GENE EXPRESSION IN THE MALE TICK SALIVARY GLANDS IS AFFECTED BY FEEDING WITH FEMALES

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BY FEEDING WITH FEMALES

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PCR	Polymerase chain reaction
cDNA	Complementary deoxyribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
RAP-PCR	Ribonucleic acid arbitrarily primed polymerase chain reaction
VMP	Variable major protein
MFWF	Male ticks fed with females
MFWOF	Male ticks fed without females
UF	Unfed male ticks
RNA	Ribonucleic acid
RMSF	Rocky Mountain spotted fever
RMWT	Rocky Mountain wood tick
SEMI	Southern Erythmia migrans –like illness
HME	Human monocytropic ehrlichiosis
HB	Human babesiosis
IgG	Immunoglobulin G
RaSH	Rapid subtractive hybridization
ssDD	Single stranded differential display
DD	Differential display
dT	Deoxythymidine triphosphate
mRNA	Messenger ribonucleic acid
NCBI	National Center for Biotechnology Information
BLAST	Basic Local Alignment Search Tool

pH	Acidity or alkalinity level
RT	Reverse transcriptase
dCTP	Deoxycytidine triphosphate
AP	Alkaline phosphatase
dNTP	Deoxynucleotide triphosphate
PTC	Programmable thermocycler
U.V.	Ultra violet
sscDNA	Single stranded complementary deoxyribonucleic acid
rpm	Revolutions per minute
IPTG	Isopropylthio-β-D-galactoside
X-gal	chloro- bromo- indolyl- β- galactoside
EB	Elution buffer
OSU	Oklahoma State University
DEPC	Diethyl pyrocarbonate
NaOH	Sodium hydroxide
CDP	Alkaline phosphatase substrate from Tropix inc.
SSC	Sodium chloride and sodium citrate
SDS	Sodium dodecyl sulfate

CHAPTER ONE

INTRODUCTION

Importance of tick study

The most important ticks of public health concern in the U.S. are *Ixodes scapularis*, *Ixodes pacificus, Dermacentor andersoni, Amblyomma americanum, Ornithodoros hermsi,* and *Ornithodoros turicata.* These ticks deserve public attention because they feed on humans and several livestock and transmit pathogens and infectious diseases to human and animal hosts. *Ixodes scapularis* and *I. pacificus* transmit *Borellia burgdorferi* which causes Lyme disease, *Amblyomma americanum* transmits *Ehrlichia chaffeensis* which causes monocytic Ehrlichiosis, *Dermacentor variabilis* and *Dermacentor andersoni* transmit *Rickettsia rickettsii* that causes Rocky Mountain spotted fever and *Ornithodoros turicata* transmits *Borrelia hermsi* that causes Relapsing fever (55). The study of ticks is of great importance worldwide in agriculture as well as in medicine. "Ticks are second only to mosquitoes as vectors of disease causing agents to humans, and they are the most important arthropod transmitting pathogens to other species" (58). Some species of very pathogenic fungus (*Metharhizium anisopliae*) taken from infected ticks were also observed to be more pathogenic in the ticks than the fungus maintained in culture (13). This may imply that ticks have a mechanism that helps boost the pathogenicity of the pathogens they may be carrying. Ticks transmit diseases mainly through feeding on a host.

A rapid increase of Rocky Mountain spotted fever in the late 1970s and human ehrlichiosis in the 1990s in the U.S., show how human populations are becoming increasingly prone to infection of these rickettsial diseases (1). Since then, annual

reported cases of Lyme disease and Rocky Mountain spotted fever and tick paralysis still occur seasonally in the U.S (4). Heavy tick infestation usually results in morbidity or mortality to livestock and wildlife (48). My project is focused on one of the most important ixodid ticks, male *Dermacentor andersoni* also known as the Rocky Mountain wood tick (RMWT). The Rocky Mountain wood ticks are so named because they were first discovered in the Rocky Mountains (55). The larvae and nymphs of RMWTs feed on a variety of hosts. The adults usually emerge in March, reach the peak of their activities in April and disappear in May. The RMWT transmits anaplasmosis, RMSF, and causes tick paralysis in the Western U.S. (55).

Anaplasma marginale.

Anaplasma marginale is a tick-borne hemoparasite that causes severe anemia in cattle in its acute stage (41). Transmission of *A. marginale* by ticks usually occurs by oral secretion during tick feeding (22). Host infection of *A. marginale* can occur when infected male ticks are transferred from infected to susceptible cattle (intrastadial transmission) or when nymphs or adult ticks infected in a previous stage feed on a host (interstadial transmission) (22). In male ticks infected as adults, *A. marginale* begins a complex developmental cycle within the midgut epithelium, gut muscle and finally the salivary glands (16).

The midgut is the first site to be infected during acquisition feeding, but the salivary glands are not infected until the beginning of transmission feeding and it takes several hours of feeding before transmission occurs (16). The time required for *Anaplasma* transmission is very important to this project because of the possible length of

time required for gene expression. Other tissues infected are the Malphigian tubules and muscle tissue (16). Male *Dermacentor* ticks become infected with *Anaplasma* over a long period and thus transmit the parasite continously to non- infected cattle (21). The persistent infection of male ticks in this manner makes them potential reservoirs of infection for ruminants over a long period (22).

Rocky Mountain Spotted Fever

This is one of the two most common tick-borne diseases in the United States. Rocky Mountain Spotted Fever, also known as tick-borne typhus, is caused by a rickettsial organism that is a gram- negative obligate intracellular coccobacillus (*Rickettsia rickettsii).* Rocky Mountain Spotted Fever is also the most common tick-borne disease among children in Oklahoma (55). This fever is most prevalent from April to October, coinciding with the peak feeding activity of the vectors *D. andersoni* and *D. variabilis.* Children between the ages of 5 to 9 years old are mostly infected. The mortality rate ranges from 2% to 4%, but 25% of patients in whom diagnosis and treatment is delayed die (55).

Classification of Ticks

Ticks are obligate blood sucking ectoparasites that are located worldwide (48). Scientists have classified ticks into two major families according to the flexibility or hardness of their cuticle, namely hard ticks (family Ixodidae) and soft ticks (family Argasidae). The hard ticks have a rigid dorsal cuticle while the soft ticks have a flexible leathery cuticle (48).

Argasidae

The *Argasidae* or soft ticks can be classified into five genera: *Argas*, *Ornithodoros*, *Otobius*, *Antricola* and *Nothoaspis*. Mating in most adult soft ticks occurs on the host during feeding. The mated female continues to feed until she oviposits. The female soft tick deposits small egg masses usually about 500 per cycle for several cycles. The life cycle of the Argasidae begins from the egg, proceeds to the larva, nymph and then to the adult. Unlike ixodid ticks, there are several nymphal stages for the soft ticks. The several nymphal stages in the *Argasidae* contribute to a much longer life cycle (several years) than in the Ixodidae (48).

Ixodidae

The hard ticks are recorded as the largest family of ticks with 13 genera and 650 species and are also the most important because of the diseases they transmit. The hard ticks have 3 main developmental stages after the eggs are hatched: larva, nymph, and adult. The ixodid ticks feed slowly because they need time for growth of their rigid integument before they can indulge further. After oviposition, the eggs hatch and the new larvae ascend vegetation to seek a host. Once a host is found, the larva feeds very slowly until it has had a complete blood meal. In the next stage, the larva drops off its host to find a secure environment where it molts into a nymph. The nymph then finds another host and repeats the process of finding a host, attaching itself and feeding until it drops again. The engorged nymph drops off its host, finds a secure niche in the environment where it undergoes its final molt into adulthood. The adult tick attacks a

host, feeds and the female drops to oviposit (48). Both *Dermacentor* and *Amblyomma* species belong to the family of hard ticks.

Tick Feeding

Ticks depend on blood meals obtained from their host for growth, egg and sperm production and also for mating (7). The regulation of host immune defenses by ticks possibly assists feeding and transmission of hemoparasites (7). Ticks have an important sensory apparatus (Haller's organ) that senses odor, heat and humidity from mammals (48). Ticks detach from their previous locations and then crawl onto their prey after sensing the prey with Haller's organ. Adult ticks have preference for large hosts such as cattle, deer, humans and sheep. Once on the prey, adult females insert their mouthparts into the carefully chosen sites on the prey. The mouthparts are anchored to the skin of the host by a cement-like material secreted by the tick. The adult females feed until they are fully engorged, while the males feed for a short time, withdraw their mouthparts then find a female and mate before continuing to feed. The tick salivary gland plays an important role in tick feeding and pathogen transmission (43). During feeding, the ticks use their salivary glands to concentrate the blood meal while returning excess fluids and ions back to the host (18, 19). Ixodid ticks attach and feed on their host longer than the Argasidae. Because of the prolonged attachment of the tick to its host, the host will be expected to induce an immune response (43). When adult ticks feed, they induce a host immune response which triggers a counter measure by the tick against the host leading to modulation of host immune responses that may affect the ability of the ticks to feed (57). A bradykinin deactivating dipeptidyl carboxy-peptidase (40, 43) and a histamine–

binding protein (33) that are present in secreted tick saliva are responsible for suppressing pain and itch in the host which may lead to grooming of the host and thus disengaging the tick from its feeding grounds (43). Most adult female ticks have a slow feeding stage followed by a rapid feeding stage (57). Mating occurs within 2-3 days of attachment, but slow feeding goes on for 7-10 days after which the mated female then accelerates her feeding to acquire enough blood supply for the development of her eggs (2). The female then detaches off the host, lays eggs and dies (51). The cement-like proteins are secreted to anchor the mouthparts onto the host during the slow feeding stage of the adult female tick (38, 57). The Ixodid ticks are resistant to starvation and dehydration and can survive without a blood meal until the next mating season. The adult male tick reattaches itself to the site of feeding females after detaching and reattaching to multiple hosts and secretes IgG-binding proteins that are proposed to help the females in successful feeding (56).

Tick and Host: Infection, Immunity and Control.

Tick interaction with hosts varies greatly depending on the host, the activities of the tick while feeding and whether it causes its host severe irritation or minimal discomfort to severe blood loss or injection of toxins into the host. Even more dangerous to the host are the numerous disease-causing agents that can be transmitted to the host during tick feeding (2). The tick is more than a hypodermic needle and syringe when it comes to transmission of tick-borne pathogens to their host (58). Most pathogen transmission and toxin secretion by ticks occurs during feeding (58). The transmitted microorganisms can express molecules during the vector phase that may not be noticeable during infection of the mammalian host (58). This may allow the infection to

progress to a severe stage before being noticed. During feeding and pathogen transmission, a number of pharmacologically active molecules including anticoagulants, inhibitors of platelet aggregation and vasodilators are secreted together with saliva (39). The secretion of these active molecules may enable the tick to regulate host immune responses and suppress the host immune system to allow efficient pathogen transmission.

When a tick infested host acquires resistance to tick feeding, there is a reduction in engorgement weight of the tick, the tick feeds much longer, number as well as the viability of ova produced by the females is reduced, molting is prevented and then death (58). This type of host-acquired resistance is not very common. Because of the numerous pathogens and toxins ticks are capable of transmitting, there should be a way of controlling tick infections and transmission of tick-borne pathogens to their host.

The use of chemical acaricides is among the most common methods of tick control, however it has several disadvantages such as high cost and difficulty of disposal (30, 59). Some of the current methods for controlling ticks and tick-borne pathogens include genetic techniques (14) while other techniques use vaccines to control the developmental cycles of the parasites as well as the ticks (22). The genetic technique introduces an isolated anti-tick gene into breeds of cattle with the intention of producing cattle with total tick resistance (14). The project is still undergoing improvement. According to Norval et al. (31), tick resistant hosts have not been successful for large-scale control of ticks (31). Anti-tick vaccines show more promise for controlling ticks in the future (22). In one case, a vaccine developed from the gut and synganglion tissue of the *Boophilus microplus* tick gave good protection for a host that was heavily infected with tick larvae (22). Various kinds of vaccines are currently being developed (22).

Gene Expression in male D. andersoni ticks is affected by feeding with females.

Bior et al. (5) observed increases in total RNA of the fed male *D. andersoni* tick relative to the unfed male tick and a further increase in total RNA in the salivary glands of males fed with females. A previous study showed new gene expression in the salivary glands of male *D. andersoni* ticks fed without females when compared to unfed males (5). Besides new gene expression in male ticks fed without females, an increase of total RNA in the salivary glands of male ticks fed with females was recorded relative to the unfed males. (5). This observation may imply that female *D. andersoni* have some effect on male *D. andersoni* tick feeding. The expression of new genes could indirectly imply a change in physiological and biochemical processes in the salivary glands of the male ticks during feeding without females and an increase of total RNA when fed with females. It is important to note however that the previous work by Bior et al. (5) showed no direct evidence of differential gene expression in male ticks in response to females, only an increase in total RNA levels (5).

Why this project?

Though at this point the male tick has been much better studied for differential gene expression, the effect of females and feeding on males has not been well understood. Bior et al. (5), in a previous study had compared differentially expressed genes in the salivary glands of unfed and fed male *Amblyomma americanum* and *Dermacentor andersoni* ticks and noted a 6 fold increase of total RNA in the salivary glands of the fed male *A. americanum* tick upon feeding, while in the fed *D. andersoni* only a 3.5 fold

increase of total RNA was observed over the unfed (5). In addition, differential display revealed clear differences between the fed and unfed male *D. andersoni*. In a cross hybridization of identified DNAs from *A. americanum* with DNA probes synthesized from *D. andersoni* total RNA, no hybridization occurred (5). The hybridization results likely indicate that the genes of the two species of ticks are too different to cross-hybridize. Based on the above, I have been interested in knowing what genes are being differentially expressed in the male *D. andersoni* ticks fed with females, as compared to males fed without females and unfed males. I then wanted to characterize the difference between male *D. andersoni* ticks fed with females and males fed without females. Although the mated female tick feeds more and increases the amount of polypeptide (32), and new gene expression (29) in its salivary glands, nothing is known about whether the presence of females, or the stimulus to mate induces the expression of new genes in males.

