion in ticks at molecular level is limited and presents a hurdle for identifying candidates for vaccine production. More fundamental research is required to identify midgut target proteins to be used for tick control and transmission blocking.

My research has identified and characterized the full length cDNA sequence of the Macrophage migration Inhibitory Factor (MIF) from the American dog tick, *Dermacentor variabilis*. The nucleotide and putative amino acid sequences from this

study shared a high level of sequence conservation with other tick MIFs. The expression of MIF in salivary glands and midgut tissues has also been confirmed for 0-96 hrs partially fed American dog ticks. MIF has been identified from many organisms including mammals, nematodes, arthropods, jawed/jawless fish, plants, cyanobacteria and parasites. Many functional studies have been performed in humans and nematodes and they have characterized MIF as an immunomodulatory protein. In ticks the expression of MIF has been studied during early blood feeding; however detailed functional information of the tick protein is not available.

Our study identified an up-regulation of the MIF gene expression during 0-96 hrs fed female *D. variabilis* ticks in both salivary glands and midgut tissues. The highest level of MIF expression was observed in midgut compared to the salivary glands. In contrast, the change in protein levels found in salivary glands and midgut tissues declined as the feeding progressed from 0-96 hrs. These results suggest that increased gene expression produces the protein and then, it is simultaneously secreted into the target region to perform its function. The salivary gland protein may be secreted into the feeding lesion to mediate the host-immune responses and enhance the blood meal acquisition. In the meantime it may also enhance pathogen transmission via the blood feeding process. The midgut protein may be secreted into the midgut lumen to avoid any host macrophage damage to the midgut epithelium by inhibiting their migration towards the epithelium (Jaworski et al., 2001). The Western blot analysis of the protein identified *D. variabilis* MIF at about 30-35 kDa in size. While we anticipated that this protein would exist in SDS-PAGE gels as a monomer, it appears that some property of our

sample shows an incomplete reduction of the polymer to monomers. This finding requires further study.

RNAi mediated gene silencing of *D. variabilis* MIF was shown to down-regulate the expression by approximately 90% compared to the buffer-injected controls in both the salivary gland and midgut. Interestingly, MIF gene silencing has not shown any phenotypic inhibition of the tick blood meal acquisition. These results indicate that tick MIF does not have a negative impact on blood meal uptake. The effect MIF gene silencing is likely to be complex and overcome by the tick utilizing other proteins for the same functions. Another interesting finding in this study was the effect of subolesin gene on MIF gene expression. In both the salivary glands and midgut, subolesin dsRNA-injection down-regulated the MIF expression by approximately 50% compared to the buffer-injected control. Subolesin is suggested to modulate the blood meal ingestion and reproduction in ticks (de la Fuente et al., 2006). Further it is an ortholog of insect and vertebrate akirin, which is a transcription factor (Galindo et al., 2008). Our results suggest that MIF may have an interaction with subolesin which may involve signal transduction and immune responses.

The bioinformatics analysis showed the across species conservation of the MIF amino acid sequence in ticks, nematodes and insects. The multiple sequence alignment identified Pro 1, 3, 55; Thr 7, 112; Asn 8, 72; Ile 64, 96; Gly 65, 110, Ser 63 and Leu 87 amino acids to be highly conserved among the sequences used for this study. Ticks do not have the unique oxidoreductase domain (Cys56XXCys59) suggesting that the tick MIF is not capable of performing as an oxidoreductase. The phylogenetic analysis revealed that tick MIFs share a closer evolutionary proximity to some parasitic nematode MIFs rather

than insect MIF, although ticks and insects belong to the same phylum. In nematodes MIFs have been shown to aid in immune evasion and homeostasis mechanisms under stressed conditions (Maizels et al., 2001 and Marson et al., 2001). The closer evolutionary proximity of MIF in ticks and nematodes is likely to be a result of positive selection pressure due to parasitism.

No crystallography data is available for tick MIF structure and it is important to have that information in the process of evaluating the suitability of tick MIF as a potential candidate for a vaccine. Homology modeling identified the closest *D. variabilis* MIF homolog as MIF from *Trichinella spiralis*, a parasitic nematode. The amino acids shared a little over 48.6% sequence identity and in the phylogenetic analysis the two sequences cluster into the two closest clades. In the identified model for tick MIF, amino acids Pro 1, Gly 65/110, Thr 7/112, Ser 63, Asn 8/72 and Ile 96 were found to be highly conserved. All these amino acids are conserved in the multiple sequence analysis as well for the *D. variabilis* putative amino acid sequence for MIF alone. The identified model exists as a trimer in *T. spiralis* and this also supported the identification and likelihood of *D. variabilis* MIF as a trimer in the immunoblot.

This study has enabled the understanding of gene expression and protein level variation of MIF during early blood feeding of American dog tick and given a foothold on the changes that may function in the blood feeding of the tick. These findings also open a gateway to a novel area where tick MIF may interact with other proteins such as subolesin and play an important role in signal transduction and immune responses. The bioinformatics study revealed the similarities and differences of *D. variabilis* MIF to MIF from other organisms and provided clues concerning their phylogenetic relationships. It

also gives basis to the possible three-dimensional structure of the protein as well as its conserved amino acid residues. Collectively, our results are a foundation for evaluating this molecule as a vaccine. Understanding the exact function of the conserved amino acid residues in active sites of the protein is important in vaccine production to manipulate tick blood feeding. Further research on tick MIF protein-protein interactions and host MIF- tick MIF interactions will be important for establishing the function of tick MIF during blood and pathogen transmission.

Table 1. Primers used for RT-PCR, RT-qPCR and RACE experiments

Product	Forward primer	Reverse primer
MIF1xQ	5'-AAGCCGCTTTCGTATGTTGTGG-3'	5'-TTCCTTGATGCCCAGGGTCTTT-3'
GSP for RACE	5'-TGTTGTGGTGCACATCAGTCCT GGCCAAT-3'	5'-CTCCTTTGGAGAGAGGCAGCC AATGCTG-3'
Human 18S rRNA	5'-TTCGAACGTCTGCCCTATCAA-3'	5'-GATGTGGTAGCCGTTTCTCAGG-3'
Tick 16S rRNA	5'-GACAAGAAGACCCTA-3'	5'-ATCCAACATCGAGGT-3'
T7MIF1xQ	5'-TAATACGACTCACTATAGGGTA CTAAGCCGCTTTCGTATGTTGTGG-3'	5'-TAATACGACTCACTATAGGGTA CTTTCCTTGATGCCCAGGGTCTTT-3'
Adaptor primer 1 (RACE)	5'-CCATCCTAATACGACTCACTATA GGGC-3'	
Subolesin	5'-GCTTGCGCAACATTAAGCGAA C-3'	5'-TTTGGTCGTACGTAAACTTGACA AATGTG-3'
Glyceraldehyde-6- phosphate dehydrogenase	5'-GGGTCATCATCTCTGCACCT-3'	5'-GGTCATAAGTCCCTCCACGA

