DIFFERENTIALLY EXPRESSED GENES IN THE SALIVARY GLANDS OF MALE TICKS, *Amblyomma americanum* (L.) AND *Dermacentor andersoni*: A COMPARATIVE APPROACH.

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PREFACE

This study was conducted to investigate differential gene expression in salivary glands of unfed and fed male ticks *Amblyomma americanum* and *Dermacentor andersoni,* in an attempt to identify and characterize novel genes induced by tick feeding. Ticks are of medical and economic importance throughout the world, but my work was concentrated on two of the most prevalent species in the Southern and Southwestern States of the United States of America (USA), that feed on cattle and humans resulting occasionally in serious diseases. As vectors of disease pathogens, ticks rank first as arthropod vectors of fungi, protozoa, rickettsiae, bacteria and viruses causing diseases in non-human vertebrates and ranking second only to mosquitoes as vectors of pathogens to humans. The use of the RAP-PCR allowed us to identify and isolate differentially expressed genes from unfed and fed male *A. americanum* and these methods may be useful in studying differential gene expression in tick species such as *D. andersoni* and identifying factors that are likely to be important in tick feeding and may have significance in the ability of the tick to transmit pathogens.

The study has shown evidence of increased gene expression and consequently increased protein synthesis during tick feeding. This was unequivocally demonstrated by both the increased total RNA and by identification of differentially expressed genes that code for transcription factors, translation initiation factors and other relevant factors involved in protein synthesis. This confirmed that changes in gene expression do occur in

the salivary glands of the male ticks, *A. americanum* and *D. andersoni* during feeding despite the differences in the feeding patterns. Further evidence for differences in the physiological and biochemical processes between the unfed and fed tick salivary glands is also observed from the increased size of the salivary acini during tick feeding. Cross-hybridization of probes synthesized from total RNA obtained from unfed and fed males, *D. andersoni* with cloned cDNA fragments from unfed and fed males, *A. americanum* salivary glands did not reveal significant similarities between the two tick species. Pathogens enter the host during feeding. Based on the results, a hypothetical model for fluid secretion by the tick salivary glands was proposed. It involves a tyrosine protein kinase substrate, ezrin, and a cAMP-activated cystic fibrosis transmembrane conductance regulator like chloride channel (CFTR-Cl- channel) that fits nicely with the salivary gland stimulatory machinery by dopamine.

ACKNOWLEGEMENT

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I would like also to express my respect and sincere appreciation to the head of our Department, Dr. James Blair for his kind, sincere and dedicated work towards the success

of our Department as the whole and his continuous attempts to make sure minorities are fairly represented in the Biomedical Sciences by trying to secure funds for that purpose. His financial support was one of the driving forces that enabled me to complete this piece of research.

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LIST OF ABBREVIATIONS

RMSF	Rocky Mountain spotted fever
HME	Human monocytic ehrlichiosis
HGE	Human granulocytic ehrlichiosis
MSP2	Major surface antigen 2
IgM	Immunoglobulin G
IgE	Immunoglobulin E
SGs	Salivary glands
RDA	Representational difference analysis
RT-PCR	Reversed transcription-polymerase chain reaction
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
RFLPs	Restriction fragment length polymorphisms
cDNA	Complementary deoxyribonucleic acid
dT	Deoxythymidine triphosphate
RAP-PCR	RNA arbitrarily primed polymerase chain reaction
ORF	Open reading frame
Taq DNA pol	*Thermus aquaticus* DNA polymerase

BLAST	Basic Local Alignment Search Tool
NCBI	National Center for Biotechnology Information
mRNA	Messenger RNA
ESTs	Expressed sequence tags
PLG	Phase lock gel
DEPC	Diethylpyrocarbonate
dATP	Deoxyadenosine triphosphate
SDS	Sodium dodecyl triphosphate
dUTP	Deoxyuridine triphosphate
$MgCl_2$	Magnisium chloride
TAE	Tris-acetate-EDTA buffer
EDTA	Ethylenediaminetetra acetic acid
SSC	Sodium dodecyl sulfate
CDP	Alkaline phosphatase substrate from TROPIX Inc.
CFTR	Cystic fibrosis transmembrane conductance Regulator
eIF2C	Eukaryotic initiation factor 2C
HSEF2	*Homo sapiens* elongation factor 2
PAGE	Polyacrylamide gel electrophoresis
ODC	Ornithine decarboxylase
TNFR2	Tumor necrosis factor receptor 2

TRAF	Tumor necrosis factor receptor-associated Factor
cAMP	Cyclic adenosine monophosphate
SPAG1	Sporozoite surface antigen 1
cMOAT	Canalicular multispecific organic anion transporter
PCD	Programmed cell death or apoptosis
PKA	Protein kinase A

CHAPTER ONE

INTRODUCTION

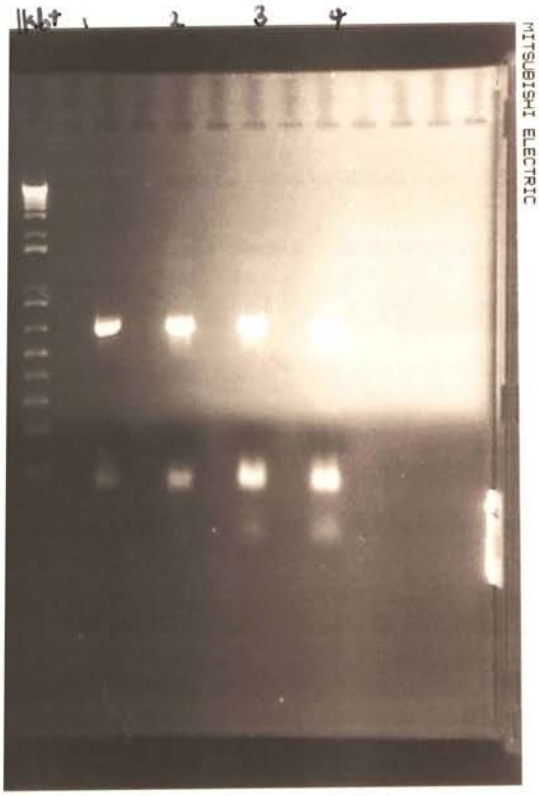
Taxonomy of the Ticks

Ticks are ectoparasites that belong to the phylum, Arthropoda, which makes up the largest population of creatures in the animal kingdom. Ticks are also the largest members of the order Acarina and consist of over 800 species. Ticks are divided into two major families, the Argasidae or soft ticks and the Ixodidae or hard ticks depending on the nature of the cuticle and other factors such as mode of feeding.

The family of Argasidae or soft ticks

Classification and life cycle

The life cycle of soft ticks begins with eggs, which hatch into six-legged larvae. Larvae take a large blood meal from a host and then molt to the nympal stage. Unlike hard ticks, the majority of soft ticks go through multiple nymphal stages (up to eight). The nymphs feed on blood and molt to the adult stage. Soft ticks are different from hard ticks in their mode of feeding in which they feed periodically, rapidly for several times during each life cycle stage and in that females lay multiple batches of eggs between blood meals.



ıan that of the hard ticks, estimated to be several
ears without a blood meal. An example for this
rsicus that is also known as the chicken tick and
ltry houses in southern and southwestern parts of
ach to humans if given the opportunity (Furman

s)

ecause of the characteristic tough, leather-like
ly. They are much smaller than the soft ticks and
lid ticks are compulsory blood feeders, found
a variety of animals including man. The females
rge blood meal while the males feed relatively
tages: eggs, larvae, nymphs and adults. Mating
ody of the host. After their blood meal, female
housands of eggs. Three different modes of
nding on whether the larval, nymphal or adult stages feed on one, two or three different hosts. Examples of one-host tick are the cattle tick, *Boophilus microplus* and the winter tick *Dermacentor albipictus*; an example of two-host tick is *Rhipicephalus evertsi*; and three-host ticks are *Amblyomma americanum, Dermacentor andersoni or Dermacentor variabilis.*

A one-host tick will spend its entire life cycle on one host; that is, it attaches to that animal as a larva, feeds and molts to the nymphal stage, feeds again and molts to the adult stage. The adults mate after which the female engorges with blood, falls to the ground and lays eggs. The new life cycle begins when the eggs hatch within thirty days and then the larvae seek a host. For two-host ticks, the larval and the nymphal stages spend their life cycle on one host, feed, molt and drop to the ground, and then wait for different animal species to complete the adult life cycle. In the three-host ticks, each stage spends its life cycle on a separate host. For instance, the larva feeds once and molts to the nymphal stage on one host and the resulting nymph drops to the ground, looks for a different host. On this second host, the nymphal stage feeds once and molts to an adult stage that will drop to the ground to look for the third host, which is usually a larger animal (Sonenshine 1993, Riberio 1996, Despommier et. al., 1995)

The Importance of Studying Ticks

Ticks are of medical and economic importance throughout the world, but my work is concentrated on two of the most prevalent species in the Southern and Southwestern States of the United States of America (USA), that feed on cattle and humans resulting occasionally in serious diseases. As vectors of disease pathogens, ticks rank first as arthropod vectors of fungi, protozoa, rickettsiae, bacteria and viruses causing diseases in non-human vertebrates and rank second only to mosquitoes as vectors of pathogens to humans. Ticks transmit diverse microorganisms that belong to the following genera: *Borrelia*, *Rickettsia*, *Francisella*, *Ehrlichia*, *Anaplasma*, *Theileria* and *Cowdria* resulting in significant economic losses worldwide. Lyme disease for instance, is a widespread debilitating disease caused by a spirochete, which affects humans and is spread

exclusively by hard ticks especially belonging to the genus *Ixodes*. The pathogen is commonly associated with white tailed deer and was first reported in Connecticut in the mid 1970s but has spread to the northeast, northern Midwest and the Western regions of the US. Rickettsiae are gram-negative intracellular bacteria transmitted by ticks belonging to the spotted fever group that causes rickettsiosis in humans. Rocky Mountain spotted fever (RMSF) is one of the most frequently reported tick-transmitted diseases in the United States. In the western hemisphere, the causative agent of RMSF *is Rickettsia rickettsii* but other strains exist throughout the world. The primary tick vectors for these pathogens are *Dermacentor variabilis* in the eastern states and *Dermacentor andersoni* in the western states. *Francisella tularensis,* the causative agent of tularemia, is a small rod-shaped gram-negative aerobic bacterium. Many distinct types with varying degrees of virulent attacks on humans are known. This pathogen is distributed worldwide and is capable of infecting a wide variety of animals. In the US, the most frequently isolated strain of *F. tularensis* is *F. tularensis var tularensis* and the common tick vector is *A. americanum* which can transmit the pathogen transovarially or transtadially (Corrier et. al., 1979, Sonenshine 1993, Eriks et. al., 1994, Kocan, 1995, Harrus et. al., 1997, Moody, et. al., 1998, Mahan 1999, Taege 2000, Blouin et. al., 2000).

Human ehrlichiosis is a disease caused by the genus *Ehrlichia.* Organisms belonging to this genus are gram-negative, obligatory intracellular bacteria that were first reported in North America in 1986 (Maeda et. al., 1987). *Ehrlichia spp.* has been demonstrated experimentally to infect humans, dogs and white-tailed deer. Within tick species, the only experimentally confirmed vector is *A. americanum.* The two types of human ehrlichiosis are human monocytic ehrlichiosis (HME) which is caused by *E. chaffeensis* and human granulocytic ehrlichiosis (HGE) caused by *E. equi* (Anderson et. al., 1991, Chen, et. al., 1994, Dawson et. al., 1991, Bakken et. al., 1994, Dumler et. al., 1995, Ewing et. al.,

1995, Bakken et. al., 2000). Theileriosis in a variety of domestic and wild ruminants in many parts of the world is caused by protozoan parasites that belong to the genus *Theileria* (Gonderia) and are transmitted by ticks. In the US, the only well-known species is *Theileria cervi*. Recent studies have shown that the organism develops in both the deer host and the tick vector. *A. americanum* is the only known vector with transtadial transmission being the only means of infection (Durham et. al., 1976, Hazen-Karr et. al., 1987, Laird et. al., 1988)

Close evaluation of several tick transmitted pathogens supports the claim that ticks are more than just simple syringes that inject the hosts with pathogens along with excess fluid and ions during blood meal concentration (Sauer et. al., 1979, Bowman et. al., 1997). The salivary glands are considered to be the major route by which pathogens enter the host during feeding. Failure to control ticks and tick-borne disease is a major factor limiting livestock production worldwide. Tick borne-diseases are particularly devastating to livestock; especially cattle with costs estimated at 7 billion-dollars/annum (Hoogstraal 1970, Riberio 1996). In the real sense the tick might participate actively with the pathogen leading to certain changes in both the vector and the pathogen during the process of feeding to enhance the pathogen's chances of transmission and survival in the vertebrate host. *A. marginale,* for instance, has a very complex cycle of development. The cycle begins with ingestion of *Anaplasma marginale* by the vector (the tick) and the transmission from tick salivary glands to the host's bloodstream where it can replicate in the red blood cells. From the two distinct morphological stages of *A. marginale* in the salivary glands of *D. andersoni*, the reticulate and the electron dense forms (Kocan et. al., 1992), it is possible that pathogen development may be promoted by certain factors in the tick. These factors may help the pathogen to undergo changes that enable it to go through all these steps and the tick, in turn, may be altered by the pathogen in order to improve its

transmission abilities and pathogen survival in the host (Kocan et. al., 1980, Kocan et. al., 1980, Oberst et. al., 1981, Kocan et. al., 1992, Kocan et. al., 1993, Kocan et. al., 1984, Kocan et. al., 1996).

The balance between host defenses and tick counter-measures that facilitate blood acquisition, including anticoagulants (Zhu et. al., 1997, Joubert et. al., 1998, Abendschein et. al., 2000,), antiplatelet aggregation factors (Keller et. al., 1993, Coburn et. al., 1993, Verstraete 1995), vasodilators (Riberio et. al., 1992, Dumler 1997, Qian et. al., 1998, Ferreira et. al., 1998, Papapetropoulos et. al., 1998), may all be important in influencing transmission and/or establishment in the host of tick-borne disease agents.

Types of Ticks Studied in This Project

Amblyomma americanum

Amblyomma americanum, commonly known as the lone star tick, is one of the most economically important tick species in the United States causing an estimated $398.9 million in losses per year (Byford et. al. 1992, Bowman et. al., 1997). The lone star tick was given its name because of the single white spot found on the back of the female. It is considered the most important tick pest of humans, pets, livestock, and wildlife in the southwestern, mid-Atlantic, and south central United States. *A. americanum* (L) belongs to the family Ixodidae or hard ticks that encompass many species that feed on vertebrate blood and use their salivary glands as a means of concentrating the blood meal by returning excess fluid and ions back to the host.

A. americanum is also a carrier of several pathogens that cause human diseases including ehrlichiosis, tularemia, Q fever and cervid theileriosis (Billings et. al., 1998,

Zikonov and Potasnan 1999, Johan et. al., 2000, Bakken and Dumler 2000, Irving et. al., 2000). Pastured cattle, *Bos taurus* when infested with large numbers of *A. americanum* ticks, lose significant amounts of weight (Barnard and Jones 1985, Barnard et. al. 1986, Ervin et. al., 1987).

