RELATIONSHIP BETWEEN ASSEMBLY BEHAVIOR AND MOLECULAR RESPONSES IN FOVEAL GLAND OF UNFED AND FED ADULT *Rhipicephalus appendiculatus*

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DECLARATION AND RECOMMENDATION

Declaration

This is original work and has not been presented before for an award of a degree/diploma in this or any other university.

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Recommendation

This work has been presented for examination with our approval as the University supervisors.

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DEDICATION

To my parents who understood the importance of education.

ABSTRACT

Ixodid ticks respond to a limited spectrum of stimuli in their search for hospitable environments, hosts and mates hence change in their behavior. This responsiveness is mediated by pheromones which are signaled by biochemical changes that occur during different stages of feeding. Genes induced during blood feeding result in the expression of new proteins secreted into tick foveal gland. Some of these proteins may be involved in the biosynthesis of these pheromones. Assembly behavior of female and male ticks appears to be mediated by an attractant signal and an assembly pheromone blend with one well characterized component. Preliminary studies suggest that when both sexes of adults acquire blood meals from host, the female enhances the production of an attractive signal and the male suppresses it. In addition, this preliminary observation suggests that with feeding there is a pheromonal switch from mutual aggregation assembly to sex attraction i.e. fed female tick attracting feeding or fed males. The aim of this study was to examine the response of adult male and female *Rhipicephalus appendiculatus* ticks to assembly pheromone in a two choice bioassay technique using guanine, tick excreta and nymphal skin washings as source of assembly signal. This study also aimed at comparing protein profiles of foveal gland extracts from fed and unfed male and female *R. appendiculatus*. Proteins were extracted from the foveal gland of unfed, 2 days, 4 days and 6 days fed ticks. The differentially expressed proteins were determined using two-dimensional polyacrylamide gel electrophoresis followed by the computer analysis of the scanned gels. Results obtained from bioassay tests were analyzed using student t-test while the gels were analyzed using Melanie software. Response of *R. appendiculatus* to guanine, tick excreta and nymphal skin washings was evident during the first hour of exposure, with little change observed between 2 and 24 hours. The assembly response was strongest to excreta treated sectors followed by guanine and nymphal skin washings. Both male and female ticks assembled at the treated sectors and there were no significant difference to their attractions. Most of the expressed proteins spots were acidic and of low to high molecular weights among all the levels of feeding. Most spots in 2D were induced in day 2 and 4 fed stages in both sexes. Conversely, in the day 6 fed populations, less spots were differentially expressed with most of them in acidic range, pH 4-6 and of high molecular weight, 90-150 kDa. Most spots in day 2 fed male and female populations were also acidic, pH 4-5 and of high molecular weight 60-150 kDa. In day 4 fed populations most spots were basic pH 8-9 and molecular weight 60-120 kDa. The results provide a basis for identification of proteins associated with pheromonal switch during feeding and lays down some ground work for the development of novel control tactic for the ticks.

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LIST OF SYMBOLS / ABBREVIATIONS

2, 4-DCP - 2,4-Dichlorophenol

2, 6 DCP - 2,6-Dichlorophenol

AAA - Attraction-Aggregation-Attachment pheromone

AI – Aggregation index

ASP - Attractant sex pheromone

ASP-Attractant sex pheromne

BM86 Antigen - *Boophilus microplus* antigen of type 86

BSA - Bovine Serum Albumin

DNA -Deoxyribonucleic Acid

DTT - Dithiothreitol

ECF- East Coast Fever

EDTA - Ethylenediaminetetraacetic Acid

FAO - Food and Agriculture Organization

HCL – Hydrochloric Acid

HPLC- High Pressure Liquid Chromatography

HSP70-heat shock proteins

IEF – Isoelectric focusing

ILRI – International Livestock Research Institute

KDa- Kilodaltons

KSDV - Kisenly sheep disease virus

LC-MS- liquid chromatography- mass spectrometry

MSP - Mounting Sex Pheromone

NP- 40 - Nonylphenoxylpolyethoxylethanol (nonionic detergent)

NSDV - Nairobi sheep disease virus

PBS- phosphate buffered saline

PCR- polymerase chain reaction

PMSF - Phenylmethylsulphonylfluoride

SDS-PAGE- Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

SPSS- Statistical Programme for Social Scientist

TBDs - Tick borne diseases

TP- Theileria parva

V -Voltage

WHO- World Health Organization

CHAPTER ONE

1.0 INTRODUCTION

Ticks are hematophaegus ectoparasites, capable of transmitting diseases to invertebrates and therefore, represent a threat to domestic, wildlife and human health (Norval, 1994). Tick and tick borne diseases have impacted negatively to the development of livestock industry in Africa (Walker *et al.,* 2003). Ixodid ticks such as *Amblyomma variegatum,* Fabriscius and *Rhipicephalus appendiculatus* Neumman 1901 (Acari: ixodidae) in particular are among the most economically important vectors of the tropics and subtropics (Bram, 1983).

Rhipicephalus appendiculatus is a hard tick, the adults of which are found in the ears of cattle, other livestock, and wild life such as antelope (Norval *et al*., 1991). This tick is considered to be a major pest in areas where it is endemic. Heavy infestations on cattle can result in severe damage to the ears, a potentially fatal toxemia, or loss of resistance to some infections. *R. appendiculatus* can also transmit a number of diseases, including East Coast Fever (ECF) (Norval *et al.,* 1991), which is the most economically important disease of cattle in Kenya and other afflicted areas of Africa. Other diseases are corridor disease, Zimbabwe malignant theilorosis, and Nairobi sheep disease.

Rhipicephalus appendiculatus transmits *Theileria parva parva* parasite, the main etiology of East Coast fever (ECF), a form of bovine theileriosis characterized by high fever and lymphadenopathy (Buisch *et al.,* 1998)**.** The disease causes high mortalities in breeds non-indigenous to the endemic areas, and is confined to eastern, central, and parts of southern Africa (Norval *et al.,* 1992). Of estimated 12.7million heads of cattle, 76% are at risk of ECF I this region (East Africa, 2003). This disease is associated with up to 10% mortality in zebu calves in ECF endemic areas and can cause up to 100% mortality in susceptible exotic and indigenous breeds (Mbogo *et al.,* 1995; East Africa, 2003). *R. appendiculatus* can also spread other protozoan parasites namely *Theileria taurotragi*, *Ehrlichia bovis*, *Rickettsia conorii*, *Babesia bigemina*, and Thogoto virus (Linda *et al.,* 1989; Walker *et al.,* 2003).

Currently, ticks are controlled primarily using acaricides. However, this method has major drawbacks, including the development of resistance by ticks, food-safety concerns and environmental contamination resulting from toxic residues (George, 2000). In addition, dipping facilities are not frequently operational because of lack of financial resources for maintenance, particularly in pastoral systems, which depend to a substantial degree on the informal economy (Lwande *et al.,* 1999). After nearly a century of acaricide utilization, it is widely believed that acaricides alone will not provide a sustainable solution to ticks and tick-borne diseases (Norval *et al.,* 1992).

Pheromones both volatile and nonvolatile are important information-carrying chemical messengers, both volatile and non-volatile, that are released by animals to alter behavior of both mono and hetero-specific animal species. Ticks use pheromones to find suitable hosts, mate recognition and defense. Assembly pheromone is associated with tick excreta, (which include the well characterized guanine) and serves as a cue signaling hospitable environments where moisture and temperature requirements are satisfied and there is increased opportunity for finding hosts (Otieno *et al.,* 1985; Sonenshine, 1991; Allan and Sonenshine, 2002). As such, the biological significance of assembly pheromone is that it concentrates ticks in favorable habitats. There is control significance related to the incorporation of assembly pheromone, its active components, or analogous compounds, into trap baits where it can be applied in combination with acaricide to prevent ticks from leaving control traps or areas (Sonenshine, 2006). The arrestment response elicited by assembly pheromone can last for several hours as ticks cease crawling, curl their legs underneath their bodies, and remain akinetic when encountering surfaces treated with the pheromone. Once the female attaches to a host and begins feeding it secretes an attractant sex pheromone which includes 2,6-dichlorophenol (Louly *et al.,* 2008) which has been detected in both male and female *R. appendiculatus* adults (McDowell and Waladde, 1985). Unfed females contain 12ng/tick, declining to 2ng/tick after six days of feeding, while unfed males contain 2ng/tick, falling to 0 after feeding (McDowell and Waladde, 1985).

So far, there is no published information showing how changes in protein content of the foveal gland relate to its function during feeding. Also, the quantitative and qualitative changes in foveal gland proteins following the attachment to the host in relation to the changes in behavior are not fully understood. To investigate changes in the protein content of foveal gland during

feeding this study endeavored to compare unfed and fed *R. appendiculatus* foveal gland extracts and possibly relate this information to the observed variation in their behavior. Such an understanding could lead to the development of new means of control and prevention of ticks, and therefore pathogen transmission.

1.1 Statement of problem

Ixodid ticks respond to a limited spectrum of stimuli in their search for hosts, mates and hospitable environments hence change in their behavior. This responsiveness is mediated by pheromones which are signaled by biochemical changes that occur during different stages of development and physiological shifts associated with feeding. Genes induced during blood feeding result in the expression of new proteins secreted into tick foveal gland. Some of these proteins may be involved in biosynthesis of these pheromones. Assembly behavior of female and male ticks appears to be mediated by an attractant signal and an assembly pheromone, a component of which is well characterized. When the adult ticks acquire blood meal from the host, the female becomes more attractive (suggesting enhanced the production of an attractant signal) while the male becomes less attractive suggesting suppression of an attractive signal) resulting in role switch. The molecular changes that occur during this stage in the foveal gland have not been well studied. This study therefore aims at providing some insights into the connection between behavior and molecular responses of *R. appendiculatus* following feeding

1.2 Main objective

To study the behavioral changes mediated by pheromones in unfed and feeding *R. appendiculatus* and investigate the changes in protein profile of foveal gland in both sexes of the tick.

1.2.1 Specific objectives

1. To examine the responsiveness of fed and unfed adult stage of both female and male *Rhipicephalus appendiculatus* to assembly pheromone.
2. To characterize foveal gland protein changes in unfed and fed female and male *R. appendiculatus* and relate it to behavioral responsiveness to assembly pheromone.

1.3 Hypotheses

1. *Rhipicephalus appendiculatus* of either sex do not significantly respond to assembly pheromone
2. There are no differential expression of proteins in either sex of *R. appendiculatus* exposed to assembly pheromone, and associated behavioral responsiveness.

1.4 Justification

East Coast Fever is a protozoan disease caused by *Theileria parva* parasite and transmitted by *Rhipicephalus appendiculatus* ticks. This disease puts 25 million cattle at risk across 11 countries in eastern, central and southern Africa, kills some 1 million cattle a year and is responsible for nearly half of calf deaths in these countries. Methods to control this disease are inadequate, particularly for pastoralists, on whom the disease has the biggest impact. The acaricide that the farmer uses to spray or dip their animals to control the ticks are expensive and are harmful to the environment. With the extended use of the acaricide, ticks can develop resistance to the acaricide. The acaricides can eliminate ticks from the host, but do not prevent continued re-infestation from the source environment which harbours 95% of tick population and also where ticks spend 90% of their life. Additionally, acaricides do not prevent the ticks already attached from transmitting the disease causing pathogens. An approach or product that continuously keeps ticks away from the host is therefore desirable. There is also need for developing new strategies aimed at reducing acaricide treatments, which not only reduces farmer dipping costs but also reduces environmental pollution and would probably also extend the duration of the viability of existing acaracides. Each of these outcomes would be a significant benefit to pastoral livelihoods. For effective management of ticks and tick-borne diseases, the development of anti-tick vaccines are inevitable since such vaccines represent to be one of the most promising prophylactic measures against tick bites and transmission of tick-borne pathogens.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Ticks

Ticks are believed to have evolved 120 million years ago and they speciated by approximately 92 million years ago into two main tick families: *Ixodidae* and *Argasidae* (Mans, 2002). Currently they are classified as belonging to the subclass Acari, the dominant subclass of *Arachinida*. The approximately 850 species are divided into the two major families and one subfamily. The family *Ixodidae* or 'hard ticks' so called because of their sclerotized dorsal scutal plate. This family is by far the largest and economically most important. It contains 13 genera and approximately 650 species (Sonenshine, 1991). Among the important genera are; *Amblyomma, Dermacentor, Hyalomma, Ixodes, Boophilus and Rhipicephalus*. They are characterized by the presence of a tough, sclerotized plate (scutum) on the dorsal body surface, which functions as the site of attachment for various muscle groups. The *Argasidae* family comprises of 5 genera and approximately 170 species (Sonenshine, 1991). In contrast to the ixodidae, the *Argasidae* lack the scutum and have a leathery cuticle The salivary glands of argasids do not produce cement and contain anticoagulant and cytolytic substances, because feeding only takes a brief time (Sonenshine, 1991). A third family, the *Nutalliedae* contains only one species, *Nuttaliella namaqua* (Sonenshine, 1991).

2.2. *Rhipicephalus* species

Rhipicephalid species occur in Eurasia and northern Africa (15 species) and also in sub-Saharan Africa (approximately 55 species) (Walker *et al.,* 2003). Adults of most species parasitize wild and domestic artiodactyls, perissodactyls, or carnivores (Aeillo and Mays, 2003). Immature stages feed mostly on smaller mammals; however, of those that parasitize rodents or hyraxes, and of those that parasitize artiodactyls, a few feed on the same host as the adults (Aeillo and Mays, 2003). The *Rhipicephalid* life cycle is typically 3-host, but in the Mediterranean climatic zone (long, warm summer with low rainfall): *R. bursa* has a 2-host cycle. In sub-Saharan Africa with long dry seasons, *R. evertsi* and *R. glabroscutatum* also have 2-host cycles (Aeillo and Mays, 2003).

Distribution of *R. appendiculatus* and other *Rhipicephalids* in Africa is by no means continuous, even in those countries in which they are known to occur commonly (Wanzala, 2009). Predictive species model and DAR-LAM climate surfaces model shows the distribution of *R. appendiculatus* in sub Saharan Africa (Erasmus *et al.*, 2002; Olwoch *et al.*, 2008). In Kenya, the CLIMEX model was used to calculate the eco-climatic index which shows the coincidence of distribution pattern of dairy cattle with that of *R. appendiculatus* (Appendix 2 and Appendix 3). *R. appendiculatus* are restricted to relatively cool and humid biotypes of less than 30^{o}C daily and at least 400mm annual rainfall with vegetation cover existing of woodlands or grasslands (Yeoman and Walker, 1967). The distribution of *R. appendiculatus* correlates closely with areas with the highest concentrations of exotic or grade cattle. The occurrence and abundance of *R. appendiculatus* are affected by several factors, the most important of which are climate, vegetation, host availability and acaricide(s) used (Norval *et al.*, 1992; Norval, 1977; Norval and Lightfoot, 1981 and Howell *et al.*, 1981). The vegetation cover affects the microclimate (Minshull and Norval, 1982), which is important for the survival of the free living stages of the tick (Branagan, 1973) whereas overgrazing and removal of trees reduce vegetation cover therefore reducing *R. appendiculatus* (Norval *et al.*, 1992). The species becomes abundant in the presence of hosts that have a low level of resistance to it (Norval and Lightfoot, 1981). Prolonged intensified acaricide treatment of livestock can cause total eradication of the tick, but the tick can spread again if he treatment measures are stopped (Norval, 1992).

2.2.1 *Rhipicephalus appendiculatus*

Figure 1: *Rhipicephalus appendiculatus* - Fed female (left), male (centre) and unfed female

Rhipicephalus appendiculatus (figure 1) are hard ticks, the adults of which are found mainly in the ears of cattle, other livestock, and wild life where it is endemic (Aeillo and Mays, 2003). Heavy infestations on cattle can result in severe damage to the ears, a potentially fatal toxemia, or loss of resistance to some infections (Aeillo and Mays, 2003). *R. appendiculatus* transmits *Theileria parva* which causes East Coast Fever (ECF), the most economically important disease of cattle in Kenya and other afflicted areas of Africa (Norval *et al.*, 1991). It can also transmit *Theileria taurotragi*, *Ehrlichia bovis*, *Rickettsia conorii*, *Babesia bigemina*, and Thogoto virus and number of diseases, including corridor disease, Zimbabwe malignant theileriosis, Nairobi sheep disease, and Kisenly sheep disease (Aeillo and Mays, 2003; Jones and Nutall, 1989). *R. appendiculatus* prefers cool, shaded shrubby or woody savannas with at least 400 mm of annual rainfall. It is endemic from southern Sudan and eastern Zaire to South Africa and Kenya and can be found from sea level to 2300 meters (Aiello and Mays 2003). Both immature and adult ticks feed in the ears of cattle and other livestock. During massive infestations, they may also be found on other parts of the body. The immature ticks also feed on small antelope, carnivores, and sometimes rodents. Cattle must be present to sustain significant populations of this tick.

2.2.1.1 Economic Importance of *Rhipicephalus appendiculatus*

Tick-borne infections of livestock are widespread in Africa and present a greatest constrain to livestock development, particularly improvement of local breeds, than in any other region of the world (Norval *et al.*, 1992). This is largely due to the fact that many different tick-borne infections occur on the continent, the most important of which are *Theileria parva parva* (T.P), *T. bovis* Neitz (Zimbabwe malignant theileriosis), *T.p. lawrencei* Neitz (corridor disease) this problem is compounded by high susceptibility of foreign breeds of livestock being used to improve livestock productivity in many African countries (Norval *et al.*, 1992). *R. appendiculatus* is the most economically important tick of the 40-70 African tick species (Norval *et al.*, 1992, Walker *et al.*, 2003). This is due the fact that it is a highly efficient vector of *T.p parva*, the pathogen of the most important and complex tick borne disease (Dolan, 1999). The prevalence of East Coast Fever is so far restricted to eastern and central Africa (Norval *et al.*, 1992). The disease causes high rates of mortality and morbidity in livestock populations as well as productivity losses in animals that recover, and is the cause of exclusion of the much desired exotic breeds of cattle of high productivity from endemic areas (Kariuki, 1989).

R. appendiculatus is an efficient vector of *T.p. lawrencei* from African buffalo to cattle, causing corridor disease and buffalo disease (Neitz, 1955). The Nairobi sheep disease virus (NSDV) and Kisenly sheep disease virus (KSDV) causes hemorrhagic gastroenteritis and high mortality in sheep and goats are transmitted by *R. appendiculatus* (Buisch *et al.,* 1998).

In wild animals such as antelopes, infestations of *R appendiculatus* have caused toxicosis problems (Norval and LightFoot, 1982). The death of eland calves is as a result of acute and chronic anaemia which is caused by heavy infestations of *R. appendiculatus* (Lewis, 1981), *Rickettsia conori* and *R. aeschlimanii* and bacteria which causes tick typhus (Larisa, 2001).

Heavy infestations on cattle can result in severe damage to the ears (figure 2) a potentially fatal toxemia or the loss of resistance to some infections and re-emergence of tick-borne diseases and may cause Tzaneen disease (Wanzala, 2009). For each fully engorged female, there is a loss of 4.0g of potential growth of cattle (Irvin *et al.,* 1996). Heavy tick infestation may also cause significant blood loss, reduced productivity in terms of poor production of milk, meat, hides and skins, reduced weight loss and restlessness (Norval *et al.,* 1997). Tick bites can cause cutaneous effects such as focal dermal necrosis (figure 3), irritation, heamorrhage, inflammatory response often involving eosinophilis and development of wounds that become infected with bacteria such as *Staphylococcus* causing local cutaneous abscesses (Wall and Shearer, 1997). Tick bite lessions also predispose animals to screw worm myiasis (Wall and Shearer, 1997). Secondary infections in wounds caused by tick bites such as infections of *Dermatophilus congolensis*, may result in high mortality of livestock (FAO, 1998).

Figure 2: *Rhipicephalus appendiculatus*, the brown ear tick, feeding on the ear of a calf.

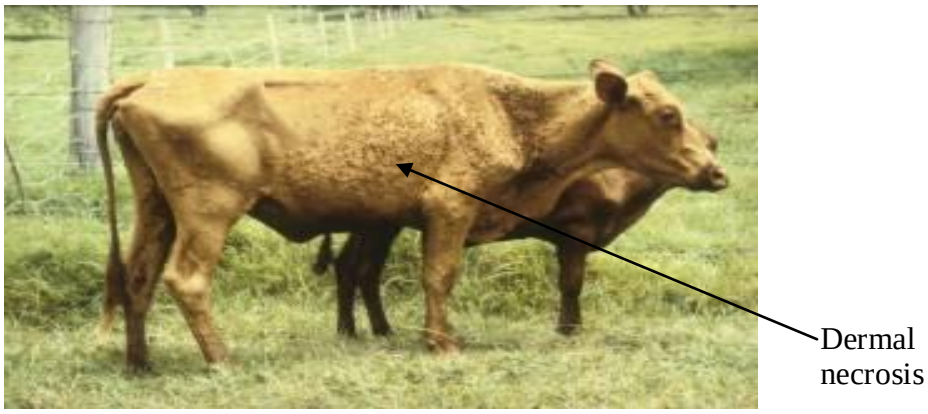


Figure 3: Cattle suffering from dermal necrosis after infestation with *R. appendiculatus*

In sub-Saharan Africa, 76 million cattle are found in the ECF affected region (Mukhebi *et al.,* 1992). ECF puts at risk the lives of about 25 million cattle in East and Central Africa (Norval *et al.,* 1992). The disease has been reported to cause half a million deaths of cattle per year in East Africa (VIE, 2002). In Kenya alone it has been estimated that 50-80% of the national cattle population are exposed to tick infestations, and of these animals 1% die of ECF each year (Mbogo *et al.,* 1995, VIE, 2002). A micro-economic analysis done on eight large and medium sized farms in Nakuru district, Rift valley, Kenya, with a total of 37,779 head of cattle showed that the cost of acaricides, production losses and, losses incurred during animal treatment and other tick borne diseases amounted to approximately US dollar 13.64 per animal per year (Kariuki, 1989).