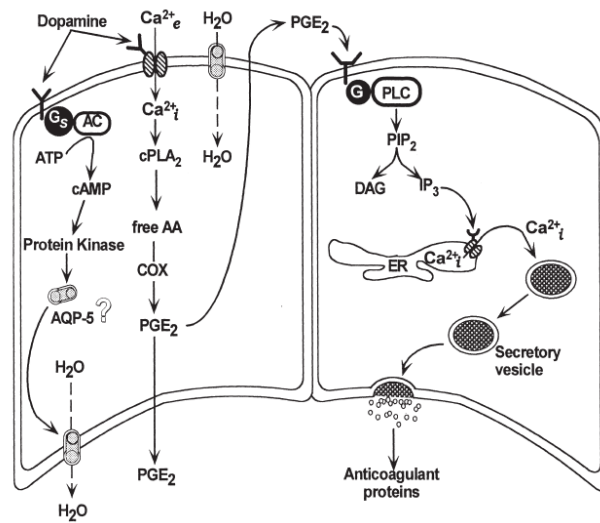


Figure 1. Control mechanism of Ixodid tick female salivary gland secretion. Dopamine released at neuroeffector junction stimulates secretion either by G protein-coupled pathway or by opening voltage dependent Ca^{2+} ion channel. (Figure reproduced from Sauer et al., 2000)

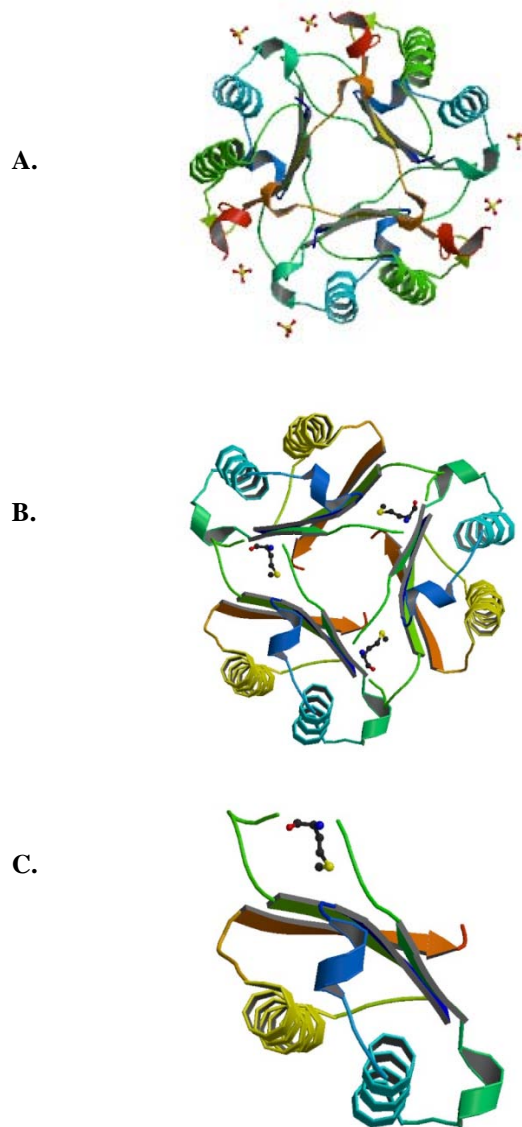
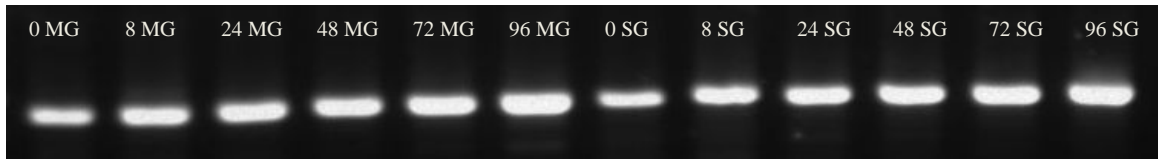
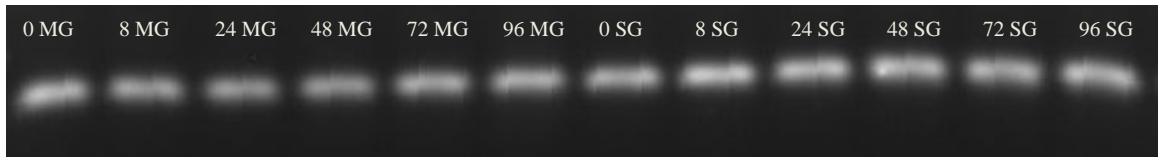


Figure 2. Structures of parasitic and mammalian macrophage migration inhibitory factor (MIF). Both MIFs exist as trimers as shown in A and B making a barrel in the middle of it and each monomer is composed of two $\beta/\alpha/\beta$ motifs. **A.** Trimer of *Ancylostoma ceylanicum* (parasitic nematode) MIF (PDB ID: 2OS5) **B.** Trimer of rat MIF isolated from the liver (PDB ID: 1FIM) **C.** Monomer of rat MIF isolated from the liver (PDB ID: 1FIM)



A) MIFq



B) H18S

Figure 3. Identification of the presence of MIF gene from 0-96 hrs fed *Dermacentor variabilis* female tick tissues. Figure 4A shows the RNA amplified with MIF1xQ primer set (product size: 180bp) and 4B shows the amplified product using H18S primer set for the same samples. The number in each lane refers to the feeding interval for midgut (MG) and salivary gland (SG) tissues.

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cggcgccgagctgccagccgtagccacttagcgcgacttgggaagacgtgtagtgcg
←
cttcttctggttgcgagtactctctgacgactcaaagccaaaatgccaactcttacgac
5'UTR → M P T L T I
aacacaaatctccccgcaagcagcattccgaacgactttctgaagacgacagcgaacggt
N T N L P A S S I P N D F L K T T A N V
gtggcggcctctttgggaaaaccgctctcgtatggtgtggtgcacatcagtcgggcaa
V A A S L G K P L S Y V V V H I S P G Q
ttgatgtcatttggagccactgacgagccatgtgccattgcaaacctgtacagcattggc
L M S F G A T D E P C A I A N L Y S I G
tgctctctccaaaggagaataagaagcattcagctgctctttttgagcacattgagaaa
C L S P K E N K K H S A A L F E H I E K
gtattgggcatcaaagggaacagaatgtacatcaacttcattgacctgccagcaacagat
V L G I K G N R M Y I N F I D L P A T D
gtgggctacagtggcaaaaacttttctggtgtagctcctggtgtggcaaaacggagaga
V G Y S G K T F A G ←
agtctgcgtatctcggatttgaataaacattcacgcttctggttgcaaaaaaaaaaaaaa
3'UTR
aaaaaaaaaaaaaaaa
→

