THE PHYSIOLOGY OF THE TICK *RHIPICEPHALUS APPENDICULATUS* IN RELATION TO THE TRANSMISSION OF *THEILERIA PARVA*

BY

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A DISSERTATION PRESENTED TO THE DEPARTMENT OF PARASITOLOGY AND ENTOMOLOGY FACULTY OF VETERINARY MEDICINE, AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA, IN FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN VETERINARY ENTOMOLOGY

PARASITOLOGY AND ENTOMOLOGY DEPARTMENT,
FACULTY OF VETERINARY MEDICINE,
AHMADU BELLO UNIVERSITY, ZARIA,
NIGERIA. MARCH, 1998

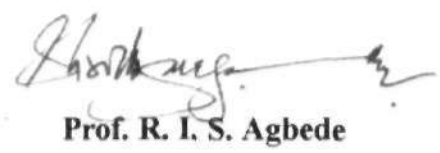
DECLARATION

This is to certify that I carried out the work reported in this Thesis at the International Center of Insect Physiology and Ecology (ICIPE), Nairobi, Kenya and The International Livestock Research Institute (ILRI), Nairobi in collaboration with the Department of Parasitology and Entomology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Nigeria, under the supervision of:



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The work of other investigators covered in this Thesis has been duly acknowledged and referred to appropriately. No part of this Thesis has been submitted elsewhere for a degree.



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CERTIFICATION

This Thesis by Mrs. Esther Nalugga Kibuuka Sebitosi meets with the regulations governing the award of the degree of Doctor of Philosophy of Ahmadu Bello University and is approved for its contribution to Scientific knowledge and literary presentation.

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ACKNOWLEDGEMENTS

I would like to extend my sincere gratitude to the Directors of the International Center of Insect Physiology and Ecology (ICIPE) Dr. Hans Herren and International Livestock Research Institute (ILRI), Dr. Hank Fitzhugh whose collaborative effort enabled to pursue my PhD.

Special thanks go to all my supervisors Dr. Godwin Kaaya of ICIPE, Prof. Roland I.S. Agbede of Ahmadu Bello Univeristy and Dr. Tony Musoke of ILRI whose advice and continous encouragement saw me through the entire PhD project. I wish to pay special tribute to the late Dr. Alan Young for introducing me to the concepts of Theileriosis and I wish to thank Dr. Subhash Morzaria of ILRI for allowing me to use the tick unit facilities.

I wish to thank the ICIPE staff members for the support especially Dr. Essuman Suleman, Miss Elizabeth Ouna and Mr.Joseph Kabi who helped me at the beginning of the project. Secondly, I thank Mrs. Joyce Mureithi and Mr. Matayo Chimutawi for the electron microscopy techniques they passed on to me. The photography services from Mr. Peter Lisamula are highly appreciated. I also thank the ARPPIS community in particular all my classmates and colleagues especially Mr. Mohammed Abakar, Mr. Jean Joseph Randriamananono and Mr. Abdulahi Nyiassy.

I also acknowledge the technical help by the ILRI staff especially those of the Lab.5 including, Mrs. Katherine Nkonge, Miss Lucy Gichuru, Mr.Dismus Lugo, Mr.Thomas Njoroge, Mr.Steven Njuguna and Mr.Alfred Tanui for the immunology and epidemiology of ECF. Very special thanks go to Mr. James Gachanja and Mr.Luka Juma for the help in the cell culture techniques. Invaluable services from members of the photography and electron microscopy unit at ILRI in particular Mr.

Clive Wells, Mr. Francis Shikubali, Mr. Joel Mwaura and Mr. Dave Elsworth are highly appreciated.

The help given to me by the Tick Unit staff at ILRI in providing the ticks and teaching me how to handle, rear and dissect ticks is greatly appreciated. Mr. Steven Mwaura, Mr.George Njihia, Mr. John Tangus, Mr. William Ratemo, Mr. George Oduol, Mr. Peter Macharia, and Mr. Peter Kimani are specially acknowledged.

The social, spiritual support and secretarial expertise in word processing given by Miss Sarah Nyongesa and Elizabeth Wairimu of Tick Unit are highly appreciated.

I extend my special gratitude to my family, relatives and friends who supported me through this project especially Miss Sandra Namugga, Master Gideon Semyalo, Master Benjamin Semugga, Miss Lucy Lawino, Dr. Margaret Lubega and Mr. Harison Maina. I thank the ARPPIS coordinator at ICIPE, Dr. Vitalis Musewe and the Education officer at ILRI, Dr. Rob Eley for their help. Lastly, I wish to ackowledge the financial support from the Netherlands Government DSO project given to me through ICIPE as well as research funds from the UNESCO courtesy of Prof. Masaquoi which helped me complete my work. The moral support from Ms. Casey Burns, dear friend of the late Alan Young is highly appreciated.

DEDICATION

This thesis is dedicated to my parents Victoria and Gideon Kibuuka and to the late Dr. Alan Young who introduced me to the world of ticks.

ABSTRACT

The factors within the tick vector *R. appendiculatus* that influence the development and transmission of the parasite *Theileria parva* were investigated and found to include the blood meal, phenoloxidase, enzymes, hormones and lectins. The cattle blood meal, when fractionated into cells and plasma components by centrifugation and provided to ticks *in vitro*, indicated that plasma alone negated the development and infectivity of *T. parva* to bovine lymphocytes leading to only 0.07% infections of *T. parva* in the peripheral blood lymphocytes, cells showed 1.02% , whole blood revealed 20% while the ticks fed in *vivo* on rabbits produced the highest infection (67.9%). For the first time *in vitro* infections of lymphocytes was carried out by sporozites obtained from ticks fed *in vitro.*

Lectins were detected in the haemolymph, gut and salivary glands from partially fed female adult *R. appendiculatus* and *R. pulchellus.* The gut lectins were agglutinated by several vertebrate erythrocytes, the highest being rabbit with a titre of 4096. No agglutination was observed when common domestic cattle, goat and sheep cells were used. The gut lectin was inhibited by fetuin, D(+) galactosamine and D(-) manosamine while the haemolymph lectin was inhibited best by xylose, N-acetyl galactosamine and B-D-fructose. The role of lectins investigated by feeding the carbohydrates and glycoproteins that inhibited agglutination to ticks *in vitro,* to ticks together with infected blood, revealed reduced or no infections in the salivary glands. Ticks fed upon immunised rabbits immunised with the lectins had also reduced infection rates (50%) compared to control (80%) which implied that these lectins were involved in the control of the development of T. *parva.* Anti-sera to lectins raised by immunising rabbit and mice with red blood cells to which an immunogen had been adsorbed reacted with purified piroplasms and bound the shizont stages of this parasite opening a new avenue in the possible intervention of Theileriosis at the piroplasm stage and underscoring the role of lectins in the development of this parasite. The digestive gut cells and lining and salivary

gland type III acini from the ticks fed upon these immunized rabbits were damaged an indication that the antibody traversed the gut, reaching target organs and remained functionally active.

Subjection of these lectins to enzyme treatments before agglutination assays revealed the lectins to be proteins, most likely glycoprotein in nature. The haemolymph was thermo-labile above 40 C, affected by freezing and thawing treatments shown by the reduction in agglutination properties, while the gut lectin was thermal stable, and withstood temperatures up to 80 °C. Optimum haemagglutination depended upon neutral pH conditions and the agglutination activity was C^{2+}/ Mg^{2+} dependent.

Among the hormone effects on ticks, topical application of ecdyson on ticks significantly reduced the moulting time as well as the engorgement weights. *In vitro* feeding of 20 hydroxyecdyson and juvenile hormones resulted into various responses among which was the significant reduction of engorgement weights of nymphs, moulting time and infection rates. The deleterious effects of hormones on ticks should be further investigated.

Enzymes particularly trypsin and protease were detected in the guts and not in the salivary glands using casein gel as substrate and results showed that there were higher levels in the infected females. These enzymes were blood meal induced and peaked at least 48 hr following a blood meal. The phenoloxidase system in *Rappendiculatus,* activated by methanol, indicated significantly low levels of phenoloxidase in the haemolymph, compared to insects (locusts) and *Amblyoma variegatum.* At least 95% of the phenoloxidase activity occurred in the plasma and was highest among the infected ticks indicating that infection with *T. parva* altered the normal levels if this enzyme activity and suggesting that the system may be involved in the "self" and "non self" recognition among ticks.

Scanning electron microscopy revealed that the salivary glands had a "grape like" appearance with type I and II acini having smooth surfaces unlike the type III which were bigger and had sculptures. The infected type III acini were bigger and had diffuse surface structures. The type III acini had distinguished morphological features which did not exist in the **other cells**

implying that the possible mechanism of entry of kinetes into the e cells was most likely specific receptors at the site of entry into these cells, coupled with chemical attraction as revealed by the glycogen deposits and was unlikely to be by chance as earlier suggested.

The investigations revealed the lectins should be looked at as possible immunogens in the blocking of transmission of *T. parva* as well as anti- tick interventions in the future of theileriosis control.

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CHAPTER ONE

1.0 GENERAL INTRODUCTION AND OBJECTIVES

1.1 Theileriosis

Theileriosis is the name given to infections caused by several species of *Theileria*. *Theileria* parasites are of great economic importance in many parts of the world where they cause disease to domestic animals (Purnell, 1977; Dolan, 1989), the two most important in Africa being *Theileria parva* and *T. annulata*. The disease in cattle has been given different names in various parts of the continent. For instance, *T. parva* infections are referred to as East Coast fever (*T. parva*), Corridor Disease or January Disease, while *T. annulata* infections are called tropical theileriosis. *Theileria parva* was first recognized in South Africa as East Coast fever following importation of cattle from East Africa (Norval *et al.*, 1992). It was reported eradicated in S.A but persisted in E.A where it causes very high mortality especially to the taurine breeds of cattle. A similar situation occurs in N. Africa where *T. annulata* causes considerable losses. *Theileria parva* is mainly transmitted by the brown ear tick *Rhipicephalus appendiculatus* Neumann, 1901. A closely related species *R. zambeziensis*, whose distribution is limited to Southern and Central Africa, is also a vector of *T. parva* (Norval *et al.*, 1992).

Transmission is transstadial (Theiler, 1911) whereby the larvae or nymphs become infected when they feed on infected cattle and transmission is by the subsequent nymphs or adults (FAO, 1983; Theiler, 1911; Norval *et al.*, 1992; Neitz, 1957; Barnet, 1968). Transovarian transmission is common in the related *Babesia* species (Young and Morzaria, 1986) but have not been proven in *Theileria* species (Norval *et al.* 1992). The infective stage of *Theileria* is the sporozoite found in the salivary glands of the tick vector. Sporozoites are introduced in cattle by the feeding ticks. They infect lymphocytes that become transformed by the developing schizont, into lymphoblastoids which divide into daughter cells (Hulliger *et al.* 1964; Irvin *et al.* 1982). The developing schizont undergoes merogony and the lymphocyte ruptures to release merozoites. The latter enter erythrocytes. The pathogenicity of several *Theileria* species is as a result of

the lymphocyte transformation and their destruction by merogony of the schizont and cytolytic lymphocytes (Norval *et al.* 1992).

According to Miller *et al.* (1977) half a million cattle die from East Coast fever each year in East Africa. Attempts made to evaluate the economic importance of theileriosis in domestic animals (Norval *et al.* 1988; Pegram *et al.* 1989; Morzaria *et al.* 1988; Mukhebi *et al.* 1992; De Castro *et al.* 1985) indicate the effects of vector ticks as well as chronic theileriosis in endemic areas to be major obstacles in assessing productivity. Morbidity and mortality of susceptible cattle may approach 100% . Acute theileriosis causes severe disease and extremely high mortality with an estimated direct cost (in beef, milk and losses due to treatment and acaricides) is estimated to be US$ 168 million per year and a mortality of 1.1 million cattle (Mukhebi, 1992). In economic terms, theileriosis is certainly a major problem in Africa, Asia and a few areas where cattle productivity is being upgraded (Norval *et al.*, 1992).

Further studies on the life cycle of *T. parva* in the tick are still required in particular with regards to the sexual cycle, and the generation of antigenic diversity. Assuming that the zygote and kinete stages are diploid compared to the sporozoite and gametocytes Morzaria (1989) and Morzaria *et al.*, (1992) showed that sexual recombination occurred between two populations. More studies in this area would have important implications in the epidemiology and control of theileriosis. Similarly, the biological development of sporozoites in the salivary glands is of particular interest because a single kinete would produce several thousand sporozoites infective to cattle. The interaction between the salivary gland acini cells and the developing parasites is of particular importance in the transmission process, and is yet to be fully established.

Physiological properties of ticks are therefore important for understanding how *T. parva* and indeed other micro-organisms circulate within tick bodies and are transmitted to vertebrates. Such investigations would elucidate the physiological adaptations to parasitism the tick undergoes in order to facilitate its survival under unfavourable conditions.

Although the physiology of the tick has been studied by many scientists (Balashov, 1972; Obenchain and Galun, 1982; Norval *et al.*, 1992) questions on the effect of the physiology of the tick on the development of *T. parva* still exist, for instance:

- What are the physiological conditions inside the tick that make them suitable for development and transmission vectors of *T. parva*?
- How do the physiological conditions in the tick affect the development of *T. parva*?
- Can these physiological conditions be manipulated to make the tick less or more susceptible to *T. parva* infection?
- How does the parasite recognize its target cells in the tick?
- To what extent does the parasite control and synchronize its life cycle in the tick?

Further work on the physiology is, therefore, required in order to understand the interaction between the tick and the *T. parva*. Dipeolu (1989) noted that while there had been remarkable advances on research on feeding behaviour of ticks, many important aspects had been neglected including development and survival under natural conditions, the phenomenon of natural resistance to tick infestation in cattle and biochemical and physiological studies. The latter could have an impact on the control of the disease. He went on to emphasise the need for further laboratory studies on tick biological systems. Against this background, the following studies were initiated with these objectives:

1.2 Objectives of the study

1. To determine the factors that affect the development of *T. parva* in the tick *R. appendiculatus* in particular:
   - Lectins
   - Enzymes
   - Hormones
   - Blood meal
2. To investigate why the kinetes selectively invade type III acini of the salivary glands.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Tick species and distribution

Ticks belonging to super family Ixodidae are a group of haematophagus arthropods that are obligate temporary parasites of mammals, birds or reptiles. About 500 species of ticks have been described in Africa (Hoogstraal, 1956) with a small proportion being of economic importance. These include: *Amblyomma variegatum* (Fabricius, 1794) that transmits cowdriosis; *Rhipicephalus appendiculatus* (Neumann, 1901) theileriosis; *Boophilus decoloratus* (Koch, 1844) Babesiosis; *Hyalomma anatolicum anatolicum* (Koch, 1844) Tropical theileriosis. *R. appendiculatus, R. duttoni, R. zambeziensis* have a definite relationship between geographical distribution and the occurrence of *T. parva* (Lessard *et al.*, 1990; Fig. 1.1).

The most widely spread tick in Africa is *B. decoloratus* which is distributed from West Africa (Dipeolu, 1984; Amoo, 1984) through Central Africa (Chizyuka and Mangani, 1986) to East Africa (Kaiser *et al.*, 1982) and Sudan (Latif,1986). This wide distribution is due to its tolerance of a wide range of climatic conditions in particular temperature. The other ticks are more limited in distribution as they are more sensitive to temperature, moisture and relative humidity.

Ripicephalus appendiculatus is known to be absent from West Africa (Maywald and Sutherst, 1986). Cattle are the predominant hosts of ticks in Africa (Punyua and Newson, 1985; Tatchell and Easton, 1986) although sheep and goats and a variety of wild animals can act as hosts (Yeoman and Walker, 1967; Walker, 1974). The African buffalo (*Syncerus caffer*) is a particularly good host (Walker, 1974; Table 1.1).

Table 1.1 Distribution of Vectors of *Theileria* parasite species and their vetebrate hosts

Vector	Parasite	Tick hosts	Distribution
R.appendiculatus *R.zambeziensis* *R.duttoni*	*T.parva*	cattle and buffalo	East & central Africa
R.appendiculatus *R.pulchellus* *R.zambeziensis*	*T.taurotragi*	cattle, sheep, goats & ungulates	East, central & S.Africa
A.variegatum *A.hebraeum* *A.astrion* *A.gemma* *A.cohaerens*	*T.mutans*	cattle & buffalo	East, central & S.Africa
A.variegatum *A.hebraeum* *A.lepidum* *A.astrion*	*T. velifera*	cattle & buffalo	wide spread within the distribution of tick vectors
Hyalomma species	*T.annulata*	cattle, Asiatic buffalo, bison	North Africa, Southern Europe & Asia
Haemaphysalis species	*T.orientalis (sergenti)*	cattle	Asia, Australia, Europe, North America & Africa
R.evertsi	*T.separrata*	cattle	East & Central Africa
Rhipicephalus species	*T.ovis*	sheep, goats	Europe, Asia & Africa
H. evertsi	*T.hirci*	sheep & goats	Europe, Asia & Africa

2.2 Vectors of *Theileria parva*

The vectors of *T. parva* in Africa and their hosts are given in Table 1.1. There are about 800 species of ticks so far described belonging to three families: Argasidae (150 species); Nuttallidae (1 genus and 1 species) and Ixodidae (650 species) (Norval *et al.*, 1992). Family Ixodidae comprises the most economically important group of ticks. Characteristically, members of this family possess a hard chitinous scutum which covers a small portion of the larva, nymph and female. There are four sub-families with 13 genera of which *Ixodes*, *Haemaphysalis*, *Rhipicephalus*, *Dermacentor*, *Boophilus*, *Hyalomma* and *Amblyomma* are of great economic importance (Norval *et al.*, 1992; Hoogstrall, 1956; Walker, 1974).

As far back as 1904, Lounsbury identified the vector of *Theileria parva* as the brown ear tick *Rhipicephalus appendiculatus* Neumann, 1901. *R. appendiculatus* transmits three sub species of *T. parva* (*T.p.lawrencei*, from buffalo to cattle, causing corridor disease; *T.p.bovis*, a mild form of theileriosis (January disease) and *T. parva* (East coast fever; Uilenberg, 1981; Grootenhuis *et al.* 1987). Whereas most *Theileria* species are transstadially transmitted, *T. annulata* is also transovarian transmitted in *Hyalomma anatolicum excavatum* (FAO, 1983). *Rhipicephalus appendiculatus*, *R. duttoni* and *R. zambeziensis* on the other hand have been shown to have a definite relationship between the geographical distribution and the occurrence of *T. parva* (Lessand *et al.*, 1990; Fig.1.1).

The distribution of *R. appendiculatus* covers 15 countries including Kenya, Uganda, Zambia, Malawi, South Africa, Central African Republic, Sudan, Zaire, Rwanda, Tanzania, Burundi, Mozambique, Swaziland, Botswana and Zimbabwe (Norval *et al.*, 1992; Fig. 1.1). *R. appendiculatus*, therefore, occurs from Sudan in the North to Natal in the South (Elbil and Anastos, 1966). *R. appendiculatus* is absent in West Africa (Hoogstrall, 1956) and occupies altitudes from sea level to about 3000 m. Its distribution is influenced by rainfall, climate and vegetation (Kettle, 1984), favouring above average rainfall, and vegetation cover, which influences the microclimate (Minshull and Norval, 1982; Short *et al.* 1989) and hence the survival of the free-living stages (Branagan, 1975;

Short *et al.* 1989). According to the ecoclimatic predictions from the *Climex* model (Maywald and Surtherst. pers Comm, 1997) the areas suitable for *R.appendiculatus* are many in Africa (Fig 1.2) and have similar ecoclimatic suitability with *A. variegatum* (Fig 1.3) in East Africa. This finding underscores the need for control of these tick species before they spread to these potential habitats.

2.3 Life cycle of ixodid ticks

The life cycle of the ixodid ticks comprises four developmental stages namely egg, larva, nymph and adult, which exist as ecto-parasites on hosts and free-living stages off the hosts. Depending on the number of hosts used to complete the cycle, there exists three developmental patterns; one-host two-host and three-host ticks.

2.3.1 One-host tick

The eggs of the one host tick, exemplified by *Boophilus decoloratus* hatch on the ground into six-legged larvae which look for a host and attach, feed and moult into eight-legged nymphs which then feed upon the same host, moults to the adult stage that feed as adults. These engorged detach and lay eggs off the host. Since the newly moulted nymphs and adults remain on the host throughout the feeding and moulting, they are well adapted to harsh environment (Soneshine, 1992).

2.3.2 Two-host ticks

The two-host tick like *Hyalomma anatolicum anatolicum* follows a similar pattern except for the nymphs that detach and moult off the host, leading the adults to find a different (second host) (Soneshine, 1992).

2.3.3 Three-host ticks

Among the three ticks, the larvae moults to nymphs and nymphs to adults off the host, hence the larvae, nymphs and adults feed on separate hosts. The three-host ticks are considered primitive in the evolution sequence. One and two host ticks moult faster thus reducing the generation time. About 600 of the 650 ixodid species have a three-host life cycle (FAO, 1984). However, one host ticks are vulnerable to unfavourable

environmental conditions whereas the three host ticks regulate their survival mechanisms to suit harsh environments.

2.4 *Theileria parva*

Theileria are tick-borne protozoan parasites which, in the mammalian host, undergo successive developmental stages, in lymphocytes and erythrocytes. There are two species (*T. parva* and *T. annulata*) which cause economically important diseases in cattle (Irvin and Morrison, 1987). Unlike babesiosis or anaplasmosis, young animals do not exhibit greater resistance to theileriosis. The classification of *T. parva* is given in Table 1.2.

Table 1.2 Classification of *Theileria* and the distinguishing features (According to Irvin, 1987)

Sub kingdom	Protozoa (single celled eukaryotes)
Phylum	Apicomplexa (apical complex present at least in some stages; reproduce sexually by syngamy).
Class	Sporozoea (Sporogamic stage producing sporozoites)
Subclass	Piroplasmia (Piroform rod-shaped or amoebic parasites in erythrocytes and other cell types)
Order	Piroplasmida (Asexual and sexual reproduction; ticks are vectors)
Family	Theileriidae (Schizont stages in lymphocytes)
Genus	*Theileria* (Piroplasm stage in erythrocytes lacks pigment)

2.5 Life cycle of *Theileria parva*

The life cycle of *T. parva* occurs in both vertebrate (cattle) and invertebrate hosts (tick). The parasite is well adapted for the two hosts through a series of several morphological changes ranging from piroplasms, gametocytes, gametes, zygote, kinetes, sporozoites, schizonts and merozoites, details of which are outlined in the section below.

2.5.1 Life cycle of *T. parva* in the vertebrate host

T. parva sporozoites, transmitted by the tick on feeding, enter the lymphoid cells through stages with the initial sporozoite and the lymphoid cell coming into close proximity, followed by zippering of the sporozoite and cell membranes. The cell to cell adhesion is Ca^{2+} independent process (Shaw *et al.*, 1993). However, the mobilisation of Ca^{2+} from the intrasporozoite stores following sporozoite binding to the host cell surface was found to be essential for successful parasite invasion (Norval *et al.*, 1992; Shaw *et al.*, 1993). Whether the process is energy dependant or not is still controversial (Fawcett *et al.*, 1982 a&b; Fawcett and Doxsey, 1982; Jura *et al.*, 1983). On entry, the sporozoite is surrounded by tightly applied host cell membrane (Schein *et al.*, 1978; Fawcett *et al.*, 1982) involving some cytochalasin- inhibitable re-arrangement of the host cell membrane or cytoskeleton (Shaw *et al.*, 1991). The major histocompatibility complex (MHC) class I molecules are essential components of the host cell surface receptor involved in *T. parva* sporozoite invasion (Shaw *et al.*, 1995).

The *Theileria* infected lymphocytes transform; the schizonts inducing lymphoblastogenesis and expand (Brown, 1987; Ole-Moi Yoi, 1989) by production of casein kinase II. The process can be reversed by Parvaquone treatment (Pinder *et al.*, 1981). Binnary fission follows (Mehlhorn and Schein, 1984) producing multinucleate shcizonts (Irvin *et al.*, 1982) which increase the number of infected cells (Jarret *et al.*, 1969; Irvin *et al.*, 1982). As the host cell divides, the schizonts also divide and get distributed to the daughter cells (Hullinger, 1965; Hullinger *et al.*, 1964). The *Theileria*-infected lymphoid cells out grow and infiltrate all lymphoid cells and the body tissues in about 14 days (Radley *et al.*, 1974); Morrison *et al.*, 1981; Dollan *et al.*, 1984a). The spherical schizonts develop into merozoites *in vivo* 8-10 days (in case of *T. annulata*) and

12-14 days in *T. parva*, *T. taurotragi* and *T. mutans*). Merozoites developing (Mehlhorn and Schein, 1984) destroy the host cell, leaving the merozoites free to enter the erythrocytes.

In other words, within three days of tick attachment, the infective sporozoites are injected from the salivary glands into the bovine host. The sporozoites enter the lymphocytes (target cell) by a receptor-ligand mediated process (Fawcett *et al.*, 1982a). Once inside the cytoplasm, the surrounding host cell membrane is dissolved and the sporozoite undergoes differentiation and asexual division producing a multinucleate schizont. The parasite induces host-cell transformation and divides synchronously with the cell (Fawcett *et al.*, 1982a) and develop into schizonts (Fig. 1.4). The lymphoid cells are transformed due to the presence of the parasite. As the lymphocytes divide, the schizont is pulled apart on the spindle resulting in the majority of daughter cells being infected with schizont. Hence, there is a clonal expansion of infected cells. The schizont is the pathogenic stage in cattle where the rapid multiplication of the infected cells in the lymphoid organs result into lymphocytolysis and death. Some of the schizonts eventually undergo merogony and the lymphocyte is ruptured to release merozoites. Merozoites penetrate the erythrocytes by unknown mechanism. However, they must have specific receptors sites for erythrocytes which may be different from those of the sporozoites since merozoites only infect erythrocytes (Norval *et al.*,1992). They invade the erythrocytes and develop into piroplasms. The piroplasm either divide readily in the erythrocytes or not depending on the species of *Theileria*. The feeding tick in either the larva or nymphal stages ingests piroplasm parasitized erythrocytes (Norval *et al.*,1992).

2.5.2 Life cycle of *Theileria parva* in the invertebrate hosts (ticks)

The development of *T. parva* has been reviewed by Melhorn and Schein (1984). Briefly, on ingestion of the erythrocytes by the tick, lysis follows, leading to many free piroplasms being released into the gut (Fig. 1.4). A variable proportion of piroplasms undergo differentiation in the lumen of the tick into morphologically identifiable micro and macro sexual stages (Norval *et al.*, 1992). In addition, strahlenkorper bodies are formed (Koch, 1906; Cowdry and Ham, 1932) whose existence was confirmed with

electron microscopy by Mehlhorn *et al.* (1975) and Mehlhorn and Schein (1976), and in culture experiments in 1984 by the same authors. These are the gametes of the parasite formed on the 2-4 th day of tick feeding depending on temperature (Young and Leitch, 1981). Spherical stages of 4-5 um diameter appear, which are considered to be macrogametes (Mehlhorn and Schein, 1984; Young *et al.*, 1980).

2.5.2.1 Development of *T .parva* in the tick gut cells and haemolymph

Syngamy follows (Young and Leitch, 1981), resulting in the formation of a spherical zygote which enters and develops in the gut epithelium of the tick, which is believed to have special receptors for zygote entering (Walker, 1990). After repletion in the tick, the zygote transforms to a kinete (Mehlhorn *et al.*, 1978) a process which appears to be synchronized with the moulting of the tick (Young *et al.*, 1980) so that the kinete stage penetrates the newly formed salivary glands of the next stage. The kinete pass through the gut wall into the haemolymph and invade the e cells of the salivary glands, where they undergo differentiation and extensive division to produce uninucleate sporozoites, which are infective to cattle.

2.5.2.2 Sporogony within the tick salivary gland

Salivary glands lie in the haemocoel of the tick. The mechanisms by which they become infected is controversial and is thought to be probably by chance (Bell, 1980; Bell and Reid, 1981), although this requires further investigation. Salivary glands are only available for infection at the time that they develop after each moult (Fawcett *et al.*, 1981; 1982b). The parasite selectively invades and develops in the e cell of type III acini although type II can also become infected when the number of kinetes produced is very high (Young, pers comm). The kinetes of *T. parva* enter the type III salivary gland acini. According to Mehlhorn and Schein (1984) and Walker (1990) the *d* cells are also infected, although Fawcett *et al.*(1981; 1982b; 1985) and Binnington *et al.* (1983) believed that *Theileria* parasites enter only granular *e* cells. Having entered the *e* cell, the kinetes are not seen to be surrounded by a parasitopharous vacuole. They round up and become polymorphic reaching the stage called a sporont. During the feeding stage of the tick, the cytoplasmic volume of the infected *e* cell, and the nuclear volume increase

(Fawcett *et al.*, 1982b; 1985; Young *et al.*, 1983) under the influence of the growing sporoblast. The structure is now called a sporoblast. At variable times, between days 3-4 after attachment of the tick, protrusions are formed which are arranged in association with organelles so that sporozoites are formed. The sporozoites are then emitted from the salivary gland acinus. One kinete produces in the case of *T. parva* 40,000 sporozoites in adult ticks (Norval *et al.*, 1992). Studies on the feeding mechanism of ticks and attachment to the mammalian host are reported by Arthur (1962) and Waladde and Rice (1982) but further investigations to determine the method of emission of the sporozoites from the ticks are required (Norval *et al.*, 1992).

2.6.0 The phenomenon of transstadial transmission

Transmission of *Theileria parva* by its ixodid vector *Rhipicephalus appendiculauts* is transstadial. The larvae or nymphs get infected when they feed on infected cattle. The parasite undergoes development from the piroplasm stage ingested in the blood meal erythrocytes into gametocyte to zygote and kinete stages. When the larvae moult into nymphs or when the nymphs moult into adults, the kinete enters the salivary gland where it develops further into sporozoites which are infective to cattle. These are passed on as the nymph/adult feeds. Transmission, therefore, is by the subsequent stages (nymphs and adults) (Norval *et al.*, 1992).

2.7.0 Tick Feeding habits

The feeding processes of ticks are complex involving host seeking, probing, attachment, feeding and detachment, all controlled by a series of behavioural, pheromoral and physiological interactions (Obenchain and Galun, 1982; Kaufman, 1989). Ixodid ticks instars may feed on hosts for periods of several days to more than two weeks. The hosts range from mammals, birds, reptiles (Arthur, 1962). Ixodid ticks generally feed for a long time and change their hosts once, twice or three times during the life cycle. One host tick like *Boophilus microplus* remains on the host through the feeding and moulting phases of the life cycle; Larvae are the only instar involved in host location. However, most Argasids spend their life time in host nests or nesting places where the host is

available with some exceptions (*Haemaphysalis*) which is a rapid feeder that drops from the host after 30 min of engorgement.

2.7.1 Blood meal digestion in ticks

According to Wigglesworth (1943) digestion is intracellular in all Acari (Balashov, 1972; Grandjean, 1984) at acid pH (Tatchell *et al.*, 1964) below the pH 6.3-6.5 of the gut contents (Tatchell *et al.*, 1972; Bogin and Hadani, 1973). This process contrasts sharply with the insects in which digestion is extracellular, much more rapid and occurs at neutral or near- neutral pH (Gooding, 1972). The process is economical (Tatchell *et al.*, 1972; Grandjean, 1983; Connat *et al.*, 1986) and hence important in the survival of ticks for prolonged periods of starvation.

Three phases are distinguishable in the blood digestion, namely continous digestion, delayed or reduced digestion and a second phase of continuous digestion (Coons *et al.*, 1986). Each is dependent upon a specific event in the life cycle of the tick. The first continuous phase is initiated by the onset of feeding and is characterised by the concentration of the blood meal by water elimination through the salivary glands (Tatchell, 1967a; 1969; Kaufmann and Sauer, 1982); red cell haemolysis and the uptake and subsequent digestion of the blood meal by the digestive cells (Coons *et al.*, 1986). A periodic acid-schiff (PAS) positive substrate secreted in the mid gut 24 hrs on the onset of feeding is thought to be responsible for lysis of cells (Hughes, 1954; Tatchell, 1964; Grandjean and Aeschlimann, 1973). Eventually, there is uptake and digestion of the blood meal by the digestive cells (Coons *et al.*, 1986).

The delayed phase of digestion is initiated by mating (Bogin and Hadani,1973; Pappas and Oliver, 1972) and happens 12-24 hrs. prior to the detachment of the ticks from the hosts (Araman,1979). At this time, enzyme inhibition and cessation of lysosome function have been proposed. Bogin and Hadani (1973) reported the presence of a proteinase inhibitor at this stage. Virgin females do not undergo full engorgement (Snow, 1967). Males of ixodidae feed for shorter periods and detach to mate. The final phase of digestion in the mated female ixodid occurs after detachment from the host. According to

Coons *et al.* (1986) the lysosomal system is activated and digestive cells accumulate numerous residual bodies as oviposition proceeds.

2.7.2 Lectins

Lectins are heat sensitive proteins or glycoproteins, which because of their polyvalent configuration and binding affinity for specific cell surface polysaccharides, can selectively agglutinate vertebrate erythrocytes (Yeaton, 1981; Renwrantz, 1983). Lectins play a role in immune defence of arthropods by enhancing clumping of parasites, bacteria and viruses and by increasing phagocytosis due to their strong opsonizing activity (Kaaya, 1990).

The activities of lectins are, however, inhibited by specific sugars, hence carbohydrates function as recognition molecules (Goldstein *et al.*, 1980; Van Driessche *et al.*, 1994). Lectins are widely distributed and are synthesized in the fat body cells (Ratcliffe, 1986; Yeaton, 1981; Richards and Ratcliffe, 1990). Their roles as recognition molecules are demonstrated by Renwrantz and Stahmer (1983).

Among ticks, lectins have been reported in the haemolymph of *Ornithodoros tartakovskyi* and *Ixodes ricinus* (Veres and Grubhoffer, 1990; Kuhn *et al.*, 1996) and the infections of *T. parva* in *R. appendiculatus* were increased by melibiose, an raffinose infused in rabbit ears on which ticks were fed (Kamwendo *et al.*, 1993). In insects, studies on lectins in *Glossina* haemolymph and the midgut have been carried out by several authors (Ingram and Molyneux, 1990; Maudlin and Welburn, 1987; Ibrahim *et al.*, 1984; Grubhoffer *et al.*, 1994; Pathak, 1993) in other invertebrates (Yeaton, 1981) *Schistocerca* and *Periplaneta* (Lackie, 1988) Sandflies, (Wallbanks *et al.*, 1986) and Phlebotomine sandflies, (Volf *et al.*, 1994). The maturation signal of trypanosomes in tsetse flies is given by lectins (Ibrahim *et al.*, 1984; Welburn and Maudlin, 1989). The duration between an infective blood meal and maturation varies and is dependant upon temperature but is genetically controlled (Maudlin and Welburn, 1989). It is believed that comparative studies in the tick would throw light on the role of lectins in the development of *T. parva* and whether refractory ticks have high levels of lectins or not.

2.7.4 Enzymes

The most important enzymes with regards to *T. parva* development in the gut would be the digestive enzymes. This is because the parasites are ingested when the tick feeds on infected blood. The gut structure, function and biochemistry of blood digestion determine the fate of vertebrate pathogens ingested into the arthropod body with host blood or lysed tissues (Balashow, 1972). The parasite is, therefore, exposed to the digestive enzymes that are involved in the breakdown of the blood meal. Little is known about the general physiology of blood meal digestion in ticks, although some studies have been done by Bogin and Hadari, 1973; Akov, 1982) and even less is known about the enzymes involved in the process (Coons *et al.*, 1986).

2.7.4.1 Proteolytic and digestive enzymes

Akov (1982) indicated that the major digestive proteinases of the tick were related to mammalian cathepsin D. This was confirmed by Vundla (1990). *Rhipicephalus appendiculatus* was shown to have 2 digestive proteinases:proteinase 1 and proteinase 2 (Vundla, 1990). These are the enzymes that are likely to act on *T. parva* when ingested. Also, pepstatin was showed to be a powerful inhibitor of both proteinases, thus proving that *R. appendiculatus* enzymes were aspartic proteinases. The role of hydrolytic enzymes, whose primary function is to hydrolyse protein other than blood proteins needs to be investigated. Vundla (1990) also found that proteases of *R. appendiculatus* proteinases were similar to other aspartic proteinases.

Both proteinases were found to be effective immunogens and showed cross reactions with each other. Vundla (1990) reported that tick physiology had been greatly neglected, and suggested that further studies should be undertaken. According to reports on proteolytic enzymes in insects, (Gooding, 1974) *G. morsitans* and *G. palpalis* contain at least seven enzymes, trypsin, carboxypeptidase A, carboxypeptidase B, two amino peptidases, a trypsin-like enzyme, proteinase VI and chymo- trypsin like enzyme (proteinase VII). Mihok *et al.* (1994) reported relationships between protease activity, host blood and the infection rates in *Glossina morsitans* species infected with different stocks of Trypanosomes, while Gingrich *et al.* (1982) postulated a relationship between

the activity of the midgut proteinases and the development of trypanosome procyclic infection after they observed increased midgut infection rates following an infective meal with trypanosomes suspended in serum-free blood. Similar studies in ticks would form a basis for understanding the role of enzymes in parasite development and transmission.

2.8.0 Phenoloxidases

The phenoloxidase (PO) enzyme system is believed to play an important role in the melanization of the insect cuticle and to be involved in the self and non-self recognition (Richards, 1951; Prestor and Taylor, 1970). PO is present in the insects *Sarcophaga bullata* larvae as inactive proenzyme form (Saul and Sugumaran, 1988; Ratcliffe *et al.*, 1984). Inactive prophenoloxidase (PRO-PO) can be activated by Zymosan but the activation process inhibited by the serine protease inhibitors, benzamidine and p-nitrophenyl-p'-guanidobenzoate. Exogenously added proteases, for example, chymotrypsin and subtilisin also activate PRO-PO in the whole haemolymph (Saul and Sugumaran, 1988).

Phenoloxidase (o-diphenol, oxygen oxidoreductase) is involved in the biosynthesis of melanin and therefore is an integral part of the immune system of insects in its inactive form in *Bombyx mori* (Ashida 1971), *Manduca sexta* (Aso *et al.*, 1985). In *Manduca sexta*, several proteases were shown to activate the PRO-PO system in the haemolymph (Saul and Suguraman, 1986; 1987).

Kaaya (1990) noted the need for studies to broaden the understanding of the interactions that occur between vectors and parasites and emphasized that understanding immunity in vectors would be of great advantage to man and animals since the manipulation of insect immunity in the vectors might render them poor vectors.

2.9.0 Hormones

2.9.1. Hormonal control of moulting in ticks

Although ticks, like insects and other arthropods grow in size and metamorphose, they do not appear to have a prothoracic gland or corpora allata, but have a functional equivalent (Obenchain and Galun, 1982). Neurosecretion in ticks follows various axon pathways, some to discrete nueroendocrine complexes like in insects. The syganglion is highly condensed into a peri-oesophageal mass (Horridge, 1965) enclosed within a sinus of the circulatory system. Equivalent of deutocerebrum is absent as ticks lack antennae. The whole system has been described by several authors (Binnington, 1972; Obenchain, 1974).

Roshdy *et al.*(1973) described a paraoesophageal neurohaemal organ in *Argus arboreus*. Investigations with insect growth regulators suggest that tick moulting and metamorphosis, vitellogenesis, oviposition, development and reproductive diapause, are under hormonal control (Obenchain and Galun, 1982). On the other hand, Cox (1960) showed characteristic histochemical changes in the peripheral ganglion cells of the synganglion of nymphs subsequent to feeding, which paralleled events leading to moulting. Such changes were not observed in adult ticks after a blood meal. Supernumerary moults in adult ticks were induced (Cox, 1960) by transplanting nymphal brains into the adults, which led to the conclusion that moulting was under hormonal control.

Incorporation of beta ecdysone or phytoecdysones into the blood meals of *O. moubata* (Kitoaka, 1972) prevented adult females from oviposition by inducing them to undergo an additional moult. Similarly, Mango *et al* (1976) induced *O. porcinus* to supermoult under similar circumstances. Among the Ixodid ticks, Wright (1969) terminated larval diapause in *Dermacentor albipictus* Packard through topical application

of beta-ecdysone; while Sannasi and Subramonian (1972) terminated larval diapause in *Rhipicephalus sanguineus* Latreille by application of alpha-and beta ecdysones. Beta ecdysone of 1-10 *ug*/nymph resulted into moderate mortality of *D. variabilis* Say. Injection of the same product (Mansingh and Rawlins, 1977) resulted in high mortality of *Boophilus microplus*. Delbecque *et al.*, (1978) isolated and identified alpha and beta ecdysone from the nymphs of *Amblyomma hebraeum* Koch, using RIA, gas liquid Chromatography and mass spectrometry.

In *Locusta migratoria*, azadirachtin, a tetranotriterpenoid seed constituent of Neem tree, *Azadirachta indica* treatment led to a reduction of ecdysteroid titres and accumulation of neurosecretory material in the corpus cardiacum (Rembold, 1989). In *Rhodinius prolixus*, the same compound disturbed the release of ecdysteroid by the prothoracic glands (Garcia *et al.*, 1986). From the foregoing, it would appear that certain ecdysteroidal hormones and their agonists have effects on the timing and outcome on the moulting process in invertebrates. Similar investigations on the ticks and *Theileria parva* would improve our understanding of the developmental cycle of this parasite. It is possible that very similar compounds may play a role in this event mimicking the control of for instance, insect cuticle moulting.

2.9.2 Physiological state and secretion of hormones

Iofee and Uspensly (1979) related "activity" within the tick neurosecretion cells with moulting by relating the volume of the material to seasonal development. Experimental evidence on the role of ecdysteroid hormones in the control of moulting has revealed that the hormone is associated with the moulting process. Ligation and transplantation experiments indicated that moulting in ticks was controlled by circulating hormones (Cox, 1960).

The effects of exogenous ecdysteroids have ranged from breaking diapause (Wright, 1969; Sannasi and Subramonium, 1972) to mortality in enlarged adults (Mansingh and Rawlins, 1977). Topically-applied ecdysterone (1-10 ug) was shown to produce moderate mortality in nymphs. Mango (1979) demonstrated that exogenous

ecdysteroids accelerated moulting in *O. porcinus porcinus* nymphs. Feeding *in vitro* of 1-8 µg ecdysterone in defibrinated pig blood to ticks produced high mortality. Cox (1960) produced super moulting adult *Ornithodorus tuniocata* as well as similar studies by Mango *et al.*, (1976). What happens to *T. parva* when the hormones change during moulting or if the moulting is too prolonged has been a subject of this investigation.

2.10.0 Current methods in theileriosis control

The control of theileriosis like many other vector-borne diseases in the world today is based upon elimination of the tick vector and the parasite within the mammalian host. Recently, an integrated approach was being sought which encompasses tick control by acaricides, biological control, parasite control by immunization with a recombinant P67 antigen, infection and treatment method, chemotherapy and breeding of resistant breeds of cattle, all of which are expected to reduce the disease incidences to below economic threshold.

2.10.1 Control of the tick vector

Since the discovery discovered that East Coast fever was transmitted by the tick *R. appendiculatus* (Lounsburry, 1904) control of theileriosis has mainly been directed towards tick vectors. Various methods used included pasture spelling based on the survival of the infected ticks in which pasture was left free of cattle for 15 months (Norval. 1977; Norval *et al.*,1992). Control of cattle movement (Yeoman, 1964) and application of acaricides (FAO, 1984). Such methods started in South Africa and have since spread to Kenya and Tanzania.

2.10.1.1 Acaricides

The conventional acaricides are applied in dips, in which animals are dipped (Norval *et al* .,1992). The high cost of the acaricide, tick resistance, environmental pollution and levels of water in arid and semi-arid regions have all rendered their use unpopular. "Spot on" synthetic pyrethroids have been developed recently and are applied on ears or perinium in specific body parts to control ticks. "Pour on" acaricides that rapidly spread on the skin now exist (Norval *et al.* 1992). Slow release devices have been

tested and acaricides impregnated ears-tags (Young *et al.* 1985; 1989; Rechav, 1987; Schroder and Van Schalkwyk, 1989). Acaricide application has been a problem because of the rising costs of acaricides and tick resistance. Due to the above problem, new methods are being developed which include vaccines against ticks, manipulation of hybrid sterility and use of pheromones. Acaricide resistance normally develops faster in one host tick (*Boophilus*) because the generation time is shorter (Baker, 1978; Nolan, 1981; Mathewson, 1984). In Africa, resistance has developed in a number of species for example *Rhipicephalus*, *Amblyomma* and *Hyalomma* (Baker, 1978; Baker *et al.* 1978; Solomon, 1983; Chema, 1984). The role of acaricides in tick control has been a subject of debate (Howell *et al.* 1981; Norval, 1983; 1989; Bezuidenhout and Bigalle, 1987). At present, it has been recognized that in the absence of effective tick control, endemic stability for tick borne diseases can exist in indigenous breeds of cattle. In such areas, cattle may acquire immunity to anaplasmosis, babesiosis, heartwater and theileriosis by exposure to the natural ticks when the animals are young and protected by an age related resistance (Norval *et al.* 1992).

2.10.2 Vaccination

There is an urgent need for the development of vaccines against protozoan diseases since the current array of vaccines is very limited (Cornelissen and Schetters, 1996). The first vaccine against Theileriosis was by Theiler (1911) who used a suspension of cells from the spleen and large lymph nodes from animals that had died of ECF in peptone to immunise susceptible animals. This method had problems in that a proportion of animals that were immunised did not became immune and it killed a considerable number of animals. Later, improvements were carried out with *Theileria* schizont- infected lymphoblastoid cell lines (Tsur and Pipano, 1966; Stagg *et al.*, 1976; Young *et al.*, 1978; Brown, 1990). developed *in vitro* which lead to *Theileria annulata* infected cell culture vaccine first tried in Israel, Iraq, Turkey and Morocco (Brown, 1990). The draw back with this method was that the parasite lost its ability to produce

merozoites when innoculated into animals and it later lost virulence. A similar tissue culture vaccine for *T. parva* was not feasible (Brown, 1987) because there was a histocompatibility barrier for cattle that received unmatched cells infected with *T. parva* schizonts, which was bovine lymphocyte antigen (BOLA) related (Dolan *et al.*, 1984a). This resulted into clinical reactions in matched and half matched animals receiving an innoculum and a failure to infect some unmatched cattle (Morrison *et al.*, 1981). Another approach of using blood transfer for *T. annulata* and *T. mutans* (Sergent *et al.*, 1924; Uilenberg *et al.*, 1977; Young *et al.*, 1985) was discontinued due to mortality and the risk of transmitting other diseases.

2.10.2.1 Infection and treatment method

This method was conceived following the discovery that the severity of *T. parva* infections was dependent on the quantum of infectious materials introduced (Lowe, 1933; Wilson, 1950; Wilde, 1967). It was apparent that low doses of an immunisation parasite stock could be titrated and given to cattle whose response varied from no infection to inapparent reaction and several or mild disease (Wilde *et al.*, 1968; Cunningham *et al.*, 1974; Radley *et al.*, 1974; Dolan *et al.*, 1984a). It was also demonstrated that administration of tetracycline over a prolonged period in *T. parva* infections resulted in cattle becoming effectively immunised without adverse effects (Neitz, 1957; Spooner, 1990; Jarret *et al.*, 1969). The infection and treatment method was improved with the production of sporozoite stabilates which allowed cattle to be infected with a particular predetermined dose (Cunningham *et al.*, 1973). A single dose of oxytetracycline (20 mg/kg) at the time of infection with the sporozoite stabilates results into the development of immunity to homologous *T. parva* stabilate with minimal clinical response (Radley,

1978; 1981; Morzaria *et al.*, 1988; Young *et al.*, 1990a; Mutugi *et al.*, 1991) has been recommended. An evaluation of other drugs such as parvaquone (Dolan *et al.*, 1984b; Young *et al.*, 1990), buparvaquone (Young *et al.*, 1990b) have shown a major difference in their usage in chemotheraphylactic immunization against *T. parva*. A combination of different immunogenic stocks (*T. parva* Muguga; *T. parva* Kiambu and buffalo- derived *T. parva* Serengeti transformed) commonly known as the "Muguga cocktail" (Mutugi *et al.*,1990; Radley *et al.*, 1975) was shown to afford protection against *T. parva* (Musisi, 1990) and is currently being used in East and Central Africa.

2.10.2.2 Chemotherapy

Early and rapid diagnosis is a pre-requisite to effective use of chemotherapy since none of the drugs are effective when given once respiratory signs have developed. Chemothgerapy for theileriosis has been developed in the past 15 years (Dolan, 1981; McHardy, 1984; 1989) particularly with the growth of *T. parva and T. annulata* schizonts *in vitro* (Malmquist *et al.*, 1970). These were made available to Wellcome Foundation which screened several compounds ranging from menoctone (McHardy *et al.*, 1976) which was too expensive to develop further, to naphthoquinones for example, parvaquone (Boehn *et al.*, 1981) marketed as Clexon and recommended for two treatments at a dose of 10mg/kg at 48hr intervals. Parvaquone inhibits electron transport within cells thus killing the schizonts and piroplasms within the lymphoid cells and erythrocytes without causing damage to the host cells (McHardy, 1984). Another napthoquinone, buparvaquone, marketed under the trade name Butalex, is 20 times more effective than parvaquone *in vitro* and eight times more effective *in vivo* against *T. parva* and is administered at a dose of 2.5 mg/kg at 48 hr intervals (McHardy and Wekesa,

1985). Other compounds developed include a quinozolline called halofuginone which was effective against *T. parva* and *T. annulata* (Schein and Voigt, 1979) but was found to have *in vivo* toxicity. Trials in Kenya (Young, 1985; Chema *et al.*, 1987) led to the development of Terit (Hoechst) administered at 1.2 mg/kg orally at 48 hr intervals. The extreme cost of chemotherapy ($40) per head has led to limited use of this method. However, ECF infected cattle treated with either parvaquone or halofuginone may become carriers of *T. parva* (Dolan, 1986) and buparvaquonone may reduce the development of the carrier state. The widespread use of chemotherapy may therefore increase the number of carriers constituting danger to susceptible in-contact cattle. Consequently, in South Africa, the use of chemotherapy was banned particularly with the risk of the carrier state developing in cattle infected with buffalo-derived *T. parva* (Potgieter *et al.*, 1988).

2.10.2.3.1 Future vaccines against theileriosis

The identification of antigens that provoke immune reactions that are important in the recovery and protection of cattle from infection are now of high priority to the development of noval vaccination strategies. This would mean that a suitable antigen delivery system capable of expressing them within cells of the vaccinated animal are found. Serum derived form animals immunised with *T. parva* have been shown to slow sporozoite neutralizing activity *in vitro* making the sporozoite surface antigens potential vaccine candidates (Hall, 1988; Nene *et al.*, 1989; Musoke *et al.*, 1993). The 67 kiloDalton (kDa) *T. parva* antigen is specific to sporoblast and sporozoites stages of the parasite, while the *T. annulata* neutralising antigens molecules with similar specificity comprise of molecules of Mr 85, 72, 63 and 54 kDa (Williamson *et al.*, 1989). The p 67

vaccine is currently under field evaluation at the Kenya coast (Musoke, pers. comm, 1997).

2.10.4 Vaccination using concealed antigens

Concealed antigens have two major characteristics, first they do not stimulate an immune response during a natural parasite infestation, typically because of their physical location. Secondly, if the host is first vaccinated with the antigen, the parasite can be damaged by the immunological response that is produced. This will happen as the concealed antigen is recognised by antibody and other effector components of the host's immune system contained in the parasite's meal with the blood or other antibody containing fluid (Willadsen *et al.*, 1993). Acquired tick- resistance in cattle results from exposure to tick salivary glands antigens as a result of tick feeding (Norval *et al.*, 1992). This, however, does not afford the complete protection (Johnson *et al.*, 1986; Agbede and Kemp, 1986; Kemp *et al.*, 1986; Willadsen and Kemp, 1988; Willadsen, 1990). The concept of concealed antigens has been demonstrated by vaccination of cattle with crude extracts of semi engorged female adult ticks (Willadsen *et al.*, 1989; Essuman *et al.*,1991). The blood meal subsequently ingested by the tick carries antibodies, compliment and cells of the immune system which have been shown to lyce the cells of the tick gut, kill a proportion of the tick population and reduce the survival and fecundity of surving ticks (Willadsen *et al.*, 1989; Essuman *et al.*,1991). In Australia, purified fractions of the antigen from *Boophilus microplus* of a 89 kDa BM 86 (Willadsen *et al.*,1989; 1993; Riding *et al.*, 1994) led to the reduction of the surviving ticks, reduction in engorgement weights and egg laying capacity of the females and the gene encoding BM

86 recombinants produced in both *Escherichia coli* and baculovirus expression system and has been marketed as Tick Guard.