Reason for studying male Dermacentor

In this thesis, I chose to study male *D. andersoni* because it is a vector of *Anaplasma marginale,* the causative agent of anaplasmosis. *Anaplasma marginale* is a tick borne hemoparasite that causes severe anemia in cattle at its acute stage (7). "Although both male and female ticks can transmit *Anaplasma marginale* intrastadially, males have been shown to be persistently infective, transfer readily among cattle and maintain high infection rates while off the host" (16). The feeding of the male ticks on multiple hosts permits efficient pathogen transfer from one host to another and enables the tick to serve as a reservoir of pathogens to infect cattle over a long period of time (22, 16).

Methods for identifying differentially expressed genes

There is currently a plethora of available molecular tools for investigating differentially expressed genes. Some of the main methods currently used to identify differentially expressed genes include rapid subtractive hybridization (RaSH) (43), single stranded differential display (ssDD) (50), multicolor fluorescent differential display, DNA micro array (10) and in-situ hybridization (9). Differential display and DNA micro array are the two most important methods used in gene expression profiling (10). Of all these methods, differential display (DD) coupled with RNA arbitrarily primed polymerase chain reaction (RAP-PCR) has emerged as the favorite choice for investigating differential gene transcripts in both eukaryotes and prokaryotes. This combination has been well favored because it is sensitive, versatile, simple and needs less RNA than some of the other methods (25). In addition to the previous advantages, differential display enables simultaneous comparison of multiple mRNA samples for identifying genes that are up regulated and down -regulated without having previous knowledge of their sequences (25). Despite the success of some these methods, significant pitfalls have rendered them less of an ideal technique and thus several researchers have minimized the pitfalls by using a combination of methods or by slightly modifying the existing techniques to suit their purpose of study. With the differential display method, one of the basic pitfalls is a high rate of false positive transcripts that are not differentially expressed in duplicate experiments (45). Also, since the oligo dT RT-PCR based differential display is restricted to investigating differences at the 3' end, the 5' end differences are usually not detected (27). This problem can usually be fixed during PCR amplification of

the cDNA fragments by using 2 primers, a 3' end and 5' end anchor primers of arbitrary sequence (9). The initial step in investigating differentially expressed gene transcripts is the synthesis of cDNA from RNA's using reverse trancriptase (47).

In this study, a pairwise combination of arbitrary primers designed for arbitrary pairing hybridize to arbitrary sequences within the mRNA in the presence of reverse transcriptase was used to synthesize a single stranded cDNA (42). In a second step, internal sequences of the single stranded cDNA were amplified by PCR using 18 mer arbitrary primers, thus the name RNA arbitrarily primed polymerase chain reaction (RAP-PCR) (42, 24). During the PCR reaction, DNA is rapidly denatured in repeated cycles. The first cycle is programmed to allow some mismatch of the arbitrary primers to anneal to similar sequences within the open reading frame of the differentially expressed gene fragments. The hybrid (cDNA and arbitrary primer) is extended by a *Thermus aquaticus* DNA polymerase resulting in a several fold increase of the cDNA sample that was originally used (47). During amplification of the cDNA, it is radioactively labeled and then resolved on a high resolution denaturing gel by electrophoresis.

The DNA bands of interest are isolated by physically cutting them out, then they are amplified, cloned and sequenced for later analysis. Two primers were used in the PCR reaction in the hopes of annealing with sequences within the RNA sequence that will not have been amplified by single primers. I expected to identify some genes mostly

from the salivary glands of male ticks fed with females and also from male ticks fed without females that might be related to tick feeding and maybe mating.

DNA Micro Array

Spotting a large number of DNA molecules on a solid substrate such as glass slides, membranes, nylon or silicon chips makes micro arrays. Each spot of DNA represents a known sequence from a different gene. Each spot is made with DNA so that it can hybridize to cDNA labeled with fluorescent probes made from mRNA. The mixed cDNA is incubated with the DNA chip or slide to allow hybridization. Some of the labeled cDNA probes in the mixture bind to some of the spots and the unbound probes are washed off. The chip containing the micro array is placed inside a dark box where it is scanned with a laser to detect bound DNA. The micro array slide is ejected and the spots are analyzed on a computer with specific programs. If only one probe contains a particular mRNA (because it is differentially expressed) then only one color will be observed, but if it is present in both probes, the computer produces a mixture of colors (eg. red+ green= Yellow). DNA micro array technology is currently used for gene discovery, gene expression analysis, gene mapping, genotyping and much more. The technique is very expensive but is able to analyze several thousands of genes (9). The reason I do not use this technique for this study is because of its limited use for detection of novel or unexpected genes (9) and it also needs a collection of cDNAs. I expect to identify novel genes in this experiment using the RAP-PCR technique.

Rapid Subtractive Hybridization (RaSH)

In this technique, cDNAs are synthesized from mRNA in a method similar to that used for cDNA synthesis in the differential display technique described previously in the thesis. The only difference in this part of the experiment is the use of primers with T-tails instead of arbitrary primers. The RaSH technique is based on the subtractive hybridization technique. Denatured double stranded complementary DNA (dscDNA) from one sample (Driver) is hybridized to denatured dscDNA from the other sample (Tracer) so that sequences common to both samples are `subtracted' from solution as shown in figure 1. This step leaves a population of cDNAs with sequences expressed in the tracer but not in the driver. Driver cDNA is photobiotinylated by irradiation with a sun lamp. After the hybridization step, both hybridized and unhybridized biotinylated driver are removed by Streptavidin precipitation and phenol-chloroform extraction. The subtracted product from the initial subtraction is used in two or three more rounds of further subtraction. The first subtractive round removes the smaller fragments that bind rapidly while the second and third round remove the DNA fragments that hybridized slowly. At the end of the procedure, only genes unique to or greatly upregulated in the tracer are left. This leaves less than 5% of the starting cDNAs. The advantages of this technique are as follows (i) Limited RNA sample size can be used (ii) Rare RNAs representing about .01% of the total RNA species can be detected (iii) Fewer false positives than differential display. The major disadvantages are (i) Demanding technique (ii) Complete removal of cDNAs common to both driver and tracer population is impossible (iii) Loss of RNA sample during the technique (9). The principle of subtractive hybridization has been used as is, as well as modified by numerous

researchers, to construct cDNA libraries in a way that suits their specific experiments. In this study, we used RNA arbitrarily primed polymerase chain reaction (RAP-PCR) to investigate differentially expressed genes in male tick salivary glands. The overall strategy for differential display depends on a combination of three techniques (i) Reverse transcription from arbitrary primers PCR (ii) Choice of arbitrary primers to determine lengths of cDNA's to be amplified by PCR. (iii) High-resolution sequencing gels (23).

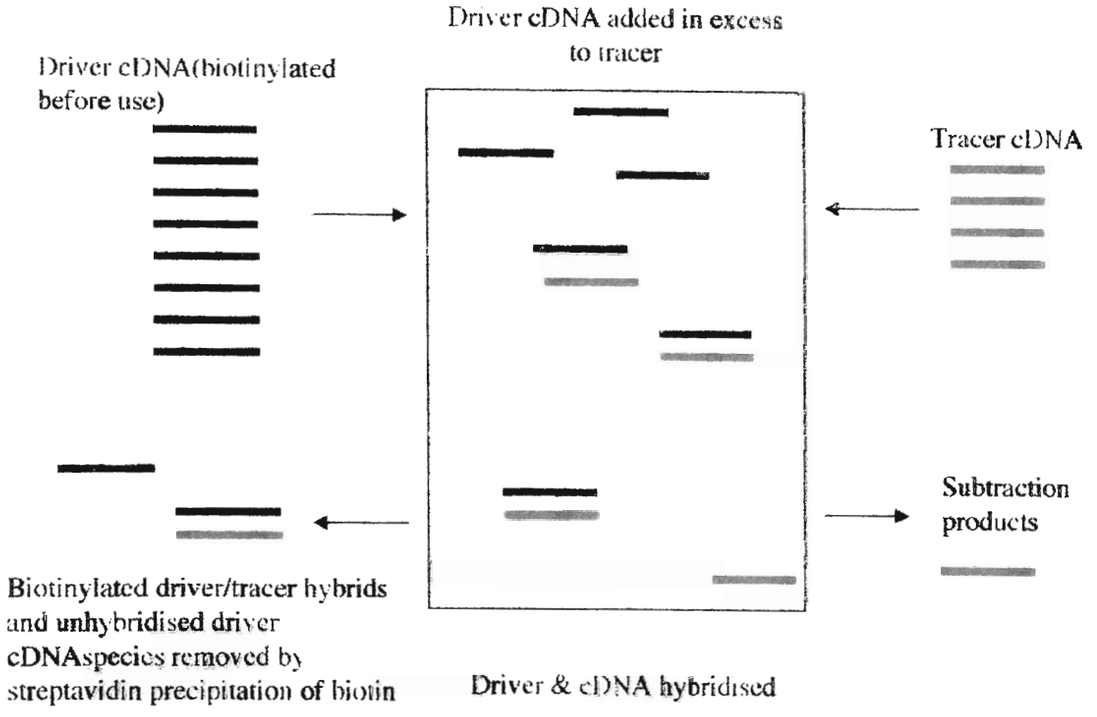

Figure1. Illustrative summary of the principle of subtractive hybridization

(Adapted from (9)). The driver cDNAs (black bands) hybridize to tracer cDNAs (red bands) to form a double stranded driver/ tracer cDNA hybrid. The hybrid cDNAs contain cDNA fragments common to both driver and tracer. The hybrid cDNAs are removed from solution by streptavidin precipitation and phenol chloroform extraction. This leaves cDNA fragments unique to only tracer cDNA.

as NCBI. Blastx is used for comparing and analyzing sequences before entry to the records that are stored for use by the investigator. The top five most significant protein alignments are stored in the PipeOnline database to be retrieved by the investigator, though the whole BLAST output can be found on a server.

Protein Alignments

This is part of "My PipeOnline" program that uses BLASTALL for batch execution of blastx to look for sequence similarity between the DNA sequence of interest translated in all possible reading frames and the public non-redundant amino acid sequence data base.

Blastx

There are several available blast searches however we will only focus on BlastX for this thesis. Blastx is useful because it translates the generated nucleotide sequence into proteins and then searches a protein database for proteins similar to the proteins generated from the query sequence. This search is particularly good for our kind of experiment because it can identify potential coding regions in newly sequenced DNA.

Blast interpretation.

Blast search usually produces results of sequences with a significant match to a similar sequence in the query database. The levels of this match is measured in terms of the probability value (P-value), also called expect or E. The P-value reflects the probability of observing by chance a score as good as that found, based on the amount of sequences in the database. The lower the P-value, the better. Normally, sequences with P-values greater than 0.0001 are not significant. The score provides the minimum percent identity that will give the closest match for aligning a random pair of amino acid residues.

CHAPTER TWO

MATERIALS AND METHODS

Ticks

Two groups of *D. andersoni* male ticks were raised on sheep according to Patrick and Hair (34). One set of the males was fed on its host without females in the sock, a second set was fed on its host in the presence of females while the last set was unfed. Fed groups were fed for 7-14 days, while the unfeds were held at 75°F in a 94% relative humidity K_2SO_4 chamber in a cycle of 14 hours in light and 10 hours of darkness. The humidity chamber was used to enable the unfed ticks to conserve internal moisture and prevent them from desiccating. Salivary glands from the three groups were isolated from the ticks and cleaned with dissection buffer within 2-3 hours of removal from their host. Cleaning the salivary glands with buffer maintains the glands at an optimum pH (54) and prevents the RNA in the salivary glands from degenerating. Immediately after harvest, the salivary glands were dipped into liquid nitrogen and stored at –80°C; this step is critical because RNA is very unstable in tissue once removed from the body of a living organism so it is important to freeze the tissue to maintain RNA stability.

Materials

Total Rapid RNA Isolation kit purchased from 5-prime → 3-prime Inc. (Boulder, CO. USA) was used for isolation of total RNA from tick salivary glands. Extracted total RNA was treated with DNase I (Message Clean Kit) from Ambion Inc. (Austin TX. U.S.A) as described by the vendor. DNase I treatment was used to degrade any genomic DNA present. Original RAP-PCR reagents were purchased from Stratagene (La Jolla,

CA.) and later from Gibco-BRL. The RAP-PCR primers were synthesized at the Oklahoma State University's DNA/ Protein Core Facility. A Moloney murine leukemia virus reverse transcriptase (RT) was purchased from Ambion Inc. (Austin, TX. USA). Mineral oil was from Sigma (St. Louis, MO., USA). Plasmid DNA and PCR purification kits were purchased from Qiagen (Valencia, CA., USA). *Thermus aquaticus* (Taq) DNA polymerase and restriction enzymes were from Gibco BRL (Rockville, MD., USA). Subcloning competent cells (DH5-α) were from Invitrogen Corp. (Carlsbad, CA. USA). α-^{32} P dCTP was purchased from New England Nuclear. X-ray film was obtained from Fuji film company (Stanford, CT.). Digoxigenin 11-dUTP probe synthesis mixture [200μM each dNTP] was purchased from Boehringer Mannheim Corporation (Indianapolis USA). Antidigoxigenin Fab fragments conjugated with alkaline phosphatase (AP) for the detection of digoxigenin –labeled compounds was also from Boehringer Mannheim Corporation (Indianapolis USA). Gel Doc utilizing Multianalyst software that captures images and performs densitometric measurements on blots on nitrocellulose or nylon membranes, X-ray film and ethidium bromide gels was from Bio-Rad Laboratories (Hercules, CA., USA). A PTC 100™ programmable thermal controller was from MJ Research Inc. (Waltham, MA., USA). A Speed Vac® Plus SC110A was from Instruments Inc., (Farmingdale, NY). An Isotemp refrigerated circulator model 9100 for performing ligation reactions was from Fischer Scientific (Pittsburgh PA.) Pico green dsDNA Quantitation reagent and kits for determining DNA concentration were from Molecular Probes (Eugene, Oregon). Fluoroskan II instrument from MTX lab systems Inc (Helsinki, Finland) commanded by Delta Soft II software from Biometallics,

Inc. (Princeton NJ.) was used for measuring DNA concentration. A U.V Stratalinker™ used for crosslinking DNA was from Stratagene (La Jolla, CA.).