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Figure 4. *D. variabilis* MIF nucleotide sequence and putative amino acid sequence. The nucleotide sequence is shown in simple letters and the encoding amino acid sequence is shown right below the respective codon (shown in blue block capitals). The start codon and stop codon are highlighted in the nucleotide sequence in yellow. Specific antibody to detect tick MIF has been raised using the peptide highlighted in blue. The 5' and 3' UTRs span in the regions shown by horizontal arrows

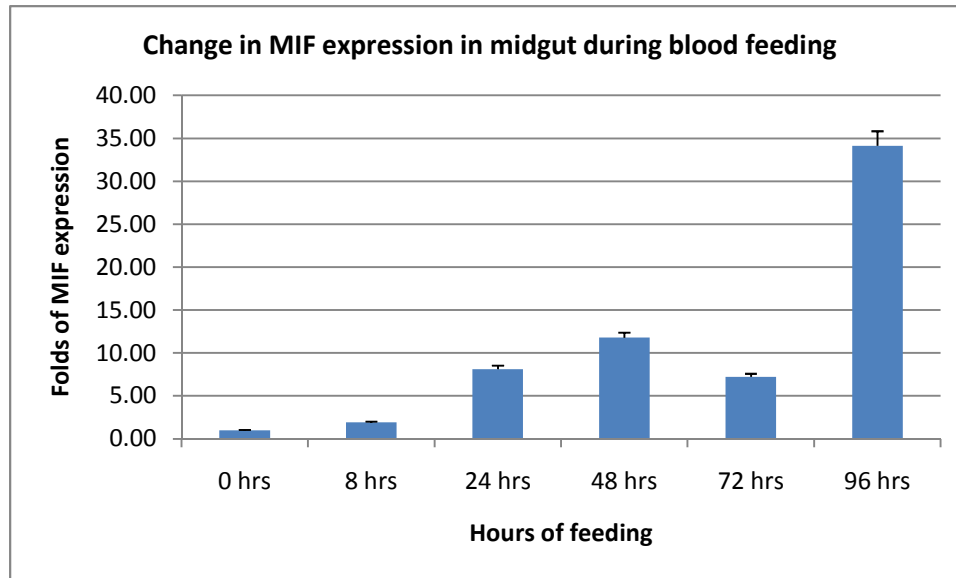


Figure 5. Change in MIF gene expression in *D. variabilis* midgut tissues during 0-96 hr feeding interval. Hours of feeding indicate the time of tick removal after attachment to the host. Fold differences are compared to 0 hrs are shown on the y-axis. Percentage error for the chart series is shown with a 5% value

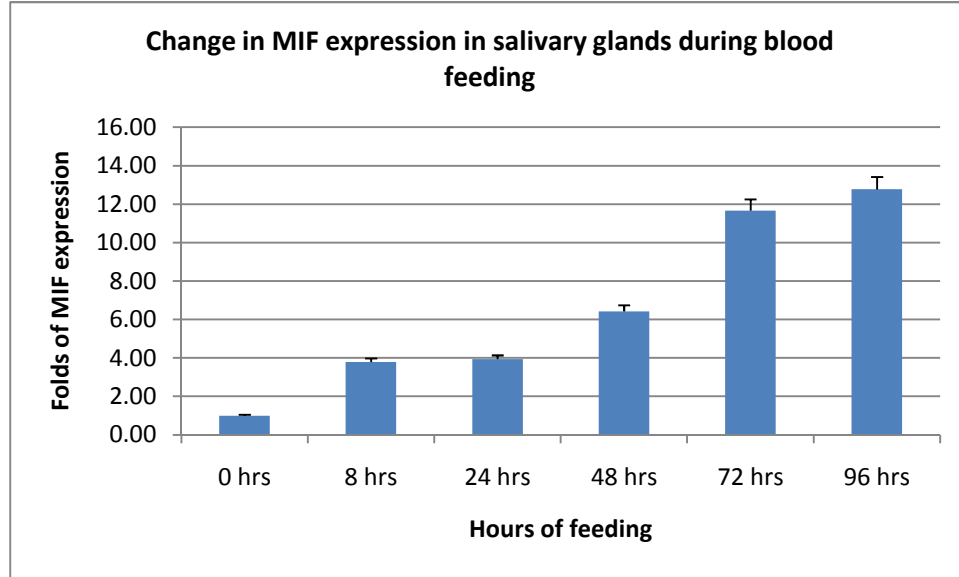
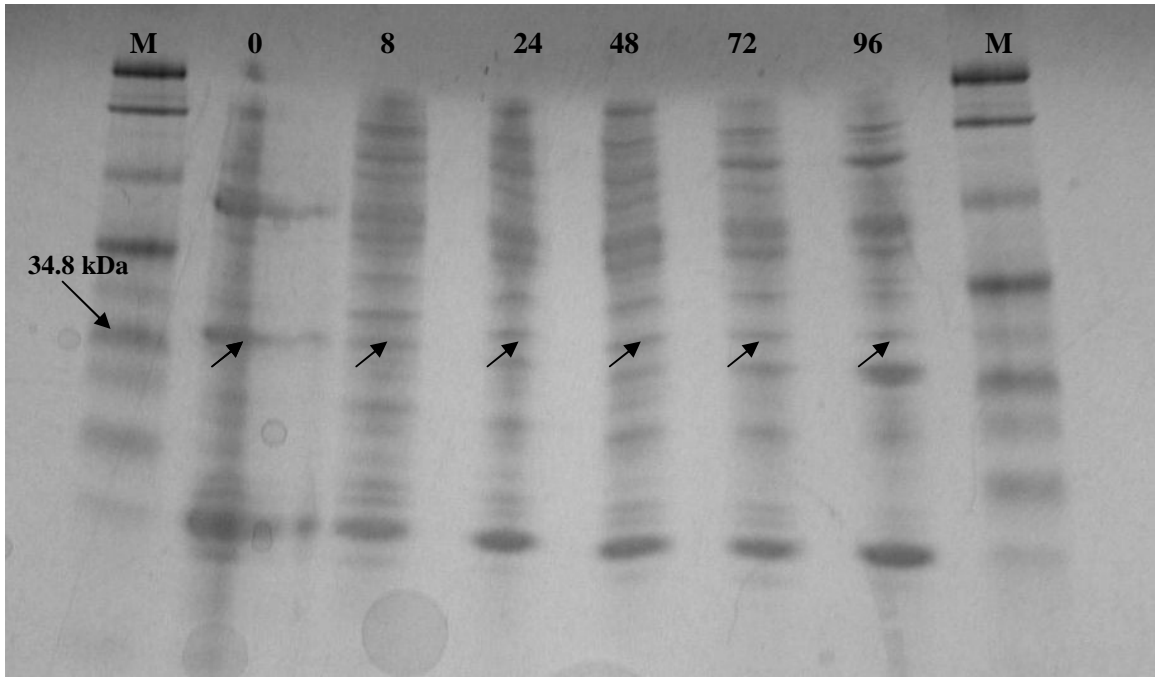
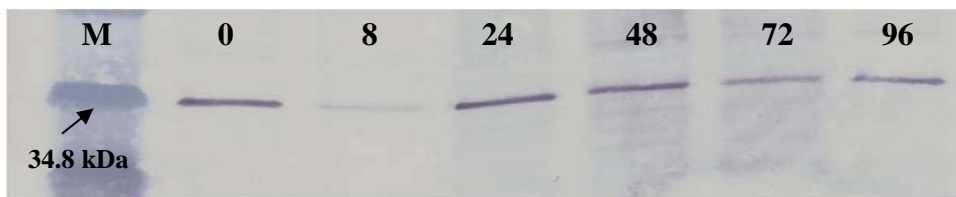