Besides cattle mortality and morbidity as well as economic losses as a result of direct and indirect tick parasitism, stock management also requires large financial inputs. For instance, cost incurred for control operations have been estimated at US dollar 7.02 per head per year (Mukhebi *et al.,* 1993). Countries exposed to the threat of ECF also face large financial burden due to importation of acaricides, training of local personnel, treatment of infected animals, maintenance of infrastructures and investments into research on appropriate solutions to ticks and TBDs. Such economic constrains have been estimated to cost millions of US dollars annually in many African countries (Mukhebi *et al.,* 1993).

2.2.1.2 Life cycle of *Rhipicephalus appendiculatus*

Ticks are obligate, blood feeding ectoparasites of mammals, birds, reptiles and amphibians, more particularly mammals and birds (Wanzala, 2009). Adults of most tick species parasitize wild and domestic artiodactyls, perrissodactyls, or carnivores (Cumming, 1998). Bovine cattle are the main domestic hosts of *R. appendiculatus*, while buffalo's, elands, waterbucks, nyala, greater kudus and sable antelopes serve as non-domestic hosts. Sheep and dogs and goats are also infested (Wanzala, 2009). *R. appendiculatus* is well adapted to domestic cattle and all stages can be maintained by feeding on cattle but immature ticks may feed on smaller antelopes and scrub hares thus showing telotropic type of behavior (three host cycle) with a tendency to the monotropic type (one host cycle). Adult *R. appendiculatus* prefer to feed on the ear pinna of bovids. In heavy infestations adults are also found around the eyelids, upper neck, in the tail-brush and around the anus. Only about 2-5 % of ticks' lifespan is spent on a host and the majority of their lifecycle is spent on the ground or vegetation (Branagan, 1973). Typically, ixodid ticks have a 3-host life cycle, with each feeding stage of the tick (larva, nymph, and adult) having a single host (Sonenshine, 1991; Spickett, 1994). Each stage of the tick seeks out a host, attaches, and then feeds over a period of several days. Once replete, the tick detaches and, after dropping from the host, finds a resting place where it can digest its blood meal and molt to the next feeding stage, or enter diapause, a state characterized by reduced metabolism and delayed development. In a few species, the immature forms may remain on the host during molting (Philippe and Didier, 2001). Generally, adult males feed only briefly and sparingly and some do not feed at all. Mating generally occurs on the host. Thereafter, the females detach and drop off

the host to digest their blood meal. They then lay their eggs, (numbering 400 to 120,000 depending on the species) in a sheltered environment and die (Sonenshine, 1991).

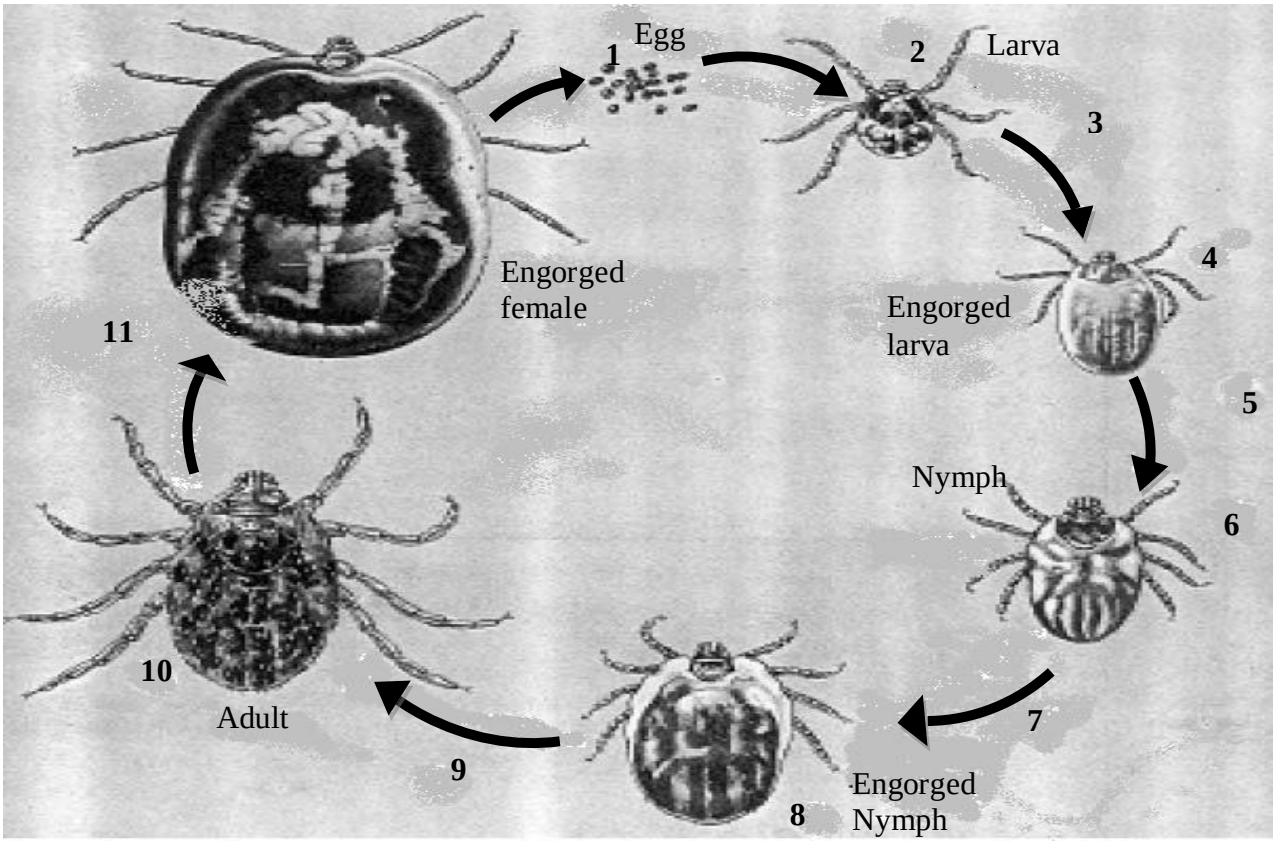


Figure 4: 3 host life cycle of ixodid tick (Adapted from Kebede, 2004)

2.3 Development of Acaricide Resistance and Possible Resistance Mechanism in Ixodid Ticks

The World Health Organization committee on insecticide resistance in 1957 defined resistance as the development of an ability in a strain of insects or other arthropods to tolerate doses of toxicants, which would prove lethal to the majority of individuals in a normal population of the same species. This ability is inherited and occurs through the selective effect of chemicals which affect selection of pre-existing resistance genes, which are present at very low frequencies in a population (Brown, 1976).

Resistance is the inevitable consequences of the use of acaricides and also as a result of the genetic selection of individuals in a population through the action of the acaricide which either

kills or affects the reproduction of the more susceptible ticks (Nolan, 1990; Stone, 1972). It is the phenotypic expression of an evolutionary process accelerated by chemical selection and often involves inherited characters (Nolan and Roulston, 1979). In order for the less susceptible ticks to survive and form the nucleus of resistant strains of ticks, the mechanism by which resistance is conferred, must be inheritable and must be passed on from one generation to the next (Stone, 1972). The process occurs primarily through the selective effect of chemicals favoring pre-existing resistant mutants which are already present in field populations of ticks at low frequencies (Mekonnen, 2005). Acaricides do not kill all the ticks on the host and mutants which survive successfully breed and become dominant resistance and the risk of this happening increases if the population of susceptible ticks is completely eliminated by the over-use of acaricides (Mekonnen, 2005). Nolan (1985) indicated that any chemical used for the control of arthropods, by interfering with some biochemical or physiological system to produce its lethal effect, must pass through several obstacles before reaching its target as an active toxicant. Any change in the nature of these barriers or extent of their activity, can also lower the effective concentration of the toxic compound (Nolan, 1985). This change may occur through a spontaneous change mutation, occurring either before or during the use of a certain pesticides by providing a few heterozygote individuals with this beneficial characteristic (Nolan, 1985).

It is increasingly common for livestock producers to experience multispecies resistance in parasite populations exposed to acaricides or insecticides (Morales *et al*., 1999). This sometimes involves multiple tick species (e.g. *Amblyomma variegatum* and *Boophilus microplus*) or multiple taxa (e.g. *Boophilus microplus* and *H. irritans*). The increasingly frequent use of acaricides in livestock production systems may accelerate this trend, not only inducing resistance in ticks but also environmental pollution.

Different mechanisms of resistance have been found for each class of acaricides studied in ticks. These include metabolic detoxification involving *cytP450* and esterases for synthetic pyrethroids resistance, respectively (Jamroz *et al.,* 2000), and target slight alterations for every class of acaricide examined (Foil *et al.,* 2004). Target site mutations are the most common resistance mechanism observed, but there are examples of metabolic mechanisms. In many pyrethroid resistant strains, a single target site mutation on the Na^+ channel confers very high resistance to all pyrethroid acaricides as well as DDT (He *et al*,. 1999). Acetylcholine esterase affinity for organophosphates is changed in resistant tick populations (Li *et al.,* 2003). A second mechanism

of organophasphate resistance is linked to cytochrome P450 monooxygenase activity (Pruett, 2002).

2.4 Current Tick Control and Management Strategies

Tick control is practiced in a wide variety of circumstances involving different tick and host species. Tick control strategies are aimed mainly to protect the host from irritation and production losses, formation of lesions that can become secondarily infested, damage to hides and udders, toxicosis, paralysis, and of greatest importance, infection with a wide variety of disease agents (Norval *et al.,* 1992). Control also prevents the spread of tick species and the diseases they transmit to uninfected areas, regions or continents (Aiello and Mays, 2003). The control of ticks has been done through chemical means, cultural measures, mechanical control, bio-control and immunological techniques.

2.4.1 Chemical Control

The first application of ixodicides to control ticks on cattle was made by treating the infested cattle with various oils including paraffin but there was no much success (Harrison *et al.,* 1973). An effective chemical control of livestock ticks began with the introduction of arsenical solutions as cattle dips in South Africa in 1893 and in Australia in 1895, and until today the use of ixodicides is being practiced. In Africa different acaricide groups have been used to control ticks which include arsenicals, organochlorines, organophosphates, carbamates, amidines, pyrethrins and synthetic pyrethroids (Mitchell, 1996). Mode of application of these acaricides have included the use of dip tanks, knapsack sprayers, hand sprayers, hand dressing, squirting acaricide on predilection feeding sites of ticks and dusting (Awumbila, 1996).

Acaricide application may be directed against free living stages in the environment or the parasitic stages on the host. The problems associated with the use of acaricides in livestock are challenging and without imminent solutions (Wanzala, 2009). Acaricides are costly and out of reach of poor rural livestock farmers who also do not have sufficient technical know how of managing and handling them (Norval *et al.,* 1992). The control of ixodid ticks by acaricidal treatment of vegetation has been done in specific sites to reduce the risks of tick infestation to susceptible hosts. However this method has not been sustainable because of associated

environmental pollution problems and high costs involved in treating large areas and therefore it has not been recommended for wider use (Aiello and Mays, 2003). In addition, inappropriate drainage of dips liquid causes water pollution and indiscriminate damage to the flora and fauna in the environment (de Haan *et al.*, 1996). Acaracides are a health hazard as well in that they cause food poisoning and residual toxicity. It has been reported that organochloride products leave residues in meat and milk (Mitchell, 1996)

Ticks have been shown to possess a genetic pool containing the potential to resist a wide range of chemical poisons (Nolan, 1990). This has been compounded by illegal cattle movement, civil unrest in some areas, poor management and inadequate maintenance of dips and poor use of manufacture's instructions (Mathewson, 1984). Indiscriminate use of acaricide may affect the tick control in the future as this has the consequences of facilitating rapid development of tick resistance to the active compounds used in acaricide formulations.

2.4.2 Mechanical Control

In certain east African pastoral communities, livestock is communally organized, brought together and held in a crutch facility where ticks are manually picked off the animals one by one and either buried or burned (Marina *et al.,* 2001). This practice is also conducted during milking and cleaning of livestock sheds by women (Marina *et al.,* 2001). The method is tedious, time consuming and involves much labor if a big herd of cattle is involved, and hence not sustainable.

2.4.3 Biological Control

Biological control of ticks is the use of natural enemies that can reduce the density of the target population or even eliminate it. More than 257 tick biocontrol agents are mentioned, comprising 100 species of pathogens, seven parasitoids and 150 predators (Samish and Alekseev, 2001). Interests in developing biological methods for tick control using birds, parasitoids, entomopathogenic nematodes, entomopathogenic fungi, arthropods and bacteria has gained momentum worldwide, because of the limited impact of these organisms on the environment (Couto, 1994; Hu *et al.,* 1998; Samish, 2000; Samish and Alekseev, 2001; Hassanain *et al.,* 1997). Commercially available nematodes which are used to control insects are also able to kill ticks. The way in which these nematodes kill ticks have been proposed. They invade ticks

through orifices or by use of digestive enzymes and penetrate the host cuticle. Once in the host haemocoel, they release mutualistic bacteria that attack and kill bacteria. There is likelihood that the use of nematodes to control ticks will be temporarily successful and in localized areas.

The application and use of bacteria as biocontrol agent is poorly documented. However, *Bacillus thuringiensis* have been reported to be lethal against ixodidea ticks

The use of fungi for the control of ticks has an advantage over acaricides because fungi can be produced easily locally and there is likelihood that they are economical and would have less target organisms (Maranga *et al.*, 2006). Obligate parasitic entomogenoues fungi, in particularly *Metarhazia anisopliae* and *Beauveria bassiana* have been used as biological control represents a fraction of entomopathogenic fungi against ticks that have been extensively studied and have shown to have lethal effects on a number of ixodid ticks (Maranga *et al.*, 2005). In field experiments conducted in Brazil and in kenya demonstrated that broadcast delivery of *Be. Bassiana* and *M. anisopliae* to pastures and vegetation by spraying resulted to reduction in population density of ticks (Bittencourt, 2000; Kaaya, 2000).

2.4.4 Vaccination

A new approach of tick control is to develop recombinant vaccines against the ticks rather than against all the microorganisms that cause the diseases they carry (Labuda *et al.*, 2006). More research has focused on identifying antigens from the whole tick macerates, tick salivary glands and mid gut as candidates for anti-tick vaccine (Trimnell *et al.*, 2005). In general, two types of antigenic target have been explored for vaccine development against ticks. The first is known as exposed antigens. These are antigens that are secreted in saliva during tick attachment and feeding on the host, the so-called exposed antigens (Allen *et al.*, 1979). Exposed antigens are taken up by host dendritic cells when the tick is feeding; these dendritic cells process these antigens and then present them to T lymphocytes, priming a cell- or antibody-mediated immune response (Larregina and Falo, 2005). The second are, called ‘concealed’ antigens. They are normally hidden from host immune mechanisms (Willadsen and Kemp, 1988). Typical concealed antigens are those found on the tick digestive tract wall and recognized by specific

immunoglobulins taken up in the blood meal. However, any antigen not normally presented to the host is a concealed antigen, and is a potential vaccine candidate if the antigen: (a) encounter immunoglobulins entering the haemolymph (or gut) and (b) is associated with some vital function of the tick.

Several exposed antigens have been expressed as recombinant proteins and evaluated as anti-tick vaccines. Generally, the results have been disappointing (Table 1). At the molecular level, immunogenicity analysis of these antigens has been used to identify exposed antigens (Tsuda *et al.,* 2001). Immunoscreening of a partially fed adult female cDNA library of *Haemaphysalis longicornis* using antiserum from a rabbit repeatedly infested with the tick identified HL 34 saliva protein. In a rabbit model, the recombinant HL 34 protein induced morbidity and mortality in adult and nymphal *H. longicornis* ticks (Tsuda *et al.,* 2001). Using immunological approach, Mulenga *et al,* (1999) was able to identify a putative cement protein known as p29 antigen from *H. longicornis*. An immunodominant cement cone protein (RIM36) of *Rhipicephalus appendiculatus* induces a strong antibody response in tick-exposed cattle, although it is not clear whether the immunological response is protective (Bishop *et al.,* 2002). Another putative cement protein, 64P from *R. appendiculatus*, was identified by random sequencing of clones from a cDNA library derived from pooled male and female salivary glands of ticks that had been feeding for 2 days on guinea pigs (Trimnell *et al.,* 2005). Calreticulin, a calcium-binding protein normally found in endoplasmic reticulum, is secreted in tick saliva (Jaworski e*t al.,* 1995). It was first identified in a cDNA library of partially fed female *A. americanum* salivary glands. A recombinant calreticulin of *B. microplus* did not induce an antibody response in cattle, although the recombinant protein was recognized by sera from *R. sanguineus*-infested dogs (Ferreira *et al.,* 2002).

Anti-tick effects are expressed as tick and egg mortality, decreased engorgement and egg mass weights, prolonged tick feeding and inhibition of moulting. Macroscopic and histological profiles of tick attachment sites differ between susceptible hosts and those that have acquired resistance (Trimnel *et al.,* 2002). Exposed antigens have mostly been identified by their ability to elicit an antibody response during tick infestations. A vaccine that targets an exposed antigen primes the host's immune mechanisms. Consequently, when a tick attaches/feeds and secretes the molecules that carry the exposed antigen, the host reacts with an anamnesti response.

However, like all haematophagous arthropods, ticks produce a pharmacopoeia of bioactive molecules (many of which are proteins and peptides) in their salivary glands (Brossard and Wikel, 2004). These molecules are secreted in saliva to counteract the host's haemostatic, inflammatory and immune mechanisms that otherwise would prevent the tick from attaching and feeding successfully. As a result, for most host species, naturally acquired immunity to ticks reduces but does not prevent tick infestation even after repeated exposure to ticks (Ribeiro, 1987).

The concept of using concealed antigens to immunize hosts against ticks was originally based on the idea of inducing antibody production to tick molecules that performed essential functions, such as developmental hormones (Galun, 1975). Uptake of the antibodies via a blood meal would then disrupt the essential functions and kill the feeding ticks and the engorged ticks. Concealed antigens do not induce an immune response during tick attachment and feeding but as shown by the Bm86 and Bm95 antigens (Table 2), are immunogenic in the form of recombinant proteins (Rand, *et al.,* 1989). These defined tick gut wall antigens interact with specific immunoglobulins taken up in the blood meal from the immunized host (Garcia-Garcia *et al.,* 2000). Antibody binding causes lysis of the gut wall, interfering with digestion of the blood and subsequent egg production.

With the advent of recombinant DNA technology which enables the production of a mass of immunogens at low cost, anti-tick vaccines, remain one of the most hopeful measures against tick bites and transmission of tick-borne pathogens (de la Fuente and Kocan, 2006). Furthermore, vaccination is likely to eliminate the problems of resistance and contamination than acaricides (Shinji *et al.,* 2008). The discovery of Bm86 antigen has led to commercialization of the first anti-tick vaccine (TickGARD^{TM}) against *B. microplus* which demonstrates protective abiltiy against tick infestation (Willadsen *et al.,* 1995; de la Fuente *et al.,* 1998).

Table 1: Exposed antigens evaluated as recombinant anti-tick vaccine

Antigen	Tick species	Comment	References
Calreticulin	*A. americanum* *D. variabilis* *B. microplus*	Low immunogenicity in cattle	(Jaworski e*t al.*, 1995)
HL 34	*H. longicornis*	Saliva protein; adverse effect on tick feeding on rabbits	(Tsuda *et al.*, 2001)
Immunoglobulin-binding protein	*R. appendiculatus*	IGBMC male-specific saliva protein; slight effect on female feeding performance on guinea pigs	(Wang *et al.*, 1998)
RIM36	*R. appendiculatus*	Cement protein; very antigenic but non-protective in cattle	(Bishop *et al.*, 2002)
Histamine-binding protein	*R. appendiculatus*	Adverse reaction in guinea pig model	(Paesen *et al.*, 1999)
64TRPs	*R. appendiculatus*	Dual action by cross-reacting with concealed antigens and potentially 'universal' affecting many different tick species	(Trimnell *et al.*, 2005)
P29	*H. longicornis*	Collagen-like; effective against all tick stages	(Mulenga *et al.*, 1999)

Table 2: Concealed antigens evaluated as recombinant vaccines

Antigen	Tick species	Comment	References
Bm86	*B. microplus*	Commercial vaccine TickGARD	(Willadsen *et al.*, 1995)
Bm91 (carboxy dipeptidase)	*B. microplus*	Increased efficacy of Bm86	(Willadsen *et al.*, 1996)
Bm95	*B. microplus*	Bm86 sequence variant used in Cuban vaccine, GAVAC; protects cattle against infestations by *B. microplus* strains from different geographical areas	(Garcia-Garcia *et al.*, 2000)
Vitellin	*B. microplus*	Effective as native protein but not the recombinant form	(Tellam *et al.*, 2002)
HLS1	*H. longicornis*	Serpin; effect on nymphs	(Sugino *et al.*, 2003)
Voraxin	*A. hebraeum*	Male gonadal protein; impairs feeding of females in a rabbit model	(Weiss and Kaufman, 2004)
P27/30	*H. longicornis*	Troponin I-like protein, impairs tick feeding on immunized mice	(Myung-Jo, 2005)
4D8	*I. scapularis*	Effective against adults when tested experimentally on sheep	(Almazan *et al.*, 2005)

Theileria parva control by infection and treatment method of immunization using tick derived sporozoites and tetracycline is being implemented in a number of countries in eastern, central and southern Africa. While vaccination using attenuated schizont-infected cell lines has been widely used for *T. annulata*. At present, one practical method of immunization is by the infection and treatment method (Radley, 1981). This involves the inoculation of cattle with a previously characterized and potentially lethal dose of sporozoites of *T. parva* and simultaneous treatment with antibiotics. This confers life- long immunity to the animal (Radley, 1981). The method has been shown to be technically efficacious in field trials carried out in different countries of the region (Robson *et al,* 1977; Morzaria *et al*, 1985; Musisi *et al,* 1989; Mutugi *et al,* 1989). The infection and treatment method of immunization has been estimated to cost US dollars 1.50-20.00 (Radley, 1981; Kiltz, 1985; Mukhebi *et al,* 1990), being the cost of producing one dose of the vaccine amounts to US$ 0.01- 0.90 and the rest is the cost of delivering the vaccine to the animal in the field (Mukhebi *et al.,* 1990). Mukhebi *et al.* (1992) assessed, *ex-ante,* the economics of immunization by the infection and treatment method in the eastern, central and southern African region affected by ECF. The analysis suggested high potential economic returns, with a benefit-cost ratio in the range of 9 to 17 based on various assumptions.