Dermacentor andersoni

This species is also an ixodid tick with the common name Rocky Mountain wood tick. These ticks gained notoriety because they transmit a rickettsial hemoparasite of cattle and other ruminants, *Anaplasma marginale,* which causes anaplasmosis resulting in significant economic losses worldwide. *A. marginale* is a unique rickettsial organism that is transmitted biologically to cattle by the ixodid tick, *Dermacentor andersoni.* In *D. andersoni,* clusters of organisms occur within parasitophorus vacuoles called colonies, within which *Anaplasma* colonizes the tick and undergoes a complex developmental cycle. Final development occurs in the salivary glands. Male ticks were chosen in this study because of their importance in transmission of *A. marginale.* Males are intermittent feeders, a behavior that promotes inter-host transfer of the pathogen allowing the tick to act as a reservoir of infection for cattle for extended periods of time (Kocan, et. al., 1980, Kocan et. al., 1984, Kocan et. al., 1992, Kocan *et. al.*, 1993, Eriks et. al., 1994, Kocan et. al., 1995, Kocan, et. al., 1996, Ge et. al., 1996, Niebylski et. al., 1997, Frieddhoff 1997).

Distinct strains of *A. marginale* have been identified based on differences in tick transmissibility, molecular size of surface antigens, differences in DNA restriction fragments and reactivity to a variety of monoclonal antibodies. Strains are also differentiated and characterized based on their virulence, antigenic composition and ability to protect against heterologous challenge (Eriks et. al., 1994). *D. andersoni* males

and females have been shown to possess an important immunosuppresant protein (Da-p36) (Bergman et.al., 2000). Da-p36 is absent in the salivary glands or saliva of *A. americanum* (L.) ticks (Bergman et. al., 2000). Most importantly, the mRNA of this novel protein is greatly reduced in near-replete females after 8-days of feeding (Bergman et. al., 1998, 2000). *D. andersoni* pathogens express specific variants of major surface antigen 2 (MSP2) within the tick salivary glands (Rurangirwa et. al., 2000). It was also shown that some strains of *A. marginale* express restricted MSP2 variants such as the one isolated from salivary glands of *D. andersoni* ticks infected with the South Idaho strains. This restricted pattern of surface antigen expression does not apply to some other strains of *A. marginale.* The authors have also demonstrated that different variants of MSP2 are expressed when the same strain is transmitted by different Der*macentor spp.* This clearly indicates that the antigenic diversity within strains is maintained in tick transmission, a fact that poses crucial constraint to the use of MSP2 as a vaccine candidate (Rurangirwa et. al., 2000).

In this study we investigated gene expression in male *A. americanum* salivary glands from unfed and feeding ticks and related this to the gene expression of *D. andersoni* male salivary glands. This is simply because *A. americanum* females have been intensively studied in the laboratory and are readily available. Some of these studies strongly indicated increased gene expression in the salivary glands of the lone star tick during feeding. *D. andersoni* on the other hand was selected for gene expression comparison due to its function as a vector of the cattle disease anaplasmosis.

Tick Feeding

Ticks feed on vertebrate blood and use their salivary glands as a means of concentrating the blood meal by returning excess fluid and ions back to the host. Ticks are active during feeding, and the salivary glands of female, *Amblyomma americanum* (L) ticks exhibit physiological and biochemical changes, notably new gene expression and protein synthesis (Hunt and Hilburn 1984, Jaworski et. al., 1992, Oaks et al., 1991).

Hard ticks have remarkable longevity and some species survive up to two years without a blood meal. Females feed slowly and become completely engorged on approximately the ninth day of feeding. The feeding site on the host is probed carefully by the tick, mouthparts are inserted and cement-like material is secreted to help fix the mouthparts to the host's skin. After engorgement the tick removes its mouthparts and detaches from the host. Tick feeding may be painless to the host and the animal is often unaware because of suppressive factors in tick saliva (Arthur 1970, 1973).

In contrast to the fast and multi-feeding bloodsucking arthropods (e.g. mosquitoes), the female ixodid ticks take a large amount of blood in a single feeding and increase in weight up to 100-fold its original body weight within a period of 7-14 days. At the completion of engorgement, this huge blood meal is ultimately converted into a large number of eggs after which the female dies (Sonenshine 1991). Female tick feeding is divided into slow feeding and fast feeding stages. In the slow feeding stage, the salivary glands secrete cement proteins to seal the mouthparts firmly onto the host's skin (Riberio 1989, Wikel et. al., 1994, Nuttall 1998). After mating, the fast or rapid stage of feeding

takes place within a few hours after which the tick drops from host and prepares for oviposition before it dies (Toutoungi et. al., 1995). Male ixodid ticks, on the other hand, frequently attach and detach from the host and can mate with multiple females. Males reattach close to the female feeding area and secrete IgG-binding proteins that are hypothesized to assist the females in successful feeding (Wang et. al. 1998).

Immunity to Ectoparasites

Although ticks cause considerable damage in their own right, they are equally, if not more, important as vectors of diseases especially if transmitting pathogens such as *Anaplasma* and *Theileria* (Chizyuka and Mulilo 1990). As a consequence, there is a fascinating interplay between the three components of this complex interaction (vector, pathogen and host). The host responds immunologically to the tick's bite by immediate hypersensitivity reactions (Lavaud et. al., 1999, Mulenga et. al., 2000) as a non-specific protection to make the host more aware of the presence of the parasite, when the latter is accessible (Wakelin, 1984). The first line of defenses which are capable of protecting the host are dendrites, antigen-presenting cells (e.g. macrophages), and Langerhans cells, capable of processing antigens presented during the feeding of the parasite. Other specialized cells such as neutrophils, basophils, eosinophils and mast cells are also involved in the protection of the host (Wikel 1996, Brossard and Wikel 1997). A cellular immune response elicited by the T-cells is more stable and effective than the humoral or antibody-based immunity (Mattioli et. al., 2000). In some hosts, the immunogenic reaction to a tick bite may be rapid and can prevent tick feeding completely or even kill the ticks by causing starvation and desiccation (Wikel 1996, Wang and Nuttall 1999).

Different breeds of cattle have developed genetic variation in their ability to develop immunity against ticks and the pathogens they transmit. Some of those breeds are more resistant to tick-borne pathogens than others. Therefore, the magnitude of animal losses due to tick attack and tick–borne microorganisms depends on the extent or the degree of breed resistance. The highest resistance has been found in *Bos indicus* (Zebu) followed by the European cattle *Bos taurus* (Mattioli et. al., 2000).

Nevertheless, some pathogens such as *Babesia* (agent of Babesiosis in cattle) can exert powerful immunosuppressive activities on the hosts, leading to more heavy tick infestation. Although, the pathogen can optimize its own survival, ticks can also exert immunomodulatory effects on their hosts, which may further confound the suppression of the host's immunity (Riberio et. 1985, 86, 87, Jaworski et. al., 1990, Wikel 1999, Paesen et. al., 1999, Nuttall 1999, Bergman et. al., 2000).

Host protective responses mentioned above notably, antigen-presenting cells, T-lymphocytes, B-lymphocytes, basophiles, mast cells, eosinophils and other variety of bioactive molecules such as cytokines, antibodies and complement, can be confounded by tick–induced immunosuppression responses that, while they are sometimes striking, appear to be irrelevant to tick rejection (Brown and Askenase 1985, Brossard and Wikel 1997). Tick feeding may take several days and is preceded by production of saliva and cement-like material that holds the mouthparts firmly in the host's skin. Saliva also contains many antigenic secretary materials that can lead to a pronounced inflammatory response, although it does not prevent engorgement.

Immunological Control of Tick Parasitic Infections

Many strategies that were developed to control ticks and tick-borne diseases include the use of chemicals (acaricides) such as organophosphorus (Redondo et. al., 1999), host diet manipulation and use of vaccines such as live and attenuated vaccines, use of tick antigens as well as genetic-based vaccines. The use of acaricides has caused environmental and residual problems apart from the fact that most tick species have developed acaricide resistance (Musoke et. al., 1997). Manipulating the host diet has boosted natural immunity by vertebrates to tick infestation. A study in which rabbits and sheep have been exposed to low- and high-protein diet and subsequently infested three times with adult ticks, the weight of the ticks fed on animals maintained at high-protein diet decreased significantly while the weight of ticks fed on animals that were exposed to low-protein diet did not change significantly. Regarding the conditions of those hosts after tick infestation, it has been demonstrated that hosts maintained on a low-protein diet failed to acquire resistance to ticks, lost weight and developed anemia, while those on a high-protein diet developed resistance, maintained weight and did not lose excess amounts of blood. These results clearly indicate that nutritional status, especially the protein content, is crucial in developing protective immunity or resistance to tick infestation by the host (Rechav and Hay 1992). Pioneer work performed by Glines and Samuel (1989), and Teel et. al., (1990) has demonstrated that cattle resistance to *Boophilus annulatus* infestation was affected by the host diet.

Immunity to ticks by the hosts is not a local phenomenon and once elicited can operate effectively at sites far from the original feeding site. Tick antigens can also elicit antibody response when processed by Langerhans cells, resulting in IgG and IgE that bind to amine-containing cells. The antigen-antibody complexes formed can also activate the

complement system. Saliva is a complex mixture and several antigens have been identified. The host may respond to many of these, with some being more important than others are. For instance, in *D. andersoni*, serum from immune rabbits recognizes a large number of antigens that are secreted early in the feeding cycle but not later, when the host's immune system responses are underway. The concept of inducing protection by immunizing the host is not against salivary antigens released during the feeding process, but against antigens associated with the intestinal epithelial cells (concealed antigens to which the host is not normally exposed (Wang and Nuttall 1999) because the former can not be regarded by the host as foreign. Therefore, the high degree of susceptibility of most animals like cattle to tick bite and their transmitted pathogens warrants the use of vaccines. Live vaccines are based on parasites derived from culture or from blood of infected animals. One of first vaccines used was developed against anaplasmosis (caused by *A. centrale*) and babesiosis (caused by *Babesia bovis*). When *Bos taurus* were immunized with these vaccines and transported to the tropics of Colombia where they were divided into two groups and placed in separate pastures, one group of cattle that was put on low-protein pasture, suffered heavy infestation with *Boophilus microplus* and the other that was put on high-protein pastures, was lightly infested. The heavily infested group suffered losses in body weight, became anemic, and about half of them died of severe ixodiasis and babesiosis while the one that was lightly infested did not show significant weight loss or anemia (O'Kelly et. al., 1971, Corrier et. al., 1979). These results may also indicate the importance of host diet in eliciting the natural immunity and acquired immunity provided by the vaccines. Despite the potential risk of accidental transmission of disease agents when using the blood-based attenuated live vaccines, various degrees of resistance to field infection have been reported with cattle immunized with those vaccines (Pipano 1995, Lawrence 1997).

Although the principal method of controlling ticks and tick-borne diseases is the use of breeds that have some degree of parasite resistance, no breed is completely resistant and all at times can be severely affected by parasites. Since complete resistance is the ultimate goal in the cattle industry, completely resistant lines have been developed by genetic means from most parasite-susceptible breeds within a commercially acceptable time frame (Frisch et. al., 2000).

Studying gene expression in the tick and in the pathogen during feeding may be important in understanding tick-pathogen-host relationships and development of genetically based vaccines.

Biochemical and Physiological Studies of Ticks

Tick salivary gland physiology has been studied extensively in females. Clear differences have been noted in unfed and fed ticks in gene expression, correlated with remarkable biochemical and morphological changes after tick attachment, mating and feeding (Sauer et. al., 1979, Wang and Nuttall 1994, Wang et. al., 1999, Sauer et. al., 2000).

The saliva of female ticks contains, in addition to water and ions, cement-like material that helps anchor the mouthparts to the host, prostaglandins, anticoagulants, immunosuppressive and anti-inflammatory protein molecules that help overcome host defense responses (Binnington and Kemp 1980, Jaworski et al. 1990, Riberio *et al.*, 1985, 1992, Sauer *et al.*, 1979, 1995, Bowman *et al.*, 1997).

The salivary glands of female ticks undergo remarkable biochemical and morphological changes after tick attachment, mating, and feeding and exhibit new gene expression and protein synthesis (Sauer *et al.* 1995). In an elegant experiment, male, *D.*

andersoni ticks were infected with *A. marginale* intrastadially by feeding them on an infected calf for 7 days (acquisition feeding), removed and then kept in a humidity chamber for 5 days before they were fed again on a susceptible calf for 10 days (transmission feeding) (Ge et. al., 1996). A generalized infection that originated in the gut tissue was observed. Final development of the organism occurred in the salivary glands, and transmission of the pathogen did not take place until after the ticks had actually fed for some days. This delay in onset of *A. marginale* transmission may imply that new gene expression in the tick salivary glands or the pathogen is required before actual transmission can take place. The multiple tissue infection was persistant in intrastadially infected male ticks. Male ticks were chosen because of their epidemiological importance in transmission of *A. marginale* and their feeding pattern when they feed intermittently, a behavior that can greatly promote inter-host transfer of the pathogen (Kocan, et. al., 1980, Kocan et. al., 1984, Kocan et. al., 1992, Kocan *et. al.*, 1993, Kocan et. al., 1995, Kocan, et. al., 1996).

Fluid secretion from the salivary glands is controlled by dopamine, a neurotransmitter at the neuroeffector junction, and as feeding advances, the rate of salivary gland secretion increases progressively to enable the tick to concentrate the blood meal (Sauer *et al.*, 1995, Sauer *et. al.*, 2000). Salivary gland proteins that change during tick feeding include dopamine-activated adenylate cyclase (Schmidt *et al.*, 1981, 1982, Schramke *et al.*, 1984), an inhibitor protein of cyclic nucleotide phosphodiestrase (McMullen et al., 1983) and Na+/K+ ATPase which enable the tick to carry out osmoregulatory functions such as concentration of the blood meal (Kaufman *et al.*, 1976).

In contrast to females, very little is known about physiological, biochemical and molecular changes in the salivary glands of male ticks before or after male attachment to

the host. Some attempts have been made to investigate male factors involved in enhancement of female feeding and egg development (Kaufman and Lomas 1996). While female ticks imbibe large volumes of blood over a lengthy period, male ixodid ticks are intermittent feeders with low blood intake (Fujisaki et. al., 1976, Madden et. al., 1996). The type IV acinus, restricted to males, is thought to have a reproductive role possibly in sperm transfer (Feldman-Muhsam et al., 1970). Wang and Nuttall (1995) have produced evidence for a male-specific immunoglobulin G-binding protein that is hypothesized to be important in protecting the "mate" during attachment and feeding by nearby females. Although feeding in males is less pronounced than in females, I hypothesized that attachment and feeding increase gene expression to provide extra proteins needed by the tick to successfully feed as in females. Differential display of eukaryotic messenger RNA (mRNA) is a powerful means of identifying and isolating those genes that are differentially expressed under altered conditions (Liang and Pardee, 1992). The purpose of this study was to assess differential gene expression in salivary glands of unfed and fed *Amblyomma americanum* and *Dermacentor andersoni* ticks. Results indicate expression of significant genes of likely importance in coding for proteins that facilitate salivary gland development, physiology and tick feeding.

The factors responsible for inducing expression of new genes in fed ticks are unknown. Nutrients are known to induce gene expression in many situations. Food availability is one of many factors that trigger developmental processes such as hatching, metamorphosis, and sexual maturity in flatfishes and eels (Adron, et al., 1978, Egginton 1986). Glucose has been implicated in transcription regulation of many genes in animal skeletal muscle and the induction of insulin gene expression (Tsao et al., 1996). Also, the liver, the principal organ of lipogensis and cholesterogenesis, is responsible for synthesis of triglycerides from excess dietary carbohydrates. A high carbohydrate diet can induce

transcription of mRNAs for a group of lipogenic enzymes such as acetyl-CoA carboxylase, fatty acid synthase, and stearoyl-CoA desaturase (Shimano et al., 1999).