Figure 6. Change in MIF gene expression in *D. variabilis* salivary gland tissues during 0-96 hr feeding interval. Hours of feeding indicate the time of tick removal after attachment to the host. Fold differences are compared to 0 hrs are shown on the y-axis. Percentage error for the chart series is shown with a 5% value

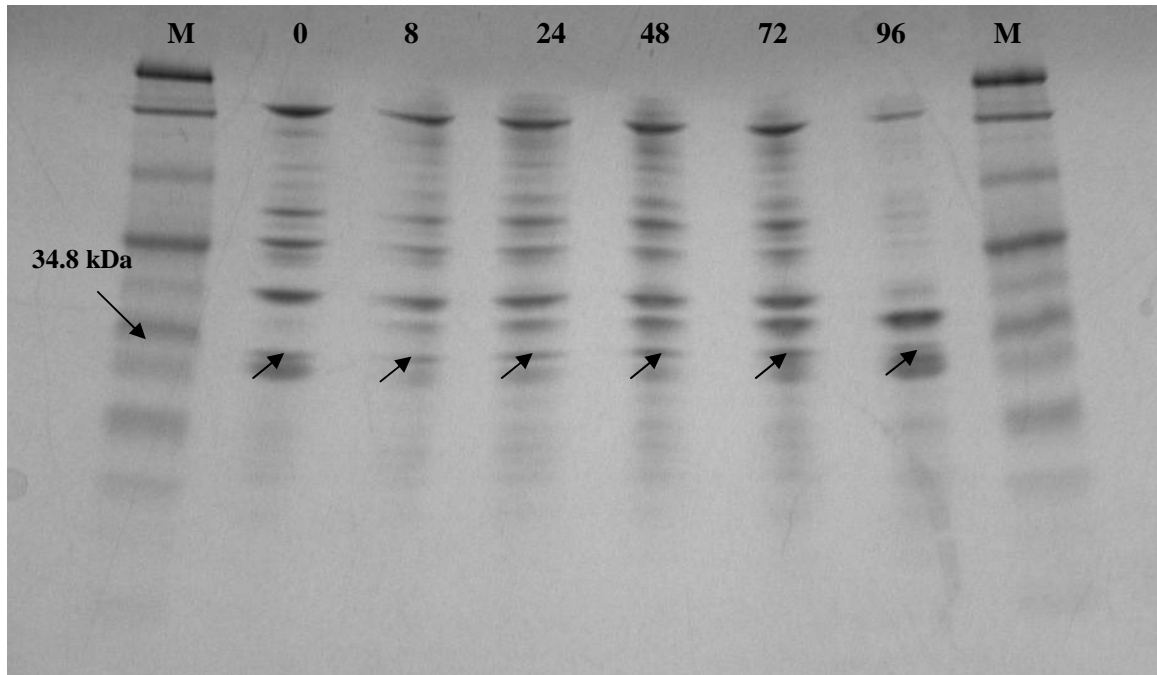


A.



B.

Figure 7. Detection of midgut proteins from *D. variabilis* females during feeding intervals from 0-96 hrs in a SDS-polyacrylamide gel (7.A) and in an immunoblot (7.B). The protein of approximately 35 kDa is shown in arrowheads for each feeding interval in the gel. The immunoblot detected the protein using a specific antibody and the amount of protein declines as the feeding progresses



A.



B.

Figure 8. Detection of salivary gland proteins from *D. variabilis* females during feeding intervals from 0-96 hrs in a SDS-polyacrylamide gel (8.A) and in an immunoblot (8.B). The protein of approximately 35 kDa is shown in arrowheads for each feeding interval in the gel. The immunoblot detected the protein using a specific antibody and the amount of protein declines as the feeding progresses

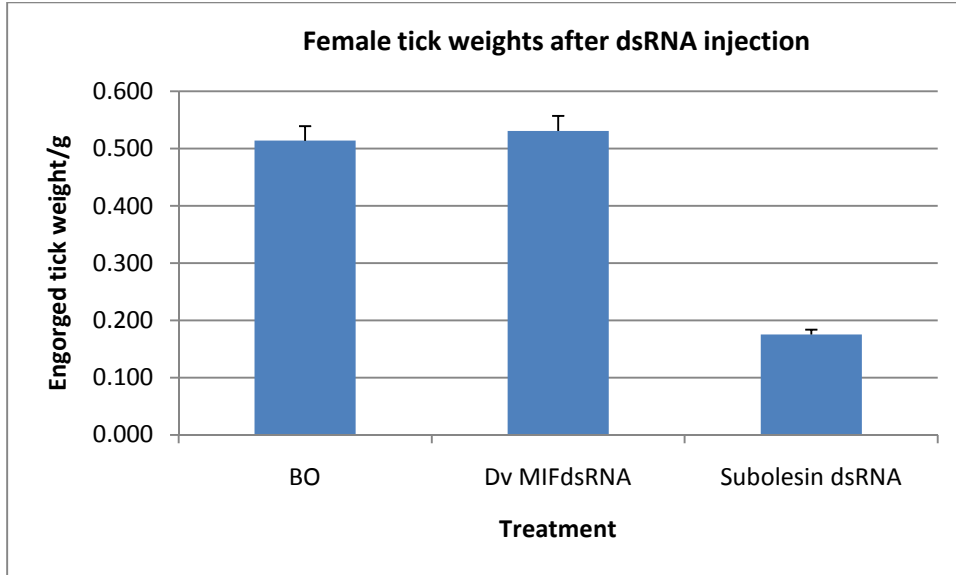


Figure 9. Effect of MIF on blood meal acquisition in *D. variabilis*. Average female tick weight after dsRNA or buffer injections and fed to reach repletion on a sheep. BO: Buffer Only, Dv MIFdsRNA: dsRNA for MIF-injected, Subolesin dsRNA-injected: the positive control for the experiment. There was no significant difference in the average body weights of buffer-injected and dsRNA for MIF injected ticks and the average body weight for subolesin dsRNA-injected ticks were significantly lower compared to the buffer-injected ticks