Total RNA

Total RNA from the three groups (MFWF, MFWOF, UF) of *D. andersoni* ticks was obtained from Bior and the RNAs were electrophoresed on agarose gel to check for RNA stability (5). 1.02 μg of total RNA per tick was isolated from the unfed males, 2.7 μg per tick from males fed without females and 3.6 μg of total RNA per tick from males fed with females. Male *D. andersoni* ticks fed with females had 3.5 times more total RNA than the unfed males, and 25% more total RNA than the males fed without females (5).

Complementary DNA synthesis

I followed a Stratagene protocol using reverse transcriptase (RT) enzyme and a chosen pair of arbitrary primers to synthesize the first sscDNA as illustrated in figure 2. The primers used for this experiment were A1+A2, A2+A3, A1+A3. The A2+A3 primer pair yielded poor results and so its use was discontinued. The sequence of 18 mer primers used in this investigation are as follows

A1: 5'-AATCTAGAGCTCCTCCTC-3', A2: 5'-AATCTAGAGCTCCAGCAG-3', A3: 5'-AATCTAGA GCTCTC CTGC-3'. I also conducted a mock reaction, also known as a negative control, in which all the reagents were present except for the RT. In a positive control reaction, we included an oligo dT and control primer set for the human β-actin gene. The results of the positive control were analyzed on a 3% agarose gel as

recommended by Stratagene. The presence of a 661 bp product depicted in figure 3 for each sample in the gel was an indication the RNA was intact and proof of successful cDNA synthesis. We proceeded to radiolabel the cDNA products from first strand synthesis with 250μCi α^{32}P dCTP by PCR using the same pair-wise primer combinations as before. The thermocycler was programmed to run one low stringency cycle and forty high stringency cycles. The low stringency cycle is to allow imperfect pairing of the primers to the daughter strand while the high stringency step is for specific annealing of the primers. The low stringency cycle was set at 94°C for 1 min, 36°C for 5 min, and then 72°C for another 5 min. The forty high stringency cycles were set for 94°C for 1 min, 50°C for 2 min., 72°C for 2 min and then 72°C for10 min and then let cool to 4°C. Four microliters of the radiolabeled cDNA's from each of the three tick samples (MFWF, MFWOF, UF) were loaded side by side and resolved on a 6% polyacrylamide gel containing 7M urea at constant power to get good gel temperature (50-60°C) until the marker dye in the samples had migrated about 3/4 or greater the length of the denaturing gel. The power was turned off and both surfaces of glass containing the sandwiched gel were rinsed with tap water to cool the gel to room temperature. The spacer on one side of the glass was carefully removed and the glass plates containing the gel were pried apart with a long metal spatula inserted between the plates. With the glass plates separated, the spacer on the other side was removed and extraneous bits of polyacrylamide around the gel were carefully removed. The glass plate containing the gel was laid flat so the gel was on top. The gel was transferred onto blotting paper by laying two pieces of blotting paper together as one piece and working slowly toward the top. The blotting paper with the gel was then peeled off the glass, covered with plastic wrap and dried for about 1 hour

at 80^{O}C under a vacuum on a gel drier. The plastic wrap was peeled away and the dried gel placed in a separate cassette where it was exposed to X-ray film in complete darkness until the film was developed. The developed X-ray film was carefully placed to match marks made on the dry gel before exposure to X-ray film to obtain perfect alignment with the bands on the gel. Bands that were differentially expressed as depicted in figures 4 and 5 were physically cut out of the dried gel with a clean sterile blade and placed in 1.5 ml Eppendorf tubes. 100 µl of DEPC treated water was added to the gel in the Eppendorf tubes and incubated at 65^{o}C for 1 hour to elute the DNA fragment. The gel suspension was centrifuged at 13,000 r.p.m at room temperature for 10 min. The supernatant from the previous step was transferred to new Eppendorf tubes where they were concentrated in a Speed Vac to 40 µl. About 10 µl of this cDNA was amplified in another round of PCR reaction as before with the same primer pairs used in first strand synthesis. The PCR products were analyzed on a 1.5% agarose gel as shown in chapter three. The visible bands were cut out and purified by a Qiagen gel extraction kit.

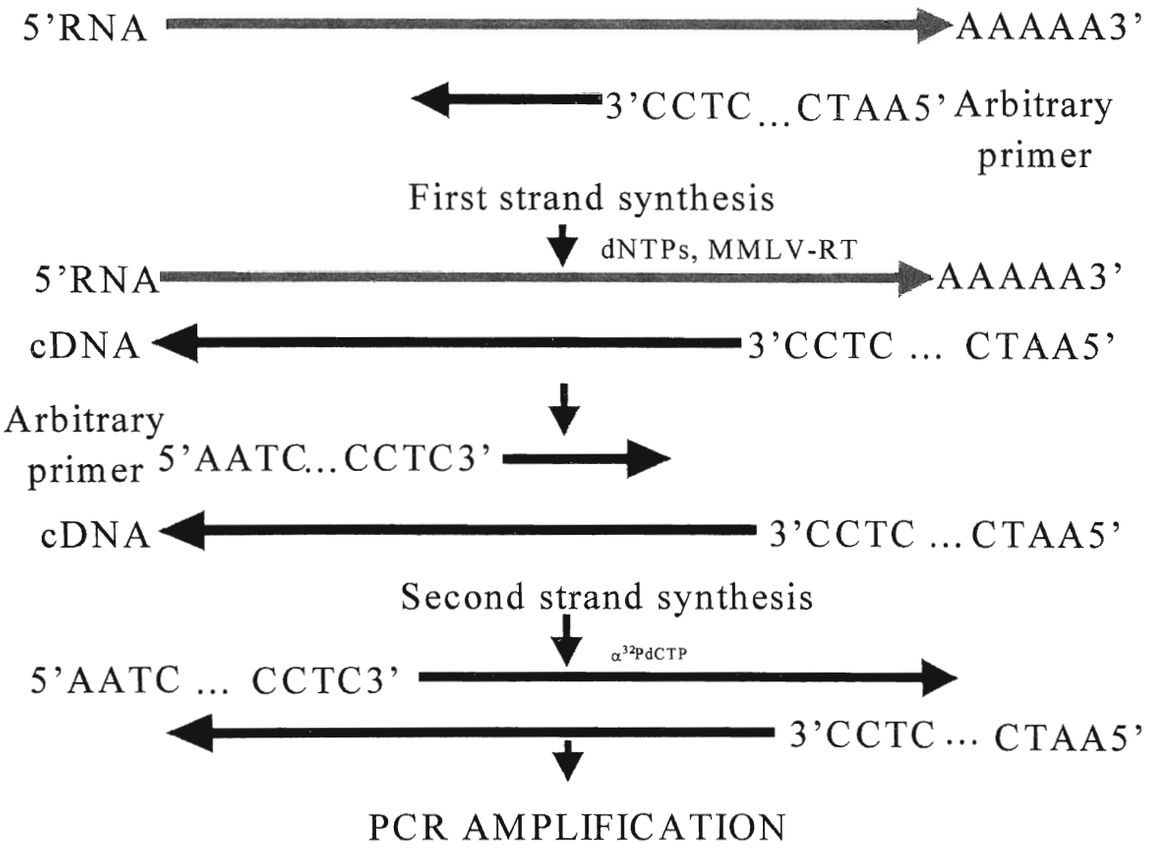

Figure 2. Summary and outline of RAP-PCR technique.

Blue arrows represent total RNA to which an A1 arbitrary primer anneals. The product of amplification is a first strand cDNA. The first strand cDNA strand is further amplified with the same A1 primer used in first strand synthesis. The product of this amplification is a double stranded cDNA fragment. During amplification, the double stranded cDNA is labeled with α^{32}P dCTP.

Cloning of cDNA's

The purified cDNA fragments of each sample (unfed males, males fed without females and males fed with females) from the previous step were ligated into a pDrive vector from Qiagen. More than one PCR product sometimes resulted from amplifying the bands isolated from the denaturing gels as depicted in figures 6 and 7. In this case, each amplified band was cut out and extracted. In the ligation step, 4 μl each of the purified PCR products were ligated into pDrive cloning vector (50 ng/μl) by a ligation protocol from Qiagen. Each ligation mixture also contained 5 μl of premixed ligation reaction mixture that was incubated for 30 min. at 14°C in an Isotemp refrigerated circulator. The ligated products were each transformed into 50 μl of DH5α competent cells by a protocol from Life Technologies. The transformation products were each plated on LB plates containing 50 μg/ml ampicillin, 20 ug/ml IPTG and 667 μg/ml X-gal. Single white colonies were selected from each cDNA library and cultured in a 1.5 ml LB broth containing 100 μl ampicillin at 37°C overnight with constant shaking.

Plasmid purification

A Qiagen miniprep kit was used for plasmid isolation. Instead of the 50μl elution buffer (EB) suggested by Qiagen, we used 200μl of EB to elute the plasmid. The eluate was evaporated to dryness in a Speed Vac and then resuspended in 50μl water; this modification enhanced the plasmid yield. The cDNA concentrations were measured by Pico-green reagent and numerically quantitated with the Delta II software. Before submitting the purified plasmids for sequencing, the plasmids were resolved on a 1.5%

agarose gel along with a linearized pDrive cloning vector to verify that each plasmid contained an insert.

Sequencing of recombinant plasmids

The recombinant plasmids were sequenced using T7 and SP6 primers by the Oklahoma State University (OSU) Protein and Nucleic Acid Resource facility. The resulting sequences were submitted to "MyPipeOnline", a local web based resource at OSU.

Making the probe for Dot blots

Complementary cDNA's were synthesized from total RNA obtained from the salivary glands of the unfed males, males fed without females and males fed with females by the same procedure as for RAP-PCR except that this was labeled with digoxigenin 11-dUTP. The cycling conditions were one low stringency cycle at 94°C for 1 min., 36°C for 5 min, 72 °C for 5 min. The low stringency cycle was followed by 40 high stringency cycles at 94°C for 1 min. 50°C for 2 min., 72°C for 2 min. and then 72°C for another 10 min. before cooling to 4 °C. The reaction mixture for making the probe contained 4 µl of sscDNA from each sample in a separate centrifuge tube, 5 µl of 10x PCR buffer, 3 mM $MgCl_2$, 2.0 units of Taq DNA polymerase, 200 µM each dNTP in PCR digoxigenin 11-dUTP labeling mixture, 2 µM each of the arbitrary primers (A1+A2) or (A1+A3). The mixture was made up to a final volume of 50 µl with diethyl pyrocarbonate (DEPC) treated water. A QIAquick PCR purification kit was used to purify the probes.

Chemiluminescent detection

The cloned DNA's were denatured in 0.5N NaOH and 20mM EDTA at 65°C for 1 hour, blotted onto nylon membranes by a Seiko model X 5000 cartesian robot (Seiko Instruments Inc., Japan). The membranes were then hybridized to specific probes described as follows. The membranes were prehybridized in 0.25 M Na_2PO_4 hybridization buffer (pH 7.2) containing 7% SDS and 1 mM ethylene diamine tetra-acetic acid (EDTA) for 1 hour at 65°C prior to hybridization. The membranes were hybridized to the probes in hybridization buffer containing 10-100 ng/ml of probe at 65°C overnight in a Biometra hybridization chamber. The hybridized membranes were then washed twice for 5 min at room temperature in 2xSSC/ 1% SDS (1ml/ cm^2). The low stringency buffer used in this step is to remove any unbound probes. In the following step, the membranes were washed twice for 15 min. at 55°C in 1x SSC/ 1% SDS (1ml/ cm^2). This high stringency wash takes off anything that is not a perfect match to the cDNA sequences in each spot. The membranes were washed twice for 5 min. in blocking buffer (1X PBS (0.058 M Na_2HPO_4, 0.017 M $Na_2H_2PO_4$-H_2O, 0.068 M NaCl), 0.2% I- Block™ Reagent, 0.5% SDS) and then incubated in blocking buffer for 10 min. The membranes with probes were treated with antidigoxigenin Fab fragments conjugated to alkaline phosphatase (diluted 1: 10,000 in blocking buffer) for 35 min at room temperature. Washing of the membrane was repeated twice for 2 min. in blocking buffer and again for three times for 3 min. in wash buffer (1X PBS, 0.5% SDS). The membrane was washed for a final time twice for 2 min. in 1X Assay buffer (20 mM Tris pH, 9.8, 1 mM $MgCl_2$). The membranes were drained by touching their corners to a paper towel and placed flat on a plastic wrap without letting them dry. A thin layer of CDP-Star (alkaline

phosphatase substrate) was pipetted onto the membranes and incubated for 5 min. The excess CDP-Star solution was drained off the membranes and they were placed in a hybridization bag. Bubbles were smoothed out of the bag and the bag sealed for later development. The standard positive controls used in the process were a gene fragment that was expressed in all three groups of experimental ticks and a tick genomic DNA sample from *D. andersoni.* The negative controls were diluted elution buffer and pBluescript vector. The sealed membranes were exposed to a phosphorimaging plate and then scanned into a phosphorimager GS-700 (BioRad). The resulting image from the phosphorimager was saved as a tiff file for further analysis.