It is well established that during feeding of the female lone star tick, RNA synthesis is increased in parallel with an increase in protein synthesis. This laboratory and others reported that previously dormant genes in the salivary glands' genome are being induced during feeding (Shelby et. al., 1987, Wang & Nuttall 1994). The work done through an *in vitro* translation of the isolated mRNAs revealed that eight proteins in the fed female tick's salivary glands were not found in the unfed tick's salivary glands. This clearly indicated that those genes were differentially expressed during the feeding state (McSwain et. al. 1982 & Oaks et. al. 1991).

This study is being conducted to identify and characterize genes that are differentially expressed in the salivary glands (SGs) of both the unfed and fed male ticks *Amblyomma americanum* and *Dermacentor andersoni*. In future studies, differentially expressed genes in male *D. andersoni* SGs that are different from those of male *A. americanum* SGs during feeding can be compared with the ones that might be identified in infected *D. andersoni* SGs and checked for possible involvement in *A. marginale* transmission. The work on this project may be important in helping identify changes in gene expression in the salivary glands of *D. andersoni* during feeding which relate to their ability to transmit pathogens such as *Anaplasma marginale*. In other words, the results from uninfected salivary glands' study may reveal some interesting differences between the two ticks during feeding. Thus my goal was to establish techniques and to investigate gene expression alteration in the uninfected tick salivary glands in male lone star ticks prior to detailed studies of *A. americanum* and *D. andersoni* interactions. Previous studies demonstrated that protein increases in salivary glands of male *A. americanum* during tick

feeding strongly suggesting the initiation of new gene expression during tick feeding (Sanders et. Al., 1996).

General Methods used to isolate Differentially Expressed Genes

Large repertoires of techniques are available for production of an inventory of differential transcripts between two populations of mRNAs. The specific methods of identification and isolation of differentially expressed mRNAs are differential display and related techniques such as representational difference analysis (RDA) (Lisitsyn et. al., 1993, Zeng et. al., 1994, Deleersnijder 1996), enzyme degradation subtraction (Akopian and Wood 1995), linker capture subtraction (Yang and Sytkowski 1996), and physical methods used to remove sequences (Von Stein et. al., 1997, Welsh and McClelland 1990). Although some of these methods are successful, they still have pitfalls. For instance, differential display (based only on oligo-dT RT-PCR) is confined to the analysis of those differences at the 3'-end of cDNAs, leaving those at the 5'-end undetected. The incidence of false positives makes it difficult to isolate rare differentially expressed transcripts. RDA (Lisitsyn et. al., 1993) requires many rounds of subtraction and fails to account for large differences in relative abundance of individual message (Welsh et. al., 1991). In other words RDA can not be used to know whether the message is abundant or rare in the system since only small amounts (representations) of the sample are analyzed (Odeberg et. al., 2000). RDA is based on the principle of subtractive hybridization, for cloning even the smallest DNA differences between two or more complex genomes and shows great promise for speeding up this rate-limiting step in genetic analysis (Lisitsyn et. al., 1993). Subtractive hybridization involves repeated rounds of hybridization of excess DNA from the source lacking a desired sequence with DNA from the source containing the sequence, followed by removal of the undesired sequences at each round.

This method has two main applications. The first involves cloning of DNA fragments present in an insertion or to clone DNA fragments that are removed by a small deletion. The second application involves identification of DNA sequence differences between individuals (Rosenberg et. al., 1994) called restriction fragment length polymorphisms (RFLPs). Two steps are used in each application; the initial step involves amplification of a relatively small fraction from each of the nucleic acid materials one wants to test (called representation). These representations are then used in a subsequent subtractive hybridization step, in which the amplified sequences of the genome lacking the target are hybridized in great excess with those from the target-containing genome. The hybridization is allowed to proceed to a point where a small percentage of the target segments re-anneal, while the large percentage of the remaining DNA fragments in common between the two fragments re-anneal because of the molar excess of the genome lacking the target. The polymerase chain reaction (PCR) is then applied using primers that allow only a small fraction of the double-stranded target segments (self-annealed tracer molecules from nonextractable fraction) to amplify exponentially. This is done in such a way that all other undesired sequences do not amplify or they might only increase linearly. Any single-stranded DNA could simply be removed by selective digestion with mung bean nuclease. The desired amplified fragments are usually enriched in the first round of RDA, while any additional round of hybridization mostly generates a few DNA fragments of different products that can be isolated in pure form by agarose gels and cloned (Myers 1993, Straus and Ausubel 1990, Patanjali et. al., 1991).

Some researchers have applied this principle to construct cDNA libraries from differentially expressed poly $(A)^+$ RNA. In this application, randomly primed cDNA fragments of selected size range were cloned into λ phage vector, and inserts were amplified by polymerase chain reaction, denatured, and self-annealed under optimized conditions. Libraries of these fragments are suitable for cDNA subtraction, screening or selection by hybridization and could be used to analyze a cDNA corresponding to species

of mRNA present at low level in a small fraction of the cells in a complex tissue (Welsh et. al., 1992, Welsh and McClelland 1994).

These methods have been extensively utilized in studying eukaryotic systems because investigators have taken advantage of eukaryotic mRNAs that are usually tailed by a polynucleotide poly (A) tail at the 3'-end. This poly $(A)^+$ tail hybridizes to a synthetic poly (dT) oligonucleotide primer and a first cDNA strand is synthesized by a reverse transcriptase enzyme through a process called reverse transcription (RT), (Sakai et. al., 1985). The polymerase chain reaction (PCR) is used to amplify the internal sequences of the first cDNA strand produced by RT reaction during the second cDNA strand synthesis. Because it uses random primers of eighteen oligonucleotides in the PCR process it is called RNA arbitrary primed polymerase chain reaction or RAP-PCR (Sakai et. al., 1988, Liang and Pardee 1992). PCR involves repeated cycles of denaturation of the DNA, annealing of the oligonucleotides homologous to sequences flanking the open reading frame (ORF) of certain gene(s). Primer extension by DNA polymerase (Taq DNA polymerase) isolated from *Thermus aquaticus* bacterium), results in a doubling of the amount of a specific DNA or cDNA fragment with each cycle (Sokolov and Prokop 1994). The RAP-PCR mentioned above is very sensitive due to the labeling of the amplified cDNAs that leads to detection of molecular weight transcripts ranging from 600 to 100 bp. Amplified fragments are detected and separated on a denaturing DNA sequencing gel electrophoresis. Particular cDNA fragments can be eluted, reamplified and characterized thereafter (Cao and Sarkar 1992). This technique for RNA fingerprinting is of particular interest since it can be applied equally to both eukaryotic and prokaryotic systems (Wong and McClelland 1994,).

The differential expression of genes in the salivary glands of unfed and fed adult males of *A. americanum* and *D. andersoni* was studied using the method of RAP-PCR. I hypothesized that attachment and feeding of male ticks would cause differential expression of genes in the physiologically different states of the tick. I also hypothesized

that some possibly novel genes relevant to salivary gland physiology and pathogen transmission would be identified.

Similarity search in the Nucleic Acids and Protein Database

Generally, sequence similarity search is the major application of the sequence database record, where sequences similar to a query sequence supplied by the investigator are identified in a sequence database. In most cases, this operation identifies potential homologues of the query sequence, in other words, database sequences sharing a common ancestral history with the query sequence. Homologous sequences can provide a clue as to the function of the query sequence, its evolutionary history and its structure. For instance, if an unknown protein sequence is found to be profoundly similar over its entire length to the sequence of a protein of known structure in the database, the two proteins are likely to have similar structure and possibly related functions. The second application of these databases involves identification of coding regions in uncharacterized genomic DNA by looking for DNA regions that can be translated into an amino acid sequence similar to that of a known protein. A notable application of BLAST with short sequences involves checking whether PCR primers are likely to match many sequences in a genomic DNA preparation and give arise to wrong PCR products.

The Types of BLAST Searches Used and Their Advantages

The advantages of the BLAST searches include: access to a large number of biological sequence databases all over the internet, among which are the central repositories of public sequence data such as NCBI, the Japanese GenomeNet Server and the European Bioinformatic Institute etc. It allows rapid searching for sequences similar to a query

sequence. There are four BLAST programs for searching nucleotide sequence databases (Blastn, Blasx, tblastn and tblastx) and two programs for searching protein sequence databases (Blastx and Blastp).

Blastn

Blastn searches a nucleotide sequence database (BLAST program) using a query nucleotide sequence to produce nucleotide sequence alignments. The typical uses of Blastn include checking whether a newly obtained DNA sequence has already been published, identifying regions in genomic DNA by searching for encoded (related mRNA) sequences and extending sequence fragments by searching collections of partially characterized sequences such as Expressed Sequence Tags (ESTs).

Blastx

Blatsx translates a nucleotide sequence into protein and then searches a protein sequence database. It is particularly useful for identifying potential coding regions in newly sequenced DNA.

Tblastn

Tblast uses a protein query sequence to search a translated nucleotide sequence database and it is useful for detecting unidentified proteins encoded in a nucleotide sequence database.

Tblastx

Tblastx compares a query nucleotide sequence with a nucleotide sequence database after translating both sequences into proteins. This approach greatly increases the sensitivity of the search when dealing with protein-coding regions but it is substantially slower than the direct comparison performed by Blastn.

Interpretation of BLAST Search Results

BLAST searches report only the significant matches that are not obtained as the result of random sequence similarity. Matches with a short sequence <30 bp are likely to have occurred by chance, and therefore, the program does not report them. While this program is useful for searching for homologous sequences, it is not useful when searching for potential candidates matching a short protein sequence (e.g. the one obtained by amino terminal sequencing of an unknown spot on a two-dimensional protein gel). The most important value given by the program is the E (or P) value, which is the statistical expectation threshold parameter. This value controls the level of statistical significance that will be reported by the program. The default value for this is 10, which corresponds to the cut- off level of similarity for reported database matches that are expected to have been found by chance alone. Maximum expectation threshold value is 1000, which is not enough for some applications involving short query sequences. It is possible to raise the statistical expectation threshold when the query sequence does not give any matches when some are expected, however, most matches with a high statistical expectation

scores are likely to be due more to chance sequence similarity than they are to biological relationship (Wu et. Al. 1997, Gaeta, 2000).

CHAPTER TWO

Materials and Methods

Experimental animals

A. americanum (L.) and *D. andersoni* males were reared and fed on host animals according to the methods of Patrick and Hair (1975). *A. americanum* males were fed on sheep with females. One group of male *D. andersoni* was reared with females (G_2), another group of *D. andersoni* for the same amount of time (7-14 days) was fed without females (G_1) to test the effect of concurrent female feeding and mating on male salivary gland RNA. Salivary glands were dissected within 2-3 hours after removal from the host, cleaned with the dissection buffer and immediately transferred into liquid nitrogen and stored at -80° C.

Materials

Rapid total RNA isolation kits were purchased from 5 prime ⟶ 3 prime Inc. (Boulder, CO USA). RAP-PCR kit including the Maloney murine leukemia virus reverse transcriptase (RT) and RAP-PCR primer sets were from Stratagene (La Jolla, CA USA). Message Clean or DNase 1 kit was from GenHunter Corporation (Sunnywale, CA USA). Plasmid DNA and PCR product purification kits were obtained from Qiagen (Valencia, CA USA) and Wizard Plus plasmid DNA purification kits were from Promega (Madison,

WI USA). Cloning vectors, pCRII, and *E. coli* supercompetent cells were obtained from Invitrogen Corp. (Carlsbad, CA USA). Restriction enzymes as well as Taq DNA polymerase were purchased from Gibco BRL (Rockville, MD USA) or Promega (Madison, WI USA). X-ray film was obtained from Fuji Film Company (Stampford, CT USA). PCR Digoxigenin 11-dUTP probe synthesis mixture [(containing 200 μM each of various deoxyribonucleoside triphosphates (dNTPs)] was from Boehringer Mannheim Corporation (Indianapolis, IN USA). Mineral oil was obtained from Sigma (St. Louis, MO USA). A PTC 100™ Programmable Thermal Controller from MJ Research, Inc. (Waltham, MA USA). GelDoc with a Multianalyst computer program (BIO-RAD Laboratories; Hercules, CA USA). Tropilon-Plus nylon membrane was from TROPIX (Bedford, MA USA). ULTRAhyb solution and BrightStar™ BioDetect™ nonisotopic Detection were Ambion, Inc. (Austin, TX USA). Anti-digoxigenin Fab-fragment conjugated to alkaline phosphatase were obtained from Boehringer Mannheim (emerged with Roche Diagnostics, Indianapolis, IN USA). Alkaline phosphatase substrate, CDP, from (TROPIX),

Isolation of total RNA

Salivary glands were obtained from 2140 unfed and 1000 fed *A. americanum* and 350 unfed and 220 fed (without females) and 70 fed (with females) male *D. andersoni* ticks. Salivary glands were dissected and placed immediately in liquid nitrogen and RNA was isolated according to the methods prescribed by 5 prime→3 prime for rapid total RNA isolation. One ml of 4 M guanidium isothiocyanate was added to approximately 100 mg of salivary glands and homogenized. The homogenate was transferred to Phase Lock Gel (PLG) tubes and centrifuged at 1500 xg for 1-2 min. One ml of 2.0 M sodium acetate, pH

4.0, was added to the homogenate, the tubes capped and the contents mixed briefly. One ml of H_2O-saturated phenol was added to the sample and mixed thoroughly. The 0.3 ml of chloroform-isoamyl alcohol (49:1) was added to the sample in the same PLG tubes and mixed again as mentioned above. Samples were incubated on ice for 10 min. and then centrifuged at 4500 xg for 5 min to separate the phases. The aqueous phase was transferred to PLG tubes and 1.0 ml of phenol-chloroform-isoamyl alcohol (50:49:1) added to the aqueous phase and thoroughly mixed as before. The samples were centrifuged at 4500 xg for 5 min and the aqueous phase collected and placed in clean RNase-free 10.0-ml tubes with an equal volume of 100% isopropanol and mixed. The samples were incubated at room temperature for 20-30 min, centrifuged at 16,000 xg for 30 min and the supernatant discarded. The RNA pellet was washed three times at 16,000 xg for 2-3 min with 5 ml of 70 % Ethanol (in DEPC-treated H_2O) and once with 5 ml of 95 % ethanol. The final wash was discarded and the RNA pellet dried at room temperature for 30 min. Isolated salivary gland RNA was dissolved in molecular biology grade water, and its concentration determined spectrophotometrically at 260 and 280 nm. The total RNA was treated with RNase free DNase 1 provided in a Message Clean Kit aliquoted and stored at –80° C before use.