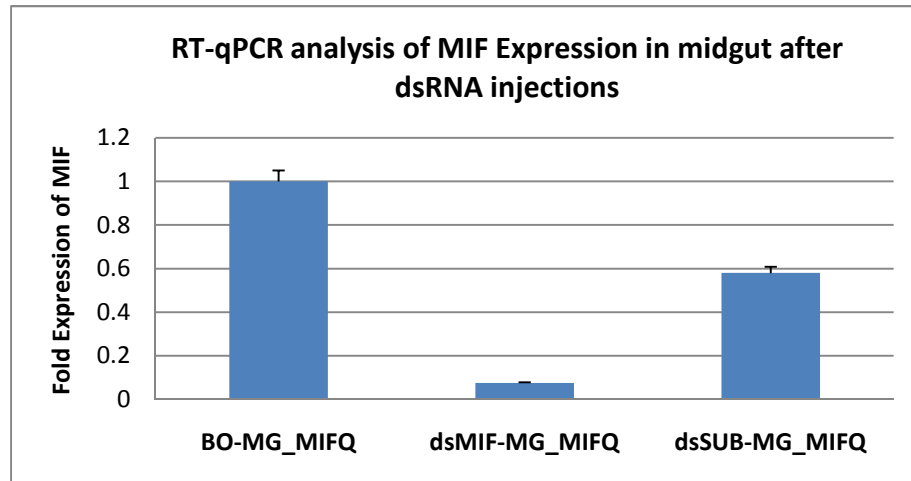


Figure 10. RT-qPCR analysis of MIF gene expression for dsRNA injected female *D. variabilis* midgut tissue. BO- buffer injected, dsMIF-dsRNA for MIF injected, dsSUB-dsRNA for subolesin injected. RT-qPCR was performed using MIF1xQ primer set and H18S primer set. Fold differences for gene expression were evaluated in reference to tissues from buffer-injected ticks

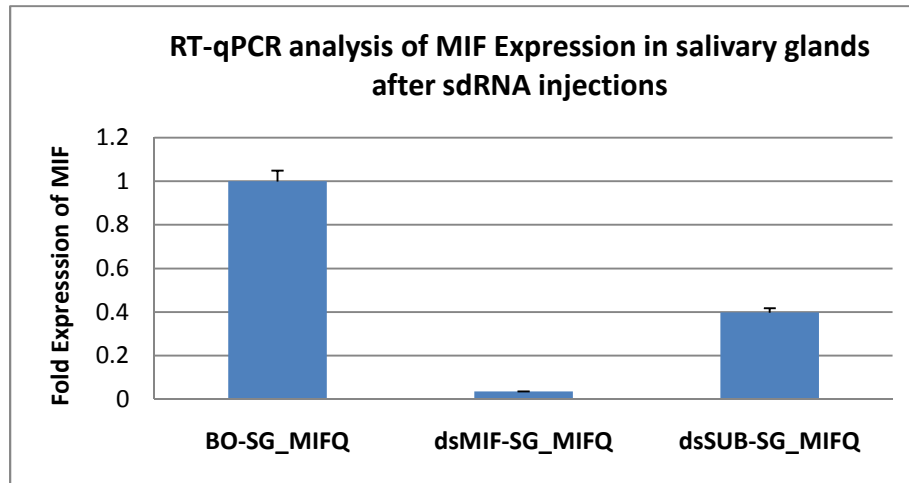


Figure 11. RT-qPCR analysis of MIF gene expression for dsRNA injected female *D. variabilis* salivary gland tissue. BO- buffer injected, dsMIF-dsRNA for MIF injected, dsSUB-dsRNA for subolesin injected. RT-qPCR was performed using MIF1xQ primer set and H18S primer set. Fold differences for gene expression were evaluated in reference to tissues from buffer-injected ticks