Immunization of cattle with a semi-purified fraction of solubilised membrane bound antigen extracted from the midgut of the tick *R. appendiculatus* resulted into decreases in engorgement weights of ticks, a 38% reduction in larval moult and increased groaming by cattle from dermal irritation (Essuman *et al.*, 1991).

2.10. 2.5 Future of integrated strategies for the control of ticks and tick-borne diseases

The philosophy behind integrated control is that the use of different methods for the control of ticks and tick-borne diseases are complimentary, economically viable and sustainable. The concept of tick eradication is unachievable in Africa as yet (Young *et al.*, 1988) and only control is feasible. The use of acaricides alone would not only be expensive but also the development of resistance will hinder their use. The Australian experience in integrated control has lead to the development of tick-resistant Zebu cattle (Sutherst, 1981) and vaccines against tick-borne diseases. A complementary approach is encouraged (Micon, 1994; Tanner and Evans, 1994). The use of population models has also led to design of management strategies for specific ecosystems and climatic zones. The models take into account the variations in livestock breeds and predictions can now be made and provided to farmers and policy makers for example when and how often to dip. Such population models include Climex (Sutherst and Maywald, 1985) that compares the relative potential for the survival and development of a species and Dymex (Sutherst and Maywald, pers comm, 1997) which are being currently used to predict population dynamics in the field before any management

strategies are implemented. Coupled with this is the need for a strong ecological data base for ticks and tick-borne diseases.

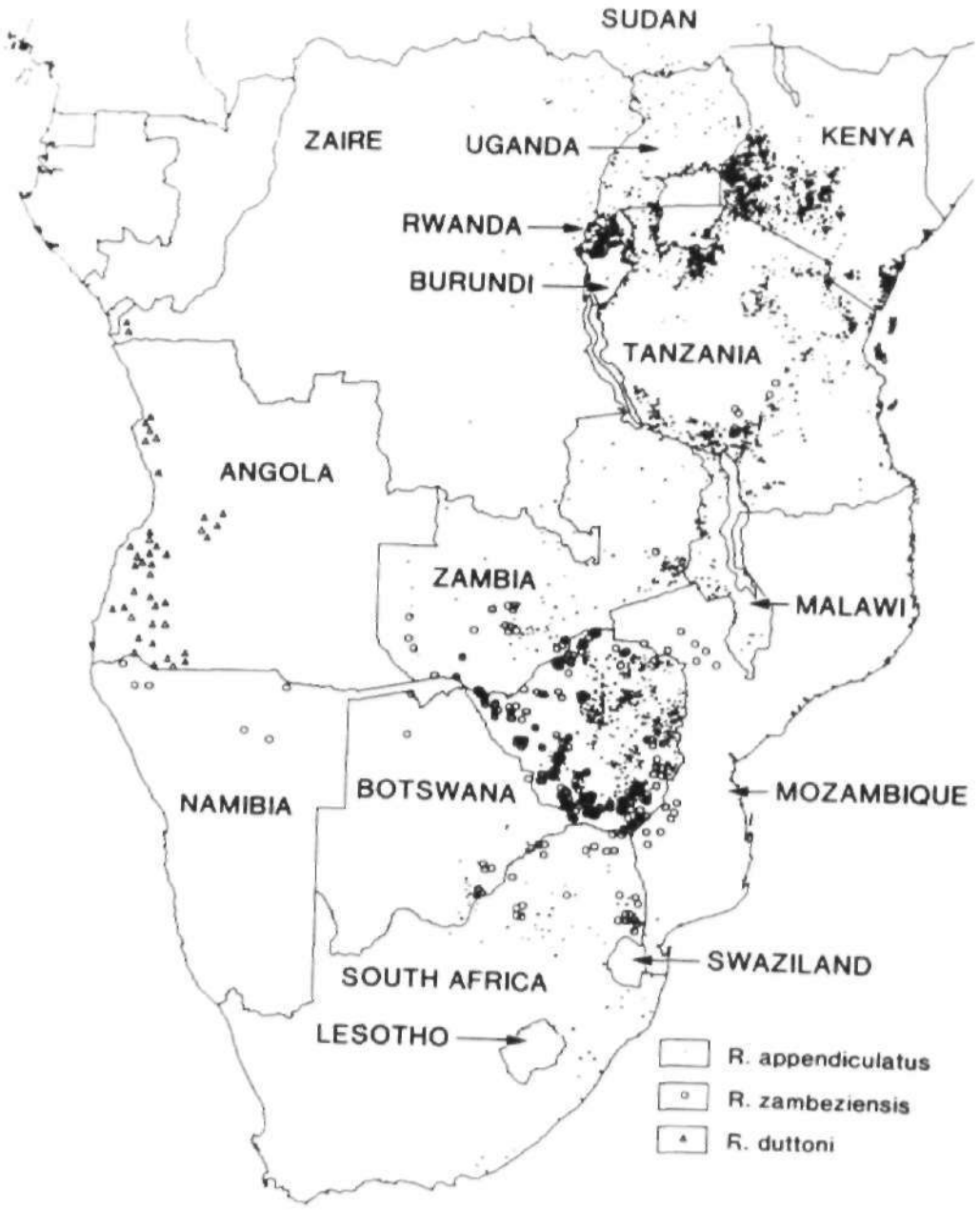
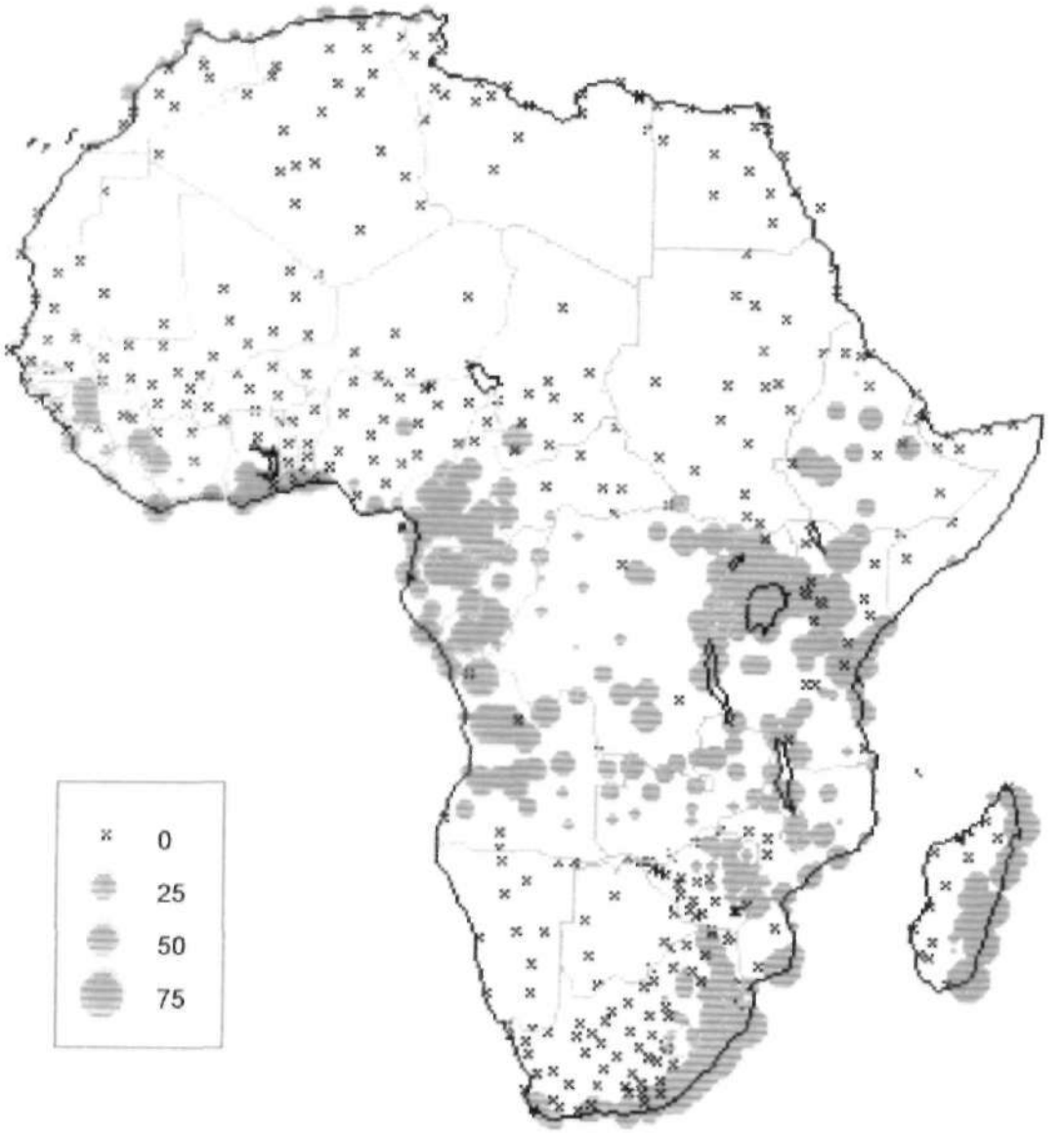
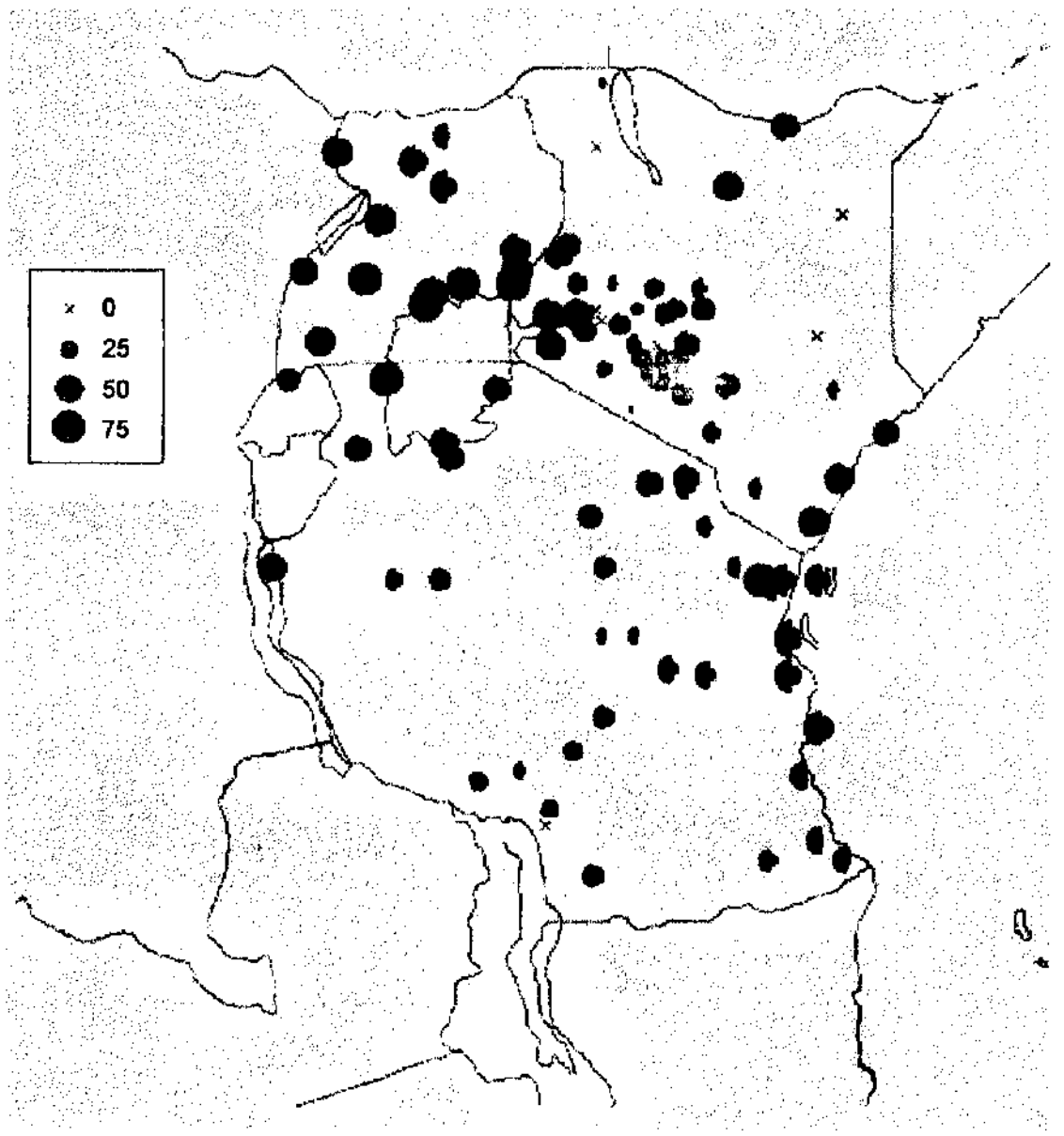


Figure 1.1 **Map showing the distribution of vectors of *Theileria* species in Africa (Norval *et al.*, 1992)**



● *R. appendiculatus*

Figure 1.2 Map showing the distribution of climatic zones suitable for the distribution of *Rhipicephalus appendiculatus* survival and development in Africa (CLIMEX;Sutherst and Maywald, 1985;1997)



● *Rhipicephalus appendiculatus*
● *Amblyomma variegatum*

Figure 1.3 Map showing the potential climatic suitability for the distribution of Rhipicephalus appendiculatus and Amblyomma variegatum in East Africa according to Climex Model (Sutherst and Maywald, 1985:1997)

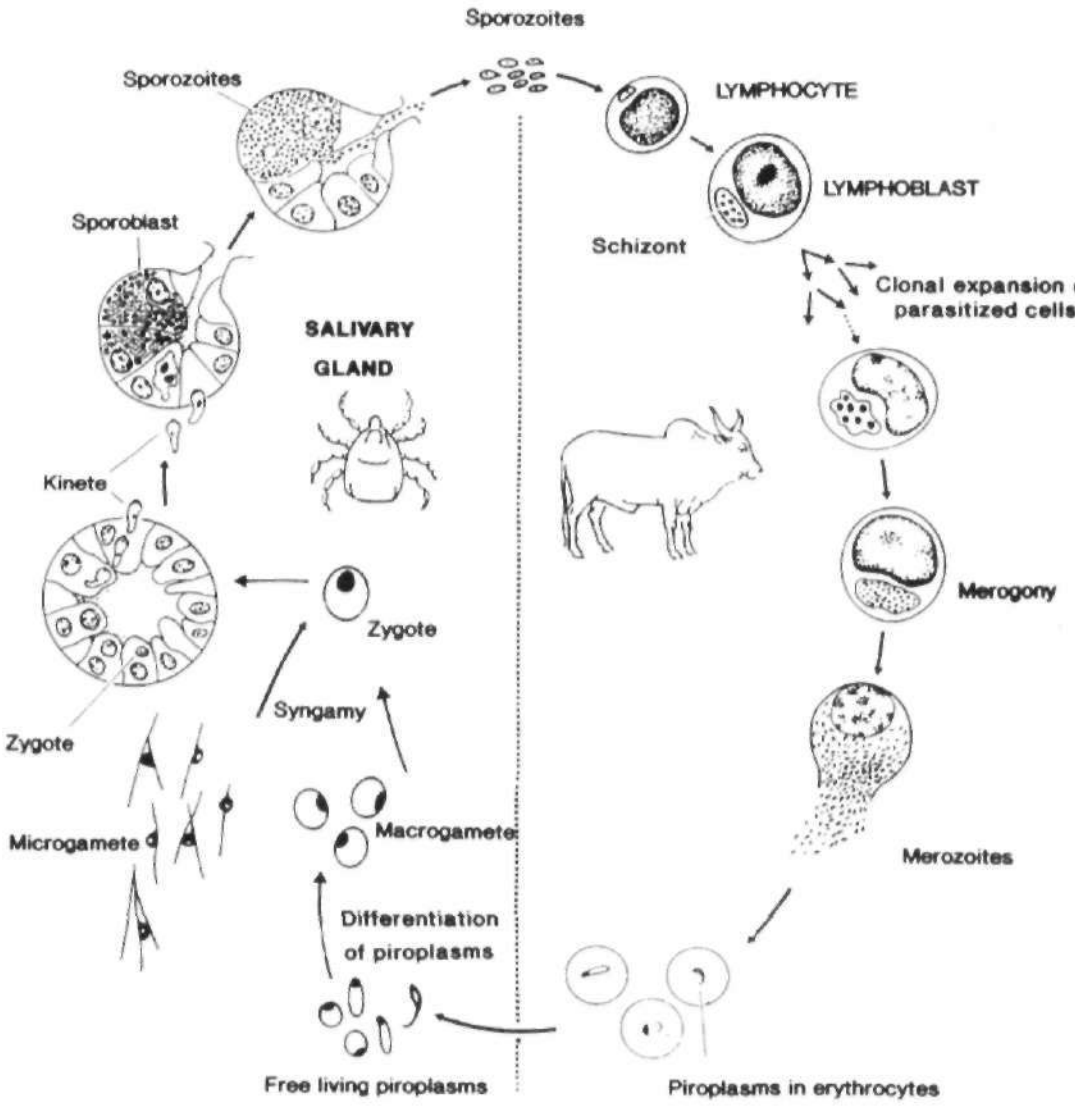


Figure 1.4 Life cycle of *Theileria parva* (Norval *et al.*, 1992)

CHAPTER THREE

3.0 GENERAL MATERIALS AND METHODS

3.1 Experimental animals, parasites and Ticks

3.1.1 Origin and maintenance of ticks

Rhipicephalus appendiculatus of the Muguga stock (RAM, Bailey, 1960) originally obtained from Kenya Agricultural Research Institute (KARI) in Muguga in 1978 was the main species investigated. For comparative purposes, *Rhipicephalus pulchellus*, a species that transmits *T. taurotragi* and not *T. parva* was used. The tick species (Fig 3.1 A&B) were obtained from the ICIPE and ILRI colonies.

3.1.1.1 Rearing and maintenance of *R. appendiculatus*

The unfed adults were maintained in 80 x 25 cm flat bottomed glass tubes plugged with cotton wool and kept in dark incubators at 20°C; 85% RH. Engorged females were maintained at 28 ^{0} C and 85% RH to lay eggs, as were all engorged instars to moult or eggs to hatch. Nymphs were kept at the above conditions until they moulted into adults. The newly moulted adults were maintained at 28°C for about three weeks for the mouth parts to harden, then put on cattle, to feed until they engorged.

3.1.1.2 Rearing and maintenance of *R. pulchellus*

R. pulchellus was kept in similar conditions as *R. appendiculatus* (unfed stages at 20 ^{0} C and fed ones at 28 ^{0} C and all at 85% RH, obtained by including a tray of water and sand in the incubator.

3.1.2 Rabbits

Feeding of ticks was carried out using New Zealand white rabbits (*Oryctolagus cuniculus*, Linneus) using ear bags (Bailey, 1960) (Fig 3.3). The same species were also used in developing polyclonal antibodies.

3.1.3 Cattle

Boran cattle (*Bos indicus)* aged between 6-18 months of either sex were used. The animals were kept off spray and contained no antibodies against *T. parva*, using the indirect fluorescent antibody test (Goddeeris *et al.*, 1982). Infected cattle were maintained in the Tick unit at ILRI, in tick secure, self contained pens, surrounded by their own water moat and fly proof netting. The animal pens were kept tick-free with dioxathion (Delnav, Coppers LTD, Kenya) and treated with high pressure steam at 130 ^{0} C from the generator (Karcher, Germany) to prevent extraneous ticks (Fig 3.1).

3.1.4 Mice

Balb C mice of either sex (6-8 weeks old) were used in the development of monoclonal antibodies.

3.2.0 Rearing *T. parva* infected ticks: Stabilate preparation and animal inoculation

3.2.1 Origin and maintenance of *Theileria* parasites

T. parva Muguga stabilate 3087 was used to infect the cattle. It was maintained in liquid nitrogen as described previously (Dolan *et al.*, 1984; Morzaria, 1989), thawed in a water bath at 37°C, and allowed to equilibrate for 30 min. It was then diluted in a

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diluent (Eagle's minimum essential medium and bovine plasma albumen (MEM/3.5; BSA/7.5 % glycerol) at 4°C to a final dilution of 1:20 (Musoke *et al.*, 1992). The cattle were injected with 1 ml on the left ear lymph node and monitored for temperature and parasitosis.

3.2.2 Preparation of ticks

The adults were selected from the batches that dropped when the cattle had rising parastaemia of greater than 10 %. This is because ticks from a dropping parastaemia would have fewer infective parasites (Alan Young Pers. Comm). The ticks selected also had high infection rates, following salivary gland examination. Infected adult *R. appendiculatus* (6 weeks to 3 months since moulting) were applied on to rabbit ears (600 per ear) for 4 days to allow the parasites to mature to sporozoites. They were carefully removed with forceps taking care not to crush the salivary glands at this moment and rinsed with tap water in a fine meshed sieve to remove rabbit debris and in 70% ethanol 3 times (1 min each) in a beaker followed by 3 times in sterile distilled water (1 min each). The ticks were then transferred to a -20 ^{0} C freezer for 5 min to immobilise them. They were weighed and counted (approximately 5 gm, made by about 1000 ticks) and ground in 100 ml Eagles' minimum essential medium (MEM) with 3.5% bovine serum albumen (BSA) in a Silverson LR2 homogeniser. The grinding vessel was placed in a beaker of crushed ice to reduce the temperature. The ground up tick suspension was sieved (gauge 60) to remove coarse debris and centrifuged in 50 ml conical tubes (Falcon 2098; 500x *g*; 5 min; 4 ^{0} C). The concentration was adjusted to 20 ticks per ml. The supernatants (which were guts) were decanted, pooled and the volume measured. An equal volume of 15% glycerinated MEM/3.5% BSA was added slowly with constant mixing on a magnetic

stirrer, and the mixture left to equilibrate (30-45 min). This gave a final guts concentration of 10 ticks/ ml. The suspension, now with the cryoprotectant, was dispensed into pre-labelled 0.5 ml AL straws marked with stabilate details and placed on globlets on crushed ice. The straws were packed loosely initially to ensure even freezing and then held in a fridge at 4 ^{0} C and later transferred to - 70 ^{0} C over night and then stored in liquid nitrogen storage tanks. When required for inoculation into cattle, the stabilate were selected randomly, put at 37 ^{0}C, mixed and inoculated 30 min later.

3.2.3 Monitoring of cattle for parasite development

The infected cattle were monitored for temperature rise daily at the same time (8.30 a.m.) which is an indication of the onset of parastaemia. This was followed by needle biopsies taken from the parotid lymphnode, on day 5 and there after. The biopsies were smeared, Giemsa stained and examined for the schizonts. Starting from the 10th day, biopsay smears from the contralateral precaspular lymphnodes and blood smears from the peripheral ear vein were examined for both schizonts and piroplasms. Parastaemia was estimated by counting of infected red blood cells per 1000. For this purpose, blood was collected from the jugular vein in EDTA coated vacutainner tubes. Packed cell volume was determined using the microhaematocrit method. From day 11 when the piroplasm parastaemia was above 4 %, ticks were applied (Fig 3.1). The animals that became highly infected with ECF were euthanized.

3.2.4 Tick application and determination of infection rates

To obtain infected ticks, the nymphs were fed on *T. parva* Muguga infected cattle, until they dropped. They were applied in ear bags or in back bags held on by glue (Fig.

3.2). Prior to application of ticks, the backs of the cattle were shaved using electric shaver (Osler manufacturing Co. Milwaake, Wis.USA). Clean *R. appendiculatus (800* nymphs*)* were applied on days 11, 13, 15 and 17 in 2 front cloth back patches, adhered to cattle using glue (Pattex, Henkel Chemical Kenya; Fig. 3.2). On engorgement (5 days later; Fig 3.2) the ticks were weighed and the females kept in glass tubes to lay eggs at temperatures described above. Nymphs were kept in tubes (200 per tube) and were incubated at 24 ^{0}C; 85 % RH. To determine infection rates in ticks, nymphs that dropped were maintained at 24 ^{0}C RH 85 % to moult into adults (3 weeks). They were kept for a further 3 weeks at 20 ^{0} C for their mouth parts to harden. About 200 adult *R. appendiculatus* (100 female and 100 males) were selected and applied to rabbit ears for 4 days. They were then removed, and 30 males and 30 females randomly selected and dissected for salivary gland infection of *T. parva*. The salivary glands were dissected in Dulbeccos' PBS and spread on slides. The salivary glands were fixed in absolute methanol for 10 mins and hydrolysed in 5M HCL for 1 hr. after which they were stained in Schiffs stained for 1 hr. They were rinsed in distilled water and air dried before examination in phases contrast microscope. Positive parasitised salivary gland acini could be identified by the periodic acid Schiff reaction presence of cells bright areas of sporozoites for *T. parva* (Fig 3.4).

3.2.4.1 Defining infection rates

The infection rates were determined and assessed according to Busher and Otim (1986). The infection was defined as follows:**Batch:** This consisted of engorged ticks harvested form the same animal on a particular day.**Prevalence:** Applied to both female

as the percentages of male and female ticks infected in a batch.**Abundance:** Defined as the mean number of infected acini per salivary gland in the particular batch examined. **Intensity:** This was taken to be the mean number of infected acini per infected tick.

3.3.0 *In vitro* feeding system for *R. appendiculatus*

The *in vitro* feeding system for *R. appendculatus* was made from Baudruche membranes attached to a feeding chamber constructed from glass beakers.

3.3.1 Baudruche membrane modification

The Baudruche membrane was made from cattle intestinal tissue processed in such a way that the soluble volatile natural compounds were extracted (Joseph Long Inc., Belleville, New Jersey 07109, USA). The other components included cotton wool, rabbit hair and an adhesive cement (Evo-stick glue®, Evode, Ltd, Staford, UK) diluted in chloroform. To achieve the correct thickness, the membrane surface was re-inforced and made rough by the addition of thin layers of cotton wool and rabbit hair. The opposite surface of the membrane which came into contact with the feeding medium was smooth and water proofed with adhesive cement. The degree of membrane thickness depended on the tick species and the instar to be fed. The membrane was hanged to dry in a hood (Flow Laboratories, UK) for several hours. The dry membrane was fixed on to the feeding apparatus called the blood chamber (Fig 3.5).

3.3.2 The tick chamber

The tick chamber consisted of a plastic ice-cream cup of 7.1 cm internal diameter and 1.5 cm depth (Fig 3.5). A 1cm diameter opening was made on the side to serve as a tick

harvesting aperture. The hole was kept sealed with plasticine all the time except when harvesting, to keep ticks from escaping from the chamber. At the bottom of the plastic chamber was black muslin cloth which provided a dark airy atmosphere. The top end of the tick chamber was left open ready for attachment on to the blood chamber.

3.3.3 Detoxification of the membranes

The toxic substances in the chambers (mainly the glue) were removed by rinsing in a water bath at least 3 times at 80 ^{0}C at least 8 hr each. Then the chambers were dried under the hood (12 hr). The interior of the chambers was sterilised with a 1:1 mixture of gentamycine, penicillin/ streptomycin and fungizone. The antibiotic/ fungicide (5ml each) was made up in a half litre of Normal saline. The external aperture was covered with zinc oxide plaster which kept it sealed until when introducing or changing blood.

3.3.4 Semiochemicals

The membrane was made attractive after tick attachment by providing a combination of suitable chemical (Semiochemicals of hosts and tick faecal material) while physical stimuli was temperature (37 ^{0} C) and humidity (85%). The external surface of the membrane was therefor treated with cattle/tick ear wash and then sprinkled with tick faecal material.

3.3.5 Preparation of ear wash

Tick attractants were extracted using two solvents methanol and di-ethyl ether from cattle ears on which 4-day fed adults *R. appendiculatus* were attached. The extracts from the 2 solvents were compared for tick attachment. Diethylether was the best solvent in

that more (>90%) attachment was observed compared to the methanol (40-50% nymphal attachment). The methanol concentration was carried out at 55 ^{0} C water bath. An attempt was made to analyse the possible components of the ear wash extract. Preliminary investigations using thin liquid chromatography (TLC) compared the extracts of methanol and diethylether using a solvent system of the ration 1 Etheyl acetate: 4 Hexane. Briefly, the hexane was less polar than ethyl acetate, therefore, extraction was started with 20 % ethyl acetate and 80% hexane which allowed increase in polarity as was necessary. Pre-coated plastic TLC sheet (Polygram, Sil GLVV 254; Germany) marked twice at 1 and 7 cm length, and doted with the extract at three points 1 cm equidistant was placed in a development tank containing the solvent, and allowed to run until the 7 cm mark (20 min). The sheet was air dried and visualised under UV light at a wave length λ=254. This revealed whether the compounds were absorbed by UV light or not if not they were sprayed with 2 % sulphuric acid in methanol, then heated in an oven at 105 ^{0} C for a few minutes (3 min). Under the UV light three bands were observed, one at methanol and at each of the dietheyl ether (coloured pink). The dietheylether had several compounds extracted compared to methanol. These were later found to be phenols, salicylaldehyde, 4-cresol,2,6 -dichlorophenol, 2, 6- dibromophenol and 2- bromo-6-chllorophenol, responsible for the attraction of ticks.

3.3.5.1 Concentration of ear wash

A large volume of the diethel ether (1 litre) was used to wash the two cattle ears and the mixture was concentrated by evaporating off the ether in a rotor evaporator. Several precautions were taken since ether is volatile and inflammable. An evaporator (Rotarvapor RE) was particularly selected for this purpose and a warm water bath to

heat the ether. The apparatus consisted of a Lie big condenser connected to it in order to condense the ether. Excess vapour was finally cooled down in a "cold trap" that consisted of dry ice (Fig 3.6). The system was put in a fume cupboard that created a vacuum by blowing air from the room out through a vent. Briefly, the ear wash extract was transferred into a 1000ml round Duran glass bowl (NS2A, Germany), which increased the surface area for the task. The flask attached to a vacuum pump was kept rotating while being heated in a water bath at 30 ^{0} C. The ether evaporated and was condensed at 4^{0} C. The liquid was collected and excess cooled in a cold trap of dry ice. The concentrate was aliquoted and kept at - 20 ^{0} C until needed. Freshly prepared ear wash gave the best attachment results.

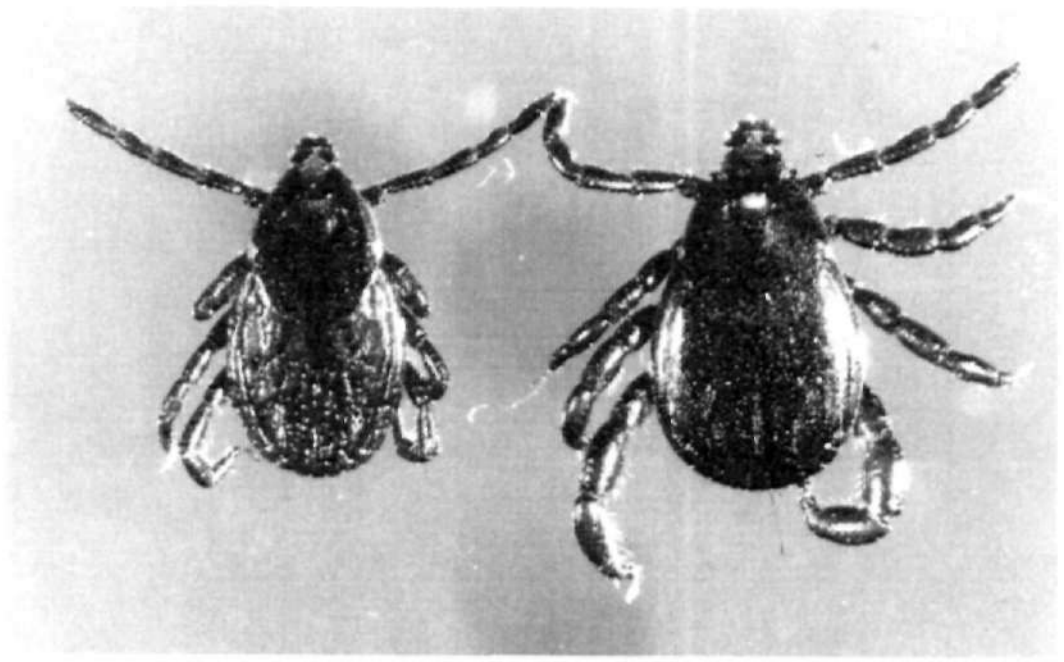
3.3.6 Tick application

Following the chamber preparation, the ear wash concentrate was carefully dubbed on to the membrane and a cluster of 500-1000 nymphs applied on to the treated membrane surface. Adult ticks were first sexed in the electronic tick trough that was heated at the sides to prevent ticks from escaping, before they were applied on to the membrane in a 1:1 sex ratio. Only a total of 60 -100 adults were used to avoid over crowding and piercing through the membrane which could lead to chamber leakage. The interface between the two components was sealed all around with parafilm and masking tape all around.

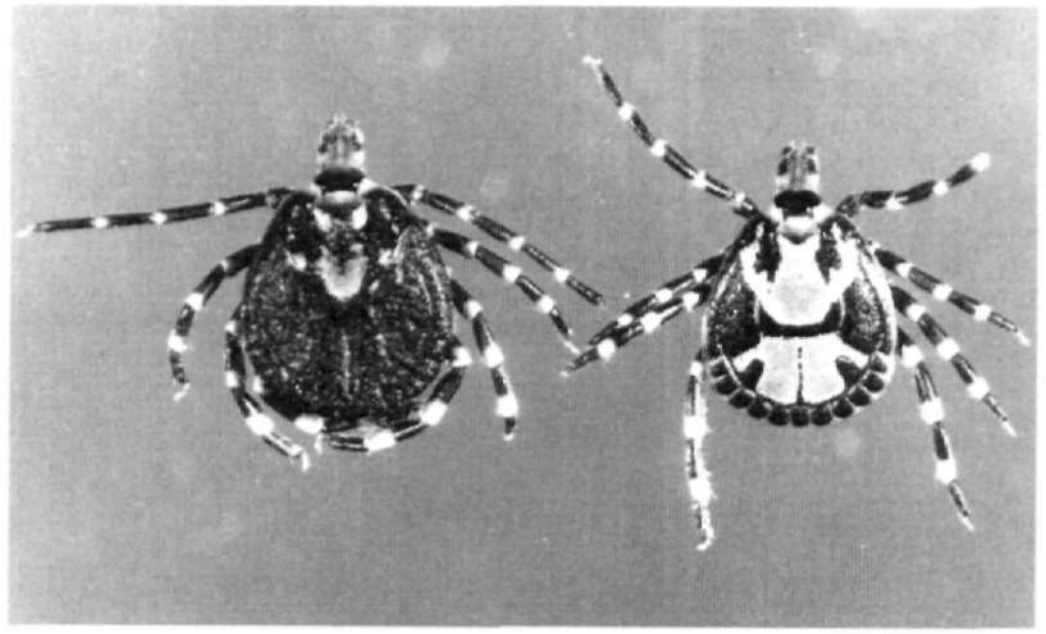
3.3.5.2 Maintenance of chambers

During the experimental period, blood was changed twice daily at 0800 and 1700 hrs, under sterile conditions described above (Fig 3.7). The chambers were rinsed with

Normal saline (9.0 g/l NaCl) before fresh blood was put into the chambers. Any chambers with very dark red or black blood suspected to be contaminated were rinsed with a mixture of penicillin/ streptomycin, gentamycine and fungizone. The blood was introduced into the chambers by opening the zinc oxide plaster on top of the blood chamber under a sterile hood, previously wiped with 70 % ethanol (Fig 3.7). Ten to twelve ml of asceptically collected heparinised cattle blood was pipeted into the chamber using the pipet aid (Drummon, UK) fitted with a 10 ml disposable plastic pipette (Costar, Cambridge USA) and the aperture immediately sealed with the plaster. The pipette tip was placed at the side of the chamber when dispensing blood to avoid piercing through the membrane (Fig 3.7) .The whole feeding system was placed in an incubator (Flow Laboratories, UK) at 37 ^{0}C with a 5 % CO_2 flow. The incubator had a relative humidity of 80-85 % obtained by placing an open tray containing saturated Sodium chloride solution, inside the incubator.



A



B

Plate 3.1 A Female and Male adult *R. appendiculatus*

Plate 3.1 B Female and Male adult *R. pulchellus*



A



B

Plate 3.2 A *In vivo* feeding of ticks on cattle in back bags and ears bags

Plate 3.2 B Engorged *R. appendiculatus* on cattle



Plate 3.3 Feeding ticks on Newzealand white rabbits in ear bags

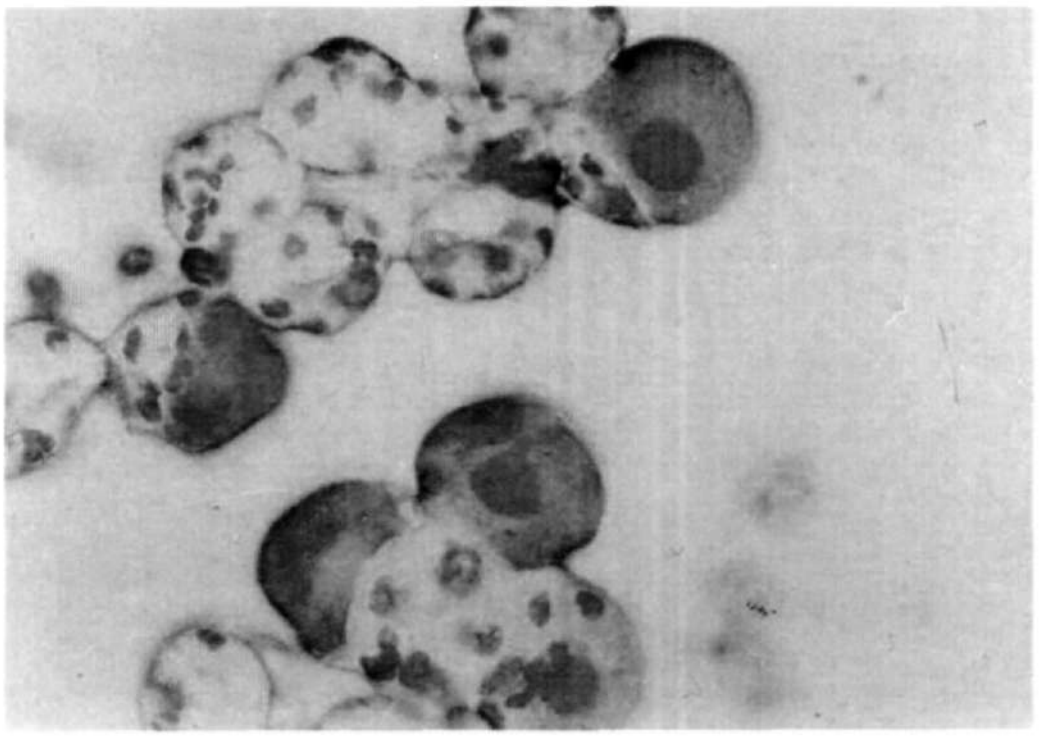
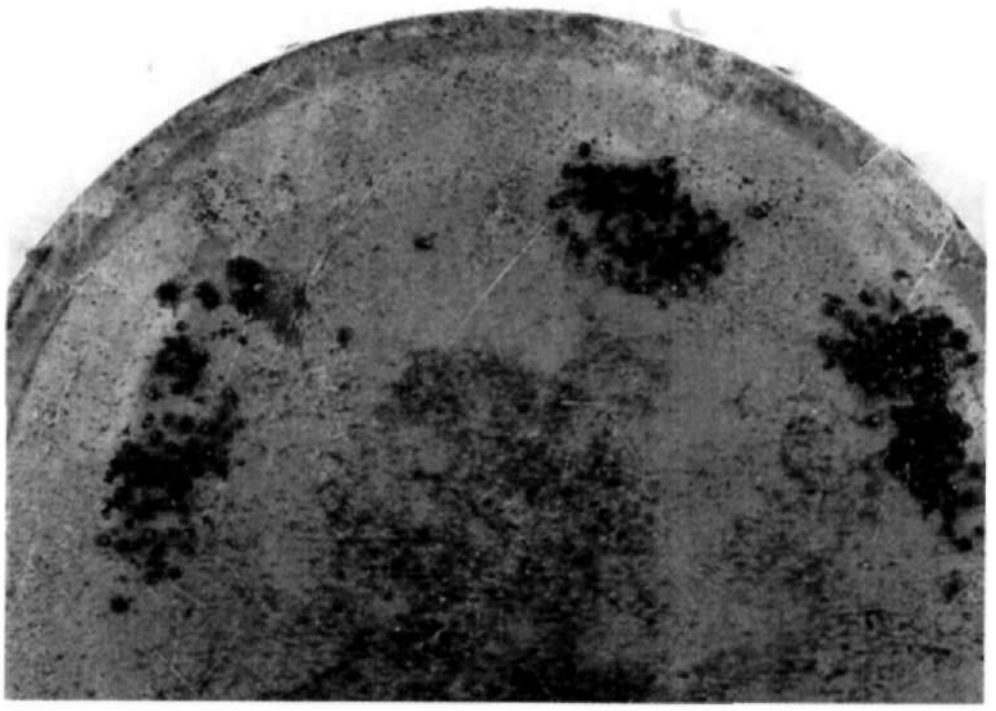


Plate 3.4 ***Theileria parva*** **infected salivary glands of** ***R. appendiclatus***

Freshly dissected salivary glands fixed in methanol and hydrolysed in HCL were stained in Schiffs stain. Note the deep pink stained infected salivary gland acini.



A

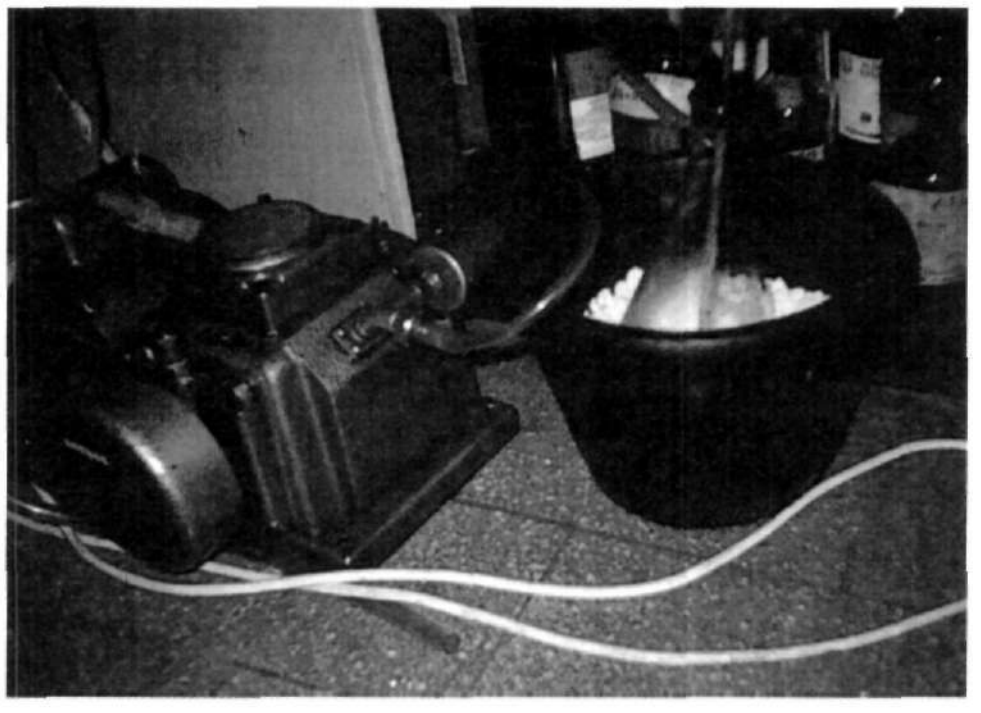


B

Plate 3.5 *In vitro* feeding chambers for ticks made from Baudriche membranes



A



B

Plate 3.6 Concentration of ear wash for tick attachment using a rotor evaporator (A) with a cold trap (B) of dry ice

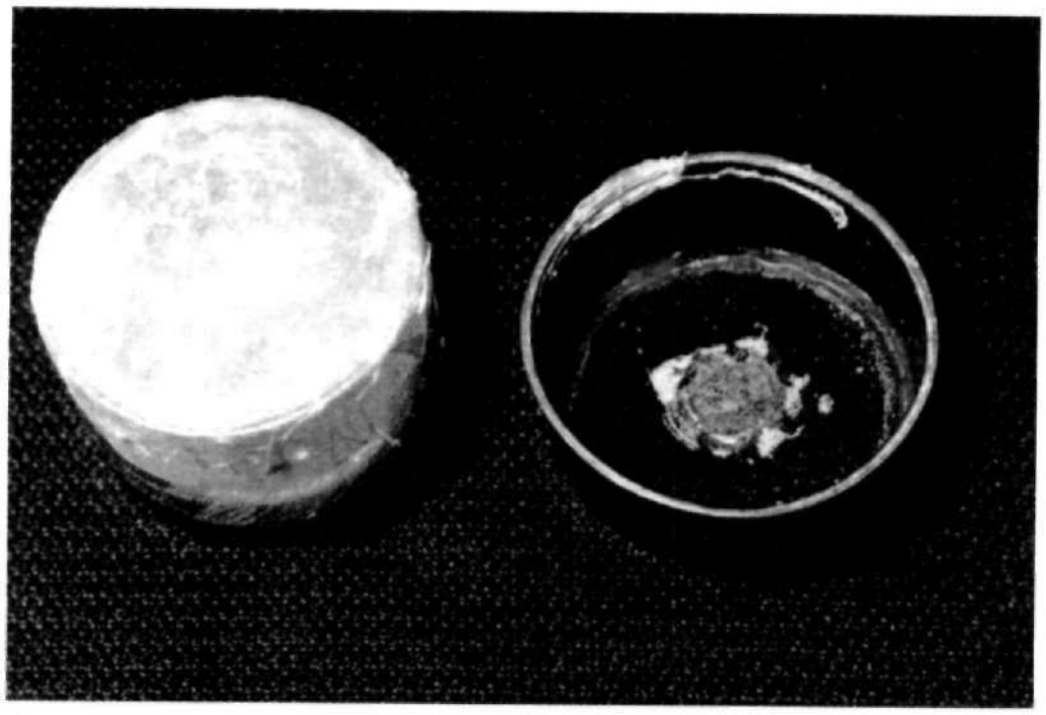
4.3.0 Results

4.3.1 Vector stages

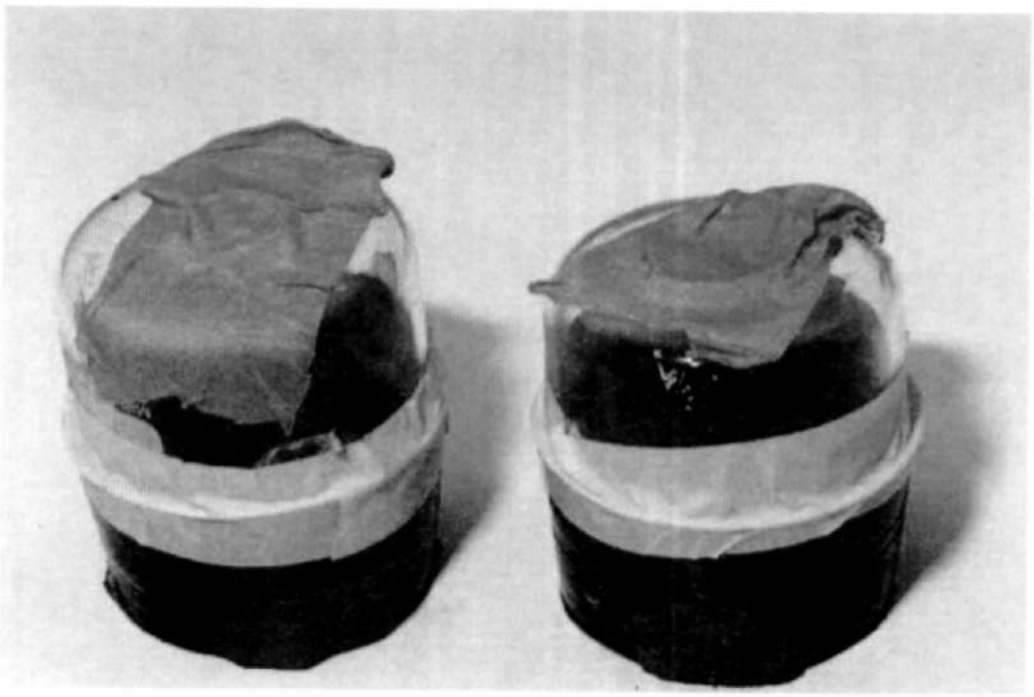
The nymph to adult moults occurred between day 11-14 post drop with females, which were heavier, dropping between day 11-12 while males moulted between day 12-14 (Fig 3.1A).

4.3.2 Parasite stages in the tick

Table 4.1 shows the various stages of the parasite life cycle and the periods they appeared after dropping from the cattle. The piroplasms (intracellular) were observed in ticks on the day the ticks dropped from cattle (day 0) in the gut smear. Following this, they were seen as concentrates 24hr (Fig 4.1). The sexual stages were seen 2 days post drop (Fig 4.2) while the zygotes (early stages) were visible from the day of detachment with large rings (Fig 4.3) appearing on day 8. Therefore, the piroplasm and the sexual stages were only seen during the first two days of nymphs drop from cattle. The kinetes (Fig 4.4) were first seen on day 11 post drop. Freshly moulted adult females also had kinetes that peaked on 12 -18 days post drop. Afterwards, the number decreased (day 19-25; Table 4.1). The kinetes were the most difficult stage to see with peaks showing on day 15 and not all ticks examined showed kinetes, depending at the time of examination. It was common to find kinetes in one tick and not in others. In the salivary glands, at least 93 % of the ticks showed sporozoites.



A



B

Plate 3.7 Maintenance of feeding chambers for *R. appendiculatus* and tick attachment

CHAPTER FOUR

LIFE CYCLE OF *THEILERIA PARVA* IN THE VECTOR *RHIPICEPHALUS APPENDICULATUS*

4.1.0 Introduction

As a prerequisite to studying factors that influence the development and transmission of *T. parva* in its vector *R. appendiculatus*, the various developmental stages the parasite undergoes were identified and followed in the tick, under laboratory conditions. Accordingly, a series of Giemsa-stained gut/haemolymph smears from freshly moulted ticks up to day 25 were prepared, the tick parasite stages identified and counted with particular attention to the timing of their appearances in relation to the ticks moulting time. The ticks were reared at 28^{oC} and 80% relative humidity. The parasite development in the salivary glands was also followed.

4.2.0 Materials and methods

Ticks were applied on cattle with rising parasitaemia above 10%. Ticks that dropped on day 15-21 were taken and followed from day 0 to 25. Smears were made each day and the slide stained by Giemsa and examined for the piroplasms, gametes, zygotes and kinetes. The resultant infection rates were obtained following schiffs stain (Young and Leitch, 1983).

4.2.1 Gut smears

These were prepared from freshly moulted adults (1-2 days) post moulting up to day 25. Ticks were held with forceps by the cephalothorax and the abdominal tip cut off with fine scissors. The body was gently squeezed and the exuding gut smeared on a clean glass slide, dried, fixed in methanol (10 min) and stained in Giemsa, before examination in a phase contrast microscope.

4.2.2 Haemolymph smears

As above, freshly moulted ticks were leg amputated, legs of adult ticks were held with forceps and cut at the coxae with fine scissors. The haemolymph was smeared on to a slide, dried and Giemsa stained as described above for the gut. The haemolymph smears were made to observe kinetes.

4.2.3 Microscope examination of various development stages

Apart from the sporozoites in the salivary glands which were stained by Schiffs stain, the rest of the developmental stages were visualised by Giemsa stain.

4.2.3.1 Giemsa staining

This was for visualising gametes, zygotes and kinetes.

Stock Giemsa preparation

Glycerol *2160ml*

4.4 Discussion

The stages in the life cycle of *T. parva* in *R. appendiculatus* has been followed and observed clearly under laboratory conditions at 28 ^{0} C. The piroplasms and the sexual stages although difficult to see, were visible only during the first two days post drop in the nymphs, while the zygotes were present up to several days after the moult. The migration of kinetes was clearly linked to the moulting of tick metamorphosis and occurred in the first 1-3 days. This would be linked to the hormone levels in the tick. It appears this stage is very critical and occurs only at particular times when the salivary glands are developed at the secretory granule level. It seems the kinetes migrate within 24-48 hr. in a particular tick and they be easily missed.

Intra specific variation in infection should be taken into consideration when assessing these results. It is normal to find some ticks with almost all the acini infected, whereas there may be no infection at all in some. Although the ticks selected were susceptible, there could have been some ticks which were completely refractory to the parasite. In addition, the parasteamia of the cattle fed upon was vital. Care was taken to attach the ticks with rising parastemia of at least 10%. The method of detection of these parasitic stages was difficult in that in the giemsa stains, the gut cellular material, haemoglobin and haematin crystals interfered with the stages especially the sexual stages which tend to accumulate in these areas. The present study enabled the author to recognise the various parasite stages and to establish the relationship between tick moulting, feeding and the development and maturation of the *T. parva* parasites, before evaluation of the factors affecting transmission were made.