Synthesis of the cDNAs

RNA was fingerprinted with reverse transcriptase (RT) and the RNA arbitrarily primed polymerase chain reaction (RAP-PCR). Primers A_1, A_2 and A_3 consisted of 5'-A-A-T-C-T-A-G-A-G-C-T-C-C-T-C-C-T-C-3' (A_1), 5'-A-A-T-C-T-A-G-A-G-C-T-C-C-A-G-C-A-G-3' (A_2), and 5'-A-A-T-C-T-A-G-A-G-C-T-C-T-C-C-T-G-G-3' (A_3). Three separate reactions were conducted with each primer: a negative control with all the components

except RT to detect DNA contaminants, a positive control consisting of oligo (dT) and a primer for the human β-actin gene; salivary gland RNA and RT enzyme. The cDNA fragments produced in the reactions were radiolabeled with α–^{32}P dATP during synthesis of the second strand of cDNA and then amplified by PCR. PCR products were separated on a 6% denaturing polyacrylamide gel, dried and exposed to Fuji X-ray film and autoradiographed. After carefully aligning the autoradiograph to the gel, the different differential bands were excised with a single-edged razor blade. The excised gel pieces were eluted in water, at 65° C for one hour and then centrifuged at 14,000 xg for 15 min. The supernatant was collected and an aliquot (10 μl) was amplified with the same primer used in the original RAP-PCR reaction. PCR amplification was performed with one low-stringency cycle at 95° C for 1 min., 36° C for 5 min. and 72° C for 5 minutes; forty high-stringency cycles at 94° C for 1 min., 60° C for 2 min. and 72° C for 2 min. with a final cycle at 72° C for 10 min A PTC 100™ Programmable Thermal Controller. The reaction mixture was held at 4° C until an aliquot (15 μl) was analyzed by 2 % agarose gel electrophoresis. The remaining reaction mixture was stored at -20° C awaiting the results of agarose gel analysis. The PCR products that were detected in agarose gel were then run on agarose gel, excised, extracted and saved for cloning. The products that were not detected in the first round of PCR were amplified in the second round to obtain the bands. These reaction products were analyzed by 2% agarose gel electrophoresis and appropriate bands were excised from the gel, extracted and saved for cloning.

Cloning of the cDNAs

After PCR amplification gel purified fragments from both unfed and fed tick salivary glands were cloned into a pCR-II vector using T/A sticky end ligation, according to the manufacturer's instructions. The insert was placed between two EcoR1 restriction sites to form the recombinant plasmid. Recombinant plasmids were then transformed into TOP 10F' One Shot Competent Cells and cultured on agar plates for selection and amplification.

Purification of the Recombinant Plasmids

White colonies, indicative of the presence of inserts (5-10) from each clone were picked from agar plates and grown in Luria Broth Base (LB) containing ampicillin. To purify plasmid DNA bacterial cells were centrifuged at 1,400 xg for 10 min and the cellular pellet was re-suspended in a solution of glucose before lysis by NaOH/SDS buffer in the presence of RNase A (Birnboim and Doly 1979; Birnboim 1983). The cell membranes were lysed and cell contents denatured by the alkaline conditions and the chromosomal and plasmid DNA and proteins released. The lysate was subsequently neutralized and adjusted with high-salt binding conditions prior to purifying with a QIAprep silica-gel membrane by adding the neutralization buffer. Before centrifugation (13,000 xg for 10 min), the solution was mixed gently to assure complete precipitation of chromosomal DNA. Soluble plasmid DNA was eluted from the column with 200 µl of Tris-HCl buffer (pH 8.5), concentrated to 50 µl in a speed vac and the optical density (OD_{260}) determined. Uncut recombinant plasmid DNA (250 ng) was examined by 2 % agarose gel electrophoresis for the presence of an insert by comparing its migration to

migration of the plasmid DNA alone. Positive clones were digested with EcoR1 and the product analyzed again by 2 % agarose gel electrophoresis to verify the presence of inserts.

Sequencing of the Recombinant Plasmids

Products were sequenced by the OSU Protein/Nucleic Acid Resource Facility from the T7 promoter. The resulting sequences were used to search the non-redundant DNA and protein databases using the National Center for Biotechnology Information database web site.

Confirmation of Differential Expression of the Cloned cDNA fragments

Synthesis of the probes

First strand cDNAs were synthesized from the same RNA used for RAP-PCR. Total RNA from unfed and fed male, *A. americanum* was reverse transcribed by reverse transcriptase (RT) using each of the three primers (A_1, A_2 and A_3). Five µl of the reaction product was amplified by PCR and cDNA labeled with digoxigenin 11-dUTP. The labeling reaction mixture consisted of 5 µl of 10X PCR buffer, 3 mM $MgCl_2$, 2.0 units of Taq DNA polymerase (5 U/µl), 200 µM of each dNTP in the PCR digoxigenin 11-dUTP labeling mixture and 1 µM of the arbitrary primer A_1, A_2 or A_3 in sterile or Diethylpyrocarbonate-treated H_2O at a final volume of 50 µl. The PCR buffer, $MgCl_2$ and H_2O were exposed to UV before the remaining components were added to eliminate

contaminating DNA. Probes were purified with a QIAquick PCR purification kit to remove primers and truncated DNA before use for hybridization.

Reversed northern analysis of the cloned cDNAs:

Recombinant plasmids (3-4 μg) were digested with EcoR1 and 0.25 μg of the digest was electrophoresed on 2 % agarose gel in Tris-acetate-EDTA buffer (TAE). The gel was prestained with ethidium bromide and photographed in a GelDoc system. After depurination, denaturation and neutralization as prescribed by DUPONT salt transfer protoco, samples were transferred to a Tropilon-Plus nylon membrane in 10X SSC (1.50 M sodium chloride, 0.015 M trisodium citrate, pH 7.0). After completion of the transfer, the membrane was treated with 0.4 NaOH solution for 1 min to denature the DNA and then neutralized with 0.2 M Tris-HCl, pH 7.5 in 1X SSC for 1 min. Nucleic acids were fixed to the membrane by automated UV cross-linking in a Stratalinker 2400. Membranes were kept moist before prehybridization in 2X SSC for 5 min before hybridization. Membranes with nucleic acids were prehybridized in 30 ml ULTRAhyb at 42° C for 30 min. Hybridization was also carried out with the same 30 ml of the ULTRAhyb containing 1 μg of the probe at 42° C for 14-24 hrs. in a water-bath with continuous shaking. Immediately after hybridization, membranes were washed twice for 5 min. at 42° C with 2X SSC, 0.1% SDS buffer and then twice for 15 min. at 55° C with 0.1X SSC, 0.1% SDS buffer before chemiluminescence detection of hybridized probes.

Chemiluminescent Detection:

Membranes with hybridized probes were treated with anti-digoxigenin fab-fragment conjugated to alkaline phosphatase diluted 1:10,000 in blocking buffer for 30-45 min at room temperature. Membranes were then incubated with alkaline phosphatase substrate, CDP-star, for 5 min. with reduced light and placed on 3 MM Whatman filter paper to remove excess CDP and transferred onto a second moistened filter paper. The membrane was wrapped in polyethylene, incubated at room temperature for 1-2 hrs. and exposed to Fuji X-ray film for either 5 min, 10 min or 15 min depending on the time needed to achieve the desired signal.

Cross-hybridization of the Cloned cDNAs Fragments with Opposite Probes:

cDNA fragments isolated from RNA in salivary glands of unfed and fed ticks were hybridized with labeled cDNA fragments derived from RNA of the salivary glands from unfed ticks and vice versa. Some cDNA fragments were hybridized with probes synthesized from total RNA obtained from the salivary glands of unfed and fed male *D. andersoni*. Total RNA from two groups of fed male *D. andersoni* was used to synthesize the probes. One group of males was fed without females and was designated as G_1. The other was fed along with females in the same cell and was designated as G_2.

As positive controls, cDNA fragments were synthesized from total RNA isolated from fed male ticks, *A. americanum* and *D.andersoni* salivary glands using oligo (dT) and control primer set for human β-actin gene and were hybridized to labeled probes produced using RT-PCR protocol provided by Stratagene except that products were labeled with Digoxigenin 11 dUTP. Also, the β-actin cDNA was hybridized to probes

produced from unfed and fed tick salivary glands using random hexamers and the RAP-PCR method.

Similarity Search in the Nucleic Acids and Protein Databases

The cloned cDNA sequences were used for homology search in Baylor College of Medicine database web site (NCBI), Macvector 6.0. The search programs include mainly Blastn and Blastx, non-redundant GenBank CDS, translations + PDB + Swissprot + Spudate + PIR.

CHAPTER THREE

Results

Total Proteins and RNA from Unfed and Fed Ticks' 'Salivary Glands

Total proteins in salivary glands of unfed and male salivary glands increased from approximately 4.3 μg per pair of salivary glands in unfed to 24.4 μg per pair of salivary glands in the fed males (results not shown). Total RNA per pair of salivary glands (i.e. per tick) was 0.039 μg for the unfed and 0.255 μg for fed *A. americanum,* an increase of approximately 6-fold (Fig. 2-A). Total RNA obtained per pair of salivary glands of male *D. andersoni* was 1.021 μg for unfed, 3.6 μg for ticks fed with females and 2.7 μg in ticks fed without females. There is an increase of about 3.5-fold in total RNA of fed (with females) over unfed in *D. andersoni* while the increase in total RNA when these ticks were fed with females is about 25% over those fed without females (Fig. 2-B). It has been observed that *D. andersoni* males, whether unfed or fed, are bigger in size than the unfed and fed *A. americanum* males. In addition, the salivary glands appear much larger in both unfed and fed *D. andersoni* than those of *A. americanum.*

Differential display of cDNAs obtained from salivary glands of unfed and fed males, Amblyomma americanum and Dermacentor andersoni

In *A. americanum* using primer A_1, 9 cDNA bands were observed in RNA from salivary glands of unfed ticks that were not obtained in RNA from salivary glands of fed ticks, while twenty-three bands were seen in RNA of fed ticks but not of unfed ticks (Fig. 3). With primer A_2, there were 10 bands specifically seen in unfed ticks and only 4 in the fed ticks. Lastly, with primer A_3, 3 bands were seen specifically in unfed ticks and 12 in fed ticks. Upon cloning and sequencing, some bands contained more than one cDNA fragment. Approximately 120 different DNA fragments were eventually identified.

Using the same RAP-PCR primer set, there were very few differences observed between DNA from salivary glands of male *D. andersoni* unfed and fed without females, (Fig. 4). Complementary DNAs were not compared but between male *D. andersoni* fed without or with females.

Confirmation of Differential Expression of the Cloned cDNA Fragments

To confirm differential expression of the cDNA fragments identified, each of the 61 bands originally isolated was cloned into the pCRII vector and at least 3 recombinant plasmids were sequenced from each. The numbers shown below each fragment (Fig. 5) indicate the number of cDNA fragments with different sequences found for that original band. Cloned cDNA fragments obtained from the salivary glands of unfed and fed (U and F respectively) male, *A. americanum* were then hybridized to probes synthesized from the same total RNA. Representative hybridizations are depicted in Fig. 5 A-D. In the hybridizations shown, cDNA fragments 2, 5 and 8 from unfed ticks (Fig. 5 A-B) hybridized to both probes, while 3, 4 and 9 hybridized only to the probes from unfed

ticks. Similarly, cloned cDNA fragments obtained from the salivary glands of fed *A. americanum* hybridized to probes synthesized from RNA obtained from fed males (Fig. 5-C) and unfed male ticks (Fig. 5-D) only F_1 (1,2), F4 (1,2,3) were differentially expressed in fed ticks. Fig. 5 E. is a positive control. Fig. 6-A, B. indicated that only F_8 1,2, 3 were expressed in both unfed and fed ticks while F_9 through F_{12} products were differentially expressed in fed ticks. Fig. 6 C, D. showed that only F_{13} 3 was expressed in both in unfed and fed ticks while the remaining were differentially expressed in fed ticks. Other hybridizations (see Fig. 7-12) confirmed that the cDNA clones originally identified by RAP-PCR were present in the salivary glands of unfed, fed or both unfed and fed ticks. The remaining cloned cDNA fragments produced by the three RAP-PCR primers were analyzed in the same way and a total of 5 from unfed and 29 fed cDNA fragments from fed ticks were differentially expressed, while 12 cDNA fragments were expressed in both unfed and fed ticks when primer A_1 was used. When primer A_2 was used the expressed and cloned cDNA fragments revealed that 5 genes from unfed ticks, 5 from fed ticks were differentially expressed and 13 were expressed in both unfed and fed ticks [(Fig 7 (C, D), Fig. 8, Fig. 9 (A, B)]. Primer A_3 did not identify any expressed genes found solely in unfed ticks, but 6 were differentially expressed in fed and only one fragment was shown expressed in both unfed and fed ticks [Fig. 9 (C, D), Fig. 10)]. Digoxigenin labeled probes were synthesized from total RNA isolated from unfed, fed without (G_1) or with females (G_2) male tick, *D. andersoni* using RAP-PCR and the three arbitrary primers A_1, A_2, and A_3. Applying the above mentioned approach used to analyze differentially expressed genes in the unfed and fed *A. americanum* salivary glands, to compare the two tick species, only the cDNAs obtained with primer A_1 from *A. americanum* salivary glands gave positive results with probes synthesized from *D. andersoni* salivary glands using the same primer (Fig. 11+ 12). Since the concentrations

of the DNA template and probe concentrations were kept the same, it is likely that the differences in band intensity are due to upregulation of those genes in G_2 during tick feeding. This can also raises the possibility that male *D. andersoni* feed more when they are fed with females, leading to more expression of the same gene (s) which is consistent with the increase in gene expression observed in the second group (refer to Fig. 1 B.). The hybridization results of cloned A_1 clones from *A. americanum* with probes from G1 showed very low intensity bands, whereas the probes from G_2 gave some bands of higher intensity.

Sequence Analysis of Some Important RAP- PCR Products (cDNAs)

After sequencing and comparing cloned cDNA fragments to known sequences in the databases, 39 cDNA fragments were highly similar to known gene sequences. The genes that showed high similarity are summarized as follows; of the genes differentially expressed soley in the unfed salivary glands only one was identifiable: the I factor 1 of the fruit fly (Table 1). Twenty-six genes of known function were differentially expressed in the fed tick salivary glands including protein synthesis proteins such as transcription factors, ribosomal proteins, translation initiation factors and translation elongation factors (Table 2). A cDNA fragment similar to cystic fibrosis transmembrane conductance regulator chloride channel-like (CFTR-Cl^- channel-like) was also in this class. There were 13 genes expressed both in unfed and fed tick salivary glands identified by this study, including genes for ribosomal proteins and the tyrosine kinase substrate, ezrin (Table 3). A surprising gene, the sporozoite surface antigen of *Theileria annulata*, was expressed in both unfed and fed ticks.

Table 1.

Complementary DNA fragments originally isolated from salivary glands of unfed and fed male, *Amblyomma americanum* using differential display approach.

	Number of bands	
RAP-PCR Primer	**Unfed**	**Fed**
A1	9	23
A2	10	4
A3	3	12

Table: 2.

Similarity representations of genes expressed specifically in salivary glands of unfed (U) male, *Amblyomma americanum*

Tissue origin of Cloned cDNAs	Similarity to sequences from the databases	E values	Accession Number
U-A1	I factor 1 (ORF1) a transposon – fruit fly (*D. teissieri*)	0.048	A36186

Table: 3.