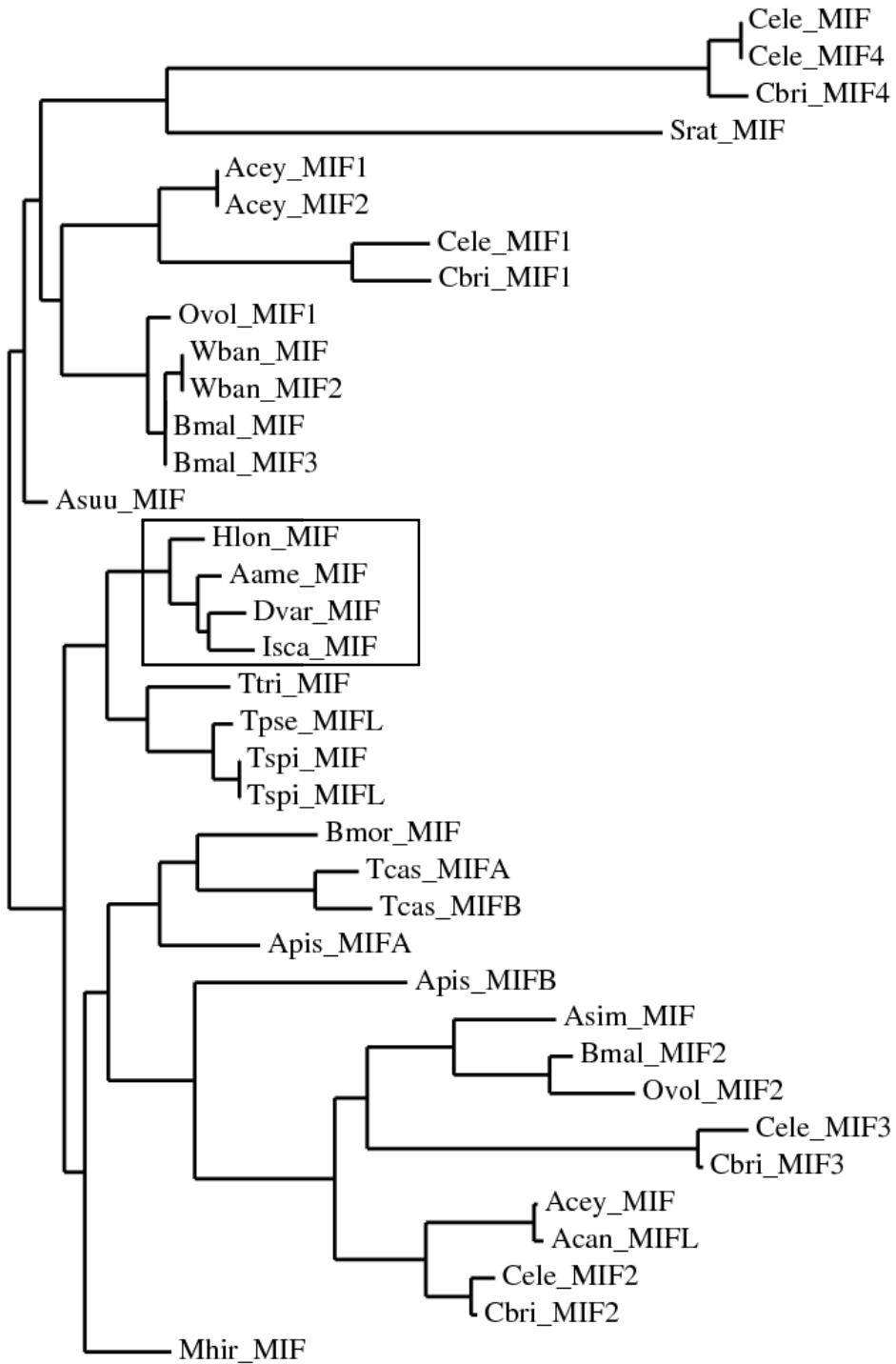
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Cbri_MIF2      MPMVRVATNLPD-EKVPQDFEIRLTDLLARSMDGKPRERIAVEVAAGA--RLVHGATHDPATVISVCRFAVFRIGSIGAVSAE-DNIRHTAAITEFCGKELGLPKDKVVIITFHDMPAVTVGVFNGTTVAETN
Acey_MIF       MPMVRVATNLPD-KDVPANFEERLTDLLAESMKNRNRRIAIEVLQAG--RITHGASRNPVAVIK-----VESIGALSAD-DNIRHTQKITQFCQDTLKLPKDKVIITFYDLQPIHVGFNGTTVAAAT
Acan_MIFL      MPPVRVATNLPD-KDVPANFEERLTDILADSMKNRNRRIAIEVMAGQ--RITHGASRNPVAVIK-----VESIGALSAD-DNIRHTQKITQFCQDTLKLPKDKVIITFYDLQPIHVGFNGTTVAAAT
Cele_MIF       MQVVRITQTNIRS-ADIPEKFEQDVIYNLSVVMELPADKQVIVVEPAVRMRIGFENKEIPVAIVN-----FQTT-RPSSRIENDSYAKKLTSLVNEQLKLPDPAHIFISFDFDKAKSFATQGKTTIASLY
Cele_MIF4      MQVVRITQTNIRS-ADIPEKFEQDVIYNLSVVMELPADKQVIVVEPAVRMRIGFENKEIPVAIVN-----FQTT-RPSSRIENDSYAKKLTSLVNEQLKLPDPAHIFISFDFDKAKSFATQGKTTIASLY
Cbri_MIF4      MQIVRIQTNIRA-ADVPEKLEQDVSYGLSVMMEMPDKFVVIIVEPAVRIRIGFEAKETTVAVVQ-----FQTT-RPSPRTENDIYAKKLTSLVNEQLKLPDPAHIFISFDFDKAKSFATQGKTTIASLY
Bmal_MIF       MPYFTIDTNIPIQ-NSISSAFLKASNVVAKALGKPEYSYVSIHVNGGQ--AMVFGGSEDPACVAV-----LKSIGCVGPK-VNNSHAEKLYKLLADELKIIPKNRCYIEFVDIEASSMAFNGSTFG---
Bmal_MIF3      MPYFTIDTNIPIQ-NSISSAFLKASNVVAKALGKPEYSYVSIHVNGGQ--AMVFGGSEDPACVAV-----LKSIGCVGPK-VNNSHAEKLYKLLADELKIIPKNRCYIEFVDIEASSMAFNGSTFG---
Wban_MIF       MPYFTIDTNIPIQ-NSISSAFLKASNVVAKALGKPEYSYVSIHVNGGQ--AMVFGGSEDPACVAV-----LKSIGCVGPK-VNNSHAEKLYKLLADELKIIPKNRCYIEFVDIEASSMAFNGSTFG---
Wban_MIF2      MPYFTIDTNIPIQ-NSISSAFLKASNVVAKALGKPEYSYVSIHVNGGQ--PMVFGGSEDPACVAV-----LKSIGCVGPK-VNNSHAEKLYKLLADELKIIPKNRCYIEFVDIEASSMAFNGSTFG---
Ovol_MIF1      MPAFTINTNIPIQ-SNVSDAFLKASSTVAKALGKPEYSYVSIHVNGGQ--AMVFGGSEDPACVAV-----LKSIGCVGPK-VNNSHAEKLYKLLADELKIIPKNRCYIEFVDIEASSMAFNGSTFG---
Asuu_MIF       MPCFTINTNIPIQ-SNVSDAFLKASSTVAKALGKPEYSYVSIHVNGGQ--QMTFSGSADPCAVCT-----LESIGAVGGS-RNNAHAEKLYKLLADELKIIPKNRCYIEFVDIEASSMAFNGSTFG---
Cele_MIF1      MPVFSINNVKVPKAEKQNEILKELSTVLGKLLNKPQYMCIIHFHEDQ--GILYAGTTEPAGFAV-----LKSIGGVSAGKQNNNAISAVVFPPIIEKHLGIPGNRLYIEFVNLGAADIAYNQGTFA---
Cbri_MIF1      MPVFSINNVKVPKAEKQNEILKELSTVLGKLLNKPQYMCIIHFHEDQ--AISFAGTTPAGFAV-----LKSIGGVSAGKQNNNAISAVVFPPIIEKHLGIPGNRLYIEFVNLGAADIAYNQGTFA---
Acey_MIF1      MPVFQLHTNSVQ-DKVTDDLKQISALVARILHKPEYSYVAVHVVPDQ--KMTFAGTDGPGCGIGI-----LKSIGGVSAGKQNNNAISAVVFPPIIEKHLGIPGNRLYIEFVNLGAADIAYNQGTFA---
Acey_MIF2      MPVFQLHTNSVQ-DKVTDDLKQISALVARILHKPEYSYVAVHVVPDQ--KMTFAGTDGPGCGIGI-----LKSIGGVSAGKQNNNAISAVVFPPIIEKHLGIPGNRLYIEFVNLGAADIAYNQGTFA---
Tsipi_MIF      MPIFTLNTNIKA-TDVPSDFLSSTALSALVGNILSKPGSYVAVHINTDQ--QLSFGGSTKPAAFGT-----LMSIGGIEPS-RNRDHSAKLFDHLNKLGIIPKNRMYIHFVNLNGDDVGVNGTTF----
Tsipi_MIFL     MPIFTLNTNIKA-TDVPSDFLSSTALSALVGNILSKPGSYVAVHINTDQ--QLSFGGSTKPAAFGT-----LMSIGGIEPS-RNRDHSAKLFDHLNKLGIIPKNRMYIHFVNLNGDDVGVNGTTF----
Tpse_MIFL      MPIFTLNTNIKA-TDVPSDFLSSTALSALVGNILSKPGSYVAVHINTDQ--QLTFFGGNTSPAAFGS-----LMSIGGIEAS-RNRDHSAKLFDHLNKLGIIPKNRMYIHFVNLNGDDVGVNGTTF----
Ttri_MIF       MPIFTLNTNIKA-TDVPSDFLSSTALSALVGNILSKPGSYVAVHINTDQ--KITFFGGTDAPAGFGQ-----LLSLGGVGE-KNRSHSAKLFKHLTDGLGIPGNRMYINFVDMRGSVDVGVNGSTF----
Tcas_MIFA      MPHFRVETNVPIQ-SKIPADLPQRLCQIVANSLSKQSYCCVTVIGDV--HMSWGGTSEPAQAQAT-----LMSIGLGEV-PNKKHAKALYEVVCKELGVSKDRMYIHFVNAPTSDVGVNGTTFHDIIF
Tcas_MIFB      MPHFRVETNVPIQ-SKIPADLPQRLCQIVANSLSKQSYCCVTVIGDV--NMCWGGTSEPAQAQAT-----LMSIGSLGIE-PNKKHAKALYEVVCKELGVSKDRMYIHFVNAPTSDVGVNGTTFHDIIF
Bmor_MIF       MPHFRLETNISR-SKIPADLVVKAIPVLAKALGKPEYCVVTVIPEM--LMSFSGSDEPCATAN-----LMSIGLGEV-QNKKHAAVLFELVEKELGVPTDRMYITFQDEPTGNVGFKGTTFHAIIF
Apis_MIFA      MPHFRLETNISR-SKIPADLVVKAIPVLAKALGKPEYCVVTVIPEM--LMSFSGSDEPCATAN-----LMSIGLGEV-QNKKHAAVLFELVEKELGVPTDRMYITFQDEPTGNVGFKGTTFHAIIF
Mhir_MIF       MPYFKLDTNVPR-SKITPDLFKSTSKLVASTLGKPEYSYVVVQVNGDQ--SIIWGGTEEPGCGYAT-----LMSIGLGEV-QNKKHAAVLFELVEKELGVPTDRMYITFQDEPTGNVGFKGTTFHAIIF
Aame_MIF       MPTLTINTNLPA-SSIPNDFLKTANVVAASLGKPLSYVVVHISPDQ--LMSFSGSDEPCATAN-----LMSIGLGEV-QNKKHAAVLFELVEKELGVPTDRMYITFQDEPTGNVGFKGTTFHAIIF
Hlon_MIF       MPTLTINTNLPA-DKLPDFLATTSKVVADSLGKPLSYVVVHISPDQ--VMSFSGSDEPCATAN-----LMSIGLGEV-QNKKHAAVLFELVEKELGVPTDRMYITFQDEPTGNVGFKGTTFHAIIF
Isca_MIF       MPTFTINTNLPA-SKVPDDFLQTTAELVARSLGKPLSYVVVHISPDQ--KMSFSGSDEPCATAN-----LMSIGLGEV-QNKKHAAVLFELVEKELGVPTDRMYITFQDEPTGNVGFKGTTFHAIIF
Apis_MIFB      MPRLSLDTNLPA-SKIPEDFLSTCTSLLSKSLGKRSYCVSTVNPVQ--IMTLGGSNDPFCGFIQ-----VTSIGSLGPE-ENPKHIEVLTDDYMHQTLGIPKERLLINLQANIQETTGYLGTTFYQFLF
Bmal_MIF2      MPLITLASNVPA-SRFPDSDFNQFTLMAKMLGKPTSRILLVMPNA--QLSHGTTENPSCFTV-----VKSIGSFSAD-KNIEYSSLISEFMKTLTDIDPAHCIHFLNLDPENVGCGNTTMKELM
Ovol_MIF2      MPLITLASNVLA-SGFPTDFSVQFTKLMAEELGKPISRITLLVTPSA--QLSRGATQDPTCLIV-----IKSIGSFSAD-KNIEYSSLISEFMKTLTDIDPAHCIHFLNLDPENVGCGNTTMKELM
Asim_MIF       MPLVTLASNVPD-QKFPDSDFNQFTLMAKMLGKPTSRILLVMPNA--RLTHGGSDEPTCLIN-----MRAIGAFSDE-LNVKYASAIAEFQMKTGVIKPEKCLIEFADLESQNVSCSGTMMKVL
Cele_MIF3      MPVIKQVQTNVKK---VSDGFVRLAIHMAKVMKRPESQIFVSLDMNS--RMTRGQLTDPLAVLD-----VTSSTVLTPLLTHEEYTVACEFFSQELALDSDAVLINYRSLSPELIGFNGHILTENR
Cbri_MIF3      MPVIKQVQTNVKK---VSDGFVRLAIHMAKVMKRPESQIFVSLDMNS--RMTRGQLTDPLAVLD-----VTSSTVLTPLLTHEEYTVACEFFSQELALDSDAVLINYRSLSPELIGFNGHILTENR
Srat_MIF       MPYVRLFSNLPE-TSFTDAFCTEFTDLLAEKLGKDKSRIVMLVQPHQ--IMSSGGVPGQPSIWI-----INNVLGQSPR-QTQELSRDLTHFVMEKTTIPRESISILYFDMSPDMVARGGITIAESI