Analysis of dot blot

The abbreviations MFWF2, MFWOF2 and UF2 will be used to represent cDNA fragments from the salivary glands of male ticks fed with females, males fed without females and unfed males respectively synthesized from A1+ A2 primers. Similarly, MFWF3, MFWOF3 and UF3 will represent cDNA fragments synthesized with A1+ A3 primers. The bound probes were detected by chemiluminescent detection on a phosphorimager. The exposed phosphorimager plate was scanned using Multi Analyst software and the resulting images were saved as tiff files and later analyzed by Gene Pix software that quantitates the intensity of the spots in each blot by using grid alignment and exports the results to a Microsoft Excel spreadsheet. The intensity of each spot was expressed as an average (F2-mean) of the pixels within each spot selected by Gene Pix software. The F-2 mean is the mean of all of the pixel intensity in a given circular region specified on the image. A portion of the image outside the spot is used to calculate the

blot background intensities. All calculations in the following parts of the experiment were done in a Microsoft Excel spreadsheet. The F2 mean values were normalized to the positive control (tick genomic DNA (Cd)) by using the formula P= F2 mean/ Cd. In the following step, we calculated new values (M ratio) using the "normalized intensities" (P-values) from the previous step eg. P_{MFWOF2}/ P_{UF2}, and P_{MFWOF3}/ P_{UF3} for A1+A2 and A1+A3 primer sets respectively. Similar ratios were calculated for P_{MFWOF2}/ P_{MFWF2}, P_{MFWOF2}/ P_{UF2}, P_{MFWOF3}/ P_{MFWF3} and P_{MFWOF3}/ P_{UF3}. In the next step, we calculated the logarithm of each M ratio eg (Log (P_{MFWOF3}/ P_{UF3})). A standard deviation value was generated for each set of the logarithmic values eg log.(P_{MFWOF3}/ P_{UF3}) values and then the standard error was calculated (S_{de}= standard deviation/ $\sqrt{n}$; n is the number of samples in each treatment). In a final step, we calculated a test statistic (t-value) using the formula $t= (x_i-\mu)/ S_{de}$ where x_i is the log value of the M ratios eg.(Log(P_{MFWOF3}/ P_{UF3}), and μ is our hypothesized value($\mu= 0$) meaning the spots are of equal intensity. The t-values were in the range of $-22 < t < 32$. Based on the calculated t-values, I was able to compare expression of gene fragments from two groups (eg. MFWF3 versus UF3, MFWF3 versus MFWOF3 and MFWOF3 versus UF3) at a time. This comparison was repeated for sequences obtained from A1+A2 primer set. All positive t-values greater than or equal to +3 were assigned to the numerator and the differentially expressed gene for a dot blot in the spot that was being compared was said to be expressed significantly more for the treatment in the numerator; in the above example, the differentially expressed gene will be specific to MFWOF3. The t-values greater than 3 standard deviations from the mean were chosen because those numbers fall outside the 99.9% confidence interval range as determined by the t- values. All negative t-values less than -

3 (ie. –3, -4, -5,…) were assigned to the denominator and the differentially expressed gene involved was said to be expressed more in the treatment involved which in the above example will be UF3. In cases where the t-value was in the given range ($-3 < t < +3$), then the genes were not differentially expressed in either groups of MFWF3 or UF3 in the example above. Using this analysis, we have been able to assign each sequence generated from PipeOnline to be differentially expressed or not in the unfed males, males fed with females or males fed without female salivary glands.

Alternative method for dot blot analysis

I used a different method to assign genes as differentially expressed in one group of male ticks or another. This method is more reliable than the previous method using t-test analysis for the following reasons. Each membrane contains three replicate spots per clone on a single blot, and this method accounts for differences within each spot in a given clone and also for the difference between individual clones. I generated an analysis of variance table in a Microsoft Excel spreadsheet that provided data for this analysis. An average value is generated for the logarithm of the intensities of the three replicate spots per each clone. The Single Anova program in Microsoft Excel computes the sum of squares (SS), degrees of freedom (df) and mean sum of squares (MS) within the three replicate spots. Similar values are also generated between the averages between clones. Total degrees of freedom (df) = n-1, where n= number of samples, degrees of freedom (df) for treatment conditions = T-1, where T= number of treatment conditions. The value of the test statistic (t) is 3.29 for 99.9% confidence interval. Other calculations performed in the table can be found in most statistics textbooks. In this analysis, we compared the difference between average values for spots within given clones to a test criterion calculated from the pooled variance (S^2p). The test criterion is also known as the lsd (least significance difference) For a comparison between clones from male *D. andersoni* ticks fed with females to males fed without females, the pooled variance is $S^2p = (SS_{MFWF}+SS_{MFWOF})$ where SS_{MFWF} is the sum of squares within spots in a clone for males ticks fed with females and SS_{MFWOF} is the sum of squares for male ticks fed without females in a paired comparison; $lsd = t^*\sqrt{\{(SS_A+SS_B)/ (df_A+ df_B)\}}$ where A and B represent the two classes of ticks being compared. The value for each difference is

compared to the lsd. If the difference of value is positive and bigger than the lsd value for the group, then the gene is differentially expressed in the clone whose average value the other average is being subtracted from, the assignment goes in the opposite direction eg. If A-B= 5 and lsd= 2, then the clone being considered is differentially expressed in A. If however, A-B= -5, the clone being considered is differentially expressed in B. In cases where a value such as +1 or –1 is obtained as the difference, then the gene being considered is not differentially expressed in either of the two groups (A or B). The two methods described use quite different criterion to decide on significance. The ANOVA analysis as used in this experiment is like the normal t-test. This is used to determine if the difference between two means is statistically significant (28) given the variability observed for replicate spots. The t-value measures how far a value is from a given center by chance. Only genes with a big change in gene expression compared to the within treatment variance are considered as a significant change (28).

CHAPTER THREE

RESULTS

Differential display

Figure 3 suggests that the RNA used in this experiment is intact and not degraded. The presence of the 661 bp products on the gel in figure 3 confirms that total RNA from the tick salivary glands is intact. The intact RNA was later used for first strand synthesis in the latter parts of the experiment. Since the credibility of results in this experiment depends mostly on the purity of total RNA used, proof of RNA purity was essential. The mock lanes in the denaturing gels of figures 4 and 5 showed few intense visible bands, meaning that since RT was left out in the cocktail of the mock cDNA synthesis, RNA amplification was not expected and the presence of several bands on the gel would have suggested contamination of the RNA sample with genomic or extraneous DNA. In addition, bands on the denaturing gels show differentially expressed cDNA fragments that are unique to one of the three groups (males fed with females, males fed without females and unfed males) of ticks, but not present in all the groups. This observation indicates that female presence and feeding affects gene expression in the salivary glands of male *D. andersoni* ticks. I repeated this experiment 8 times and each time, there were bands uniquely present at the same positions as in the other displays. The differential display shows a number of cDNA fragments (represented in table 1) unique to either male *D. andersoni* ticks fed with females, males fed without females or unfed males. I observed some cDNA fragments that were present in two classes of ticks shown in table 2. A third group of cDNA fragments was observed in all three classes of ticks represented in table 3.

Confirmation of the Differentially Expressed Genes

I isolated 62 fragments (including some that showed more than one fragment when amplified and electrophoresed on 1.5% agarose gel) shown in figures 6 and 7 that tested for differential expression. Occasionally, it was observed that two or more different sequences originated from the same cDNA fragment obtained from the denaturing gel. This observation is due to some of the original cDNA fragments amplified from the denaturing gel revealing more than one significant band after being electrophoresed on 1.5% agarose gel. Each of the bands observed from the multiple sets was purified, cloned and then sequenced. The clones were electrophoresed on a 1.5% agarose gel to verify inserts as depicted in the Gel Doc picture in figure 8. The new bands were identified by sub lettering the original bands with lower case alphabets. E.g., for an F1 band, if two new bands were observed when electrophoresed on a 1.5% gel, they will be labeled as F1a and F1b. The gel docs for the amplified differentially expressed cDNA fragments show 56 original cDNA bands that were successfully purified and cloned. Some of the 56 original bands displayed multiple bands (2 or 3) when electrophoresed, however only the most intense bands were isolated for sequencing. There were multiple bands, not all of which could be isolated for sequencing, thus explaining the mismatch between the numbers in the tables and the gel doc pictures. Chemiluminescence of the dot blots in figures 9, 10, 11, 12, 13 and 14 indicate that hybridization did occur between given probes and the corresponding cDNAs (cDNAs from salivary glands from MFWF, MFWOF and UF) thus confirming that the genes that were isolated as being differentially expressed were indeed from the tick salivary glands.

Some of the probes also hybridized to cDNA from other stages. Also, plots of frequencies versus the logarithmic ratios of the normalized dot blot intensities (histograms) of cDNAs from the salivary glands of two groups of ticks compared at a time as shown in figures 15 A through 15 F, except for figure 15 B, follow a normal distribution curve as expected for a large population of normalized data points. Figure 15 B is skewed, but it is not bimodal. Since some bands in the differential display were so close to each other, it was easy to isolate cDNA bands from one lane and assign it to another. Dot blot analysis was important in sorting out this kind of incorrect gene assignment. The most intense spots were present where probes from the salivary glands from one class of male ticks had hybridized to corresponding cloned cDNAs, e.g probes synthesized from males fed with females had corresponding hybridization with the cDNAs of males fed with females in figures 9 and 10. Spot intensity outside the rectangular area indicates that the probe made from RNA from the salivary glands of male *D. andersoni* ticks fed with females had hybridized to some cDNAs present on the nylon membrane from the salivary glands of male ticks fed without females or unfed male ticks or both. Further analysis of the intensities generated by hybridization of the probes to the blots on the nylon membrane by using the calculated t-values or the ANOVA described in chapter two was used to assign sequences of the differentially expressed gene fragments as uniquely expressed in one of two groups of ticks compared at a time. At other times, given gene sequences were assigned as differentially expressed in two treatments based on the data, e.g., the putative senescence associated protein gene from the F1 band in the differential display of A1+A2 primers is expressed in both *D. andersoni* male ticks fed with females and also fed without females. The differential

display shows a faint band at the adjacent location to the F1 band for males fed with females as for the males fed without females. Some of the gene sequences were not differentially expressed according to the data. Tables 4, 5, 6, 7, 8. and 9 are standard deviation tables containing t-values that were used in characterizing the identified differentially expressed genes for each pair of tick classes that were compared at a given time. In addition, tables 10, 11, 12, 13 and 14 are standard deviation tables for the unidentified genes. The confirmation results agree fairly well with the original gene assignments. This result is confirmed in table 15, for which the t-test analysis and ANOVA procedure was used to show existence of genes whose expression is changed by feeding or females. The gene assignments due to ANOVA and modified t-test analysis are shown in table 15. Tables 16, 17, 18, 19, 20 and 21 are ANOVA tables containing essential values used for the analysis.

Sequences

Of the 62 genes that were isolated, 33 gene sequences had hits (sequence similarity in the DNA database). Thirteen of those gene sequences could be identified as differentially expressed by dot blot analysis. Based on the t-values as we used it, 6 genes were differentially expressed in the salivary glands of *D. andersoni* male ticks fed with females, 2 genes expressed in males fed without females, three genes were expressed in the salivary glands of both males fed with females and males fed without females and 2 gene sequences were present in all three classes. According to ANOVA analysis, 7 genes were differentially expressed in the salivary glands of *D. andersoni* male ticks fed with females, 3 genes were expressed in males fed without females, a single gene was

expressed in both males fed with females and unfed male tick salivary glands and 2 gene sequences were again present in all three classes as in table 18.

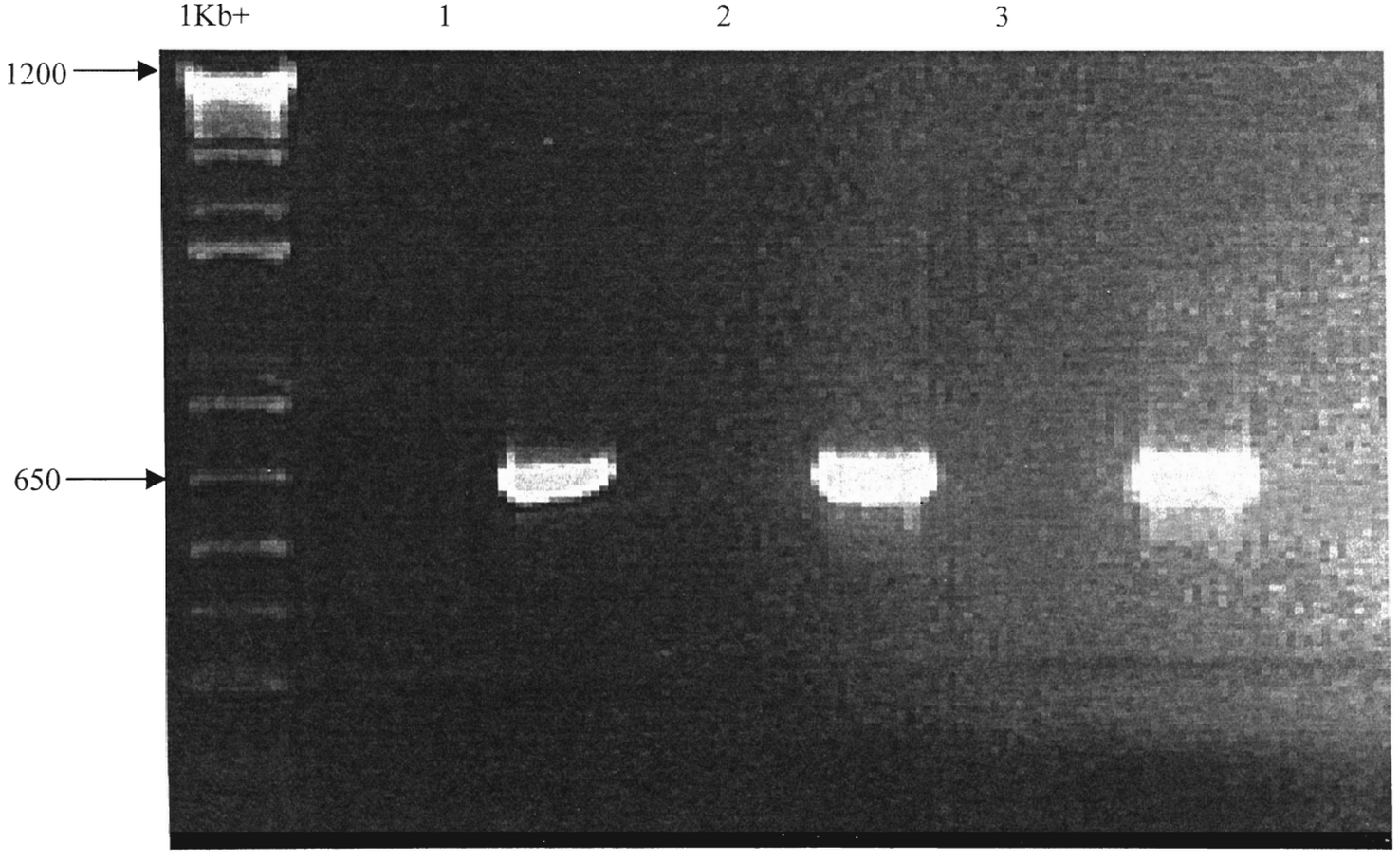

Figure 3. 661bp product of the β-actin gene.