Similarity of genes expressed only in salivary glands of fed (F) male, *Amblyomma americanum*

Tissue origin of Cloned cDNAs	Similarity to sequences from the databases	E value	Accession Number
F-A1	Serine-arginine-rich splicing regulatory protein SF (*Rattus norveggicus*)	0.021	AF234765
F-A1	60S ribosomal protein L11-cytosolic fruit fly (*Drosophila melanogaster)*	1 e-24	U15643
F-A1	A putative G-protein (*Homo* sapiens)	1e-16	AF065393
F-A1	Cerebroside sulphate activator protein - prosaponin (*Homo sapiens*)	1e-13	M60258
F-A1	Nonfunctional folate binding protein (*Homo sapiens*)	1e-11	AF000381
F-A1	Open reading frame1 (ORF1) (*D. virilis*)	1e-10	U49102
F-A1	Hypothetical KIAA0616-human (*Homo sapiens*)	2e-05	AB014516
F-A1	High density lipoprotein-binding protein (*Homo sapiens*)	7e-16	M64098
F-A1	Clottable protein from Tiger shrimp (*Penaeuus mondom*)	9e-06	AF089867
F-A1	Latent transforming growth factor β-binding Protein-4-(a Ca2+-binding protein (*Homo sapiens*)	0.098	Y13622
F-A1	Inhibitor of apoptosis proteins 1 & 2 (*Homo sapiens*)	4e-06	L49431
F-A1	Putative SUDD-like protein (*Arabidopsis thaliana*)	7e-19	AC006585
F-A1	Ezrin (a protein tyrosine kinase- *M. musculus*)	5e-03	X60671
F-A1	Translation initiation factor-2C (eIF2C- *Oryctolagus cuniculus*)	5e-07	AF005355

F-A1	Translation initiation factor 3 (β-subunit) (*Homo sapiens*)	1e-35	U78525
F-A2	Regulator of nonsense transcripts stability (*Homo sapiens*)	6 e-41	U65533
F-A2	Ornithine decarboxylase (*Bos taurus*)	9e-21	BTU18531
F-A2	Elongation factor-2 (HSEF-2) (*Homo sapiens*)	3e- 42	D21163
F-A2	U5 small nuclear ribonucleoprotein 116 KDa (*M. musculus*)	4e-42	U97079
F-A3	Ubiquitous TPR motif X isoform (*Homo sapiens*)	7e-33	AF000992
F-A3	Cystic fibrosis transmembrane conductance regulator (CFTR-Cl- channel- *Homo sapiens*)	8e-09	AF00027
F-A3	Development related protein (*Rattus norvegicus*)	3e-09	AF045564
F-A3	Protein tyrosine phosphatase epsilon cytoplasmic isoform (*Homo sapiens*)	9e-03	U36623
F-A3	60S ribosomal protein L6 (*Mesembryanthenum crystallinum*)	3e-26	X69378

Table: 4.

Similarity of genes expressed in salivary glands of both unfed (U) and fed (F) male, *Amblyomma americanum.*

Tissue origin of Cloned cDNAs	Similarity to sequences from the databases	E values	Accession Number
U-A1	*Amblyomma americanum* 18S ribosomal RNA		19633
U-A1	60S ribosomal protein L7a (*Gallus gallus*)	2e-07	D14522
F-A1	Ribosomal protein L22 (*Gadud mordua*)	4e-18	GMU49123
F-A1	Hypothetical protein of (S. cerevisiae)	1 e-10	AF067624

F-A1	60S ribosomal protein L22 (*Mus musculus*)	1e-19	D17654
F-A1	Ubiquitously transcribed X-chromosome Tetratricopeptide repeat, a gene that confers H-Y antigenicity to male cells (*Homo sapiens*)	7e-33	AJ002730
F-A1	Importin β-subunit (a nuclear protein) (*Homo sapiens*)	5e-04	U18916
F-A1	Ezrin (a protein tyrosine kinase) (*Homo sapiens*)	2e-40	X51521
F-A1	Ezrin (*Bos taurus*)	4e-41	M98498
F-A1	60S ribosomal protein L11 (*Rattus rattus*)	2e-24	X62146
U-A2	Williams Syndrome (WS) basic helix-loop-helix- Leucine zipper protein (*Homo sapiens*)	2e-16	AF056184
U-A2	Sialidase – a highly conserved gene (*Actinomyces viscosus*)	3.2e-03	L06898
F-A2	Probable cathepsin B-like cysteine proteinase (EC 3.422) a precursor of a 29 KDa proteinase of flesh fly (*Sarcophaga peregrina*)	2e-26	D16823
F-A2	Suppressor of potassium transport defect 3 (*Mus musculus*)	5e-21	U09874
F-A2	Ezrin protein (a member of highly conserve family of proteins-ezrin-radixin-moesin (*M. musculus*)	4e-63	X60671
F-A2	Suppressor of potassium transport defect 3 –SKD3 (*Rattus norvegicus*)	4e-22	AB027570
F-A2	Leucine zipper protein (DNA-binding protein) (*Homo sapiens*)	1e-14	AF056184
F-A3	Sporozoite surface antigen SPAG-1 (*Theileria annulata*)	3e-04	M63017

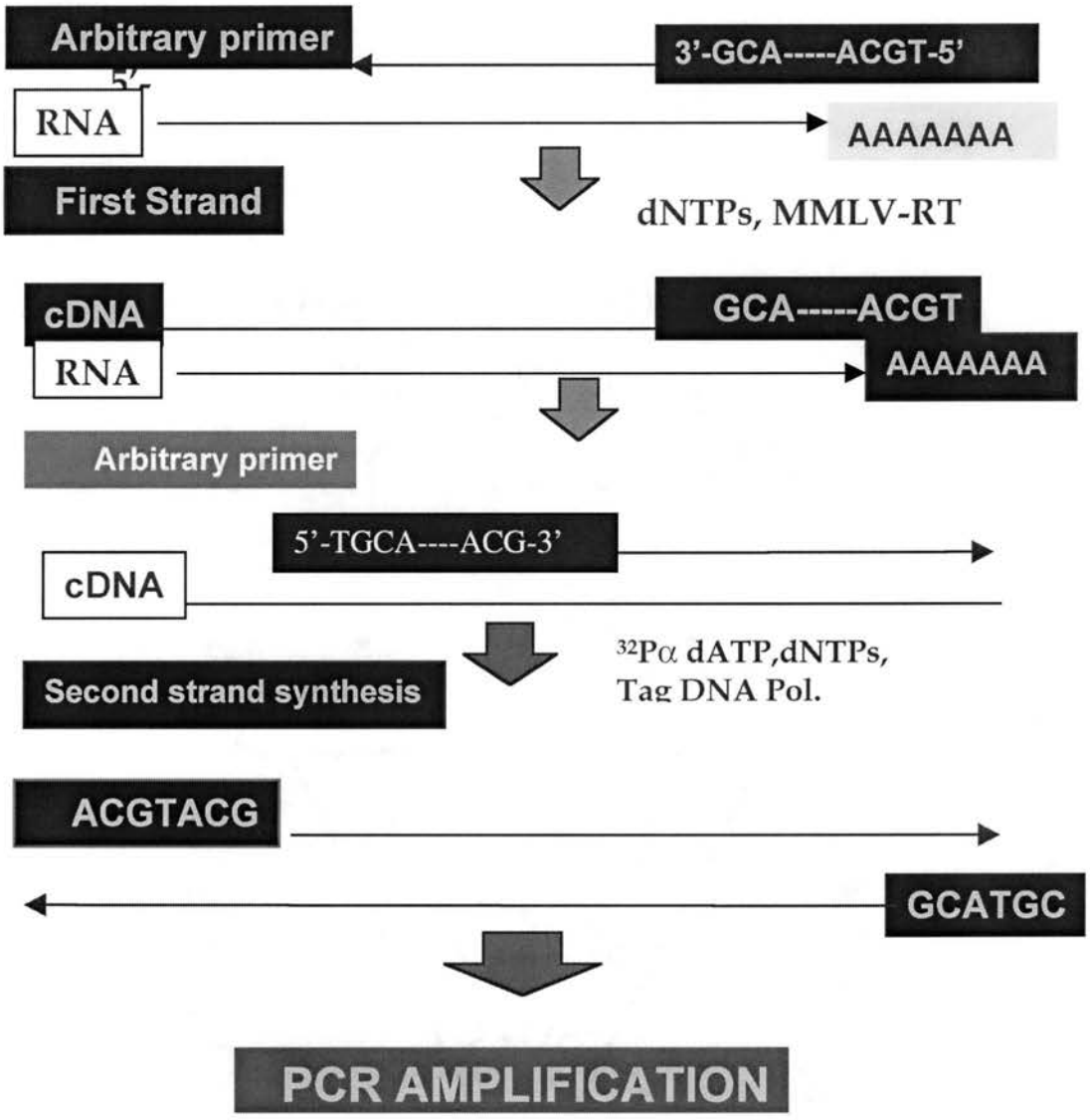


Figure 1. RNA Arbitrarily Primed Polymerase Chain Reaction (RAP-PCR)

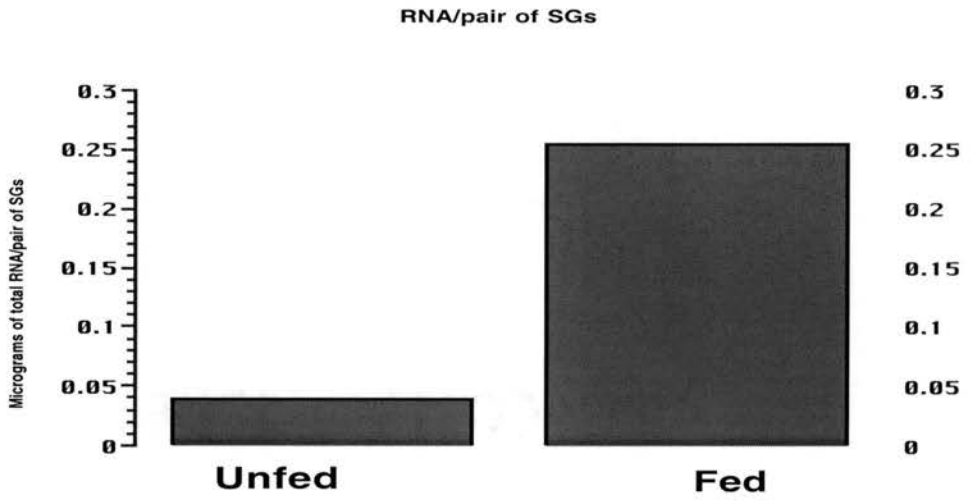


Figure 2 (A). Total RNA isolated from the salivary glands of unfed and fed male tick, *A. americanum*. Gene expression is increased in the fed tick salivary glands to about 6-fold compared to the unfed salivary gland

Figure 2 (B) Total RNA isolated from the salivary glands of unfed and fed male tick, *D. andersoni*. Male ticks were either fed without females or along with females. Gene expression was always increased in the fed tick salivary glands when females are present in the same feeding cell compartment. Compared with the unfed tick's tissue gene expression was almost increased 4-fold during feeding.

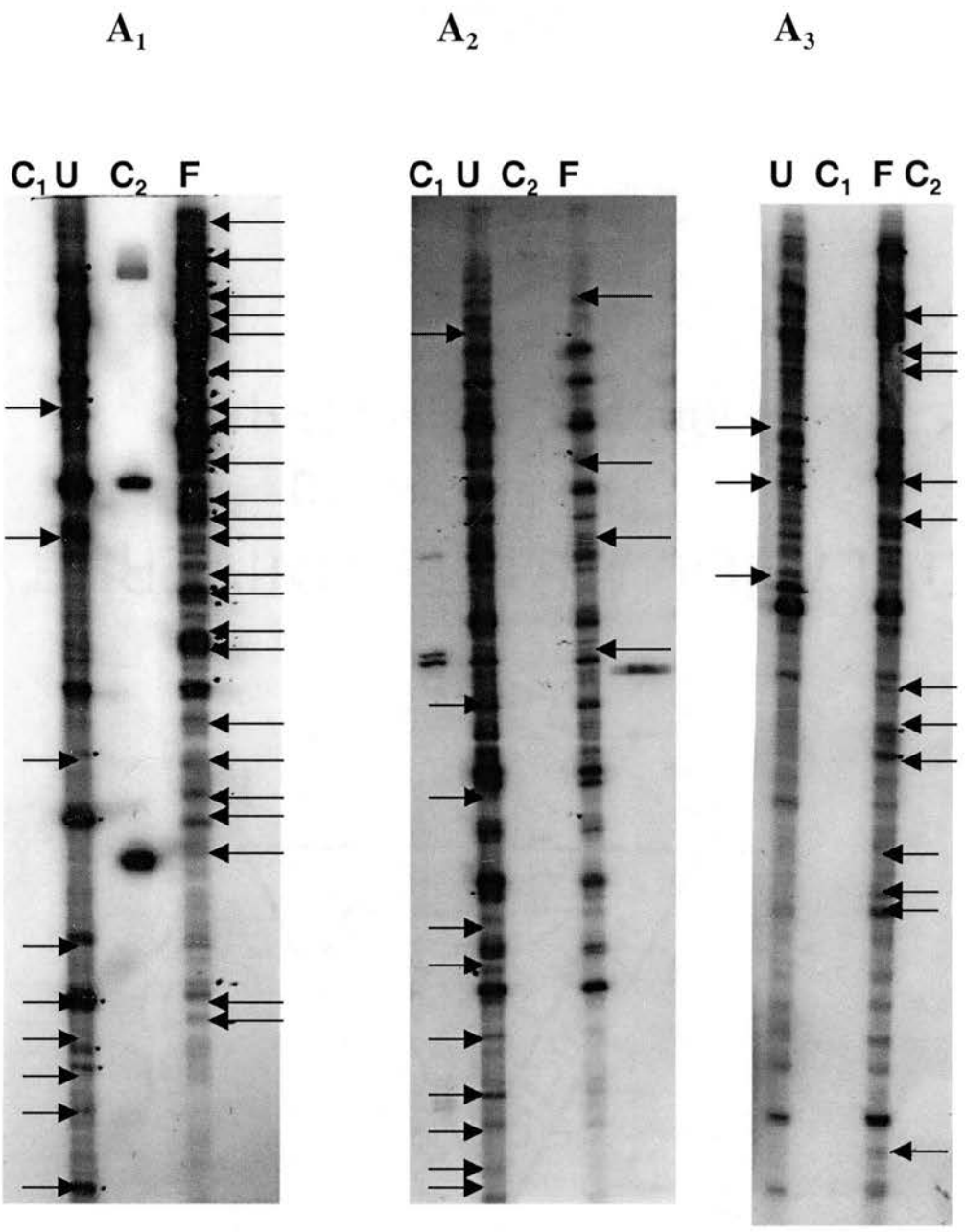


Figure 3. Differential display patterns of the cDNAs synthesized from mRNA obtained from salivary glands of unfed and fed male, *A. americanum* ticks using arbitrary primers A_1 and A_2 and A_3in reverse transcription, second strand synthesis, PCR amplification and labeling with [α-^{32}P] dATP. Samples were analyzed by denaturing PAGE, dried and autoradiographed. Mock (C) reaction is referred to as a negative control (-RT or without the reverse transcriptase), Arrows indicate cDNAs bands obtained from salivary glands of unfed (Un) ticks but not in fed (Fe) ticks and vice versa.