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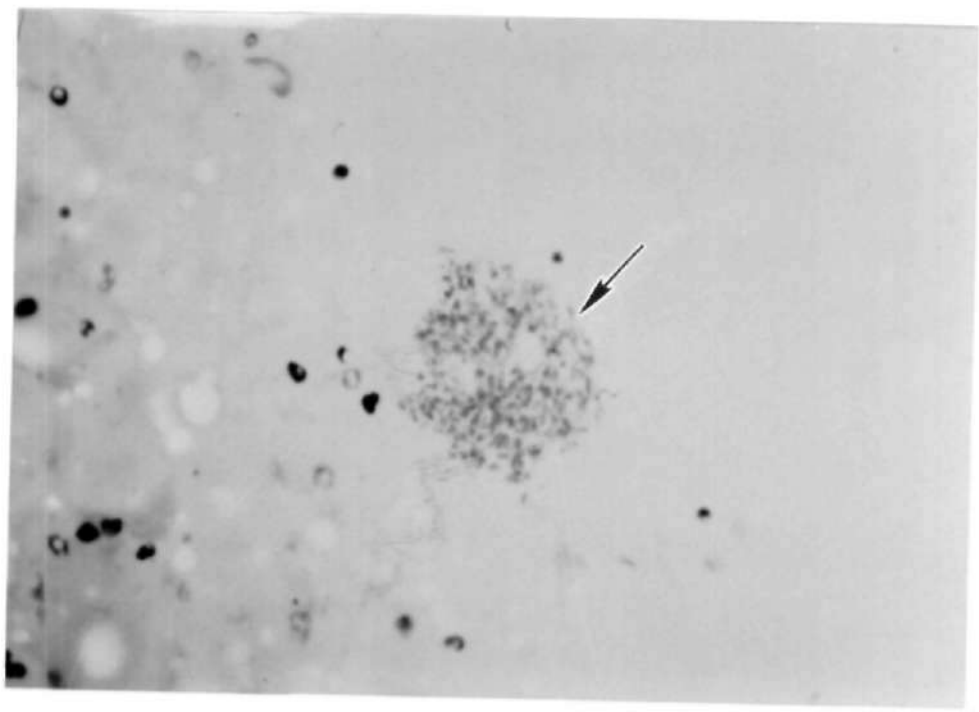


Plate 4.1 Light microscope photograph of *T. parva* piroplasm from the gut of *R. appendiculatus (x 2400).*

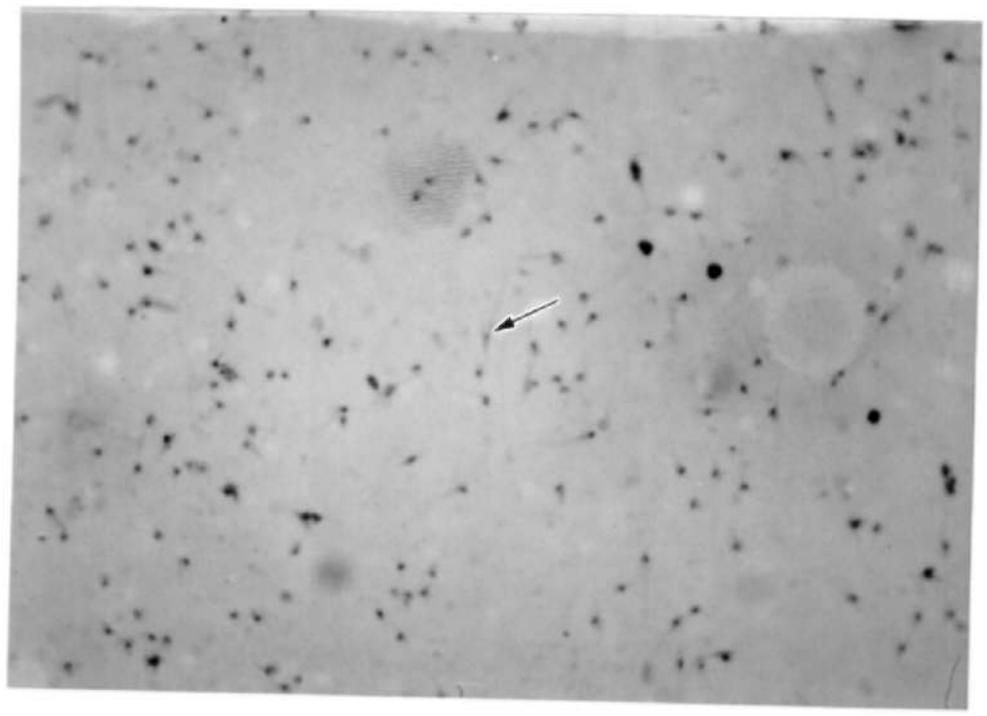


Plate 4.2 Light microscope photograph of sexual stages of *T. parva* from the gut smear of *R. appendiculatus* . The microgametes are seen as ray bodies or strahlenkoper bodies (Koch, 1906) in a smear preparation soon after repletion of the tick *(x 2400).*

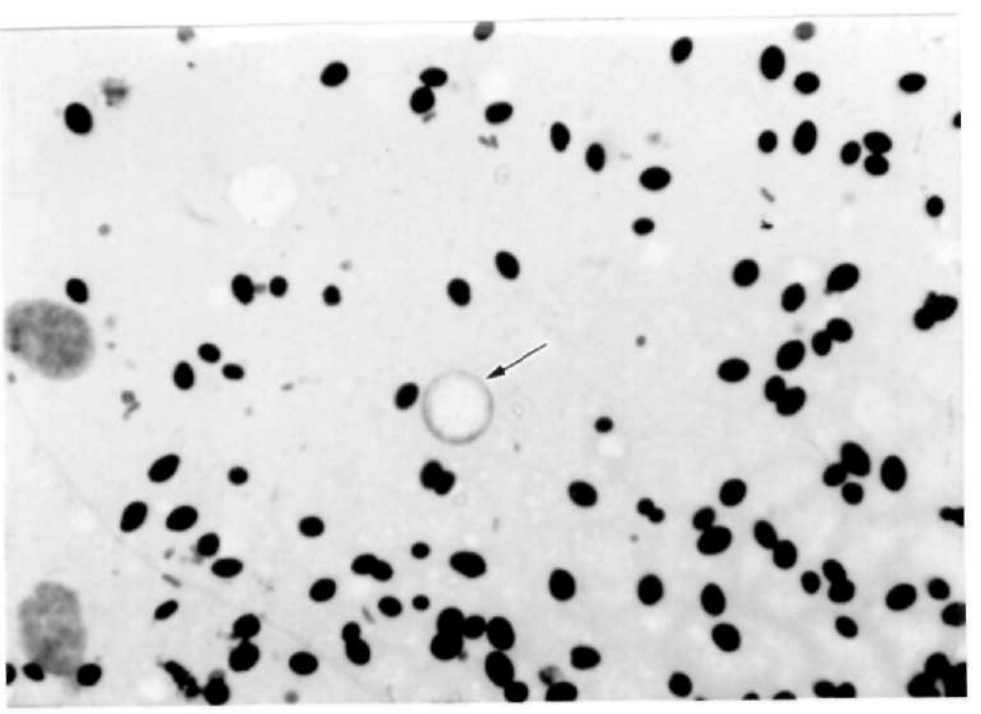


Plate 4.3 Light microscope photograph of zygotes of *T. parva* from gut smear of *R.appendiculatus (x 2400)*

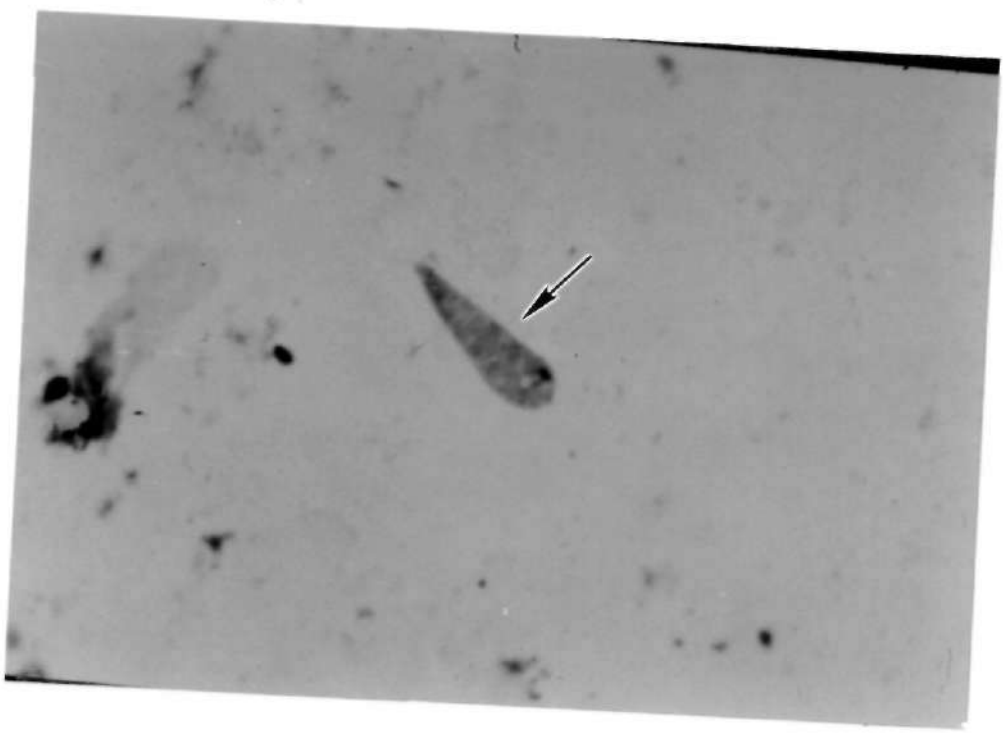


Plate 4.4 Kinete of *T. parva* from the haemolymph smear of *R. appendiculatus* *(x1000)*.

CHAPTER FIVE

5.0 DETECTION, ISOLATION AND CHARACTERISATION OF NEW LECTINS FROM THE TICK *RHIPICEPHALUS APPENDICULATUS*

5.1 Introduction

Lectins have been defined as polyvalent, structurally diverse proteins or glycoproteins that bind carbohydrates, agglutinate cells (for example erythrocytes, bacteria, fungi and viruses) or precipitate polysaccharides, glycoproteins or other glyco-conjugates (Sharon and Lis, 1989; Ingram and Molyneux, 1991). They exhibit specific and reversible sugar binding activity (Sharon and Lis, 1972). Lectins react with glycosylated macromolecules and cells and may be utilised *in vivo* either as homing factors and recognition molecules or as effector factors with enzyme activity (Sharon and Liz, 1989). Lectins occur in plants and animals and have been reported in the fluids of many invertebrates (insects, molluscs and Crustacea). Soluble lectins have been known to occur in a variety of species (Gold and Balding, 1975; Yeaton, 1982, Ey and Jenkin, 1982, Vasta and Marchalonis, 1987; Ratcliffe 1986). Agglutination of parasites by lectins has been reported in Diptera: Glossinidae (Ingram and Molyneux, 1988; Maudlin and Welburn, 1994) in Sandflies (Wallbanks *et al.*, 1986), in Mosquitoes (Mohammed and Ingram, 1994) and in Reduviid bugs (Pereira *et al.*, 1981; Gomes *et al.*, 1988; Barrocco and Loch., 1988). The ability of lectins to agglutinate parasites plays a role in the immunity as well as self and non self recognition (Kaaya *et al.*, 1986; Ingram and Molyneux.,1991; Welburn *et al.*, 1989).

Defining the binding specificity of lectins and identification of the receptors that mediate adhesion may lead to development of a novel type of control, whose mode of action would be based on competing with the endogenous ligands for binding to the pathogens receptor or preventing adhesion to host tissues, thereby preventing infection. This chapter describes the significance of haemolymph, gut and salivary gland lectins in the transmission of *T. parva.*

5.2 Materials and methods

5.2.1 Haemolymph collection

Haemolymph was collected from partially- fed (day 4) *R. appendiculatus* female adults maintained at 28°C, 95% relative humidity and 12h photoperiod. The haemolymph was collected in 10µl micropipettes following gentle pressure, expelled and pooled in eppendorf tubes containing a few crystals of phenylthiourea (PTU;Sigma, UK) to prevent melanization. Throughout the process, the tubes were maintained on ice. Haemaolymph was frozen at -70 ^{0} C until required.

5.2.2 Gut dissection and preparation

Guts from partially- fed (day 5) adult female *R. appendiculatus* were dissected in a petridish flooded with distilled water and immediately stored in liquid nitrogen. Eight grams of gut was homogenised in 10 ml homogenisation buffer (0.1M Phosphate buffered saline, pH 7.2) containing inhibitors, 0.1 M ethyl diamine tetra-acetic acid (EDTA), 0.1M Phenylmethyl sulphonyl fluoride (PMSF) and 0.01% Sodium azide. The mixture was centrifuged at 900 x *g* for 30 min and the pellet solubilised in detergent (0.01% Triton x100 (Pierce, Rockford) and hand homogenisation on ice. It was then sonicated for 2 min with 30 sec rest in between the sonication (Branson Sonifier, Model B-30, USA) followed by Ultra centrifuged at 3000 x *g* for 1 hr (Beckman) at 4 ^{0} C. The supernatant was stored in 1 ml aliquots at - 70 ^{0} C until required.

5.2.3 Preparation of the salivary glands

Salivary glands were obtained from *R. appendiculatus* and *R. pulchelus* females. The ticks were pinned in wax containing physiological saline. Using a sharp blade, a slit along the posterior lateral margin of the scutum was made along the ventral line. The salivary glands were removed and stored as above. They were then homogenised in homogenisation buffer (0.2MNa - Phosphate buffer; 5M NaCL 0.2MEDTA; 0.001M PMSF) and centrifuged at 1,000 x *g* 4 ^{0}C; 5 min. Both the supernatant and pellet were tested for agglutination using various erythrocytes. The pellet was then solubilised in the

same buffer containing detergent (0.001 % triton x 100) and tested after centrifugation as above. Samples of salivary gland were freeze-thawed three times in liquid nitrogen and thawed at 25 ^{0} C and supernatant and pellet tested for agglutination.

5.2.4 Detection of lectins by haemagglutination

Haemolymph: Haemagglutination was carried out using different host red blood cells (rbc) namely, rabbit, mouse, rat, horse, goat, sheep, cow, eland, buffalo, rhino, zebra, oryx and giraffe. The blood was collected in heparin. The rbc were cleaned by washing in Tris-HCl buffer (pH 7.2) two times by spinning at 100 x *g* for 15 min to remove cell debris and the third time in buffer containing 2% bovine serum albumin (BSA). The rbc were then resuspended in BSA to give a final rbc suspension of 3 %. Doubling dilutions of haemolymph each 10 µl in volume were prepared in Tris-HCl buffer in "V" shaped microtitre plates to give final dilutions ranging from 2^{-1} to 2^{-11} . Ten µl of 3% were added to all the wells and incubated overnight at 4^0 C or for 2 hr at room temperature. Cotton wool was placed inside the plate to provide moisture. The plates were examined under an inverted microscope and agglutination scored at an arbitrary scale of 3+ (100 % agglutination), 2+ (50 %), 1+ (10 %) and 0 (no agglutination). The end point titres were reciprocals of the dilution that just caused visible agglutination. Controls consisted of buffer alone with rabbit rbc.

Gut: The blood from the various hosts was washed in PBS (pH 7.2) by centrifuging at 100 x *g* for 15 min until the supernatant was clear. A 3% suspension of the rbc was made in PBS with Ca^{2+}/Mg^{2+} pH 7.2. The crude gut samples were slowly thawed from the freezer at -70°C (over ice). Fifty µl of PBS was put in the "U "shaped 96 well microtitre plates and 50 µl of samples added and serially diluted. Fifty µl of a 3% rbc suspension was added to all wells and incubated at 4°C overnight or for 2hr at room temperature. The agglutination titres were scored as described for the haemolymph. Controls comprised PBS with rabbit rbc.

5.2.5 Sugar specificities of Haemolymph and gut lectins

Carbohydrate and glycoproteins inhibition tests were carried out with concentrations up to 0.2 M and 2% respectively, of these substances in 20mM tris-HCl pH 7.4 with Ca^{2+} / Mg^{2+} or PBS for haemolymph and gut lectins, respectively. Serial, two fold dilutions of 25 µl aliquots of haemolymph were made in wells containing the inhibitors, incubated for 1-2 hr, rbc added and end points read as described above for haemagglutination assays.

5.2.5.1 Sugar specificity minimum concentration

For the threshold end points determination for inhibitors, 25 µl samples of the haemolymph and gut diluted from 1:4 to 1:125 were added to each row of the microtitre plate. The carbohydrates and glycoptoteins were then serially diluted in the extracts and the mixture incubated for 2 hr at room temperature. The rbc were then added and end points determined as above.

A total of 31 sugars plus glycoproteins were screened for inhibition of lectins. These were:N- acetyl-D- galactosamine, N-acetyl D- glucosamine, α-D (+) xylose, β-lactose, maltose, β-D(+) gluctose, D(+) glucosamine, N-acetyl-β-D-mannosamine, N-acetyl- glucosamine, sucrose, stachose, trehalose, α- lactose, D(+) raffinose, L(+) arabinose, D (-) arabinose, D (+) xylose, β-D (-) fructose, D(-) fructose, α-D(+) melibiose, β-lactose, D(-) ribose, D (-) mannitol, D(+) galactose, D (+) turanose, D(+) mannose, D(+) trehalose, D (L) arabinose, D- mannosamine, maltose, D - sorbiol α- L-rhaminose, D (+) malibiose and α- methyl- D- mannoside and glycoproteins fetuin, mucin. These were prepared in PBS (pH 7.2). Twenty five µl of PBS was added to each well of U- bottomed microtitre plates; 25µl of the lectin were serially diluted and then 25 µl of the sugar (0.2M) or 1% glycoproteins added to each well. The plates were then incubated at room temperature for 1hr and then 50µl of rbc added to each well.

Incubation was overnight at 4°C or for 2h at room temperature. The agglutination was scored as described above.

5.2.6 Physical/Chemical characterisation of tick lectins

5.2.6.1 Effect of temperature

Aliquots of gut extract, purified fraction or haemolymph were heated at 40, 60, 80 or 100 ^{0} C for 45 min in a water bath. Samples were also subjected to consecutive freezing (-20^{0} C) and thawing (25^{0} C) prior to the agglutination assay.

5.2.6.2 Requirement of divalent cations

Midgut and haemolymph samples were incubated overnight at 10^{0}C with equal volumes of either 0.015M ethylenediaminetetraacetic acid (EDTA), EDTA chelates both Ca^{2+} and Mg^{2+} ions)] or 0.015M ethyleneglycol-bis b-amino ethyl ether N,N,N,N - tetraacetic acid (EGTA, that chelates only Ca^{2+}) in PBS, pH 7.4. Doubling dilutions of untreated, EDTA, EGTA treated samples were prepared in PBS. In addition, agglutination was tested with and without these metallic ions separately or jointly in the buffers and examined for haemagglutination. Furthermore, the rbc to be used in haemagglutination were washed in PBS with or with out these divalent ions and the effect on agglutination assessed.

5.2.6.3 Effect of pH

To examine the effect of pH, samples were prepared in PBS ranging from pH 3 to 9 prior to the haemagglutination assays). For the low pH treatments, 5M Glycine-HCl pH 2.5 was mixed with equal volume of lectin samples, while in others, the pH was tested with litmus paper and later put to neutral with Di-Sodium Phosphate.

5.2.6.4 Effect of enzymes on tick lectins

To ascertain the possible chemical nature of tick lectins, midgut extracts and haemolymph were treated with enzymes. Solutions of 1mg/ml of ribonuclease, trypsin,

chymotrypsin and protease in PBS were incubated with tick samples and incubated at 37 ^{0}C for 2 hr. Enzymatic activity was stopped by appropriate inhibitors (0.1mg/ml) before agglutination assays. Controls included PBS alone or with erythrocytes and inhibitor, or active or heat inactivated enzyme.

5.2.7 Purification of lectins in the tick *Rhipicepalus appendiculatus*

Attempts were made to purify the gut lectins using several methods including gel filtration and affinity chromatography .

5.2.7.1 Gel filtration

The gut extract obtained as previously described was loaded on to Sephacryl S200 gel (Biorad) column size 2 x 170 cm with ribonuclease A (13,700), chymotrypsinogen A (25,000), ovalbumin (43,000), albumin (67,000), blue dextran (2,000,000) from Biorad as molecular weight markers. The gel was equilibrated with PBS pH 7.2 containing detergent 0.01% Triton x 100 since the detergent was used in solubilising the gut extract during isolation. Two millilitres of sample was loaded, the flow rate was adjusted to 15ml/hr and chart speed 0.2mm/min. Elution was with the same buffer. The fraction peaks were assayed for haemagglutination assay separately and later pooled, concentrated using polyethylglycol (PEG) at 4 ^{0} C and aliquots stored at -70 ^{0} C. A second experiment involving gel filtration was carried out on sepharose 6B equilibrated and eluted with PBS, pH 7.2 with triton x100 at 4 ^{0} C. Fractions were assayed for agglutination activity.

5.2.7.2 Affinity Chromatography

Affinity chromatography is a type of adsorption chromatography in which the molecule to be purified is specifically and reversibly adsorbed by a complementary binding substance, the *ligand* having been immobilized on an insoluble support *matrix*, whose functions include purifying substances from complex biological mixtures, separating native from denatured forms of the same substance and removing small amounts of

biological material from amounts of contaminating substances. A successful separation requires that a biospecific ligand is available and that it can be covalently attached to a chromatographic bed material, the matrix. Also, the immobolized ligand should retain its binding affinity for the substance of interest and that methods are available for selectively desorbing the bound substance in an active form, after washing away unbound material.

5.2.7.3.1 Fast Protein Liquid Chromatography (FPLC)

FPLC (Pharmacia) was used in an attempt to purify the gut lectin on a MONO Q column. Several buffers were used including HEPES (N-(2-Hydroxyethyl) piperazin N'-2-ethansulfonsaure; Serva), PBS, Tris-HCl and MES (2N-morpholino) ethanesulfonic acid with different molarities and KCl content. The buffers' influence on lectin activity was tested using the crude material as well as pure lectins such as Concanavalin A (Con A; Sigma) and a lectin from *Arachis hypogaea* (peanut lectin).. Buffer A (50mM HEPES pH 7.2) and buffer B (50 mM HEPES; 1 MKCl pH 7.0) were prepared and filtered through a filter (0.45μm pore; Sartorius) to remove any particulate matter. A MONO Q HR 5/5 column (Pharmacia) was pre-equilibrated with 20 ml of a 1:1 mixture of buffer A and B. The flow rate was 1ml/min and peaks read at wave length 280 nm. Crude samples were filtered through a 0.45μl pore filter prior to application onto the anion exchange column. The samples were loaded (1.2 ml made up to 10ml with PBS). The column was run on a linear gradient of 0-100mM NaCl which was used to elute the fractions. Each of the fractions (1ml) was examined for agglutination activity and the positive ones pooled. The pooled fractions were dialysed in molecular porous membrane tubing (Spectra\POR; molecular weight cut off 12,000-14,000) against PBS pH 7.2 overnight at 4^{0}C, and the activity analysed.

Cleaning of a MONO Q column: The tick gut sample often left a dark green colour on the column necessitating chemical scrubbing before the next sample was applied The column was ran in reverse phase (upside down) at a rate of 1-2ml/min following several steps in sequence, ensuring that each time the monitor peaks were identical in size before proceeding to the next step. First, 2ml of 2M NaCl was injected followed by a rinse with

buffer A (50mM HEPES). Two millilitres of NaOH was then injected and the column washed with buffer A, following which 2ml NaCl was injected. This was followed by another rinse with buffer A, a 2 ml injection of acid (1M HCl) and 2ml NaCl solution which has the same caunter-ion as the solution used for elution. The column was then equilibrated with the appropriate buffer before use.

5.2.7.3.2 Fetuin affinity chromatography used to purify lectins

Fetuin from fetal calf serum (Sigma) previously found to inhibit the gut lectin agglutination activity was selected for use in the purification of this lectin. A gel consisting of fetuin (lympholised and insolubilized on a 4% beaded agarose and activated by cynogen bromide (Pharmacia) was poured and equilibrated with different buffer systems or processed differently to evaluate the best method of lectin purification.

In the first instance, it was equilibrated with Ca-TN3 buffer (0.033M Tris-HCL;0.5M NaCL; 0.01M$CaCL_2$ pH 8.0). A 50% saturation of the gut extract, achieved by mixing the gut extract with solid ammonium sulphate overnight at 4^0C, was prepared. The precipitate was collected by centrifugation (2,500 x g; 5 min) dissolved in 2 ml distilled water, equilibrated with Ca-TN 3 buffer pH 8.0 and the resulting volume divided into 3 aliquotes. They were chromatographed in three consecutive runs on the Fetuin affinity Column (1 x 10 cm) pre-equilibrated with Ca-TN3 at a flow rate of 20ml/hr. Following sample application, the column was extensively washed with Ca-TN3 buffer and the bound material sequentially eluted with 0.01M and 0.025M EDTA in 0.033M Tris-HCl, 0.5M NaCl , pH 8.0. Fractions (0.7 ml) were collected and assayed for haemagglutination. Those found positive were pooled and dialysed against 0.15M Ammonium carbonate buffer pH.8.0.

In the second experiment, the gut extract lectin samples were filtered through a 0.2 μm pore filter (Sartorius, Minisant) and 1 ml sample loaded on to a 1.5 ml Fetuin agarose bead gel column, pre-equilibrated with Ca-TN3 buffer. The column was washed with the same buffer and fractions eluted sequentially with 0.01M EDTA and 0.025M EDTA. Fractions (0.7 ml) were collected at a flow rate of 1.88 ml/ min such that tubes 1-10 consisted of the wash ; 11-20, 0.01M EDTA; 21-30, 0.025M EDTA in 0.033M Tris-

HCl, pH 8.0. The optical densities were obtained at wavelength λ = 280 and their haemagglutination activity assessed.

Thirdly, a 2 ml Fetuin column was loaded with Fetuin agarose beaded gel, washed excessively (20ml) with PBS pH 7.2 and a 1ml gut extract sample loaded. The column was washed with 10 ml PBS to collect the unbound 1ml fractions (tubes 1-10) and the elution carried out with 0.01M EDTA, 0.025M EDTA followed by a wash (tubes 21-24) and further elution with 2M NaCl. The fractions were all tested for agglutination with PBS as blank.

5.2.7.3.3 Immunoabsorbent Column used in the purification of lectins

In another experiment an immunoadsorbent column, composed of Cyanogen bromide Sepharose 4B was used in the purification of the lectin. Prior to the immunoadsorbent column application, the lectin was subjected to acidity test because low pH values were to be used in the purification. In this respect, the crude gut was mixed with equal volumes of buffer (0.5M Glycine-HCl buffer; pH 2.5) making the molarity 0.1M. The pH was tested with litmus paper (indicator, BDH) and immediately brought to neutral pH by adding anhydrous Di-Sodium hydrogen phophate before agglutination assay. Although the acid treatment decreased the agglutination titres by 2, nevertheless, the assay could be used to indicate the presence of the lectin. Consequently, it was decided to carry out the purification using the Glycine-HCl in the immunoadsorbent column. Prior to immunoadsorbent column purification lectin antibodies were prepared and purified as described below:

5.2 7.3.3.1 Preparation of polyclonal immune sera to lectins

5.2.7.3.3.1.1 Lectin innoculation

Lectins were adsorbed on to red blood cells before being used to immunise rabbits. A 3 % suspension of rabbit rbc was incubated with the gut lectin for 1 hr at room temperature and left to agglutinate. Pre-sera was collected from each rabbit before injection. The agglutinated rbc were emulsified with Freunds complete adjuvant and 4.0 ml injected intraperineally into 3 sets of 3 rabbits (each rabbit with its own rbc).

Boosters were carried out 2 weeks intervals in incomplete adjuvant, in order to obtain a high titre of polyclonal antibodies. Test bleeds were assayed by haemagglutination inhibition test.

5.2.7.3.3.1.2 Detecting the presence of lectin antibodies: Haemagglutination inhibition

The rational for selecting haemagglutination inhibition for testing the antibodies to the lectin was that lectins are known to agglutinate vetebrate erythrocytes and can be inhibited by sugars. Anti-lectin antibody would therefore, be able to inhibit the agglutination of the cells. Blood was collected from rabbit ears in vacutainor tubes. It was left to coagulate at 37 ^{0} C for 30 min, centrifuged at 300 x *g* (Heraeus) for 30 min and sera obtained. The sera were heated at 56 ^{0} C in a water bath for 30 min to remove non specific inhibitors of haemagglutination. Fifty microlitres PBS were dispensed in a "U" bottomed micro titre plates and fifty microlitres of sera was serially diluted in PBS. The same amount of lectin previously doubly diluted in PBS was added to each well and incubated for 1 hr at 37 ^{0} C. Fifty microliters of 3 % rabbit rbc was added (separate for each rabbit) and further incubated for 1 hr at room temperature. The rabbit sera that inhibited the highest titre was selected for further antibody purification.

5.2.7.3.3.1.3 Purification of lectin antibody: Ammonium precipitation

The sera (15ml per rabbit) was subjected to ammonium sulphate (30%) precipitation by drop wise addition of equal volume of ammonium sulphate pH 7.0. The mixture was stirred and incubated for 30 min on a roller at 4 ^{0} C, followed by centrifugation (350 x *g*; 15 min; Heraeus). The pellet was resuspended in half the original volume PBS (5ml) and dialysed in a large volume of PBS and concentrated to its original volume in polyethyl glycol (PEG) in the cold room (4 ^{0} C). The dialysis tubings (Spectrafpor, Molecular weight cut off 6-8,000; Texas) were cleaned by soaking them for 1 hr at 60 ^{0} C in 1% $NaHCO_3$ solution, rinsing thoroughly in distilled water, followed by soaking in 0.1% SDS solution (1 hr at 60^{0} C) after which they were rinsed extensively in distilled water, before samples were applied.

5.2.7.3.3.1.4 Purification of the lectin antibody on protein A

The sera, previously subjected to ammonium sulphate precipitation described above and dialysed against Dulbeccos' PBS pH 7.4, was microfuged to remove any precipitate. A weight of 1.5g Protein A Sepharose in 1 ml PBS was swollen for 1 hr at room temperature and packed in a small chromatographic column in the cold room (4 ^{0}C).

The protein A column was equilibrated with Dulbeccos' PBS and the sample loaded (1ml). Elution was carried out with 0.3M Glycine-HCl pH 2.5 and 1 ml fractions collected. The pH values of the fractions were brought to near neutral by addition of anhydrous di -sodium ortho phosphate. All fractions were tested for haemagglutination inhibition. The agglutination inhibition was found in the run though hence the purification was carried out further by gel filtration on sepharose 6B. No agglutination was detected in the bound fractions.

5.2.7.3.3.1.5 Protein estimation

The protein content of the samples was estimated using Pierce Bicinchoniric acid (BCA) with bovine serum albumin (BSA) as standards. Briefly, 50 µl PBS was dispensed in flat bottomed microtitre plates (Costa). Similar volumes (50µl) BCA (1mg/ml) standards and that of the samples were added and serially doubly diluted. The samples were read on ELISA reader (multiscan MCC/340 Labsystems Finland) spectrophtometer at 414nm.

5.2.7.3.3.1.6 Purification of the lectin antibody by gel filtration on Sepharose 6B

The gel (Sepharose 6 B. Pharmacia, Fine chemicals, Sweden) was washed in buffer (0.5M Dulbeccos' PBS, pH 7.2), degassed by suction pressure and allowed to pack overnight using a pump (LKB, Broma,Sweden) in a column (1.5 cm X 60 cm) at a flow rate of 20 ml/hr. The sample from the protein A column was loaded (2 ml) and tubes collected and tested for haemagglutination inhibition. The peaks were concentrated separately using Amicon ultrafiltration membranes (DIAFLO, Ireland) using nitrogen gas. The protein concentration was determined (Pierce) with BCA as above .

5.2.7.3.3.1.7 Purification of the lectin using immunoadsorbent column

Sera containing the lectin antibody was first subjected to ammonium sulphate precipitation followed by gel filtration on a size column (sepharose 6B). The size column was selected because the activity (agglutination inhibition) was found in the run through following protein A purification (peak 3;Fig 5.10. The gel selected for use was cyanogen bromide activated sepharose 4B because of the following reasons:

- hydroxyl groups on the sugar residues could be easily derived for covalent attachment of a ligand
- open pore structure
- exclusion limit was wide (mw= 20 X 10^6) which made interior of matrix available for ligand attachment and ensures good binding capacities even for large molecules.
- extremely low non -specific adsorption
- bead-form provides excellent flow properties, ensuring rapid separation
- stability under wide range of pH conditions and dissociating agents
- rapid immobilization of ligands containing primary amino groups
- coupling of controlled amounts of ligands
- efficient, mult-point attachment of proteins giving a stable absorbent

The reaction:The Cyanogen bromide reacts with hydroxy groups on sepharose. The Cyanogen Bromide converts the groups to imido carbonate groups which react with nucleophiles. The activated groups of the ligand react with primary amino groups of the ligand to form isourea ligands. Results showed that the activity was optimum in the third peak (titre 32) although some activity was recorded in peak two (titre 4). It was, therefore, decided to purify peak three (P_3).

5.2.7.3.3.1.8 Swelling and washing the gel

One gram CNBr- activated sepharose 4B was swollen in 1 mM HCl for 15 min (giving 3.5 ml gel volume) while stirring with a plastic pipette because the magnetic stirer would have broken the beads. The gel was then washed and reswollen in 200 ml of the same buffer, in a sintered glass filter. The HCl preserved the activity of the reactive groups which hydrolyse at high pH. The gel was quickly washed with 5 ml coupling buffer (0.1M $NaHC_3$; 0.5M NaCl; pH 8.3). This stage was carried out quickly in order to prevent hydrolysis of the reactive groups at coupling pH. The sera was added to the coupling buffer and mixed with the gel. The mixture was left on an end- over- end mixer for 2 hr at room temp. Titration was carried out to illustrate whether the protein had coupled to the gel. The protein content of the sera was 18 mg/ ml and had heamagglutination inhibition titre of 32. Following the coupling, the mixture was again passed through the sintered glass and tested for any presence of protein. This had trace proteins, indicating that most of the protein had coupled in the two hours. The couple was transferred to the blocking buffer (0.2M Glycine; PH 8). A number of active groups remain on the gel after coupling. These are hydrolysed by the primary amine glycine. The glycine introduces a small number of charged groups into the gel, whose effect is overcome by the use of a high salt concentration (0.5 M NaCl) in the washing buffer. The blocking was carried out at 4^0 C over night.

5.2.7.3.3.1.9 Washing away excess adsorbent

The aim was to remove the excess uncoupled ligand that remains after coupling. The adsorbent was washed alternately with high (0.1M acetate buffer; 0.5 M NaCl; pH

4) and low pH buffer solution (Coupling buffer: 0.1M NaHC03; 0.5 M NaCl; pH 8.3) four times.

5.2.7.3.3.1.10 Maintenance of the adsorbent

The coupled mixture was transferred to PBS pH 7.2 containing sodium azide as preservative and stored at 4°C.

5.2.7.3.3.1.11 Dissociation of the complex

The immunoadsorbent was poured in a chromatography column (Pharmacia) flow rate 25.8 ml/hr and washed with PBS pH 7.2 to remove excess unbound protein. Glycine-HCl buffer was added (0.1 M Glycine- HCl; pH 2.5) and the fractions collected. The peaks were tested for agglutination and purity checked by gel electrophoresis.

5.2.7.3.3.2 Sodium dodecyl sulphate Polyacrylamide gel electrophoresis (SDS-PAGE)

The proteins in the gut extract were separated by SDS PAGE according to Laemnli (1970). Gels of 1.5mm thickness were cast and run on a Hoefer SE 600 Dual cooled vertical slab unit (Hoefer Scientific Instruments, California, USA). The 100% or straight 7.5-17.5% gradient gels (annex 1) were prepared and the mixture poured into the pre-assembled glass plates using a volume gradient maker, SC Series (Hoefer Scientific Instruments, California, USA). A small amount of water (1-2 ml) was poured on the surface to prevent bubbles. The separating gel was left to polymerise for about 1 hr at room temperature and 3 % stacking gel was poured gently on top of the separating gel and a comb inserted. The stacking gel was then allowed to polymerize for another 30 min after which the samples were boiled for 3 min in sample buffer and loaded into the wells, which had been previously rinsed with electrophoresis buffer. The gels were stained with coomassie brilliant blue (0.05% in isopropl) for 3 hr and destained in 0.0020% isoprop (4-6) hrs, or stained with Silver (see appendix 1).

5.2.7.3.3.3 Western blotting

The proteins were transferred to nitro-cellulose paper (14cm x 14 cm) at a voltage of 80mA for 2 hrs at room temperature, followed by blocking of the remaining protein binding sites on the nitro-cellulose paper (10% skimmed milk; 30 min or 5% milk overnight). The blots were reacted with the primary antibody diluted 1:40 in 5% skimmed milk overnight at 4^0 C . The strips were then washed six times with 5% skimmed milk (15 min) after which they were reacted with the secondary antibody (anti-rabbit labelled with Iodine 125 (I_{125}; 1μ Ci per sample diluted in 5% milk) incubated at room temp for 1hr and the strips washed six times again (5% milk; 2.5% milk in PBS;15 min). After drying, the blots were (10-20 min) between filter paper, they were wrapped in cling film and inserted into an X-ray cassette with autoradiography film. The film was developed according to manufactures instructions.

5.2.8 Preparation of Monoclonal antibodies

5.2.8.1 Inoculation of mice with lectin antigen

Balb/ C mice (6-8 weeks old) of either sex were immunised with lectin which had been previously adsorbed on to mouse red blood cells by haemagglutination. The blood was collected in heparin, washed three times in PBS (pH 7.2;150 x *g*, 10 min; 4^0 C) before the agglutination assay was carried out in a "*U* " bottomed micro titre plate. Following incubation at room temperature, the cells were emulsified with Freuds complete adjuvant and 0.5 ml injected subcutaneously into Balb/C mice. The mice were boosted with the antigen in incomplete adjuvant at 2-week intervals. The fusions were carried out 4 days following an intravenous injection (IV) of lectin in the mice. Details of the fusion and hybridoma production, are given below.

5.2.8.2 Fusion basis

Monoclonal antibodies, which are homogeneous populations of antibody molecules produced by the descendants of a single B lymphocyte, were produced by B lymphocyte hybridomas (hybrid cells made by fusing B lymphocytes with myeloma cells. The lymphocytes contributed the specific antibody-producing genes to the hybridomas

while the myelomas provided the genes that allowed the hybridoma to divide indefinitely and express the immunoglobulin genes. Lymphocytes were obtained from the lectin immunised Balb C mice while mouse myeloma cells (X63) were obtained from ILRI cell culture unit (courtesy of James Gachanja). The myeloma cells are deficient in hypoxanthine-guanine phosphoribosyl transferase (HGPRT) and can not incorporate the antimetabolite 8-azaguanine in their purines, hence are resistant to this drug. However, the enzyme deficiency makes them sensitive to the hypoxanthine-aminopterin-thymidine (HAT) medium used in monoclonal antibody procedures. The fusion of the X 63 Hypoxanthine guanine phosphoribosyl transferase (HGPRTase) deficient and immortal, which provided the genetic material for the required antibody and the lymphocytes produces hybridoma cells that can grow out from the HAT medium because DNA from the normal parent provides the information to synthesise HGPRT and the tumor cell DNA provides the message for unrestricted proliferation (Hudson and Hay, 1989; Letchworth and Appleton, 1984).

5.2.8.3 Fusion

Hybridomas were prepared by a modified method of Kohler and Milstein (1975) in a sterile chamber (Gelaire). Fusion of spleen cells with the myeloma cells X63 (9 X 10^7 cells) at a ratio of 5:1 was performed in 42% polyethylene glycol 1550 (Serva) with 15% dimethylsulphoxide. The resulting cells were cultured in RPMI -1640 media (500ml) (Sigma; prepared by ILRI, Central core) supplemented with 20% foetal calf serum (Hyclone), HEPES (15 ml/l), and hypoxanthine, aminopterine and thyminidine (Sigma), gentamycine (5mg/ml), glutamine (200mm), 2mm mercapethanol, penstrep (5ml), in 24-well plate (Costa) with thymus feeder cells (7 x10^7) cells. The cells were then given HAT media by pipetting out half the volume of the growth media and replacing it with HAT (0.5ml) 24 hrs following the fusion. Supernatants from individual wells were assayed for production of specific antibodies by heamagglutination inhibition and ELISA. Producing hybridomas were cloned by limiting dilution technique in 96-well plates (Costa) with feeder cells. The plates were maintained at 37 ^{0} C and 5% CO_2 and the supernatants were testes at least 10 days after fusion.

5.2.8.4 Testing for antibodies

5.2.8.4.1 Haemagglutination inhibition test

Fifty microlitres of the monoclonal supernatants were incubated with the 50 µl of lectin which was previously diluted in PBS for 1 hr at room temperature. A similar volume of a 3 suspension of mouse rbc were added and further incubated for 1 hr. at room temperature. Controls included Normal mouse sera before the immunization, pre heated for 1hr min at 56 ^{0} C; a positive control consisting of mice sera just before the spleen were removed and the antigen positive control that agglutinated the mouse rbc.

5.2.8.4.2 Determination of antibody class and subclass

Supernatants from the MoAbs were collected and concentrated (1ml to 200 µl) in polyethylene glycol (PEG) in a membrane (Spectra molecular porous membrane;12-14,000). A 1% agar (Technical Oxoid) was made in Barbitone buffer, heated in a microwave to boil and poured on to glass slides which had been rinsed in alcohol. The gel was allowed to polymerise (1hr) in the cold room and wells punched by vacuum suction. The supernatants were tested against anti-mouse IgM, IgG_1, IG_{2a}, IgG_{2b}, IgG_3 and antimouse-specific antisera (Sigma).

5.2.8.4.3 Development of an ELISA to detect lectin antibodies (Indirect ELISA)

An Indirect Enzyme Linked immuno sorbent assay (ELISA) was developed in order to screen monoclonal antibody supernatants as well as to quantify the amount of lectins present in different tick species.

5.2.8.4.3.1 Preparation of buffers

Reference is made to annex 1.

5.2.8.4.3.2 Antigen preparation

The lectin antigen solution, prepared as previously described (refer to Chapter three) and stored at - 70 ^{0} C was gently agitated before the protein concentration

estimate was made using BCA protein assay kit (Pierce). The concentration was 0.2 mg/ml. The required concentration was made in coating buffer using the following formula:

$\frac{RV}{O}$ where R= required concentration;

V= required volume;

O= original concentration. The value obtained was the volume taken from the original antigen solution and made up to the required volume necessary for the number of plates.

5.2.8.4.3.2 Removing detergent from the antigen preparation

Detergent (Triton X-100) used in the extraction of antigen (lectin) from the gut, was removed using *Extracti-Gel™D* detergent removing gel (Pierce). The *Extracti-D* support is a versatile affinity matrix for the extraction of unwanted detergent from solutions containing proteins or other macromolecules. This affinity support allows relatively small detergent molecules to enter the gel matrix, where they interact with a special ligand which removes them. The low exclusion limit of this gel overcomes the possibility of non specific binding by not permitting the larger macromolecules to enter the gel. The triton X -100 was removed as follows:

Packing the column: The gel and buffer (Dulbeccos' PBS) were degassed to remove all entrapped air bubbles. A Pierce's diposable polystyrene 2ml column, placed on a stand, was fitted with a porous polyethylene disc at the bottom that exhibits a stopped flow action. Three ml of buffer was passed through the column followed by careful packing of 1-2 ml gel (under atmospheric pressure). The gel was washed and equilibrated with PBS at room temperature.

Loading the sample: The sample (1ml) was loaded gently. The proteins were eluted in the void volume (0.7-1.0 ml/ml of gel). Buffer was applied on the column to allow the sample to pass through the gel. The eluted detergent -free sample was collected in 1 ml eppendorf tubes.

5.2.8.4.3.3 Selecting suitable ELISA plates

A variety of microtitre ELISA plates on the market were screened in order to select the best one for lectin binding properties. The five manufactures microtitre plates were:

- Polysorp Nunc (plain)
- Dynatech (Lactdyte)
- Dynatech immulon (USA lactimmum)

5.2.8.4.3.4 Coating ELISA plates with antigen

ELISA plates were coated with 100µl lectin antigen at a concentration of 50ng/well diluted in Dulbeccos PBS with magnesium and Calcium. The sides of the microplates were tapped to ensure that the antigen was evenly distributed over the bottom of each well. The plates were then covered, sealed and incubated overnight at 4^0 C on a shaker (Labfacility Shaker incubator IS89; Insel, England). The plates were covered with pre-cut transparent microplate sealers (Greiner Labortechnik Ltd., Dursley, UK). Following incubation, the plates were washed in the 2M NaCl washing buffer using a Nunc Immunowash 12 (Nunc Inter Med, Denmark) channel bar fitted to a vacuum pump. In between the washes, 10 min of incubation with diluting buffer was necessary for through washing of the plates.

5.2.8.4.3.5 Blocking of the antigen coated microtitre plates

The excess antigen was discarded by sucking with the Nunc twelve multichannel suction pipettes. The uncoated sites on the walls of the microplates as well as the non-specific sites were blocked with 100 µl of 0.25% casein. The plates were covered and incubated at room temperature for 30 min.

5.2.8.4.3.6 Addition of test sera :antibody dilutions

Preliminary experiments were carried out to find the optimal dilution or working dilution of the sera. One hundred microlitres aliquots (1:100) of the test samples was added to the wells of the micro plates in at least two replicates. The control sera (M_1 and M_4) diluted (1:100) in diluting buffer 1:200, in Dulbecco's phosphate buffered saline pH 7.4, with Mg^{2+} /Ca^{2+} containing 0.1% tween 20, without skimmed milk were added in replicates (100 µl each).

Control wells

- Negative control was sera obtained from 'Naive' BalbC mice
- There was a conjugate control consisting of the Dulbecco's phosphate buffered saline alone.
- RPMI media containing 20% fetal calf serum alone

The plates were incubated at room temperature for 30 minutes, to allow the antibodies to bind to the antigen (Prior experiments were carried out to establish the optimum reaction time). During this period, the plates were gently agitated on a micro-agitator (Heidolph Bioblock Scientific, Germany). The unbound antibodies were removed by washing the plate extensively with washing buffer (0.9% w/v saline with

0.05% w/v tween 20). To wash the plates, the sera was discarded by suction using the Nunc 12 channel washing bar and the wells washed by filling and aspiration of the washing buffer and drained by inverting and banging on to lint-free paper hand towels. The wells were filled with washing buffer and left to stand for 20 min at room temperature followed by filling and aspiration as described above.

5.2.8.4.3.6 Addition of the anti-mouse IgG conjugate

Following the washing, 100µl of anti-mouse Ig HRP conjugate, diluted 1:5000 in Dulbecco's PBS containing 0.1% tween 20 and 2.5% skimmed milk was added to the appropriate wells. The plate was incubated at room temperature for 30 minutes with continuous shaking.

5.2.8.4.3.7 Addition of substrate/Chromogen solution

According to the required volume (chart 1) the following proportions were used. For example, a volume of 25 µl substrate buffer, 125 µl of 40 mM ABTS (2,2'- Azino-bis {ethylbenz-thiazoline-6-sulphonic acid}) and 100µl of 1% hydrogen peroxide in water as substrate/chromogen solution was added to the plate (100µl).

Chart 1: ELISA substrate /Volume substrate

Buffer pH 4 (ml)	Vol.ABTS (µl)	Vol. H_2O_2 (µl)
50	250	200
45	225	180
40	200	160
35	175	140
30	150	120
25	125	100
20	100	80
15	75	60
10	50	40
5	25	20

The plate was incubated at room temperature for 30 min in the dark with gentle agitation to allow optimum colour development. The reaction was assessed following the reading of optical density (OD) read at wave length (λ) = 414 nanometers (nm), in an ELISA reader (Titertek Multiscan Mcc/340 spectrophotometer; Labsystems, Finland).

5.2.8.4.3.8 Evaluating the role of fetal calf serum (FCS): Inhibition ELISA

Preliminary experiments of growing hybridomas in culture media (RPMI -1640 containing 20% FCS revealed a problem in assays. This was because the controls such as media inhibited haemagglutination. It was thought that this was due to the presence of Fetuin in the FCS, which had been found to inhibit lectin action. There was need, therefore to establish whether the FCS does inhibit lectin action thereby interfering with the lectin- antibody reaction. In the case, 100 μ l FCS was added to the test wells after blocking in replicates, and incubated for 30 min. The test antibody was then applied (incubated 30 min) followed by the anti-mouse conjugate (30 min). The substrate was then added (30 min) and tested for colour development as described above.

5.2.9 The role of tick lectins

The role of lectins in the tick *R. appendiculatus* was investigated following three approaches; one directly with the purified lectin, the second using antibodies to these lectins and a third indirect method of feeding the ticks *in vitro* with inhibitory carbohydrates, glycoproteins or antibodies and assessing their influence on ticks and parasite development. The effect of the lectins on the parasite was a priority as they are known to be involved in the self and non self recognition as well as defence (Ratcliffe, 1986). Earlier reports from tsetse flies had also indicated the role of lectins in the

maturation of parasites (Maudlin, 1991). In this respect, the piroplasm stage as well as the schizonts were studied.

5.2.9.1 Piroplasm-Lectin Interactions: Agglutination of the piroplasms

In order to visualise whether lectins could agglutinate the piroplasm stage of *T .parva*, agglutination of infected rbc was carried out. In addition, an investigation was carried out on the purified piroplasms.

5.2.9.1.1 Purification of piroplasms

Blood from calves (6-9 months old) with at least 20 % parasitaemia of *T. parva* was collected in Alsever's solution containing heparin (final conc. 50 i.u/ml), centrifuged three times (350 x *g* ; 30 min; Sorval) and the buffy coat removed each time using a pasture pipette and a water vacuum pump. Following a fourth centrifugation, the cells were resuspended in TEN buffer, pH 8.0 (20mM Tris; 10 mM EDTA; 100mM NaCl;appendix 1). The packed cell volume was estimated by taking blood in 75 µl haematocrit capillary tubes (Cristaseal, Hawsley & Sons, Ltd, Sussex) and spinning it for 5 min in a micro-haematocrit centrifuge (12,000 rpm). The packed cell volume was read off from the micro-haematocrit reader in percentages. The cells were then lysed using a detergent saponin (Sigma). The detergent and the cells were both warmed separately in a water bath at 37 ^{0} C for 30 min. Equal volumes of warm saponin (1mg/ml) and cells were lysed for 30 sec. The mixture was diluted four times the volume with TEN buffer and centrifuged (350 x *g*; 20 min; Sorval) and the supernatant saved. This was followed by a further spin (1,000 x *g*; 30 min; Beckman ultra centrifuge). The supernatant was sucked off and discarded. The pellet was resuspended in TEN buffer and re-centrifuged (1,000 x *g*; 30 min; Beckman) until the pellet was white (only the soft pellet was taken) which constituted the purified piroplasms. A smear was made to check for any "ghost" cells in which case the piroplasms were sonicated (30 sec) on ice to release the piroplasms a smear of which was made and stained with Giemsa.

5.2.9.1.2 Agglutination of piroplasms inside red blood cells

Blood from infected cattle of at least 20% parastaemia was collected in heparin/Alsevers was processed in TEN buffer as previously described, except that the cells were not lysed in saponin. The cells were diluted in PBS and spotted on a microscope slide (Multispot, Supeorior, Germany). The excess was sucked off leaving a thin film. The antigen was added and incubated for 1 hr at room temperature. The agglutination was checked under a microscope.

5.2.9.1.3 Agglutination of purified piroplasms

The purified piroplasms were diluted in PBS (1:400) spotted on slides (Multispot Supeorior, Germany) and incubated with similar volume of the lectin, in serial dilution, for 1 hr at room temp. The agglutination was scored under a microscope. Controls included blood from a naive cow as well as normal rabbit sera which gave an idea about the non-specific binding.