The 661 bp cDNA fragments were synthesized from total RNA isolated from *D. andersoni* tick salivary glands of males fed with females, males fed without females and unfed males using oligo-dT and the control primers for the human β-actin gene. Lane 1=males fed with females, Lane 2 = males fed without females and Lane 3= unfed male *D. andersoni* ticks.

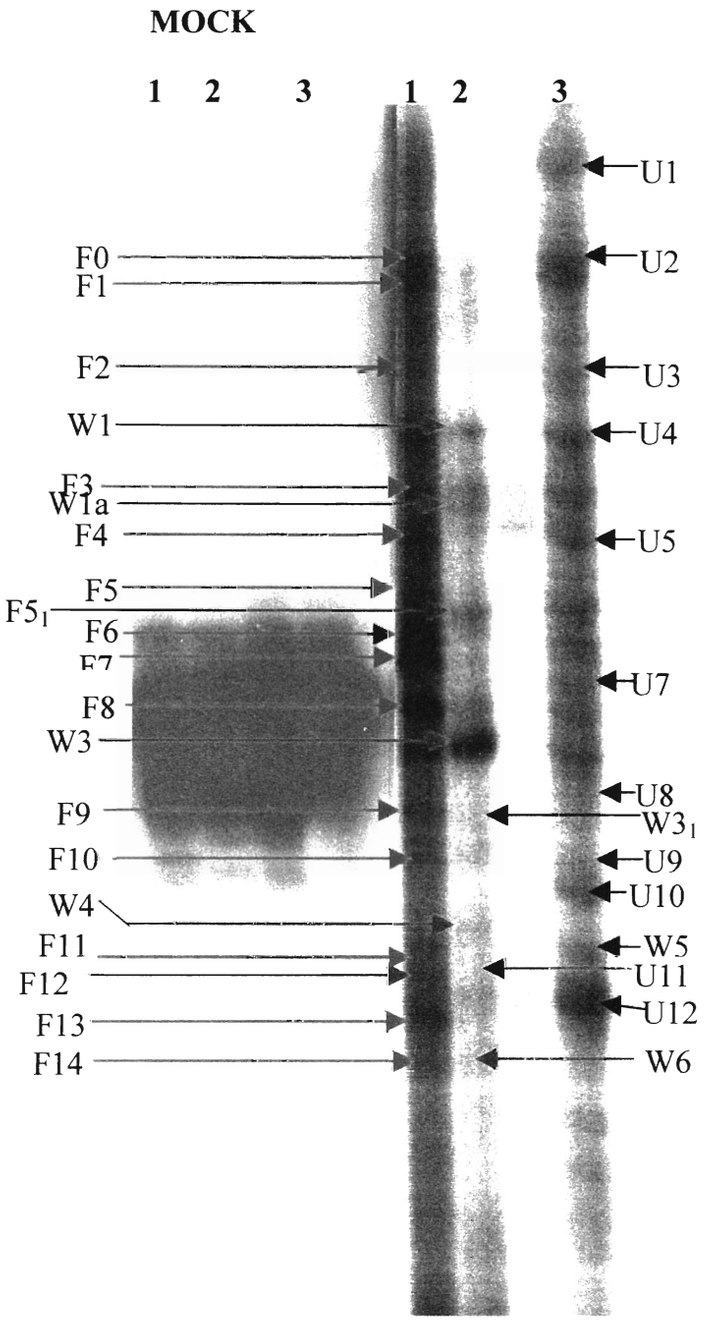

Figure 1. Differential display of cDNAs synthesized from A1+A2 primers.

The cDNAs were synthesized by reverse transcription from total RNA isolated from salivary glands of male *D. andersoni* fed with females, males fed without females and unfed males using the arbitrary primer pair A1+A2. The same primers were used in first strand synthesis and also in second strand synthesis during which the cDNAs were labeled with α^{32} P-dCTP. The mock lanes contain negative controls of cDNA synthesized without RT.
Lane 1= F: *D. andersoni* male ticks fed with females
Lane 2= W: *D. andersoni* male ticks fed without females
Lane 3= U: Unfed male *D. andersoni*

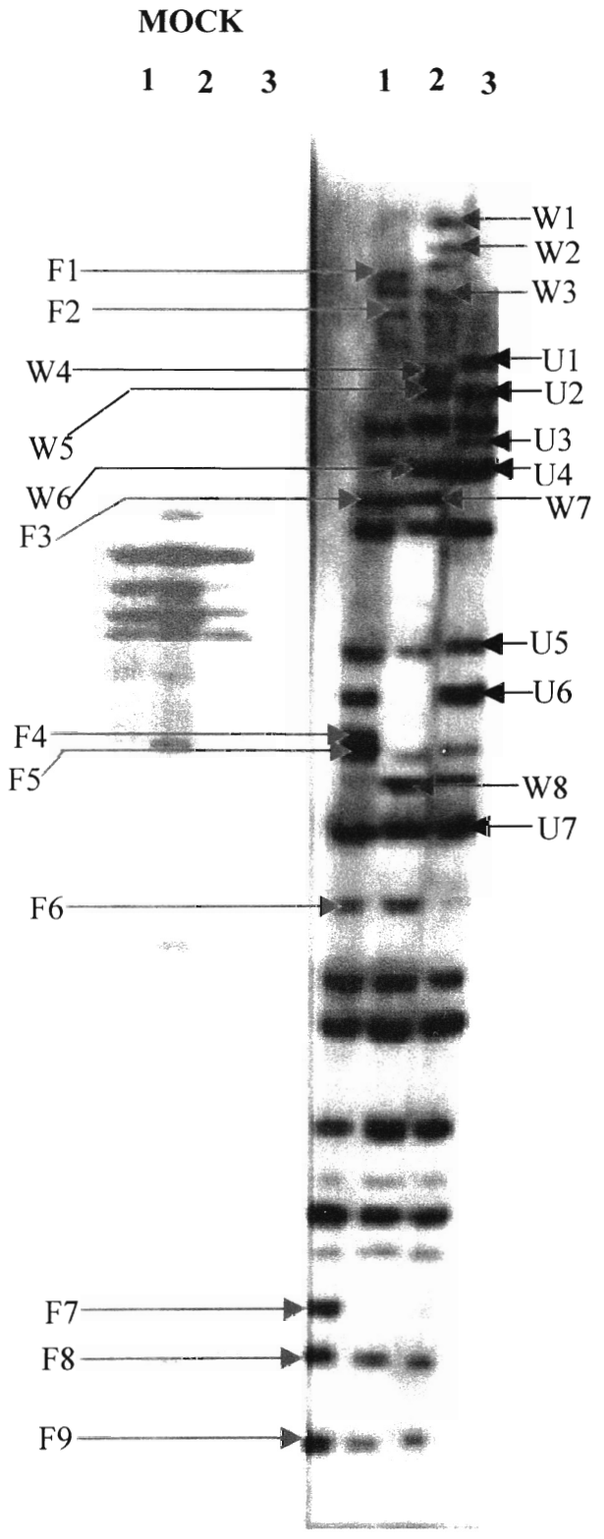

Figure 2. Differential display of cDNAs synthesized from A1+A3. primers. Lanes as in figure 4.

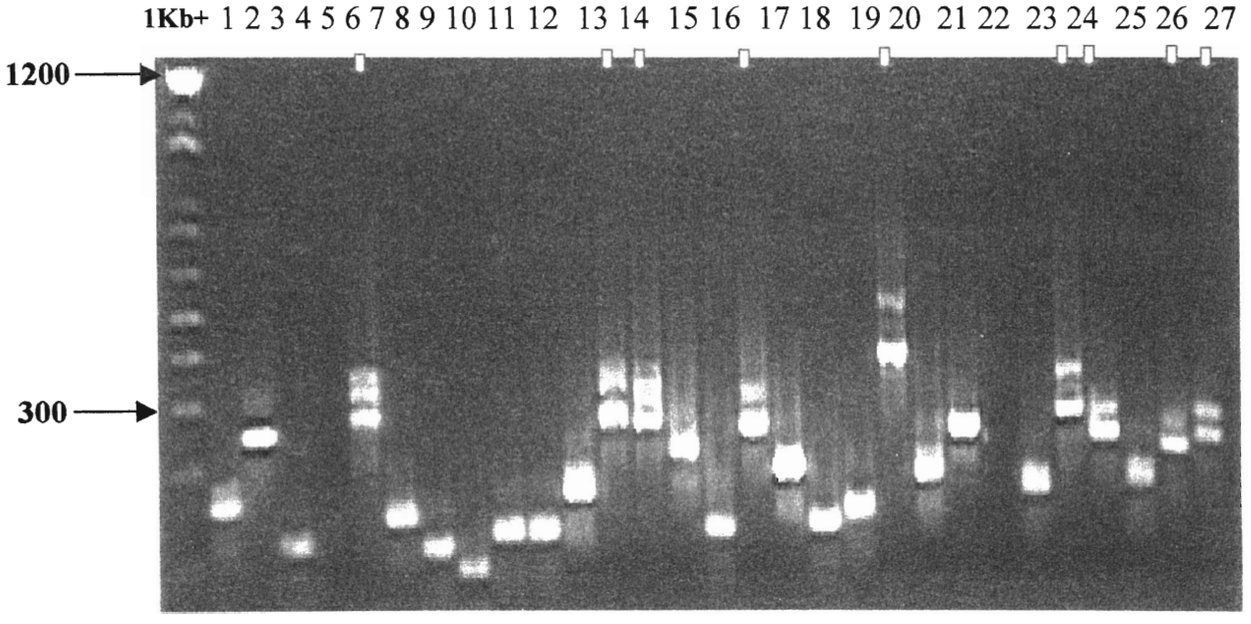

Figure 6. Amplified differentially expressed cDNA fragments from A1+ A2 arbitrary primer pair on 1.5% agarose gel.

The cDNA fragments are differentially expressed cDNAs isolated from the cDNA patterns on the denaturing gel in fig. 4. The cDNA fragments were isolated by physically cutting the bands out from the gel and then extracting the bands using a Qiagen gel extraction kit. The extracted DNAs were amplified using the original arbitrary primer pairs (A1+A2) used in first strand synthesis and then resolved on a 1.5% agarose gel as shown in Figure above. The marked lanes contain single cDNA bands from the denaturing gel that displayed more than one band when resolved on the 1.5% agarose gel. Each band was purified and later cloned for sequencing.

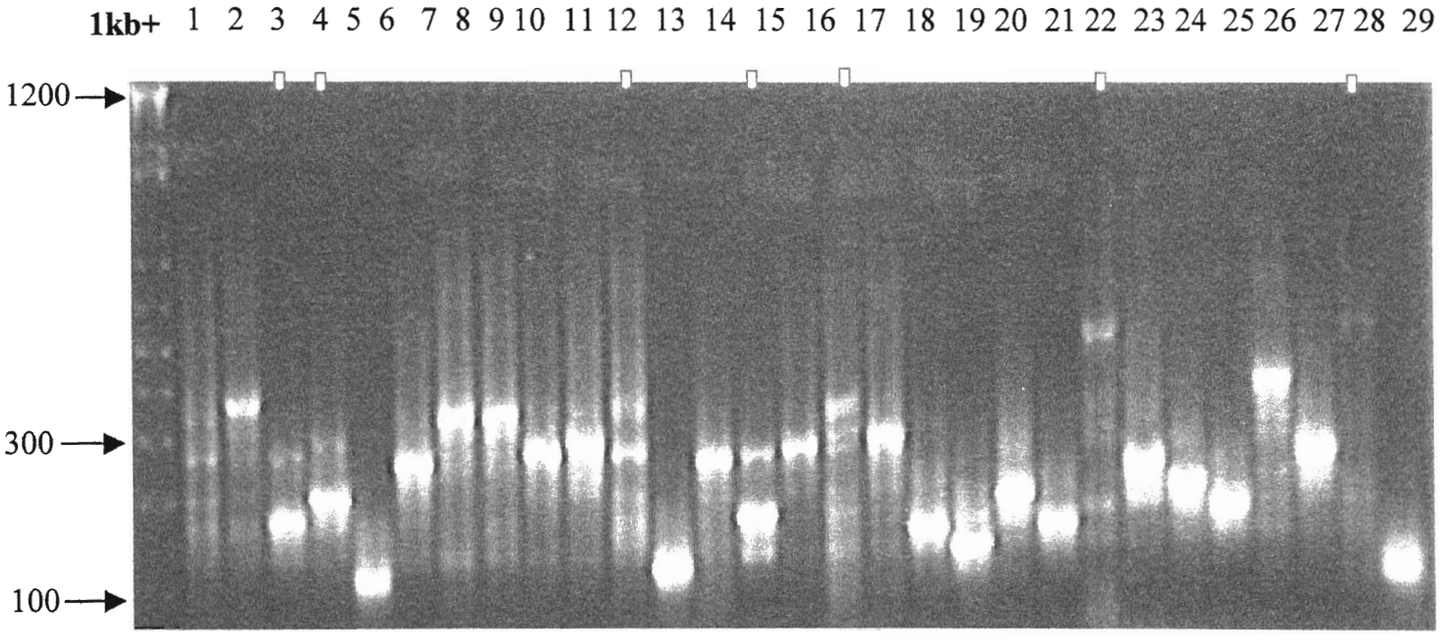

Figure 7. Amplified differentially expressed cDNA fragments from A1+ A3 arbitrary primer pair on 1.5% agarose gel.