The infection and treatment method of immunization, however, has some drawbacks. Although this method allows considerable reduction of acaricide use, it does not completely eliminate the use of acaricide due to the potential existence of other tick-borne diseases (Mukhebi and Perry, 1992). In addition, the use of live parasites in the vaccine poses some safety limitations for large-scale immunization purposes. This is compounded by uncertainty about the spectrum of different species, strains and antigenic types of *Theileria* parasites in different areas, variation in the sensitivity of different parasite isolates to therapeutic drugs and the development of a potentially infective carrier state in immunized animals (Mukhebi and Perry, 1992). Furthermore, the use of this vaccine requires cold storage, during transportation and during the pilot application stage for an extended monitoring period post-immunization to detect and treat any breakthrough infections. Preferably, liquid nitrogen system is needed. Both these aspects currently constitute high cost items in the delivery of the vaccine.

From economic, social and environmental point of view, the need for an alternative, such as a subunit vaccine against ECF, cannot be emphasized enough. These limitations ended in efforts to develop and optimize a subunit vaccine for ECF based on *T. parva* surface protein p67. This surface protein plays a central role in the entry process of the parasite in to the cell. Immunization using recombinant p67 based vaccines has only resulted in reduction of 77% in severe ECF (Kaba *et al.,* 2005). Moreover, a lot of efforts are paying attention on the identification of schizont-specific components for inclusion in a second-generation multicomponent product (Morzaria *et al.,* 2000). In addition, the completion of the genome sequence of *T. parva* (Nene *et al.,* 2000) will advance the schizont antigen identification process. Into the bargain, it offers new chance in fighting infection and diseases by understanding the biology of these parasites and their hosts in depth.

Chemotherapy has and is being used to treat *T. parva* and *T. annulata*. The first commercial drug against *Theileria* was halofuginone lactate (Terit®) followed by parvaquone (Clexon®). Subsequently, a more active analogue of parvaquone, buparvaquone (Butalex®), was synthesised. The three drugs are available but are extremely expensive. Costs of treatment of small indigenous breeds of low productivity may equal the value of the animal and only early treatment has a high probability of success (Muraguri *et al.,* 2006).

2.5 Tick Pheromones

Pheromones are external chemical messengers that pass between individuals of the same species and control intra-specific interactions (Evans, 1984). Pheromones are thought to be the most frequent and prevalent of all semiochemicals as manifested in ticks and contain a single compound or a mixture of compounds (Sonenshine, 1985). Pheromones facilitate intra-specific interactions such as mate identification and location,aggregation and assembly. While non-pheromonal semiochemicals mediate food and host finding and other activities. It is vital to the benefit and survival of a population that a pheromone must stand out in a world cluttered with chemical stimuli in order to reach the target species (Evans 1984). Chemical communication system in ticks are mediated by three different types of pheromones these include: assembly pheromones, sex pheromones, and aggregation-attachment pheromones (Sonenshine *et al.,* 1982; Sonenshine, 1985)

2.5.1 Assembly Pheromones

Assembly pheromones are widespread among species of both *Argasidae* and *Ixodidae.* Assembly pheromones mostly occur in species that inhabit arid environments for example most A*rgasidae and* Rhipicephalids (Sonenshine *et al.,* 1985). They are found on the tick's natural substratum off the host when the ticks come on contact; they arrest the ambulatory activity of actively wandering ticks leading to the formation of tight clusters of akinetic ticks. The arrestant response elicited by assembly pheromone can last for many hours as ticks cease wandering, curl their legs beneath their bodies and remain akinetic (Yoder *et al.,* 2008). These facilitate non-parasitic phases of its being and to perk up its chances of finding its host when members return to their previous habitats under favorable conditions (Leahy, 1979).

Leahy *et al*, (1975) in his study recognized that tick assembly pheromones are nonvolatile, insoluble in less polar solvents, and relatively stable in the environment. A prominent feature is their relative lack of specificity, eliciting responses from different life stages of the same species as well as those of other species (Leahy *et al.,* 1975; George, 1981). Assembly pheromones provoke ticks to cease movement and cluster in groups. However, not all constituent compounds of assembly pheromone are known. Ticks excrete substances on surfaces they come in contact with. This suggests that these compounds are found in excreta and forms part of excretory products (Sonenshine, 1991). The principle component of tick assembly pheromone is guanine (Sonenshine, 1991) but arrestment behavior is not only prompted guanine but also by xanthine, hypoxanthine and probably uric acid (Allan and Sonenshine, 2002; Yoder *et al.,* 2003). These clustering of ticks occur in natural habitats in their environment such as cracks, crevices caves (Sonenshine, 1991). This kind of assembly behavior is most likely in a rejoinder to ensure survival of the population in response to harsh environmental conditions in order to avoid desiccation (Aiello and May, 2003). Large numbers would also ensure the recruitment of sexual partners for mating (Petney and Bull 1981). Moreover, clustering of ticks would prop up host contact and allow for blood meals to be taken (Sonenshine, 1991). These views are in tandem with the findings by Hassanali *et al.* (1989) that a sustained positive response to guanine by nymphals and adults *A. persicus* is linked with sustained exposure of the ticks to low relative humidities of about 25% relative humidity. The indifference of the tick to purines appears to be influenced by metabolic water, which is likely to be high in recently moulted ticks, and is

consistent with the pheromone's role as an assembly stimulus when conservation of water becomes very crucial (Hassanali *et al.,* 1989). Evidence indicates that assembly pheromone in *A. americanum* elicits responses from only non-fed stages in the life cycle and immature stages (Yoder *et al.,* 2008).

2.5.2 Sex Attractant Pheromones

Mating in ticks follows a ritualistic pattern that is characteristic of all species. A male that come in contacts with sexually active female mounts onto its dorsum, puts its legs and mouthparts to the body surface, turns, and crawls over the posterior edge onto the female's ventral surface. Once the male locates the female's genital pore, it inserts its mouthparts into the female's vulva and commences to form a spermatophore. When ready, the copulating male inserts the spermatophore to inseminate the female (Sonenshine, 2006).

In the *Ixodidae,* mating is delayed until both female and male adults have commenced blood feeding on their preferred hosts (Sonenshine, 2006). The sexually immature males and females seek hosts and commence feeding independent of one another. However, once feeding begins, spermatogenesis in males and oogenesis in females begins, and within 2 days of feeding, females begin to secrete the attractant sex pheromone (ASP) 2,-6-dichlorophenol (2,-6-DCP) (Sonenshine *et al.,* 1985). Males feeding nearby are stimulated to stop and commence crawling in search for the pheromone-secreting females. This chemical compound, which is volatile, has been reported from seven genera of ticks, as well as in at least 16 different species (Sonenshine, 1991). Males perceive the pheromone and creep over the host until they come in contact with the producing female. A compound 2,-4-dichlorophenol (2,-4-DCP), of the same class was also found in extracts of fed female *Dermacentor variabilis,* although at a 1:9 ratio of 2,- 4-DCP to 2, -6-DCP. 2,-4-DCP was also found to be attractive to mate-seeking males (Hanson *et al.,* 2002). Following male-female contact, detection is facilitated when the males sense cholesteryl esters on the female's body. The mixture of compounds, which comprise the mounting sex pheromone (MSP), stimulates the male to mount the dorsal surface, grasp the female, turn to the female's venter, and search for the genital pore (Sonenshine, 2006). Variations in the composition of the cholesteryl esters on the females of different species facilitate species recognition and determine whether courtship continues (Sonenshine *et al.,* 1992). MSP is essential for the mating process. If the female is washed with a lipid solvent, mating is aborted. However, when lipid extracts (made by

washing fed females) were applied to the carcasses of delipidized females, the mounting response was restored. Males could even be induced to mount inanimate objects, e.g., plastic beads, in which a mixture of 2, 6-DCP and the cholesteryl esters was applied. The chemical composition of the MSP is very important to the fulfillment of the mating process. Different mixtures of cholesteryl esters have been identified in different species and genera of these ticks. Even though attracted by ASP from a feeding female, males will not mate with a female unless it recognizes the MSP (specific for that species) upon contact with the female body surface. However, species-specific discrimination with this pheromone is imperfect. Male ticks showed the greatest ability to distinguish between ticks of different genera but only a limited ability to distinguish between the MSP extracts or artificial mixtures of species within the same genus.

2.5.3 Attraction-Aggregation-Attachment Pheromone

The attraction-aggregation-attachment (AAA) pheromone is actually a mixture of three or more specific compounds that mediate different behaviors leading to the formation of species-specific feeding clusters on their preferred vertabrate host. In contrast to other pheromones, the AAA pheromone is produced exclusively by males but elicit attractive responses to both unfed males and females of the same species. It is secreted by large glands, the Type II dermal glands positioned on the ventral surfaces of the feeding males. Both *o*-nitrophenol and methyl salicylate were identified by high-pressure liquid chromatography (HPLC) in extracts of these glands removed from fed male ticks (Diehl *et al.,* 1991). The best known example is the AAA pheromone of the tropical bont tick, *Amblyomma variegatum*, comprising a mixture of two substituted phenols, *o*-nitrophenol and methyl salicylate and a fatty acid, nonanoic acid (Schoni *et al.,* 1984). Surprisingly, 2, 6-DCP which was initially thought to be only a sex attractant was also identified in the tick extracts and also elicits positive response to unfed ticks (Price *et al.,* 1994; Norval *et al.,* 1991), but its precise role in the aggregating or attachment response is unclear. Another unstable compound known as 1-octen-3-ol was also found to exert a pull on *A. variegatum* adults that formed aggregations around tick waste material or molted skins. This compound was a major constituent of secretions from *A. variegatum* adults, as well as from tick exuviae, fecal wastes, or even dead ticks (McMahon *et al.,* 2001). Differences in the relative load of methyl-salicylate and *o*-nitrophenol, plus the presence or absence of benzaldehyde, are understood to throw in to the formation of species-specific aggregations in which *A. variegatum*

and its close relative, *A. hebraeum*, are sympatric (Price *et al.*, 1994). In spite of the extensive distribution of *A. variegatum*, geographic range does not show to have any effect on the effectiveness of the AAA pheromone; male extracts or feeding males from several Caribbean islands, Kenya, Zimbabwe, and other localities at different parts of the tick range were similarly attractive (Sonenshine *et al.*, 2000). The strangely large amounts of pheromone secreted by feeding male ticks (Diehl *et al.*, 1991; Pavis and Barre, 1993; Price *et al.*, 1994), combined with CO_2 emissions from their huge vertebrate hosts permit the pheromone to attract unfed males and females from distances of approximately 5 to 10 m (Norval *et al.*, 1991). For effective stimulation and attraction of these ticks, large amounts of CO_2 are indispensable. The amount of CO_2 produced over eight hours from a 500g block of dry ice which is comparable to the amount emitted by one 500kg cattle attracted adults from two to eight meters apart, depending on the direction of wind blow (Barre *et al.*, 1997).

2.6 The Foveal Gland and Pheromone Biosynthesis

To this pointing moment, there is no published information regarding the biosynthetic pathway of assembly pheromone and 2,-6-DCP in ticks. The occurrence of this molecule in ticks is remarkable. No other chlorinated organic compounds in terrestrial animals have been reported. However, biosynthesis of halometabolites in plants and microbes is illustrious (Van, 2001). Chlorometabolites are predominantly produced by terrestrial microbes, mainly by enzymes like chloroperoxidases, which, in presence of $FADH_2$ coenzymes, catalyze the construction of carbon-halogen bonds.in plants for instance kidney bean, chloroperoxidases produce a assortment of chlorinated hydrocarbons (Yenoyama *et al*., 2004), diverse groups of fungi (Simons *et al.*, 1995), and bacteria (Preobrazhenskaya *et al.*, 2003). An fascinating possibility is that the biosynthesis of 2,6-DCP may have started with a commensal or mutualistic microbe, conceivably by capture and integration of one or more microbial genes into the tick's genome. Ticks harbor a wide range of microorganisms within their body tissues (Yoder *et al.*, 2003) and one such microbe, the fungus *Scopulariopsis brevicaulis*, can also synthesize 2,6-DCP. However, not any are known to take place in the pheromone glands. Nevertheless, this captivating possibility certainly merits further study.

2.6.1 Foveal Gland

Adult *ixodid* ticks are identified to produce sex pheromone (Sonenshine *et al.,* 1985), but only the female emits 2,-6-Dichlorophenol (McDowell and Waladde, 1985), the sex attractant stir up mating response in the male (Sonenshine *et al.,* 1985). The source of the sex pheromone in the female ixodid ticks is a pair of foveal glands lying beneath the foveae dorsales (Samir and El Shoura, 1987). In *Argasids,* however, the source of the sex pheromone is believed to be the coxal glands (El Shoura and Roshdy, 1985). Sonenshine in 1985 reported the presence of foveal glands in males and females of at least six species of *Ixodidae*. The paired foveal glands and foveae dorsales are found underneath the dorsum, approximately 1 mm posterior to the scutum. They open on the extensible alloscutal area posterior to the nonextensible scutum at the level of the last pair of legs (Samir and El Shoura, 1987). These sex pheromone glands are very different from the sex pheromone glands of insects. Instead of a single pore and simple connecting duct, or an eversible sac widespread in many insects, the ixodid foveal glands have many pores in two discrete clusters on the dorsal body surface. Each fovea may contain from 15 to 50 pores (Samir and El Shoura, 1987). The fovea and the corresponding foveal gland below it form a complex system consisting of three parts, (a) foveal pores, (b) ducts, and (c) secretory lobes.

The pheromone glands go through growth cycle, activity, and disintegration related to the physiological occurrences that exemplify the life processes of developing and reproducing adults. They form nymphal primordia and proceed to completion in their development just before appearance of the imago (Sonenshine, 1985). At this stage, even though they are structurally the same to the mature adult glands, they do not yet produce 2,-6-DCP, and the secretory droplets seems to differ from those in the mature glands and lack the highly osmiophilic oil droplets which are feature of the latter. The pheromone appears during female maturation (Sonenshine *et al.,* 1984). Pheromone synthesis proceeds during feeding, presumably replenishing the supply of secreted material. In *Dermacentor variabilis,* feeding led to reduced pheromone concentrations to about 0.2 ng/tick, which remained stable during the feeding period of the virgin female. The biochemical changes that do occur in the foveal gland during the unfed and fed stages have not been well understood.

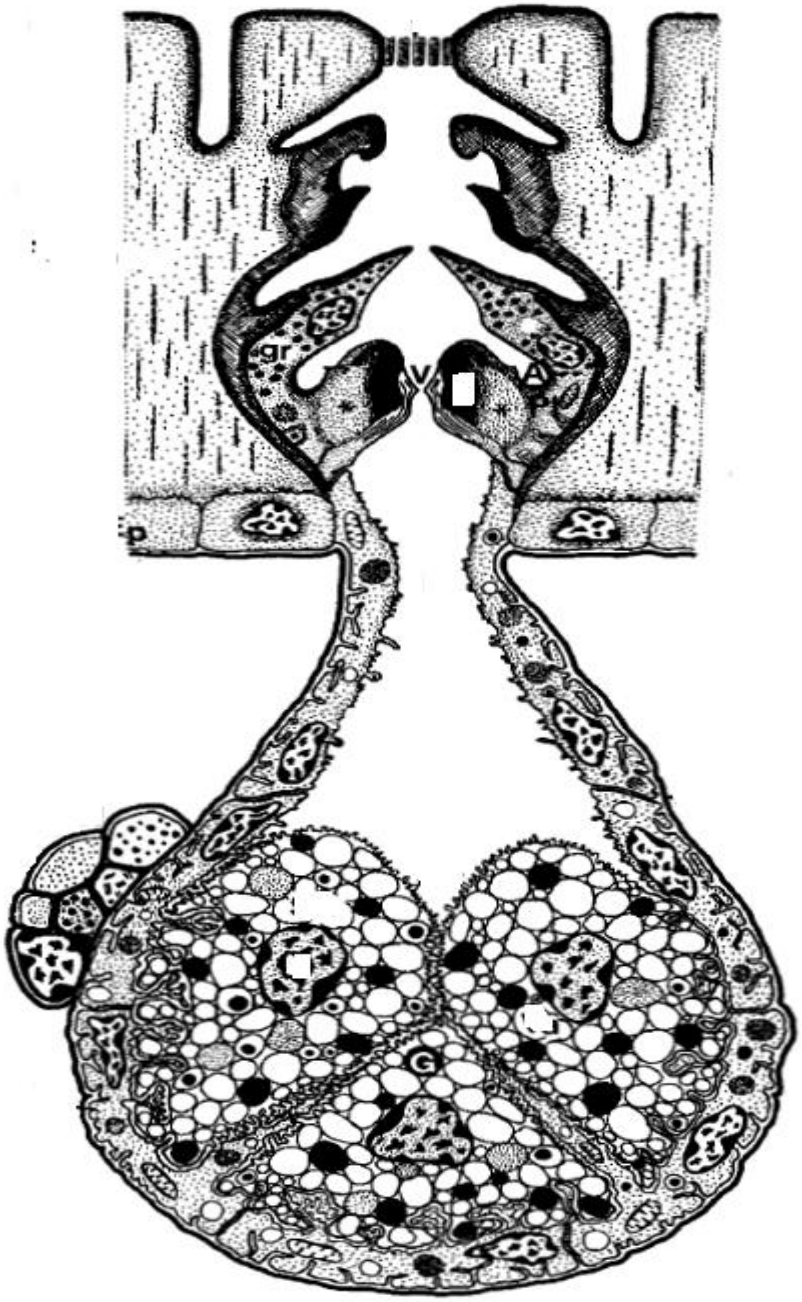


Figure 5: Foveal gland of *R. appendiculatus* tick.

2.7 Integration of Semiochemicals in Tick Management

Unlike in insects where pheromones have been used to control certain crop orchard pests (Judd and Gardiner, 2005) pheromonal based strategies for controlling tick has gained little progress. A wide range of innovative strategies employed to control selected insect pests uses pheromones as a key element in their components. These measures have met significant success, in which

pheromone-baited traps are now in existence for control of gypsy moths, Japanese beetles, and other important pests (Sonenshine, 1985). Sonenshine, (1984) and Ziv *et al*, (1981)in their reviews described the status of pheromone-assisted techniques for control of mites and ticks. Different strategies have included the use of lures, sexual confusants, antagonists of pheromone activity, and repellants to kill ticks and/or to disrupt their mating success. A good example is the use of allomones which have been highly effective in protecting stored food products against damage by acarid mites.

Application of pheromones alone in an effort to control ticks was not effective. Rather, they must be used in a blend with a toxicant. For effective use, these blends of compounds must be included into a slow-release delivery gadget otherwise any development in efficacy is fleeting (Sonenshine, 2006). Assortments of technologies are existing to retard emergence of pheromone compounds, e.g., incorporation into plastic, adhesive materials, paraffin, or gelatin microcapsules.

2.7.1 Pheromone-Assisted Matrix for Application to Vegetation

When blacklegged ticks (also called deer ticks) perceive components of the arrestment pheromone they form clusters when they encounter one another in the natural environment and recognize components of the arrestment pheromone. Components of this assembly pheromone include guanine, xanthine, and hematin. Guanine and xanthine are essential for nymphal assemblies (Allan and Sonenshine 2002), while adult assemblies are stimulated by guanine, xanthine, and hematin (Sonenshine *et al*., 2003). The incorporation of these compounds and an acaricide (permethrin) into oily droplets led to the development of a patented technology that, when applied to vegetation, kills ticks that come in contact with the droplets (Allan *et al.*, 2001). This type of gadget offers the prospect to control ticks with a single application, since the active compounds are released gradually over long periods. In a laboratory trial, formulations of the oily material with (control) and without pheromone were tested against female *I. scapularis*. The results indicated that the addition of the pheromone components increased the efficacy of the oily droplets from 70% for droplets with acaricide alone to 95% with both acaricide and pheromone. Encouraged by these results, researchers incorporated these compounds into a commercial

device, Last Call (IPM Technology, Inc., Portland, Oregon), a product comprising small adhesive droplets that can be delivered in large quantities from a hand-held pump sprayer.

2.7.2 Tick Decoy

Disruption of tick reproduction rather than killing was found to be effective. A pheromone-acaricide-impregnated device was developed to attract and kill male ticks before they could mate with feeding females (Hamilton and Sonenshine, 1989). A mixture of organophosphorus acaricides, attractant sex pheromone, 2,-6-dichlororphenol and an, propoxur, polyvinylchloride resin and molded into numerous small plastic spherules resembling feeding female ticks. Following curing by heating at 100°C for 20 min, the spherules were layered with an oily extract containing cholesteryl oleate, the mounting sex pheromone (MSP) of *D. variabilis*. When completed, the devices resembled the shape and pheromonal composition of a partially fed female tick. Emission of 2, 6-DCP from these plastic decoys was 0.9 ng min^{-1} when subjected to gas chromatography analysis and emission of the acaricide was estimated to be 152.5 ng min^{-1}. Mate-seeking *D. variabilis* males were attracted and were quickly killed. Further experiments were carried to check the efficacy of the device; decoys were attached to the hair coat of a rabbit host with cement and dispersed at a ratio of 10 decoys per each live female tick. Male ticks were killed before they could mate with female indicating that at this ratio, the decoys were highly effective. In a span of thirty minutes after application to a tick-infested host, 89% of the males released onto the animal were killed after an attempt to mate with the decoys; the remaining ticks finally died while trying to attach nearby. Fewer males attempted to mate with the single-pheromone decoys or attach beside them; most attached elsewhere or mated with live females. In a period of thirty minutes after release, 36% of the males had died. Most females on the tick-infested animal also died; the remaining failed to engorge, dropped off and died without laying eggs. With the use of this technique tick reproduction was completely disrupted. In Egypt, this same technique was also used to control camel ticks, *H. dromedarii*. For this purpose, the 2,- 6-DCP- and acaricide-impregnated decoys were mixed with cholesteryl esters from this tick species before they were lined to the hair coat of the animals. The response of the males was similar to that seen with *D. variabilis*, i.e., they preferred the copious decoys to the feeding females, attempted to mate with these devices, and died soon afterward. Test results showed an efficacy of 85.3% as well as eventual kill of the unmated females (Abdel-Rahman *et al.*, 1998).