5.2.9.1.3 Visualising lectin/piroplasm interactions with dyes

5.2.9.1.3.1 Carboxyfluorescein diacetate (CFDA)

Agglutination of live piroplasms was visualised using the 5-carboxyfluorescein diacetate (%CFDA) method. Briefly, the CFDA stock (5mg/ml) was diluted 1:50 and 100 µl added to 250 µl piroplasm mixture, incubated (15 min) in the dark at room temperature, then washed once for 5 min at 1000 x *g* at 4 ^{0} C to remove the CFDA. The pellet was resuspended in 500 µl PBS using a 26 gauge needle and washed (1000 x G; 5 min; 4 ^{0} C). The pellet was resuspended in 90 µl PBS then ten µl were put on a slide. Lectin (10 µl) was added and the mixture incubated at room temperature (30 min). A cover slip was put and the slide was examined under the fluorescent microscope (

Orthoplan) using filter 4 (blue) that filters all other colours except green). Only the alive cells fluorescent under this dye.

5.2.9.1.3.2 Use of Fluorescent isothiocyanate (FITC)

5.2.9.1.3.2.1 Preparation of antibody

The test sera was precipitated with 50% ammonium sulphate and dialysed against PBS (Dulbeccos) at pH 7.4 in the cold room. A protein A column was equilibrated with PBS pH 7.4 and the sample loaded. Fractions were eluted with glycine-HCl (2M) pH 2.6 and neutralised with Disodium. One ml fractions were collected and tested for agglutination inhibition. The "run through" contained the haemagglutination inhibition activity, which meant that the anti- lectin is in the IgM since the Protein A binds IgG. Further purification was, therefore by size and was carried out by gel filtration on sepharose 6B. The sample found in the second peak was conjugated with FITC.

5.2.9.1.3.2.2 Conjugation of anti-lectin antibody with FITC

The antisera was dialysed against 0.25M Carbonate/bicarbonate pH9.6. The protein content of the above fraction was determined and then mixed with fluorescein isothiocyanate FITC) (0.5 mg/1mg protein) in 10 times the volume of the dye for 15-24 hr or overnight at 4 ^{0} C. This was carried out using carbonate/bicarbonate buffer pH 9.6. Separation of the conjugated protein from the free fluorechrome was carried out on Sephadex G 50 column equilibrated with PBS. Since the fluorochrome: protein ratio is only an average determination, some protein molecules would have more fluorochrome and others less. As each fluorochrome molecule is added to the protein molecule, there is a net decrease in charge. Consequently, conjugated antisera was fractionated by ion exchange chromatography using an elution gradient of increasing ionic strength. This was carried out on DEAE 52 ion exchanger using elution buffer of increasing ionic strength (Carbonate/bicarbonate buffer pH 9.6; 0.1M. 0.3; 0.4 and 0.8 M NaCl).

5.2.9.1.3.2.3 Effects of lectins on piroplasms using FITC

The piroplasms were incubated with the lectin (1hr; room temp) were cytospinned on a slide, air dried and fixed in 3.7 % formaldehyde (10min). They were then rinsed in PBS to remove the fixative and the conjugate was applied. The conjugate was diluted 1:50 - 1:5000) in order to select the best working dilution, which was 1:10,000. Briefly the conjugate was diluted in Evans blue and applied to piroplasms previously incubated with lectins. They were incubated for 30 min in a moist chamber in the dark and then washed three times in PBS to remove excess conjugate. A drop of glycerol (50% glycerol: PBS) was used as mounting media and piroplasms were observed on a a Leitz Orthoplan fluorescent microscope fitted with a ploemopak 2 vertical illuminator and a HBO 100 mercury lamp (Leitz, Wetlar, Germany).

5.2.9.1.3.2.4 Effects of lectins on infected lymphocytes (Schizonts)

5.2.9.1.3.2.4.1 Preparation of purified schizonts from *T. parva* infected lymphoblastoid cell line culture cells

Lymphocytes were grown in culture L15 media supplemented with 15 % foetal calf serum) and infected *in vitro* with *T. parva* sporozoites. The infected culture cells were separated on Ficol-Hypaque sedimentation (250 x *g*; 30 min; Beckman; 4°C) without brakes. They were washed to remove the ficol 150 x *g*) and washed twice in Alsevers with the last wash in L15 media. They were cytospinned on to slides (Multispot; superior, Germany) and the antigen added. Following a 1 hr incubation in a moist chamber, the conjugate diluted (1:1000) in PBS and evans blue was added. A further incubation (1 hr) was carried out after which the slides were washed three times in PBS by immersion in a Goblins' jar (10 min; 4 ° C; on a rocker). Mountant (50% glycerol in PBS) was applied together with a cover slip and the slides examined under a fluorescent microscope.

5.2.9.2 Effect of lectins on parasite maturation

5.2.9.2.1 In vitro feeding of Carbohydrates and glycoproteins that inhibited lectin activity

From the list of the sugars described above, those that inhibited lectin activity were selected and used *in vitro* experiments. For instance, D(+) glucosamine, D(+) mannosamine, D (+) galactosamine and fetuin that inhibited the gut lectin, xylose, N-acetyl D-galactosamine and D-fructose which inhibited the haemolymph lectin were used. Ticks (nymphs) were fed on parasitised freshly collected heparinised blood (cattle BM 248 parasitaemia 10.4% and BM 316 of 8.6%) for two days before sugars were added to a final concentration of 0.5M or 2% for the glycoproteins, and fed to ticks using membranes in replicates (Waladde *et al.*,1993). Glucose, D(+) galactose and N-acetyl glucosamine were used as a positive controls since they were not inhibitory. The resultant infection rates were compared with controls fed upon carbohydrate-free blood. In addition, about 400 nymphs were fed to engorgement on infected cattle. They were allowed to moult and then fed on carbohydrates for 4 days before their infection rates were assessed.

5.2.9.2.2 *In vitro* feeding of ticks to anti-lectin antibodies

Anti sera from immunised rabbits were supplied to ticks *in vitro* and the resultant infection rates and survival assessed. The guts and salivary glands from such ticks were also examined by transmission electron microscopy.

5.2.9.2.3 *In vivo* feeding of ticks to immunised rabbits

T. parva infected nymphs and adult *R. appendiculatus* were fed on lectin immunised rabbits and their infection rates assessed. Similarly, their salivary glands were

examined under transmission electron microscopy. The rabbits were immunised as previously described with lectin adsorbed on to rbc.

5.3 Results

5.3.1 Detection of lectins in *R. appendiculatus* haemolymph

Lectins were detected in the haemolymph of *R. appendiculatus* as demonstrated by agglutination of rbcs of different animal species at various degrees (Table 5.1A). The rhino and horse gave the highest titres (4096) followed by horse (1024), rabbit (512), giraffe (256) and zebra (128) whereas Thomson's gazelle gave a low titre of 2. However, no agglutination was obtained for oryx, buffalo, cow and sheep rbc.

5.3.2 Detection of lectins in the tick gut

Free lectins were not detected in the gut lumen with all the erythrocytes showing no agglutination. However, following homogenisation and solubilisation with a detergent triton x 100, lectins were detected in the integral membrane part (Table 5.1A). The highest agglutination titres were observed with rabbit (4096) followed by mouse (512) rbc. Among wild life, the buffalo gave a titre of 512, while oryx, giraffe and Thomson gazelle were agglutinated only up to titre of 16. The agglutination titre with horse rbc was 4 and little or no agglutination occurred with sheep, goat and cattle rbc. Subsequent experiments were carried out using rabbit erythrocytes.

5.3.3 Detection of lectins in the haemolymph and gut of *R. pulchellus*

Looking at the *T. parva* refractory species *R. pulchelus*, the heamolymph gave a high titre of 1024 with mouse rbc (Table 5.1B) which was four times higher than the un - infected *R. appendiculatus* haemolymph and twice that of the infected *R. appendiculatus* haemolymph. The rabbit rbc demonstrated a titre of 8 while the cow rbc whether

infected or not (Table 5.1B) were not agglutinated. The *R pulchellus* gut lectins demonstrated a titre of 1024 with rabbit rbc, 256 with mouse and none for cow indicating that the lectins are probably different (Table 5.1B).

5.3.4 Detection of lectins in the salivary glands

The lectins in the salivary glands of *R. appendiculatus* agglutinated only mouse but not rabbit or cattle rbc (Table 5.2). The salivary gland lectins that had been obtained by freeze/thawed showed no agglutination with rabbit rbcs but revealed a titre of 64 with mouse rbc. No agglutination was detected with infected or clean cattle blood using any of the above extraction methods (Table 5.2). The homogenisation /sonication and freeze/thawing isolation methods for the salivary gland lectin were quite successful in extraction of this lectin.

5.3.5 Detection of lectins in infected ticks

A comparison of agglutination in haemolymph derived from infected ticks revealed that there were higher quantities of lectins in the positive ticks (512) compared to the uninfected *R. appendiculatus* (256), which was almost a two fold increase (Table 5.3) with mouse rbc. No agglutination was observed with either clean or infected ticks when infected or clean cattle rbc were used (Table 5.3).

5.3.6 Sugar specificity's of Haemolymph lectin

The carbohydrates prepared at various concentrations or 1-2 % (for glycoproteins) and used to characterise both the haemolymph and tick gut lectins showed that the haemolymph lectin was inhibited by Xylose, N-acetyl galactosamine and β-D-Fructose and partially inhibited by D(-) Mannose (16) L(+) Arabinose(8), D(-) Arabinose (16) and D(+) Raffinose(4) (Table 5. 4).

5.3.7 Carbohydrate inhibition characteristics of the tick gut lectin

Out of a total of 35 sugars and glycoproteins screened for inhibition of this lectin (Table 5.5) the following sugars partially inhibited the tick gut lectin: D (+) glucosamine

(64), N-acetyl glucosamine (256), D (+) Galactosamine (16), D- mannosamine (64) and Fetuin (16).

5.3.8 Minimum carbohydrate/ glycoprotein concentration for inhibiting the tick gut lectin

Fetuin was found to be the best inhibitor at a dilution of 1: 256 (2% Fetuin; PBS pH 7.2) where by complete gut lectin agglutination activity was inhibited. For galactosamine, a dilution of 1:64 0.5M galactosamine: PBS inhibited the gut lectin of 1: 128 dilution implying that fetuin was 4 times better as an inhibitor (Table 5.6;5.7).

5.4.0. Physical/chemical characterisation of tick lectins

5.4.1 Protein content

The protein content of the extracts was as follows:

Solubilised gut : 920 µg /ml

Salivary gland: 680 µg/ml

5.4.2 Effect of temperature

The temperature had little effect upon the activity of gut lectins, since heating up to 80^0 C gave an agglutination titre of 1:2048, a 2 fold decrease from the control (Table 5.8) against rabbit erythrocytes. However, the haemolymph lectins were thermo-labile above 40^0C and recorded a titre of only 4, indicating an 8 fold decrease in activity. Higher temperatures coagulated the haemolymph and gut. Freezing and thawing processes did not appear to have a significant effect on the gut lectins, since end point titres were similar to the untreated samples. These processes however, reduced the agglutination end points of the haemolymph 8 times from 512 to 2. When the fraction was heated from 40 to 100 ^{0}C, a progressive reduction in titres occured. Heamolymph titres were the most affected or negatively significantly ($p<0.001$) with increasing temperatures or freezing followed by immediate thawing.

5.4.2 Requirement of divalent ions

Following treatment with EDTA or EGTA, agglutination levels were reduced or remained the same. Compared to the PBS control, EDTA lowered the gut haemagglutination titres by 4 to 8 fold depending on the rbc type used. The gut was reduced 4 fold with rabbit and 8 with mouse erythrocytes (Table 5.9). Following EGTA treatment, the fraction was lowered 2 fold while the crude extract was unaffected. Addition of excess Ca^{2+} to EDTA treated samples increased titres 4 fold while Mg^{2+} inclusion resulted in little titre variation. However, inclusion of both Ca^{2+}/ Mg^{2+} enhanced the titres 2 fold. Agglutination was not significantly affected by washing rabbit rbc with buffer containing these ions or without Ca^{2+}/Mg^{2+}.

5.4.3 Effect of pH

Treatment of gut samples with acid in the glycine -HCl buffer (pH 2.5) reduced the agglutination titres significantly ($p<0.001$) 5 fold with mouse rbc and 8 fold ($p<0.001$) with rabbit rbc and neutralising them with di Sodium phosphate restored the activity up to 512 and 128 for rabbit and mouse rbc, respectively. The agglutination activity appeared to be optimal around neutral pH (Table 5.10).

5.4.4 Effect of enzymes

Treatment of the crude extract and isolated lectins with proteolytic enzymes resulted in no change or reduced agglutination titres (Table 5.11). The fraction was significantly ($p<0.05$) reduced by trypsin, chymotrypsin and protease a 3 fold decrease, while the crude was little affected with titres ranging from 2048 to 4096 (Table 5.11).

5.4.5 Purification of tick lectins

5.4.5.1 Gel Filtration

Three peaks were obtained from the sephacryl S200 (Biorad) gel filtration (Fig 5.1) and the haemagglutination activity using rabbit rbc recorded a titre of 256 from the 3rd peak only (tubes 15-35; Fig 5.1). The protein analysed by SDS -PAGE revealed 2 bands at 66,000 and 52,000kd (Fig 5.2) from the concentration of peak 3 and staining with silver. The sepharose 6B gel filtration produced three peaks (Fig. 5.3). The first small peak (tube 1-9; Fig 5.3) had lysis properties in high concentrations (Table 5.12). The first well (1:2) was lysed followed by agglutination optimum in tube 4 and 5 with titre 64 followed by a peak in tube 7 where lysis was observed in the first well, but the agglutination titre was high (1024). The second peak was bigger (tube 10-27) and had maximum activity in 18 and 20 (Fig 5.4; Table 5.12) demonstrating a titre of 256, with lysis occurring in the first three wells together with an opsonising effect. The third peak (tube 28-34; Fig 5.4) was also small and had only agglutinating properties.

5.4.5.2 FPLC Purification of the tick gut lectin

Several buffer systems used in the purification of the tick gut extract on FPLC resulted into different lectin activity as the experiments explained below revealed.

5.4.5.2.1 PBS

The PBS utilised to isolate the lectin from the integral membranes of the gut, contained non ionic detergent triton x 100. This, however, interfered with the purification system resulting in the lectin not binding on to the MONO Q column. The agglutination activity was only present in the run through (Fig. 5.5), making this buffer unsuitable for purification.

5.4.5.2.2 HEPES

HEPES (buffer A 50mM HEPES and buffer B (50mM HEPES + 1M KCl, pH 7.0) demonstrated a loss of 84% in the lectin activity (Table 5.13). The crude extract loaded on the *MONOQ* and eluted with linear gradient of NaCl 0-100mM revealed a total of 24,576 agglutination titre units (Table 5.13) compared to 3890 from the fractions from the FPLC. The unbound fraction gave a maximum titre of 32 while the highest from the eluted fractions was 16 (Fig 5.6).

5.4.5.2.3 Tris -HCl

The elution profile of the FPLC using Tris-HCl with increasing ionic strength (Fig 5.7) revealed 5 peaks whose agglutination properties given in Fig 5.8 showed low activity in the eluted fractions. Tris-HCl (Buffer A 50mM Tris-HCl and buffer B Tris-HCl + 1M KCl), pH 7.0 demonstrated loss of activity spread through the run through (Tubes 1-20; Fig 5.8) and throughout the eluted fractions with KCl (Fig. 5.8). Comparing the total agglutination titre units, there were significantly more specific activity in the run through than in the fractions (1523.7 and 283.8, respectively, Table 5.14) . This loss of lectin activity on purification on FPLC coupled with the fact that the gut extract left a dirty green colour on the *MONOQ* column that required elaborate chemical scrubbing (see materials and methods) lead further search into other purification methods such as Fetuin, a glycoprotein that inhibited this lectin as well as the immunoadsorbent column.

5.4.5.3 Fetuin affinity chromatography

The gut sample subjected to filtering only, loaded and eluted sequentially with EDTA of increasing ionic strength, revealed agglutination activity in the run through but not in eluted samples (Fig. 5.9A; Table 5.15). Judging from the optical densities, elution with 0.01M EDTA and 0.025M EDTA produced some proteins whose agglutination activity was not detected (Fig.5.9A). The lectin activity in the run through was significantly more (98714 titre units; $p<0.05$; Table 5.15) that in the crude (20480 titre units; Table 5.15) as was the specific activity in the run through (5,333,589.7) compared

to the crude (15,753.84). Among the EDTA eluted fractions indicated by arrows (0.01M and 0.025M; Fig 5.9A), no lectin activity was detected although optical densities revealed the presence of protein molecules (Fig 5.9A; Table 5.15). SDS-PAGE analysis of the samples eluted with 0.025M EDTA had 2 bands at 47 and 67 kDa (Fig 5.9B). When the EDTA eluted fractions were pooled, concentrated on PEG and analysed with SDS_PAGE, two bands of pure gut lectin were observed at 69 and 52 kDa (Fig 5.9C). There was, however no agglutinating activity associated with the fractions.

5.4.5.4 Immunoadsorbent column

5.4.5.4.1 Lectin antibody purification

Ammonium sulphate precipitation of the immunoglobulins was successful and these were further purified on protein A.

5.4.5.4.2 Protein A

The highest haemagglutination inhibition activity (titre 256) was found in the run through from the Protein A column. No agglutination inhibition was detected in the bound fractions. Since protein A binds IgG, these findings indicated that the lectin antibody was found in the IgM, hence the purification was to be by molecular sieving (gel filtration). The protein content of the run through was very high (5mg/ml) while the bound fractions was trace (0.25 mg/ml). The column (0.5 ml) of Protein A had a capacity to bind 80 mg/ml.

5.4.5.4.3 Gel filtration of lectin antibody on Sepharose 6 B

The antibody that had been collected from the run through of Protein A (which contained IgM) when loaded on sepharose 6B produced 3 peaks (Fig.5.10). Fractions (12 & 13) and peak 2 (16&17) hardly showed any haemagglutination inhibition (titre 2). The inhibition was highest in tubes 25-29 in the 3rd peak (Fig. 5.11) at titre 8. Peak 1 had mainly fatty acids and was white in colour. Peak three was used in the immunoadsorbent column.

5.4.5.4.4 Immunoadsorbent Column

The coupling ability of the lectin antibody to cyanogen bromide activated sepharose 4B used in the purification indicated that there was sufficient coupling (Fig 5.12) following titration to determine the protein concentrations of the lectin antibody before and after coupling in the immunoadsorbent column which indicated trace protein in the run through (0.25 mg/ml; Fig 5.12) compared to the original 2.5mg/ml concentration. The profile from the immunoadsorbent column revealed 2 peaks (Fig 5.13) the first big sharp peak was obtained with the run through. The addition of the Glycine-HCl, pH 2.5 resulted into a small peak. The agglutinating properties of the lectin from the immunoadsorbent column revealed most activity in the run through (Fig 5.14; tubes 1-15) . The elution with Glycine HCl (tubes 12-19) demonstrated little activity compared to the run through with titres ranging from 4 to 8 with the highest occurring in tube 17 (Fig 5.14). The rbc especially the first well of the microtitre plates, also appeared lysed at dilution 1:2. The fractions showed a greenish colour .The cell lysis was followed by agglutination indicating a possibility of the pure lectin molecule possessing both agglutinating and lysis properties. The SDS gel electrophoresis of the concentrated fraction 17 and 18 with silver staining showed bands at 197, 46 and 97 kDa (Fig 5.15A). Results from the tubes that showed agglutinating and lysis properties revealed 3 bands at 32, 45 and 67 (Fig 5.15B) while lysis only showed bands at 37 and 45 kDa (Fig 5.15C). The western blot on a 7.5%-17.5% gel showed bands at 69, 97 and 200 kDa with rabbit as well as mouse anti sera (Fig 5.16). Immunodiffusion showed that immunoglobulin to be in the IgM further confirming the activity observed earlier from the protein A column.

5.4.5.4.5 Lectin antibody screening

Polyclonal sera screening of the gut lectin extract by western blotting revealed bands at 47, 53, 97 and 200 kDa (Fig 5.17).

5.4.5.4.6 Ammonium precipitation of gut extract

Lectin molecules precipitated out of the gut extract by a 50 % solution of Ammonium sulphate, precipitated by centrifugation (1200 x g; 30 min) and analysed by SDS-PAGE on a 10% gel showed 2 bands at 69 and 52 kDa.

5.4.5.6 The role of lectins in the tick *R. appendiculatus*

5.4.5.6.1 Agglutination of piroplasms

Agglutinated piroplasms were observed as orange clumps following lectin application when enhanced by carboxyfluorescein diacetate fluorescence dye (Fig 5.19). A longer incubation time led to lysis of piroplasms when viewed under the Axiophot microscope (Fig 5.20). Control slides incubated without lectin were seen to contain scatted piroplasms.

5.4.5.6.2 FITC conjugated anti lectin antibody

Treatment of purified piroplasms with FITC conjugate anti-lectin antibody showed fluorescence of the piroplasms (Fig 5.21). However, no fluorescence was observed on the surfaces of the red blood cells that contained piroplasms. On the other hand, fluorescence was observed in the *T. parva* infected lymphocytes at the schizonts stage (Fig. 5.22).

5.4.5.6.3 Ticks fed on lectin antibodies *in vitro* and on immunised rabbits

Infection rates observed among ticks fed upon lectin antibodies *in vitro* were significantly lower (58%;$p<0.05$) than control that had been fed on clean blood (69.9%; Table 5.16). Similarly, the ticks that fed upon the immunised rabbits showed lower infection rates (48%) than the control (80%). The abundance in these ticks (mean number of infected acini per tick) was 32.8 for females, 9.1 for males while the intensity was high (147) among the females compared to the males (15). From the same batch of ticks, those fed upon clean rabbits had significantly higher infection rates among females (80%); 63.3 for males; an abundance of 94.6 and 16.2 for females and males respectively. The intensity (mean number of infected acini per infected tick) was 118.25 for females and 25.5 for males. Compared to the control, the infection rates amongst the females were significantly lower in the ticks fed upon immunised rabbits ($p<0.001$) than those of clean rabbits. The abundance was also significantly different. The situation among the males followed a similar trend (table 5.16). Electron microscopy studies of the gut

revealed disintegration (Fig 5.23) and the salivary glands type III acini cells petrified (Fig 5.24) compared to those derived form ticks fed upon clean rabbits (Fig 5.25).

5.4.5.6.7 Ticks fed on carbohydrates/glycoproteins

Nymphs that were fed on carbohydrates started dropping from the membranes on day 5. Fetuin fed ticks attached high in number compared to all the other sugars and had high mean engorgement weights (0.003mg). However, about 5-10% of nymphs attached without fully engorging (partially fed). On the other hand, some ticks just died following the sugar feeding. The chambers containing carbohydrates had problems with fungal contamination, hence they were frequently rinsed with antibiotics and fungizone before the next sugar blood meals were supplied. The colour of the blood was also changed by the sugars in some cases (Table 5.17A). The infection rates among these ticks depended on the type of sugar they fed upon. For example, those fed upon Fetuin showed no infection at all (Table 5.16), D (+) galactose 76.6% while galactosamine gave none all of which were significantly different from the control ($p<0.05$; Table 5.16). The moulting success of the ticks fed upon carbohydrates was low compared to the control (Table 5.17B).

5.4.5.6.8 Development of an ELISA for testing MoAbs

5.4.5.6.8.1 Plate selection

Results from the determination of the best plate to use for the lectin ELISA showed that a concentration of 50ng/µl of the antigen and antibody (1:100) was the best for coating the plates. There was a significantly higher binding in the Dynatech lactdyte plate (mean= 0.76 $P < 0.05$) compared to lactopolynun $x = 0.253$ (Table 18). Although there was no significant difference between the dynatech immunol and dynatech lactydyte ($x = 0.713$) $P > 0.05$), the dynatech were cheaper and higher so selected for the assays.

5.4.5.6.8.2 Detergent

The detergent was clearly lowering the readings by interfering with the binding of the lectin to the plates. It was decided to get rid of the detergent in the antigen.

5.4.5.6.8.3 Blocking

Normal Balb/C showed a mean OD of 0.214 and the immunised mouse ($M_{4)}$ a mean of 0.558. With the detergent removed and blocking with casein, a mean OD value for normal Balb/ C of 0.0195 and M_4 = 0.271 was obtained (Table 5.19). Clearly, the background was brought down significantly ($p<0.05$) hence the necessity to block. Blocking of 30 min was found to be the best for the assay. FCS inhibited wells were not significantly lower in OD than the immunised mice hence FCS had no effect on the lectin -Antibody interaction and ELISA could be used as an assay to detect the lectin antibody.

5.4.5.6.8.4 Monoclonal antibodies

When tested against anti-whole mouse and the different subclasses, MoAbs were detected that reacted with IgG_1. During the growth of the hybridomas, there was problems encountered with the haemagglutination inhibition assay to show the presence of the anti body, probably due to the presence of foetal calf serum in the culture media which was inhibiting the antibody. Several alternative sera tried indicated that the cow, goat and sheep did not inhibit at all, while the rabbit showed a titre of 16 rat 256 and horse the highest (4096). The cells were grown in FCS -free media namely Iscoves media with essential amino acids. The hybridomas cultured in this foetal calf serum -free media, however, did not produce enough quantities of the MoAbs. The test assay was changed to ELISA.

5.5 Discussion

Lectins have been detected in both the gut, salivary glands and haemolymph of *R. appendiculatus* and *R. pulchellus* using haemagglutination of the erythrocytes. Similar detection of lectins in the guts of invertebrate disease vectors has been shown in the mosquitoes (Mohammed *et al.*, 1992), tsetseflies (Ingram and Molyneux.,1991; Maudlin and Welburn., 1994; Welburn *et al.*, 1989) in Argazid and Ixodid ticks transmitting spirochetes (Grubhoffer *et al.*, 1993) and in *Rhodnius prolixus* the vector of *Trypanosoma cruzi* (Pereira *et al.*, 1981). The haemolymph lectin also agglutinated the various rbcs to very high titres. Suprisingly it agglutinated the horse rbcs unlike the gut lectin. Mohammed *et al.*, (1992) demonstrated agglutination in the haemolymph and gut extracts of female and male *Anopheles gambiae* mosquitoes. Earlier, Chiesa and Palatnik (1990) had showed anti-human ABO agglutinins in the gut extracts of *Ae. albifasciatus* and *Culex pipiens*. The variation if titres of agglutination may imply a variation in the amounts of lectins with different affinities or perhaps, different binding affinities for the various rbc which is common among invertebrates (Fries, 1984). The lectins from the haemolymph and gut of *R. appendiculatus* showed different sugar specificity's which led to the agglutination of some species' erythrocytes by both lectins but to different titres and with different absorptive capabilities against erythrocytes of other species. Such a phenomenon is common among invertebrates (Ishiyama *et al.*, 1974).

The inability of the tick gut and haemolymph lectins to agglutinate bovine erythrocytes would suggest that there has been a host-vector-parasite evolutionary adaptation. The parasite seems to be well adapted in that it is passed on to the invertebrate host where it is deposited in the gut where further development occurs. In the gut, the rbc are lysed and the piroplasms released (Norval *et al.*, 1992). They further differentiate into micro and macro gametes (Melhorn and Schein, 1984; Young and Leitch, 1981) and develop into male and female gametes. Fusion takes place and a zygote is formed in the gut lumen. It then undergoes development in the gut epithelium (Walker, 1990). Had the lectin agglutinated the bovine rbc, perhaps haemolysis and

subsequent release of piroplasms would have been prevented. On the other hand, buffalo blood, where the parasite produces no clinical symptoms, was agglutinated by the gut lectins implying the role of lectins in modulating parasite infection. The ability of the parasite to survive in some hosts and not in others is due to several factors. In the African buffalo, plasma and serum for instance, there exists a factor that kills Trypanosoma brucei brucei, *T.b.rhodesiense, T.b.gambiense, T.evansi, T. congolense* and *T.vivax in vitro* (Reduth *et al.*, 1992). Similar trypanocidal serum was observed in the eland where as serum from oryx, waterbuck, yellow back duiker, cattle, sheep, goat, horse, mouse, rat and rabbit was not trypanocidal. This may imply that the tick gut lectin is not able to agglutinate the rbc containing the parasite, which may have implications on the transmission of the disease.

Looking at the carbohydrate specificities of lectins helps define the structures of the lectins. Agglutinating properties that could be inhibited by sugars and glycoproteins have been demonstrated confirming that these are indeed lectins. The inhibition of the gut lectin activity was demonstrated by Fetuin, a glycoprotein, and the sugars, D(-) mannosamine, D (+) glucosamine and D(+) galactosamine. On the other hand, the haemolymph lectin was inhibited by Xylose, N-acetyl galactosamine and β-d-Fructose. This difference in carbohydrate-binding specificity of lectins with respect to simple sugars is common (Sharon and Lis, 1989) with regard to the physiological functions haemolymph lectins have been reported to act as opsonins which enhance phagocytosis against foreign particles by haemocytes (Suzuki and Mori, 1990). This may help explain why some of the rbc were agglutinated and others were not and suggests that the lectins recognise carbohydrate structures on the surface of the cells that are present in some cells and absent from others, revealing tick lectins to be heterogeneous with regards to their reactivities towards erythrocyte surface carbohydrates. These properties have also been observed in insect lectins (Pathak, 1993). The haemolymph and gut lectins have also been shown to be inhibited at the minimal concentrations, by (1-1), (1-4) or 1-6) linked glucose based disaccharide or glucose and its linkages to fructose. Feting, a sialylated glycoprotein and aminated sugars were also inhibitory. Lectin amounts seemed to vary in different tick batches as observed from the slight differences in agglutination titres even with the same erythrocytes. This variation in lectin quantity may be reflected in the

differences in infection rates among ticks from the same batch same locality or different ecological zones. Further research is required in this area as it may have implications in the epidemiology of the disease.

The chelation of Ca^{2+} and Mg^{2+} ions by EDTA resulted in a significant decrease in agglutination titres, implying a dependency of the gut lectin on these ions. Addition of excess Ca^{2+} resulted in increased titres while Mg^{2+} caused a slight decrease. Among Dipterans, for instance the levels of Mg^{2+} ions are usually higher than that of Ca^{2+} ions (Wigglesworth, 1972). On the other hand, some insects Ca^{2+} ions or other divalent ions are an important requirement for haemagglutination function while in others they seem to be non essential (Renwrantz and Stahmer, 1983; Richards *et al.*, 1988; Anderson *et al.*, 1972). In this case both these ions seem to be required for maintenance of the structural configuration of agglutinin under normal conditions.

The tick guts are normally neutral to slightly acidic pH resulting from the presence of red blood cells which have been lysed by extracellular hemolysins (Obechain and Gallun, 1982). Through a process called receptor mediated endocytosis, macromolecules, specially hemaglobin, are bound to clathrin coated pits on the surface of the cells (Soneshine, 1992). Akov (1982) suggested that the protein moiety of the hemaglobin molecules is digested by cathepsin D whose pH optimum is 3, implying that the gut pH conditions may be ranging from slightly acidic to neutral pH. The optimum pH for heamagglutination activity was between pH 3.0 to 7.4 with optimum at 7.2, which is within the physiological pH range of insects, which is pH 6.2-8.2 (Soneshine, 1992). Above pH 9.0 the activity decreased. The influence of pH on heamaglutination activity is an indication that the gut lectin is probably protein in nature.

Haemagglutination activity was obtained in the gut lectin even after heating at 80^{0} C, implying that this lectin is thermal stable and this could have been possible due to protein agglutination in which a number of binding sites may have been protected in protein degeneration. The haemolymph lectin, on the other hand, was thermal labile as in many insect lectins (Ingram *et al.*, 1990). This heat sensitivity is indicative that the haemolymph lectin is protein or glycoprotein in nature.

Proteolytic enzymes trypsin, chymotrypsin and protease lowered the agglutination titres in the isolated lectin which implied that the lectin is proteinaceous in nature. The significant reduction with chymotrypsin, which is specific for aromatic amino acids and residues and basic amino acids lysine and arginine, which trypsin acts upon, strongly suggest the proteinacious nature of this lectin. Physical and chemical characterization of lectins in other animals has shown that different lectins have different characteristics which greatly influences their mode of action (Miller *et al.*, 1972; Mohammed and Ingram, 1994).

A major problem in applying affinity chromatography to receptor isolation is the difficulty in solubilising membranes so that receptors remain in an active form and retain the ability to bind with the immobilised ligand. Tick gut lectins appeared to be located in the lipid bilayer or associated to the inner surfaces and showed a strong tendency to aggregate and therefore were difficult to purify by conventional chromatography procedures. Before these could be applied, the gut membranes had to be solubilised, which could have been in three ways: mechanical, organic solvent or detergents. Selective extraction with non-ionic detergents was carried out especially as the FPLC was to be used.Triton x 100 and NP 40 were tested and the latter found to be most suitable to isolate proteins. To this end, mild ionic detergents were selected to disperse membranes so that the soluble glyco-protein components still retained their ability to bind. During the purification of the lectins, it was necessary to include detergent in the buffer to isolate the integral membrane bound lectins. This meant that in the buffers of for instance, gel filtration, contained detergents (Marcia and Fettener, 1994). Affinity chromatography has been presented as a possible means of isolating the tick lectins. It is feasible and a simple method as earlier demonstrated by covalent coupling of specific polysaccharides or glycoproteins to cyanogen bromide activated beads (Lis *et al.*, 1974; Renwrantz and Stanmer, 1983). Purification of lectins especially of plant origin has been reported by Allen and Johnson (1977) who isolated L- fucose- binding lectins from *Ulex europaeus* and *Lotus tetragonolobus*. Among other invertebrates, affinity chromatography has since been used in the isolation of lectins, for instance, in sponge

Aaptos papillata (Bretting *et al.*, 1976; Castro *et al.*, 1987; Chalk *et al.*, 1993; David *et al.*, 1990; Dorai *et al.*, 1981; Drifand and Brehelin, 1994; Fornster and Parath, 1975).

Results from the Fetuin affinity column revealed most of the activity in the run through. This could mean that either that the lectin did not bind in great amounts to this column, or it is binds so tightly that the excess ran off and the lectin remained bound and was not fully eluted. Elution with EDTA of increasing strength produced some proteins whose agglutination activity was not detected probably due to the chelating process of EDTA. The EDTA chelates Ca^{2+} and Mg^{2+} ions which influence the activity of this lectin, rendering it unable to recognise the cell surface, hence inhibiting the activity in the that already small quantity proteins, judging from the optical densities. It was not therefore, not possible to get any agglutination in the pure lectin fractions that were of molecular weights 69 and 52 kDa (Fig 5.9C). Prior to the haemagglutination assay, divalent ions should have been added to the lectin.

The lectin obtained in the peak following Glycine-HCL on the immunoadsorbent column was very small in quantity with little protein content which may imply that very little binds to the column. However, there was little protein content in the run through, although it showed high agglutination properties. This may mean that the lectin may not be entirely a protein but may contain other molecules such as carbohydrates. One interesting fact has been that the run through contained more lectin activity that was purer than the crude. For instance the crude lysed the cells in the first and second wells which was completely absent in the run through. The lysis activity combined with agglutinating activity has also been observed in fluids of Anelida. There was also a prozone effect in the wells following the lysis. The gel used, therefore, was useful in clearing the crude, hence purifying the lectin.

It is difficult to speculate on the possible role of the gut lectin since it is most improbable that this lectin would agglutinate cattle erythrocytes *in vivo*, since the study has shown that it is an integral membrane protein, not present in the lumen. The possible role, therefore, would be to fix the parasite on to the gut wall following lysis of the rbc where further differentiation would occur. In the present research, lectins were shown to

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agglutinate purified piroplasms but not those present in the red blood cells, and their antibodies bound on to the piroplasms, which is the stage of the parasite ingested by the tick. Lectin parasite interactions have been reviewed by (Jacobson and Doyle, 1996). A bipartiste model where midgut lectins promote cell death, but are essential for trypanosome maturation was proposed (Jacobson and Doyle, 1996). Furthermore, the ticks that fed upon inhibitory sugars and glyco proteins were found to develop no infections. This signifies possible role of lectins in *T. parva* parasite development in the ticks. Such a role of lectins has been observed in insects (Ingram and Molyneux, 1991; Lehane and Msangi, 1991; Maudlin and Welburn, 1994). It also clearly shows that lectins are indeed involved in the parasite development and points out points of intervention in the disease. Recent reports indicate that an important role for lectins is the interaction between vectors and the pathogens they transmit (Ingram & Molyneux, 1991). In the tick gut, for instance, the red blood cells are lysed by enzymes such as hemolysins and the piroplasms released (Norval *et al.*, 1992). They further differentiate into micro and macro gametes (Melhorn and Schein, 1984; Young and Leitch, 1981) and develop into male and female gametes. Fusion takes place and a zygote is formed in the gut lumen. It then undergoes development in the gut epithelium (Walker, 1990). Had the lectin agglutinated the bovine rbc, perhaps haemolysis and subsequent release of piroplasms would have been prevented. On the other hand, buffalo blood, where the parasite produces no clinical symptoms, was agglutinated by the gut lectins implying that some carbohydrate structures were recognised on these cells, which may reflect on the role of lectins in modulating parasite infection. The lectins, therefore, may play a role in vectorial capacity of ticks for *T. parva* infections as well as establishment into the bovine hosts. Looking at parasites in the same order Haemosporida, *Plasmodium* in mosquitoes, seems to be modulated by lectins. It has been reported that lectins in the haemolymph and midgut extracts in mosquitoes may play a role in susceptibility/ refractoriness of mosquitoes in exflagellatin of gametes, in gut orientation or in development of malarial parasites (Mohammed *et al.*, 1992). Furthermore, a particular chemical factor in the stomach of *Cx. pipiends* which greatly activated the microgametocytes of *Plasmodium* reticulum exflagellation while a similar substance in *Ae. aegypti and An. quadrimacualtus* inhibited the process. Only gametocytes survive in the stomach of susceptible mosquitoes while all other blood forms of the malarial parasites are destroyed (Service, 1986;1988).

Lectin - binding has also been considered to play a role in the orientation of *Plasmodium* parasites on their way out of the midgut lumen (Rudith & Hecker, 1989) to the distal parts of the mosquito salivary glands lobes (Perrone *et al.*, 1986; Mohammed *et al.*, 1991). In addition, lectins have been shown to play a role in merozoites attachment to host rbc prior to their invasion (Jungery *et al.*, 1983) following the recognition of Sialoglyco conjugates and glycophorus (Cox, 1983). Among the insects, lectins in *Glossina* have been shown to kill trypanosomes as well as signal their maturation and establishment (Maudlin and Welburn, 1987; Welburn and Maudlin, 1990). The events triggering maturation are reported to involve stimuli from lectins of both the midgut and haemolymph and various strains of tyrpanosomes seem to require different levels of lectins to induce maturation. Volf *et al.*, (1994) surveyed the haemagglutination activities in the gut and head extracts of various species and geographical populations of Phlebotomine sandflies by comparing their lectin quantities and sugar binding specificity and found that there was a species specific interaction between lectins and the *Leishmania* parasites. Similar results were reported by other authors (Wallbanks *et al.*, 1986; Ingram & Molyneux, 1991).

Antibodies to lectins fed *in vitro* to ticks or *in vivo* on lectin immunised rabbits, revealed reduced infection rates. Electron microscopy showed that the type III acini where the parasite develops to infective forms were damaged and their guts as well. Active antibody is known to pass through the arthropod gut wall into the haemolymph and reach target organs (Nogge and Gianneti, 1980; Ackerman *et al.*, 1981; Mongi *et al.*, 1986). The antibodies could have a destructive effect on internal tick organs. If the observed tick gut and salivary gland destruction is antibody dependant, then means that transmission of the disease could be blocked using lectins has been identified. Furthermore, vertebrate immunoglubulins have been shown to cross the midgut epithelium of invertebrates and enter the haemolymph without breakdown (Sauer *et al.*, 1994; Wang and Nuttal, 1994). The efficacy of a vaccine-derived antibodies will depend partly, on whether the various developmental stages in ticks express antigen recognition ingested by the tick (Kocan *et al.*, 1996). In this study, the anti-lectin antibodies recognised the piroplasm as well as the schizonts of *T. parva*. The sporozoite stage of *T. parva* has been reported to be neutralised by a monoclonal antibody directed against a

sporozoite -specific antigen (Musoke *et al.*, 1992; Nene *et al.*, 1992; Knight *et al.*, 1996), which is conserved among sporozoites of different stocks (Musoke *et al.*, 1984). A recombinant vaccine based on the sporozoite protein induces protection cattle (Musoke *et al.*, 1992; 1993). With increasing attention on vaccination of cattle against ticks (Willadsen *et al.*, 1988) there is a need to show possible interactions between the immunogens and tick organs. There is indeed great potential for immunological targeting of lectins, hormones and enzymes and an understanding of the fate of anti bodies in the salivary gland or gut is important. Such knowledge is relevant to the efficacy of vaccines that target antigens within the tick (Willadsen *et al.*, 1989). This study has clearly illustrated to sites, the salivary gland and the gut. This is the first report on the effect of anti-lectin antibody on the development and transmission of *T.parva*. The study of parasite surfaces with fluorescein dyes has been reported (Mutharia and Pearson, 1987). The antibodies to the lectins were demonstrated not only to bind to piroplasms but also to the schizont stages found in cattle. This means that lectins may be used as disease interventions at the piroplasm stage and possibly the schizont stage.

In this study, we tested whether lectin inhibitory sugars and glycoproteins are inhibitory to the continued development and transmission of *T. parva* by ticks. Rabbits immunised by purified lectins developed anti- *T. parva* surface antibodies as indicated by agglutination of purified piroplasms and the presence of antibody markers on the piroplasms and schizonts. Tick transmission of *T. parva* was reduced either by ingestion of antibodies and inhibitory sugars by previously infected ticks. It has been assumed that the antibodies ingested remain functionary intact. The quantity and reactivity of these antibodies was not confirmed. However, recent studies have shown that antibodies from vertebrate hosts enter ticks and pass through the salivary gland secretions (Wang and Nuttal, 1994). Future experiments should concentrate on the localisation of the antibodies when ingested in ticks and looking for vaccines against protozoan diseases as previously observed (Allison and Elsie, 1981; Dolan and McKeever, 1992; De Castro and Mewson, 1993; Cornelissen and Schetters, 1996).

This study has demonstrated three important aspects, that different lectins exist in the tick, *R. appendiculatus*, they have an important role to play in the

development of the parasite *T. parva* and finally the lectins should be urgently targeted as sub-unit vaccine molecules in the control of Theileriosis. The lectins of the gut have been partially purified and characterised and further work should be carried out to characterise and clone the genes.

TABLE 5.1A: Agglutination titres of haemolymph and gut lectins in the tick *R. appendiculatus*

Host blood	Haemolymph lectins Agglutination titres	Membrane bound gut lectins Agglutination titres
Rabbit	512	4096
Mouse	1024	512
Cattle	0	0
Sheep	0	0
Goat	0	2
Horse	4096	4
Buffalo	0	512
Eland	ND	0
Oryx	0	16
Giraffe	256	16
Thomson's gazelle	2	16
Zebra	128	ND
Rhino	4096	ND

End point titres expressed as the reciprocals of the dilution (1/n); 0, negative at 1:2 dilution; ND, not determined.

Table 5.1 B: Agglutination titres of haemolymph and gut lectins in the tick *R. pulchellus*

Host blood	Gut	Haemoly
Rabbit	1024	8
Mouse	256	1024
negative cow	0	0
positive cow	0	0

End point titres expressed as the reciprocals of the dilution (1/n); 0, negative at 1:2 dilution

Table 5.2 Agglutination titres of salivary glands lectins of *R. appendiculatus* and *R. pulchellus*:Salivary glands

Salivary gland	*T. parva* infection in tick	Host erythrocytes	*T. parva* exposure to host	Agglutination titr	
				F/T*	H/S *
R.appendiculatus	negative	rabbit	none	0	0
R.appendiculatus	positive	rabbit	none	0	0
R.appendiculatus	negative	mouse	none	0	0
R.appendiculatus	positive	mouse	none	64	64
R.appendiculatus	negative	cow	negative	0	0
R.appendiculatus	positive	cow	positive	0	0
R.pullchellus	refractory	cow	negative	0	0

***F/T=Freeze/thawed samples;H/S=homogenised/sonicated samples**

Table 5.3 Comparison of agglutination properties between infected and clean *R. Appendiculatus* haemolymph lectins with various erythrocytes

Haemolymph source	*T.parva* infection in tick	Host erythrocytes	*T.parva* exposure to host	Agglutination titre
R.appendic	negative	rabbit	none	512
R.appendic	positive	rabbit	none	4
R.appendic	negative	mouse	none	256
R.appendic	positive	mouse	none	512
R.appendic	negative	cow	negative	0
R.appendic	negative	cow	positive	0
R.appendic	positive	cow	negative	0
R.appendic	positive	cow	negative	0
R.pulchellus	negative (refractory)	rabbit	none	8
R.pulchellus	negative (refractory)	mouse	none	1024
R.pulchellus	negative (refractory)	cow	none	0
R.pulchellus	negative (refractory)	cow	positive	0

Table 5.4: Sugar inhibition characteristics of haemolymph lectin of *R. appendiculatus*

Sugar	Agglutination titre
D(-) Mannose	16
L(+) Arabinose	8
D(-) Arabinose	16
D(+) Raffinose	4
Xylose	0
N-acetyl galactosamine	0
β-D-Fructose	0

End point titres expressed as the reciprocals of the dilutions (1/n); 0, negative at 1:2 dilution;.

Table 5.5: Sugar / glycoprotein inhibition characteristics of *R. appendiculatus* gut lectin

Sugar	Agglutination titre	Sugar	Agglutination titre
β-D(+) glucose	4096	α-D(+)Melibiose	4096
D(+) glucosamine	64	β-Lactose	4096
N-acetyl-β-D-Mannosamine	4096	D(-) Mannitol	4096
N-acetyl Glucosamine	4096	D(-) Ribose	1024
Sucrose	4096	D(+) Galactose	1024
Stachyose	4096	N-acetyl-D-galactosamine	1024
D(+) galactosamine	16	D(+)Turanose	4096
D(+)Trehalose	4096	D(+) Mannose	4096
α-Lactose	4096	D(L) Arabinose	4096
D(+) Raffinose	4096	D-Mannosamine	64
(-)Arabinose	4096	Maltose	4096
D(+) Xylose	4096	D-Sorbitol	4096
β-D(-) Fructose	4096	α-L-Rhaminose	4096
L-glucose	4096	D-L-Arabinose	4096
Mannitol	4096	α-D(+) Fucose	4096
Myo-Inositol	4096	Mucin	4096
D(-) Fructose	4096	Fetuin	16

Table 5.6: Minimum sugar concentration required of inhibiting of the tick gut lectin

Sugar	Concentration	Titre inhibited
D(+) Glucosamine	0.5M	512
N-acetyl Glucosamine	0.5M	256
D(+) Galactosamine	0.25M	2048
N-acetyl glucosamine	0.25M	4096
D(+) Glucosamine	0.25M	2048
D(-) Mannosamine	0.25M	2048
D(+) Glucosamine	0.125M	4096
D(+) galactosamine	0.125M	4096
N-acetyl glucosamine	0.125M	256
N-Mannosamne	0.125M	4096
D(+) Glucosamine	0.0625M	4096
D(+) Galactosamine	0.0625M	4096
N-acetyl-glucosamine	0.0625M	4096
D(-) Mannosamine	0.0625M	4096
D(+) Galactosamine	0.03125M	4096
D(+) Glucosamine	0.03125M	4096
D(-) Mannosamine	0.03125M	4096
N-acetyl glucosamine	0.03125M	4096
N-acetyl galactosamine	0.5M	1024
α-D(+) Fructose	0.5M	256
β-D(+) Glucose	0.5M	256
β-Lactose	0.5M	4096
Maltose	2%	4096
D(+)Maltose	0.5M	4096
D(-)Mannose	0.2M	512
D(+)Melibiose	0.5M	4096
D(-) Mannosamine	0.5M	128*
D(+) Galactose	0.5M	1024
Fetuin	2%	0***
Mucin	2%	4096
N-acetyl-mannosamine	0.5M	1024
D(+) Galactosamine	0.5M	16***
α-methylD-manose	0.5M	512

Table 5.7:Threshold dilutions of the sugars and glycoproteins for gut lectin

Fetuin dilution → / lectin↓	1	2	3	4	5	6	7	8	9	10	Constant
	11	12									
A	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
	+++	+++	1:2								
B	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
	+++	+++	1:4								
C	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
	+++	+++	1:8								
D	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
	+++	+++	1:16								
E	-	++	++	+++	+++	+++	+++	+++	+++	+++	
	+++	+++	1:32								
F	-	-	-	-	++	+++	+++	+++	+++	+++	
	+++	+++	1:64								
G	-	-	-	-	+	+	+	+	+++	+++	
	+++	+++	1:128								
H	-	-	-	-	-	-	-	-	-	-	- -
Next plate											
A	-	-	-	-	-	-	-	-	-	-	- -

Table 5.8. Influence of Temperature on *Rhipicephalus appendiculatus* haemolymph and gut lectin haemagglutination titres (1/n)

	Temperature ° C					
sample	Untreated control	Freeze/thaw -20/25 ° C	40	60	80	100
Haemolymph	512	4*	2***	-	solidified	-
Gut	4096	4096	4096	4096	2048	solidified
Fraction	512	512	512	256	128	128

*Level of significance compared., ***$p<0.001$; *$p,0.05$.*

Table 5.11: Effect of various enzymes on *Rhipicephalus appendiculatus* gut and semi- purified fraction agglutination titres

Treatment	crude	fraction
Control (thawed from -70 ^{0}C)	4096	2048
Untreated control (incubated at 37 ^{0}C)	2048	256**
Ribonuclease	4096	2048
Trypsin	4096	512**
Chymotrypsin	4096	256**
Protease	4096	256**

*Level of significance compared to PBS pH 7.4 control; * *p< 0.05.*

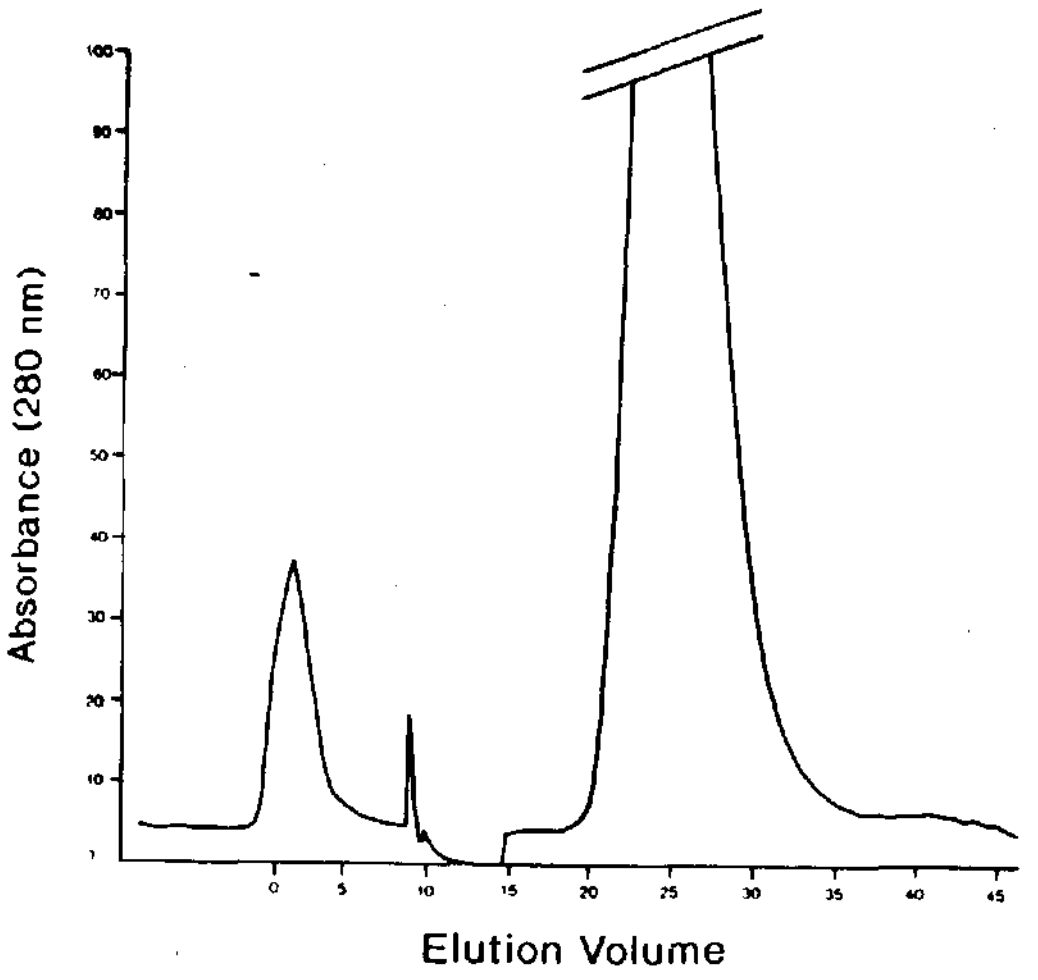
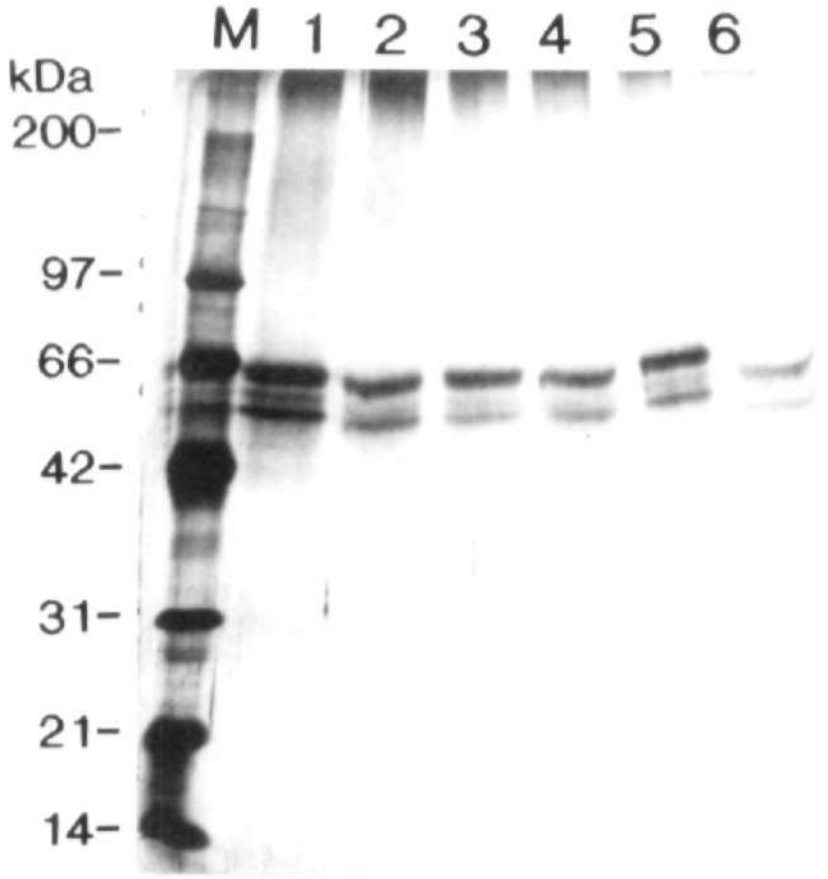


Figure 5.1 Gel filtration of gut lectin extract on sephacryl S200.