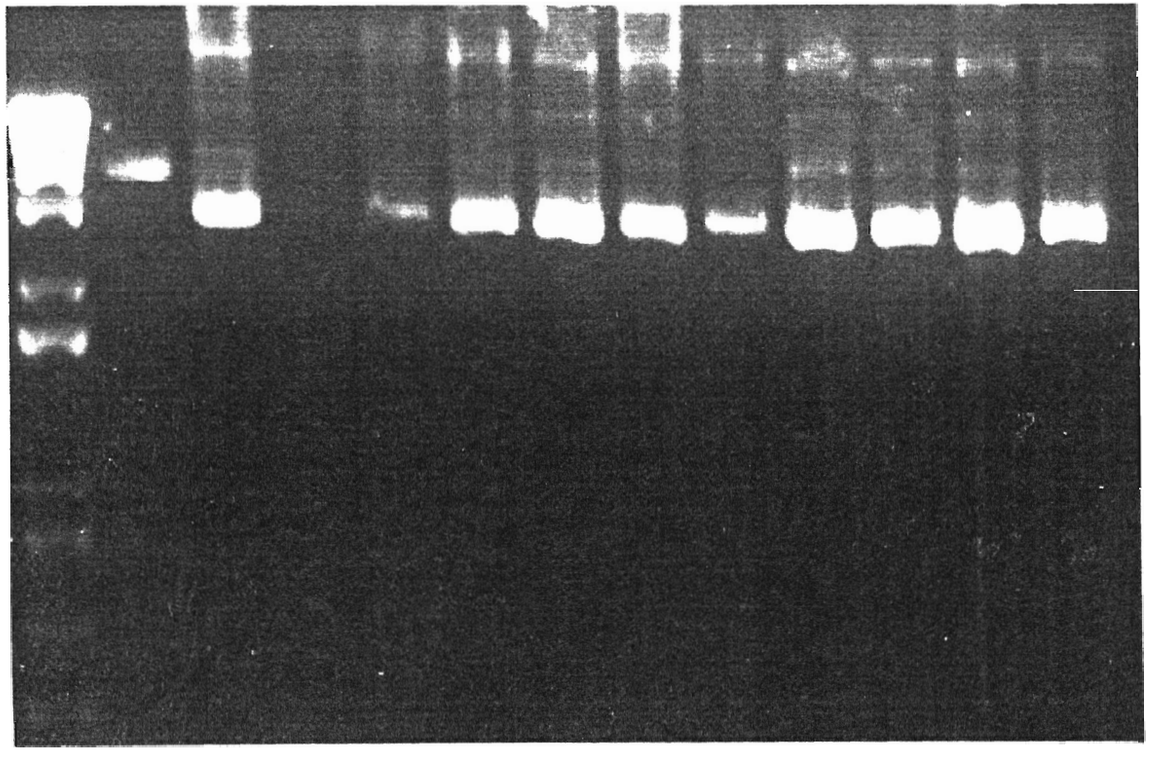

Figure 8. Verification of inserts in plasmids on 1.5% agarose gel.

The inserts in lanes 1 to through 11 are cDNA fragments purified (Qiagen gel extraction kit) from the amplified cDNA fragments on 1.5% agarose gels from figs. 6 and 7. The inserts were each cloned into a pDrive cloning vector, plated on LB plates and screened for white colonies (colonies with inserts). The plasmids containing inserts were further purified and resolved on a 1.5% agarose gel as shown in the figure above. The remaining stocks of the purified inserts were submitted for sequencing. This figure only represents a few random samples of purified plasmids with inserts from A1+ A2 and A1+A3 primer pairs.

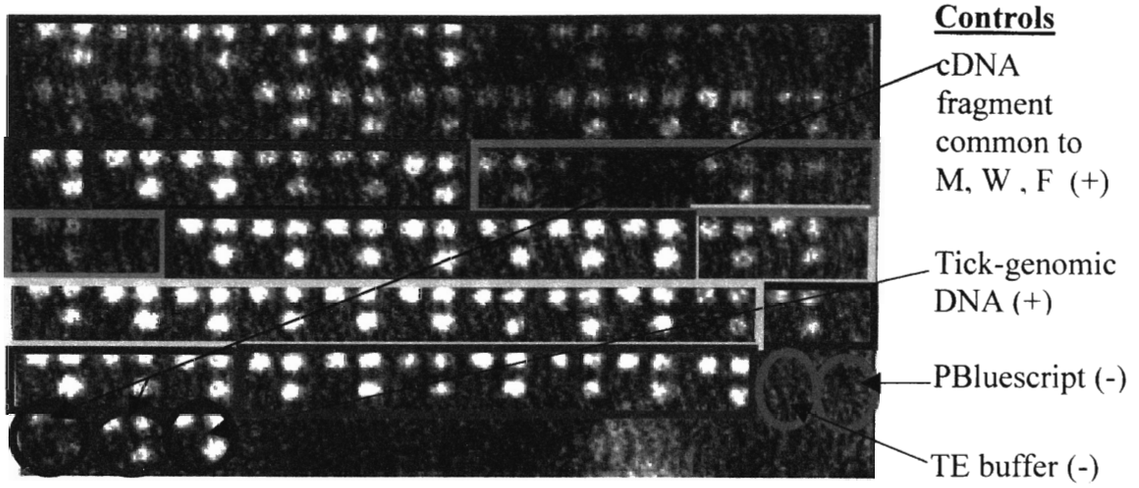

Figure 1. Potentially differentially-expressed cDNA fragments hybridized against cDNA from male ticks fed with females using A1+A3 primers.

The probe was also synthesized from total RNA from salivary glands of male ticks fed with females and labeled with Dig-11dUTP during the PCR reaction. The cDNA probes were visualized by chemiluminescence in a reaction using antidigoxigenin Fab fragments conjugated to alkaline phosphatase.
There are two red circles, one of which is the control vector and the other is TE buffer (both are negative controls). The three dark green circles represent positive controls, one of which is tick genomic cDNA and the others are cDNA fragments common to all three classes (M, W, F) of ticks. The area enclosed by the blue rectangle represents dot blots of cDNAs from the salivary glands of *D. andersoni* male ticks fed with females using A1+A3 primers. Other areas enclosed by the rectangle are as follows.
Green rectangle: Dot blots of cDNAs from the salivary glands of *D. andersoni* male ticks fed with females from A1+A3 primers.
Yellow rectangle: Dot blot of cDNA's from the salivary glands of *D. andersoni* male ticks fed without females from A1+A3primers.
Black rectangle: Dot blot of cDNA's from the salivary glands of *D. andersoni* male ticks fed without females from A1+A2 primers.
Violet rectangle: Dot blot of cDNA's from the salivary glands of unfed *D. andersoni* male ticks from A1+A3 primers.
Orange rectangle: Dot blot of cDNA's from the salivary glands of unfed *D. andersoni* male ticks from A1+A2 primers.

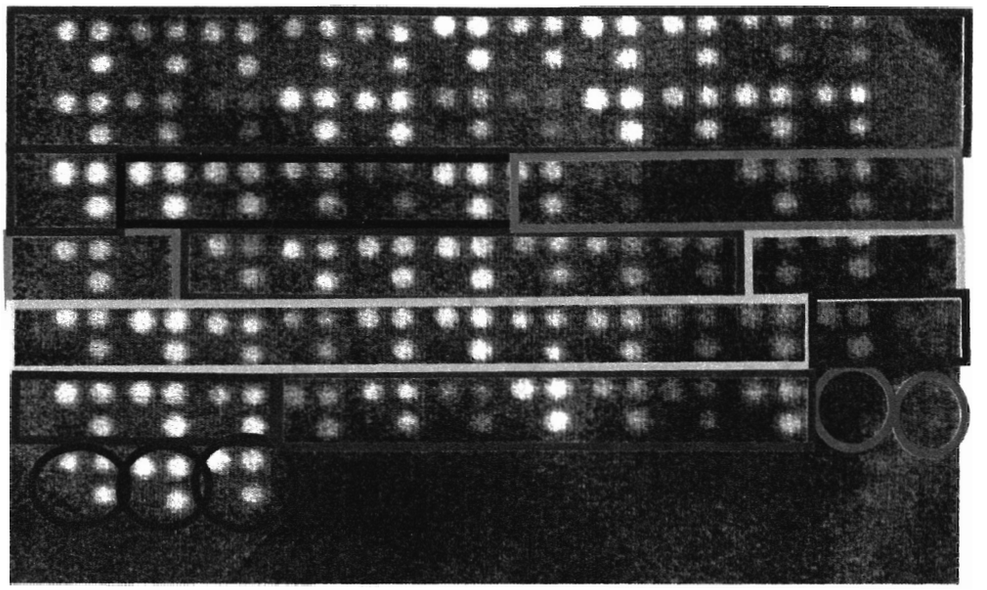

Figure 2. Potentially differentially expressed cDNA fragments hybridized against cDNA probe made from the salivary glands of *D. andersoni* male ticks fed with females using A1+A2 primers.

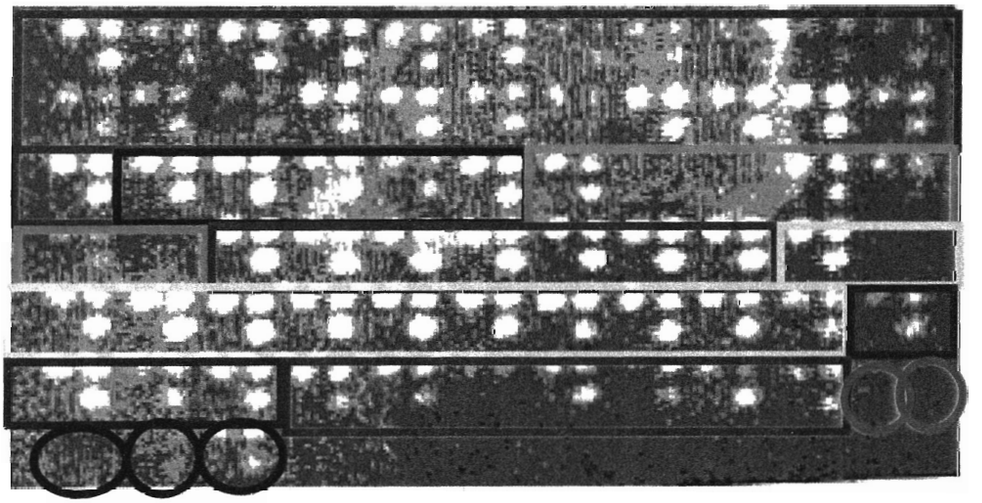

Figure 3. Potentially differentially expressed cDNA fragments hybridized against cDNA probe made from the salivary glands of *D. andersoni* male ticks fed without females using A1+A3 primers.

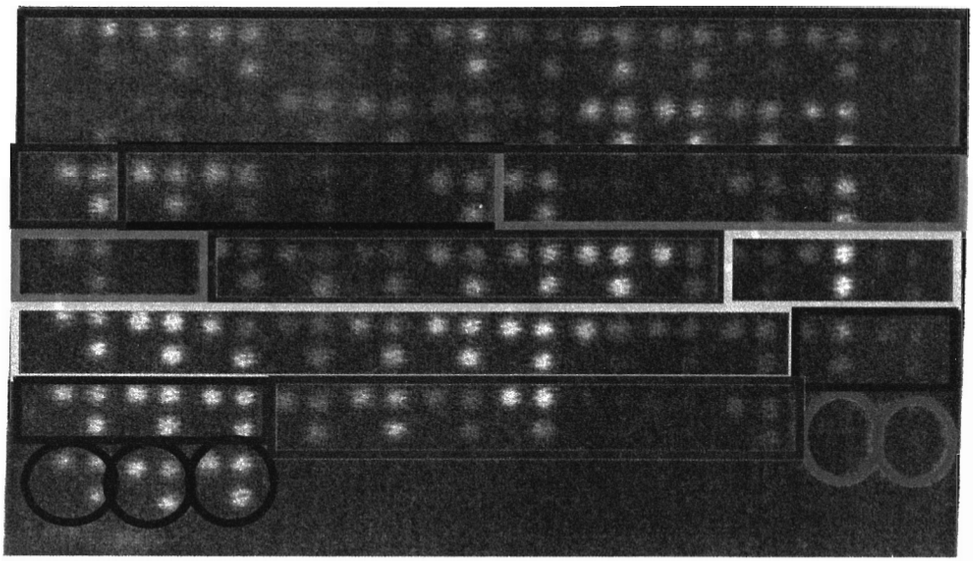

Figure 4. Potentially differentially expressed cDNA fragments hybridized against cDNA probe made from total RNA from the salivary glands of *D. andersoni* male ticks fed without females using A1+A2 primers.

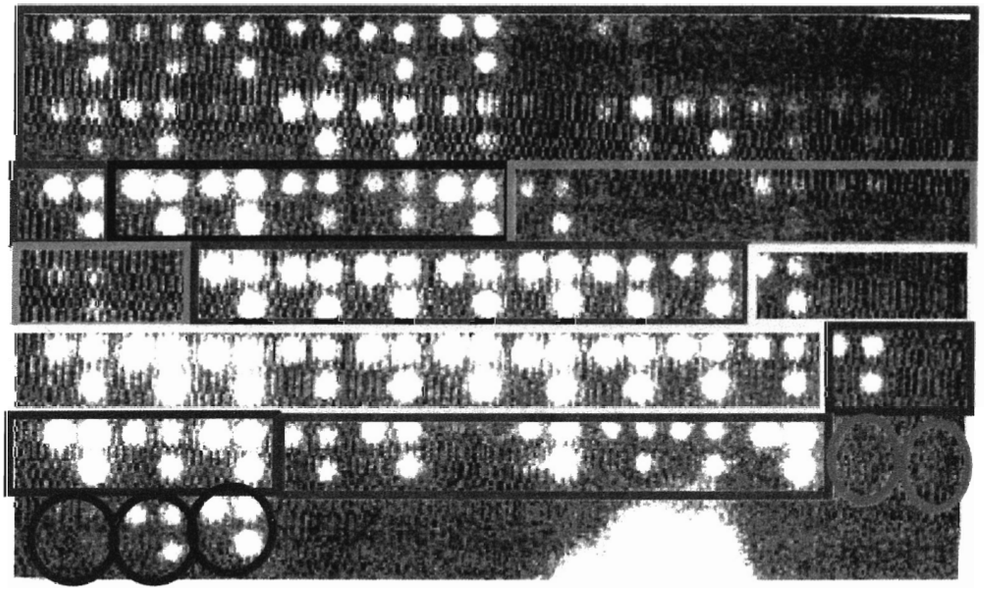

Figure 5. Potentially differentially expressed cDNA fragments hybridized against cDNA probe made from total RNA from the salivary glands of unfed *D. andersoni* male ticks using A1+A3 primers.