Evidently, this technology offers an opportunity to eliminate a tick population in a semi-enclosed environment, for example farm, ranch, home, or kennel, with just a few applications. This same goal has been achieved rarely with acaricides alone only after massive consequence and cost. The efficacy of this technique lasted for up to 3 months hence tick control can be achieved with only two or three applications per year (Sonenshine, 2006). This is particularly helpful in tropical and subtropical regions where conventional tick control must be applied regularly.

2.7.3 Bont Tick Decoy

A specialized modified decoy concept for use on cattle to control the ticks *Amblyomma hebraeum* and *A. variegatum* has been developed. Ticks from these group form clusters on their hosts in response to Attraction-Aggregation-Attachment-pheromone emitted by the male (Norval *et al.*, 1991). In this adoption, methylsalicylate, *o*-nitrophenol, 2, 6-DCP, and phenylacetaldehyde, which were earlier shown to induce attraction and aggregation and attachment in this species (Norval *et al.*, 1991), were integrated into a plastic strip attached to the animal's tail. Also incorporated in the tail tags was a pyrethroid acaricide. Results from laboratory trials showed that the volatile pheromone components were released gradually, at a rate of 1% per day, and could provide uninterrupted tick control for a period of more than three months. Attraction of bont ticks from their hiding places in the surrounding grazing was more effective when AAA pheromone was combined with CO_2. CO_2 was provided in huge amounts by the herds of cattle to which the tail tag decoys were attached. In a three-month field trial involving hundreds of animals, including a control group, efficacy for cattle treated with cyfluthrin plus pheromone-impregnated tags was 94.9% and efficacy for cattle treated with the flumethrin plus pheromone-impregnated tags was 87.5%. When the test was repeated for a second three-month trial period, long-term efficacy for the same treatments increased to 99.3% and 95.1%, respectively. However, tags impregnated with pheromones plus α-cypermethrin was considerably less effective (Norval *et al.*, 1996). Maranga *et al.* (2003) used this model to attract *A. variegatum* to a treated site in the center of a circular field plot. Different concentrations of the AAA pheromone were dissolved in paraffin oil and the response of ticks released from varying distances was determined. When AAA was combined with a source of CO_2, within three hours 90% of ticks released into the field plot were attracted to the treated site. When used in

combination with an acaricide in a cage, or other animal enclosed space, this method afforded to attract and kill large numbers of bont ticks before they can infest the animals.

2.7.4 Confusants

Another way of distracting sexual activity between ixodid ticks is to "baffle" the males by saturating their surroundings with a glut of pheromone. With 2,- 6-DCP all over the host, males are not in a position to distinguish differences in concentration and find the feeding females. The longer they move in search of females, the greater the possibility that they will acquire a toxic dose of acaricide and die. To realize this objective, Sonenshine *et al.* (1985) dispensed a water solution of 2,- 6-DCP and acaricide impregnated into gel microcapsules onto dogs infested with ticks. Microscopic observations of the hair of these animals indicated microcapsules attached to the hair, causing constant discharge of the active ingredients for up to three weeks. Most of the male ticks were killed, and the a small number of surviving females laid less eggs compared to that produced by the controls (Sonenshine *et al.,* 1985).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Ticks

Rhipicephalus appendiculatus adult male and females were obtained from colonies maintained at International Livestock Research Institute (ILRI), Nairobi, Kenya. To obtain active males and females, the ticks were fed on rabbits (*Oryctolagus cuniculus*) for 2, 4 and 6 days. The rabbits were infested with a maximum of 100 adults of *R. appendiculatus*. After feeding, the ticks were removed and washed in tap water to remove blood.

3.2 Assembly Pheromone Sample Preparation

Nymphal cast skins and tick excreta were extracted with 0.95% saline as described previously by Allan *et al,* (2002) 1g of nymphal cast skins were placed in 5ml of 0.95% NaCl, vortexed three times for 20 sec, allowed to stand for 2hrs, and then all liquid was transferred to a separate vial. The skins were then washed twice with 100µl of 0.95% NaCl, and washes were added to the original extract. Unfed tick excreta were obtained from the vials where the ticks were held for 2 months. 1g of the excreta was put in 5ml of 0.95% saline and mixed thoroughly by votexing; it was then left to stand for 2 hrs. In addition, 1mg/ml of guanine (Sigma) was used and was prepared by dissolving 2mg of guanine was dissolved in 2ml of 0.95% saline and allowed to stand for 2 hours.

3.3 Two - Choice Assembly Bioassay

Ticks were tested for assembly behavior in a two–choice bioassay set up using the four quadrant technique as described by Arlian and Vyszenski-Moher (1995). Tests were conducted on a 9.5-cm diameter filter paper arenas fitted within a Petri dish. The filter paper was divided into quadrants and within each quadrant a 2 cm diameter circle was drawn 3 cm from the centre of the disc. The guanine, tick excreta and nymphal skin washings (assembly pheromone and sources) were applied in 25µL aliquots within each 2cm-diameter circle and air-dried prior to use. Application of test and control (saline) solutions alternated between quadrants (i.e. control, test, control, test) such that a test quadrant was adjacent to a control quadrant. In each trial, ticks

were exposed to only one test material at a time. Ticks were released in groups of 10 into the centre of the arena and each trial was replicated 10 times. Observations were made during the first 20 minutes of an experiment for behavioral assessment and at 1 hour, 2 hours and 24 hours later for counting purposes. Only ticks that were found at the treated and control quadrants were counted. Ticks that were found at the edges of the Petri dishes were not counted.

3.4 Protein Analysis of Foveal Glands Extracts

3.4.1 Protein Extraction

Foveal glands from each group were dissected from either unfed or fed ticks in ice-cold, phosphate-buffered saline (PBS: 5mM $Na_2HPO_4.12H_2O$, 1.6mM KH_2PO_4, 131mM NaCl, pH 7.2). The foveal glands were pooled, and proteins extracted using phenol extraction method of Hurkman and Tanaka (1986) with some modifications. Briefly, the foveal glands were suspended in 0.5 ml of extraction buffer (0.7 M sucrose, 0.5 M Tris, 30 mM HCl, 50 mM EDTA, 0.1 M KCl, 2% DTT, and 2 mM PMSF) in a 1.5ml eppendorf tube and homogenized. 250µl of the extraction buffer was added to the sample and incubated for 10 min at 4^oC. An equal volume of Tris-saturated phenol was then added. After 10 min with shaking at 4^oC, the phases were separated by centrifugation at 10000xg for 5 minutes. 200µl of phenol phase was recovered and re-extracted with an equal volume of extraction buffer. Phenol phases from both extraction steps were pooled. Proteins were precipitated from the phenol phase by addition of 5 volume of 0.1 M ammonium acetate in methanol and incubated at -20^oC overnight then centrifuged at 10000×*g*. The pellet was washed three times with ice-cold ammonium acetate in methanol and once with acetone. The pellet was then dried at room temperature for 10 minutes and dissolved in PBS. The protein samples were centrifuged at 18400×*g* to remove insoluble material and stored at -80^oC until used. Further, the samples were desalted and concentrated using the microspin column (microcon, amicon bioseparation, millipore) of molecular weight cut off 3000 Daltons according to manufacturer's instructions.

3.4.2 Determination of Protein Concentration

Total protein concentration of the extracts were determined using the colorimetric method at 750nm according to Lowry *et al.* (1951), with bovine serum albumin as the standard with range

of 0.2- 2.0 mg/ml (Appendix 1). Protein sample solution were pipetted (10 µl) into 1.5ml eppendorf tubes and volume adjusted to 100µl with the PBS buffer. 100µl of Lowry reagent1 (2% Na_2CO_3, 2% NaKtartrate, 1% $CuSO_4 \cdot 5H_2O$) and contents mixed by vortexing and incubated at room temperature for 20 minutes. 200µl of Folin reagent was added and votexed to mix and incubated in dark at room temperature for 30 minutes. Absorbance at 750nm was measured against a reagent blank prepared from 100ul of the PBS buffer, 100µl of Lowry reagent and 200µl of folin reagent. Concentration of BSA was plotted against corresponding absorbance to obtain the standard curve used to determine the protein in the sample.

3.4.3 Two- Dimensional Gel Electrophoresis

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was performed as described by Mireji *et al.* (2006). The protein samples were solubilized in urea buffer (7M urea, 2M Thiourea, 4% CHAPS, 5% Triton X-100, 100mM DTT 2% ampholytes pH 3-10). For each tube gel isoelectric focusing, the gel was pre-run for 15 min at 200V, 30 min at 300V and 60 min at 400V. Protein sample (50 µg) were loaded and isoelectrically focused for 1 h at 150V, 200V for 1 h, 400V for 17 hrs and finally 800V for 1h. Gels were equilibrated in equilibration buffer one (375mM Tris–HCl (pH 6.8), 2% SDS, 100mM DTT and 10% glycerol) for the first 15 minutes then for another 15 minutes in equilibration buffer two: (6M urea 2% SDS, 375mM Tris- HCl (pH 6.8) 10% glycerol, 60mM iodoacetamide and few crystals of Bromophenol blue). Separation in the second dimension were performed in 12% polyacrylamide (SDS-PAGE) mini gel and elecrtrophoresed at 20mA for 30min and at 30mA until the tracking dye has reached 1cm from the bottom. The gels were silver stained to reveal protein spots. The gel images were digitized with digital camera (Panasonic 6X optical zoom; 7.2 mega pixel, model DMC-127).

3.4.4 Two-Dimensional Gel Image Processing

Second dimension gels were analyzed using Melanie 2D gel analysis software (GENEBIO VERSION 7.0). Apparent molecular weights of proteins were determined by analysis against the co-electrophoresed 10–200 kDa SDS-PAGE molecular weight standards (Fermantas). Apparent isoelectric points (pIs) of proteins were determined by calibration against the 3–10 pH gradients of IEF electrophoresis. Results obtained by computer-aided evaluation were compared with

visual analysis of the original gels. Changes in specific polypeptides were recorded only when they occurred in all the replicated gels. Quantitative comparisons between gels were achieved using normalized spot volumes. Differences less or equal to ±1.5-fold in any of the treatments against control among matched protein spots were considered significant. The spots were therefore categorized as up- or down-regulated if the differences were ≥1.5 or ≤1.5, respectively or unaffected (if the differences were within ± < 1.5-fold change. Both differentially and constitutively expressed protein spots with pH less than, equal to or greater than seven were categorized as acidic, neutral or basic, respectively. The spots were also categorized as having low, medium or high molecular weight if they were 10 – 69, 70 –133 and 134 –200 kDa, respectively.

3.5 Data Analyses

Data generated from behavioral experiment were expressed as the percentage of ticks in a test quadrant and also as the aggregation index (AI) , AI = 100 × [(No. attracted to treated − no. attracted to control)] / (total no. of ticks). The control in the AI calculation was derived from the number of ticks in 0.95% saline-treated quadrants and used to rule out potential left- and right-hand bias. Data were analyzed using student t-test. Patterns of distributions of differentially expressed proteins against constitutively expressed proteins were analyzed using Melanie 2D gel analysis software (GENEBIO version 7.0). All statistical analyses were conducted through SPSS (SPSS Corporation, Chicago, Illinois, Statistical Package version 11.5).

CHAPTER FOUR

4.0 RESULTS

4.1 Tick Response to Assembly Pheromone

The wandering periods for *R. appendiculatus* were 3 days for fully fed females, characterized by rapid crawling activity. At the end of wandering, presumably in preparation for egg laying, the ticks settled with their anterior end buried at the edge of the Petri dish and remained completely immobile. While crawling, they waved their front pair of legs and paused for 2 minutes with front legs raised between bouts of regular crawling activity. On occasion, they gathered in small groups at the edge of the Petri dish, for as long as 20 min, during which some ticks would leave the cluster and resume crawling, would pause, or would join a different cluster.

For each non-fed tick, ticks stopped momentarily directly on or in the close vicinity of spots treated with guanine, excreta or nymphal skin wash. At these points the tick would lower its body, retract its legs and become akinetic. There was resultant cluster formation as arrestment occurred in other ticks that passed by and encountered the treated areas (figure 4, 5 and 6). This took place within 30 min. With few exceptions, no additional movements were observed thereafter, as the ticks remained aggregated for up to a day without ticks re-positioning in the cluster or crawling to adjacent quadrants.

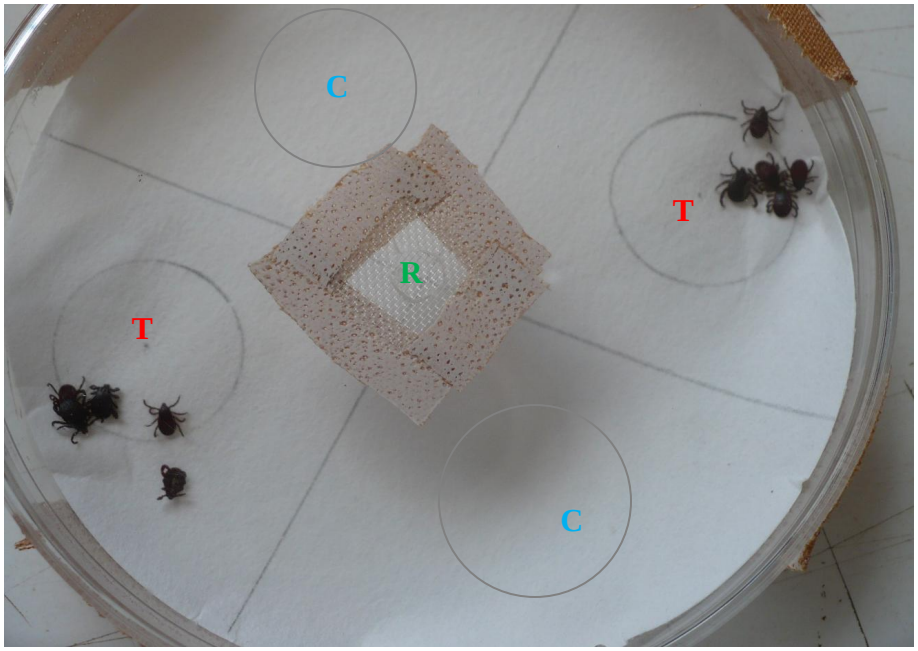


Figure 6: Response of unfed *R. appendiculatus* adult ticks to guanine treated quadrants. R- Point of release, T- Treated surface and C- Control surface

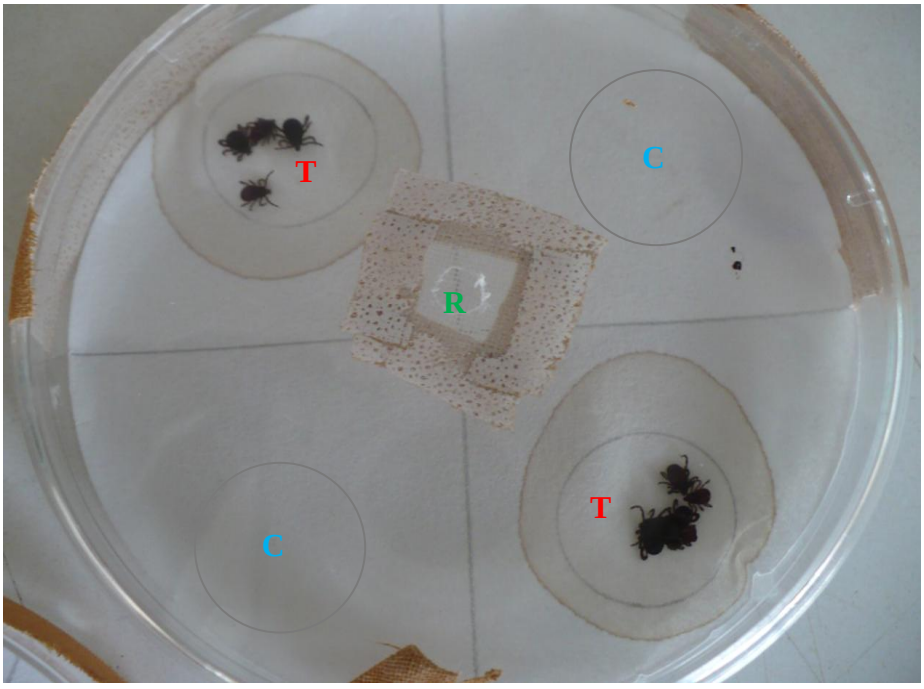


Figure 7: Response of unfed *R. appendiculatus* adult ticks to tick excreta treated quadrants. R- Point of release, T - Treated surface and C- control surface.

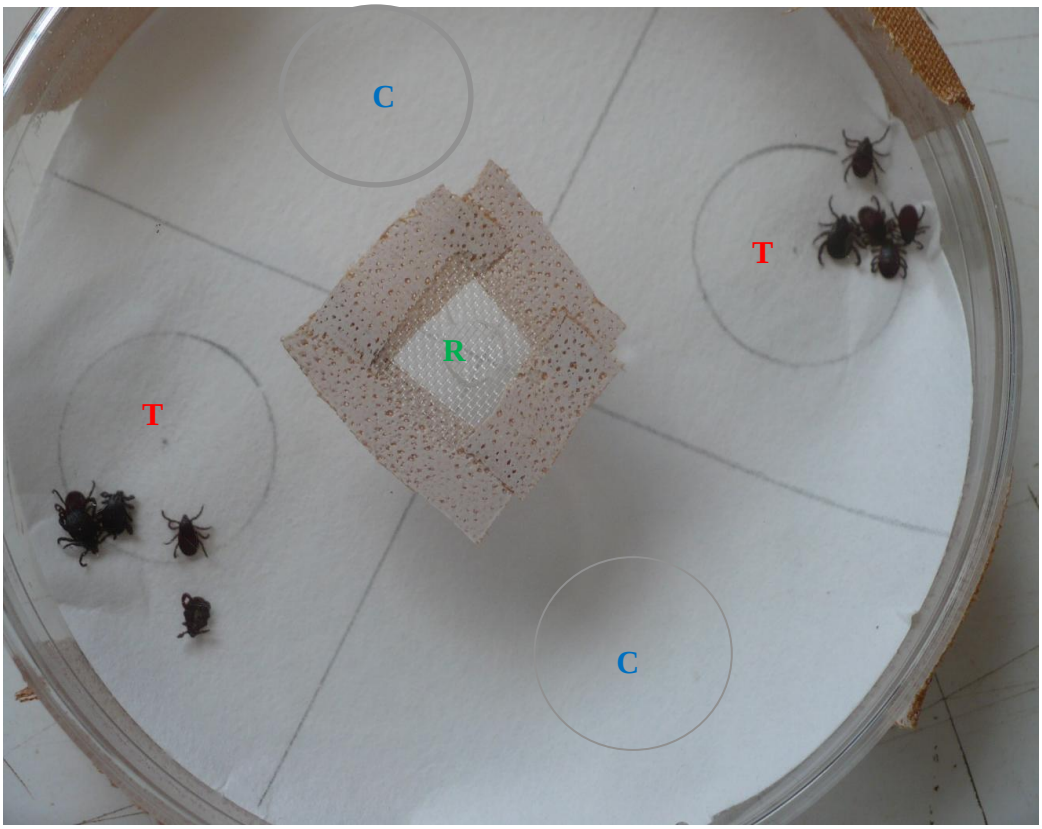


Figure 8: Response of unfed *R. appendiculatus* adult ticks to nymphal skin wash treated quadrants. R- Point of release, C- control surface and T- Treated surface.

Tick excreta, guanine and nymphal skin washings accounted for arrestment of an average of 74% of non-fed stages, which was significantly different from the number of ticks counted in untreated control quadrants. Guanine, nymphal skin washings and excreta, elicited significant arrestment activity averaging 63% compared with control 4% responses across the non-fed stages (Table 3). Within 1 hr, there were more ticks in sectors with nymphal skin washing (t=-*2.418*, df = 38, $p < 0.05$), tick excreta (t =-4.834, df = 38, $p < 0.05$), and guanine (t =*-1.853* df =38, $p < 0.05$) compared to controls. Similarly, by 2 and 24 hrs there were more ticks in the sectors with nymphal skins washings (t = -2.042, df =38, $p < 0.05$; t = - 3.907, df=38, $p < 0.05$, respectively), tick excreta (t=*-1.244*, df=38, $p < 0.05$; t =*-5.836*, df =38, $P < 0.05$, respectively), and guanine (t =-1.853, df =38, $p < 0.05$; t =-1.853 df = 38, $p < 0.05$, respectively) than in the controls. There was significant difference in tick response between 1 and 24 hr in sectors containing nymphal skins washings (t=*-5.836 df =38 p <0.05*), excreta (t =*3.907*, df = 38, p<*0.05*), guanine (t = -1.853, df =38, p <0.05), than in controls where there was no difference (t = 2.494, df = 38, p< 0.05).

Table 3: Assembly of unfed *R. appendiculatus* adults in petri dish quadrants containing nymphal skin washings, tick excreta and guanine. Each mean represents 10 replicates with 10 adults per replicate**.**

	Ticks in treatment sector (Mean ± S.E)			
Time (hr)	Control	Nymphal Skin Washings	Guanine	Excreta
1	0.2± 0.136	1.8± 0.270 ^{b}	2.3± 0.228 ^{b}	5.0± 0.308 ^{b}
2	0.2± 0.136	2.0± 0.256 ^{b}	2.3± 0.219 ^{b}	5.1± 0.315 ^{b}
24	0.18 ± 0.153	1.9± 0.244 ^{b}	2.5± 0.235 ^{b}	5.0 ± 0.276 ^{b}

b Significantly different from the control, *t* test, $p < 0.05$.