The colunm was equilibrated with PBS pH 7.2 at a flow rate of 15ml/hr.



5.2

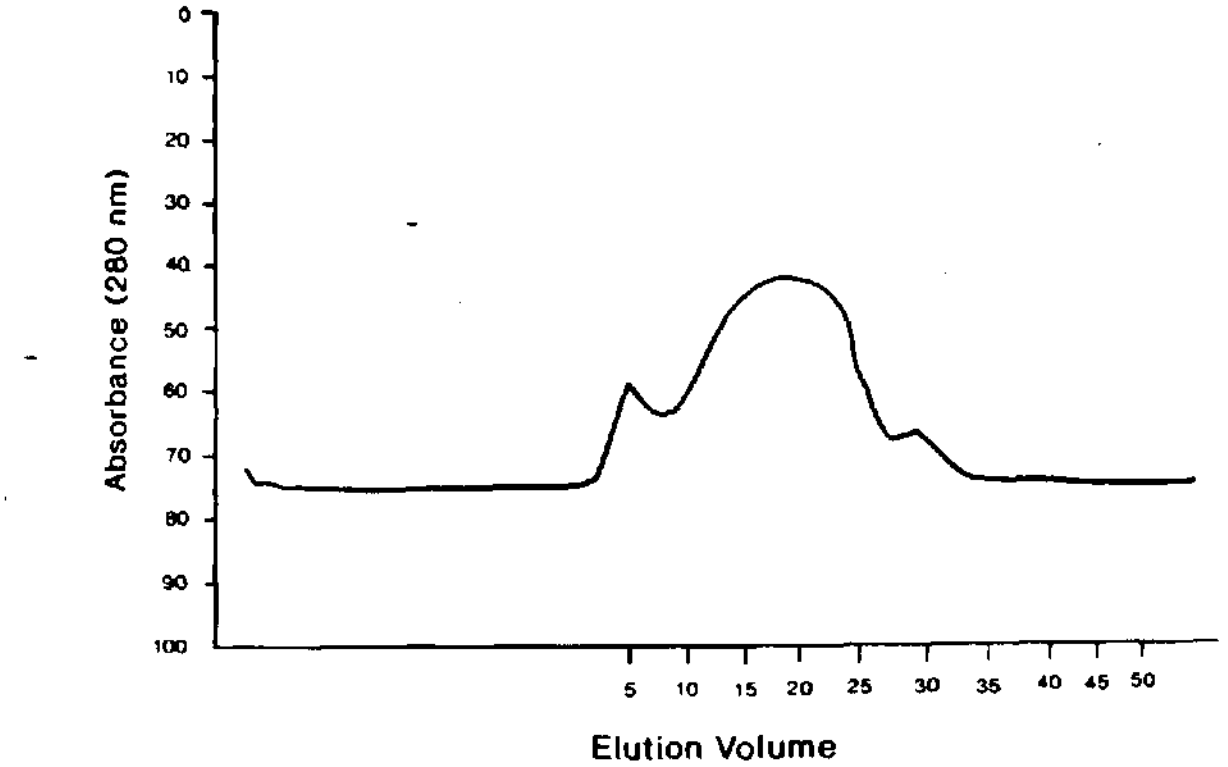


Figure 5.3 Elution profile of gel filtration of gut lectin on sepharose 6B. The column (83 ml) was equilibrated with PBS pH 7.2 with 0.01% triton x 100 detergent and the sample loaded at flow rate of 20ml/hr.

TABLE 5.12 Agglutination properties of tick gut extract from gel filtration on Sepharose 6B

Tube no.	Maximum agglutination titre	Observations
1-4	64 (tube 4 &5)	Agglutination only
5-19	1024 (tube 7)	lysis in 1st well
13-21	256 (tube 18 &20)	lysis in 1st well; prozone effect in 2 wells in tube 20
22-24	64 (tube 22)	agglutination only without lysis
25-27	8	lysis in the firs well;trace agglutination
28-34	8	agglutination only

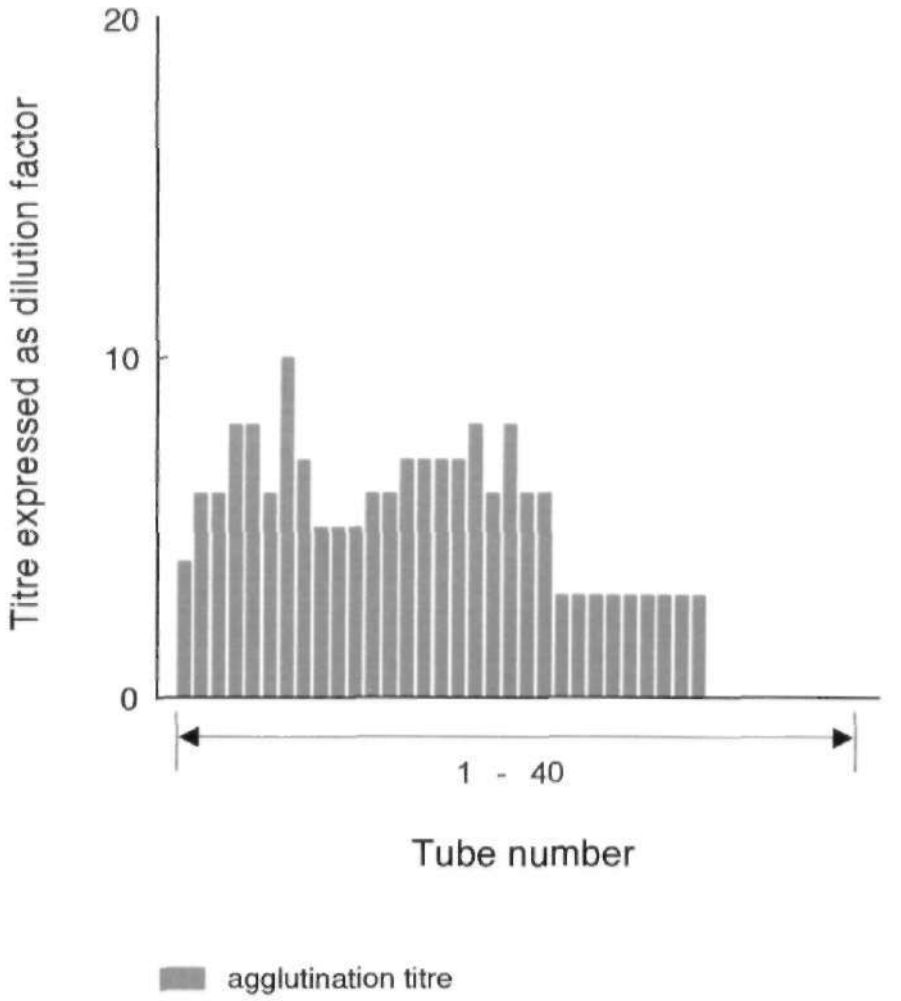


Figure 5.4 Agglutination properties of gut lectin following gel filtration of sepharose 6B

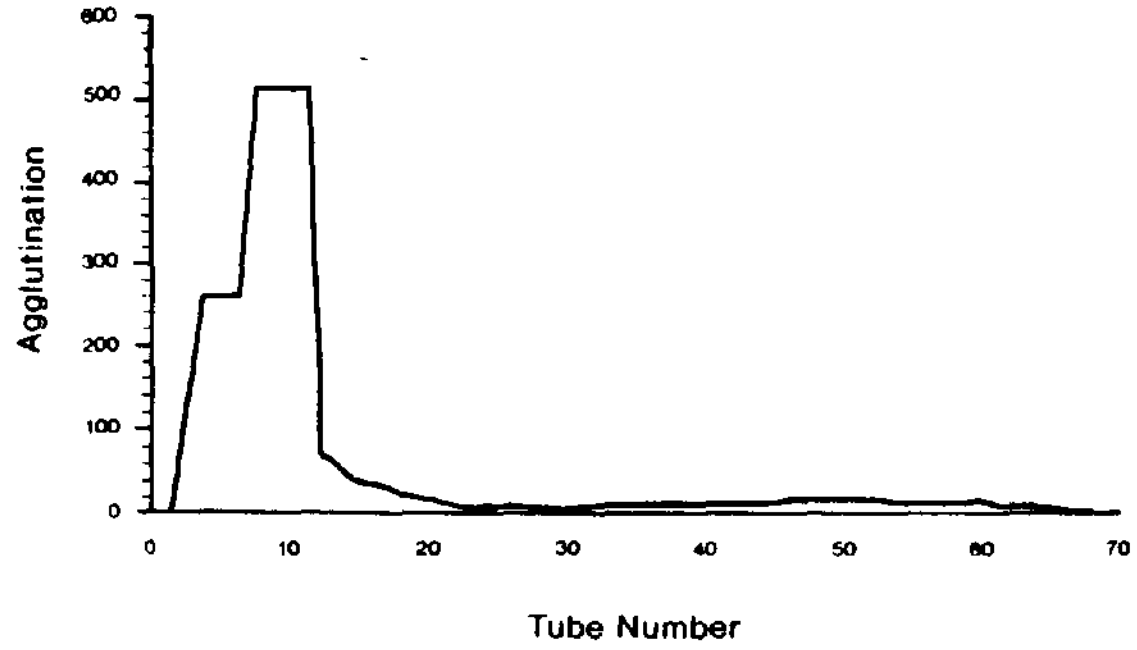


Figure 5.5 Agglutination activity of gut lectin fractions from FPLC on MONOQ column equilibrated with PBS buffer system

TABLE 5.13 Purification of the tick gut lectin on FPLC using HEPES buffer system

Purification (ml)	Volume (ml)	Protein (mg)	Titre units (mg/ml)	Total titre limits	Specific activity Titre unit/protein
Initial extract	1.2	1.3	1.104	24576	18.6×10^3
Loaded sample (diluted in 10ml on monoQ)	10.2	1.3	1.104	3890	29.9×10^2

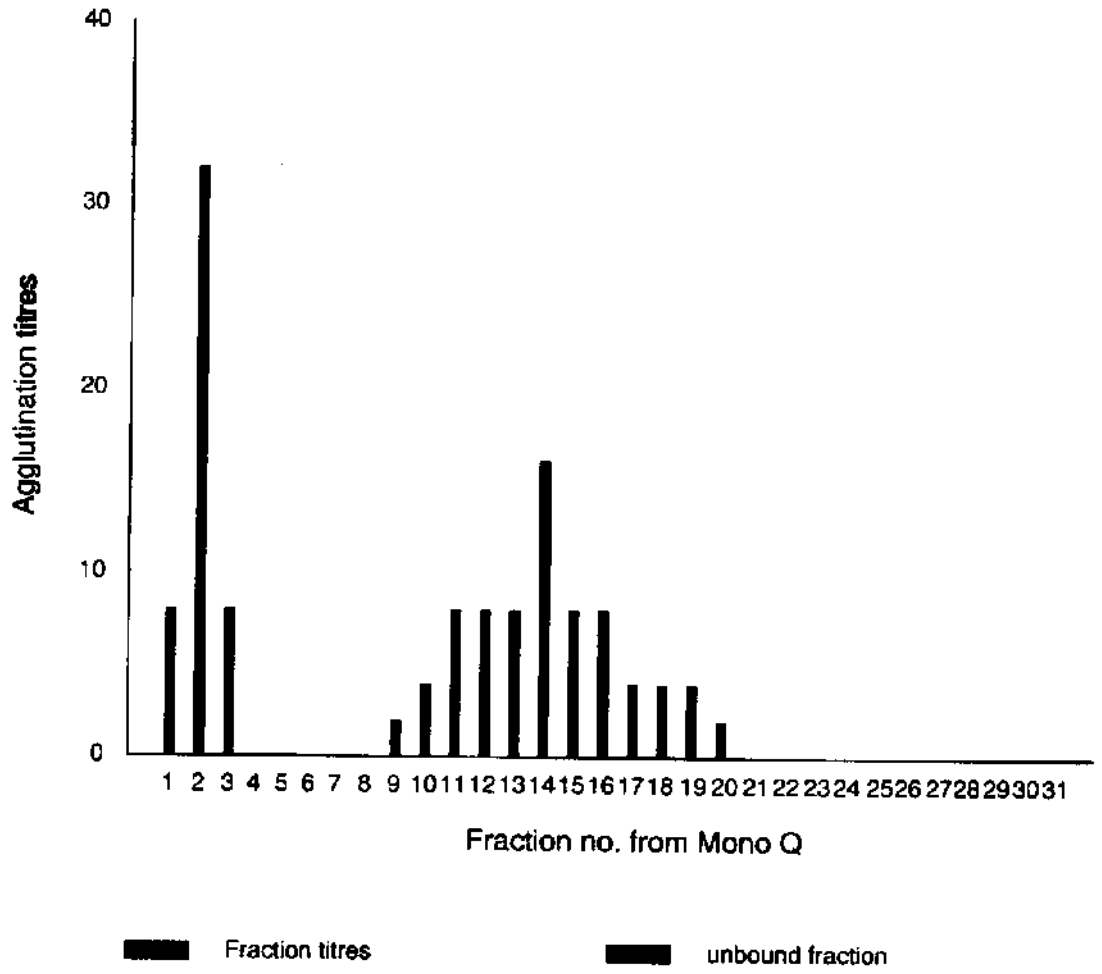


Figure 5.6 Agglutination properties of *R appendiculatus* lectin from FPLC equilibrated with HEPES buffer system. A MONOQ 5/5 ml was loaded with 10.2 volume of sample and subjected to a linear gradient (0-100 mM KCL). Differences in activity among the eluted fractions (red) compared to the run through (green) are illustrated

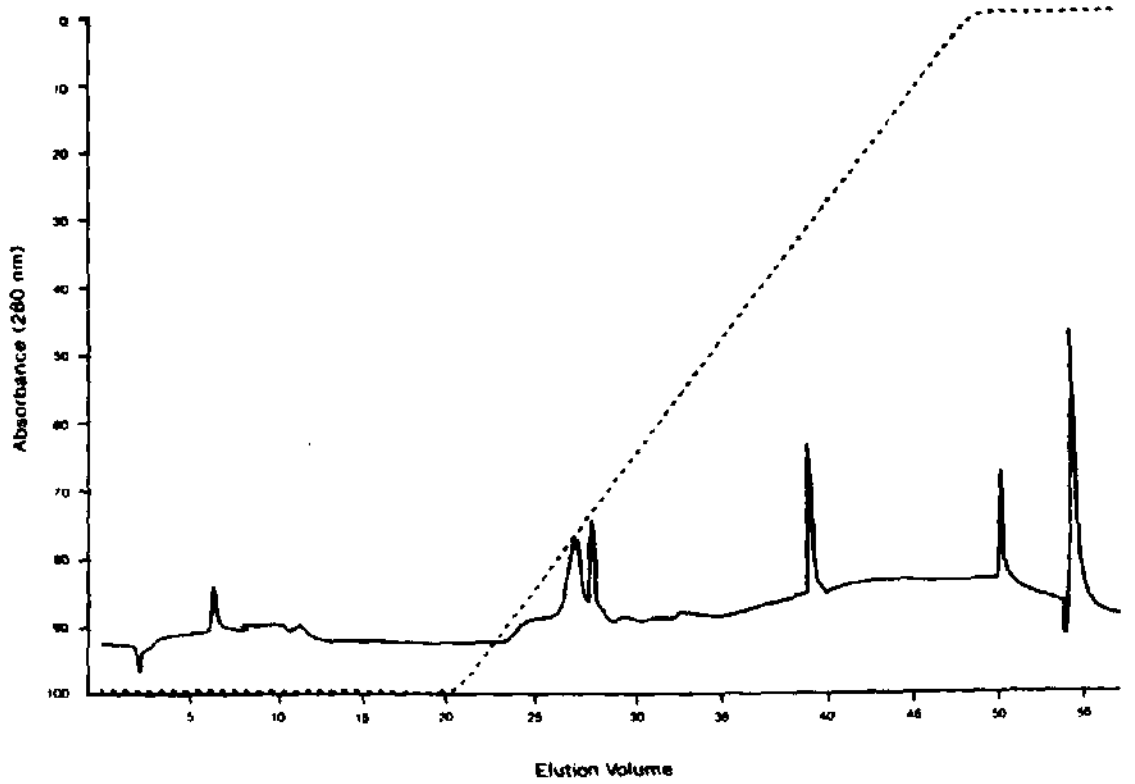


Figure 5.7 Elution profile of tick gut lectin on FPLC on MONOQ column using Tris-HCL buffer system. A sample of 10.2ml was loaded on to the column equilibrated with 50 mM Tris-HCL (buffer A) and 50 mM Tris-HCL + 1MKCL (buffer B). Elution was with 0-100mM KCL.

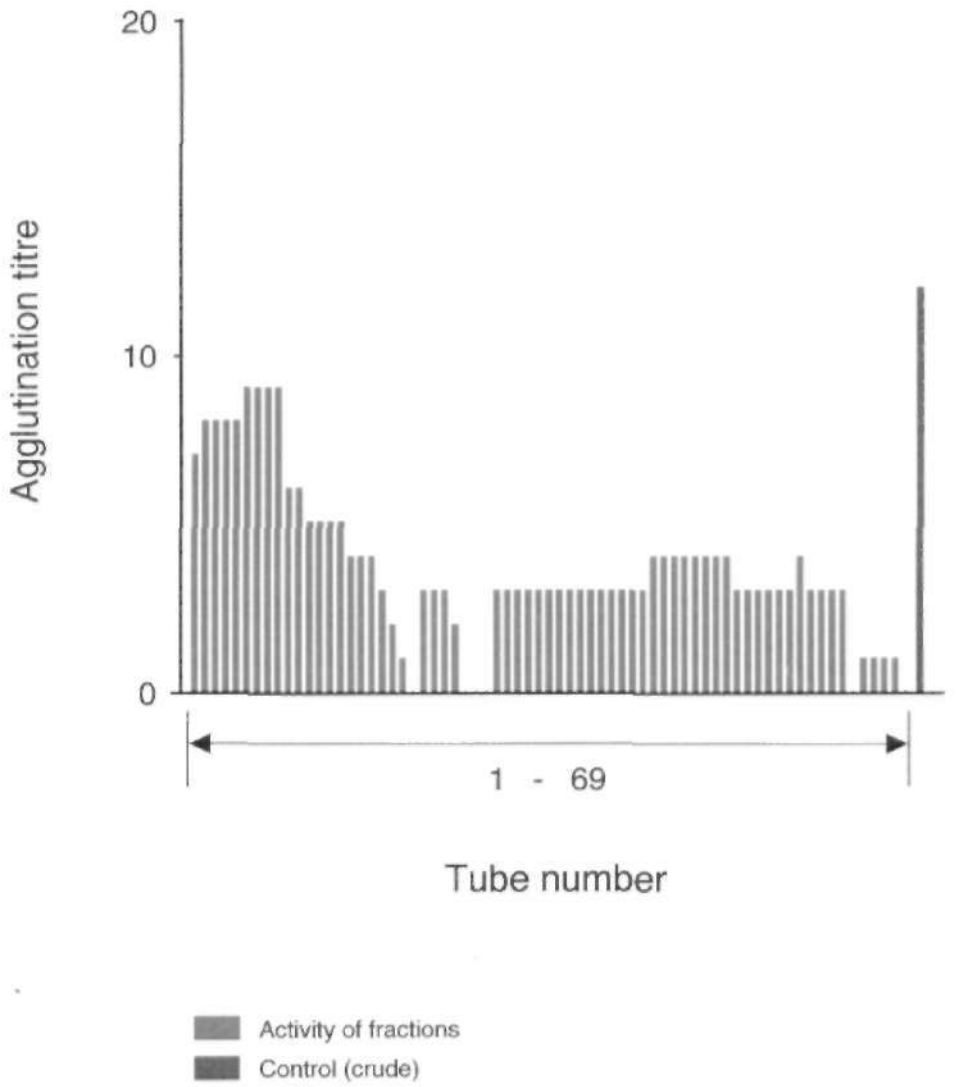


Figure 5.8 Agglutination properties of tick gut lectin following purification on FPLC using Tris-HCL buffer system.

Table 5.14 Fast Protein Liquid Chromatography (FPLC) Purification of the Tick gut Lectin using Tris-HCL buffer system

Purification sample	volume (ml)	Protein (mg)	Titre units (mg/ml)	Total titre units	Specific activity
Initial extract	1.2	1.10	1.1	1980	1523.7
Loaded sample diluted in 10ml buffer A	10.2	1.10	1.1	369	283.8

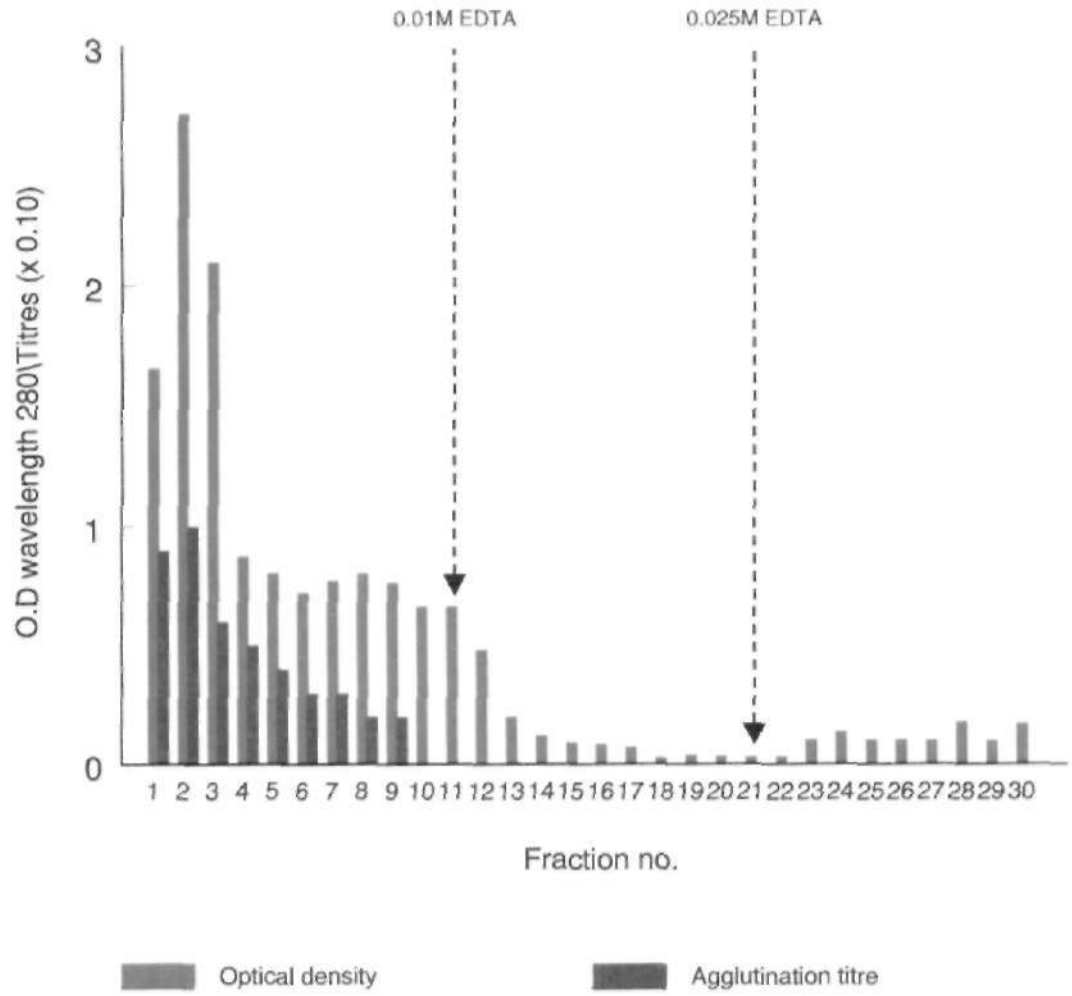
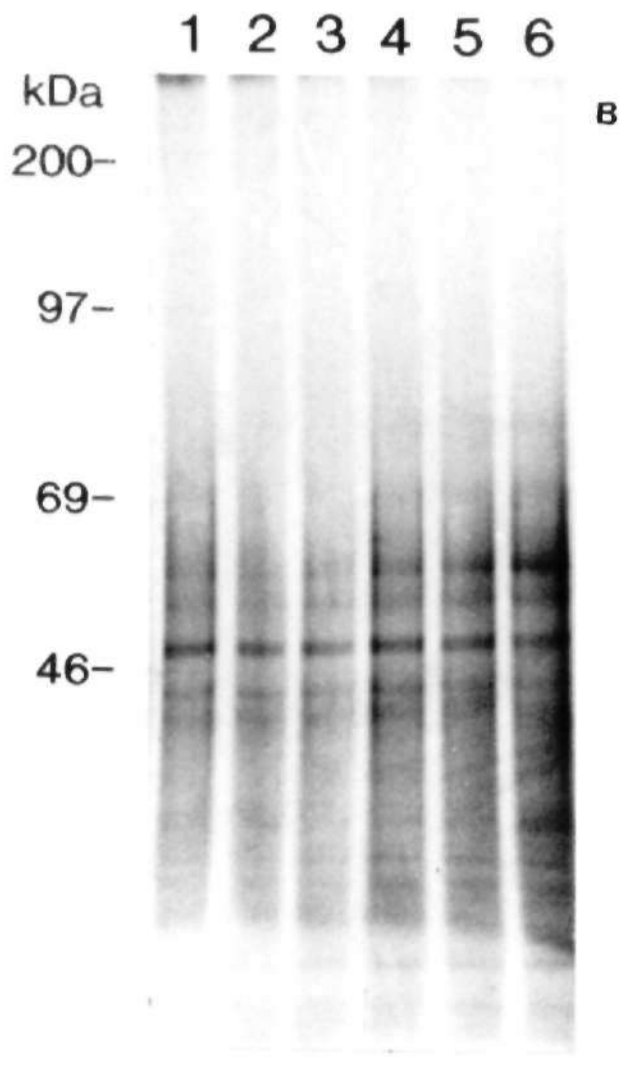


Figure 5.9A. Agglutination properties of gut lectin purified on Fetuin affinity column. The column was equilibrated with PBS pH 7.2. Elution was with EDTA of increasing ionic strength (0.01M and 0.025M EDTA).

TABLE 5.15 Purification of the tick gut lectin by Fetuin/EDTA affinity chromatography

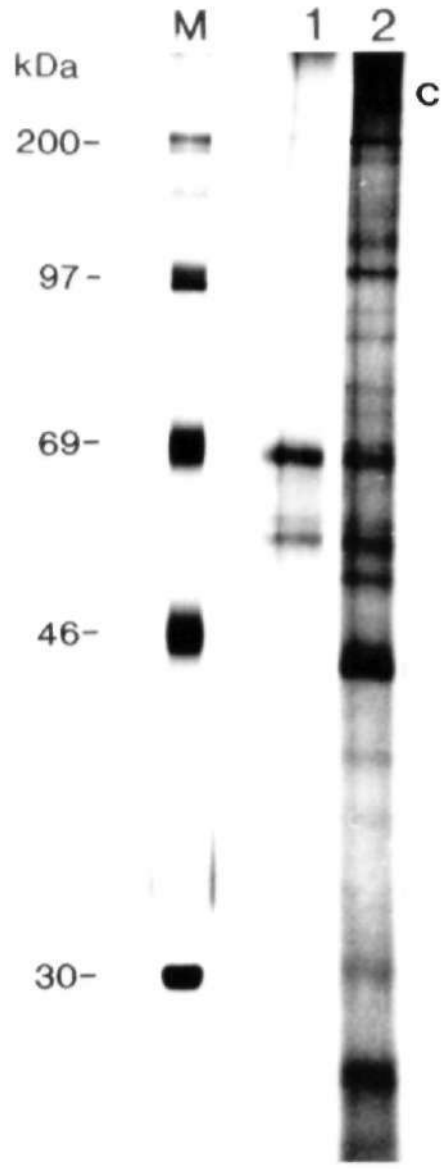
Purification sample	Volume (ml)	Protein (mg)	Titre units (mg/ml)	Total titre units/ml	Specific activity
Initial extract	0.60	1.30	1.160	20480	15,753.84
Run through	7 (10.7ml fractions)	1.30	0.185	98714	5,333,589 .7
EDTA- Eluted samples	20	-	-	0	0

Figure 5.9B. SDS-PAGE analysis of sample from Fetuin affinity column eluted with EDTA. The lectin was equilibrated with PBS and eluted with EDTA (0.01; lane 1-3 and 0.025M; lane 4-6).



593

Figure 5.9C. SDS-PAGE analysis of pooled concentrated fractions from Fetuin/EDTA affinity chromatography column. The column was equilibrated with PBS and eluted with EDTA (0.01M and 0.025M). Bands are shown at 69 and 52.


M
1
2
kDa
200-
97-
69-
46-
30-
C

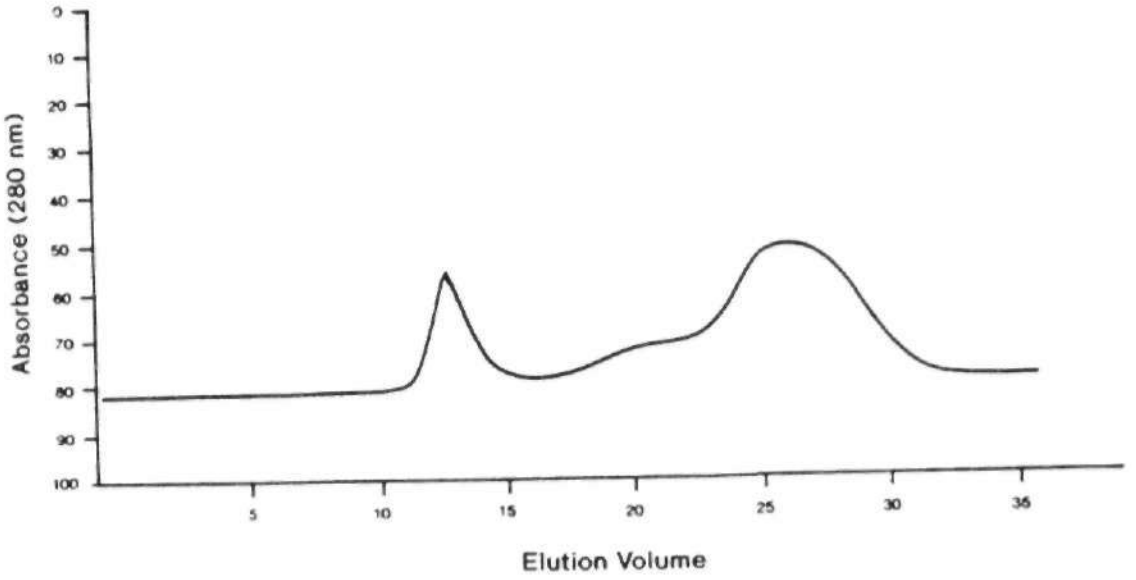


Figure 5.10 Elution profile of lectin antibody on sepharose 6B. Sera subjected to 30% Ammonium sulphate precipitation was loaded on to protein A column equilibrated with PBS pH 7.2 buffer. The run through was re-chromatographed on sepharose 6B equilibrated with the same buffer.

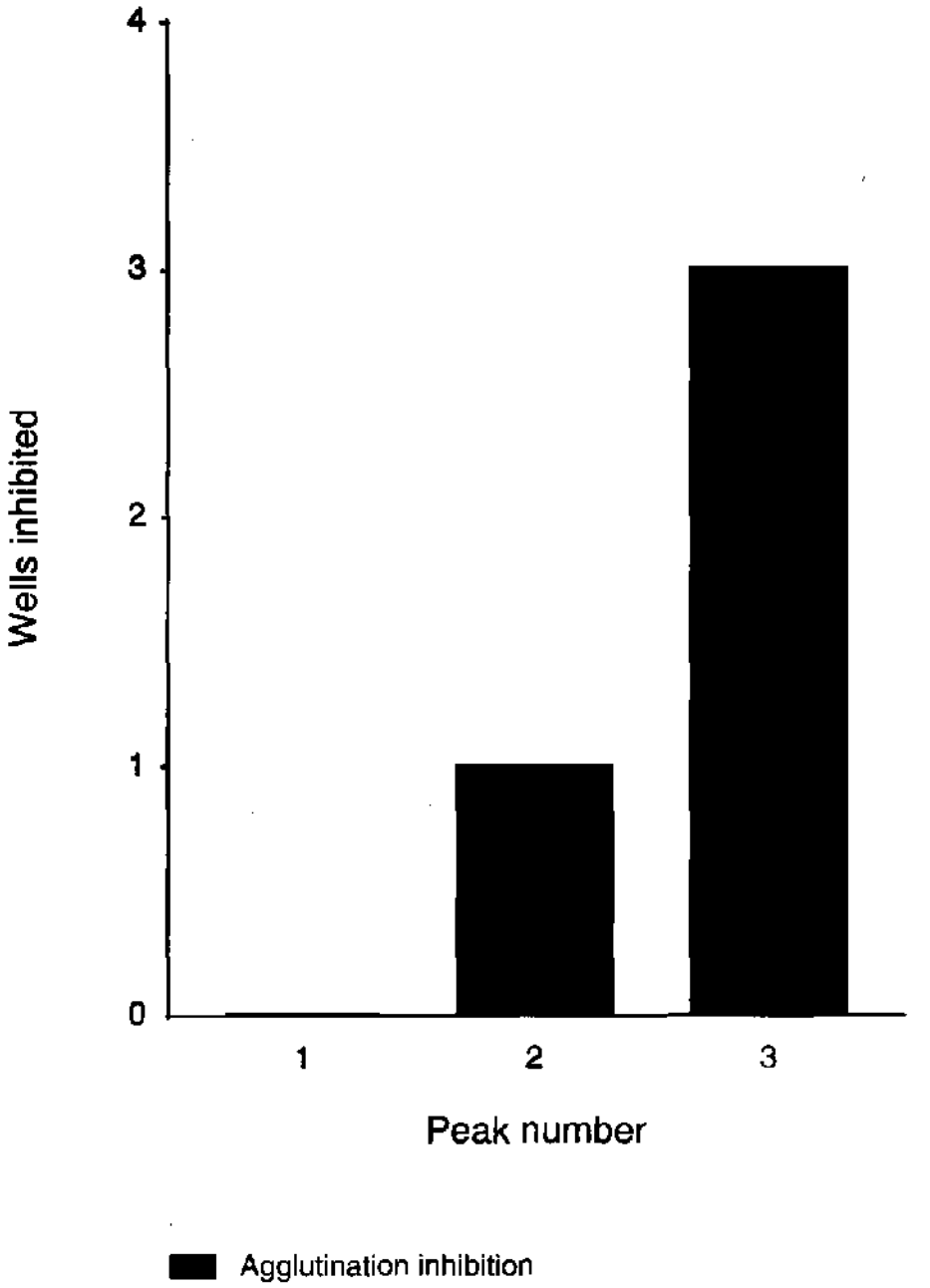


Figure 5.11 Haemagglutination inhibition of lectin antibody fractions from sepharose 6B

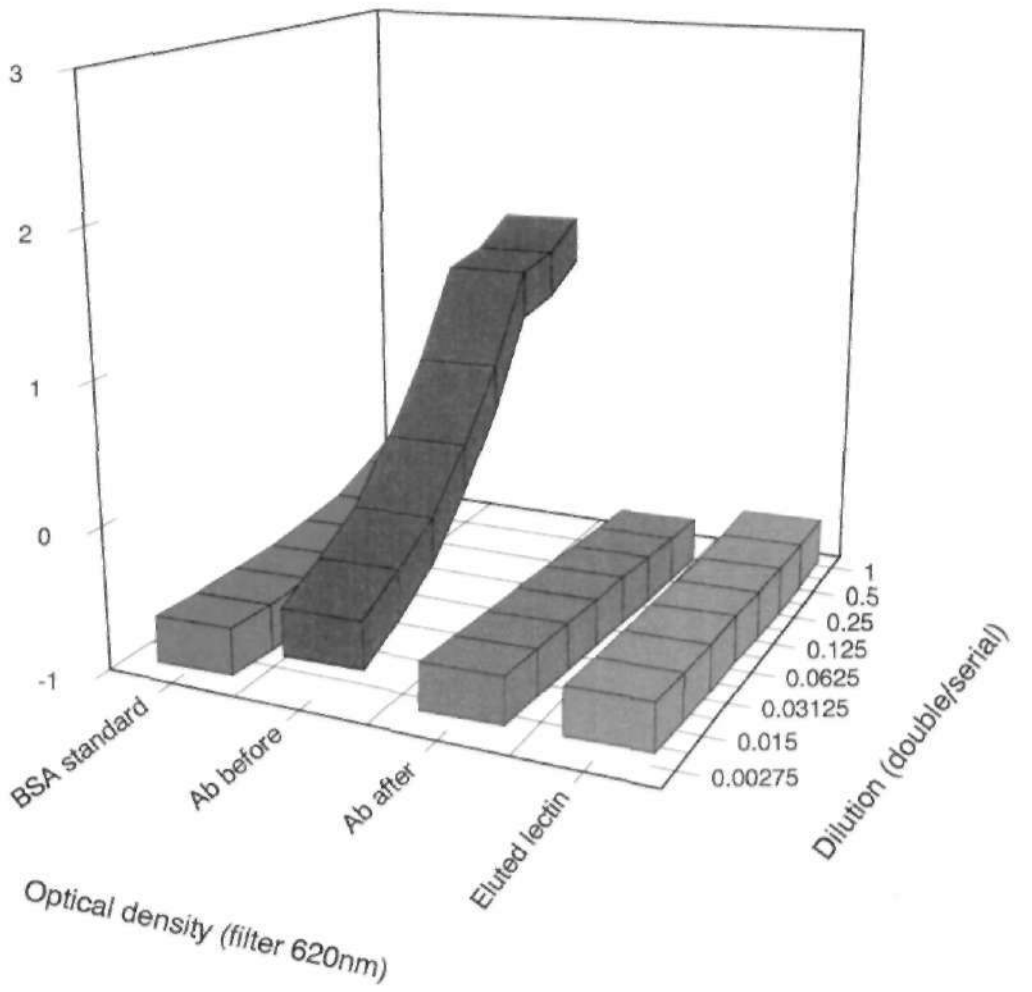


Figure 5.12 Coupling lectin antibody on Cyanogen Bromide (CNBr) activated sepharose 4B. Titration to determine the protein concentration of lectin antibody before and after coupling on CNBr sepharose 4B immunoadsorbent column carried out with bovine serum albumin (BSA) as standard doubly diluted in PBS pH 7.2 showed reduced concentrations indicating coupling had occurred successfully. The eluted lectin protein content is also shown.

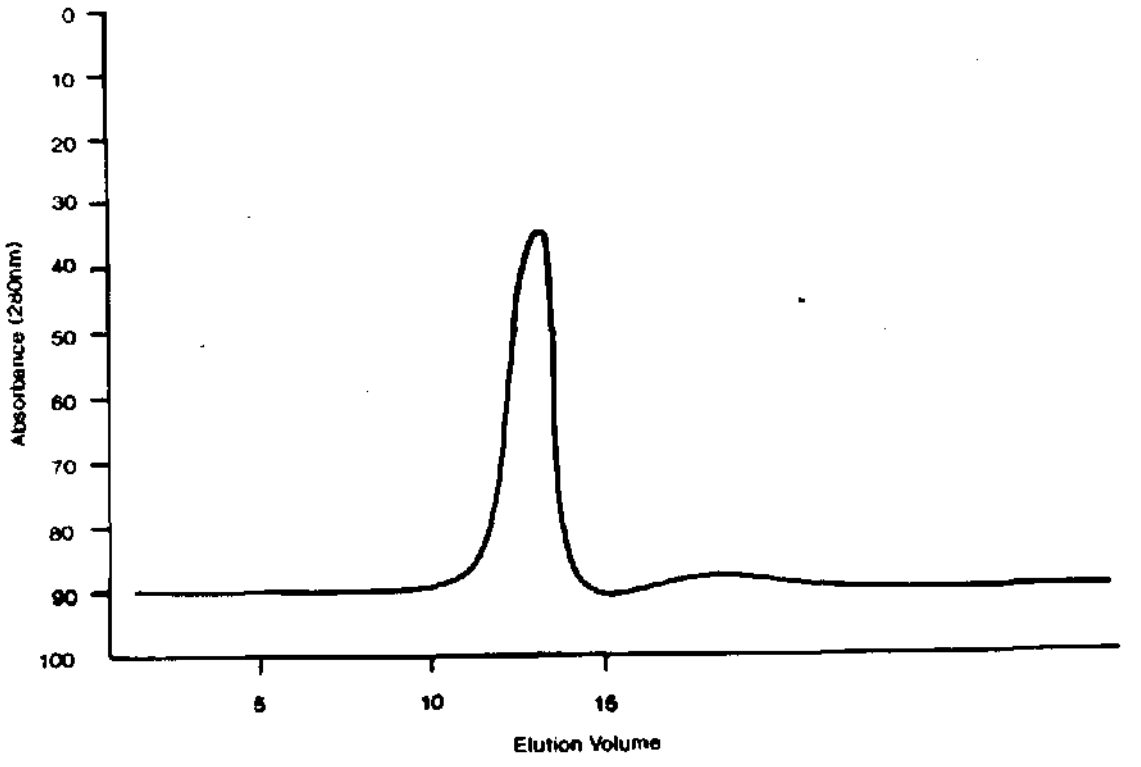


Figure 5.13 Elution profile of lectin from cyanogen bromide (CNBr) activated sepharose 4B immunoadsorbent column. The column was equilibrated with PBS and lectin eluted with glycine-HCL.

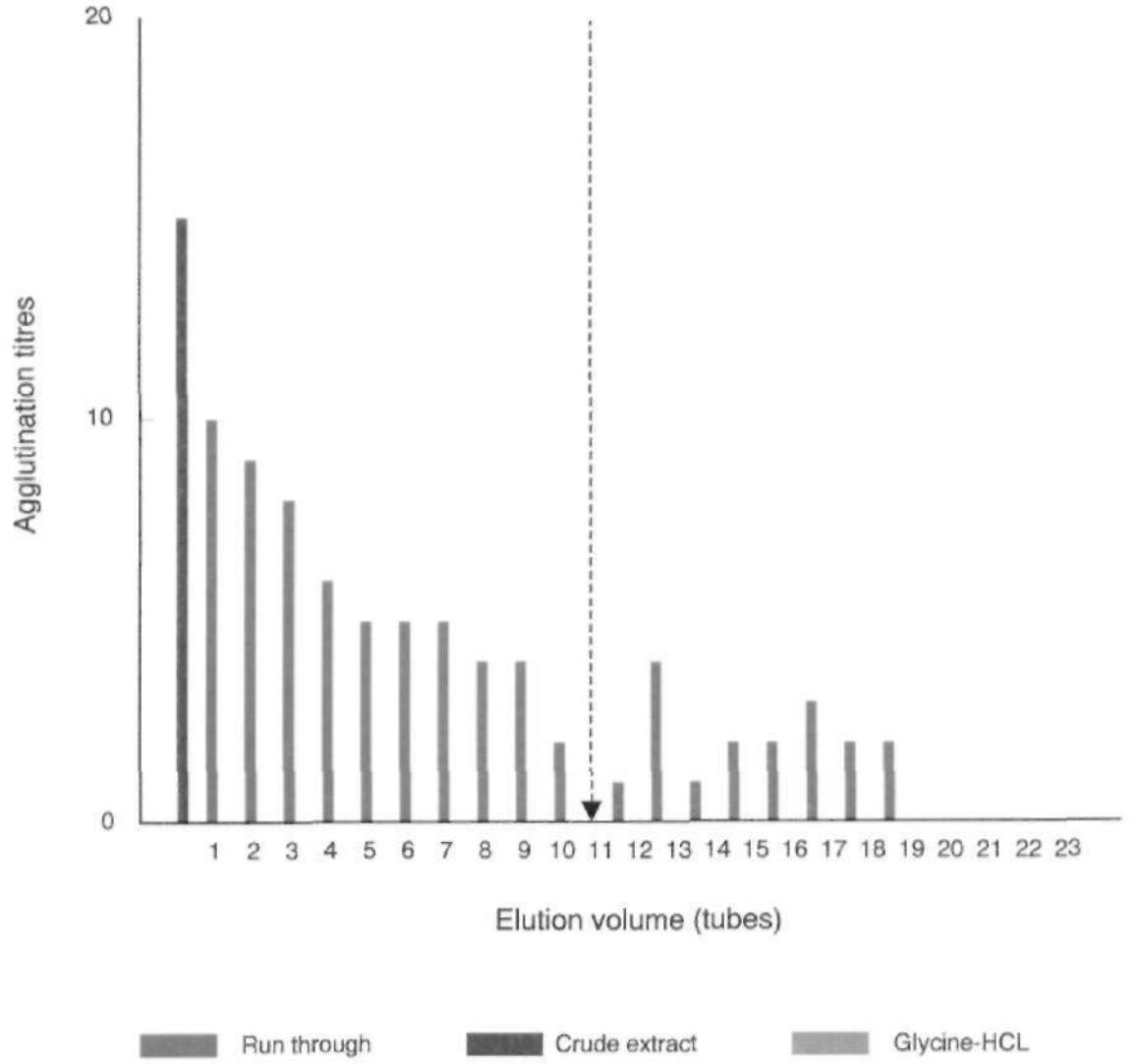
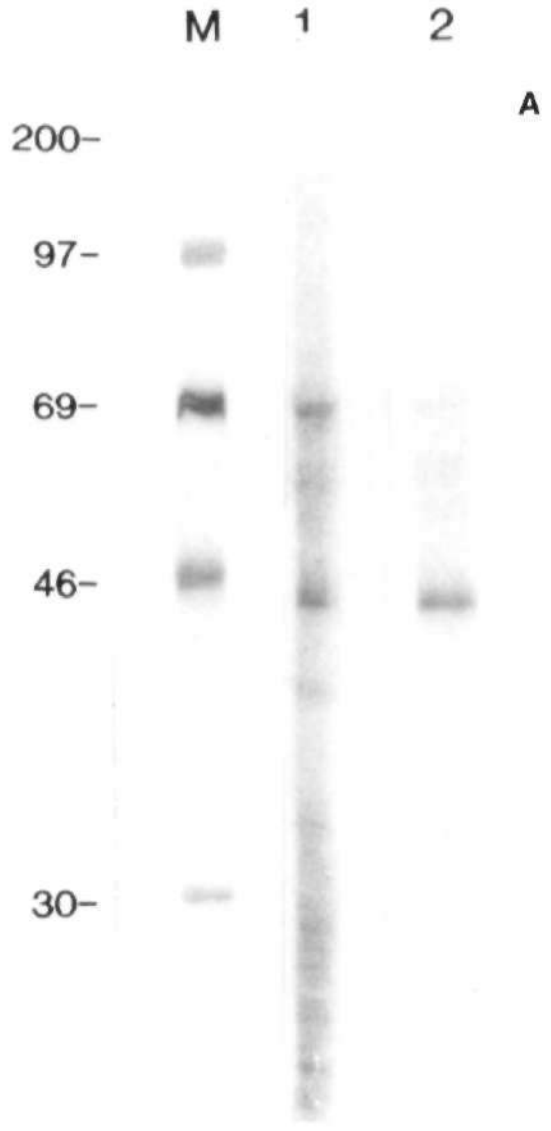
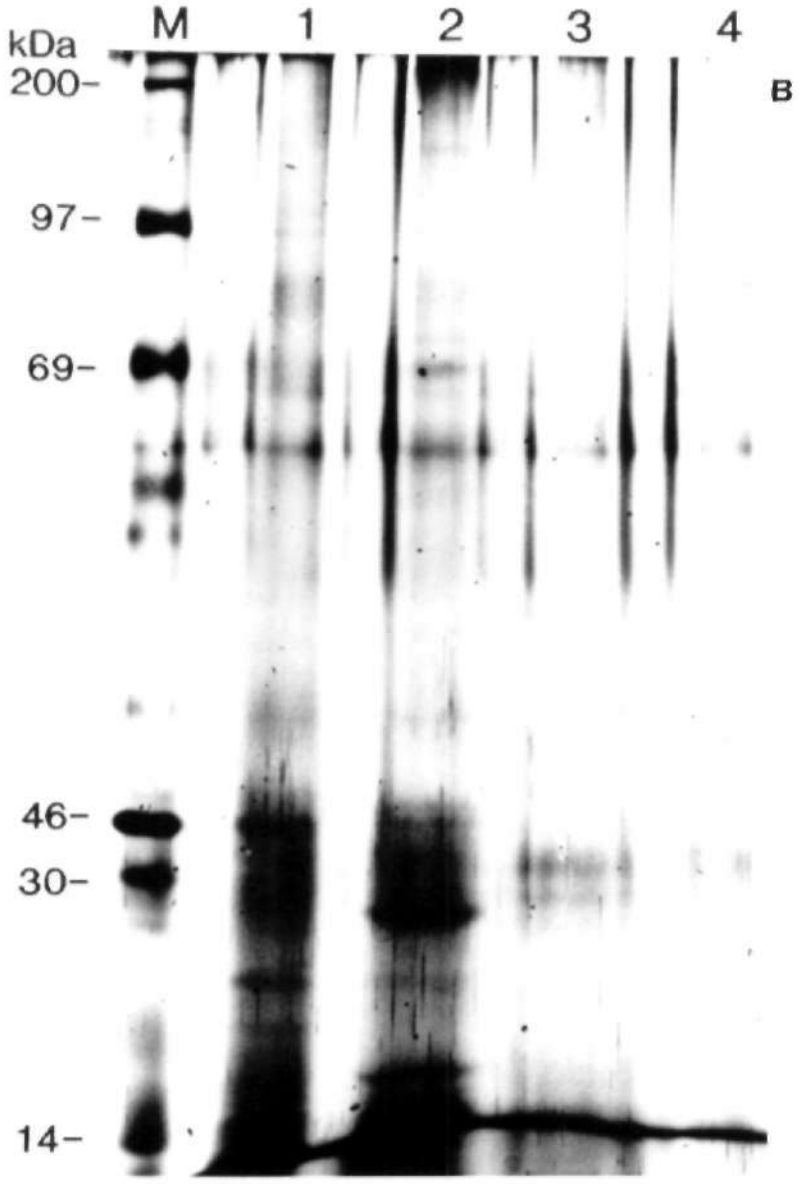
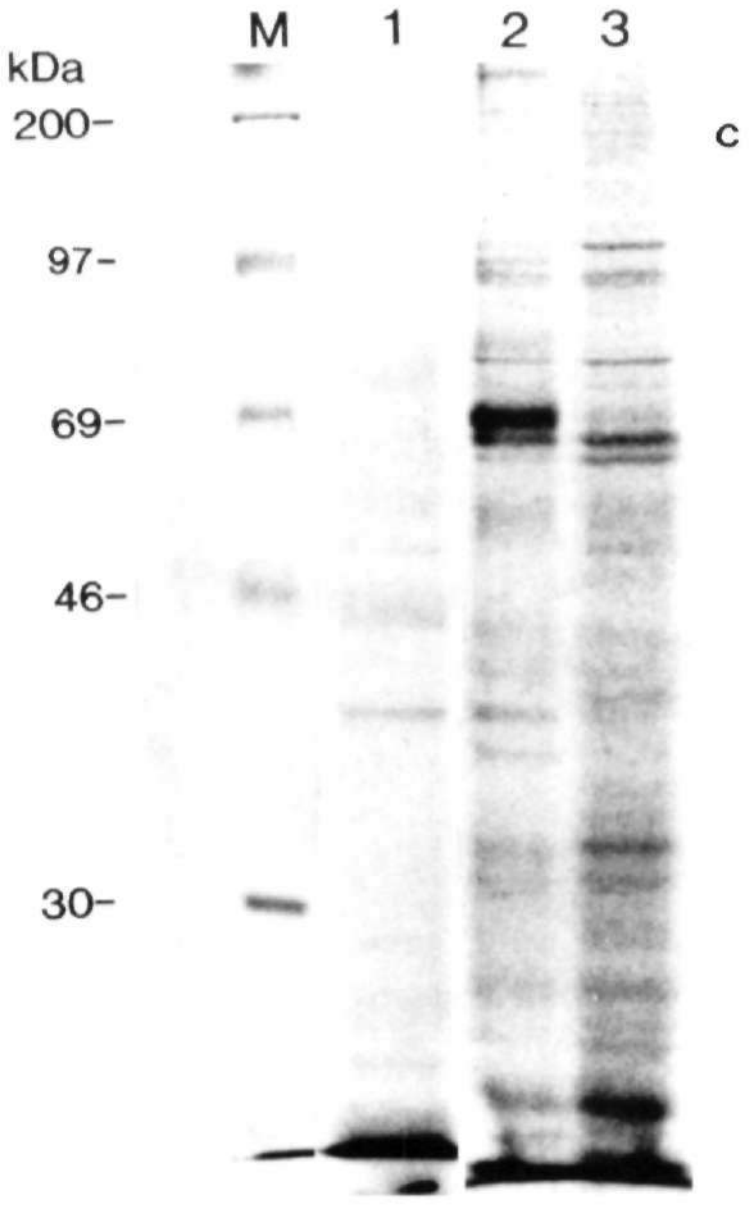
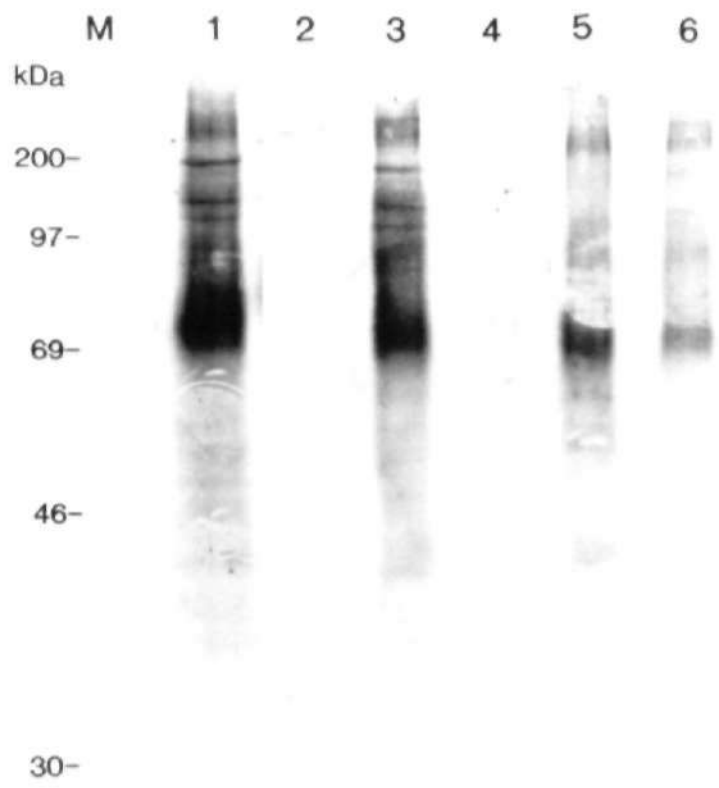


Figure 5.14.Agglutination of eluted fractions from CNBr activated sepharose 4B immunoadsorbent column. The lectin antibody was coupled on to the sepharose and eluted with glycine-HCL pH 2.5. The fraction were immediately neutralised with Disodium before the haemagglutination assay.