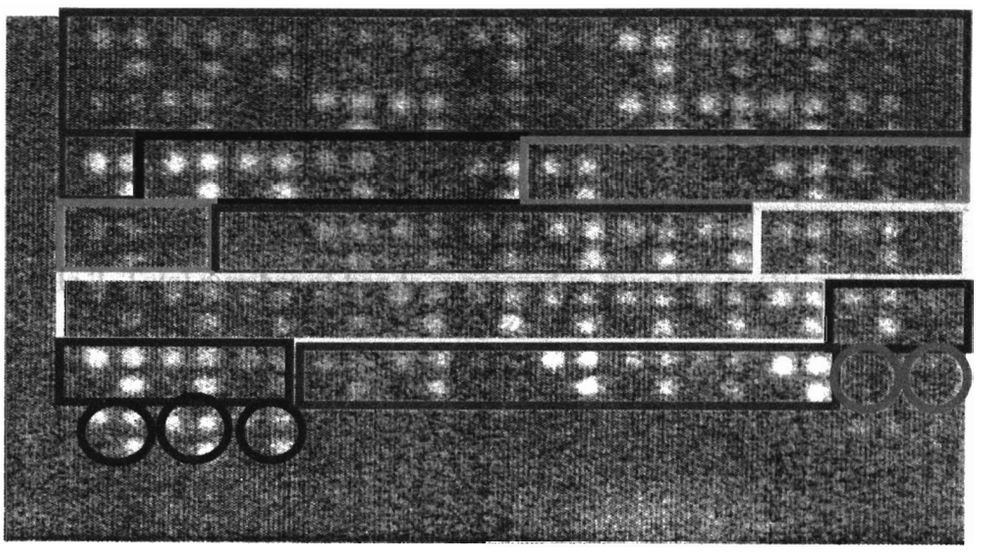

Figure 6. Potentially differentially expressed cDNA fragments hybridized against cDNA probe made from total RNA from the salivary glands of unfed *D. andersoni* male ticks using A1+A2 primers.

Table 1. Total number of differentially expressed bands from differential display expressed in only one class (F or W or U).
These bands (cDNA fragments) were synthesized from A1+A2 and A1+A3 primer pairs and are unique to one of three groups (males fed with females, males fed without females and unfed male ticks).

Band origin	A1+ A2	A1+ A3
F1, F3, F4, F5, F6, F7, F8, F9, F11, F13, F14, W2, W4, W5, U7, U8, U10, F1, F4, F7, W1, W2, W4, U2, U3	17	8

Table 2. Total number of bands expressed in two classes (W and F, W and U or F and U).

	Band origin	A1+A2	A1+A3
F+W	F1, F14, F2, F3. W3, W6, W8, W7.	4	4
F+U	F3, F5. U5, U4, U1, U6.	2	4
W+U	W1, W2, W6, W5.	2	2

Table 3. Total number of bands common to all three groups of male ticks (F, W, U) from differential display in figures 5 and 6.

A1+ A2	A1+ A3
5	13

Table 4. Values of normalized intensities of blots from MFWF3 and UF3, logarithms of ratios of the normalized intensities, the standard deviation (Sd= 0.6188), and t-values used in characterizing genes as being expressed in MFWF3 or UF3: n= 96.

Gene name/ origin	P_{MFWF3}	P_{UF3}	P_{MFWF3}/P_{UF3}= (M)	Log. M	t
Putative senescence associated protein (F1)	0.0359	0.0157	2.2960	0.3610	5.7156
Putative senescence associated protein (F7)	1.4702	1.2292	1.1961	0.0778	1.2313
Elongation factor-2 (U3)	1.3263	0.7688	1.7272	0.2374	3.7582
Guanine nucleotide regulatory protein (W2)	1.3672	0.9128	1.4978	0.1755	2.7782

Table 5. Values of normalized intensities of blots from MFWOF3 and MFWF3, logarithms of ratios of the normalized intensities, the standard deviation (Sd= 0.5227) and t-values used in characterizing genes as being expressed in MFWOF3 or MFWF3: n= 96.

Gene name/ origin	P_{MFWF3}	P_{MFWOF3}	P_{MFWOF3}/P_{MFWF3} = (M)	Log. M	t
gD glycoprotein (W1)	1.3089	4.6623	3.5619	0.5517	10.3416
rRNA intron encoded homing endonuclease (F2)	1.4430	4.1104	2.8486	0.4546	8.5225

Table 6. Values of normalized intensities of blots from MFWF2 and UF2, logarithms of ratios of the normalized intensities, the standard deviation (Sd= 0.5766) and t-values used in characterizing genes as being expressed in MFWF2 or UF2: n= 96.

Gene name/ origin	P_{MFWF2}	P_{UF2}	P_{MFWF2}/P_{UF2} = (M)	Log. M	t
XP_O69680.1 hypothetical protein (F3)	0.1027	0.0068	14.9876	1.1757	19.9781
rRNA intron encoded homing endonuclease (U2)	0.8460	0.2740	3.0880	0.4897	8.3205
Eukaryotic polypeptide chain release factor 3 (F2)	0.3593	0.1301	2.7609	0.4410	7.4943

Table 7. Values of normalized intensities of blots from MFWF2 and MFWOF2' logarithms of ratios of the normalized intensities, the standard deviation (Sd= 0.4487) and t-values used in characterizing genes as being expressed in MFWF2 or MFWOF2: n= 96.

Gene name/ origin	P_{MFWF2}	P_{MFWOF2}			
Septin (F2)	1.1316	3.7439	2.8441	0.4539	8.5095

Table 8. Values of normalized intensities of blots from MFWOF2 and UF2, logarithms of ratios of the normalized intensities, the standard deviation (Sd= (0.6436) and t-values used in characterizing genes as being expressed in MFWF2 or MFWO2: n= 96

Gene name/ origin	P_{MFWOF2}	P_{UF2}	P_{MFWOF2}/ P_{UF2} = (M)	Log. M	t
CG10925 gene product (U7)	0.7391	0.5616	1.1316	0.1193	1.8157
Utrophin (U2)	0.2019	0.0068	29.4721	1.4694	22.3712

Table 9. Values of normalized intensities of blots from MFWOF3 and UF3, logarithms of ratios of the normalized intensities, the standard deviation (Sd= (0.8585) and t-values used in characterizing genes as being expressed in MFWFO3 or UF3: n= 96.

Gene name/ origin	P_{UF3}	P_{MFWOF3}	P_{MFWF3}/P_{UF3} = (M)	Log. M	t
Hypothetical protein XP_0706536 (F9)	1.3672	0.9128	2.7542	0.4399	5.0214

Table 10. Values of normalized intensities of blots from MFWF2 and MFWOF2, logarithms of ratios of the normalized intensities, standard deviation (Sd= 0.4487), and t values for unidentified genes: n= 96.

Gene origin	P_{MFWF2}	P_{MFWOF2}	$P_{MFWOF2}/ P_{MFWOF2}= M$	Log M	t
	0.7717	0.3929	0.5091	-0.2932	-6.4019
F1a	0.4832	0.6957	1.4397	0.1583	3.4559
F1b	0.7982	0.2019	0.2529	-0.5971	-13.0367
F0	1.4779	0.6367	0.4308	-0.3657	-7.9857
F5	0.8460	0.2531	0.2992	-0.5241	-11.4429
F2C	0.4743	0.3804	0.8020	-0.0958	-2.0919
F12	0.3592	0.4736	1.3182	0.1199	2.6194
F15	0.1558	0.1584	1.0169	0.0073	0.1589
F9	0.2885	0.2065	0.7159	-0.1452	-3.1698
F8	1.4389	0.8913	0.6194	-0.2080	-4.5419
F6	0.6460	0.7205	1.1153	0.0474	1.0347

Table 11. Values of normalized intensities of blots from MFWOF2 and UF2, logarithms of ratios of the normalized intensities, standard deviation (Sd= 0.6463), and t values for unidentified genes: n= 96.

	P_{MFWOF2}	P_{UF2}	$P_{MFWOF2}/ P_{UF2}= M$	Log M	t
W5	0.9689	2.6369	0.3674	-0.4348	-6.6198
W4	0.5109	1.3356	0.3825	-0.4173	-6.3543
W2	0.6086	1.6849	0.3613	-0.4422	-6.7321
W3a	0.1584	0.8630	0.1835	-0.7363	-11.2099
W6a	1.0823	0.9178	1.1792	0.0716	1.0900
W6b	0.2096	0.0342	6.1211	0.7868	11.9792

Table 12. Values of normalized intensities of blots from MFWOF3 and UF3, logarithms of ratios of the normalized intensities, standard deviation (Sd= 0.8585), and t values for unidentified genes: n= 96.

	P_{MFWOF3}	P_{UF3}	$P_{MFWOF3}/ P_{UF3}= M$	Log M	t
W9a	2.2472	0.9305	2.4152	0.3829	4.3705
W9b	3.4238	1.7277	1.9817	0.2970	3.3900
W1c	3.7042	0.7679	4.8239	0.6834	7.7994
W7	3.5033	1.2126	2.8892	0.4608	5.2587
W6a	2.5101	1.0617	2.6468	0.4227	4.8244
W6b	1.5408	0.5779	2.6664	0.4259	4.8609
W4	1.9470	1.1548	1.6861	0.2269	2.5893
W3b	1.3333	0.3976	3.3530	0.5254	5.9966
W4	1.4857	0.6846	2.1700	0.3364	3.8399
W2a	0.9735	0.9931	0.9802	-0.0087	-0.0989
W7	0.5607	0.3869	1.4493	0.1611	1.8393
W2b	0.9426	1.3604	0.6929	-0.1593	-1.8186

Table 13. Values of normalized intensities of blots from MFWF3 and MFWOF3, logarithms of ratios of the normalized intensities, standard deviation (Sd= 0.7875), and t values for unidentified genes: n= 96.

Gene origin	P_{MFWF3}	P_{MFWOF3}	$P_{MFWOF3}/ P_{MFWF3}= M$	Log M	t
F3	1.6569	1.3333	0.8407	-0.0944	-1.1743
F6	0.9683	1.4857	1.5342	0.1859	2.3128
F5	1.7994	0.9735	0.5409	-0.2668	-3.3195
F7a	0.4829	0.5607	1.1612	0.0649	0.8078
F2	2.1689	0.9426	0.4346	-0.3619	-4.5027

Table 14. Values of normalized intensities of blots from MFWF2 and MFWOF2, logarithms of ratios of the normalized intensities, standard deviation (Sd= 0.5766), and t values for unidentified genes: n= 96.

Gene origin	P_{MFWF2}	P_{UF2}	$P_{MFWF2}/ P_{UF2}= M$	Log M	t
U2	0.6885	1.6849	0.4086	-0.3887	-6.6045
U4a	0.0195	0.0205	0.9475	-0.0234	-0.3980
U5	0.9416	0.3219	2.9249	0.4661	7.9203
U4b	0.0796	0.0137	5.8142	0.7645	12.9902

Table 1. Summary of similarity of genes.

The following defined symbols would be used through out the tables.

+ Means gene is differentially expressed in a given stage,

- Means gene is not differentially expressed at that stage

Gene origin	Primer pair used in synthesizing cDNA	Sequence description from top alignment	GenBank accession number	F	W	U	F	W	U	Score	P-value
F1	A1+A2	Putative senescence – associated protein [*Pisum sativum*]	gi| 13359451	+	+	-	+	+	-	335.0	3e-31
F2	A1+A2	Eukaryotic polypeptide chain release factor 3 [*Oryctalogus cuniculus*]	gi| 707714	+	-	-	+	-	-	196.0	6e-15
W2	A1+A2	(AF263243) SocE [*Myxococcus xanthus*]	gi| 9652070	+	-	-	+	-	-	104.0	1e-3
F7	A1+A2	Putative senescence-associated protein [*Pisum sativum*]	gi| 13359443	+	-	-	+	-	-	168.0	5e-12
U2	A1+A2	NP_037202.1| **Utrophin** (homologous to dystrophin) [*Rattus norregicus*]	gi| 6981696	+	+	-	-	+	-	162.0	4e-11

Table 1. Summary of similarity of genes.

The following defined symbols would be used through out the tables.

+ Means gene is differentially expressed in a given stage,

- Means gene is not differentially expressed at that stage

Gene origin	Primer pair used in synthesizing cDNA	Sequence description from top alignment	GenBank accession number	F	W	U	F	W	U	Score	P-value
F1	A1+A2	Putative senescence-associated protein [*Pisum sativum*]	gi| 13359451	+	+	-	+	-	-	335.0	3e-31
F2	A1+A2	Eukaryotic polypeptide chain release factor 3 [*Oryctalogus cuniculus*]	gi| 707714	+	-	-	+	-	-	196.0	6e-15
W2	A1+A2	(AF263243) SocE [*Myxococcus xanthus*]	gi| 9652070	+	-	-	+	-	-	104.0	1e-3
F7	A1+A2	Putative senescence-associated protein [*Pisum sativum*]	gi| 13359443	+	-	-	+	-	-	168.0	5e-12
U2	A1+A2	NP_037202.1| **Utrophin** (homologous to dystrophin) [*Rattus norregicus*]	gi| 6981696	+	+	-	+	+	-	162.0	4e-11

F3	A1+A2	XP_06968.1 (XM_09680.1) hypothetical protein XP_069680 [*Homo sapiens*]	gi| 1746575213	+	-	-	+	-	-	117.0	5e-5
U3	A1+A2	(AF240821) elongation factor 2 [*Limulus polyphemus*]	gi| 13111498	+	+	-	-	+	+	154.0	2e-10
F2	A1+A3	NP_ component of 10 nm mother-bud neck; CDC 11p (*Saccharomyces cerevisiae)* gi| 416760| sp| P324581 CC11_Yeast cell division control protein 11 gi| 482158| pir| S40911 **Septin** CDC 11-Yeast (*Saccharomyces cerevisiae)*	gi| 6322536	+	-	-	+	-	-	178.0	3e-12
U2	A1+A3	rRNA intron homing endonuclease [*Oryza sativa*]	gi| 13171103	-	+	-	-	-	+	190.0	4e-14
U1	A1+A3	gD glycoprotein [*BABOON herpesvirus*]	gi| 559564	+	-	-	+	-	-	113.0	1e-4
F9	A1+A3	XP_070653| (XM_070653) hypothetical protein [*Homo sapiens*]	gi| 1746752	-	+	-	-	+	-	142	6e-9
F1	A1+A2	Putative senescence associated protein [*Pisum sativum*]	gi| 13359443	+	+	+	+	-	-	330.0	1e-30
U8	A1+A2	CG10915 gene product [*Drosophila melanogaster*]	gi| 7302657	+	+	+	+	+	+	214.0	3e-17

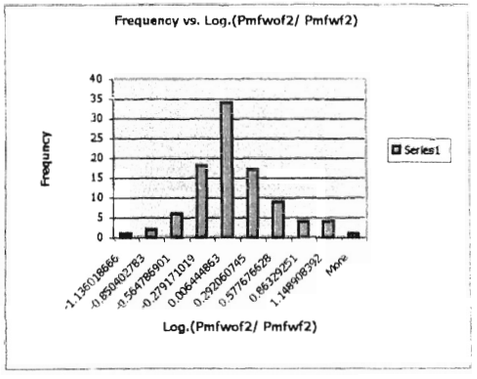

Figure 15 A.