Counts of fed stages by day in treated quadrants (excreta, guanine or nymphal skin washings) were not significantly different from the control (Table 4). The counts in the various treatments at different days were; excreta treated quadrants two day fed(*t= 3.329, df= 19, p<0.05),* four day fed (*t= 0.052, df=19, p<0.05*), six day fed (*t= 0.067, df=19, p<0.05)*; guanine two day fed(*t= 1.000 df= 19 p<0.05),* four day fed (*t=1.000, df=19, p<0.05),* six day fed (*t= 0.453 df=19 p<0.05);* nymphal skin washings two day fed(*t= 1.228, df= 19, p<0.05),* four day fed (*t= 0.438, df=19, p<0.05),* six day fed (*t= 0.428, df=19, p<0.05)* from counts of ticks in the control quadrants (two day fed *(t= 3.329, df= 19, p<0.05;* four day fed *t= 0.04,2 df=19, p<0.05,* six day fed *t= 0.567, df=19, p<0.05)*

Table 4: Assembly of fed *R. appendiculatus* adults in petri dish quadrants treated with 0.95% saline (control), nymphal skin washings, tick excreta and guanine. Each mean represents 10 replicates.

	Number of ticks in treatment sector (Mean ± S.E)			
Days	Control	Nymphal Skin Washings	Guanine	Excreta
0	0.35±0.30	0.47±0.18*	2.10±0.56*	0.73±0.39*
2	0.38±0.29	0.20±0.19	0.55±0.46	0.3±0.27
4	0.02±0.73	0.05±0.20	0.55 ±0.54	0.2±0.14
6	0.05±0.09	0.95±0.11	0.52±0.33	0.1±0.19

* Denotes significant difference *t* test; ($p < 0.05$) from the control.

On average, both male and female ticks were likely to be attracted to all types of exposures equally. This is reflected in the time of exposure with sex type (Table 5 and 6). In the guanine-treated surface there were significant differences after 1 hour in the number of the male and female ticks (*t= -4.385, df =18, p < 0.05; t= -5.237, df = 18, p < 0.05*) respectively compared to controls. Similarly, at 2 and 24 hours there were more males attracted (*t= -5.073, df =18, p < 0.05;* (t= *-5.161, df =18, p < 0.05*) respectively compared to control. In the surface treated with excreta there was significant difference in the number of male ticks after 1 hour (*t= -8.910, df =18, p< 0.05*) 2 hours (*t = - 10.713, df= 18, p < 0.05*) and 24 hours (*t= -8.677 df= 18, p < 0.05*) of exposure compared to the control. Equally in the female population, there was significant difference in the excreta-treated surface at1 hour 2 hours and 24 hours post-exposure (*t = df = p< 0.05; t = df = p< 0.05; t = df = p< 0.05*) respectively. In the nymphal skin washing treated surface at 1 hr there was difference in the number of males attracted (*t= -2.426, df= 18, p < 0.05*); 2 hours (*t= -3.834, df= 18, p < 0.05*) and 24 hours (*t= -3.184, df= 18, p < 0.05*). There were also differences in the number of females present in the sector at 1 hour 2 hours and 24 hours (*t= -3.320, df = 18, p < 0.05; t=-3.015, df= 18, p < 0.05); t= - 3.737, df= 18 p < 0.05*) respectively, compared with control.

Table 5: Assembly of unfed *R. appendiculatus* female adults to assembly pheromone in petri dish bioassay. Each mean represents 10 replicates.

	Ticks in treatment sectors (Mean ± SE)			
Time (hr)	**Control**	**Guanine**	**Excreta**	**Nymphal skin washings**
1	0.40 ± 0.16	2.0 ± 0.26*b*	5.2 ± 0.47*b*	1.8 ± 0.39*b*
2	0.50 ± 0.22	2.2 ± 0.25*b*	5.3 ± 0.40*b*	1.9 ± 0.41*b*
24	0.50 ± 0.17	2.0 ± 0.26*b*	5.4 ± 0.45*b*	2.4 ± 0.37*b*

b significantly different from the control, independent sample test, p< 0.05

Table 6: Assembly of unfed *R. appendiculatus* male adults to assembly pheromone in petri dish bioassay. Each mean represents 10 replicates.

	Ticks in treatment sectors (Mean ± SE)			
Time (hr)	**Control**	**Guanine**	**Excreta**	**Nymphal skin washings**
1	0.6 ± 0.22	2.5 ± 0.37*b*	4.8 ± 0.47*b*	1.7 ± 0.40*b*
2	0.4 ± 0.22	2.7 ± 0.40*b*	4.7 ± 0.34*b*	1.8 ± 0.29*b*
24	0.5 ± 0.22	2.6 ± 0.34*b*	4.8 ± 0.44*b*	1.9 ± 0.38*b*

b significantly different from the control, independent sample test, $p < 0.05$

4.2 Two Dimensional Gel Electrophoresis of Foveal Glands Proteins

2-Dimensional gel electrophoresis analysis of foveal glands proteins showed that most of the differentially expressed spots had acidic charge and were of low molecular weight among all stages of feeding and between the sexes. The number of acidic induced spots increased in unfed and 2 day fed stages of feeding except in the 4^{th} and 6^{th} day of feeding population in both sexes. The proportion of proteins increased with the level of feeding; however, there were less differentially expressed proteins in male populations than in female population.

Overall, more protein spots were differentially expressed in the fed populations than in control populations. Only five protein spots (nos 1-5) were down-regulated, where spot 1 and 2 were basic and of molecular weights 40 and 33 kDa respectively, spot 3, 4 and 5 were acidic and of molecular weight 25, 15, and 90 kDa respectively (Fig. 3) this is in contrast to 2day level of feeding where- 15 spots were up-regulated in female population (Fig. 4). Most of the up-regulated spots were acidic and of low molecular weights (Fig. 4). Most notably, were spots numbered 10 (pI 7.0; Mr 65 kDa) and13 (pI 7.0; Mr 10 kDa) (Fig. 4). Comparison of 2 day fed female group against 4day fed female group indicated that only 3 spots were down-regulated (Fig. 4), while 8 spots were up-regulated among the female group (Fig. 5). Most of the up-regulated were basic and were categorized as low molecular weights proteins. The most prominent spots were spot 3, (pI 9.8; Mr 69 kDa), spot 4 (pI 9.0; Mr 62 kDa) (Fig. 5). Equally, comparison of 4day fed female group against 6day female group showed that 4 spots were down regulated and 6 spots were up-regulated (Fig. 5 and 6 respectively). Among the differentially expressed protein spots, all the down regulated spots were basic with varying molecular weights

ranging from 12-120 kDa (Fig. 5), while in the up-regulated spots; most were classified as acidic with high molecular weights (Fig. 6). However, spots 5 and 6 with pI 5.5 and 4.6 and of molecular weights 30 and 14 kDa respectively were the most prominent in the up-regulated group.

A similar trend was observed in the male population where 5 spots were down-regulated in the unfed (control) group (Fig. 7), and 11 spots were up-regulated when compared against 2 day fed group most of which were classified as acidic and of low molecular weight (Fig. 8). Most significantly up-regulated spots were nos. 4 (pI 7.0; Mr 60 kDa), 8 (pI 4.0; Mr 65 kDa), 9 (pI 4.0; Mr 17 kDa) and 11 (pI 5.0; Mr 11 kDa) (Fig. 8). When 2 day fed male group was compared to 4 day fed male group, 4 spots were found to be down-regulated while 6 spots were up-regulated (Fig. 8 and 9 respectively). In the down-regulated spots pool all the spots were acidic with molecular weights 10, 14, 18, and 65 kDa respectively. Within the up-regulated spots pool most spots were categorized as basic with low molecular weights with the most prominently being spot 2 of pI 8 and Mr 12 kDa (Fig. 9). Fewer number of spots were down-regulated (Fig.9) and 5 spots were up-regulated in a comparison between 4 day fed group and 6 day fed group (Fig. 10). Spots 1 and 2 that were down-regulated were categorized as acidic and of low molecular weight with (14 kDa). Up-regulated spots showed acidic characteristics with high molecular weights ranging between 120-180 kDa. Most notable spot was spot 2 with pI 6.0 and of Mr 180 kDa (Fig. 10).

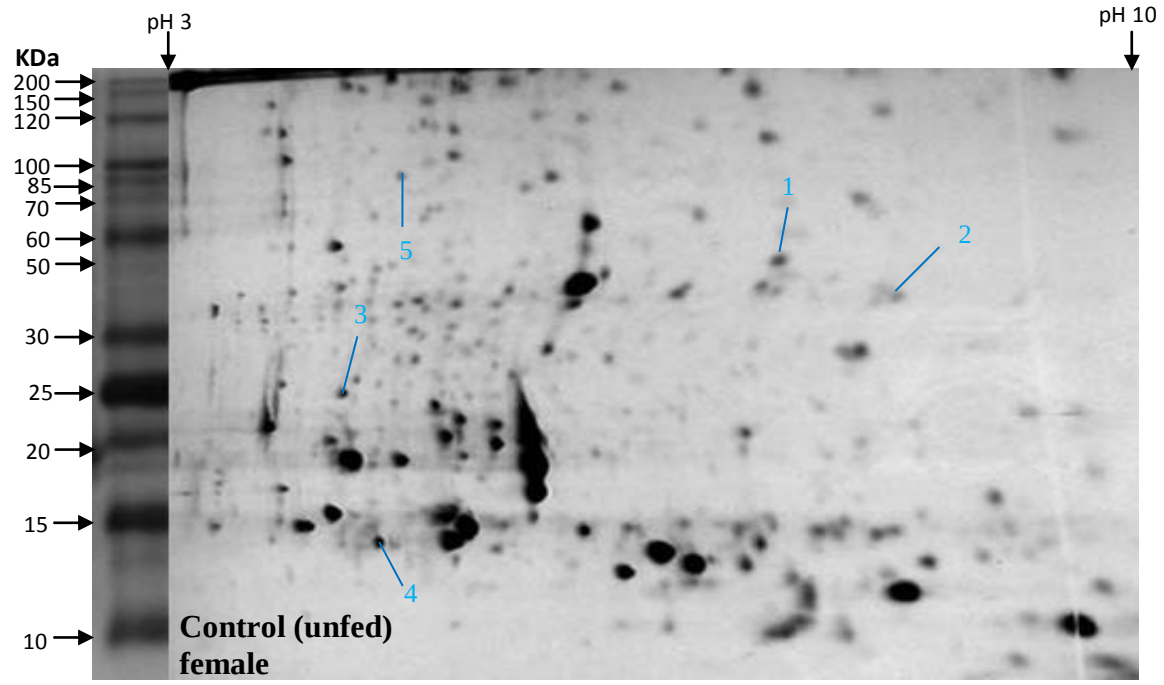


Figure 9: Two-dimensional separations of foveal gland proteins of unfed female adult *R. appendiculatus*. Spots indicated by lines and numbers are differentially expressed in response to feeding. Spots indicated in blue colour are down-regulated.

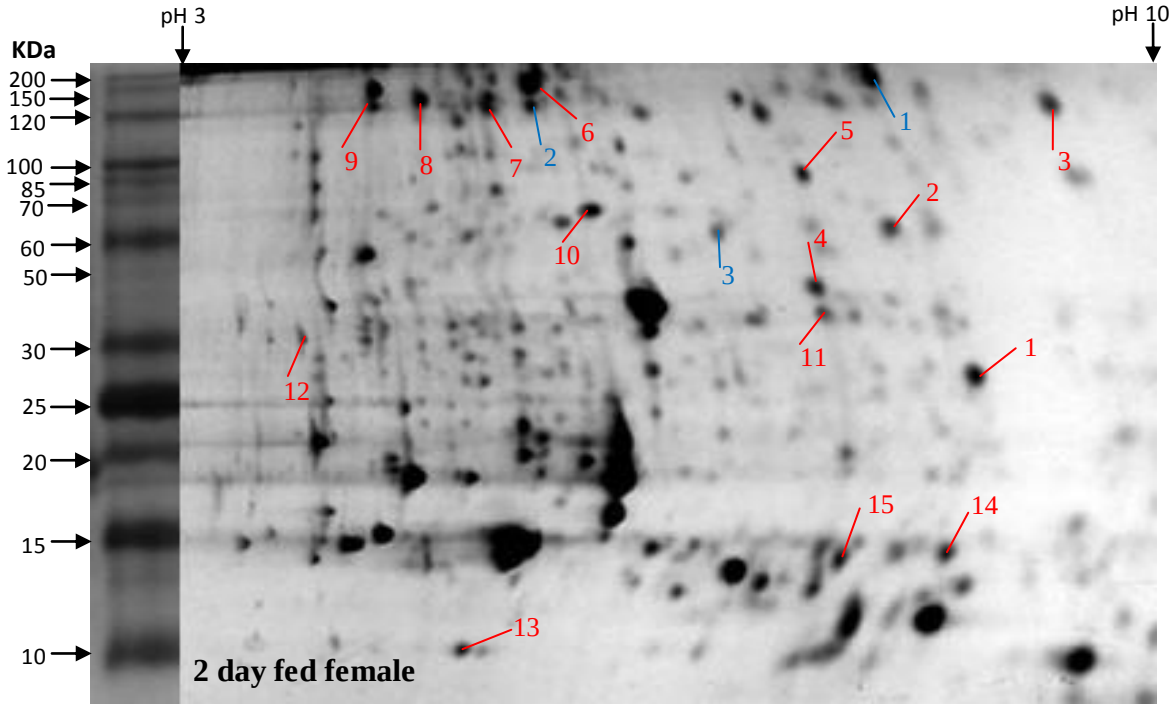


Figure 10: Two-dimensional separations of foveal gland proteins of 2 day fed female adult *R. appendiculatus.* Spots indicated by lines and numbers are differentially expressed in response to feeding. Spots indicated in red are up-regulated while in blue are down-regulated.

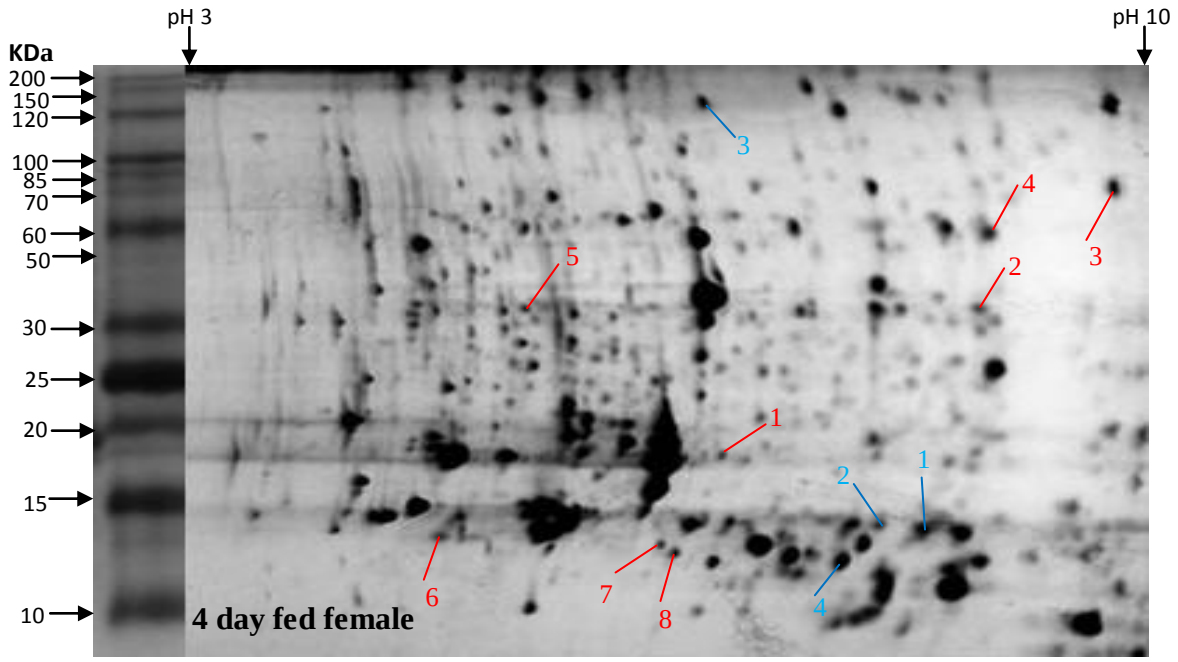


Figure 11: Two-dimensional separations of foveal gland proteins of 4 day fed female adult *R. appendiculatus.* Spots indicated by lines and numbers are differentially expressed in response to feeding. Spots indicated in red are up-regulated while in blue are down-regulated.

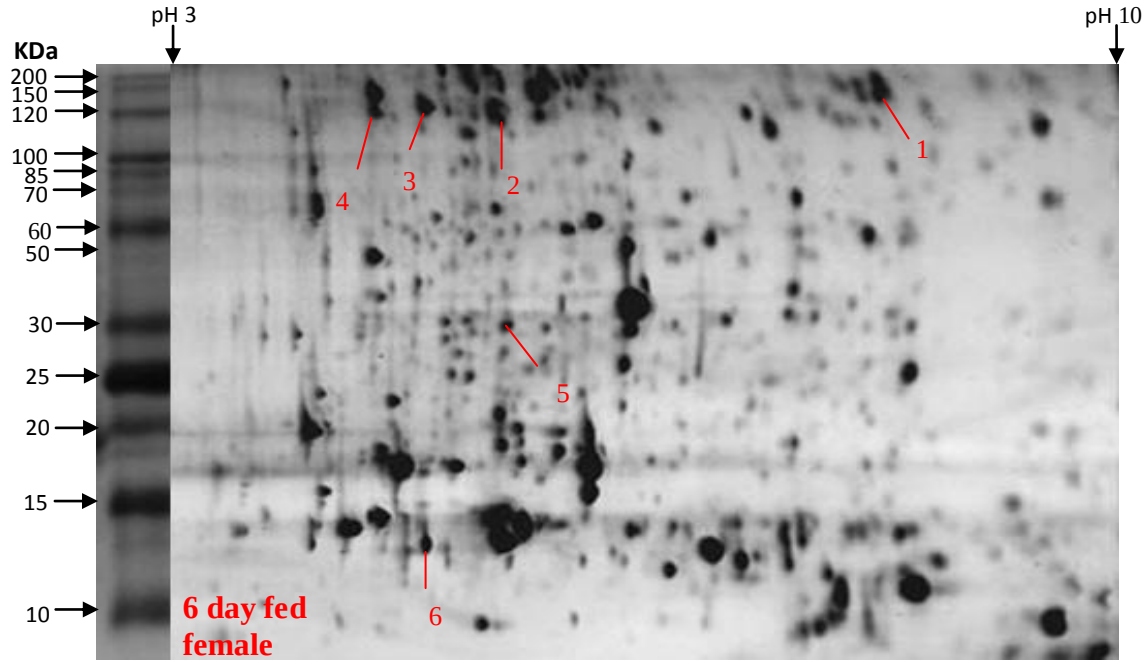


Figure 12: Two-dimensional separations of foveal gland proteins of 6 day fed female adult *R. appendiculatus.* Spots indicated by lines and numbers are differentially expressed in response to feeding. Spots indicated in red are up-regulated.

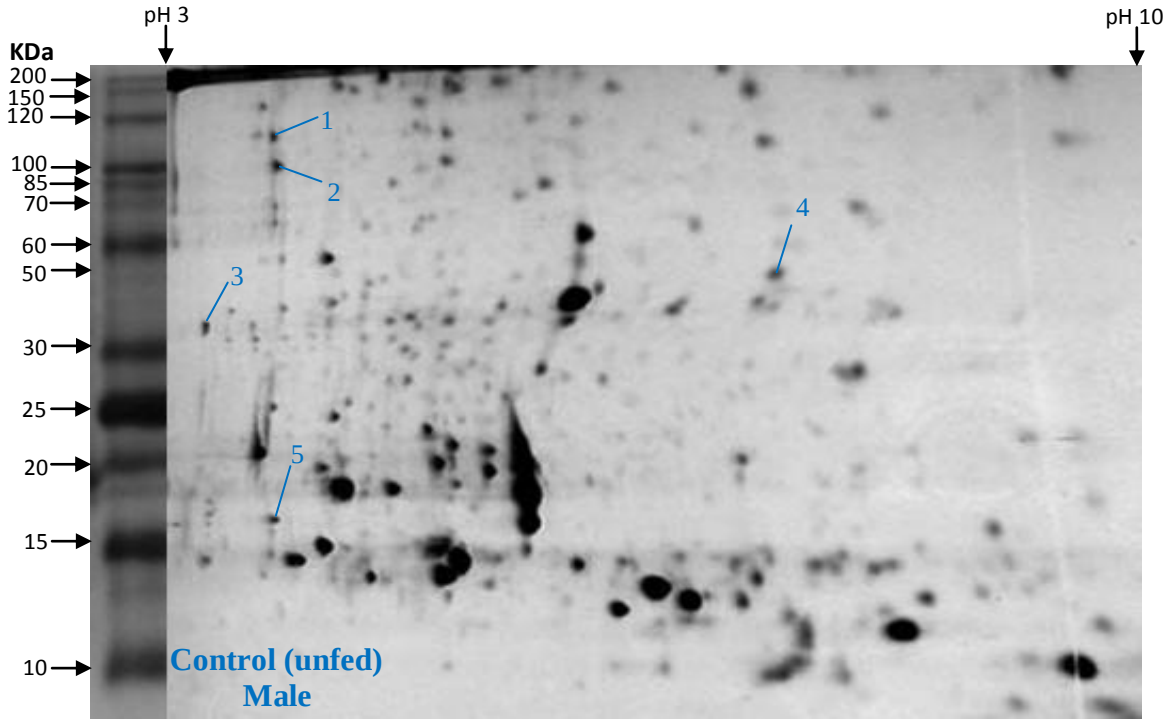


Figure 13: Two-dimensional separations of foveal gland proteins of unfed male adult *R. appendiculatus*. Spots indicated by lines and numbers are differentially expressed in response to feeding. Spots indicated in red are up-regulated while in blue are down-regulated.