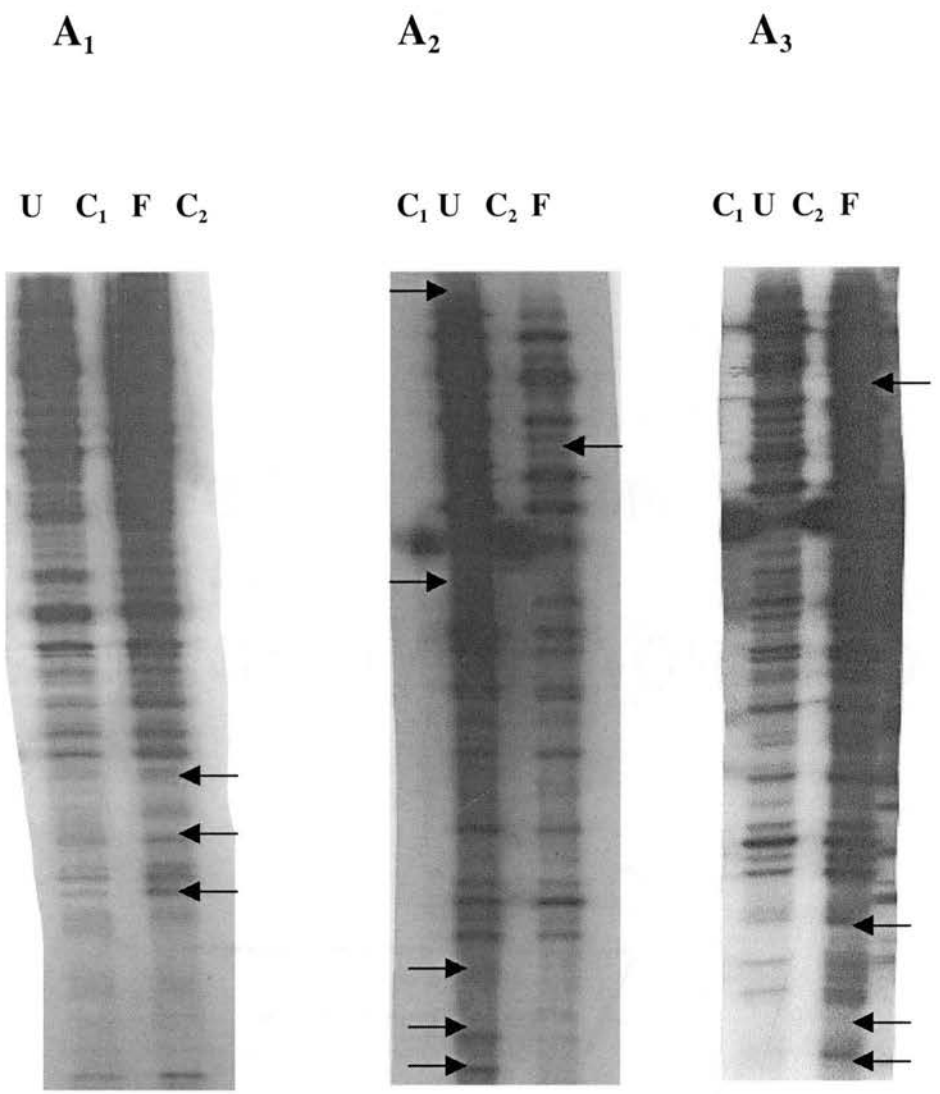


Figure 4. Differential display patterns of the cDNAs synthesized from mRNA obtained from salivary glands of unfed and fed male, *D. andersoni* ticks using arbitrary primers A_1 and A_2 and A_3in reverse transcription, second strand synthesis, PCR amplification and labeling with [α–^{32}P] dATP. Samples were analyzed by denaturing PAGE, dried and autoradiographed. Mock (C) reaction is referred to as a negative control (-RT or without the reverse transcriptase), Arrows indicate cDNAs bands obtained from salivary glands of unfed (U) ticks but not in fed (F) ticks and vice versa.

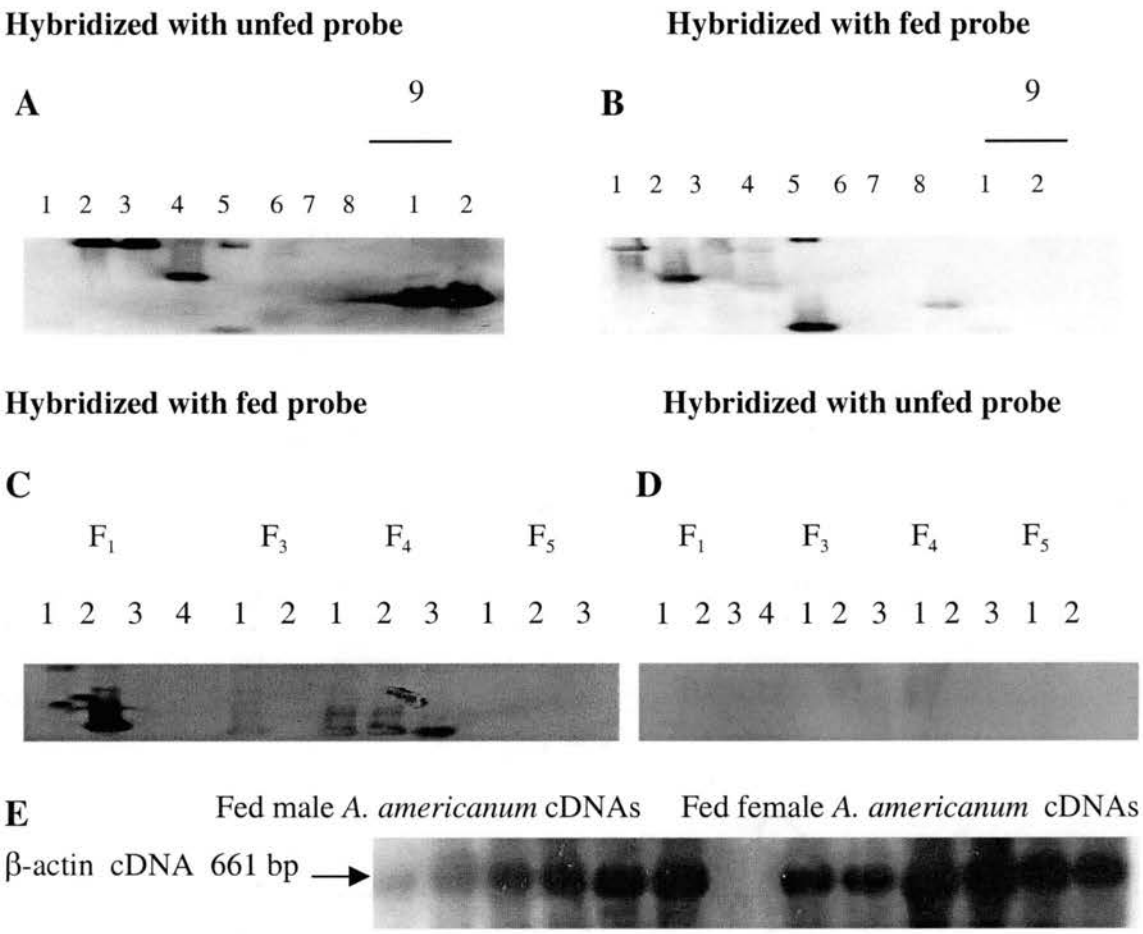


Figure 5. Cloned tick salivary glands cDNA fragments obtained with the primer A_1 from unfed and fed male, *A. americanum*. Probes were synthesized from unfed and fed ticks' RNA by RT-PCR and labeled with Dig-11dUTP using the A_1 primer. The cDNA probes were detected by an immunoenzymatic reaction using anti-digoxigenin-antibody Fab Fragments conjugated to alkaline phosphatase and visualized by chemiluminescent reaction. Fig. 5-A, B represent unfed cDNA fragments (U) whie C, D are Fed fragments (F). Fig. 5-E shows different concentrations of β-actin cDNA fragments synthesized with oligo dT and a control primer set for human β-actin gene (Stratagene) from total RNA isolated from fed male and female *A. americanum* salivary glands. These fragments were used as templates and hybridized to labeled probes synthesized from the same total RNA obtained from both fed male and female *A. americanum*.

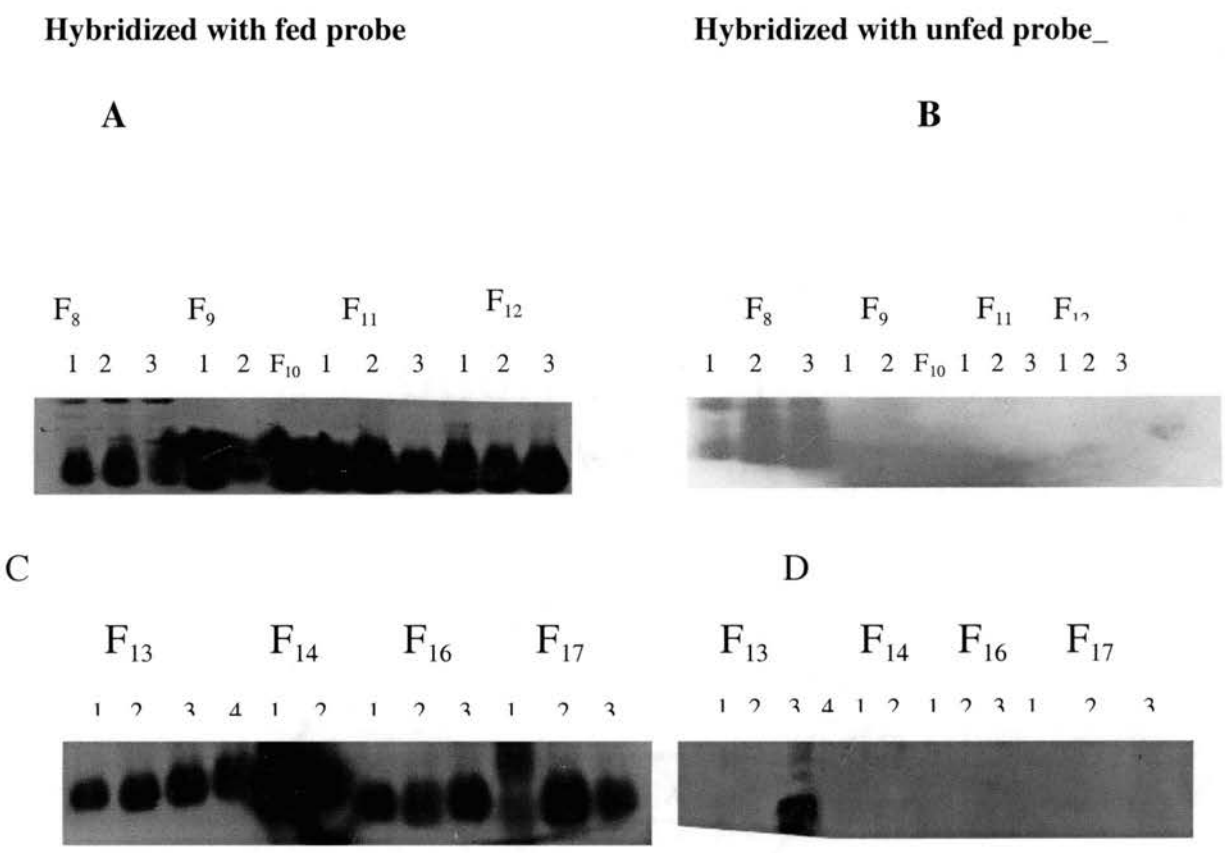


Figure 6. Cloned tick salivary glands cDNA fragments obtained with the primer A_1 from fed male, *A. americanum*. Probes were synthesized from unfed and fed ticks' RNA by RT-PCR and labeled with Dig-11dUTP using the A_1 primer. The cDNA probes were detected by an immunoenzymatic reaction using anti-digoxigenin-antibody Fab Fragments conjugated to alkaline phosphatase and visualized by chemiluminescent reaction.

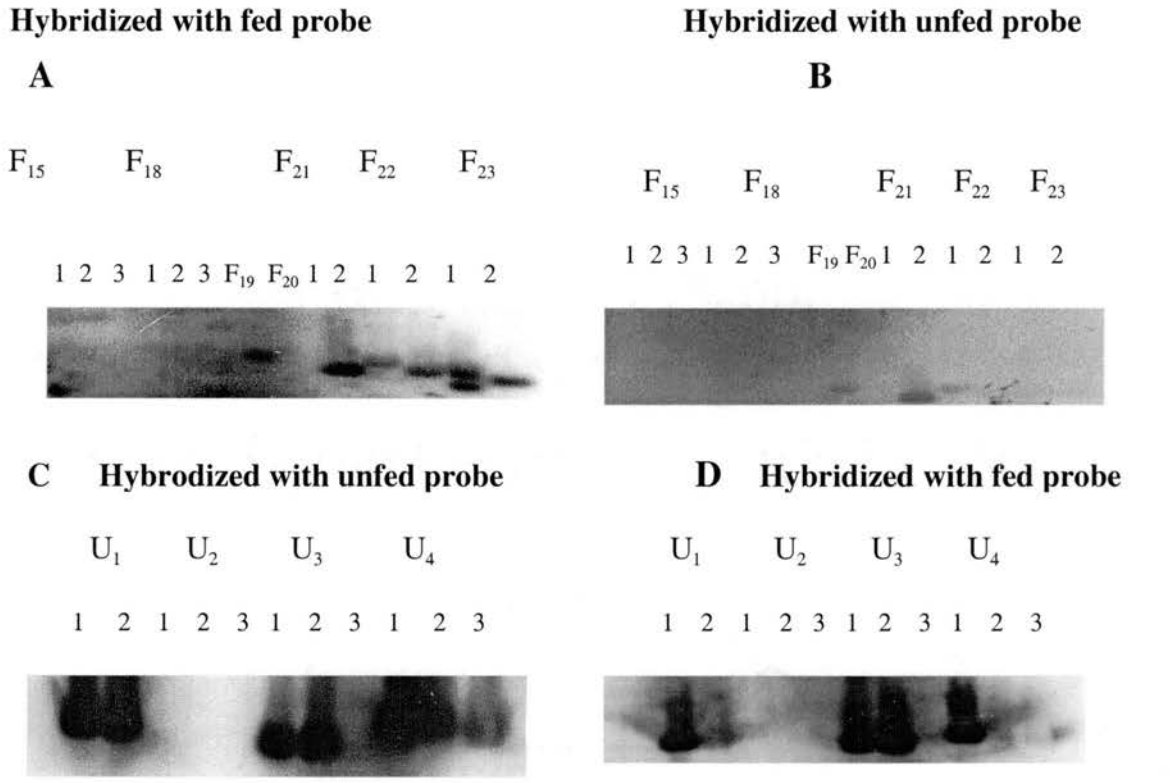


Figure 7. Cloned tick salivary glands cDNA fragments obtained with the primer A_1 from fed male, *A. americanum* (A & B). C & D show cDNA fragments obtained with the primer A_2 from salivary glands of unfed male, *A. americanum*. Probes were synthesized from unfed and fed ticks' RNA by RT-PCR and labeled with Dig-11dUTP using the A_1 primer. The cDNA probes were detected by an immunoenzymatic reaction using anti-digoxigenin-antibody Fab Fragments conjugated to alkaline phosphatase and visualized by chemiluminescent reaction.