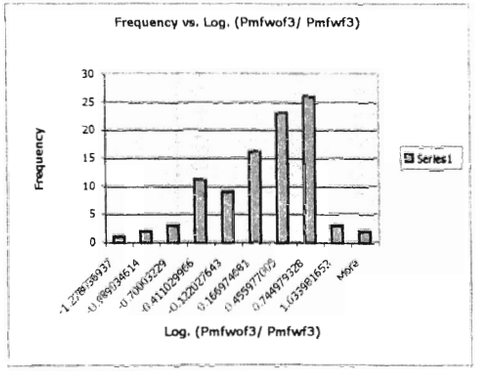

Figure 15 B.

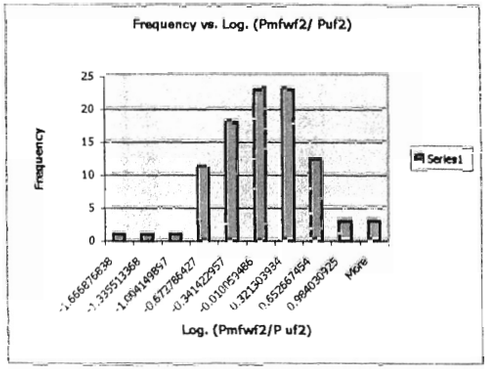

Figure 15 C.

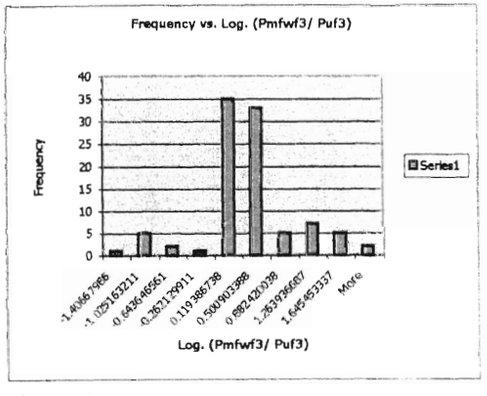

Figure 15 D.

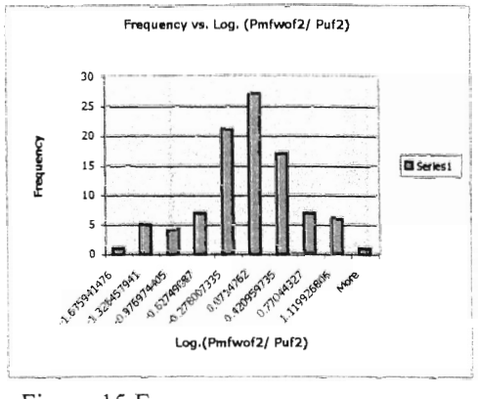

Figure 15 E.

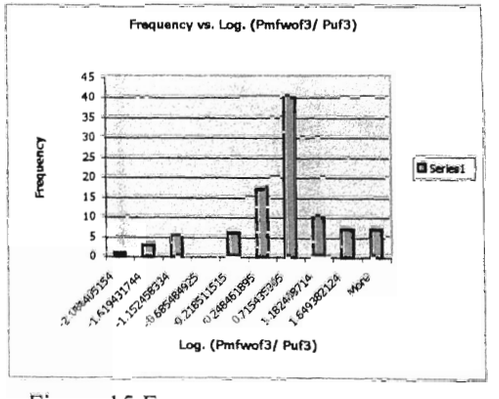

Figure 15 F.

Figure 15. A, B, C, D, E, and F are plots of frequencies vs. the logarithms of the ratios of the normalized dot blot intensities of cDNAs from the salivary glands of two groups of *D. andersoni* male ticks synthesized with A1+A2 or A1+A3 primers. P_{mfwf3}, P_{mfwf2}, P_{mfwof3}, P_{mfwof2} and P_{uf3} and P_{uf2} are defined in chapter three.

Table 16. Analysis of variance table for average intensities of spot per clone from the salivary glands of males *D. andersoni* ticks fed with females from A1+A2 primers.

ANOVA			**MFWF 2**			
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	48.7909046	75	0.65054539	61.9270592	1.8151E-85	1.37612233
Within Groups	1.59676402	152	0.01050503			
Total	50.3876686	227				

Table 17. Analysis of variance table for average intensities of spot per clone from the salivary glands of males *D. andersoni* ticks fed with females from A1+A3 primers.

ANOVA		**MFWF3**				
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	44.3193694	75	0.59092493	102.3139	1.991E-101	1.37612233
Within Groups	0.87789233	152	0.00577561			
Total	45.1972617	227				

Table 18. Analysis of variance table for average intensities of spot per clone from the salivary glands of males *D. andersoni* ticks fed without females from A1+A2 primers.

ANOVA		**MFWOF 2**				
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	39.7925567	75	0.53056742	24.8871133	1.2775E-57	1.37612233
Within Groups	3.24048222	152	0.02131896			
Total	43.0330389	227				

Table 19. Analysis of variance table for average intensities of spot per clone from the salivary glands of males *D. andersoni* ticks fed with females from A1+A3 primers.

ANOVA		MFWOF3				
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	78.1765177	75	1.04235357	58.2729859	1.4782E-83	1.37612233
Within Groups	2.71888835	152	0.01788742			
Total	80.8954061	227				

Table 20. Analysis of variance table for average intensities of spot per clone from the salivary glands of unfed male *D. andersoni* ticks from A1+A2 primers.

ANOVA		**UF2**				
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	97.7973783	75	1.30396504	140.781598	1.056E-111	1.37612233
Within Groups	1.40787354	152	0.00926233			
Total	99.2052518	227				

Table 21. Analysis of variance table for average intensities of spot per clone from the salivary glands of males *D. andersoni* ticks fed with females from A1+A3 primers.

ANOVA		**UF3**				
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	154.168429	75	2.05557905	123.067431	2.306E-107	1.37612233
Within Groups	2.53883593	152	0.01670287			
Total	156.707265	227				

CHAPTER FOUR

DISCUSSION

The most widely used computational approach for analyzing data for differentially expressed genes is cluster analysis. Cluster analysis however, is not a suitable approach for this study because it focuses on group similarities instead of differences within each individual gene (49). The t-test as used in this experiment and analysis of variance (ANOVA) procedure using lsd are other procedures most often used to characterize differentially expressed genes. The ANOVA analysis as used in this study is like the normal t-test. This test "assumes normality and constant variance for every gene across all samples" which is not appropriate for a subset of genes regardless of any transformation (49). In other words, ANOVA requires the variance to be equal, while the t-statistic as used does not (though it assumes a normal distribution). The ANOVA procedure however, accounts for differences within a set of replicated data (as in the case of dot blots) and also between samples (49). Both methods are appropriate for comparing two treatments simultaneously but I favor results produced by the ANOVA procedure for the following reasons. Despite the efficiency of t-values when variances are assumed to be equal, the t-values yielded slightly different test statistic and ordering than the ANOVA procedure. As for ANOVA, the larger the sample size, the more reliable the information produced and the error rate is reduced assuming the assumptions are true. If the sample sizes are large enough, even small differences in means may be significant. If I should repeat this experiment, I will increase the sample size by performing several replicate blots. The replicates will include more blots with the same RNA -probes used initially. I may also try some new probes. Increasing the sample size may reduce the

error rate significantly and thus increase the accuracy of characterizing the differentially expressed genes. The genes characterized by the ANOVA procedure for this study are therefore considered to be the legitimate differentially expressed genes and will be discussed.

It has been shown that that tick feeding stimulates differential gene expression in developing salivary glands of ticks (32). In addition to new gene expression, protein changes also occur in the tick salivary glands during feeding (44). Results from other experiments indicate that mating in females stimulates additional feeding and thus an increase in the amount of polypeptide detected in the tick salivary glands (29). Even though differential gene expression has been better studied in male ticks than in females, little is known about the effect of feeding and females on male *D.andersoni* ticks. Results from this study indicate that new gene expression occurs in the salivary glands of male *D. andersoni* ticks when fed, and an additional increase in gene expression when fed with females. Some of the sequenced genes that may be important in tick feeding include a gene for septin CDC 11, a utrophin gene and two G-proteins, eukaryotic polypeptide chain release factor 3 (eRF3) and elongation factor 2. The septins are a highly conserved family of membrane-associated GTPases that were first identified in yeast and have recently been identified in other animals (52). Originally, septins were only known to be involved in yeast cell division; however, recent studies show other roles of septins in mammalian cells (52). Some functions of septins that may have some correlation with tick feeding and females are as follows. The c-terminal of the Afr 1 p protein interacts with CDC 12 septin; this Afr 1 p protein is induced by sex hormones and is known to negatively regulate pheromone receptor signaling (52). The interaction of a

pheromone induced protein with septin CDC 12 is likely to have a correlation with feeding with females. Septins are known to interact with several other proteins, whose binding mechanisms are yet to be studied. In a recent study, it has been shown that septins contain a binding motif that binds to phosphatidyl inositol. Septins bind directly to the SNARE protein syntaxin to regulate vesicle dynamics (3). Syntaxin is part of a machinery that has been thought to regulate fusion of vesicles to their target membrane, and is also a major part of the SNARE hypothesis. According to the hypothesis, septins bind to syntaxin and also to SNARE proteins found in both the vesicles and plasma membranes. This could mean that the effect of septins on secretion may be due to their ability to sequester vesicles to the membrane and also towards each other thus moving the vesicles or components involved towards the outside of the cell. The major role of septins may be to keep vesicle concentration stable near their site of release to optimize spatial and temporal control (52). By being able to interact in the way described, the role of septins in ticks may be to move necessary proteins and maybe small molecules like transmitters into specific sites in ticks during feeding. It is important to mention that the septin gene was differentially expressed only in male *D. andersoni* ticks fed with females. Utrophin is another important gene identified in this study, however there is no study correlating the utrophin gene with tick feeding. The utrophin gene is a 400kD protein that is similar to dystrophin protein and they both share a 73% amino acid sequence similarity (26). Dystrophin is the protein product of Duchenne muscular dystrophy (DMD) gene. Because of the high similarity between dystrophin and utrophin (16), dystrophin can be replaced by utrophin in muscle deficient in dystrophin in humans (6). Live muscles deficient in dystrophin lose their stiffness (34), meaning that

dystrophin maintains the rigidity of muscles. Dystrophin in muscle cells is for maintaining structural support and strengthening of the muscle cells (8). In the absence of dystrophin, the cell membrane of muscles becomes permeable thus allowing components from outside the cell to enter the cell; this causes pressure in the muscle cells to increase until the muscle cell bursts and dies. Utrophin is usually found at crests of neuromuscular junctional folds together with acetylcholine receptors, whereas dystrophin is localized deep in the folds (11). Utrophin has also been identified in normal mouse brain, kidney, spleen, liver, and lung and mRNA has been isolated from human placenta, liver, smooth intestines and adult and foetal skeletal muscles (50). In this study, the utrophin gene was differentially expressed in the salivary glands of male *D. andersoni* ticks fed with females and without females. The effect of utrophin on male tick feeding is unknown, however, since this gene was expressed in only the feeding male ticks I speculate that it may have something to do with depleting muscles around the area of attachment, thus permeabilizing the host muscle at the site of tick feeding to probably aid in feeding and maybe pathogen transmission without eliciting host response. G-proteins have been so named because they bind guanosine triphosphates (GTP) (12). Several kinds of G-proteins exist and each is activated by specific receptors and in turn stimulates specific systems (12). Elongation factor 2, a member of the G-protein superfamily is mainly involved in protein synthesis and also serves as a catalyst for ribosome movement along mRNA in a reaction driven by GTP hydrolysis (37). A study by Qian et al.(36) show that dopamine stimulated adenylate cyclase turns on fluid secretion the salivary glands of *Amblyomma americanum* ticks (36). The dopamine receptors interact indirectly

through specific monomeric G-proteins to stimulate fluid secretion in the salivary glands. G-proteins may also play a critical part in expression of genes during tick feeding.

CONCLUSION

Based on the differential display, I have evidence that feeding and females affect gene expression in male *D. andersoni* ticks. Figures 4 and 5 show genes that are uniquely expressed in one of the three groups of male *D. andersoni* ticks. Among the gene sequences obtained for *D. andersoni* are utrophin and a septin gene. Since these genes are turned on in response to feeding (and maybe mating), one would expect they have important functions in the tick in these conditions however the sequences do not give us much hint what their function is or why it is important. The expressed genes that were isolated, cloned and sequenced did originate from the salivary glands of the three groups of ticks (MFWF, MFWOF, UF). This observation was confirmed by hybridization of probes made from total RNA that was isolated from the male *D. andersoni* ticks fed with females, males fed without females and males fed with females. In addition to identifying differentially expressed genes such as mentioned above, I have shown that differentially expressed genes in the salivary glands of male *D. andersoni* ticks show a significant difference between fed males and unfed males and also between males fed with females and males fed without females. A septin gene was found to be expressed in the salivary glands of feeding males which were not expressed in the unfed male; A utrophin gene was expressed in the salivary glands of males fed with females and in the salivary glands of males fed without females. A gene for eukaryotic polypeptide chain release factor 3 was expressed in the salivary glands of male *D. andersoni* ticks fed with females while an elongation factor 2 gene was expressed in both males fed with females and males fed without females. Genes were uniquely expressed in the salivary glands of the feeding male ticks in the presence of females. For the first

time, I have shown that mating and feeding has an effect on gene expression in the salivary glands of male *D. andersoni* ticks. Future studies will focus on isolating new genes expressed in male ticks fed with and without females and their roles in tick feeding and mating.

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VITA 2

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