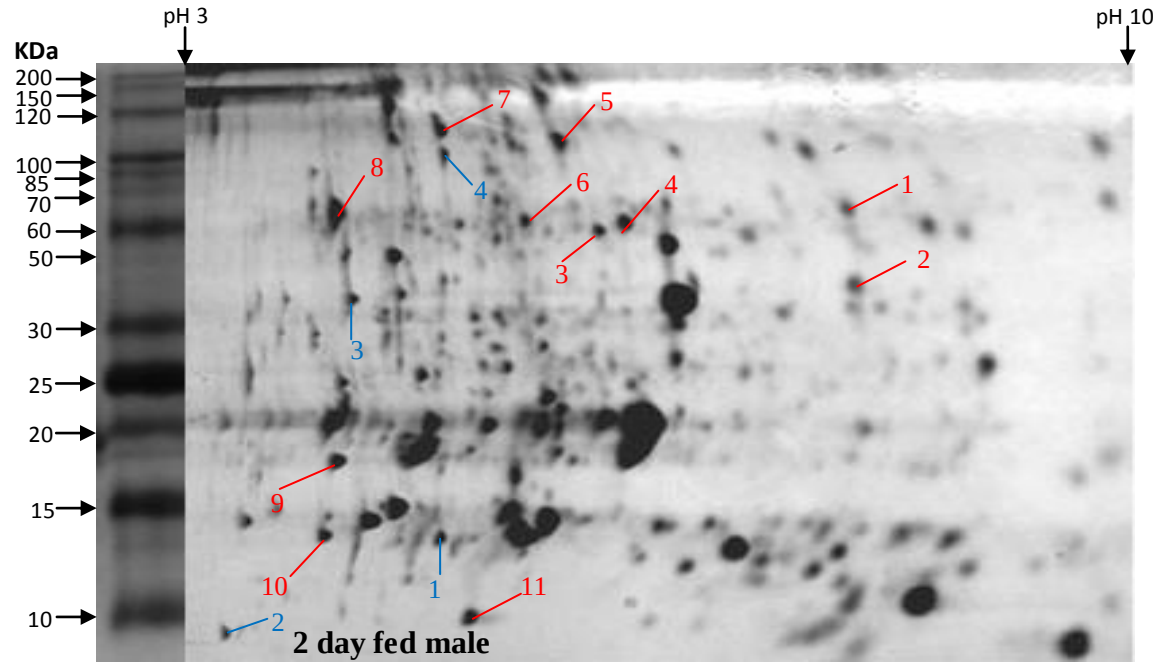


Figure 14: Two-dimensional separations of foveal gland proteins of 2 day fed male adult *R. appendiculatus*. Spots indicated by lines and numbers are differentially expressed in response to feeding. Spots indicated in red colour are up-regulated and in blue are down-regulated.

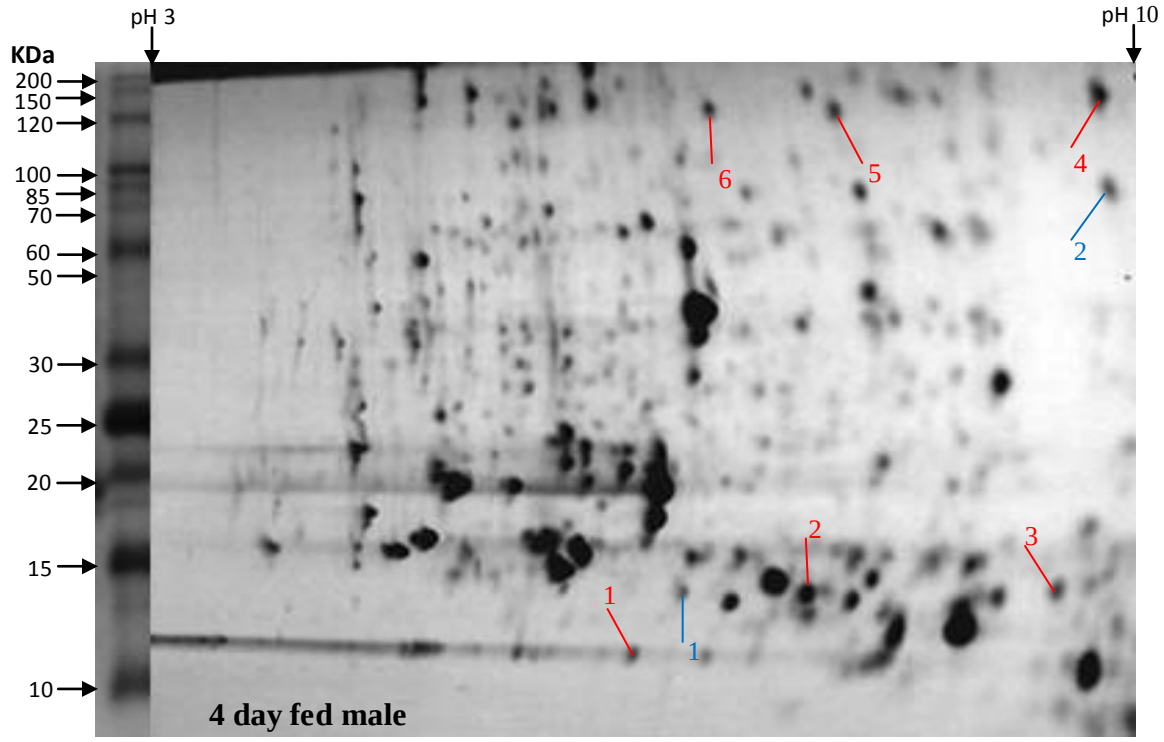


Figure 15: Two-dimensional separations of foveal gland proteins of 4 day fed male adult *R. appendiculatus*. Spots indicated by lines and numbers are differentially expressed in response to feeding. Spots indicated in red colour are up-regulated and in blue colour are down-regulated.

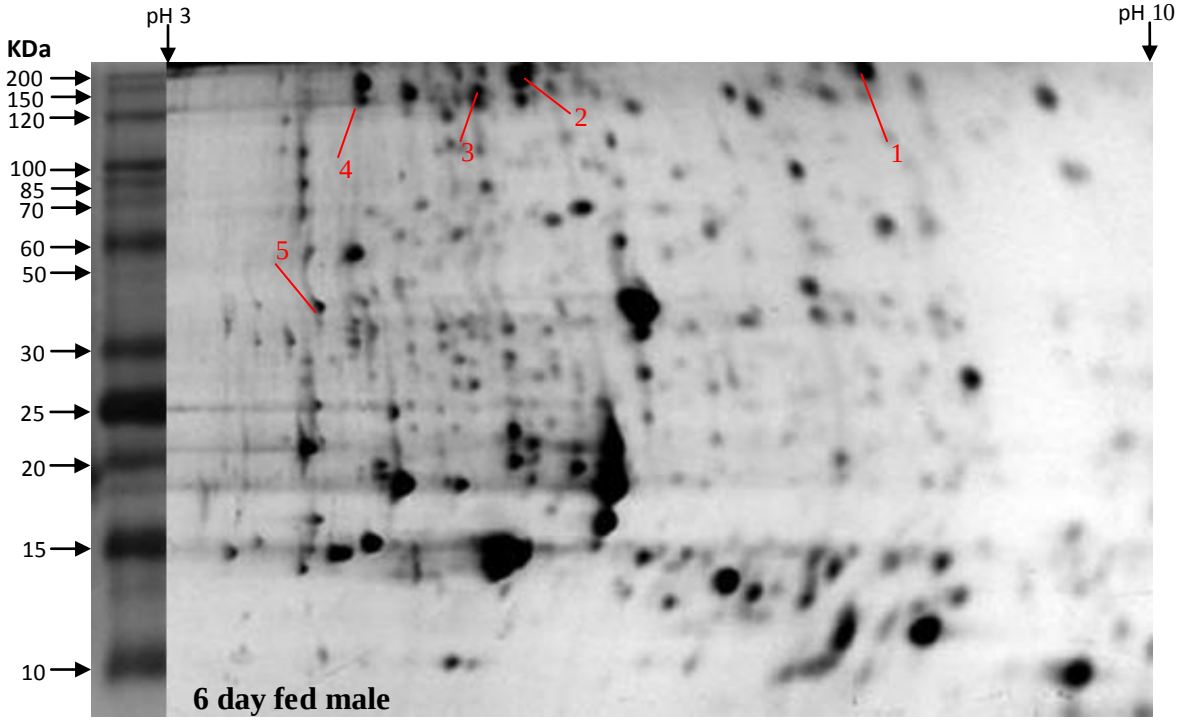


Figure 16: Two-dimensional separations of foveal gland proteins of 6 day fed male adult *R. appendiculatus*. Spots indicated by lines and numbers are differentially expressed in response to feeding. Spots indicated in red are up-regulated.

CHAPTER FIVE

5.0 DISCUSSION

5.1 Response of *R. appendiculatus* to Assembly Pheromone

The assembly of ticks to assembly pheromone-treated surfaces indicates that in *Rhipicephalus appendiculatus*, elicits response from only non-fed stages in the life cycle, complementing work on adults by Leahy *et al.,* (1981) which showed that assembly of unfed, adult *Hyalomma dromedarii* tick was triggered by assembly pheromone. Lack of perception of assembly pheromone by engorged stages is not unusual, and the condition has been described as being 'switched off ' after feeding in the camel tick *H. dromedarii* Koch (Leahy *et al.*, 1981), bont tick *A. hebraeum* Koch (Rechav, 1978), argasid *Ornithodoros moubata* Murray (Leahy *et al.*, 1975) and *A. americanum* (Yoder *et al.,* 2008). Indeed, assembly pheromone and associated compounds that induce arrestment may be useful in control efforts because they can be applied as retainers to prevent ticks from escaping traps or to keep ticks on acaricide treated surfaces. However, because of their non-volatility the mediating compounds (guanine, xanthine and hypoxanthine) display no attractant properties over space and would be ineffective at luring ticks into traps (Sonenshine, 2006).

Excretion of nitrogenous waste products by ticks generally occurs after blood feeding and immediately after molting thus contaminating the cast skins. This perhaps explains the assembly response of ticks to quadrants treated with nymphal skin washings. Locations with an accumulation of excretory products are likely to be conducive to tick survival as these indicate successful completion of blood feeding and molting and have appropriate environmental conditions for tick survival (Otieno *et al.,* 1985). Although there may be differences in sites of molting of different stages of *R. appendiculatus*, arrestment of tick movement in sites containing excretory products may result in assemblies of ticks of different stages since assembly pheromones are nonspecific in activity.

The assembly behavior in response to guanine, the key active assembly pheromone component, of some tick species (Otieno *et al.,* 1985) was lower than that of tick excreta and nymphal skin washings of *R. appendiculatus* showing that the tick assembly pheromone of this tick species,

like that of most species are multi-component (Sonenshine *et al.,* 2003). Indeed, tick excreta involve a heightened level of response by approximately 10% more ticks than guanine alone. The highly interspecific nature of assembly pheromone suggests that use of excreted guanine as an active ingredient of assembly pheromone is probably widespread in ticks with additional excretory products (such as related purines, haematin hypoxanthine and xanthine) serving in combination to elicit synergistic responses (Allan and Sonenshine, 2002). Other compounds such as xanthine, hypoxanthine, and uric acid also elicited significant responses with response levels greater than 75% (Otieno *et al.,* 1985). Though, this was not tested in this experiment, in addition to guanine, Dusbabek *et al.* (1991) also reported significant assembly responses (>55%) of *A. persicus* males to xanthine, hypoxanthine, and inosine.

Feeding and mating complement location of off-host environment during assembly. Accordingly, when opportunity avails itself at appropriate juncture responses to host-derived kairomones and sex pheromones take precedence over assembly (Petney and Bull, 1981). It follows then that assembly pheromone detection is 'switched off' for fed stages because they no longer need to conserve moisture or require a host. Despite being bloated with blood, one distinguishing feature of fed stages is their remarkable, albeit only for a few days, capacity to crawl quickly. Because fed stages are no longer retained by assembly-induced arrestment, this enables fed stages to leave the host to seek cool, moist reprieves for egg laying, thereby contributing to expanding the clustering range within the habitats.

Periods of the most intense excretory production in ticks occur within a few days after moulting (nymphs and adults) and hatching (larvae) (Yoder *et al.,* 2008) which is probably when the bulk of assembly pheromone blend (guanine) is laid down. Thus, assembly pheromone serves as a signal of successful development (Yoder *et al.,* 2008). Because maintaining water balance is the major off host problem for ticks (Wharton, 1985), assembly pheromone functions to keep ticks in sites selected by preceding fed stages that relied on minimizing water stress for proper development (Yoder *et al.,* 2004). Assembly pheromone has the dual effect of maintaining a concentrated population of *R. appendiculatus by* retaining ticks in habitats where hosts are prevalent. However, these effects are not observed in the unrestrained, highly mobile fed stages that actively seek out moisture-rich and protected sites for moulting and oviposition. Resultant

non-fed stages are retained by elimination of their own excreta (with assembly pheromonal blend) which serves to keep them within attainable distances of potential hosts.

5.2 Molecular Responses

One of the notable features of ixodid ticks is the fact that in both their resting and questing stages they are not fully developed (Slovak *et al.,* 2000). Indeed, the development of their foveal glands occurs simultaneously with the initiation and commencement of blood feeding on their host. At the same time, the protein content increases rapidly from unfed to the fed stages.

The assembly response by the unfed tick and the failure of the fed tick to respond to the assembly pheromone clearly demonstrates that there is change in behavior of the *R. appendiculatus* following blood meal, which is accompanied by biochemical changes as illustrated by the differentially expressed proteins in the present study. This suggests that this behavioral change is mediated by differentially expressed proteins. These behavioral changes in *R. appendiculatus* in response to assembly pheromone in the fed populations is accompanied by the biochemical changes in the foveal gland resulting in pheromonal switch from assembly pheromone to mutual aggregation mediated by sex attractant signal. Biochemical analysis of the foveal gland protein extract showed the complexity of this particular biological organ which is illustrated by the differentially expressed proteins. These differentially expressed proteins may arise from altered gene expression, protein modification, degradation or changes in protein stability (Shang and Wang, 2009).

There were protein profile variations among the stage of feeding, reflecting changes in the tick population. The variations in induction and expression of the genes between days of feeding can be a reflection of dynamics underlying gene expression in response to a blood meal. This can be in a bid to commence pheromonal switch by regulation of genes responsible for pheromone production.

Some of the up-regulated proteins expressed in response to a blood meal may be enzymes, which play important role in cellular biosynthesis of pheromones. The relatively equal expression of these proteins in the 4 and 6 fed day populations could be because the biosynthesis of

pheromones has reached completion. However, the underlying mechanisms mediating reduced expression of low molecular weights proteins concomitant with level of feeding is not clear.

The differentially and constitutively expressed proteins with similar patterns of molecular weight and isoelectric point may be enzymes in the cellular signal transduction in metabolic pathways that also mediate responses to feeding or proteins that provide structural component of the ribosomal stalk of the large subunit like ribosomal protein PO (Rachinsky *et al.,* 2008).

Variation in patterns of differentially expressed proteins between the sexes and their duration after feeding can be attributed to differences in the feeding periods and amount of blood meal taken. Some of the up-regulated proteins may be those that mediate pheromone biosynthesis switch from assembly pheromone to mutual attractant sex signal. Others may be involved in process of protein folding and protein translocation from the cytoplasm into endoplasmic reticulum lumen like endoplasmic reticulum signal peptidase. ER signal peptidases are associated with the protein translocation machinery, the tranlocon, of the endoplasmic reticulum (Johnson and Waes, 1999). These peptidases cleave the N-terminal signal sequence of secretory or membrane proteins while these proteins are translocated in to the lumen of the ER, thereby controlling protein transport and localization within the cell (Paetzel *et al.,* 2002). Proteins up-regulated perhaps could be involved in glycosylation of membrane by catalytic subunit of oligosaccharyltransferase (OST) (Wilson and High, 2007). For many proteins, glycolsylation is a requirement for correct folding and function. Other differentially expressed proteins may be the HSP70 group. The major physiological functions of members of the HSP70 superfamily are protein folding, unfolding and translocation, and assembly and disassembly of oligomeric protein complexes (Kaufman, 1990).

As noted, several differentially expressed proteins has multiforms with different M_r and pI for example, proteins having the same molecular weight 180 kDa but different PIs were spots 7, 8 and 9 (Fig. 4); spots 2 and 5 with M_r 32 kDa but different pIs (Fig. 5); spots 4, 5 and 6 with M_r 120 kDa with different pIs (Fig. 9); Spots 1 and 2 (Fig10) which are basic and acidic respectively with the same M_r of 180 kDa. It is likely that these multiforms represent post-translational modification. Although not tested in this study, the different charges carried by these proteins may be caused by phosphorylation, since phosphorylated forms of these proteins have been observed and are known to modulate protein activity (Hancock *et al.,* 2004).

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

From behavioral study, it is clear that assembly behavior of *R. appendiculatus* tick is influenced by chemical messengers (pheromone). This pheromone is found in the tick excreta, and nymphal skin washings. This assembly behavior was not exhibited by the fed stages suggesting pheromonal switch from response to assembly pheromone to sex attractant pheromone. This kind of phenomenon serves as a mechanism of/for increasing the chances of a tick finding a mate following feeding suitable. The identification of individual assembly pheromones components, kairomones constituents that guide the ticks to their preferred sites in the host ears and sex attractant pheromone in *R. appendiculatus* through LC-MS and GC-MS respectively can provide the basis for development of an alternative targeted control with the potential to reduce overall acaricide use and non-target effects. The potential of one such approach was demonstrated by initial studies by Dusbabek *et al.* (1997), who reported the enhancement of efficacy of permethrin against immatures and adults of *A. persicus* when used in conjunction with guanine hypochloride.

During period of feeding, adult female ticks of other species have been reported to produce sex attractant pheromone which responsible for mating behavior. This phenomenon has not been previously reported in *R. appendiculatus* ticks and hence carrying out mating behavior studies during feeding and characterization of the mediating pheromone would provide another basis for development of control strategies for the ticks.

In the endeavor to understand the molecular mechanisms underlying pheromone production in the *R. appendiculatus* tick it is clear that the change in behavior in *R. appendiculatus* in response to assembly pheromone after a blood meal was rapid and appears to have been mediated by differential expression of some specific proteins. The pattern of this expression perhaps relate to change in behavior from assembly before feeding to dispersion and mate location in feeding stages mediated by different pheromonal signal. This characteristic protein induction can potentially serve as an insight to pheromone biosynthesis. Sequencing of the induced proteins isolated from the gels, together with information from annotated *R. appendiculatus* genome can

further facilitate characterization of the proteins. This will provide additional information on the specific functions of these proteins in pheromone biosynthesis. It is probable that other important molecular techniques will also be applied to future studies of pheromone biosynthesis and, especially, regulation. These include any of the PCR-based methods for differential or subtractive screening of nucleic acid libraries to examine life stage-, sex-, or species-related differences linked to pheromone biosynthesis, e.g. differential display (Liang *et al.,* 1993) or representational difference analysis (Lisitsyn *et al.,* 1993; Hubank and Schatz, 1994), and *in situ* hybridization and immunochemistry to localize cellular sites of synthesis. Ultimately, just as behavioral chemicals have been extended to pest management, research on pheromone biosynthesis and its regulation may be directed toward application. This might include the culturing of tick tissues or cells, or the transfer of relevant genes into expression systems, for production of behavioral chemicals of high stereochemical purity. Perhaps eventually, the isolated genes could be transgenically introduced into microorganisms for area wide-treatments, or into agriculturally important organisms to produce semiochemicals to disrupt assembly, mating or otherwise interfere with the reproductive biology and host finding.

REFERENCES

Abdel-Rahman, M. S., Fahmy, M. M. and Aggour, M. (1998). Trials for control of ixodid ticks using pheromone acaricide tick decoys. *Journal of Egypt Society of Parasitology.* 28:551–557.

Aeillo, E.S and Mays, A. (2003). Ticks: tick control. *The Merck Veterinary Manual (MVM) 8th edition Merck and Co.Inc., Whitehouse Station, NJ,USA.*

Allan, S. A., Sonenshine, D. E and Burridge, M. J. (2001). Tick pheromones and uses thereof. *U.S. Patent No.* 6331297

Allan, S.A. and Soneshine, D.E. (2002). Evidence of an assembly pheromone in the black-legged deer tick, *Ixodes scapularis. Journal of Chemical Ecology*, 28: 15 – 27.

Allen, J., Khalil H and Wikel, S. (1979). Langerhans cells trap tick salivary antigens in tick-resistant guinea pigs. *Journal of Immunology*, 122: 563.

Almazan, C., Kocan, K.M., Blouin, E.F and de la Fuente, J. (2005). Vaccination with recombinant tick antigens for the control of *Ixodes scapularis* adult infestations. *Vaccine* 23: 5294-5298

Arlian, L.G and Vyszenski-Moher, D.L. (1995). Response of *Sarcoptesscabiei* var. *canis* (Acari: Sarcoptidae) to lipid of mammalian skin. *Journal of Medical Entomology*, 32**:** 34 – 41.

Awumbila, B. (1996). Acaricides in tick control in Ghana and methods of application. *Tropical Animal Health and Production* 28:50-52.

Barre, N., Garris, G.I., Lorvelec, O. (1997). Field sampling of the tick *Amblyomma variegatum* (Acari: Ixodidae) on pastures in Guadeloupe: attraction of CO_2 and/or tick pheromones and conditions of use. *Experiment Applied Acarology* 21:95–108.

Bishop, R., Lambson, B., Wells C., Pandit, P., Osaso J., Nkonge, C., Morzaria, S., Musoke, A. and Nene, V.(2002). A cement protein of the tick *Rhipicephalus appendiculatus*, located in the secretory e cell granules of the type III salivary gland acini, induces strong antibody responses in cattle. *International Journal of Parasitology,* 32: 833–842.

Bittencourt, V. (2000). Trials to control South American ticks with entomopathogenic fungi. *Annals of the New York Academy of Sciences , 916*, 555-558.

Bram, R.A (1983). Tick borne livestock diseases and there vectors the global problem In: tick and tick borne diseases, FAO animal production and health. Food and Agriculture Organization. Rome, pp 7-11.

Branagan, D. (1973). The development periods of the *oxidid R. appendiculatus* under laboratory conditions. *Bulletin of Entomology Research 63: 155-168.*

Brossard, M and Wikel, S.K. (2004). Tick immunobiology. *Parasitology* 129: 161–176.