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Figure 12. Multiple sequence alignment of tick, nematode and insect MIFs. The MSA shows high level of sequence conservation in MIF among different species of invertebrates. Pro 1, 3,55; Thr 7,112; Asn 8,72; Ile 64, 96; Gly 65, 110, Ser 63 and Leu 87 amino acid Residues found to be the highest conserved ones across the species. Highlighted in blue is a CXXA motif identified in ticks.

The oxidoreductase domain represented by CXXC pattern is observed in some nematode sequences, but this sequence varies in ticks and all the ticks and show CXXA pattern. Some nematodes and arthropods do not exhibit any of these patterns. Highlighted grey region refers to a highly conserved amino acid sequence observed only in ticks and mites. This sequence was used to raise anti-tick MIF antibodies. The percentage identity of this region varies from 73- 91 % among ticks and it is well below 50% in other arthropods and nematodes. (The abbreviations for the species are as follows **Ticks:** Aame – *Amblyomma americanum*, Dvar – *Dermacentor variabilis*, Haelon – *Haemaphysalis longicornis*, Isca – *Ixodes scapularis*; **Insects:** Tcas – *Tribolium castaneum*, Bmor – *Bombyx mory*, Apis – *Acyrtosiphon pisum*, Mhir – *Maconellicoccus hirstus*; **Nematodes:** Ovol – *Onchocerca volvulus*, Bmal – *Brugia malayi*, Asuu – *Ascaris suum*, Ttri – *Trichuris trichiura*, Tspi – *Trichinella spiralis*, Cele – *Caenorhabditis elegans* , Cbri – *Caenorhabditis briggsae*, Wban – *Wuchereria bancrofti* , Acey – *Ancylostoma Ceylanicum*, Tpse – *Trichinella pseudospiralis*, Asim – *Anisakis simplex*, Srat – *Strongyloides ratti*, Acan – *Ancylostoma caninum*, Ovol – *Onchocerca volvulus*, Ttri – *Trichuris trichiura*, Tspi – *Trichinella spiralis*)