M
1
2
A
200–
97–
69–
46–
30–


M
1
2
3
4
kDa
200-
97-
69-
46-
30-
14-
B


M
1
2
3
kDa
200–
97–
69–
46–
30–
c


M
1
2
3
4
5
6
kDa
200–
97–
69–
46–
30–

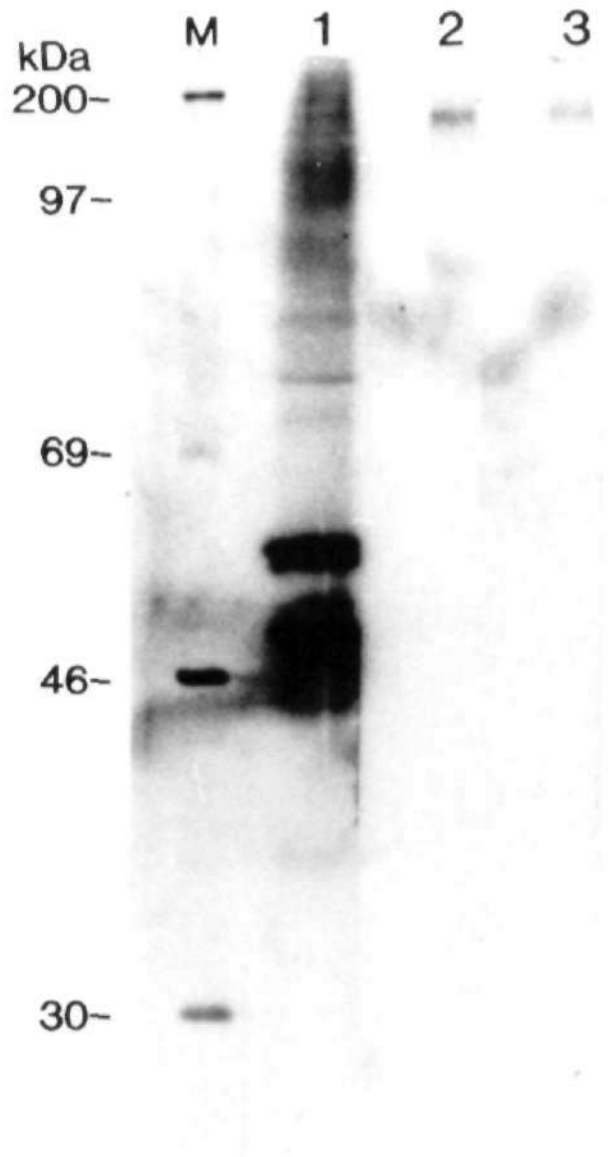

kDa
M
1
2
3
200–
97–
69–
46–
30–

Table 5.16 Role of lectins on parasite development:

Infection rates in *R. appendiculatus* fed *in vitro* upon anti lectin anti body carbohydrates and *in vivo* upon immunised rabbits

Treatment	Infection rates (%)		abundance		intensity		Number	
	F	M	F	M	F	M	F	M
Lectin anti sera *in vitro*	58.0		26.2				30	30
Anti sera free blood *in vitro*	69.9		26.8		77.8		30	30
Immunised rabbits	48.0	59.9	32.8	9.1	147	15	90	90
Clean rabbits	80.0	63.3	94.6	16.2	118	25.5	90	90
Fetuin (glyco protein) *in vitro*	0	0	0	0	0		90	90
D(+)galactose	76.6	73.9	30.1	11.5	36.1	15.6	30	23
Galactosamine	0	0	0	0	0		14	15

Table 5.17 A. *In vitro* feeding of ticks upon carbohydrates /glyco proteins

Sugar	blood appearance	tick attachment	nymph engorgement
Control (*in vivo* cattle)		>50%	(mean=0.0092;n=54)
D (+) glucosamine	dark brown & sticky	>50%	Partially fed (<20%)
Galactosamine	dark brown	<20%	engorgement (<10%); day 6=0.059g; partially fed (2%)
Galactose	bright red	no attachment	
Fetuin	dark brown	>50%	engorgement (90%); wt=0.00396g;n=109.
D(+) mannose	dark red	>40%	partial feding (>50%)
Xylose	very dark red	<10	partial feeding and death
N-acetyl-D-galactosamine	dark red	>50%	deaths (>20%);fungal contamination problem
N-acetyl-D glucosamine	dark red	>60%	partially fed (>60%)
Glucose	dark red	>50%	fungal contamination problem

Table 5.17 B Moulting success of ticks fed upon carbohydrates

Carbohydrate	%moult	%mortality/non moulted
Fetuin	55.10% (n=147)	Dead (2%); non moulted (31.9%)
D(+) glucosamine	7.14 (n=14)	7.1
Glucose	7 (n=17)	42
Xylose	17.64 (n=18)	5.8
N-acetyl-D glucosamine	8.33 (n=12)	66.6
Control	83.7 (n=54)	dead (11.9); non moulted (2.7)

TABLE 5.18: Comparison of lectin binding properties in different ELISA plates based on optical densities (OD λ=414)

Plate make	Mean OD
	λ=414
Dynatech immunol	0.25
Dynatech Lactdyte	0.76
Lactopolynunc	0.01

TABLE 5.19 Optical densities of ELISA with detergent removed with or without blocking

Without blocking		With blocking		Blank	
Normal Balb C	Immunised Balb C (M_4)	Normal Balb C	Immunised Balb C (M_4)	PBS	RPMI
0.214	0.558	0.0199	0.271	0.000	0.01

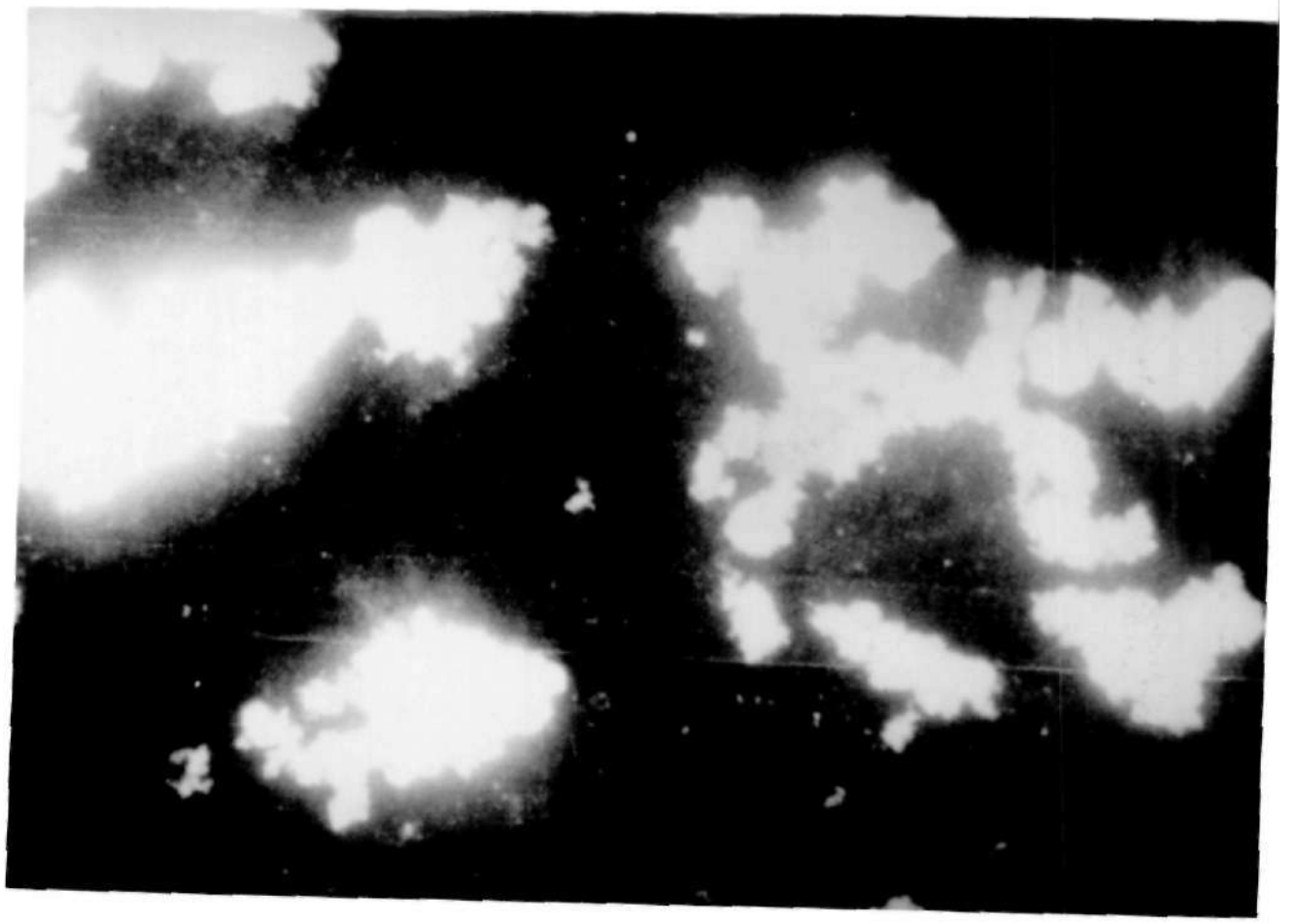


Plate 5.1 Agglutination of purified *T. parva* piroplasms following treatment with tick gut lectin and carboxyfluorescein diacetate dye (phase contrast *x 120*).

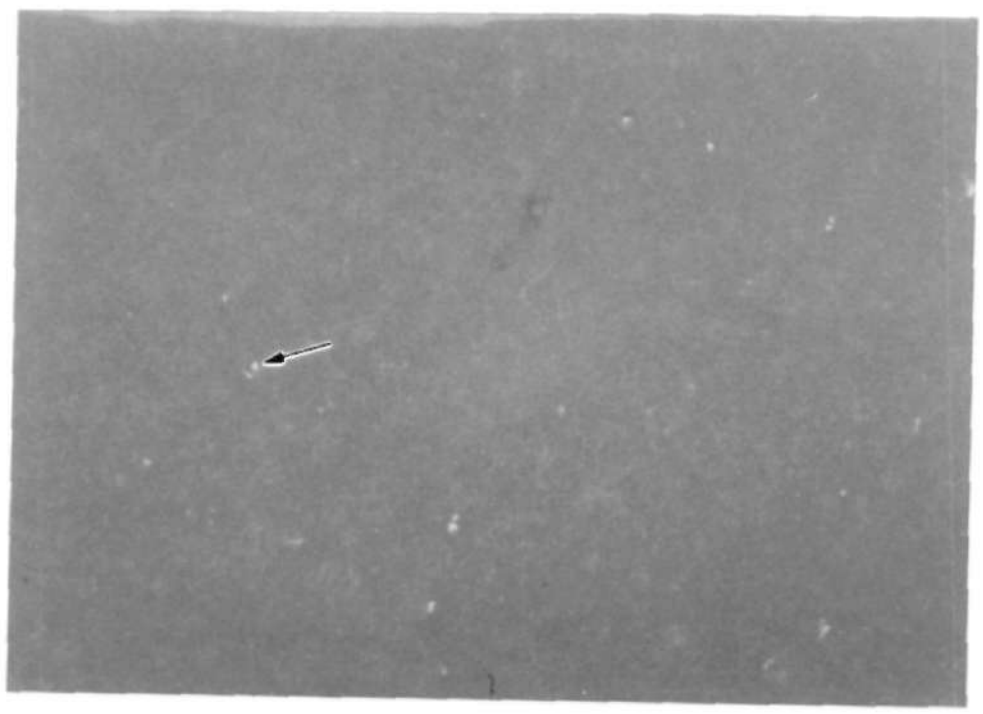


Plate 5.2 Lysis of piroplasms following long incubation period with tick gut lectin (phase contrast *x 120*).

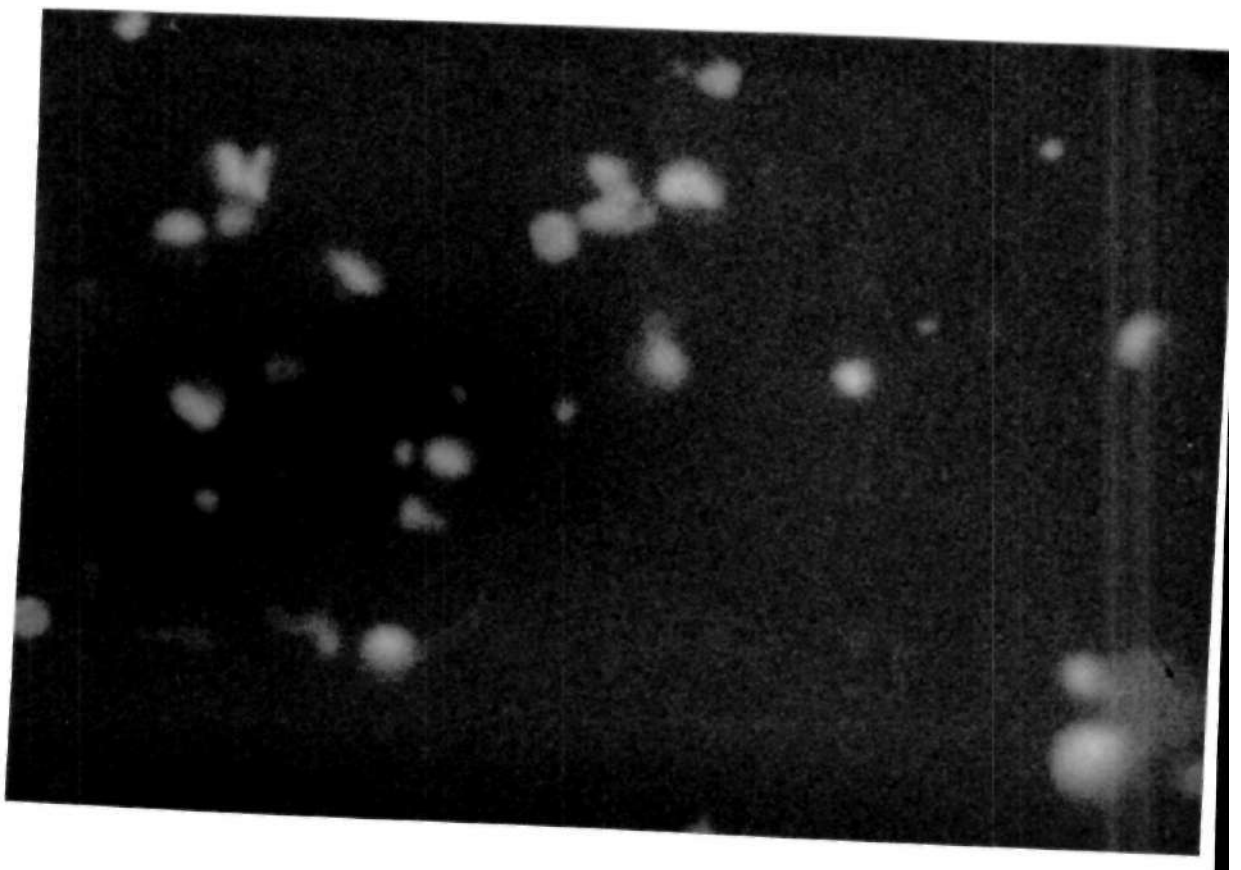


Plate 5.3 Fluorescence of *T. parva* piroplasms with Fluorescein isothiocyanate (FITC) conjugated anti-lectin antibody *(x1200)*.

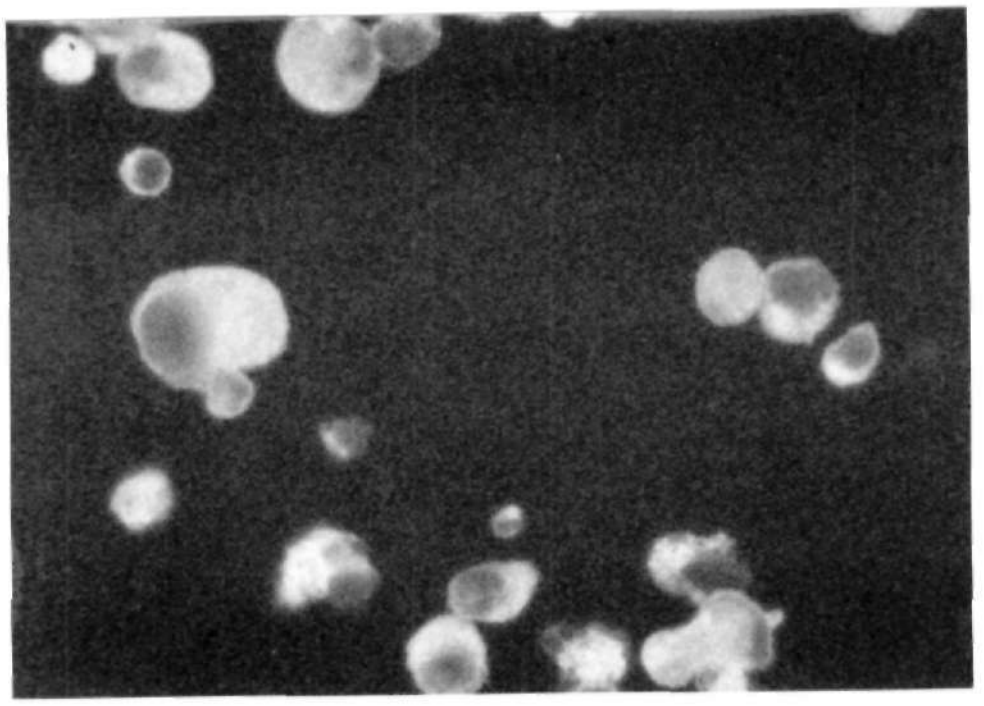


Plate 5.4 Fluorescence of *T. parva* schizonts with Fluorescein isotiocyanate (FITC) conjugated anti-lectin antiboby *(x1200)*

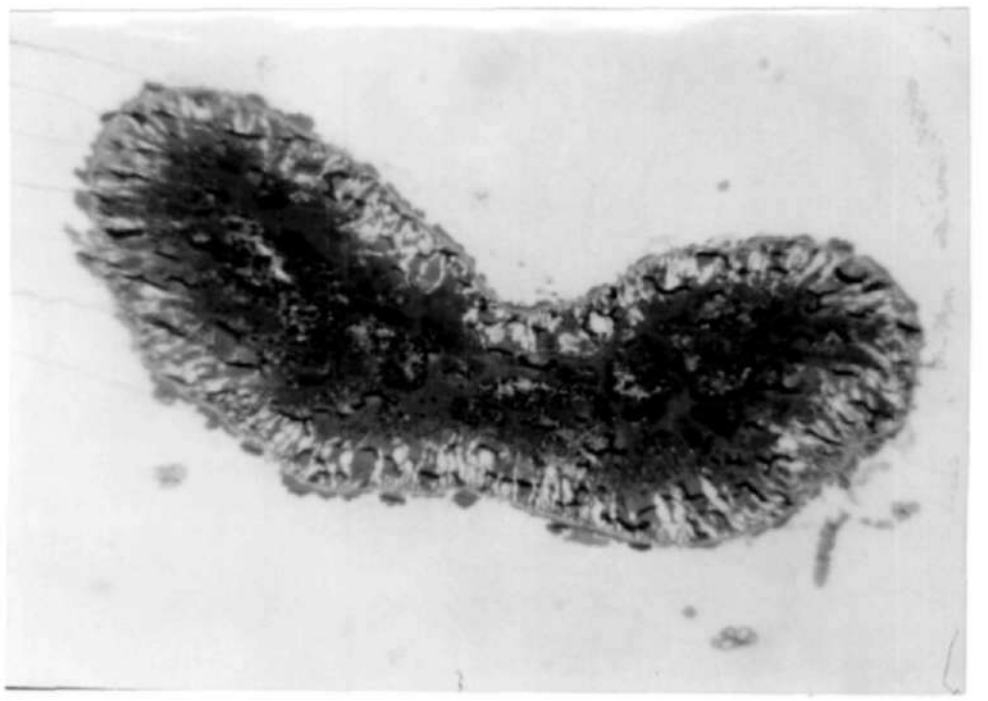


Plate 5.5 Transmission electron micrographs of the gut of *R. appendiculatus* female fed upon lectin immunised rabbits *(x1200)*.

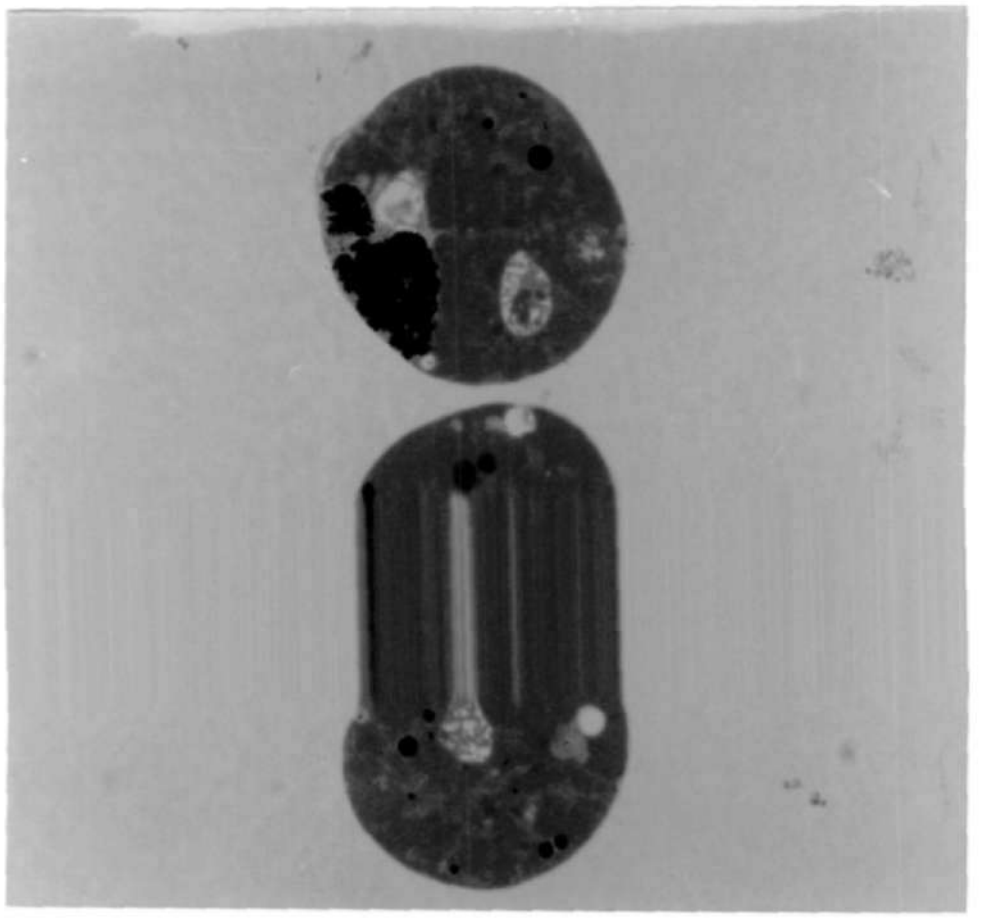


Plate 5.6 Light micrograph illustrating the appearance of salivary glands of type III acini of *R. appendiculatus* fed upon lectin antibody *(x 1200)*.

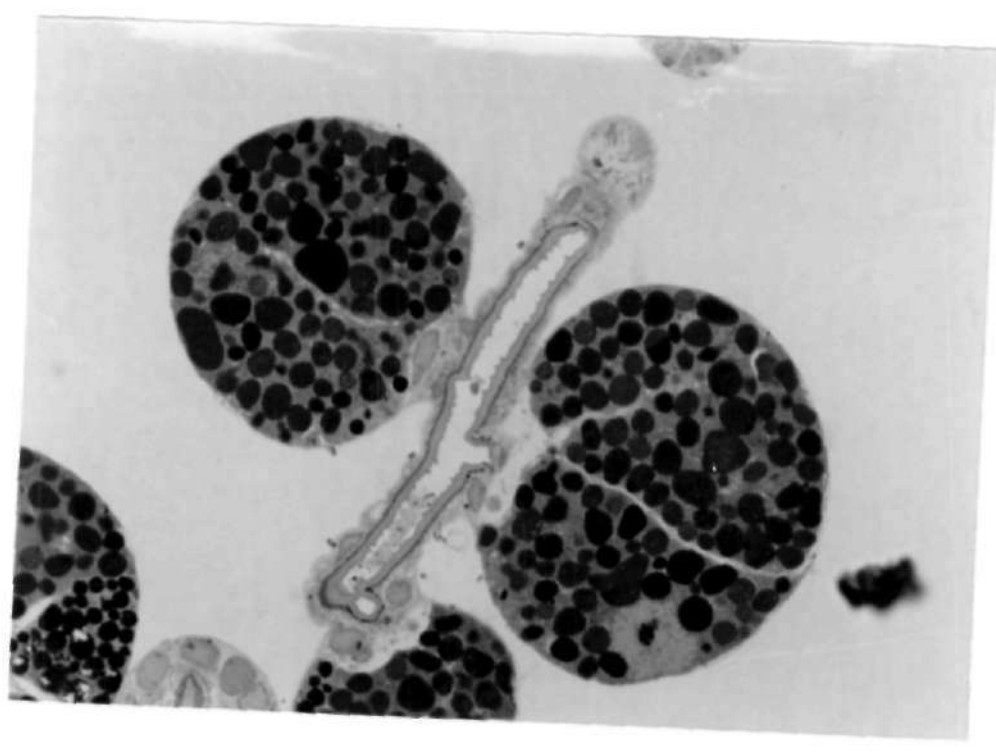


Plate 5.7 Transmission electron micrograph of the salivary gland type III acini from ticks fed upon clean rabbits (control) ***(x 16000.***

CHAPTER SIX

6.0 THE INFLUENCE OF THE BLOOD MEAL ON THE PARASITE *THEILERIA PARVA* INFECTIVITY

6.1 Introduction

Ticks are obligate haematophagous ecto-parasites of several vertebrate hosts including cattle, goats and wildlife. They utilise blood in growth, general metabolism and reproduction. Vitellogenesis and oviposition in females for instance, cannot occur unless females have taken a blood meal (Akov, 1982). Ticks take several millilitres of blood depending on whether they are "hard" or "soft" ticks and on their developmental stage. The blood digestion is slow and intracellular (Arthur, 1965; Balashov, 1972; Akov, 1982; Araman, 1979). Among the *ixodids*, the adults females take up to 10 times their body weight. During feeding, ticks inject the parasite *T. parva* sporozoites into susceptible lymphocytes resulting in the progressive lymphoproliferative disease initiated by the transformation of parasitized mononuclear cells in the lymph nodes. Once inside the lymphocytes, the sporozoites develop into mononucleate (trophozoite) and into multinucleate stage- the macroschizonts (Jura *et al.*, 1983; Preston *et al.*, 1992). In the following experiments the role of the blood meal components in the development of the parasite *T. parva* and the subsequent invasion of the cattle host lymphocytes is described.

6.2 Materials and Methods

In order to study the role of the blood meal and the maturation of the sporozoites in the tick salivary glands, an *in-vitro* system was set up according to Wallade *et al.* (1993). The details of membrane construction and chamber maintenance are given in Chapter 3 of General Materials and Methods.

6.2. 1 Fractionation of blood components for the *in vitro* system

Blood samples from cattle (6 months old Boran) were collected in heparin (1:5000 units) asceptically by jugular venipuncture and fractionated into three components:

- Whole blood
- Plasma
- Cells

Plasma was obtained by centrifugation (1000 x *g;* 4°C; Heraeus sepatech megafuse). After removing the clear plasma, the cells were cleared of the buffy coat layers by sucking with a pasture pipette. A portion of the blood referred to as whole blood was left uncentrifuged. The cells, whole blood as well as the plasma were all warmed at 37°C in a water bath (Kotterman labortechnik, Germany) for 15 minutes before they were transferred into the feeding chambers. Three replicates comprising 2 chambers each were set up for 3 different tick batches. The chambers were maintained at 37°C, 5% carbondioxide and 77 - 80% relative humidity. Such relative humidity was obtained by including a tray of saturated sodium chloride in the incubator (Flow laboratories). Blood samples were changed twice daily and chambers rinsed with normal saline between the changes.

6.2.2 *In vivo* feeding system

As a control, ticks were fed *in vivo* on rabbit ears for 4 days. To ensure homogeneity in the samples, the ticks put on the rabbit were from the same batch as those applied *in vitro*.

6.2.3 Selection of tick batches

T. parva (Muguga stock) and the tick *R. appendiculatus* maintained in the laboratory at ILRI were used in these studies. Ticks were infected as nymphs by feeding them on experimentally infected cattle when the erythrocyte piroplasm counts were high

(at least > 10%). Engorged nymphs were maintained at 23-25 ^{0} C and 80 % relative humidity, and allowed to moult to adult stage.

The adult ticks which were used in the sporozoite infectivity of the peripheral blood lymphocytes (PBLs) were first screened for their infection rates (percent number of infected ticks per number examined) by Fleugen staining of whole salivary glands after ticks had fed for 3 days on rabbits. This was necessary in order to standardise the number of infected acini used in the interaction of the sporozoites with the PBLs. Tick batches that showed at least 80% infection rates were used (Table 6.1). Following the infection rates, the abundance (mean number of infected acini per tick) was calculated. Since the required number of infected acini was 1000 and the abundance was 94.6, the number of ticks dissected were obtained by dividing the required number of infected acini by the abundance (1000/94.6). Therefore, only 11 ticks were required for dissection.

6.2.4 Synchronisation of the feeding *In vitro* and *In vivo* systems

In order to compare the sporozoite maturation and their ability to infect lymphocytes, the two feeding systems, *in vitro* and *in vivo* were synchronised so that fresh sporozoites were obtained the same day. This was carried out by applying at least 60 - 100 adults (both sexes) on the feeding chambers at least 24 -48 hr prior to the application of ticks on rabbit ears (Table 6.2). This time period allowed the *in vitro* fed ticks to attach, since ticks put on rabbits attached faster. In the feeding chambers, either plasma, whole blood or cells were separately added and the ticks were allowed to feed up to 4 days.

6.2.5 Tick dissection for sporozoites

Ticks were removed by forceps, washed in detergent (Divoclean, Diversey, Mombasa) for a few minutes followed by a rinse in distilled water. They were sterilised by rinsing in 70% ethanol and finally in RPM + HEPES media. The salivary glands were dissected out under sterile conditions (reference made to Chapter 3 General materials and methods) under a Laminar flow hood (Gelair, Flow laboratories).

6.2.6 Handling of *T. parva* Sporozoites for *in vitro* infection

Viable sporozoites (Muguga stock) were prepared from the salivary glands of 4-day fed *R. appendiculatus* adults fed on different blood portions or on rabbits described above. They were dissected into special media whose composition is given below to keep the parasites alive:

Sporozoite dissection Media composition

- RPMI 1640 (Flow laboratories) + HEPES
- L-glutamine
- Fetal calf serum (5%)
- Gentamycine (Flow laboratories)
- Penstrep (antibiotics) 5 ml.

The sporozoites from the three groups were separately homogenised in the above media in a 0.1ml homogeniser (Melag) and the volume made up to 0.1ml. All these processes were carried out under a hood. The homogenate was centrifuged at 150 x *g* for 5 min to remove salivary gland debris, and the supernatant used without further purification in the infection of lymphocytes.

6.2.7 Preparation of Bovine peripheral blood lymphocytes (PBLs) for *in vitro* infection

6.2.7.1 Source of the PBLs

Cattle blood was obtained from uninfected Boran cattle (*Bos Indicus)* of 6-9 months old sceptically from the jugular vein with a 16 gauge needle into a syringe containing an equal volume of Alsevers solution and heparin. The cattle were kept on a free East Coast Fever farm, and at the age of 1-2 weeks brought to the ILRI farm where they were maintained. They were screened for antibodies into *T. parva* before the experiment by the indirect innunofluorescent antibody test (IFAT), immunobloting and ELISA and neutralisation of sporozoite infectivity and found to be negative (Goddeeris *et. al.* 1982; Musoke *et.al.* 1984; Katende *et. al.* 1990).

6.2.7.2 Processing of the PBLs

The preparation of PBLs was carried out according to Goddeeris and Morrison (1988) with modifications. All the processing occurred under a horizontal laminar airflow hood. Using a pipette, an aliquot of 30ml of the blood was carefully layered into 20ml Ficoll Hypaque gradients (Pharmacia, Fine chemicals, Sweden) contained in a 50ml polypropylene conical tube and centrifuged at 900 x *g* for 30min in Beckman centrifuge without brakes at room temperature. The centrifugation, without brakes, ensured that the cells settled properly.

The PBLs were aspirated from the interface using a pipette and mixed with an equal volume of Alsevers solution in a 50ml polypropylene conical tube. The cells were pelted by centrifugation at 450 x *g* at room temperature for 10 minutes. The slower speed at this time ensured that the ficoll was removed without letting the platelets into solution. Following this, the PBLs were washed three times in 50 ml Alsevers' solution for resuspension and centrifugation (180 x *g* ; 10 min) to remove platelets. The final wash was carried out in L-15 media. Finally, the pellet was resuspended in L-15 culture media, the composition of which is given below:

Composition of media for Peripheral Blood Lymphocytes

- L-15 media
- FCS (20%; Gibco Laboratories, Paisley, Scotland)
- tryptophosphate broth (10%)
- 20mm HEPES
- L-glutamine (200mM ; Gibco)
- penistrep and gentamycin (50mg/ml)

6.2.7.3 Standardising the lymphocytes (cell counting)

The cells were counted in a hemacytometer chamber (Neubauer, Germany) of depth 0.1mm - 0.0025mm^2, to standardise the number for each experiment. Prior to this, 20µl PBLs were mixed with 180µl Trypanol blue on a vortex (Type 167000). Twenty microlitres (20µl) of this was added to another 180µl Tryanol blue and counted.

6.2.7.4 Divisions of the haemocytometer chamber

The haemocytometer chamber was divided into 9 large squares by triple white lines, and the centre square was divided into 25 squares which, in their turn were subdivided into 16 squares. The large square had each an area of 1 mm^2. A chamber was created by putting a cover slip over the grid until interference patterns appeared ensuring tightness. The depth of the chamber thus formed was 0.1 mm. The total volume over each large square was therefore:
$= 1 \times 1 \times 0.1 = 0.1\ mm^3$ ($0.0001\ cm^3$) or 10^{-4} ml.

At least 50-100 cells were observed over each large square of the chamber. This was carefully carried out with a pasture pipette by touching the edge of the cover slip with the pipette tip and allowing the well to be filled by capillary action without over flooding the chamber. Cell counts were made in the centre square as the four large corner squares. Cells touching the upper and right hand perimeter lines were ignored; while those touching the lower and left hand perimeter lines were counted. Dead cells stained blue and alive ones were colourless, which gave an indication of the cell viability.

The Total cell count was = cell count x 10^4 x dilution factor.

6.2.8 Preparation of feeder cells

6.2.8.1 Obtaining thymocytes from calf foetus

Feeder cells were obtained from a bovine embryo thymus under sterile conditions. The animal was anaesthetised by injecting 1ml Nembutal (Abbot laboratories) per 2kg body weight intraperitoneally. The skin was swabbed with 70% ethanol and access was gained to the foetus using sterile instruments. Using another set of sterile instruments, the thymus was removed and transferred to a sterile petridish and cut into cubes of 2-3 mm. The tissues were washed twice with Earle's balanced salt solution without calcium and magnesium containing a double dose of penicillin/streptomycin solution. This was followed by more Earle's balanced salt solution without calcium and magnesium containing 10% (v/v) trypsin solution (Sigma) and 4% (v/v) penicillin/streptomycin solution (Trypsinising solution). The contents were swirled, allowed to settle and the

6.2.9 *In vitro* infection of lymphocytes with *T. parva* by sporozoites

Infection of lymphocytes by *T. parva* sporozoites was carried out in a 96 well cell culture plate (Costa). A hundred microlitre (100 µl) sporozoites suspension at a concentration of 100 acini/ well, for maximum infection, were mixed with a similar amount of PBLs and incubated at 37 ^{0} C and 5% CO_2 /air mixture for 1 hr to interact freely. The plates were removed from the incubator and 200µl of the mixture was transferred into a 24 well cell culture plate (Costa) containing 500 µl feeder cells in RPMI + 10 % FCS . The plates were set up in duplicates as indicated below and incubated at 37 ^{0} C in 5 % CO_2 /air mixture.

Well	Contents	Code
A	Cell -fed	(C)
B	Plasma	(P)
C	Whole blood	(W)
D	Rabbit	(R)

The sporozoites and the lymphocytes were left to interact for 10 days, with constant microscope examination to check for schizonts. From the start of the neutralisation assay (Day 0) up to day 4, the mixture was left alone without disturbance. On day 5, 1ml of the media was removed and replaced with fresh media (L-15 plus 20% FCS) that also contained 1 % mycostatin and 0.1 % gentamycin. The cells were left undisturbed for day 6 and day 7 and on day 8, cytospins were made.

6.2.10 Checking for sporozoite infectivity among lymphocytes

6.2.10.1 Cytospins

It was necessary to make cytospins of infected lymphocytes because this process, at the right concentration, produces flat cells on a glass slide for easy counting. Briefly, the cytospins were made by spinning the cell suspension on to a glass slide

(Objekttrager, Maltrand, Germany) which was lined with filter paper cards (Shandon; Pittsburgh). Using a 1 ml pipette, the contents of each well were mixed and aliquots from 2 wells were transferred into a glass tube (Röhren Tubes, Sarstedi, Germany) and centrifuged at 150 x *g* for 5 min at 0 ^{0}C to settle the cells without pelting them. Fifty (50µl) of the supernatant was resuspended into a similar amount of PBS and vortexed (Super-mixer). In the cytospin, a volume of 50µl cell suspension was centrifuged at 70 X 10 rpm cytospin (Cytospin 2, Shandon, Southern products Ltd. Cheshire, U.K.) using low acceleration for 6 min. The slides were removed from the cytospin, air dried for 2 min, fixed in methanol (5min) on a rank and then Giemsa stained (30 min). They were then washed in distilled water and examined in microscope SM-Lux Zeiss, Germany) for the presence of intra-lymphocytic schizonts.

6.2.10.3 Schizont scores

All slides were viewed carefully for schizonts. Both the number of cells in the field as well as the infected ones were counted and the percentage infected calculated out of a total of 400 cells.

6.3.0 Results

6.3.1 Attachment and feeding of ticks fed *In vitro* on different blood meal components

6.3.1.1 Plasma feeding

In vitro feeding chambers containing plasma were characterised by frequent leakages and self sealing. They also showed slow and poor tick attachment and over 20 % adult mortality. The Baudruche membrane appeared bleached.

6.3.1.2 Whole blood meal feeding

The adult ticks attached (70-80%) within 24 -48 hr to the chambers and fed well.

6.3.1.3 Cell- feeding

There was plenty (about 90%) of attachment of ticks within 24 hr. Ticks fed well and took on a characteristic reddish colour unique from all the other adults. The cells

produced the best attachment visualised as "cones" on the membrane in the first 24 -48 hr .

6.3.1.4. *In vivo* (Rabbit) feeding

These ticks had very good attachment (about 90-100 %) and fed well . These served as the control batch and were applied 48 hr. after the *in vitro* fed ticks batches.

6.3.2 Cell appearance

Lymphocytes (PBLs) that were not infected by the *T. parva* sporozoites were oval shaped (Fig 6.1). Those which were parasitised had the nucleus and cytoplasma expanded and schizonts were visible as pink dots in a blue cytoplasm (Fig 6.2). The sporozoites were viable as predicted from their batch (Table 6.1; Fig 6.3).

6.3.3 Influence of blood meal components on sporozoite infectivity

The sporozoite infectivity was highest among ticks fed on rabbits (67.9.%) followed by whole cattle blood given *in vitro* (20 %), cells (1%) and almost none among the plasma fed ticks (Table 6.3).

6.4 Discussion

An important stage in the transmission of theileriosis is the entry of sporozoites injected by ticks while feeding, into the bovine lymphocytes. The sporozoites, however, must be well developed from the kinete that invades the type III acini "e" cells into sporozoites. Once the tick feeds, it takes in a lot of piroplasms (about 1 million;Young pers. comm) whose development depends upon the several midgut factors the parasite encounters during its development such as lectins and enzymes (Maudlin, 1991). The number of parasites that develop from the initial number of piroplasms engulfed, depends upon the type and quantity of these factors. Since the midgut factors are blood meal induced, the resultant infections would depend upon the type of host blood meal at the time of infection as was the case among tsetse flies (Moolo, 1981; Mihok *et al.*, 1993). Using the *in vitro* system and tissue culture techniques to study the various fractions of the blood meal and subsequent infectivity, some insight into the blood meal

components influencing the parasite development have been established. An assay has also been set up in which ticks can be fed upon different components and their ability to transmit the parasite assessed *in vitro*.

Firstly, whole blood from cattle supplied *in vitro* gave the highest level of sporozoite infectivity followed by cells, whereas the plasma gave almost no parasite infectivity. The presence of red blood cells seem to be necessary in *T. parva* maturation. Earlier studies in the tsetse flies showed that *T. brucei* transformation was only supported by midgut homogenates derived from tsetse flies that had been fed on whole blood (Imbuga *et al.*, 1992) suggesting that blood played a crucial role in parasite development. On the other hand, Gingrich *et al.* (1982) reported high infections by trypanosomes in the midgut of tsetse that had been fed on serum-free meals. Furthermore, Maudlin *et al.* (1984) demonstrated that removal of certain components from pig or cow blood greatly reduced the maturation rate of *T. congolense* in *Glossina morsitans*, fed on such diets. Similarly, pig red cells produced a greater proportion of mature infections (13%) than cow cell diet (3%). They concluded that erythrocytes had an effect on trypanosome maturation which was independent of, but not as profound as serum-contained factors. It is therefore possible that the plasma contains a factor that inhibits parasite maturation, where as the cells possess the essential factors. The complete removal of serum from cow red cells resulted in very high infections rates of *T.congolense* midgut infections (>90%). However low levels of maturation (3-6%) were observed (Maudlin *et al.*, 1984). Such observations could imply that serum has inhibitory factors found in the various bloods. Above all, ticks that were fed upon plasma did not attach effectively and detached frequently. This may have been as a result of lack of a vital element in the plasma necessary for attachment, which was lacking among the whole meal fed adults, that also attached properly. The cell fed ticks on the other hand, showed great number of attachment implying that it is perhaps the cells that attract the ticks to attach. However, the cells alone, despite attracting large numbers of ticks to attach, could not produce a number large enough of mature viable sporozoites (only 1% compared to 20% from whole blood meal) to infect the lymphocytes that would eventually transform into schizonts detected in this experiment.

Studies on the influence of blood meals on parasite development in other invertebrates have shown a link between the type of blood meal and the development of the parasite (Ngu *et al.*, 1996). Among tsetse flies, approximately 50% of the blood stream form trypanosomes transformed into either transition or procyclic (midgut) forms after 10 hr of incubation at 27 ^{0}C in tsetse flies fed upon rat and goat blood (Ngu *et al.*, 1996). On the other hand, buffalo blood supported a slower transformation rate with the same percentage transforming after 18 hr. Lysis of the trypanosomes from flies fed on whole eland blood was observed. Furthermore, transformation of trypanosomes occurred only in rat blood fractions that contained cells. Plasma or serum alone or a mixture of the two did not support transformation. These results are in agreement with those in the present study where plasma prevented maturation of *T. parva* sporozoites and cells attracted tick feeding and supported parasite maturation into infective stages.

The mechanism by which host blood modulates *T. parva* infections is poorly understood. Similarly, the reason why plasma fed ticks showed no infectivity is not clear. This is, however related to the blood meal. It is for example known that blood from buffalo, eland and water buck have been shown to have trypanolytic molecules in the plasma fraction that have a direct contribution to the low infection prevalence in tsetse fed upon such samples (Mulla & Richman, 1988; Reduth *et al.*, 1992;). This implies that within the blood meal, certain factors inhibit or "neutralise" parasite infectivity. In studies involving the identification of cell surface molecules involved in the process of parasite invasion, monoclonal antibodies raised against the lymphocyte surface molecules showed almost total inhibition (>90%) of invasion by sporozoites. These MoAbs appeared to inhibit sporozoite entry by preventing initial recognition/binding event, which could be interpreted as meaning that class I molecules were involved in the process of sporozoite invasion. Other reasons such as coating the surface membrane with antibodies/ and or a general disorganisation of other membrane proteins could have been possible (Shaw *et al.*, 1991).

Neutralisation of sporozoites infectivity *in vitro* by antibodies has been, for instance, observed (Musoke *et al.*, 1992; Knight *et al.*, 1996) and has been attributed to inhibition of sporozoite recognition of bovine leukocytes, inhibition of the entry

mechanism or a combination of both (Knight *et al.*, 1996). The mechanism by which plasma fed ticks had low infectivity is not clear but could be the result of the inhibition of the sporozoite recognition of the leukocytes as was the case with the antibodies (Musoke *et al.*, 1992) .

The entry mechanism of the sporozoite into lymphocytes has not been clearly elucidated, although there is evidence that *T. parva* sporozoites entry is mediated by binding to MHC class I molecules on the leukocytes surface (Morrison, 1986; Shaw *et al.*, 1991) and endocytosis depends upon a ligand-receptor interaction of the parasite and the host cell membranes via a "zippering" process. (Fawcett *et al.*, 1982b). The sporozoite enters a subpopulation of bovine T and B lymphocytes (Morrison *et al.*, 1989) where it differentiates into the multinucleate schizont stage and in the process induces and subsequently maintains host cell blastogenesis leading to a rapid clonal expansion of parasitised cells. The mechanism that limited the sporozoites entry into the lymphocytes especially in those derived from plasma fed ticks as evidenced by lack of schizont parasitosis therefore needs further study. However, it is likely that vital ingredients in the plasma were lacking that are important in sporozoite maturation process and establishment. This could have prevented the sporozoite in the vector to mature to a crucial stage necessary for penetrating the lymphocytes or the receptor-ligand interaction between the parasite and the lymphocytes were inhibited. More explanations on neutralization of infection by antibodies are given by Musoke *et al.* (1984;1992) who reported on the P 67 sub-unit molecule whereby several theories were put forward. First of all, the NS1-p67- specific antibodies probably enhanced phagocytosis of sporozoites by opsonization; this was because of the development of bovine IgG_1 and IgG_2 generated during the immunisation which induced phagocytosis by homologous neutrophils and macrophages. On the other hand, the antibody may have protected through antibody-dependent cell-mediated cytotoxicity as evidenced in its role of trypanosome clearance in cattle (Ferrante and Allison, 1983) .

Theileria entry into susceptible lymphocytes and development has been reported to be inhibited by several factors amongst which are cytokines were observed by

Preston *et al.,* (1992) among *T. parva* and *T.annulata* trophozoite infected cells. On the other hand, serum lipids were found to be responsible for the elimination of midgut infections or preventing these infections from becoming established. The serum contained essential substrates for trypanosome maturation or was possibly affecting the flies'metabolism in such a way to induce trypanosome maturation (Maudlin *et al.,* 1984). Other explanations involving the enzyme theory, could be held for the ticks in that the ticks fed on plasma alone, probably produced proteinases in response to the plasma and this were responsible for the killing of the parasites. The only problem with this explanation could be the fact that the parasites in adults are either in the kinete stage or in early sporozoite stages in the haemolymph and salivary glands, respectively, and therefore would be unlikely to be harmed by proteases produced in the midgut.

Finally, the role of complement in elimination of sporozoites *in vivo* has to be considered. Related studies on the African trypanosomes (Ferrante and Allison, 1983) showed that trypanosomes that lacked a surface coat (procyclicks) were lysed by human serum. The trypanolytic activity of serum was removed by heating at 56 ^{0} C for 30 min or by the presence of EDTA, which chelates both Mg^{2+} and Ca^{2+}. Since the classical pathway compliment activation requires the presence of both Ca^{2+} and Mg^{2+} (Levine et al., 1953). The lysis of these parasites must have been by the alternative pathway. This route is activated by a particulate fraction of *T.b.brucei*, which probably contained plasma membrane components (Musoke and Barbet, 1977). The sporozoites used in this experiment were viable, intact as evidenced by their parasitosis of the lymphocytes. They could not have been activated the complement through the alternative pathway. Intact parasites do not activate complement in the absence of antibody against variant-specific glycoproteins, presumably potential complement-activating sites are not accessible on the surface of the parasite (Musoke and Barbet, 1977; Ferrate and Allison, 1983). It seems that a combination of several factors may have been responsible. The cells together with the plasma constitute the favourable environment in which sporozoites mature and are able to infect. If they do not mature to a certain critical stage, then they lack the necessary receptors to bind and penetrate into the lymphocytes.