Brown, A.W.A (1976). Genetics of insect vectors of diseases. In: J.W. Wright and R. pal (eds). Elsevier,Amsterdam 794 pp

Buisch, W.W., Hyde, L.J and Mebus, A.C. (1998). Foreign pests and vectors of arthropod-borne disease (vector borne diseases and arthropod vectors foreign animal diseases, the gray book, 6th edition. www.vet.uga.edu/vpp/gray book02/fad/ecf.php as retrieved on 20th April 2009

Couto, J. T. (1994). Operation oxpecker. *The Farmers 10-11*.

Cumming, S.G. (1998). Host preference in African ticks (Acari: oxidida) a quantitative data set. *Bulletin of Entomological Research* 88: 379-406.

de haan, C., steinfield, H and Blackburn, H. (1996). Livestock and the environment: find a balance. European commission directorate-general for development: development policy, sustainable development and natural resources, pp. 115. www.fao.org/docrep/x5303e/x5303e00.htm#contents as retrieved 17th April, 2009.

de la Fuente, J. and Kocan, K. M. (2006). Strategies for development of vaccines for control of Ixodid tick species. *Parasite Immunology 28: 275-283.*

de la Fuente, J., Rodriguez, M., Redondo, M., Montero, C., Garcia-García, J.C., Méndez, L., Serrano, E., Valdes, M., Enriquez A., Canales M., Ramos, E., Boue, O., Machado, H.,

Lleonart R., de Armas, C.A., Rey S., Rodríguez, J.L., Artiles, M and García, L. (1998). Field studies and cost-effectiveness analysis of vaccination with Gavac against the cattle tick *Boophilus microplus. Vaccine* 16: 366–37.

Diehl, P., Guerin, P.M., Vlimant, M. and Steullet, P. (1991). Biosynthesis, production site and emission rates of aggregation-attraction pheromone in males of two *Amblyomma* ticks. *Journal of Chemical Ecology*, 17: 833–47.

Dolan, T. T., (1999). Dogmas and misunderstandings in east coast fever. *Tropical Medicine and International Health*, 4:3-11.

Dusbabek, F., Rupes V., Simek, P and Zahradnickova H. (1997). Enhancement of permethrin efficacy in acaricide-attractant mixtures for control of the fowl tick *Argas persicus* (Acari: Argasidae). *Experimental Applied Acarology. 21:293–305.*

Dusbabek, F., Simek, P., Jegorov, A and Triska, J. (1991). Identification of xanthine and hypoxanthine as components of assembly pheromone in excreta of argasid ticks. *Experimental Applied Acarology*, 11:307–316.

East Africa. (2003). Tick modeling management and control in eastern and southern Africa. www.csiro.au/research/pestmgmt/tick/east.htm retrieved on 9th March, 2009.

El Shoura, S.M and Roshdy, M.A. (1985). The subgenus *Persicargas* (Ixodoidea: Argasidae: *Argus). 42.* Ultrastructure of the coxal organ in female *A. (P.) arboreus. Journal Medical Entomology*, 22:675-682.

Erasmus, B.F.N., Van Jaarsveld, A.S., Chown, S.L., Kshtriya, M and Wessels, K.J. (2002). Vulnerability of South African animal taxa to climate change. *Global Change Biology*, 8: 679-693.

Evans, H.E. (1984). *Insect Biology.* Addison Wesley publisher, London. PP 114-131, 424.

FAO. (1998). Carribean *Amblyomma* eradication programme, CARICOM/FAO/IICA programme on tropical bont tick (TBT). www.fao.org/docrep/vi650t/vi650Tow.html as retrieved on 16th April, 2009.

Ferreira, C.A.S., Da Silva Vaz Jr, I., Da Silva, S.S., Haag, K.L., Valenzuela, J.G and Masuda, A. (2002). Cloning and partial characterization of a *Boophilus microplus* (Acari: Ixodidae) calreticulin. *Experimental Parasitology*, 101: 25–34

Foil, L.D., Coleman, P., Eisler, M., Fragoso-Sanchez, H., Garcia-Vazquez, Z., Guerrero, F.D., Jonsson, N.N., Langstaff, I.G., Li, A.Y., Machila, N., Miller, R.J., Morton, J., Pruett, J.H and Torr S. (2004). Factors that influence the prevalence of acaricide resistance and tick-borne diseases. *Veterinary Parasitology*, 125: 163–181.

Galun, R. (1975). Research into alternative arthropod control measures against livestock pests (Part I). In *Workshop on the Ecology and Control of External Parasites of Economic Importance on Bovines in Latin America*. Columbia: Centro International de Agricultura Tropical (CIAT).

Garcia-Garcia, J.C., Montero, C., Redondo, C., Vargas, M., Canales, M., Boue ,O., Manuel RodroAguez, M., Joglar, M., Machado, H., Iliana, L. lez, L., Mario, Valde, A. M., Luis MeAndez, L and de la Fuente, J. (2000). Control of ticks resistant to immunization with Bm86 in cattle vaccinated with the recombinant antigen Bm95 isolated from the cattle tick, *Boophilus microplus*. *Vaccine* 18: 2275–2287.

George, J.E. (1981). The influence of aggregation pheromone on the behavior of *Argas cooleyi and ornithodoros concanensis* (Acari: Argasidae*) Journal of Medical Entomology*, 18:129-133.

George, J.E., (2000). Present and future technologies for tick control. *Annal New York. Academic of Science*, 916, 583–588.

Hamilton, J. and Sonenshine, D.E. (1989). Methods and apparatus for controlling arthropod populations. *U.S. Patent No.* 4884361

Hanson, P., Yoder, J.A., Pizzuli, J.L and Sanders, C.I. (2002). Identification of 2,4-dichlorophenol in females of the American dog tick, *Dermacentor variabilis* (Acari: Ixodidae), and its possible role as a component of the attractant sex pheromone. *Journal of Medical Entomology,* 39:945–947.

Harrison, R.I., Palmer, H.B and Wilmshurst, C.E. (1973). Chemical control of cattle ticks-resistance problems. *Pesticide Science,* 4:531-542.

Hassanain, M.A., El-Garhy, M.F., Abdel-Ghaffar, F.A., El-Sharaby, A and Megeed, A. K.N. (1997). Biological control studies of soft and hard ticks in Egypt. The effect of *Bacillus thuringiensis* varieties on soft and hard ticks (oxididea). *Parasitology Research,* 83: 209-213.

Hassanali, A., Nyandat, E., Obenchain, F.A., Otieno, D.A and Galun, R. (1989). Humidity effects on response of *Argas persicus* (Oken) to guanine, an assembly pheromone of ticks. *Journal of Chemical Ecology*. 15: 3.

He, H., Chen, A.C., Davey, R.B., Ivie G.W and George, J.E., (1999). Identification of a point mutation in the para-type sodium channel gene from a pyrethroid-resistant cattle tick. *Biochemical and Biophysical Research Communication*, 261: 558–561.

Howell, C. J., De Vos, A.J., Bezuidenhout, J.D., Potgieter, F.T and Barrowman, P. R., (1981). *The role of chemical tick eradication in the control or prevention of tick transmitted diseases in cattle. Tick biology and control* (ed. By whitehead, B.G. and Gibson,). D.J) pp. 61-66. The proceedings of an international conference held in Grahams town on 27-29th January, 1981. Tick research unit, Rhodes University, Graham's town, South Africa.

Hu, R., Hyland, K.E and Oliver, Jr. (1998). A review on the use of ixodiphagus wasps (*Hymenoptera:Encyrtidae*) as natural enemies for the control of ticks (Acari: ixodidea). *Systemic and Applied Acarology,* 3: 19-28.

Hubank, M. and Schatz, D.G. (1994). Identifying differences in mRNA expression by representational difference analysis of cDNA. *Nucleic Acids Research,* 22: 5640–5648

Hurkman, W. J and Tanaka, C. K. (1986). Solubilization of plant membrane proteins for analysis by two dimensional gel electrophoresis. *Plant Physiology,* 81: 802–806.

Irvin, A.D., McDermott, J.J and Perry, B.D. (1996). Epidemiology of tick and tick borne diseases in Eastern, Central and Southern Africa. Proceedings of a work shop held in Harare 12-13 March 1996. International Livestock Research Institute (ILRI), Nairobi, Kenya and Addis Ababa, Ethiopia.

Jamroz, R.C., Guerrero, F.D., Pruett, J.H., Oehler D.D and Miller, R.J. (2000). Molecular and biochemical survey of acaricide resistance mechanisms in larvae from Mexican strains of the southern cattle tick, *Boophilus microplus*. *Journal of Insect Physiology*. 46: 685–695.

Jaworski, D.C., Simmen, F.A., Lamoreaux, W., Coons, L.B., Muller, M.T and Needham, G.R. (1995). A secreted calreticulin protein in ixodid tick (*Amblyomma americanum*) saliva. *Journal of Insect Physiology*, 41:369–375.

Johnson, A.E and Van Waes, M.A. (1999). The translocon: a dynamic gateway at the ER membrane. *Annual Review Cell Development Biology,* 15: 799–842.

Jones, L.D and Nutall, P.A. (1989). Non- viraemic transmission of Thogoto virus: influence of time and distance. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 83: 712-714.

Judd, G.J. R and Gardiner, M.G.T. (2005). Towards eradication of codling moth in British Columbia by complementary actions of mating disruption, tree banding and sterile insect technique: five-year study in organic orchards. *Crop Protection,* 24: 718-733.

Kaaya, G. P. (2000). Laboratory and field evaluation of entomogenous fungi for tick control. *Annals of the New York Academy of sciences, 916*, 559-564.

Kaba, S. A., Musoke, A.J., Schaap, D., Schetters, T., Rowlands, J., Vermeulen, A.N., Nene, V.,Vlak, J.M., Oers M.M.V.(2005). Novel Baculovirus-derived p67 subunit vaccines efficacious against East Coast Fever in cattle. *Vaccine,* 23:2791-2800

Kariuki, D.P. (1989). Theilerosis in Kenya. Theileriosis in Eastern, Central and Southern Africa (ed. by Donal, TT). Proceedings of a workshop on East Coast Fever immunization held in Lilongwe, Malawi 20-22 September. The International Laboratory for Research and Animal Diseases, Nairobi, Kenya. Initiatives ltd., Nairobi Kenya.

Kaufmann, S.H.E. (1990). Heat shock proteins and the immune response. *Immunology Today*, 11: 129–136.

Kebede, A. G. (2004). Seasonal dynamics of ticks (*Amblyomma cohaerens* and *Boophilus decoloratus)* and development of a management plan for tick and tick born diseases control on cattle in Jimma zone, Southwestern Ethiopia. PhD. thesis, *Georg-August-University Gottingen.*

Kiltz, H. H. (1985). Theileriosis in Burundi. In: Irvin, A D (ed), *Immunization against Theileriosis in Africa*. ILRAD (International Laboratory for Research on Animal Diseases), Nairobi, Kenya. pp. 12-15.

Labuda, M., Trimnell, A. R., Lickova, M., Kazimirova, M., Davies, G.M., Lissina, O., Hails, R. S and Patricia, N. A. (2006). An anti-vector vaccine protects against a lethal vector-borne pathogen. *PLoS Pathogen*, 2: 27.

Larisa, V. (2001). Back ground information on the biology of ticks. *Department of Entomology, University of California, Davis, USA.*

Larregina, A.T and Falo, L.D. (2005). Changing paradigms in cutaneous immunology: adapting with dendritic cells. *Journal Investigative Dermatology*, 124: 1–12.

Leahy, M.G. (1979). Pheromones of Argasid ticks PP. 297-308, in J.G. Rodriquez (ed.). Recent Advances in Acarology, Volume II Academic Press, New York USA.

Leahy, M.G., Hajkova, Z and Bouchalova, J. (1981). Two female pheromones in the metastriate tick, *Hyalomma dromedarii* (Acarina: Ixodidae). *Acta Entomologica Bohemoslovaca*, 78**:** 224 – 230.

Leahy, M.G., Karuhize, G., Mango, C and Galun, R. (1975). An assembly pheromone and its perception in the tick *Ornithodoros moubata* Murray (Acari: Argasidae). *Journal of Medical Entomology*, 12: 284 – 287.

Lewis, A.R. (1981). The pathology of *Rhipicephalus appendiculatus* infestation of eland *taurotragus orxy*. Proceedings of an international conference on tick control and biology held on 27-29 January 1981. Tick research unit, Rhodes University, Grahamstown, South Africa.

Li, A.Y., Davey, R.B., Miller, R.J and George, J.E. (2003). Resistance to coumaphos and diazinon in *Boophilus microplus* (Acari: Ixodidae) and evidence for the involvement of an oxidative detoxification mechanism. *Journal of Medical Entomology,* 40: 482–490.

Liang, P., Averboukh. L and Pardee, A.B. (1993). Distribution and cloning of eukaryotic mRNAs by means of differential display: refinements and optimization. *Nucleic Acids Research,* 21: 3269–3275.

LightFoot, C.J and Norval, R.A.I. (1982). Tick problem in wildlife in Zimbabwe. 1. The effect of tick parasitism on wild ungulates. *South African Journal of Wildlife Research,* 11: 41-45.

Linda, D., Jones, C R., Gordon D., Steel, M and Nuttal, A. (1989). Vector capacity of *Rhipicephalus appendiculatus* and *Amblyomma variegatum* for Thogoto and Dhori viruses. *Medical and Veterinary Entomology,* 3: 195-202

Lisitsyn, N., Lisitsyn, N and Wigler M. (1993). Cloning the differences between two complex genomes. *Science,* 25: 946–951

Louly, C.C.B., Silveira, D. N., Soares, S.F., Ferri, P.H., de Melo, A. C.C and Borges, L.M.F. (2008). More about the role of 2,6–dichlorophenol in tick courtship: identification and olfactometer bioassay in *Amblyomma cajennense* and *Rhipicephalus sanguineus. Mem Institute of Oswaldo Cruz, Rio de Janeiro,* 103: 60-65.ute

Lowry, O.H., Rosebrough, N.J., Farr, A.L and Randall, R.J. (1951). Protein measurement with folin phenol reagent. *Journal of Biochemistry,* 193: 265.

Lwande, W., Ndakala, A.J., Hassanali, A., Moreka L., Nyandat, E., Ndungu, M., Amiani H., Gitu, P.M., Malonza, M.M and Punyua D.K. (1999). Gynandropsis gynandra essential oil and its constituents as tick (*Rhipicephalus appendiculatus*) repellents. *Phytochemistry* 50: 401-405

Mackonnen, S. (2005). Acaricide resistance profiles of single and multi-host ticks in commercial and communal farming areas in the Eastern Cape and North-west provinces of South Africa. Msc. Thesis University of Pretoria.

Mans, B.J. (2002). Functional perspectives on the evolution of *Argasid* tick salivary gland protein super families. PhD thesis. university of Pretoria, Pretoria.

Maranga, R. O., Hassanali, A., Kaaya, G. P., and Mueke, J. M. (2006). Performance of a prototype baited-trap in attracting and infecting the tick Amblyomma variegatum (Acari:Ixodidea) in field experiments. *Experimental and Applied Acaralogy , 38*, 211-218.

Maranga, R. O., Kaaya, G. P., Mueke, J. M., and Hassanali, A. (2005). Effects of combining the fungi Beauveria bassiana and Metarhizium anisopliae on the mortality of .the tick Amblyomma variegatum (ixodidae) in relation to seasonal changes. *Mycopathologia , 159*, 527-532.

Maranga, R., Hassanali ,A., Kaaya, G.P and Mueke, J.M. (2003). Attraction of *Amblyomma variegatum* (ticks) to the attraction-aggregation-attachment pheromone with or without carbon dioxide. *Experimental Applied Acarology*. 29:121–30

Marina, M., Mathias, E and McCorkle, M.C., (2001). Ethnoveterinary medicine: an annotated Bibliography of community animal Health care, 2nd edition. *Practical action ITDG publishing, London, UK.*

Mathewon, M D., (1984). The future tick control: a review of the chemical and nonchemical options. Impact of disease on livestock production in the tropics. (ed. By Riechmann H.P. and Burridge M.P.), pp. 559-568.

Mbogo, K.S., Kariuki, P.D., McHardy, N and Payne, R. (1995). Training manual for veterinary staff on immunization against East Coast Fever using the ECFiM system. Kenya Agricultural Research Institute and overseas development administration of UK.

Mcdowell, P.G and Waladde, S.M., (1985). 2, 6-dichlorophenol in the tick *Rhipicephalus appendiculatus* Neumann, a reappraisal. *Journal of Chemical Ecolog,y* 12:69-81.

McMahon, C., Guerin, P.M and Syed, Z. (2001). 1-octen-3-ol isolated from bont ticks attracts *Amblyomma variegatum*. *Journal of Chemical Ecology.* 27:471–86.

Minshul, J.I and Norval, R.A.I. (1982). Factors influencing the distribution of *Rhipicephalus appendiculatus* in kyle recreational park, Zimbabwe. South African. *Journal of Wildlife Research*, 12: 118-123.

Mireji1, P.O., Keating, J., Eucharia, K., Mbogo, C., Nyambaka, H., Osir, E., Githure, J and Beier, J. (2006). Differential induction of proteins in *Anopheles gambiae* sensu stricto (Diptera: Culicidae) larvae in response to heavy metal selection. *International Journal of Tropical Insect Science*, 26: 214-226.

Mitchel, M. (1996). Acaricide resistance–back to basics. *Tropical Animal Health and Production*, 28: 53-58.

Morzaria, S. P., Irvin A.D., Taracha, E and Spooner, P. R. (1985). East Coast fever immunization trials in the Coast Province of Kenya. In: Irvin A D (Ed), Immunization against theileriosis in Africa. ILRAD (International Laboratory for Research on Animal Diseases), Nairobi, Kenya. pp. 76-78.

Morzaria, S., Nene, V., Bishop, R., and Musoke, A. (2000). Vaccines against *Theileria parva*. *Annal NewYork Academic Science*, 916: 464 - 473.

Mukhebi, A W., Morzaria, S. P., Perry, B. D., Dolan, T. T and Norval, R. A. I. (1990). Cost analysis of immunization for East Coast Fever by the infection and treatment method. *Preventive Veterinary Medicine*, 9:207-219.

Mukhebi, A. W., Perry, B. D and Kruska, R. (1992). Estimated economics of theileriosis control in Africa. *Preventive Veterinary Medicine*, 12:73-85.

Mukhebi, A.W., Curry, J., Perry, D.B., Reid, S.R., Thorpe, W., Laker, C.R., Onchoke, D. Hassan, Z., Kariuki, D.P., Munyombwe, T. and Ushewokunze-obatolu, U. (1993). Economic impact of theilorosis and its control in Africa. www.cgiar.org/ilri/research/impacts/imtrh2.cfm as retrieved on 17th March, 2009.

Mulenga, A., Sugimoto, C., Sako, Y., Ohashi, K., Musoke, A., Shubash, M. and Onuma, M. (1999). Molecular characterization of a *Haemaphysalis longicornis* tick salivary gland-associated

29-Kilodalton protein and its effect as a vaccine against tick infestation in rabbits. *Infection and Immunity*, 67: 1652–1658.

Muraguri, G.R., Ngumi, P.N., Wesonga, D., Ndungu, S.G., Wanjohi, J.M., Bang, K., Fox, A., Dunne, J and McHardy, N. (2006). Clinical efficacy and plasma concentrations of two formulations of buparvaquone in cattle infected with East Coast fever *(Theileria parva* infection) *Research in Veterinary Science,* 81: 119–126

Musisi, F. L., Quiroga, J. C., Ngulube, B and Kanhai, G. K. (1989). An East Coast fever immunization field trial at Kasoba, Malawi. In: Dolan T T. (ed), *Theileriosis in Eastern, Central and Southern Africa*. ILRAD (International Laboratory for Research on Animal Diseases), Nairobi, Kenya. pp. 71-76.

Mutugi, J. J., Young, A. S., Lampard, D., Ndungu, S. G., Ngumi, P. N., Mining, S. K., Maritim, A. C., Kariuki, D. P., Awich, J. R and Kwena, A. (1989). *Immunization of cattle against East Coast fever in the Coast Province of Kenya: Pilot immunization trials on government farms.* In: Dolan T T (ed), Theileriosis in Eastern, Central and Southern Africa. ILRAD (International Laboratory for Research on Animal Diseases), Nairobi, Kenya. pp. 39-45.

Myung-Jo, Y. (2005). Immunization of mice with recombinant P27/30 protein confers protection against hard tick *Haemaphysalis longicornis* (Acari: Ixodidae) infestation. *Journal of Veterinary Science*, 6: 47–51.

Neitz, W.O. (1955). Corridor disease. A fatal form of bovine theilorosis encountered in Zululand. *Bulletin of Epizootic Diseases of Africa*, 3: 121-123.

Nene, V., Bishop R., Morzaria, S., Gardner, M., Sugimoto, C., Ole-Moi-Yoi, O., Fraser, C. and Irvin, A. (2000). *Theileria parva* genomics reveals a typical apicomplexan genome. *International Journal of Parasitology*, 30: 465 – 474.

Nolan, J. (1985). Mechanisms of resistance to chemicals in arthropod parasites of veterinary importance. *Veterinary Parasitolgy* 18: 155-166.

Nolan, J. (1990). Acaracide resistance in single and multi host ticks and strategies for control. *Parasitogolia,* 32: 145-153

Nolan, J. and Roulston, W.J. (1979). Acaricide resistance as a factor in the management of Acari of medical and veterinary importance. In recent advancs in acarology. *Vol.2 (Ed.J.G. Rodriguez) academic press, New York* pp. 3-13.

Norval, I. A. R and LightFoot, C.J. (1981). Tick problems in wildlife in Zimbabwe. Factors influencing the occurrence and abundance of *Rhipiciphelus appendiculatus*. *Zimbabwe Veterinary Journal,* 13: 11-20

Norval, I. A. R. (1977). Tick problems in relation to land utilization in Rhodesia. *Rhodesia Veterinary Journal,* 8: 33-38.