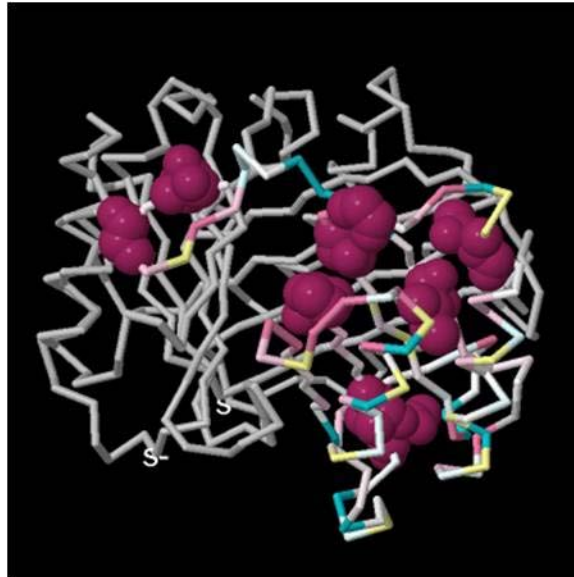


0.8

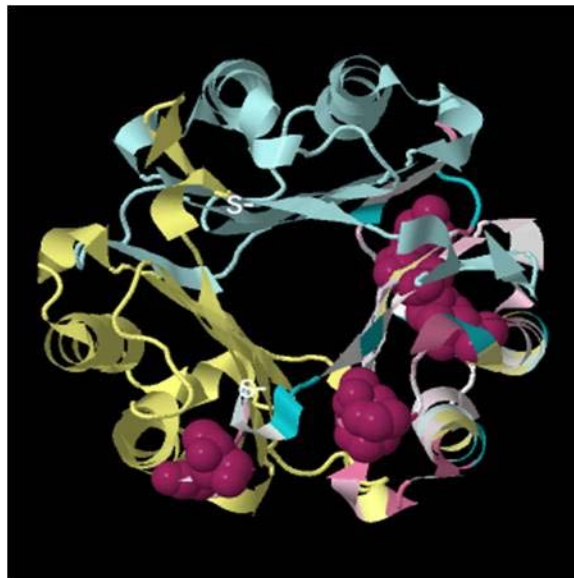
Figure 13. Phylogenetic analysis of MIF from ticks, nematodes and insects. Shown inside the rectangle are tick MIF sequences. Note that the tick MIF sequences show a closer phylogenetic relationship to the nematode MIF sequence than to the other insect MIF sequences. (The abbreviations for the species are as follows **Ticks:** Aame – *Amblyomma americanum*, Dvar – *Dermacentor variabilis*, Haelon – *Haemaphysalis longicornis*, Isca – *Ixodes scapularis*; **Insects:** Tcas – *Tribolium castaneum*, Bmor – *Bombyx mory*, Apis – *Acyrtosiphon pisum*, Mhir – *Maconellicoccus hirstus*; **Nematodes:** Ovol – *Onchocerca volvulus*, Bmal – *Brugia malayi*, Asuu – *Ascaris suum*, Ttri – *Trichuris trichiura*, Tspi – *Trichinella spiralis*, Cele – *Caenorhabditis elegans* , Cbri – *Caenorhabditis briggsae*, Wban – *Wuchereria bancrofti* , Acey – *Ancylostoma Ceylanicum*, Tpse – *Trichinella pseudospiralis*, Asim – *Anisakis simplex*, Srat – *Strongyloides ratti*, Acan – *Ancylostoma caninum*, Ovol – *Onchocerca volvulus*, Ttri – *Trichuris trichiura*)

TARGET	2	PTLTINTNLP	ASSIPNDFLK	TTANVVAASL	GKPLSYVVVH	ISPGQLMSFG
lhfoE	1	piftlnlnik	atdvpsdfsls	stsalvgnil	skpgsyvavh	intdqqlsfg
TARGET		sssssss	hhhh	hhhhhhhhhh	sssss	ss
lhfoE		sssssss	hhhh	hhhhhhhhhh	sssss	ss
TARGET	52	ATDEPCAIAAN	LYSIGCLSPK	ENKKHSAALF	EHIEKVLGIK	GNRMYINFID
lhfoE	51	gstnpaafgt	lmsiggieps	rnrhdhsaklf	dhlntklgip	knrmyihfvn
TARGET		ssss	sss	hhhhhhhhh	hhhhhhh	ssssssss
lhfoE		ssss	sss	hhhhhhhhh	hhhhhhh	ssssssss
TARGET	102	LPATDVGYSG	KTF			
lhfoE	101	lngddvgwng	ttf			
TARGET		s				
lhfoE		s				

Figure 14. Sequence alignment between *D. variabilis* MIF (Target) and its homology model identified using automated modeling mode at SwissModel. Two sequences shared 48.673% identity. The amino acids labeled as ‘s’ refer to a β -strand in the 3 dimensional protein structure and ‘h’ refer to α -helix



A.



B.

Figure 15. A schematic presentation of the results from the ConSurf analysis to find the conserved amino acids in the homology model for *D. variabilis* MIF. **A.** Stick diagram of the 1hfo trimer. Shown in spheres are the highly conserved amino acid residues localized in chain E of the trimer (Pro 1, Gly 65/110, Thr 7/112, Ser 63, Asn 8/72 and Ile 96) **B.** A cartoon diagram of 1hfo showing the solvent accessible channel located in the middle of the trimer. Each monomer is shown in different colors and highly conserved amino acids that are localized in chain E are shown in spheres.

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VITA

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Thesis: IDENTIFICATION AND CHARACTERIZATION OF MACROPHAGE
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Scope and Method of Study: This study identified and characterized macrophage migration inhibitory factor (MIF) from the American dog tick, *Dermacentor variabilis*. The gene expression, protein level changes and function of MIF was studied using a number of biochemical and molecular biology methods. The study further identified the phylogenetic relationship of MIF from different species using high throughput bioinformatics tools

Findings and Conclusions: The current study has identified and characterized the full length cDNA sequence of the macrophage migration inhibitory factor (MIF) from the American dog tick, *D. variabilis* and an up-regulation of the MIF gene expression during 0-96 hrs fed female *D. variabilis* ticks in both salivary glands and midgut tissues. The highest level of MIF expression was observed in midgut compared to the salivary glands. In contrast, the change in protein levels found in salivary glands and midgut tissues declined as the feeding progressed from 0-96 hrs.

RNAi mediated gene silencing of *D. variabilis* MIF was shown to down regulate the expression approximately by 90% compared to the buffer-injected controls in the salivary gland and midgut but gene silencing has not shown a negative impact on the ticks' ability to uptake blood meal. In both salivary gland and midgut, subolesin dsRNA injection down-regulated the MIF expression approximately by 50% compared to the buffer-injected control.

The bioinformatics analysis showed the across species conservation of the MIF amino acid sequence in ticks, nematodes and insects. The phylogenetic analysis revealed that tick MIFs share a close evolutionary proximity to some parasitic nematode MIFs rather than insect MIF. The homology modeling identified the closest *D. variabilis* MIF homolog available by the analysis was performed was from *T. spiralis* and the identified model and *D. variabilis* shared the same highly conserved amino acids.

ADVISER'S APPROVAL: Dr. D.C. Jaworski