In this study, the *invivo* system has been demonstrated to so far supersede the *in vitro* system by almost a factor of two in sporozoite infectivity, as is evident in the high parasitosis among the rabbit fed compared to the whole blood fed *in vitro*. This is the first time that sporozoites have been obtained from ticks *in vitro* and used to infect lymphocytes *in vitro*. It is an important new avenue opened for the study of pathogen transmission, and from the stand point of evaluating potential immunogens in controlling the disease by vaccination against the parasite.

Table 6.1 Details of *T. parva* sporozoites used in infecting cattles

Animal number	Infection rate	Abundance	Intensity
BN334	80%	94.6	118.25
Total acini	2838		

Total acini used = 1000 acini from 11 ticks

Ticks per treatment = Total acini required/ Abundance = 10.57

Table 6.2 Synchronisation of the *In vivo* and *In vitro* feeding systems for *R. appendiculatus*

Day	Activity	
	Invitro	***Invivo***
0	Ticks applied on feeding chambers plasma, cells and whole blood from cattle added	-
1	Ticks attachment and feeding	Ticks applied on rabbit ears
2-3	Feeding of ticks on membrane	Feeding of ticks on rabbit ears
4	Removal of ticks from membrane	Removal of ticks from rabbit ears.

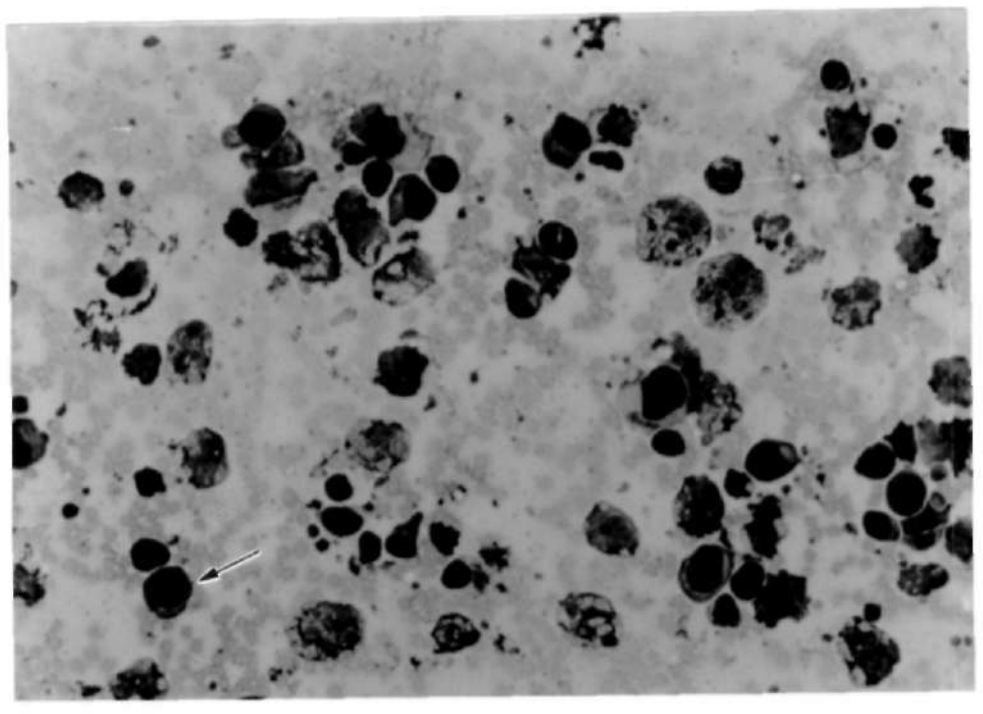


Plate 6.1 Light microscope photograph of uninfected bovine lymphocytes. The cells were derived from the cell culture plates containing sporozoites from *R. appendiculatus* fed *in vitro* on plasma (*x 1600*).

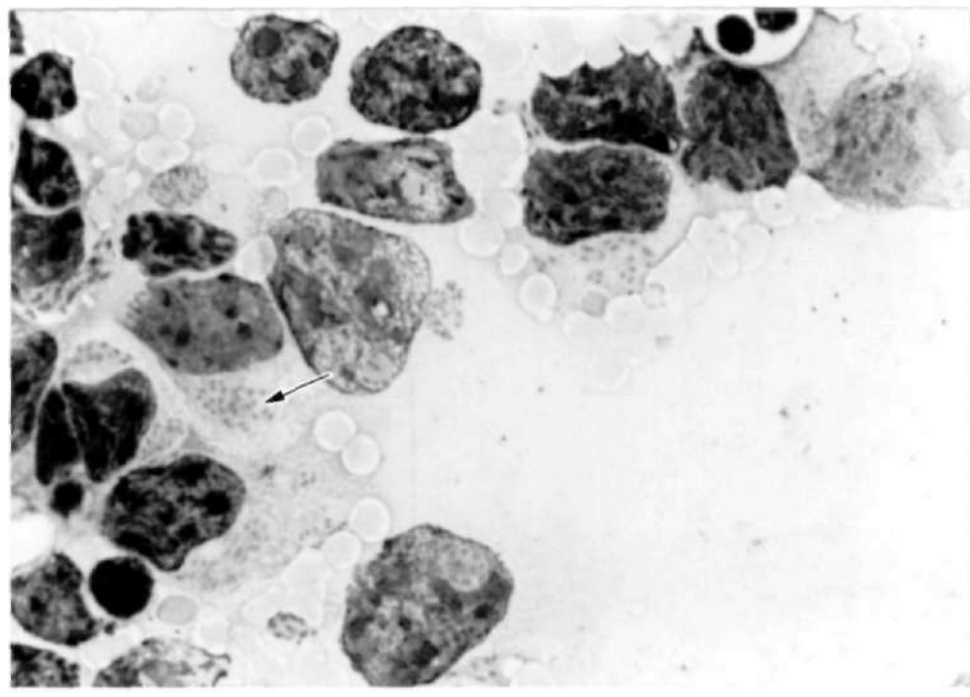


Plate 6.2 ***Theilera parva*** **schizonts obtained from** ***in vitro*** **infection of lymphocytes with sporozoites deriverd from** ***R. appendiculatus*** **fed** ***in vitro*** **on whole cattle blood** ***(x1600).***

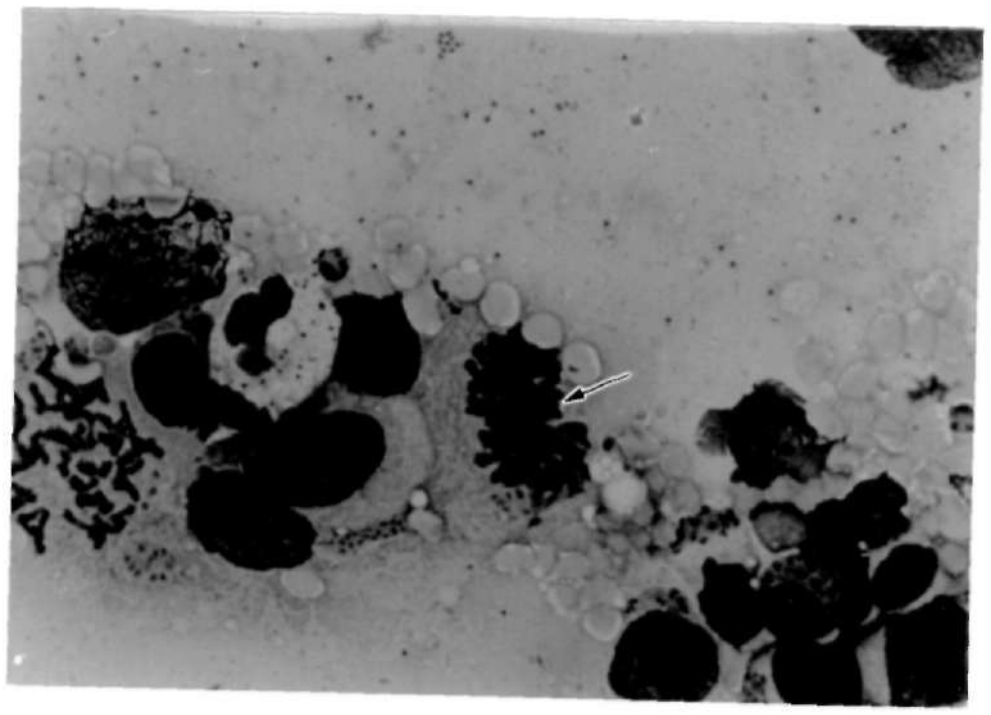


Plate 6.3 ***Theileria parva*** **dividing schizonts obtained from** ***in vitro*** **infection of lymphocytes with sporozoites derived** ***R. appendiculatus*** **fed** ***in vitro*** **on cells** ***(x1600).***

CHAPTER SEVEN

7.0 THE INFLUENCE OF HORMONES ON *RHIPICEPHALUS APPENDICULATUS* AND THE TRANSMISSION OF *THEILERIA PARVA*

7.1 Introduction

Ticks have been observed to synchronise their moulting process with the formation and migration of kinetes from the haemolymph to the newly moulted instar salivary glands (Young *et al.*, 1980). In looking for ways to block or influence *T. parva* transmission, this study was set up to determine whether the moulting time of the tick could be altered and if so what influence this would have on the maturation (kinete formation and invasion of the salivary glands) and the resultant infection rates. The study was carried out in stages, first of all, it was necessary to investigate the influence of hormones on the feeding behaviour of ticks in terms of engorgement weights, which would influence moulting. This was followed by an investigation into the moulting success and the subsequent infections in the salivary glands of the adult ticks. Work to determine whether the moulting time was related to the size of the tick was also carried out. For this reason, nymphs were divided into three categories (small, medium and big) and their moulting periods studied.

In the experiments below, determination of the best solvents for the hormones was carried out. In addition, the best mode of hormone application was investigated amongst injection, topical application and *in vitro* feeding.

Successful *in vitro* feeding of ixodid ticks and in particular *R. appendiculatus is* a relatively new method (Waladde *et al.*, 1996) which is currently facilitating the research into factors that influence transmission and development of pathogens in ticks. Such experiments were being hampered by the lack of a suitable artificial pathway into the vector/parasite relationships. The following, therefore, is a pioneer *in vitro* study into the role of hormones on the moulting and the effect on development of a protozoan parasite.

7.2 Materials and methods

7.2.1 Hormones

The following hormones were investigated:

- Juvenile hormone III
- Ecdysone
- 20-Hydroxy ecdysone

Given in Table 7.1 gives a brief description of the above hormones.

7.2.2 Solvents

Preliminary experiments were carried out to determine the best solvent which dissolved the hormone and caused minimum mortality to the ticks.

7.2.3 Methods of hormone application

Three methods of hormone application to the ticks were used:

- Topical application
- Injection
- *In vitro* feeding

7.2.3.1 Topical application

Nymphs and adults were topically treated with 1µl of a 1-8ng/µl concentration of hormone dissolved in ethanol as found to be the best solvent in preliminary studies, on the dorsal cephalothorax. Adults were hand held without prior anaesthesia and kept at 20°C following topical treatment.

Larvae were treated *in masse* by putting them in a glass tube and dropping the hormones from above. The tube was gently shaken to ensure that all were in contact with the hormone. Three hundred nymphs were left to serve as the untreated controls. The mortality rates at 24 hr. and 48 hr were calculated and $_{LD}50$ assessed. Their mortality, moulting time, parasite development and infection rates were monitored.

7.2.3.2 Injection method

The engorged nymphs were injected with hormones 24 hrs post drop from cattle. Prior to the treatment, nymphs were weighed immediately they dropped. For injection, ticks were tapped on to a double sided tape and injected through the abdominal tip with hormone through a 10µl Hamilton Precision syringe (Bonaduz, Switzerland). They were maintained at 20°C in glass tubes and mortality rates assessed 24 and 48 hr later.

7.2.4 Susceptibility studies

7.2.4.1 20-Hydroxy ecdyson

Following treatment with 20-hydroxy ecdyson and solvents, the ticks were put in glass tubes and maintained at 20° C. Their mortality rates, were assessed 24 and 48 hr later and subjected to probit analysis to evaluate the immediate effects of the treatments.

7.2.4.2 Juvenile hormone III

Ticks were divided into three categories according to their engorgement weight. The small ones had a mean weight of 2.10± 1.85mg (n= 70); medium 3.68± 2.33 mg and big ones 6.26± 3.24mg (n= 91). They were topically treated with Juvenile hormone and their mortality rates or the number moribund at 24 and 48 hr recorded.

7.2.5.0 *In vitro* feeding of hormones to ticks

7.2.5.1 Preparation of feeding chambers

The feeding system was constructed according to Waladde *et al.*, 1996 as described in chapter three.

7.2.5.2 Experiment 1:Hormone-free blood

Blood was collected asceptically from the jugular vein of cattle in heparin (5000 unit per ml). It was then transferred into the chambers and the blood for changing at 1700hrs kept at 4^{0} C. When required, it was warmed at 37^{0} C before being introduced into the chambers. Warming the blood before giving it to ticks did not only simulate the natural host temperature, but prevented the already attached ticks from detaching. At least three chambers of control untreated blood were set up in each treatment. On dropping, the ticks were harvested through the hole at the side of the chamber. The weights were then taken and the moulting time observed.

7.2.5.3 Experiment 2: *In vitro* feeding of hormones to nymphs

The hormones were added to the blood in the chambers at a concentration of 5 µg/ml and 10 µg/ml. Over 200 nymphs were put per chamber. On day one, normal blood was put in the chamber. From day 2-7, hormones were added to the blood and fed on until the ticks dropped. The ticks were weighed to establish the engorgement weight and the effect of the hormone on their feeding. They were maintained at 20°C; RH 80% until they moulted into adults. The moulting success in relation to the treatment was assessed.

7.2.5.4 Experiment 3: Infection rates of hormone fed ticks

Cattle were infected with *T. parva* by injection of 1 ml of 1/20 dilution of stabilate 3087 subcutaneously in front of the right ear. The development of the infection was monitored by daily temperature and needle biopsies from the parotid lymph node,

which were smeared, stained with Giemsa stain and examined for Schizont presence. On day 10 following inoculation, needle biopsies taken from the contralateral prescapular lymph node and stained with Giemsa, were examined for the presence of piroplasms. The parastaemia of piroplasms was attained by counting the number of infected erythrocytes per 1000. About day 13-14, the cattle were ready for donating parasitised blood for the experiment. The blood was collected from the jugular vein in heparin and introduced into the chambers as stated above. Hormones were added to the blood thereafter. Clean nymphs were attached and allowed to engorge. The nymphs were kept until they moulted into adults. These adults were fed for 4 days upon rabbits for the sporozoites to mature and then the salivary glands were dissected, smeared on to glass slides, Giemsa stained and examined for infection rates.

7.3 Statistical methods

The engorgement weights were subjected to correlation and General Linear Modules Procesure (GLM) of the SAS system, a software system for data analysis. The GLM uses a method of least squares to fit general linear models in which regression, analysis of variance and covariance was carried out. In the analysis of variance (ANOVA) a continuous response variable, known as a dependant variable, is measured under the experimental condition identified by classification variables, known as the independent variables. The variation in the response is explained as being due to effects in the classification with random error accounting for the remaining variation. The ANOVA procedure of the SAS system utilised was designed and used to handle balanced data (with equal number of observations for every combination of the classification factors) while the GLM procedure was used to analyse balanced and unbalanced data. The

Duncans' multiple range tests or the Student-Newman-Kleuls test (SNK) rankings were also performed using the SAS system to demonstrate the significance of the differences among experimental group means.

The effects of hormones on the engorgement weights were tested with the analysis of variance. The weights of the nymphs were correlated with the dose of the hormones used as well as the length of time the nymphs spent on the membrane feeding. Correlation measures the closeness of a linear relationship between two variables. If, for example, one variable x can be expressed exactly as a linear function of another variable y, then the correlation is -1 or 1, depending on whether the two variables are directly related or inversely related. If a correlation of zero between two variables is obtained, it means that there is no linear predictive ability for the other. When the values are normally distributed, then a correlation of zero means that the variables are independent of one another.

The mortality rates were analysed using the probit statistical package under the SAS system. The moulting rates of the nymphs exposed to various hormones either by topical application or incorporation in the *in vitro* blood meals, were plotted as cumulative percentages against the days post- blood meal for comparison.

7.4 Results

7.4.1.1 Juvenile hormone III

Juvenile hormone III topically applied to engorged *R. appendiculatus* nymphs 24- hr post drop at doses ranging between 1-4 μg/ tick in 95% ethanol showed mean mortality

rates between 11.58 % to 52.28 % which increased to 71.15%, 48 hr post treatment (Table 7.2A). Probit analysis of the treatment on a small nymphs produced non significant mortality (r= -1.19; p>0.08; Table 7.2B). Similarly, the medium and big sized effects were non significant (r=1.32; p>0.37) and r=0.79; X^2=0.18; p>0.67, Table 7.2B). Overall, the pooled nymphal weights demonstrated a non significant effect (r= -0.23; X^2 = 0.90; p>0.34) which meant that the size of the nymphs did not influence mortality. The estimated lethal doses that would cause a mortality of 50 % of the population (LD_{50}) were 3.06, 2.48 and 0.44 µg/µl for the small, medium and big nymphs respectively, with the mean for the pooled data being 1.76 µg/µl.

7.4.1.2 Topical application of 20-Hydroxy Ecdyson to engorged *R. appendiculatus* nymphs

Treatment of 20-hydroxy ecdysone (1µl) per tick of doses ranging between 1µg/µl to 10µg/µl on to fed nymphs led to no mortality 24 hr later. However, the ticks were moribund and the susceptibility studies demonstrated in Fig 7.1 showed a significant effect (r=2.23;p<0.0001;Table 7.2B). The LD_{50} was 0.53µg/µl. The ticks, although moribund 24 hr following treatment, recovered from the hormone treatment unlike those treated with Juvenile hormone.

7.4.1.3 Hormone injected ticks

Injection of hormones with either Juvenile hormone III or 20-Hydroxy Ecdysone or the solvent ethanol led to erratic mortalities as high as 95% 24hr in all tick batches (Fig 7.2). This method of hormone application was, therefore, discouraged and no further investigations were performed.

7.5.0 Effects of *invitro* fed hormones to ticks

7.5.1 Influence of 20-hydroxy ecdyson on engorgement weights

Analysis of the data on blood meals containing exogenous 20 hydroxy ecdyson at doses ranging from 0-10μg/ml in cattle blood with GLM and Duncans' multiple range test showed significant differences between the ticks fed upon high doses of hormone and those fed with low doses. The nymphs that had been fed upon whole cattle blood *in vitro* had a mean engorgement weight of 7.59 mg while those that were fed upon 20 hydroxy ecdyson at 5 μg/ml showed a mean weight of 4.68 mg. Doubling the amount of hormone to 10 μg/ml produced nymphs which weighed 3.49 mg (Table7.3A&B) which were significantly smaller ($P<0.05$) than control nymphs.

From the GLM procedure analysis of the weights of the 921 nymphs, there were significant effects due to duration of feeding (days) as well as treatments ($F = 604.82$; $P< 0.001$) and days ($F= 14.18$;$P<0.0001$; Table 7.3A) respectively. The nymphs that fed upon hormone- free blood weighed significantly higher (7.59mg; n = 221) compared to those fed upon 5 μg/ml and 10 hormones (4.68 mg; n=412; 3.49mg; n=296; $p<0.0001$) as shown by the Duncans'groupings (Table 7.3B).

7.5.2 Feeding duration

A comparison of the feeding duration of the ticks (Table 7.4) demonstrated that nymphs attached on membranes on day zero (0) started dropping on day 5 and completed dropping on day 8. Those on cattle (fed *in vivo*) attached on day 13 post

infection and started to drop on day 16 right through to day 21 (Table 7.4). The nymphs fed upon the hormones, however, dropped on day 5, 6 and 7 (Table 7.4) with the smallest weights on day 7.

There was no significant differences in weights ($p > 0.001$) between the nymphs that dropped on day 5 and 6 (mean weight= 5.50 and 5.74 mg, respectively, Table 7.5). However, those dropping on day 7 were significantly smaller (mean weight = 4.49 mg $p < 0.001$; Table 7.5). The interaction between the hormone treatment and the days that the nymphs fed ($F = 161.73$; $P<0.0001$) was also significant ($P<0.0001$). It appears that the longer the nymphs remained on the membrane, the less they weighed. This was reflected by the negative correlation between the weights and the days ($r= -0.26$; $n= 929$) which was significant ($p<0.001$; Table 7.5).

The overall mean weight for the days 5-7 was 4.99 mg. Considering each treatment, there was a highly significant correlation between the weights of the control nymphs fed upon clean cattle blood ($r = -0.33$; $p<0.0001 = 221$; Table 7.6) with a mean eight of 7.59 $\pm$ 1.76 mg. Among the nymphs fed with 5 μg/ml ecdysteron in the blood, there was a mean engorgement weight of 4.67 $\pm$ 1.37mg and a significant negative correlation between the days and weights ($r = -0.12$; $P<0.0001$; $N = 412$). However, the nymphs that fed *in vitro* upon blood containing 10ug/ml ecdysterone showed a low mean of engorgement weight of 3.48 $\pm$ 0.96mg and no significant correlation between the day spent on the membrane and the resultant of the nymphs ($r = 0.02$; $P>0.73$; $N = 296$; Table 7.6).

In order to determine whether the engorgement weights depended upon how many days it took the nymphs to drop, relative frequencies were computed for each of the weights by assigning categories. If the weight was less that 3 mg then it was assigned category 1; between 3 and 6 mg category 2; between 6 and 9 mg category 3; between 9 and 12mg and category 4. This was carried out for each of the days 5, 6 and 7 on which the nymphs dropped (Table 7.7A&B). Chi-square analysis was highly significant (X^2=80.78; P<0.001; N=929; Table 7.7A) which confirmed that weight was dependant on days taken to drop. There was, however, an exception in the nymphs fed upon a high dose (10 µg/ml), where there was no significant difference observed (P>0.0001).

The weights of the nymphs depended upon how many days the ticks took to drop among the controls and those fed on 5 µg/ml (X^2 = 29.42; P<0.0001;Table 7.7B). Those fed upon 10µg/ml there was no significant effect (X^2 = 3.61 P>0.46; Table 7.7C).

The following hypotheses were tested:

- H_0: Weight is independent on days taken to drop from the membrane
- H_1: Weight is dependent upon the days taken to drop from the membrane.

The alternative was adopted in all cases except in the 10µg/ml treatment. The engorgement weights of *R. appendiculatus* nymphs were dependent on how many days it took to drop from the *in vitro* chambers.

7.5.3 Moulting Success of *R. appendiculatus* nymphs

Following treatment of either topical application, injection or *in vitro* feeding, the *R. appendiculatus* nymphs maintained at constant temperature of 20 ^{0} C and RH of 80-85

% until moulted were recorded and death as well as moulting success evaluated. Analysis of the data using the GLM procedure on arcsine transformed data revealed that the day on which the nymphs dropped from the membrane had no significant effect over the moulting success (Table 7.8) for the 2 treatments 5 and 10 μg/ml).

Taking into account the days on which the nymphs dropped analysis of the moulting success of the nymphs treated with low and high doses of hormones produced 49.8% for those that dropped on day 5 and 35.89% on day 6 for 10 μg/ml treatment (Table 7.8).. Only 13.48% moulted considering nymphs treated with 5 μg/ml and dropping on the fifth day. Relatively more ticks that dropped on day 7 moulted (45.93%; Table 7.8A), compared to those dropping on other days 34.24 and 29.33 % (day 5 and 6, respectively; Table 7.8B).

A large number of ticks fed upon high hormone levels (10μg/ml) moulted significantly more ($p<0.05$) than those fed upon low levels of hormones (5μg/ml). Over fifty nine percent (59.76 %) ticks that dropped on day 7 moulted among those fed upon a high dose, while 45.93% of the ticks that dropped of day 7 from the low dose group moulted (Table 7.9B). Among those that received 5 μg/ml only 27.53% moulted compared to 49.47% of those that fed upon 10μg/ml 20 hydroxy ecdyson ($p<0.05$;Fig.7.9C). On transforming the data using arcsine, cumulative moulting showed significant effects due to treatments ($F=7.37$; $p<0.005$;Table 7.10).

Ticks fed on high doses of hormones moulted in relatively higher numbers than those fed with lower doses *in vitro*. From the analyses above, there was a significant effects

resulting from both the hormone treatments (F=5.59; p<0.03 and days dropping (F=7.06;p<0.002; Table 7.10) as well as the interaction (F=8.38;p<0.0016).

7.5.4 Effect of death

There was no significant influence of deaths occurring in nymphs fed *in vitro* upon hormones (F=1.15;p>0.38;Table 7.11 A) as confirmed by the interaction between treatment and days (F=1.55;p>0.24;Table 7.11B).

7.5.5 Moulting time

The hormones ingested with the blood meal hastened the moulting time of the nymphs by 7 days. Whereas the ticks that fed upon clean blood moulted within 28 days, those that fed upon hormones *in vitro* moulted in 21 days on average. The ticks that fed upon a low dose of 5µg/ml and dropped on day 7 demonstrated that 50 % would moult by the 23rd day , 20 % of those dropping on day 6 would moult by day 21 and 70 % of the ticks fed upon a high dose of 10 µg /ml dropping on day 7 moulted after 23 days (Fig 7.3). A high percentage of ticks topically treated with ecdyson moulted faster compared to the control (Fig.7.4).

7.6 *Theileria parva* infections among *in vitro* fed ticks

Among the ticks fed upon infected blood containing hormones, very low infections rates to almost nothing were observed. Whereas the untreated control that comprised a prevalence (percentage of ticks infected in a batch of males and females) of 86.6 % , those fed on 20-hydroxy ecdyson had only 33.3 % which is almost 3 times less. Furthermore, the corresponding mean number of infected acini per tick (abundance) in

the salivary glands of those fed upon hormones was 2 fold less (Table7.12). In addition, among the membrane fed ticks, the ecdyson treatment produced no infections at all (0%) prevalence.

Among the solvent treated batches, both females and males recorded high infections with methanol and acetone topical application having infections ranging between 79-93% (Table 7.12). This was comparable to those ticks harvested from cattle with a prevalence of 100% if females and 80% in males. In addition to the foregoing, in membrane and cattle fed ticks, male batches showed a consistently lower prevalence, abundance and intensity of *T. parva* infection in the salivary glands (Table 7.12).

7.7 Discussion

A new avenue into investigations into the physiological factors affecting parasite development and transmission have been developed successfully in which external factors can now be incorporated in the diet and fed to ticks *in vitro*. *R. appendiculatus* nymphs have been successfully fed upon hormones, to study the factors that influence the transmission of parasites.

Nymphs that fed upon hormones weighed significantly less than the controls that fed on clean blood which means that either the hormone was a repellent that reduced the amount of blood the nymphs engorged or the hormone induced some metabolic changes in the tick that prevented them from engorging. This was supported by the fact that doubling the concentration of the hormone significantly reduced the engorgement weight.

It is important therefore, from these studies, that further research into the deleterious effects of hormones to ticks be instituted.

The period (number of days) taken to drop significantly reduced affected the weight. This perhaps implied a longer period of exposure to the hormone, since hormones were incorporated daily in the blood meal and these ticks took more amounts of hormones into their bodies. In addition, the hormones had a longer time to act upon the metabolism (this time engorgement). The hormone may have interfered with the gut permeability to the blood meal for example so that the ticks digest or assimilate the meal. All these factors contributed to the subsequent weights displayed. The hormonal effects also could have been in terms of attachment to the membrane (attach/detach mechanism) which disrupted in the actual feeding or in digestion, absorption and influence on the diluent rates.

Topically applied hormones did not seem to be as effective as those ingested from these studies. The activity of JH III was erratic compared to that of 20 hydroxy ecdyson. It caused mortalities to ticks although no significant effects on weights and moulting was observed. Mortality following hormone application is a common phenomenon. Insect mortality after topical application of ecdysone and phytoecdysones was reviewed (Williams 1968; Solomon *et al.*, 1983). In similar experiments, McDaniel and Oliver (1978) topically applied beta-edysone to *D. variabilis* nymphs and observed moderate mortality levels.

Ecdyson, on the other hand, resulted into quick moulting times and its effects on ticks should be investigated further. Among the three hormones, the best response was from *in vitro* fed 20-hydroxy ecdyson. The topical method of application was found to be infective. Ecdyson and phytoecdyson was observed to be more active when ingested by arthropods than when topically applied (Ruscoe, 1974). Furthermore, ticks have been demonstrated to show supernumerary moulting when hormones are included in the diet (Kitacha, 1972). The author recorded supernumerary moulting in adults and mortality in both nymphs and adults fed upon a blood meal containing beta-ecdyson or ponasterone A.

Hormones applied topically to *R. appendiculatus* or provided *in vitro* had influenced the feeding, moulting success and prevalence of *T. parva* in the salivary glands of adults. The engorgement weights for nymphs fed *in vitro* upon blood free of hormones were high (mean = 7.59mg) and comparable to those *in vivo* on cattle (mean= 8.92mg). This means that *in vitro* feeding system has been improved and optimised to feed *R. appendiculatus*. Such a system could lead to further research into the transmission, Epidemiology and control. Moreover, it can substitute the use of live animals for tick feeding. Similar results were obtained by Wallade *et al.*, 1996 who recorded a mean of 8.5 and 8.9 mg for membrane and cattle fed respectively from ticks that dropped on day 5.

The infection rates in hormone treated ticks were observed to be lower, which, coupled with the shortened the moulting time in hormone treated ticks was probably due to lack of synchronisation of the moulting time and the maturation (migration of the

kinete from the haemolymph to the salivary glands. This study has therefore thrown light on the possibility of using hormone-based immunogens as a tick control strategy. Kaufman (1991) also found a significant reduction in fluid secretory competence of salivary glands of *Amblyomma americanum in vitro.* This study is further evidence that structural degeneration is acquired after ecdysteroid activity on arthropods as reported by other authors (Harris and Kaufman, 1981;1985; Lindsay, 1988).

Table 7.2A. Mortality rates of *R. appendiculatus* following treatment with Juvenile hormone III

Percentage mortality (%)									
Treat (ug/ul)	**big 24hr**	**medium 24hr**	**small 24hr.**	**big 48hr**	**medium 48hr**	**small 48hr.**	**mean 24hr**	**mean 48hr**	**No. (n)**
1	77.27	35.00	30.00	81.77	44.09	45.00	11.58	46.88	30
2	35.00	50.00	35.00	40.00	71.43	55.00	22.39	41.18	28
3	75.00	26.00	55.00	77.78	47.37	55.00	39.00	57.33	43
4	94.7	85.00	53.00	94.70	92.85	69.00	52.28	71.15	37

Engorged larvae of different sizes were compared in their response to Juvenile treatment (small=2.10±1.85mg; medium=3.68±2.33mg; big 6.26±3.24).

Table 7.2 B. Probit analysis of the mortality rates of *R. appendiculatus* nymphs treated with Juvenile hormone III and 20-hydroxy ecdyson

Variable	LD_{50} µg/µl	correlation (r)	X^2	Pr>chi	Significant/not
JHm intercept		-0.52	0.86	0.35	ns
JHm log 10	2.48	1.32	0.80	0.37	ns
JHs intercept		-0.58	4.59	0.032	s
JHs log10	3.06	-1.19	3.05	0.0804	ns
Jhb intercept		0.28	0.14	0.70	ns
Jhb log 10	0.44	0.79	0.18	0.67	ns
JH mean	1.76	-0.23	0.90	0.34	ns
20-Hydroxyecdyson intercept		0.56	9.60	0.0001	s
20-Hydroxyecdysone log 10	0.53	2.23	17.91	0.0001	s

alpha=0.05;s=significant;ns=not significant
JHm=Juvenile Hormone III treated medium sized nymphs
JHs=Juvenile Hormone III treated small sized nymphs
Jhb= Juvinile hormone III treated big nymps

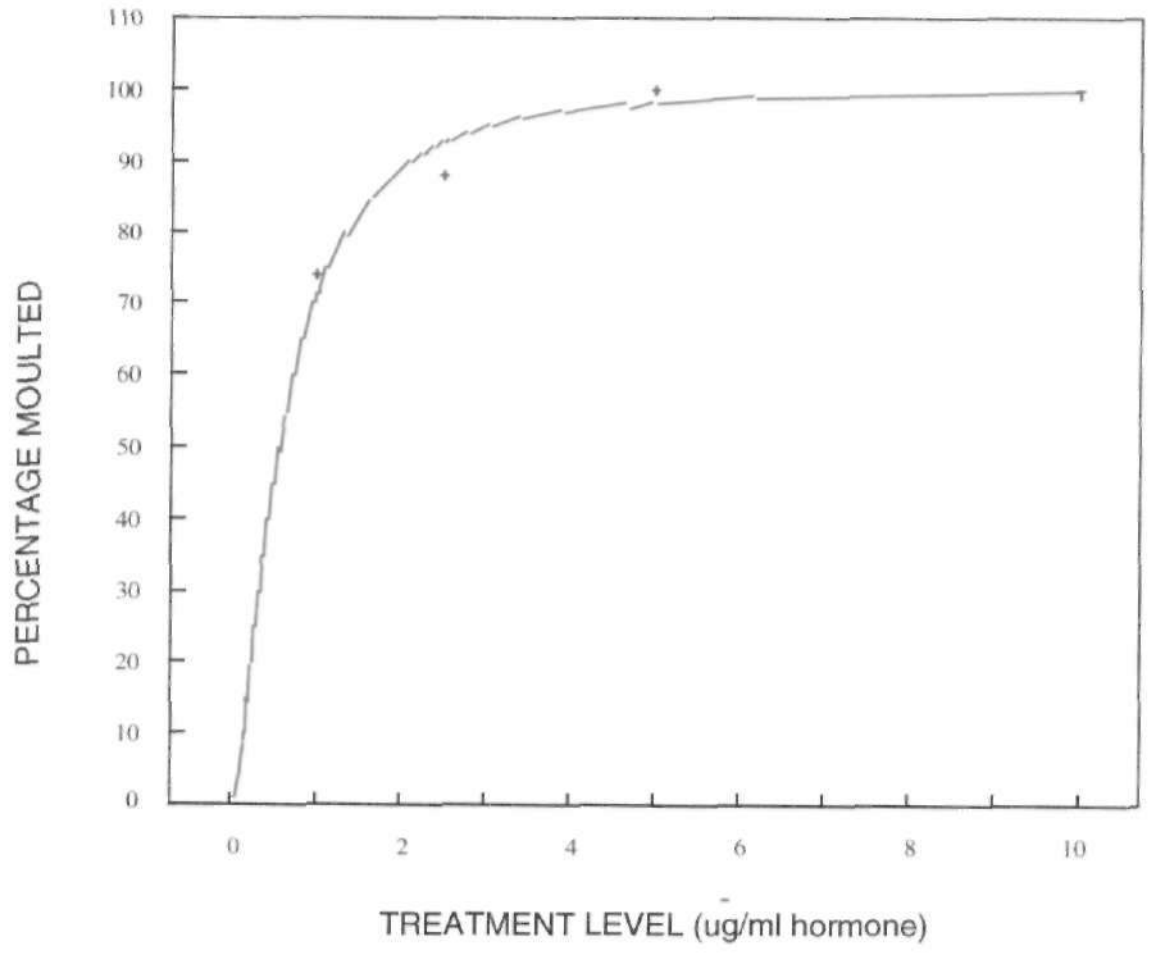


Figure 7.1 Susceptibility of *R. appendiculatus* engorged nymphs to 20-hydroxy ecdyson

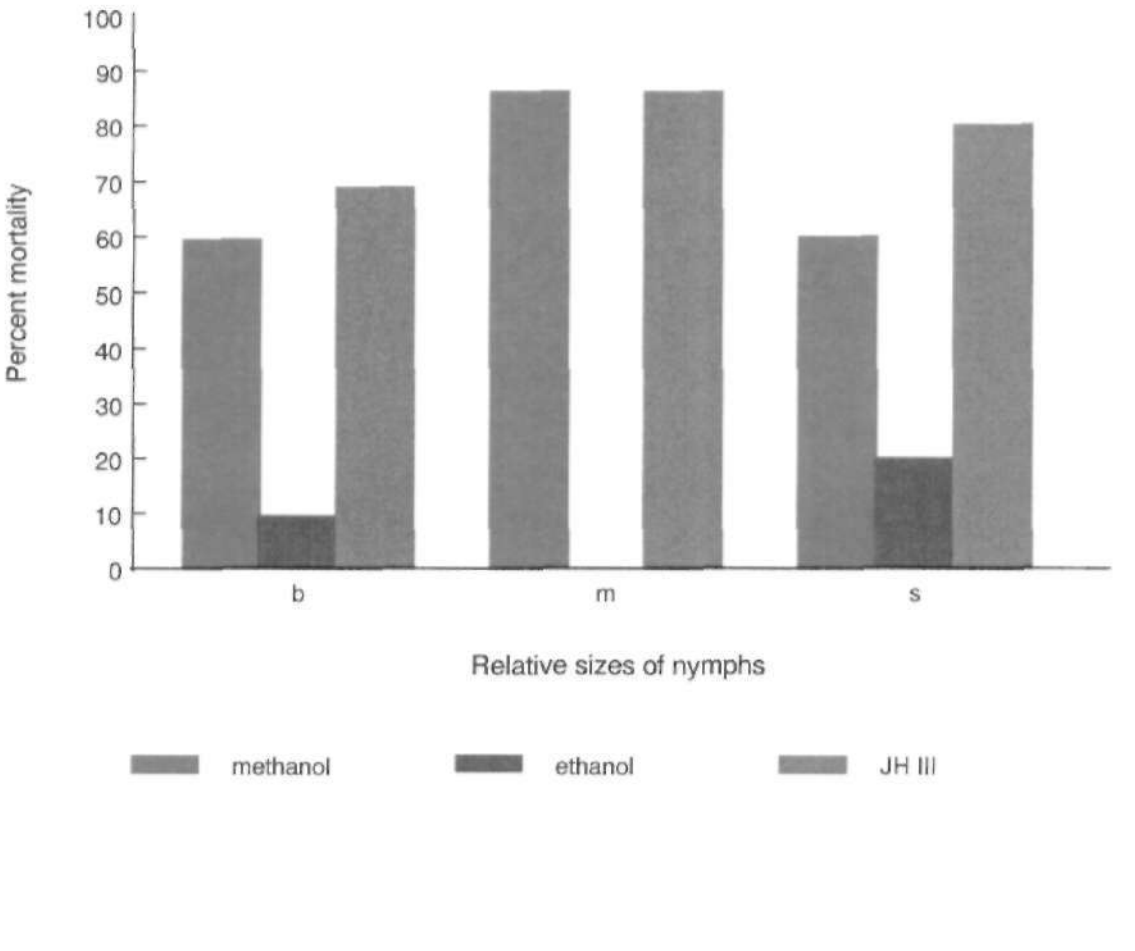


Figure 7.2 Mortality rates in hormone injected *R. appendiculatus*

Table 7.3A General linear models procedure for engorgement weights of *R. appendiculatus* nymphs fed upon 20-Hydroxy ecdysone *in vitro* A:Dependant variable: weight

Source	DF	Typel sum of squares	Mean square	F value	Pr>F
Treat	2	2203.31	1101.66	604.82	0.0001
Day	2	51.65	25.82	14.18	0.0001
Error	923	1681.22			
cv=27.04					

Table 7.3B: Duncans' multiple range test groupings for the *R. appendiculatus* nymphs engorgement weights following treatment with 20-Hydroxyecdysone

Treatment (μg/ml)	Mean engorgement weight (mg)	Number of nymphs analysed	Duncan's groupings
0	7.59	221	A
5	4.68	412	B
10	3.49	296	C

Means with the same letter are not significantly different from each other. Alpha = 0.05.

Table 7.4 Comparison of duration of events involved in *in vivo* and *in vitro* feeding of *R. appendiculatus*

	***In vivo* feeding**	***In vitro* feeding**	
Day	*T. parva* stabilate infected cattle	Baudriche membrane chamber	Day
0	sporozoite stabilate innoculation in cattle	-	-
13	Clean nymphs attached	Clean nymphs attached; fresh clean blood supplied	0
14	Nymps attaching	Nymphs attaching	1
15	Nymphs attaching	Nymphs attaching	2
16	Few engorged nymphs start dropping	Nymphs changed to infected blood .	3
17	Dropping of infected nymphs continue	Few engorged nymphs start dropping	4
18	"	A large number of engorged nymphs drop	5
19	"	Continuation of dropping	6
20	"	"	7
21	Completion of dropping from cattle	Completion of dropping from the membrane	8

Table 7.5A: Influence of feeding duration on engorgement weights of *R. appendiculatus* nymphs fed on 20-hydroxy ecdyson

Feeding duration (days)	No. observed	mean	Standard deviation	Duncan's groupings
5	68	5.50	± 2.61	A (ns)
6	308	5.74	± 2.23	A (ns)
7	552	4.49	± 1.71	B (s)

Means with the same letter are not significantly different; Alpha = 0.05.
ns=not significant;s = significant.

Table 7.6 Correlation analysis between the engorgement weight of the *R. appendiculatus* nymphs fed on hormones and the day they dropped from the *in vitro* membranes

Treament	Mean weight	Std deviation	Mean day	Std. deviation	Pearson correlation coefficient (r)	Probability	N
Control (0)	7.59	±1.76	6.31	±0.65	-0.33	0.0001	221
5µg/ml	4.67	±1.37	6.51	±0.61	-0.12	0.02	412
10 µg/ml	3.48	±0.96	6.69	±0.59	0.02	0.73	296
Combination	4.99	±2.06	6.52	±0.63	-0.26	0.0001	929

Table 7.7 A: Relative frequencies of weights of nymphs fed *in vitro* on 20-hydroxy ecdyson dropping on different days:

A. Combination frequencies of the weights and days

Day/Category	1	2	3	4	Total
5	11	29	19	9	68
6	29	146	107	26	308
7	98	353	94	8	553
Total	138	528	220	43	929

chi-square $X^2 = 80.78$; Df=4; Probability = 0.0001;
sample size = 929.

Table 7.7B: Relative frequencies of weights of nymphs dropping on different days for *R. appendiculatus* fed upon 5ug/ul hormone in blood

Day/Category	2	3	4	Total
5	0	15	9	24
6	8	70	26	104
7	24	63	6	93
Total	32	148	41	221

X2= 29.421; Df=4; Probaility=0.0001;

sample size= 221.

Table 7.7C Frequencies of weights of *R. appendiculatus* nymphs fed upon 20 Hydroxy ecdyson (10μg/ml) dropping on different days

Day/Category	1	2	3	Total
5	10	10	0	20
6	16	36	0	52
7	70	152	2	224
Total	96	198	2	296

X^2 =3.61; Df=4; Probability=0.461; sample size=296

Table 7.8 General linear modules procedure Analysis of variance for the days taken to feed on the membrane and the moulting success of *R. appendiculatus* nymphs

Source	Dependable variable	df	Sum of squares	Mean square	F-value	pr>F	Significant /not
Model	No. moulted	8	2.37	0.29	1.42	0.27	ns
Model	Cumulative % moulting	8	1.92	0.24	7.37	0.0005	s
Interaction	treatment* daydrop (cumulative)	3	0.82	0.27	8.38	0.0016	s
Model	No. dead	8	0.41	0.05	1.15	0.39	ns
Model	% moulted	7	0.63	0.09	3.30	0.02	s

Table 7. 9 Moulting success of 20 hydroxy ecdyson treated *R. appendiculatus*

A

Treatment	day dropped	mean % moulted	SD
10	5	49.81	17.87
10	6	35.89	8.90
10	7	59.76	14.76
5	5	13.48	5.62
5	6	24.42	7.30
5	7	35.55	19.80
5	8	50.00	

B Effect of days :

Days taken to drop	mean	SD	SNK grouping
5	34.24	23.39	A
6	29.33	9.53	A
7	45.93	20.80	A

C Effect of treatment:

Treatment	N	mean	SD	SNK grouping
10µg/ml	11	49.47	15.73	A
5 "	12	27.53	15.93	B

Table 7.10 General linear modules procedure analysis of transformed cumulative moulting success

Source	df	Sum of squares	Mean square	F-value	Pr>F
Model	8	1.92	0.24	7.37	0.0005
Error	15	0.49	0.03		
Corrected total	23	2.40			

Type I SS Analysis of variance

Source	df	TypeIss	Mean square	F-value	PR>F
Treatment	1	0.18	0.18	5.59	0.0319
Daydrop	4	0.92	0.23	7.06	0.0021
Treatment*daydrop	3	0.82	0.27	8.38	0.0016

Table 7.11 Effect of death on the moulting success of hormone treated *R. appendiculatus*

A:General linear models procedure : dependant variable number dead

Source	df	Sum of squares	Mean square	F-value	Pr>F
Model	8	0.41	0.05	1.15	0.3873
Error	13	0.67	0.04		
Corrected total	23	1.07			

B:Analysis of variance (Type I SS)

Source	df	Type I SS	mean square	F value	Pr>F
Treatment	1	0.04	0.041	0.93	0.3511
daydrop	4	0.16	0.040	0.91	0.4837
Treat*daydrop	3	0.21	0.069	1.55	0.2435

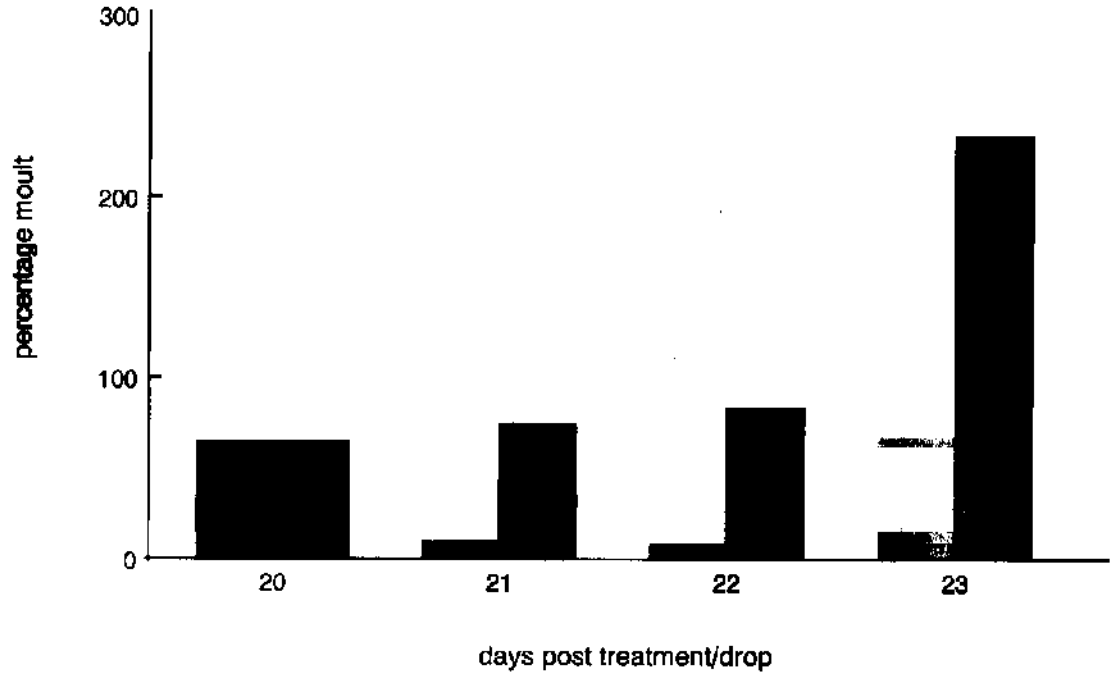


Figure 7.3 Moulting success in hormone treated *P. appendiculatus* (20-hydrocy ecdyson)

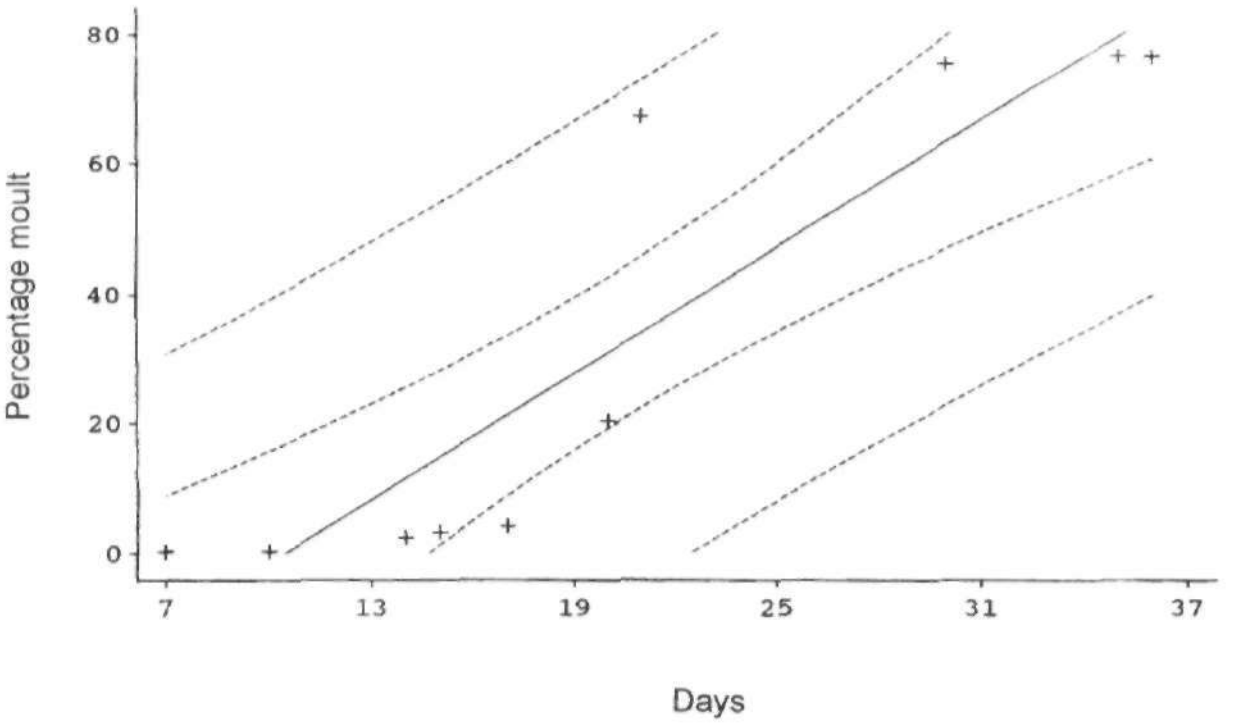


Figure 7.4 Moulting success of *R. appendiculatus* treated with ecdyson

Table 7.12. Comparison of *Theileria parva* infections in *R. appendiculatus* ticks fed *in vivo* and *in vitro* upon hormones

Treat	Prevalence		Abundance		Intensity	
	female	male	female	male	female	male
In vivo cattle BN 318	100%	80 %	104.70	40.66	104.7	46.92
in vitro hormone -free blood	90%	86.66 %	44.50	3.00	51.30	2.40
in vitro 20-hdroxyecdysone	33.3%	50%	31.80	5.25	95.50	10.50
Solvents methanol	79.3%	73.9%	31.30	12.80	39.33	17.40
Solvent acetone	92.9%	20%	29.20	25.40	31.46	31.75
Juvenille hormone III	50%	34.5%	17.30	5.30	34.5	11.40

CHAPTER EIGHT

8.0 THE INFLUENCE OF THREE ENZYMES: TRYPSIN, PROTEASES AND PHENOLOXIDASE ON *T. PARVA* IN *R. APPENDICULATUS*

8.1 Introduction

During the course of its development, the protozoan parasite *T. parva* encounters several factors in the vector *R. appendiculatus* which modulate its development. In the gut, the piroplasm stage of *T. parva* found in the red blood cells ingested by the tick while feeding encounters a physiologically hostile environment that includes various digestive enzymes. Similar processes occur in insects such as tsetse flies (Gooding, 1975). Other factors the parasite comes across include lectins (Welburn *et al.*, 1989) or agglutinins (Ingram and Molyneux, 1988) and trypanolytic proteins (Stiles *et al.*, 1989). However, the mechanism by which the parasite overcomes these factors is not clearly understood, but it is generally accepted that most parasites are killed by one or more of these factors and those which manage to survive, transform into the next stage in development, which is the gametes that fuse to form zygotes. The zygotes transform into the next stage in development, the kinetes, which attach to the gut wall and later migrate through the haemolymph into the salivary glands from where the parasite is transmitted as sporozoites into the next host (Norval *et al.*, 1992). The enzymes most importantly encountered by the parasites during this stage include digestive enzymes for example trypsin and protease. Studies on digestive enzymes include those on the digestive aspartic proteinases in the midgut of *R. appendiculatus* (Vundla *et al.*, 1992). On the other hand, an esterase from

the *Boophilus microplus* larvae that was important both for the feeding of the parasite on the bovine host and as a target for a protective immunological response by the host was reported by Willadsen, *et al.* (1987). However, no study has yet addressed the question as to how the parasites manages to evade the enzyme degradation in *R. appendiculatus*.