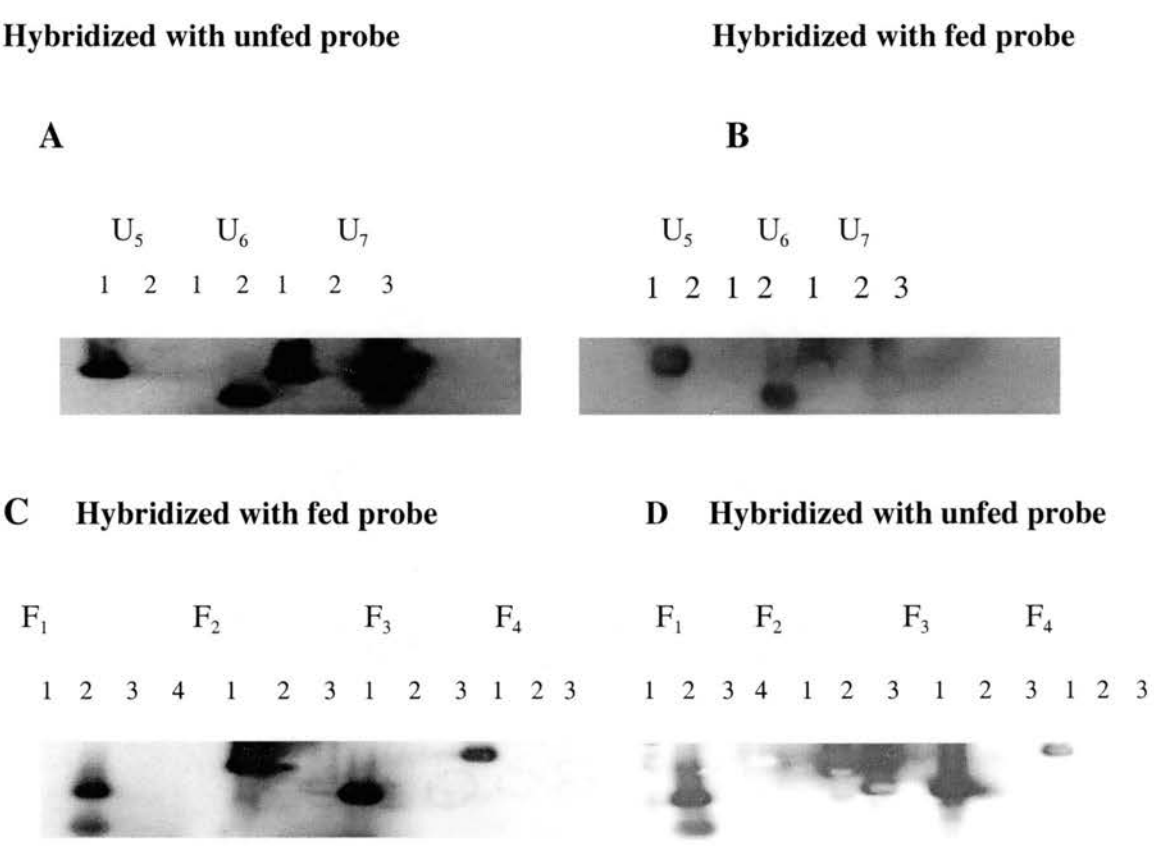


Figure 8. Cloned tick salivary glands cDNA fragments obtained with the primer A_2 from unfed and fed male, *A. americanum* Probes were synthesized from unfed and fed ticks' RNA by RT-PCR and labeled with Dig-11dUTP using the A_1 primer. The cDNA probes were detected by an immunoenzymatic reaction using anti-digoxigenin-antibody Fab Fragments conjugated to alkaline phosphatase and visualized by chemiluminescent reaction.

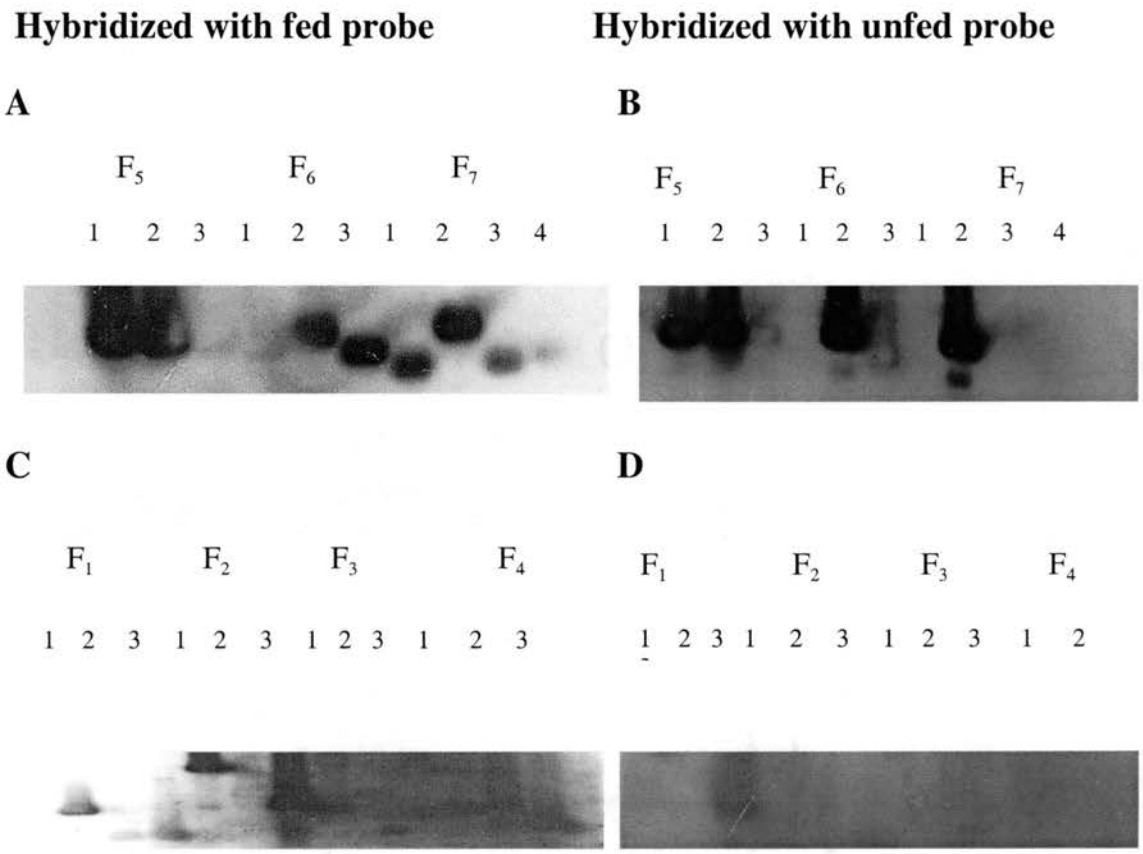


Figure 9. Cloned tick salivary glands cDNA fragments obtained with the primer A_2 from fed male, *A. americanum* (A & B). C & D show cloned cDNA fragments obtained with primer A_3 from fed male, *A. americanum*. Probes were synthesized from unfed and fed ticks' RNA by RT-PCR and labeled with Dig-11dUTP using the A_1 primer. The cDNA probes were detected by an immunoenzymatic reaction using anti-digoxigenin-antibody Fab Fragments conjugated to alkaline phosphatase and visualized by chemiluminescent reaction.

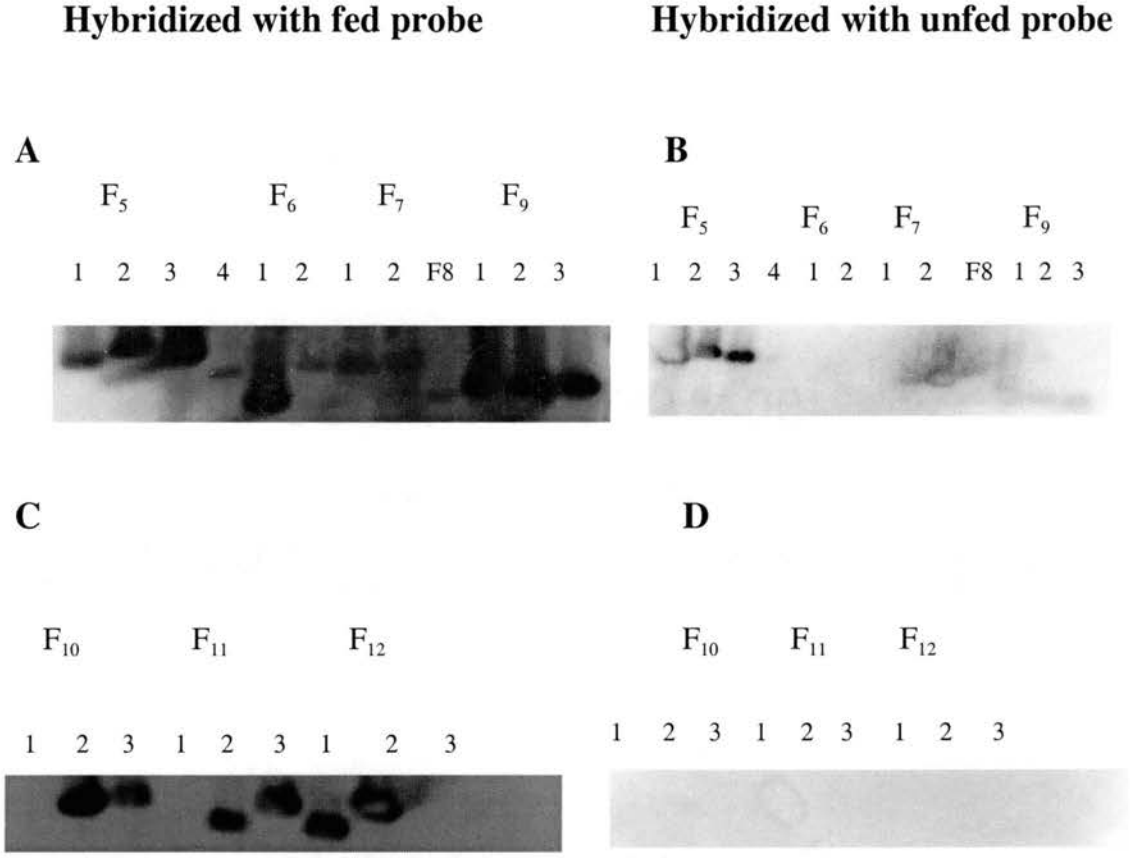


Figure 10. Depicts cloned cDNA fragments obtained with primer A_3 from fed male, *A. americanum*. Probes were synthesized from unfed and fed ticks' RNA by RT-PCR and labeled with Dig-11dUTP using the A_3 primer. The cDNA probes were detected by an immunoenzymatic reaction using anti-digoxigenin-antibody Fab Fragments conjugated to alkaline phosphatase and visualized by chemiluminescent reaction.

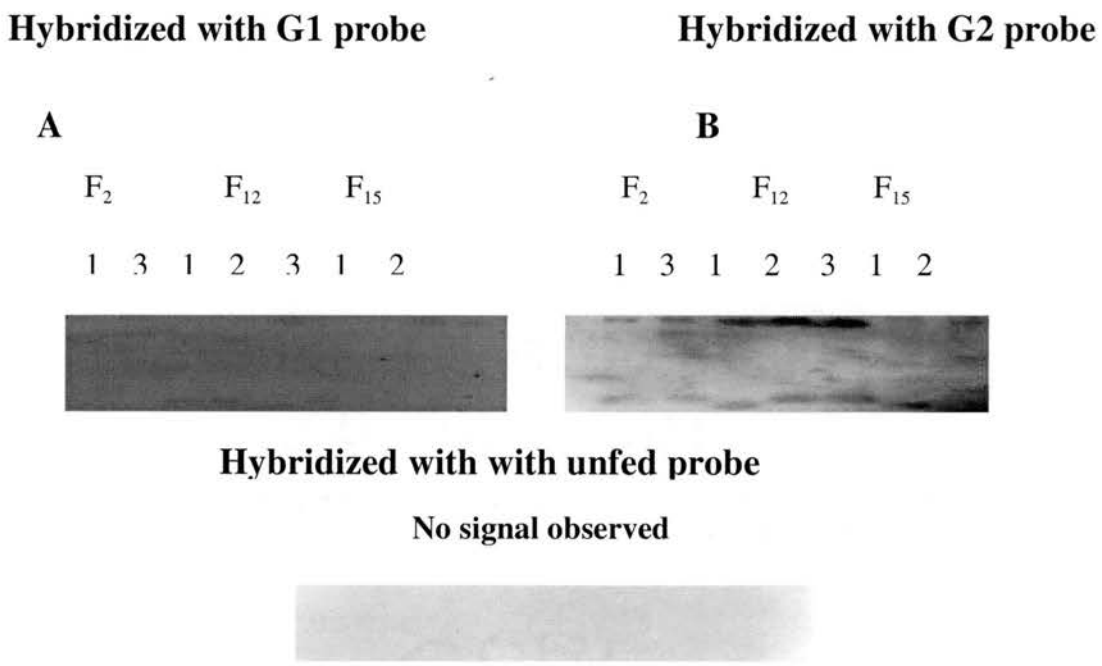


Figure 11. Comparison between primer A_1 cloned cDNAs from unfed and fed total RNA of male tick, *Amblyomma americanum* salivary glands with cDNA probes synthesized from total RNA obtained from unfed and fed male tick, *Dermacentor andersoni* salivary glands labeled with Dig.11dUTP. Clones were hybridized using Ultrahyb solution and detected with BrightStar nonisotopic BioDetectKit from Ambion Inc. G_1 represents ticks fed without females while G_2 are those fed along with female ticks.

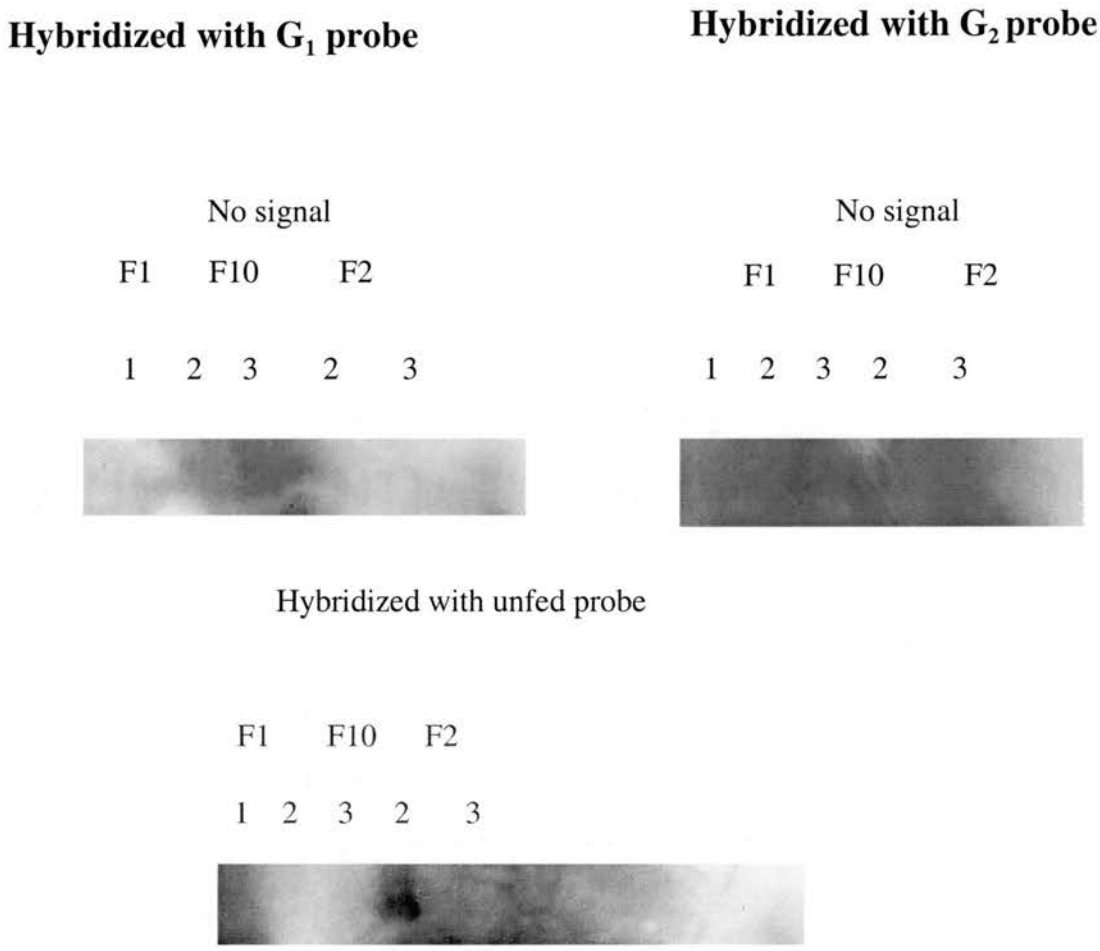


Figure 12. Comparison between primer A_3 cloned cDNAs from unfed and fed total RNA of male tick, *Amblyomma americanum* salivary glands with cDNA probes synthesized from total RNA obtained from unfed and fed male tick, *Dermacentor andersoni* salivary glands labeled with Dig.11dUTP. Clones were hybridized using Ultrahyb solution and detected with BrightStar nonisotopic BioDetectKit from Ambion Inc. There was no signal observed with G_1 and G_2 probes while unfed probe gave a positive signal for F2(2) that seems to be differentially expressed under unfed condition in male tick, *Dermacentor andersoni* salivary glands.