Norval, I. A. R. (1989). Tick control in relation to epidemiology of theilorosis. Theilorosis in East, Central and Southern Africa (Ed. by Donald T.T). Proceedings of a workshop on East Coast Fever immunization, held in Lilongwe Malawi 20-22 September 1988. *The International Laboratory for Research and Animal Diseases. English press Nairobi Kenya*

Norval, I. A. R. Perry, D. B and Young, S.A. (1992). The epidemiology of theilorosis in Africa. *Academic press, London, UK.*

Norval, R. A. I., Sonenshine, D. E., Allan, S. A and Burridge, M. J. (1996). Efficacy of pheromone-acaricide impregnated tail-tag decoys for control of bont ticks, *Amblyomma hebraeum*, on cattle in Zimbabwe. *Experimental Applied Acarology.* 20:31–46

Norval, R. A. I., Sutherst, R. W., Gibson, J. D., Kerr, J. D., Thorne, l . M and Ellen, H. A. (1997). The effects of the brown ear-tick, *Rhipicephalus appendiculatus,* on milk production in dairy cattle *Medical and Veterinary Entomology,* 11: 155-158.

Norval, R.A.I (1994). Vectors: Ticks. In. Coetzer, J.A.W Thomson, G.R and Tustin, R.C (Eds), infectious Diseases of livestock with special reference to southern Africa, Vol. 1. Oxford university press, Cape Town. 3-24.

Norval, R.A.I., Peter, T., Yunker C.E., Sonenshine, D.E and Burridge, M.J. (1991). Responses of the ticks *Amblyomma hebraeum* and *A. variegatum* to known or potential components of the aggregation-attachment pheromone. 1. Long-range attraction. *Experiment Applied Acarology*, 13:11–18.

Olwoch, J. M., Reyers, B., Engelbrecht, F. A. and Erasmus, B.F.N. (2008). Climate change and tick-borne disease, Theilorosis (East Coast Fever in sub Saharan Africa). *Journal of Arid Environments*, 72: 108-120.

Otieno, D. A., Hassanali, A., Obenchain, F. D., Steinberg, A and Galun, R. (1985). Identification of guanine as an assembly pheromone of ticks. *Insect Science and its Application*, 6:667–670.

Paesen, G. C., Adams, P. L., Harlos, K., Nuttall, P. A and Stuart, D. I. (1999). Tick histamine-binding proteins: Isolation, cloning, and three dimensional structure. *Molecular Cell*, 3: 661–667.

Paetzel, M., Karla, A., Strynadka, N. C. J and Dalbey, R. E (2002). Signal peptidases. *Chemical Review* 102: 4549-4579.

Pavis, C. and Barre, N. (1993). Kinetics of male pheromone production by *Amblyomma variegatum* (Acari: Ixodidae). *Journal of Medical Entomology*. 30:961–965.

Petney, T.N. and Bull, C. M. (1981). A non-specific aggregation pheromone in two Australian reptile ticks. *Animal Behavior*, 29:181 – 185.

Philippe, P and Didier, R. (2001). Ticks and Tickborne Bacterial Diseases in Humans: An Emerging Infectious Threat. *Clinical Infectious Diseases*, 32:897–928

Preobrazhenskaya, Y., Voskoboev, A and Burd, V. N. (2003). Phosphatase activity of non-heme chloroperoxidase from the bacterium *Serratia marcescens*. *FEBS Letter*, 536:41–44

Price, T., Sonenshine, D. E., Norval, R. A. I., Yunker, C.E., Burridge, M. J. (1994). Pheromonal composition of two species of African *Amblyomma* ticks: similarities, differences and possible species specific components. *Experiment Applied Acarology*, 18:37–50.

Pruett, J. H., (2002). Comparative inhibition kinetics for acetylcholinesterases extracted from organophosphate resistant and susceptible strains of *Boophilus microplus* (Acari: Ixodidae). *Journal of Economic Entomology. 95: 1239–1244*.

Rachinsky, A., Guerrero, F. D and Scoles, G. A. (2008). Proteomic profiling of *Rhipicephalus (Boophilus micropolus* midgut responses to infection with *Babesia bovis*. *Veterinary Parasitology*, 152:294-313.

Radley, D. E. (1981). Infection and treatment method of immunization against theileriosis. In: Irvin A D, Cunningham M P. and Young A. S (eds), Advances in the control of theileriosis. Martinus Nijhoff Publishers, *The Hague, The Netherlands. pp.* 227-237.

Rand, K. N., Moore, T., Sriskantha, A., Spring, K., Tellam, R., Willadsen, P and Cobon, G. S. (1989). Cloning and expression of a protective antigen from the cattle tick *Boophilus microplus*. *Proceedings of the National Academy of Science of United States of America,* 86: 9657–9661.

Rechav, Y. and Whitehead, G. B. (1978). Field trials with pheromone-acaricide mixtures for control of *Amlyomma hebreaum. Journal of Economic Entomology,* 71:149-151.

Ribeiro, J. M. C. (1987). Role of saliva in blood-feeding by arthropods. *Annual Review of Entomology,* 32: 463–478.

Robson, J., Pedersen, V., Odeke, G. M., Kamya, E. P and Brown, C. G. D. (1977). East Coast fever trials in Uganda: Field exposure of zebu cattle immunized with 3 isolates of *Theileria parva*. *Tropical Animal Health and Production,* 9:219-231

Samir, E. M. (1987). Fine structure of the foveae dorsales and foveal glands in the female tick *Amblyomma americanum* (Acari: Ixodoidea: lxodidae*) Journal of Morphology,* 193:277-284.

Samish, M. (2000). Biocontrol of ticks. *Annals of the New York academy of sciences,* 916: 172-178.

Samish, M. and Alekseev, E. (2001). Arthropods as predators of ticks (ixodidea). *Journal of Medical Entomology* 38: 1-11

Schoni, R., Hess, E., Blum, W. and Ramstein, K. (1984). The aggregation-attachment pheromone of the tropical bont tick *Amblyomma variegatum* Fabricius (Acari: Ixodidae): isolation, identification and action of its components. *Journal of Insect Physiology* 30:613–618.

Sheng, W.Y and Wang, T.C.V. (2009). Proteomic Analysis of the Differential Protein Expression Reveals Nuclear GAPDH in Activated T Lymphocytes. *Plos One,* 7: 6322.

Shinji, Y., Yuko I., Saiki, I., Satoru, K., Takuya, I., Misao, O. and Kazuhiko, O. (2008). *Rhipicephalus appendiculatus:* Characterization of a testis-associated protein. *Experimental Parasitology,* 120: 337–342.

Simons, B., Barnett, P., Vollenbroek, E. G., Dekker, H. L and Muijsers, A. O. (1995). Primary structure and characterization of vanadium chloroperoxidase from the fungus *Curvularia inaequalis*. *European Journal of Biochemistry,* 229:566–574

Slovak, M., Hajnicka, V., Labuda, M. and Fuchsberger, N. (2000). Comparison of the protein profiles of salivary gland extracts derived from three species of unfed and partially fed Ixodid ticks analysed by SDS-PAGE. *Folia Parasitological,* 47:67-71.

Sonenshine, D. E (1985). Pheromones and other semiochemicals of the acari. *Annulal Review of Entomology,* 30: 1-28.

Sonenshine, D. E. (1984). *In Pathogen Host-Vector Research*. ed. K. F. Harris. New York: Praeger Science.

Sonenshine, D. E. (2006). Tick pheromones and their use in tick control. *Annual Review of Entomology,* 51: 557-580.

Sonenshine, D. E., Hamilton, J. G. C., Phillips, J. S., Ellis, K. P. and Norval, R. A. I. (1992). Innovative techniques for control of tick disease vectors using pheromones. In: *Tick Borne Pathogens*, Conference Proceedings, St Paul, USA. pp. 308-313. Eds. Munderloh, U. G. and Kurtti, T. J.

Sonenshine, D. E., khalil, G. M., Homesher, P. J and Manson, S.N. (1982). Dermacentor *variabilis and andersoni*: Genital sex pheromone. *Experimental Parasitology*. 54:317-330

Sonenshine, D. E., Silverstein, R. M and West, J. R. (1984). Occurrence of the sex attractant pheromone, 2-6-dichlorophenol, in relation to age and feeding in the American dog tick, *Dermacentor variabilis* (Say)(Acari: Ixodidae). *Journal of Chemical Ecology,* 10:95-100

Sonenshine, D. E., Silverstein, R. M., Collins, L. A., Saunders, M., Flynt, C and Homsher, P. J. (1977). The foveal gland: source of sex pheromone in the Ixodid tick, *Dermacentor andersoni* Stiles. *Journal of Chemical Ecology*. 3:697-706.

Sonenshine, D., Adams, T., Allan, S.A., McLaughlin, J. R and Webster, F.X. (2003). Chemical composition of some components of the arrestment pheromone of the black-legged tick, *Ixodes scapularis* (Acari: Ixodidae), and their use in tick control. *Journal of Medical Entomology,* 40:849–859

Sonenshine, D., Allan, S. A., Peter, T. F., McDaniel, R. and Burridge, M. J. (2000). Does geographic range affect the attractant-aggregation-attachment pheromone of the tropical bont tick, *Amblyomma variegatum* (Koch)? *Experiment Applied. Acarology,* 24:283–299.

Sonenshine, D., Hamilton, J. G. C and Lusby, W. R. (1992). The use of cholesteryl esters as mounting sex pheromones in combination with 2, 6-dichlorophenol and pesticides to control ticks. *U.S. Patent No.* 5149526

Sonenshine, D., Taylor, D., and Corrigan G. (1985). Studies to evaluate the eefectiveness of sex pheromone impregnated formulations for control of populations of American dog tick, Dermecentor andersoni Stiles (Acari: Ixodidea). *Experimental Applied Acarology* 1:23-34

Soneshine, D. E. (1991). Biology of ticks. Volume 1. *Oxford university press, New York NY, USA.*

Spickett, A. M. (1994). Tick ecology. *International Journal of Parasitology,* 24:845–849.

Stone, B. F. (1972). The genetics of resistance by ticks to acaricides. *Australian Veterinary Journal,* 48: 345-350

Sugino, M., Imamura, S., Mulenga, A., Nakajima, M., Tsuda, A., Ohashi, K and Onuma, M. (2003). A serine proteinase inhibitor (serpin) from the ixodid tick *Haemaphysalis longicornis*;cloning, and 23: preliminary assessment of its suitability as a candidate for a tick vaccine. *Vaccine,* 21: 2844–2851.

Tellam, R.L., Kemp, D., Riding, G., Briscoe, S., Smith, D., Sharp, P., Irving, D and Willadsen, P. (2002). Reduced oviposition of *B.microplus* feeding on sheep vaccinated with vitellin. *Veterinary Parasitology,* 103: 141–156.

Trimnell, A.R., Davies, G.M., Lissina, O., Hails, R.S and Nuttall, P.A. (2005). A cross-reactive tick cement antigen is a candidate broad-spectrum tick vaccine. *Vaccine,* 23: 4329–4341.

Trimnell, A.R., Hails, R.S and Nuttall, P.A. (2002). Dual-action ectoparasite vaccine targeting 'exposed' and 'concealed' antigens. *Vaccine,* 20: 3560–3568.

Tsuda, A., Mulenga, A., Sugimoto, C., Nakajima, M., Ohashi, K and Onuma, M. (2001). cDNA cloning, characterization and vaccine effect analysis of *Haemaphysalis longicornis* tick saliva proteins. *Vaccine,* 19: 4287–4296.

Van, P. K. (2001). Microbial biosynthesis of halometabolites. *Archives of Microbiology.* 175:250–258

VIE. (2002). Veterinary informatics and epidemiology at the university of Glasgow and university of strathclyde. www.vie.gla.ac.uk/project/ecfxpert.html as retrieved on 17th April, 2009

Walker, R.A., Bouattour, A., Camicas, J.L., Estrada- Pena, A., Horak, I.G., Latif, A. A., Pegram, R. G and Preston, P.M. (2003). Ticks of domestic animals in Africa. A guide to identification of species. *Bioscience report. University of Edinburgh. Edinburgh, UK.*

Wall, R. and Shearer, D. (1997). Ticks. Veterinary entomology: arthropod ectoparasites of veterinary importance. *Kindle Edition Springer, London, UK and New York, NY, USA.*

Wang, H., Paesen, G.C., Nuttall, P.A and Barbour, A.G. (1998). Male ticks help their mates to feed. *Nature,* 391: 753–754.

Wanzala, W. (2009). Ethnobotanicals for management of the brown ear tick *Rhipicephalus appendiculatus* in western Kenyan. PhD Thesis. *Wageningen University Netherlands.*

Weiss, B.L and Kaufman, W.R. (2004). Two feeding-induced proteins from the male gonad trigger engorgement of the female tick *Amblyomma hebraeum*. *Proc Natl Acad Sci USA*, 101: 5874–5879.

Wharton, G.W. (1985). Water balance of insects. *Comprehensive Insect Physiology, Biochemistry and Pharmacology* (ed . by G.A . Kerkut and L.I. Gilbert), Vol. 4 , pp . 565 – 603, Pergamon Press, Oxford.

WHO expert committee on insecticide (1957). Seventh report, Geneva. World health organization technical report. No. 125

Willadsen, P and Kemp, D.H. (1988). Vaccination with 'concealed' antigens for tick control. *Parasitology Today*, 21: 196–198.

Willadsen, P., Bird, J., Cobon, G.S and Hungerford, J. (1995). Commercialization of a recombinant vaccine against *B. Microplus*. *Parasitology*, 110: 43–50.

Willadsen, P., Smith, D., Cobon, G and McKenna, R.V. (1996). Comparative vaccination of cattle against *Boophilus microplus* with recombinant antigen Bm86 alone or in combination with recombinant Bm91. *Parasite Immunology,* 18: 241–246.

Wilson, C.M and High, S. (2007). Ribophorin I act as a substrate-specific facilitator of N-glycosylation. *Journal of Cell Science,* 120: 648-657.

Yeoman, G.H. and Walker, J. B. (1967). The ixodid tick of Tanzania. *The Commonwealth Institute of Entomology, London UK.*

Yoder, J. A., Ark ,J. T and Farrell, A. C. (2008). Failure by engorged stages of the lone star tick *Amblyomma americanum,* to react to assembly pheromone, guanine and uric acid. *Medical and Veterinary Entomology,* 22:135–139

Yoder, J. A., Benoit, J. B and Opaluch, A. M. (2004). Water retention in eggs of the lone star tick, *Amblyomma americanum,* with experimental work on the capacity for water vapor absorption . *Experimental and Applied Acarology.* 33**:** 235 – 242.

Yoder, J. A., Domingus, J. L and Luerman, G.C. (2003). Uric acid-induced arrestment as a possible bird host cue (kairomone) in nymphs and adults of the lone star tick *Amblyomma americanum* (L.). *International Journal of Acarology,* 29**:** 399 – 402.

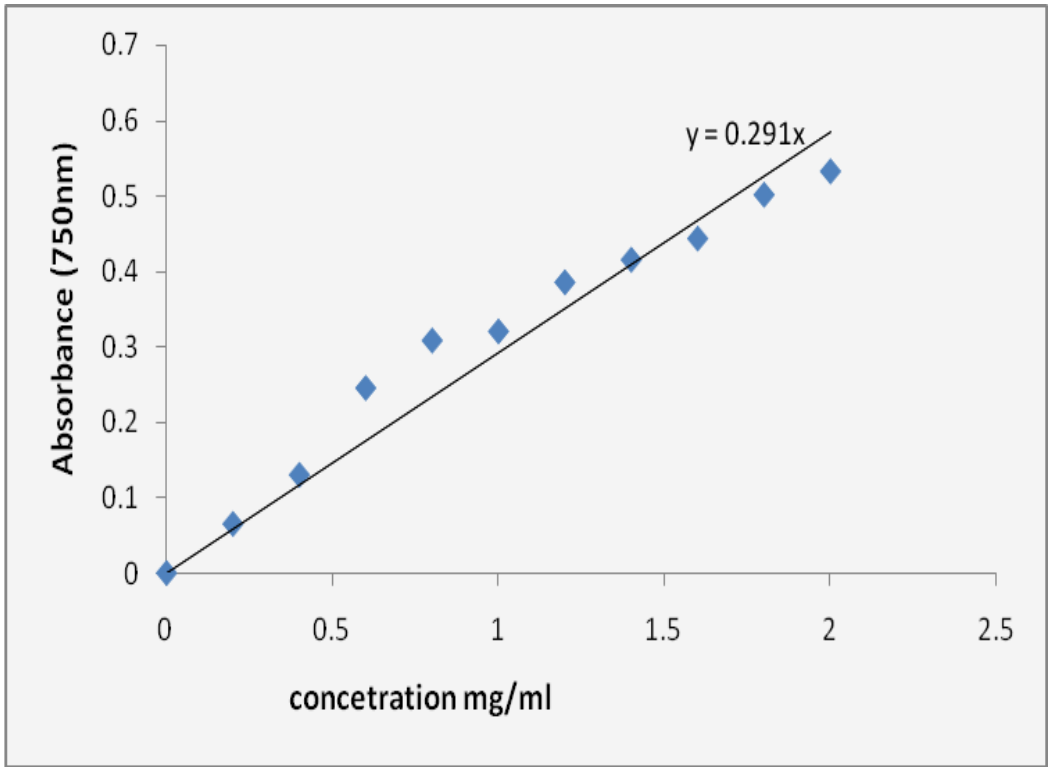
Yoder, J., Hanson, P.E., Zettler, L.W., Benoit, J.B., Ghisays, F. and Piskin, K.A. (2003). Internal and external mycoflora of the American dog tick, *Dermacentor variabilis* (Acari: Ixodidae), and its ecological implications. *Applied Environmental Microbiology,* 69:4994–4996

Yoneyama, T., Shiozawa, M., Nakamura, M., Suzuki, T and Sagane, Y. (2004). Characterization of a novel acid phosphatase from embryonic kidney bean exhibiting vanadate-dependent chloroperoxidase activity. *Journal of Biochemistry,* 279:37477–37484.

Ziv, M., Sonenshine, D. E., Silverstein, R. M., West, J. R and Gingher, K. H. (1981). Use of sex pheromone 2, 6- dichlorophenol to disrupt mating by the American dog tick Dermacentor *variabilis* (Say). 1. *Journal of Chemical Ecology*, 7:829- 840.

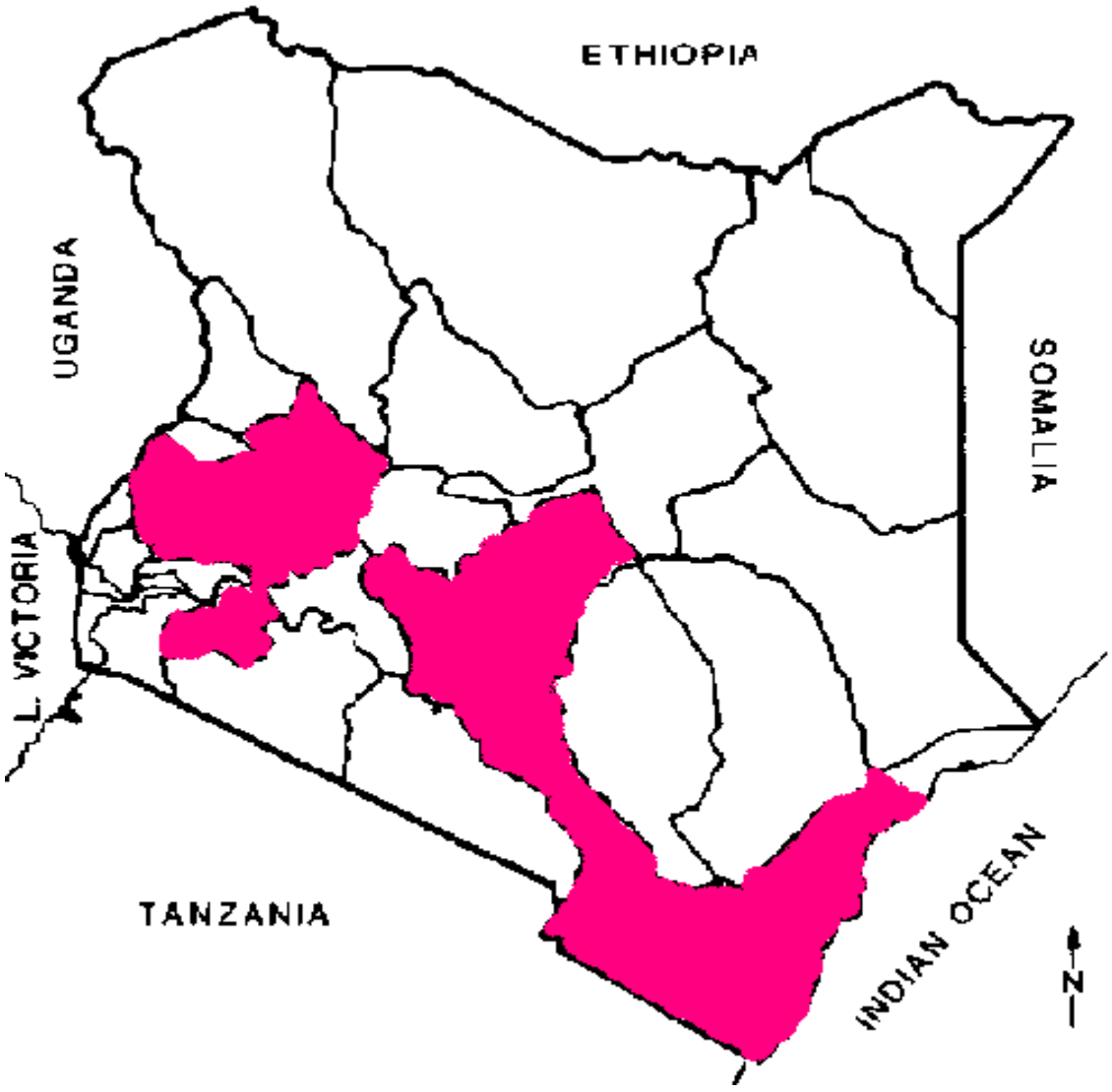
APPENDICES

Appendix 1



Appendix 2

Distribution of *Rhipicephalus appendiculatus,* in Kenya



Appendix 3

The distribution of exotic beef and dairy cattle breeds in Kenya, showing the percentages by district.