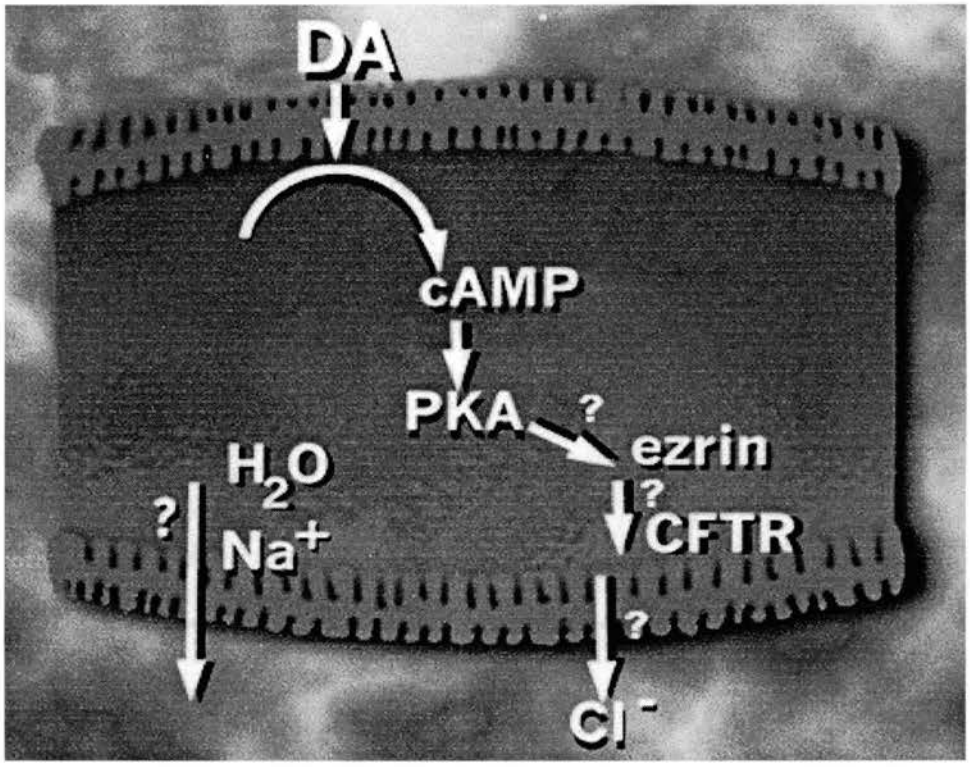


Fig. 13: A possible mechanism of fluid secretion by salivary glands of ixodid ticks, *Amblyomma americanum*. Dopamine is a neutransmitter at neuroeffector junction, which binds to its receptors D_1, and the receptors activate adenylate cyclase. Adenylate cyclase then synthesizes cAMP from ATP. The increased intracellular second messenger activates protein kinase A (PKA) which in turn phosphorylates ezrin. Ezrin brings PKA to close proximity with cystic fibrosis transmembrane conductance regulator (CFTR) and helps in phosphorylation and activation of CFTR by PKA. Opening of the chloride triggers efflux of Cl^- and electrochemical gradient leads to efflux of Na^+ followed by water.

CHAPTER FOUR

DISCUSSION

It is well established that RNA and proteins are synthesized in the salivary glands of feeding ixodid female ticks. This laboratory and others have reported salivary gland genes that are induced during feeding in females (Hunt and Hilburn 1985, Jaworski et. al., 1992, Wang and Nuttall 1994). *In vitro* translation of isolated mRNAs revealed that at least eight proteins were induced in the fed female tick salivary glands (McSwain et al., 1982, Oaks et al., 1991). Unlike females, little is known about the expression of new gene activity in males. Male ixodid ticks, unlike females, attach and detach from the host prior to mating with multiple females.

The present results demonstrate that new gene expression occurs in male tick salivary glands. We found a 6-fold increase in RNA and proteins in salivary glands of fed male *A. americanum* and 3-5-fold increase in salivary glands of male *D. andersoni* as compared to that in the salivary glands of unfed ticks. It was speculated that absence of females could affect the feeding behavior of males and possibly gene expression. This hypothesis was confirmed as male *D. andersoni* fed with females had 25% more RNA than males fed without females (Fig. 2-B).

Salivary glands from male *A. americanum* demonstrated at least 61 differentially expressed genes in unfed and fed ticks in the present study using three arbitrary primers. The factors responsible for inducing expression of new genes in fed ticks are unknown.

Nutrients are known to induce gene expression in many situations. Food availability is one of many factors that trigger developmental processes such as hatching, metamorphosis, and sexual maturity in flatfishes and eels (Andron, et al., 1978, Egginton 1986). Glucose has been implicated in transcription regulation of many genes in animal skeletal muscle and the induction of insulin gene expression (Tsao et al., 1996). Also, the liver, the principal organ of lipogensis and cholesterogenesis, is responsible for synthesis of triglycerides from excess dietary carbohydrates. A high carbohydrate diet can induce transcription of mRNAs for a group of lipogenic enzymes such as acetyl-CoA carboxylase, fatty acid synthase, and stearoyl-CoA desaturase (Shimano et al., 1999). Fatty acids are also known to regulate gene transcription (Duplus et. al., 2000).

Similarity searches in various databases have suggested possible identities for 39 of the 61 genes isolated from unfed and fed *A. americanum* salivary glands. Many of the genes identified are associated with increased protein synthesis, not unexpected since the salivary glands increase in size and protein content during tick feeding. These genes include 60S ribosomal protein L11, a serine-arginine-rich splicing regulatory protein (Barnard & Patton 2000), a regulator of nonsense transcript stability (Perlick et. al. 1996), a translation initiation factor 2 C (eIF2C) (Zou et. al. 1998), and a translation elongation factor-2 (EF2) (Grinblat et. al. 1989, Kim et. al. 1991, Nakanishi et. al. 1988).

Genes were identified that may be involved in cell functions such as development, growth and differentiation. Amongst those, a cDNA fragment from fed male tick salivary glands that has high homology to ornithine decarboxylase (ODC) gene was identified. It is possible that the ODC gene may be induced during tick feeding to enhance the growth of the salivary glands. A gene for ODC was shown to be induced by dietary intake in male mouse kidney (Suka et. al., 1999).

Similarly, cDNA fragments similar to latent transforming factor β-binding protein 4 (Giltay et. al. 1997), apoptosis inhibitor, or tumor necrosis factor receptor 2 and TNF receptor-associated factor (TNFR2-TRAF) signaling complex of proteins (Rothe et. al. 1995, Rothe et. al., 1995), high density lipoprotein binding protein (McKnight et. al. 1992) were found. Other cDNAs of interest included cDNA fragments for a suppressor of potassium transport defect 3 protein CLP (HSP 104 family), and cathepsin B-like cysteine proteinase (Takahashi et. al., 1993, Merckelbach et. al. 1994, San Segundo 1985, Guenette et. al. 1994, Cygler et. al. 1996, Jia et al., 1995).

The antiapoptosis cDNA fragments that were identified in salivary glands of fed male *A. americanum* and *D. andersoni* are quite similar to human inhibitor of apoptosis proteins 1 and 2 (IAP1 & IAP2). These proteins were originally identified in baculoviruses and are recruited to tumor necrosis factor receptor 2 (TNFR2) signaling pathway through their association with tumor necrosis factor receptor-associated protein 1 and 2 complex (Rothe et. al., 1995). Apoptosis or programmed cell death (PCD) is often used to describe cell death occurring in developing embryos and in metamorphosing insects. It is characterized by a predictable sequence of events or steps in target cells that are determined by developmental or hormonal stimulation. PCD also implies a genetic control and differential expressions of genes that may either regulate the activation and progression of cell death or inhibit the whole process. Among the apoptosis inducers are the caspases that create a proteolytic assemblage within the target cell resulting in DNA and membrane fragmentation and eventually cell death (Jochova et. al., 1997, Maccarrone et. al., 2000). In tick salivary glands a phenomenon similar to apoptosis known as salivary gland degeneration is also occurring and is stimulated by hormones. The salivary gland degeneration in female ixodid ticks is stimulated by high titre of ecdysteroids elicited in the very early stages of post-engorgement stage (Kaufman

1976, 1990). A salivary gland stimulating factor can be detected in the nervous system only, which results in the salivary degeneration after feeding once for an extended period and laying eggs and the death of female tick after oviposition. The male feeding pattern is different: they attach, feed once, detach and mate and then feed again for multiple times (also 7-14 days). Although male salivary gland glands are sensitive to 20-hydroxyecdysone, it is uncertain if the hormone causes the tissue to degenerate, as was the case with female tick salivary glands (Kaufman 1990). Other findings have implicated a protein from male tick gonad that is secreted after mating to be the key in accelerating the appearance of ecdysteroids in the female hemolymph that eventually causes salivary degeneration within four days of detachment from the host (Lomas and Kaufman 1992, Mao and Kaufman 1998). Since salivary gland degeneration in ixodid ticks at present is triggered by ecdysteroid hormone (Mao et. al., 1995, Lomas et. al., 1998, Mao and Kaufman 1999), further investigation is needed to see what effect the inhibitors of apoptosis identified in male salivary glands have on the hormone receptors. Inhibition of those receptors by IAP1 and IAP2 through the association with TRAF2-TRAF1 complex mentioned above (Rothe et. al., 1995) would be interesting to find out. This may indicate inhibition of apoptosis (degeneration) and ability of males to feed several times after mating.

Some cDNA fragments were identified that may relate to fluid secretion. Included in this group are cDNA fragments for ezrin, a member of a family of three tyrosine kinase substrates, ezrin-radixin-moesin, that are concentrated at specific regions where actin filaments are densely associated with plasma membranes and are regulated by cAMP (Sato et. al. 1992, Bergson et. al. 1993, Andreoli et. al., 1994). Cyclic AMP is known to induce fluid secretion in tick salivary glands (McMullen and Sauer 1978, Needham and Sauer 1979, Hume et. al., 1984). Furthermore, a cDNA fragment for a cystic fibrosis

transmembrane conductance regulator (CFTR) (Singer et. al., 1998, Ikuma and Welsh, et. al., 2000) was identified. Ezrin has been implicated in the interaction between type II protein kinase A (PKA), and CFTR, which is a Cl^- channel. Chloride channels are integral membrane proteins responsible for the selective movement of chloride ions across the cell plasma membranes (Melvin et. al., 1987, Melvin 1999, Turner et. al., 1998). CFTR is an epithelial Cl^- channel expressed in luminal membranes of secretary and reabsorptive epithelia, whose activity is controlled by a number of intracellular factors such as cAMP-dependent protein kinase A. Ezrin serves as PKA-anchoring protein (AKAP) for the PKA-mediated phosphorylation of CFTR ((Kunzelmann et. al., 2000, Sun et. al. 2000). Tick salivary gland fluid secretion is stimulated by dopamine. Dopamine is the neurotransmitter at the neuroeffector junction regulating fluid secretion via an increase in cellular cAMP (Sauer et. al., 2000) and the salivary glands contain cAMP-dependent protein kinases (Mane et al. 1985). Earlier studies revealed that cAMP and dopamine were among those agents that stimulated the uptake of ^{36}Cl in glands obtained from adult females of *A. americanum* undergoing the rapid engorgement (Sauer et. al., 1974). The results suggested that the Cl^- movement across the cells might be one of the essential events responsible for the generation of fluid. Female tick saliva contains a high concentration of Cl^- (Sauer et. al., 1974). Although most of the research on fluid secretion in ixodid ticks has been done on females, dopamine injected into the tick hemocoel can induce a small amount of fluid secretion in males (Kaufman 1976, Schmidt et. al., 1981, 1982). From these findings, it seems possible that the mechanism of fluid secretion during tick feeding may involve a CFTR-like Cl^- channel and ezrin that serves to anchor the cAMP-dependent protein kinase A (Fig. 13).

A surprising gene identified was one similar to sporozoite surface antigen (SPAG-1) for *Theileria annulata,* which is the causative agent of tropical theileriosis. *Theileria spp.*

belongs to a group of protozoans called piroplasms. They are characterized by an exoerythrotic cycle and have been found to have a sexual cycle in the tick, based on ultrastructural evidence (Rudzinska et. al., 1979). Genetic evidence has confirmed this cycle to be occurring in the tick midgut (Morzaria et. al., 1992). However, *T. annulata* is not known to be vectored by *A. americanum* ticks which do, however, vector *T. cervi,* the infestation of which is well established in the lone star ticks (Durham et. al. 1976, Hazen-Karr et. al. 1987, Kocan et. al. 1987, Laird et. al. 1988)). Moreover, the well-known vector of *T. annulata* is the ixodid tick, *Hyalomma anatolicum anatolicum* (Reid and Bell 1984). Thus, the presence of a cDNA for *T. annulata* in the salivary glands of fed male, *A. americanum* may be due to the presence of *T. cervi* conserved sequences showing similarity to that of *T. annulata.* More research will be required to confirm this hypothesis.

In this study, we have seen that the differences between the two tick species exist. For instance, cDNAs cloned and sequenced from the fed male *A. americanum*, including the CFTR-like Cl^- channel, that were confirmed by hybridization experiments in the salivary glands of fed male *A. americanum,* were not seen with probes synthesized from the total RNA obtained from the salivary glands of the two groups of fed male *D. andersoni.* Differences between tick species are expected. Wang et. al., (1999), observed a significant degree of molecular polymorphism between ticks and among individuals in the sampled population within the same tick species. It is possible that the sites for arbitrary primers may be quite different depending on the genome organization.

Conclusions

My studies have shown evidence of increased gene expression and consequently increased protein synthesis during tick feeding. This was unequivocally demonstrated by both the increased total RNA and by identification of differentially expressed genes that code for transcription factors, translation initiation factors and other relevant factors involved in protein synthesis. This confirmed that changes in gene expression do occur in the salivary glands of the male ticks, *A. americanum* and *D. andersoni* during feeding despite the differences in the feeding patterns. Further evidence for differences in the physiological and biochemical processes between the unfed and fed tick salivary glands is also observed from the increased size of the salivary acini during tick feeding. Cross-hybridization of probes synthesized from total RNA obtained from unfed and fed males, *D. andersoni* with cloned cDNA fragments from unfed and fed males, *A. americanum* salivary glands did not reveal significant similarities between the two tick species. Pathogens enter the host during feeding. The use of the RAP-PCR allowed us to identify and isolate differentially expressed genes from unfed and fed male *A. americanum* and these methods may be useful in studying differential gene expression in tick species such as *D. andersoni* and identifying factors that are likely to be important in tick feeding and may have significance in the ability of the tick to transmit pathogens.

The RAP-PCR (Fig. 1.) can be modified by performing the RT reaction with random hexamers and PCR amplification using different combinations of the arbitrary primers. This strategy has been found to increase the size of the cDNA fragments to about 3 Kb. Research is needed further to investigate the impact of feeding on *D. andersoni* males under different physiological conditions, unfed and fed. Feeding of these males in presence and absence of females also needs further investigation.

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