Although arthropods do not posses an immunoglobulin based defence system, they have a variety of defensive processes both hummoral and cellular designed to recognise the foreign body as non-self and protect themselves. The primary response to foreign bodies for example parasites, glass beads by insects for instance, is deposition of melanin around the non-self object (Nappi, 1975; Ratcliffe *et al.*,1984) making melanization and the enzyme that biosynthesises process an integral part of the immune system. Phenoloxidase (O-diphenol, oxygen oxidoreductase) is normally present among insects in an inactive proenzyme form (Comb and Jenkin, 1984; Gotz and Boman, 1985; Ashida, 1971).

Phenoloxidase is a bifunctional copper containing enzyme which catalyses both the O-hydroxylation of monophenols and the oxidation of diphenols to quinones (Ratcliffe *et al.*, 1984). The enzyme therefore convents tyrosine to DOPA as well as DOPA to DOPA quinone. These processes are followed by non-enzymatic spontaneous reactions forming melanin a brownish pigment (Aspan and Soderhall, 1991). Among the arthropods, the pigment is usually produced in the cuticle following a wounding or as a result of parasite attack (Ratcliffe *et al.*,1985).

Despite the presence of the immune system in arthropods, several pathogens thrive in their bodies and are transmitted to other organisms. A clear understanding of the molecular mechanisms of tick immunity could unravel the survival strategies of vector/ parasite interactions and also lead to the development of new vector control measures. In this chapter, trypsin, protease and phenoloxidase enzymes are discussed with reference to *T. parva* infections.

8.2 Materials and methods

8.2.1 Ticks maintenance

R. appendiculatus the vector of *T. parva* and *R. pulchellus* a non vector of the above parasite adults were used and maintained on cattle and rabbits as blood meal sources. They were fed on rabbits for varying periods of time (between 1 - 5 days and kept for 24- 48 hr before dissection. In order to compare the enzyme activities from ticks fed for various periods of time, their attachment to the rabbit host were synchronised (Fig. 8.1) so that all the dissections were carried out on the same day.

8.2.2 Enzyme detection

8.2.2.1 Protease

8.2.2.1.1 Preparation of midgut lysate as a source of protease

Twenty ticks were surface sterilised by washing in detergent and 70 % ethanol and sterile distilled water. The guts and salivary glands were then dissected in 60 µ l Dulbeccos' PBS pH 7.4 and kept on ice throughout the process. They were then washed in PBS and homogenised in a tissue grinder (Melags, Germany) and centrifuged (1200 x

g; 15 min; 4^0 C) in a microfuge (Beckmann). The supernatant solution was used as the protease enzyme source for subsequent experiments.

8.2.2.1.2. Preparation of casein gel substrate for the protease assay

One tablet of commercial casein gel (Pharmacia) was dissolved in 5 ml distilled water, boiled for 5 min and the resulting gel mixture poured on to diffusion plates (7 cm X 8 cm; Biorad). The gel was left to set for half hour at room temperature. Each tablet dissolved yielded a 10 % agar gel containing bovine casein in buffered saline, pH 7.4 for protease detection. The gel was punched and wells created by vacuum suction through a water tap vacuum.

8.2.2.1.3 Protease assay

Ten microlitres were placed in 0.3 cm wide wells cut in a casein gel substrate in 8.5 X 6.5 cm diffusion plates. The plates were covered with plastic leads and incubated at 27 ^{0} C for 16 hr. The resultant lytic regions (zones) were enhanced by treatment with 3% acetic acid, which also stopped the reaction. The zones were measured with vernier callipers. A standard curve was obtained by serial dilution of trypsin (Bovine pancreatic type III, Sigma) in PBS pH 7.4 (Na_2HPO_4 , 13.48g; $NaH_2PO_4.2H_2O$, 0.28g; NaCL, 4.25g in 1 litre distilled water). Control wells were filled with buffer. Protease levels are reported as per ticks basis as international units (IU) based upon the standard curve for commercial trypsin (Fig 8.2).

8.2.2.2 Trypsin

8.2.2.2 .1 Preparation of trypsin enzyme solution for trypsin detection

Guts from adult partially fed (4 day-fed) *R. appendiculatus* were dissected in 20 mM Tris-HCl pH 8.0 and kept on ice. They were homogenized in the same buffer using a hand homogenizer (Pellet Peste, Motor, Kontes) for 1 min and centrifuged at 12,000 g for 15 min; at 4 ^{0} C in a microfuge (Beckmann). The supernatant was kept and assayed for trypsin activity.

8.2.2.2.2 Preparation of substrate for trypsin assay

The trypsin activity was assayed using a chromogen substrate, carbobenzoxy-val-gly-arg-4-nitranilide-acetate (Chromozym-try; Boehringer-Mannheim).

8.2.2.2.3 Trypsin assay

In a glass cuvette, 0.95 ml of 20mM Tris-HCL, pH 8.0 buffer was mixed with 5µl of the sample and incubated for 5 min at 30°C in a water bath. The blank was read at wave length A_{405} in a spectrometer (Spetrophase GEMCO). Immediately afterwards, 50µl of the substrate chromozyme was added. The reading was immediately taken at time zero (t_0) and at 2, 5 and 10 min for each of the samples. Infected and clean tick samples were compared. Activity was measured by calculating the change in absorbance per minute.

8.2.3 phenoloxidasse Assay

8.2.3.1 Haemolymph collection

Haemoplymph samples were collected from at least 250 ticks previously fed upon rabbits for 4 days and from the same batch (that had dropped the same day from cattle). The haemolymph was obtained in capillary tubes as previously described (Chapter 3) by leg amputation. Throughout the collection, the samples were kept on ice in chilled microfuge tubes without PTU crystals. The haemolymph was prepared separately in order to assess the different components for PO activity.

8.2.3.1.1 Whole haemolymph (WH)

The haemolymph which was not centrifuged was taken as for PO activity whole haemolymph (WH).

8.2.3.1.2 Haemocyte- free haemolymph (plasma; P)

The haemolymph was cold centrifuged (300 x *g*; 5min) in microfuge tubes, after which the supernatant consisting of the plasma (P) was transferred into a clean chilled microtube tubes and kept once. The aliquots of centrifuged haemolymph was analysed in quadruplets immediately after the haemolymph collection to avoid coagulation and melanisation.

8.2.3.1.3 Preparation of haemocyte lysate and supernatants

The pellet (haemocytes) from above were washed twice with 10 mM Sodium cacodylate buffer pH 6.9 without Calcium. This was carefully carried out using a pasture

pipette through flushing the pellet and sucking the solution without resuspending the cells to avoid cell rapture. A volume of sodium cacodylate equal to that of the plasma removed was introduced into the tube and the pellet homogenised in a glass homogeniser. The homogenate was centrifuged (1000 x *g*, 10 min) and the resultant supernatant (haemocyte lysate; HL) and haemocytelysate pellet (HP) assessed for phenoloxidase activity. The total amount of protein in the haemolymph was about 2mg/ml.

8.2.3.2 Buffers

The buffers used included phosphate and sodium cacodylate with or and without calcium. The Phosphate buffer (0.01M; pH 5.9) was prepared from monosodium 0.01M $NaH_2PO_4.H_20$ (901 ml) and 0.01 M $Na_2HPO_4.12H_20$ (99 ml). The sodium cacodylate (0.01 M; 10 mM Ca^{2+} ; pH 6.9) prepared by adding 0.147 g $CaCL_2.2H_20$ to 100 ml of the sodium cacodylate.

8.2.3.3 Phenoloxidase substrate L-DOPA

A saturated solution of l-3, 4- dihydroxyphenylalanine (L-DOPA; Sigma) was prepared in Phosphate buffer to a final concentration of 2 mg/ml.

8.2.3.4 Phenoloxidase system activators

The Phenoloxidase ((PO) activators prepared freshly before use in sodium cacodylate buffer which contained Calcium included:

- LPS from *Escherichia coli* (Sigma)
- Laminarin, a cell wall carbohydrate from fungi (sigma) prepared at a concentration of 1mg/ml

- methanol
- Enzymes (trypsin)

8.2.3.5 Phenoloxidase assay

Phenoloxidase activity was assayed using a modification of Horowitz and Shen (1952) method. For the proteinase-free prophenoloxidase preparation, a sample (10 µl) was incubated with 10 µl of activator for a varying periods of time depending on the activator. To this mixture, 100 µl L-DOPA (2 mg/ml) in 0.01M phosphate buffer (pH 5.90) was added at 5 min intervals, for a period of 20 min. Absorbency was read at 490 nm in a Microtitre plate reader (Titertek, Multiskan MC).

8.2.3.6 Protein determination

The protein level of whole heamolymph was determined using a Biorad Kit to assay total protein concentration. For each sample, three aliquots of 50 µl heamolymph were analysed using the standard assay and bovine serum albumin (Biorad) as the standard. Assays were carried out in microtitre plates and optical densities measured by an ELISA reader (Titertek , Multiscan, MC).

8.2.3.7 Comparison of phenoloxidase in ticks and insects

The activity was compared in the freshly collected haemolymph of *A.variegatum*, *R.appendiculatus* and the desert locust *Schistocerca gregaria.*

8.3. Results

8.3 1. Protease activity

Protease activity measured as the diffusion zone diameter in the casein substrate was shown to increase linearly with the logarithm of trypsin concentration at concentrations beyond 1.3 µg/ml (0.12 IU/ml; Fig 8.2). Based upon the standard curve, the protease activity in the tick guts were calculated from the measurements of 20 guts suspended in 60 µl buffer. The protease activity in terms of diffusion zones created by the gut of *R. appendiculatus* fed for 4 days; 48 hr after feeding is depicted in Fig 8.3. No protease activity was detected in the salivary glands of the ticks either fed for 1- 5 days or engorged, in either species.

Peak protease activities in the guts of *R. appendiculatus* occurred 48 hr after a blood meal (Fig 8.4). Minimum protease activity was found in the ticks 24 hr after a blood meal, regardless of the feeding duration. Furthermore, no protease activity was detected in unfed ticks in either species which implied that the enzyme was blood meal induced. More protease activity detected as diffusion diameter was recorded in the infected *R.appendiculatus* adults fed for similar time periods compared to clean 4-day fed ticks.

8.3.2 Trypsin activity

The presence of trypsin in the gut of infected or clean *R. appendiculatus* was detected between zero to 24 hr after feeding (Table 8.1). There was no significant difference in the amount of trypsin between positive and negative *R. appendiculatus* or between the haemolymph and guts of *R. appendiculatus*. However, there was a significantly higher content of trypsin in the haemolymph of *R. pulchellus (*Table 8.1*;* $p<0.05$) indicated by the highest absorbance (0.144) measured at λ 405.

The above enzyme activity as calculated by change of absorbency revealed that although trypsin was present in the guts, its activity could not be established using the substrate chromozyme 0 - 24 hr after removal from rabbit host. However, after 24 hr, trypsin activity was detected in 1-5 days fed ticks. The highest amount of trypsin activity was detected in the solubilised gut of *R.appendiculatus* (Fig 8.5).

8.2.3 Phenoloxidase system in ticks

8.2.3.1 Activation of the prophenoloxidase system in ticks

In case of methanol activation, the incubation period of 3-5 min was found to be appropriate. For LPS, the incubation period was 1 hr and laminarin 30 min. Because of the quick reaction methanol was adopted for the subsequent experiments. No significant auto-activation of the phenoloxidase system was observed in the freshly collected haemolymph of *R. appendiculatus*. However, following 24 hr incubation, there was auto-activation of the phenoloxidase in the plasma (Fig. 8.6). Phenoloxidase activity in

the haemolymph was easily induced by methanol treatment (Fig 8.7). The activation efficiency depended upon the concentration of the methanol, the period of incubation, and the haemolymph sample before addition of the of the L-DOPA substrate. The optimum concentration was 50 % methanol (1 part haemolymph and 1 part absolute methanol) which was adopted in the subsequent experiments. The reaction sharply increased from the beginning (time 0) to a maximum in 1 min and the reaction remained constant for the next 5-10 minutes (Fig 8.7). Enzyme treatment (trypsin) of the haemolymph resulted in high phenoloxidase activation (Fig 8.8), compared to the methanol activation. The reaction peaked 20min after activation.

8.2.3.2 Comparison of phenoloxidase activity between ticks and insects (locusts)

There was a significantly lower Phenoloxidase activity in the *R. appendiculatus* haemolymph ($t=7.96$; $p<0.0001$) compared to the locust *Shistocerca gregaria* (Fig. 8.9). Similarly, there was a significantly lower phenoloxidase activity in the haemolymph of *Amblyoma variegatum* ($t=8.76$; $p<0.0009$; Fig 8.9) compared to *S. gregaria.* A low but significant phenoloxidase activity was observed in the *R. appendiculatus* haemolymph compared to *A. variegatum* (Fig 8.9).

8.2.3.3 Phenoloxidase activity in different components of the haemolymph

Significant differences were detected in the plasma (without haemocytes) compared to those with haemocytes among the engorged infected ticks (Fig 8.10). Similarly, higher PO activity was observed in the plasma compared to the lysate and pellet of uninfected engorged ticks (Fig 8.11) leading to the realisation that at least 95%

detectable PO activity occurred in the plasma. There was no significant differences in activity between the heamocyte lysate and the haemocyte lysate pellet (Fig 8.10).

8.2.3.4 Phenoloxidase activity among infected and non infected ticks fed for different number of days

8.2.3.4.1 Engorged infected ticks

A higher activity rate of conversion of tyrosine to DOPA in the plasma (supernatant) in the haemolymph of infected engorged ticks compared to that of clean (uninfected) engorged adult ticks, showing that infections clearly altered the normal rate of haemolymph enzyme activity (Fig 8.10 and 8.11). Among the engorged but negative of uninfected adult *R.appendiculatus*, a significantly higher activity was observed (t=p,0.05) in the haemocyte lysate than in the plasma and haemocyte pellet (Fig 8.11).

Among the partially fed adult *R. appendiculatus*, there was similarly a higher activity of the conversion of tyrosine to DOPA in the plasma of infected ticks compared to that of clean ticks. Activity was monitored *in vitro* in both engorged (5 day fed and partially engorged (4 day fed) ticks and expressed as normal DOPA formed per min. The colour changes from light orange brown to purple were noted at wave length 497 nm and expressed as optical density (Fig 8.10). There was higher PO activity among the positive females haemocyte lysate than in the pellet from the negative engorged females (Fig 8.10 and 8.11). On the other hand, no significant difference in PO activity was observed in the partially fed among the infected or non infected.

8.4 Discussion

Protease activity in the two tick species *R. appendiculatus* and *R. pulchellus* has been successfully detected using gels containing protease substrate. The use of gels with substrates has been reported by several authors (Sevier, 1976; Lowenstein and Ingrid, 1976). Similar detection of protease activity was carried out by Stiles *et al.*, (1991) in tsetse flies who detected the activity in small quantities (1.2 $\mu g/cm^3$). The salivary glands from fed or unfed. *R. appendiculatus* have been shown to no detectable proteases specific to this particular substrate. Comparing the two groups of fed and unfed ticks, it appeared that the quantity of casein- substrate- specific proteases was considerably higher among the infected ticks than in the clean or uninfected *R. appendiculatus*. Similarly, considerably less protease activity was detected among the *R. pulchellus*, a non vector of *T. parva*. Interspecific variation in the quantities and types of proteases among arthropods is a common phenomena. Gooding *et al.*, (1984) described such differences in the tsetse flies which produced different quantities of protease to digest a given blood meal.

The study has also shown that protease activity peaked 48 hr after a blood meal. The fact that little or no protease was detected immediately or 24 hr after the ticks were detached from the rabbit host implies that the protease synthesis in the gut is probably a time dependant process peaking at 48 hr after a blood meal. Since deferent levels of

protease were detected in ticks fed for different days, it means that varying quantities of these protease are produced by each tick batch for a given blood meal.

The mechanism of protease release in insects for instance has been dealt with by various authors (Langley, 1966; Gooding, 1947; Akov, 1972) who showed that the amount of midgut protease correlated with the blood meal size. Gooding (1975) then explained this enzyme secretion mechanism to be controlled by a secretorygogue mechanism with one or more serum proteins providing the normal stimulus to the midgut secretory cells in the tsetse flies. In the present study, no protease activity was found in the unfed ticks which supports these possibilities.

Studies on midgut protease in insects (Gooding, 1972;1975; Cheesman and Gooding, 1985) have not included the consideration of their possible involvement with ingested parasites. This could be due to the complexity of protease assay methods (Stiles *et al.*, 1991; Borovsky and Schlein, 1987). In *Glossina* several different proteolytic enzymes have been shown to exist (including carboxy peptidasse, amino peptidases, trypsin, chymotrypsin and trypsin-like enzymes (Gooding *et al.*, 1984). In this study, blood meal induced trypsin and protease have been shown to occur in the midgut whose peak activity occur at least 48 hr after a blood meal. The possible involvement in the ingested *T.parva* parasites lays in their lysis activity of the red blood cells. A simple, rapid and reproducible assay for detecting and assaying activity in microquantities of individual or pooled guts in ticks has been also illustrated, and confirms earlier observations (Stiles *et al.*, 1991;Schumacher and Schill, 1972) that radial diffusion in gel can be a method of enzyme determination.

The fact that the infected ticks showed higher phenoloxidase activity than unaffected ticks is probably because the PO system is ticks is also involved in self and non-self recognition. The presence of *T. parva* in the haemolymph of the ticks may also activate the PO system, leading to the high activity. In arthropods, melanin produced a s a result of the PO system is produced as a result of parasite attack (Ratcliffe *et al.*, 1985) or wounding. The phenoloxidase is present prior to this as a pro-enzyme in an inactive precursor in the haemolmph (Ashida, 1981; Saul and Sugumaran, 1988) or in the blood cells (Soderhall *et al*, 1979; Soderhall and Hall, 1984; Leornard *et al.*, 1985). The precursor Pro-phenoloxidase is converted to phenoloxidase and melanin is subsequently produced during wound healing and during the elimination of parasites in the haemolymph. This process is known as pro-phenoloxidase activity cascade (Ashida *et al.*, 1982; Soderhall *et al.*, 1986; Ratcliffe *et al.*, 1985; Soderhall and Smith, 1986). This system is inactivated by a variety of polysaccharides example ß-1,3-glucan (cell wall carbohydrates from bacteria and fungi) or lipopolysaccharides (Soderhall *et al.*, 1986). Results have shown that incubation of trypsin with haemolymph induced PO enzyme activity. Similar results have been obtained earlier (Preston and Taylor, 1970). In addition, serine proteases have been reported to be directly involved in the conversion of phenoloxidase (Soderhall *et al.*, 1986; Ashida *et al.*, 1982). Melanin and its precursors have been found to inhibit bacterial enzymes (Kuo and Alexander, 1967) and are fungistatic (Soderhall and Ajaxon, 1984). Proenzymes of *Bombyx mori* (Ashida, 1971) *Manduca sexta* (Aso *et al.*, 1985), *Musca domestica* (Tsukamoto *et al.*,1 1986) have been characterised. Ashida (1971) reported that *ß, 1 3* glucans could activate the silk

worm. Pro-phenoloxidase in the whole haemolymph indicating the direct link between foreign matter presence and the pro-phenoloxidase activation in a cascade system.

Visual observation of the tick haemolymph revealed a quick colour change from almost colourless or light brown to dark brown. This suggested that the melanisation rate of the haemolymph conversion of tyrosine to DOPA and melanin was very fast. Multiple factors could have contributed to this including problems related to activation of pro-pro-phenoloxidase and cleavage of peptides fragments to generate an active PO molecule (Ashida and Ohnishi, 1967; Broockman, 1988; Beckage *et al.*, 1990). On the other hand, the serine proteinases secreted on feeding as a result of blood meal ingestion have long been implicated in the conversion of pro-phenoloxidases to phenoloxidase in the (Soderhall *et al.*, 1986).

Several proteases in addition, have been found to activate the prophenoloxidase system (Saul and Sugumaran, 1986; 1987) and in higher organisms controlled by protease inhibitors (Sugumaran *et al.*,1985).

Since no statistically significant differences in Phenoloxidase activity were detected between samples incubated with or without haemocytes, this implied that at least 95% of the detectable phenoloxidase activity occurred in the plasma. Similar results were observed by Saul and Sugumaran (1987).

No statistically significant differences were detected between samples of the haemocyte lysate and haemocyte lysate pellet. On the other hand, high activity was

observed among the haemocyte-free plasma which indicated that about 95% of detectable phenoloxidase activity is carried out in the plasma. These results were in agreement with several studies (Saul and Sugumaran.,1987; Johnson and Soderhall, 1985) carried out in the crayfish *Pacifastacus leniusculus*. Several proteins of *P. leniusculus* are contained within circulating blood cells (Johnson and Soderhall, 1985). Protease inhibitors within the plasma seem to act as regulatory factors preventing the undesired activation of the pro-phenoloxidase system (Aspan *et al.*, 1990). Later (Aspan and Soderhall, 1991) a homogeneous preparation of a 36 kDa serine proteases from blood cells of the cray fish could convert phenoloxidase by proteolytic cleavage.

Generally, the ticks were observed to have very low PO activity as compared to insects. The desert locust *Shistocerca gregaria* was compared and had ten fold PO activity in the same span of time (10 min). The presence of low detectable PO activity in the ticks could be to a number of reasons for example the fast rate of melanisation, which could also have been triggered by the method of collection of the haemolymph. Haemolymph was collected in capillary tubes (1µl) and immediately pipetted into microfuge tubes placed on ice. In the process, the haemocytes could have been damaged leading to triggering of the cascade causing melanisation an coagulation (Dularary and Lackie, 1985; Soderhall and Smith, 1986; Soderhall and Hall, 1984).

The high activity of PO found in the plasma compared to the haemocyte lysate or haemocyte pellet can be explained by considering the fat that proteins of the prophenoloxidase activating system are contained within the circulating blood cells in arthropods. When such cells are exposed to lipopolysaccharides (LPS) or ß-1,3, glucans

from bacteria and fungi, they are released by exocytosis into the plasma and subsequently activated outside the cells by elicitise of the pro-phenoloxidase activating system; finally, the prophenoloxidase system is converted into the active form phenoloxidase (Johnson and Soderhall, 1985).

There was higher PO activity in the infected tick haemolymph which may be part of the defence system. Defensive mechanisms invertebrates include antimicrobial activity in insect haemolymph such as lysosomes (Powning and Davidson, 1976) and protease inhibitors (Aspan *et al.*, 1990). Cecropins, first described in the Cecropia moth (*Hyalophora cecropia;* Hultmark *et al.*, 1980) are low molecular weight (4 kda) that fight against gram positive and gram negative bacteria while attacins located on the outer membranes of *E.coli* cause lysis (Boman and Hultmak, 1987). Other mechanisms include coagulation of the haemolymph which serves as a physical barrier to prevent entry of a variety of pathogens (Brehelin, 1986), diptericins (Keppi *et al.*, 1986; Ratcliffe and Rowley, 1983;) Bowman *et al.*, 1972), encapsulation (Ratcliffe *et al.*, 1985) and anti-microbial activity involving attacins (Hultmark *et al.*, 1980).

In insects, on of the defence routes against parasites is encapsulation by heamocytes followed by melanisation in which tyrosine and DOPA are converted to melanin through a cascade of toxic quinones intermediates (Aspan *et al.*, 1990). It is likely that similar processes occur in ticks, especially since high levels of PO activity were detected in the infected ticks as compared to uninfected ones. The PO system, therefore seems to play a role in the establishment of the parasite in the ticks. The parasite *T. parva* may be evading it after its initial activation.

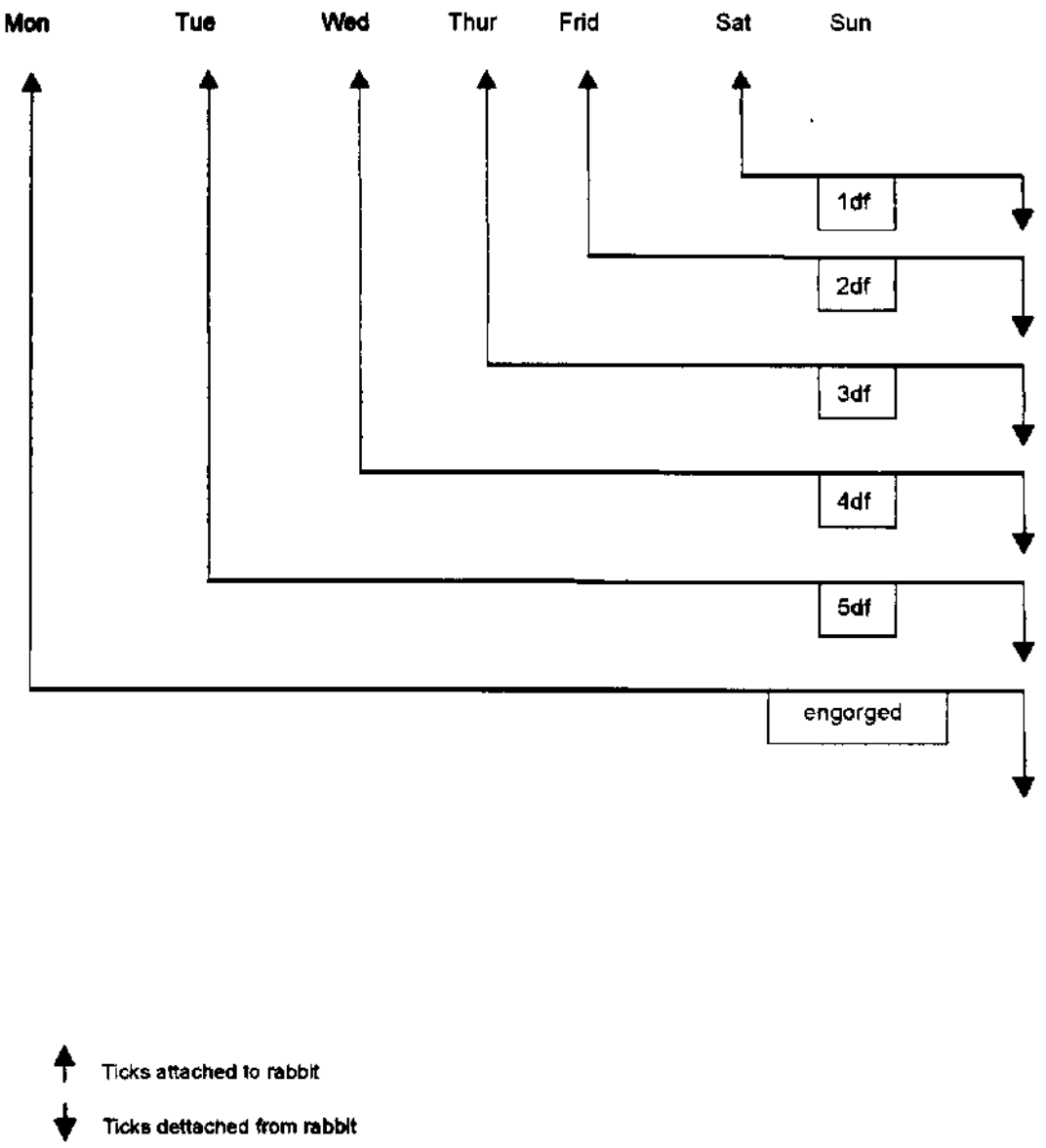


Figure 8.1. Synchronisation of attachment and drop off of ticks for enzyme studies

Table 8.1: Detecting the presence and activity of trypsin in the gut and haemolymph of *R. appendiculatus* and *R. pulchellus*

Species	Sample	Sex	Infected/ clean	Post feeding	Absorbence A_{405} (t)
R.appendiculatus	Gut	Female	+ve	Day 0	0.0465
R. appendiculatus	Gut	Female	-ve	Day 0	0.047
R. pulcehllus	Gut	Female	-ve	Day 0	0.042
R. appendiculatus	Gut	Female	+ve	24 hrs	0.043
R. appendiculatus	Gut	Female	-ve	24 hrs	0.046
R. pulchellus	Gut	Female	-ve	24 hrs	0.039
R. appendiculatus	Haemolymph	Female	+ve	Day 0	0.056
R. pulchellus	Haemolynph	Female	-	-	0.144

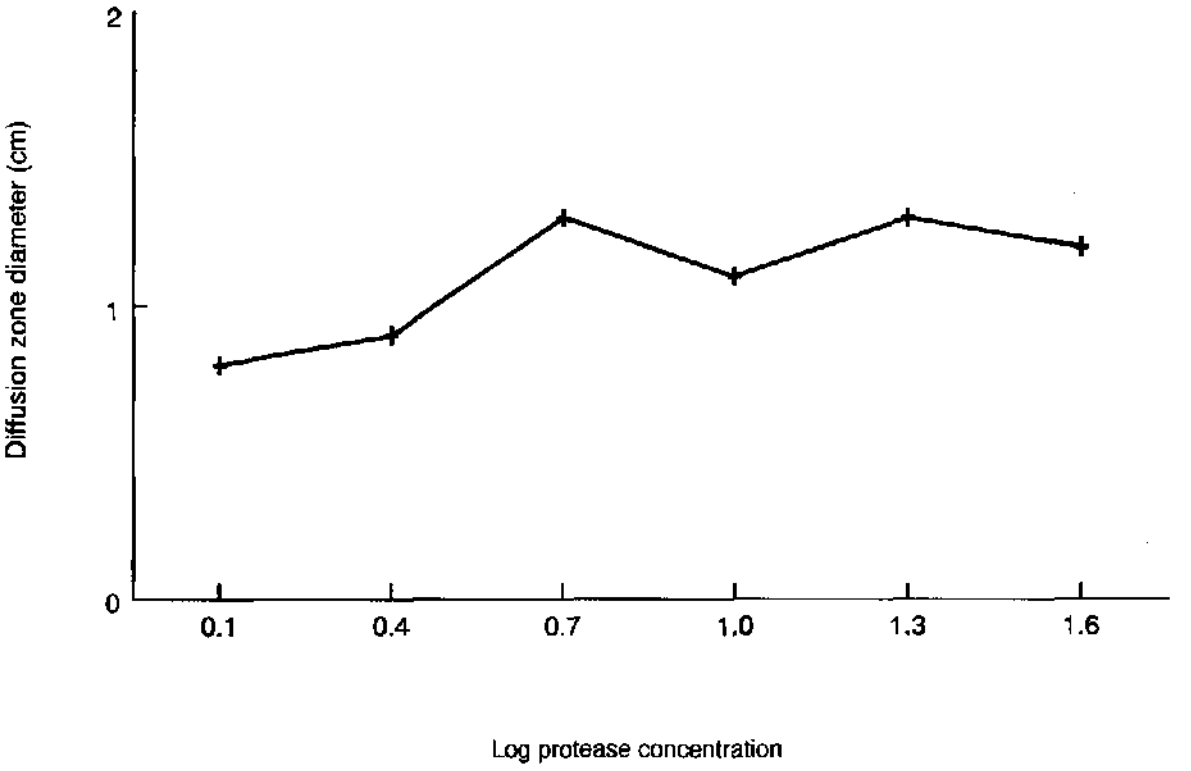
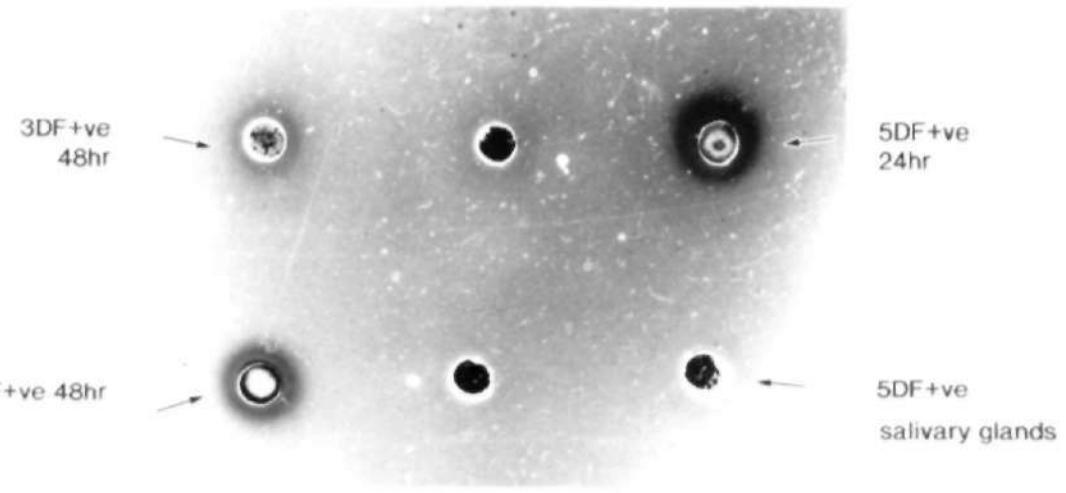


Figure 8.2 Protease standard curve relating the diffusion zone diameter (y) against log concentration (log x)

Detection of enzymes in the gut and salivary glands of ticks



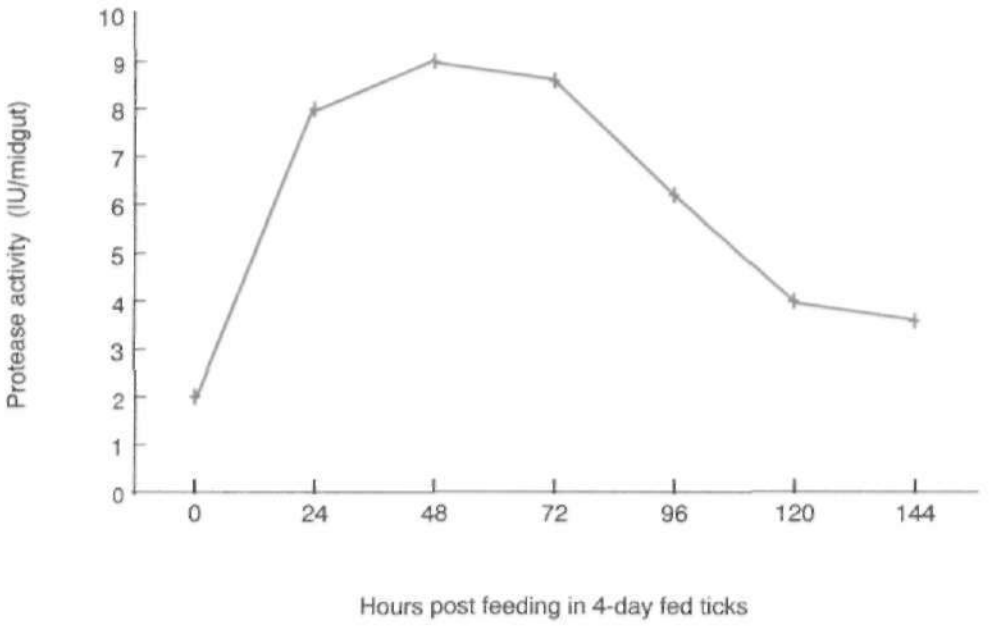


Figure 8.4 Protease activity in relation to period after feeding in *R. appendiculatus* guts

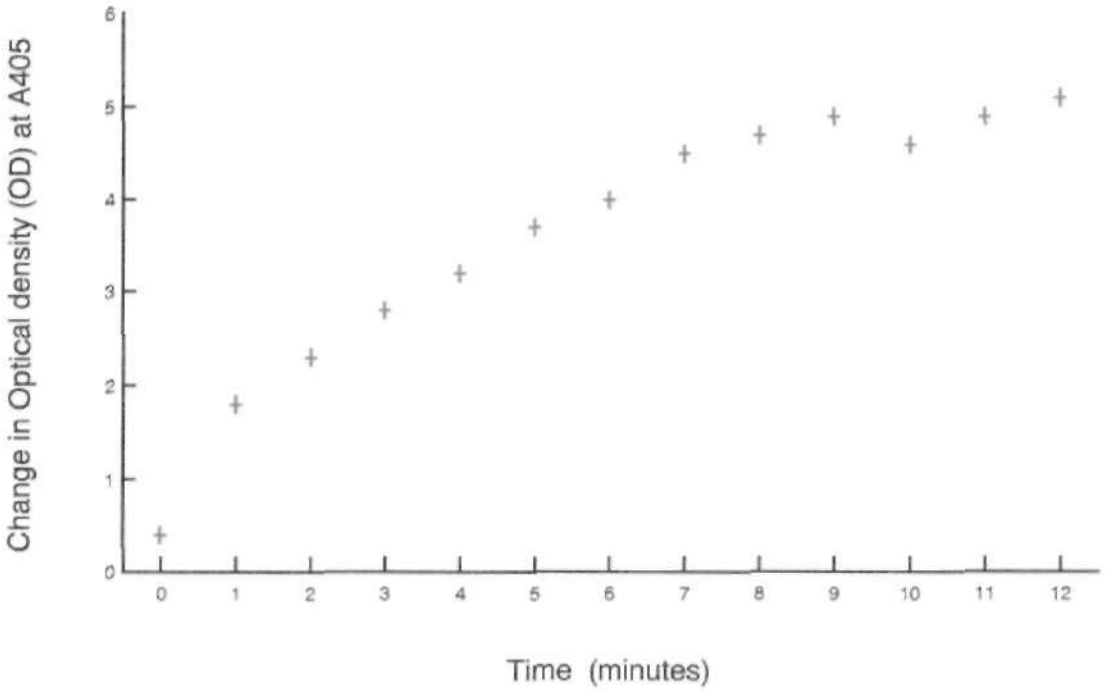


Figure 8.5 Trypsin-activity in the detergent solubilised *R. appendiculatus* gut

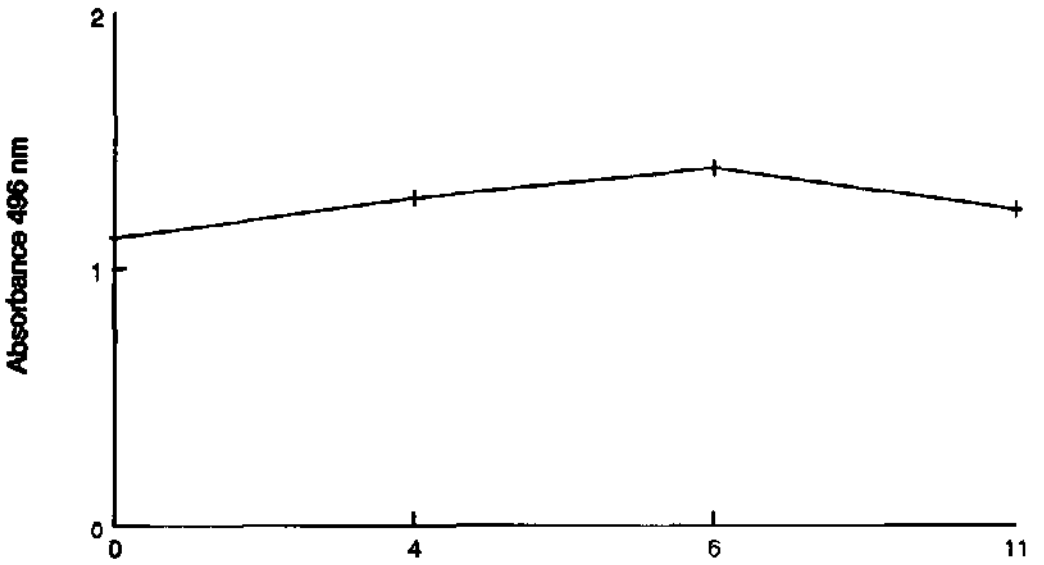


Figure 8.6 Auto-activation of phenoloxidase activity in freshly collected whole tick haemolymph

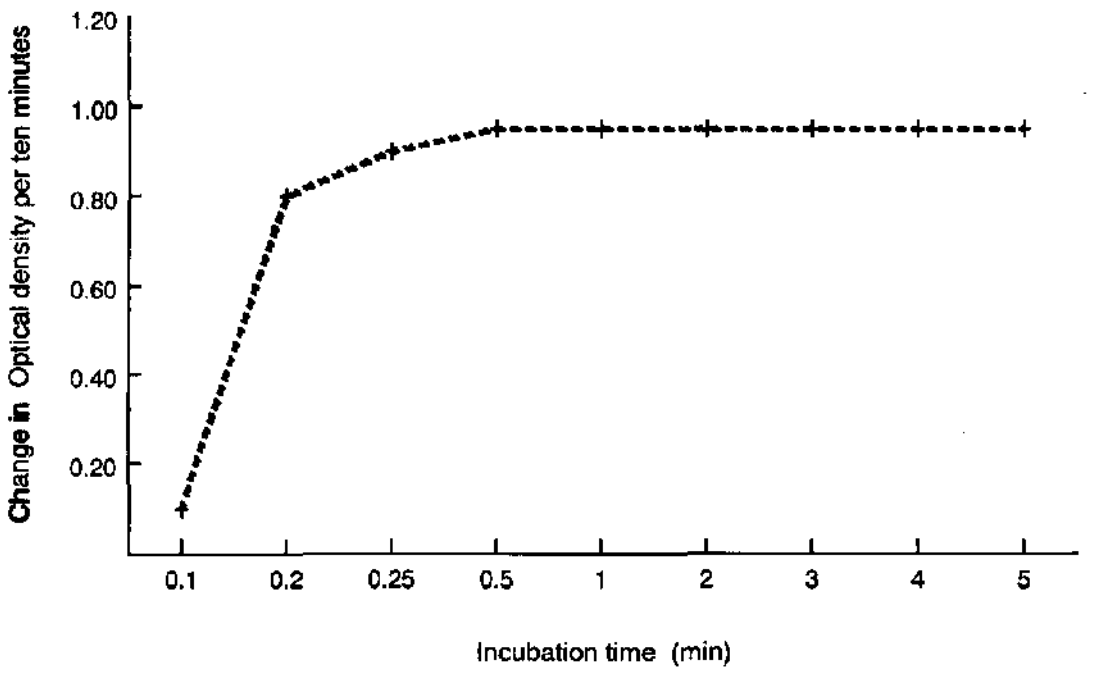


Figure 8.7 Phenoloxidase activity in the haemolymph of *R.appendiculatus* following activation by methanol

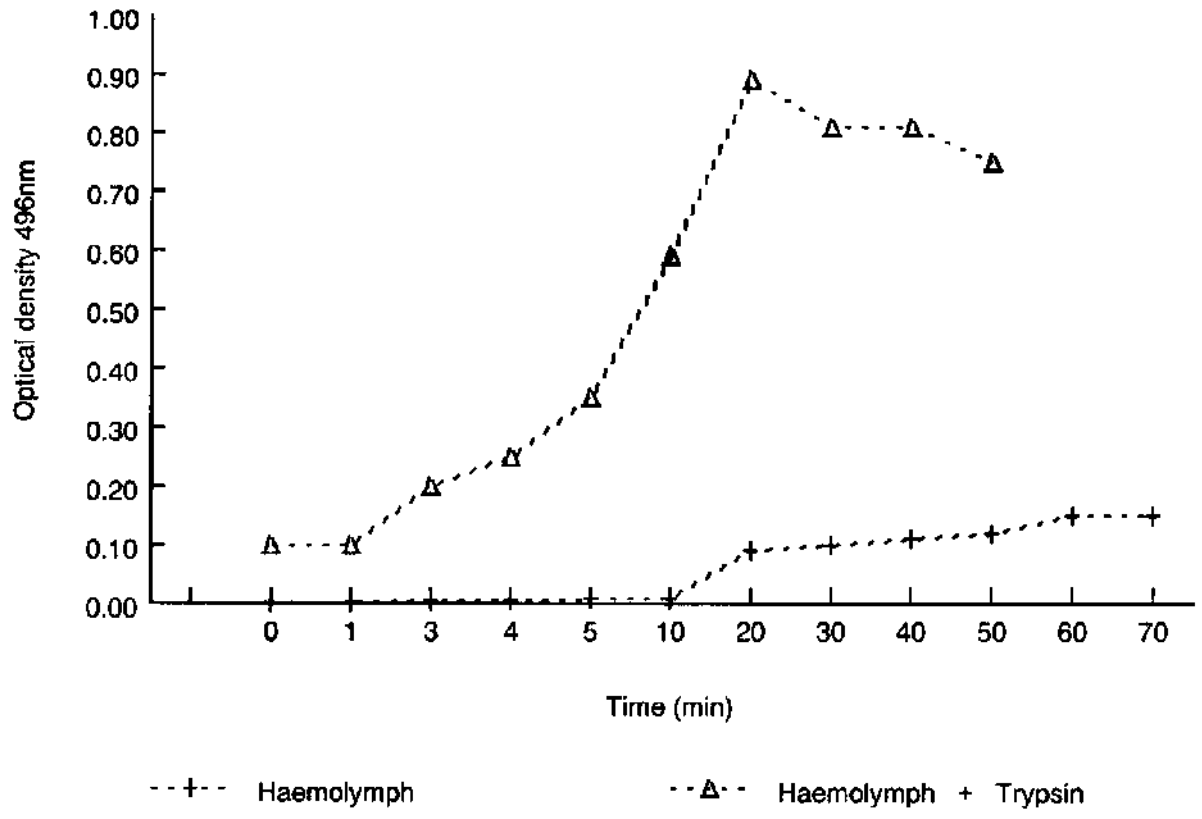


Figure 8.8 Enzyme (trypsin) activated phenoloxidaase activity in the haemolymph of *R. appendiculatus*

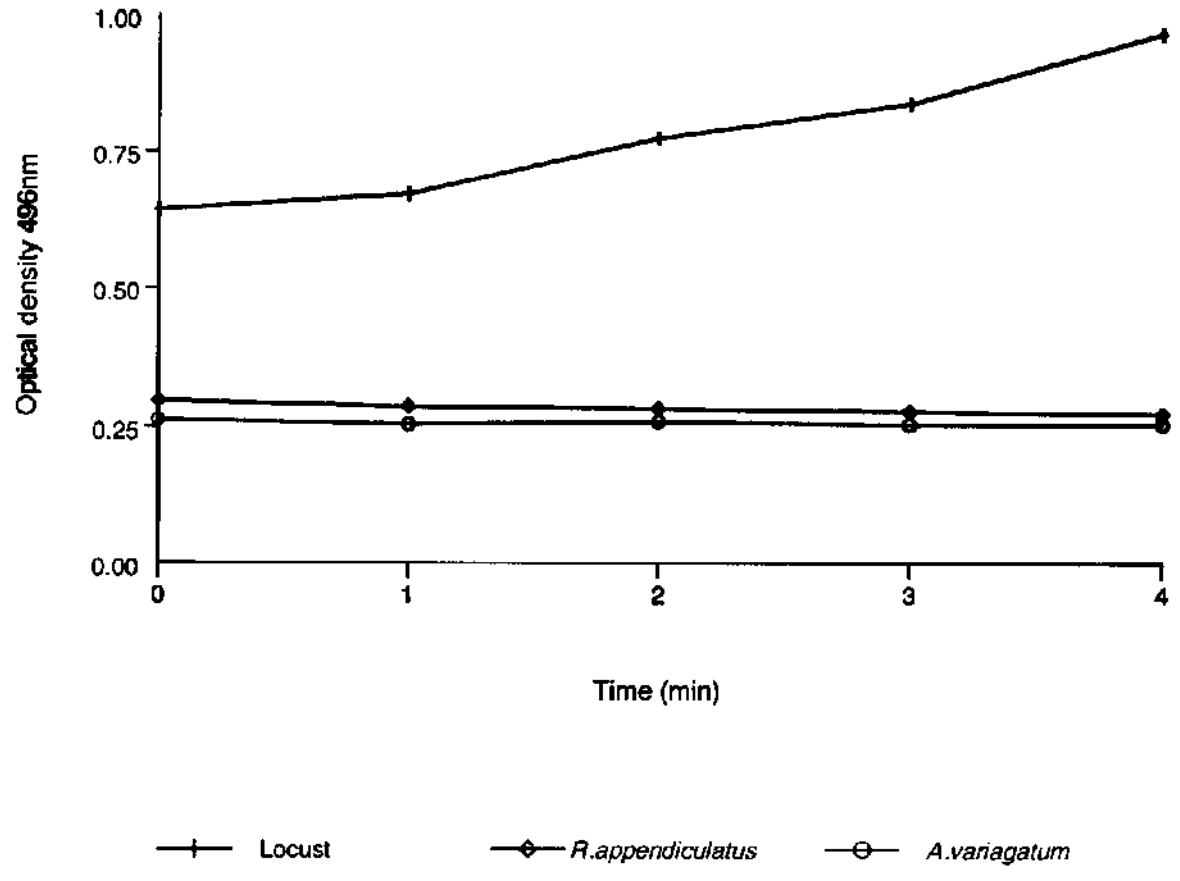


Figure 8.9 Comparison of phenoloxidase activity in ticks and insects

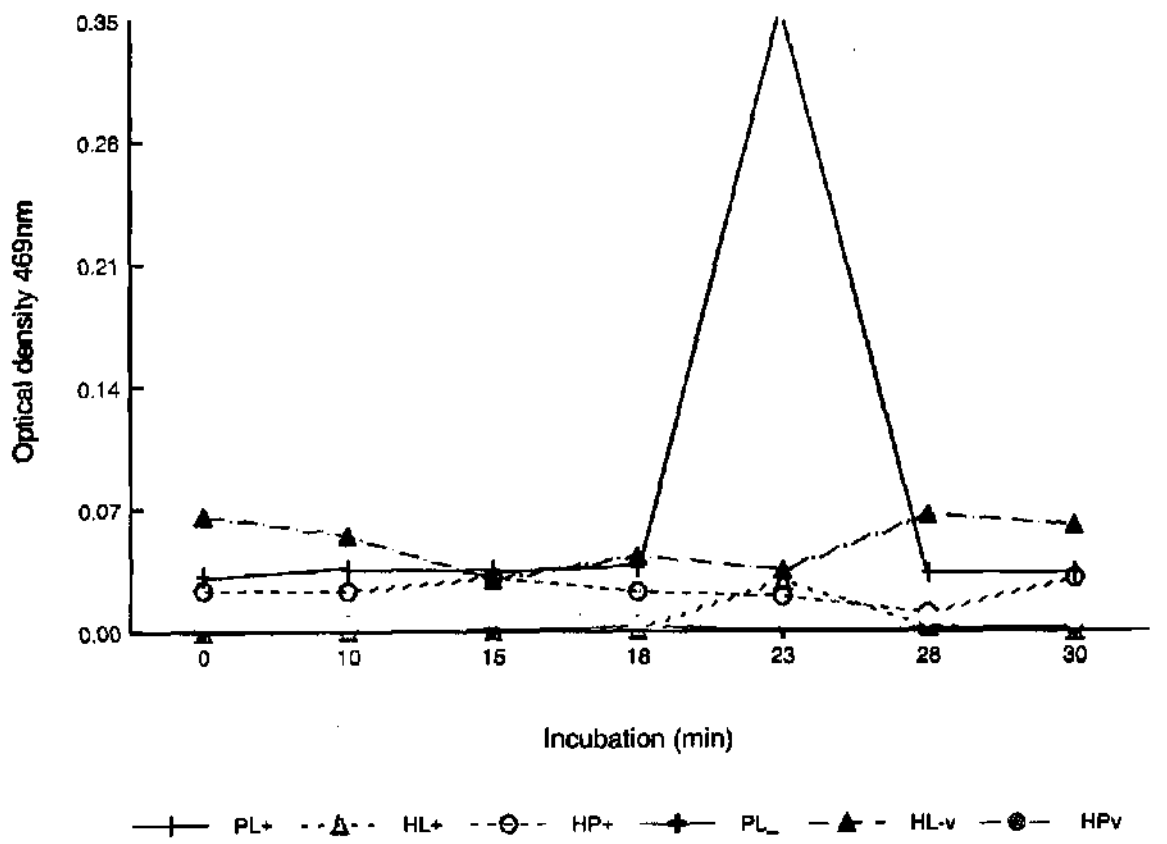


Figure 8.10 Phenoloxidase activity in *T. parva* infected engorged *R. appendiculatus* adults. Haeamolymph was centrifuged 900 x *g*; 5 min) and the supernatant (Plasma;PL) activated with methanol prior to addition of L-DOPA at 2mg/ml. Following homogenisation and centrifugation in 0.1M Sodium cacodylate buffer pH 6.9 the haemocyte lysate (HL) and haemocyte pellete were treated as above, optical density was read at 497 nm and ploted against reaction time. PL+ (Plasma from positive females); HL (Haemocyte lysate of +ve female); HP (haemocyte pellete of positive females).

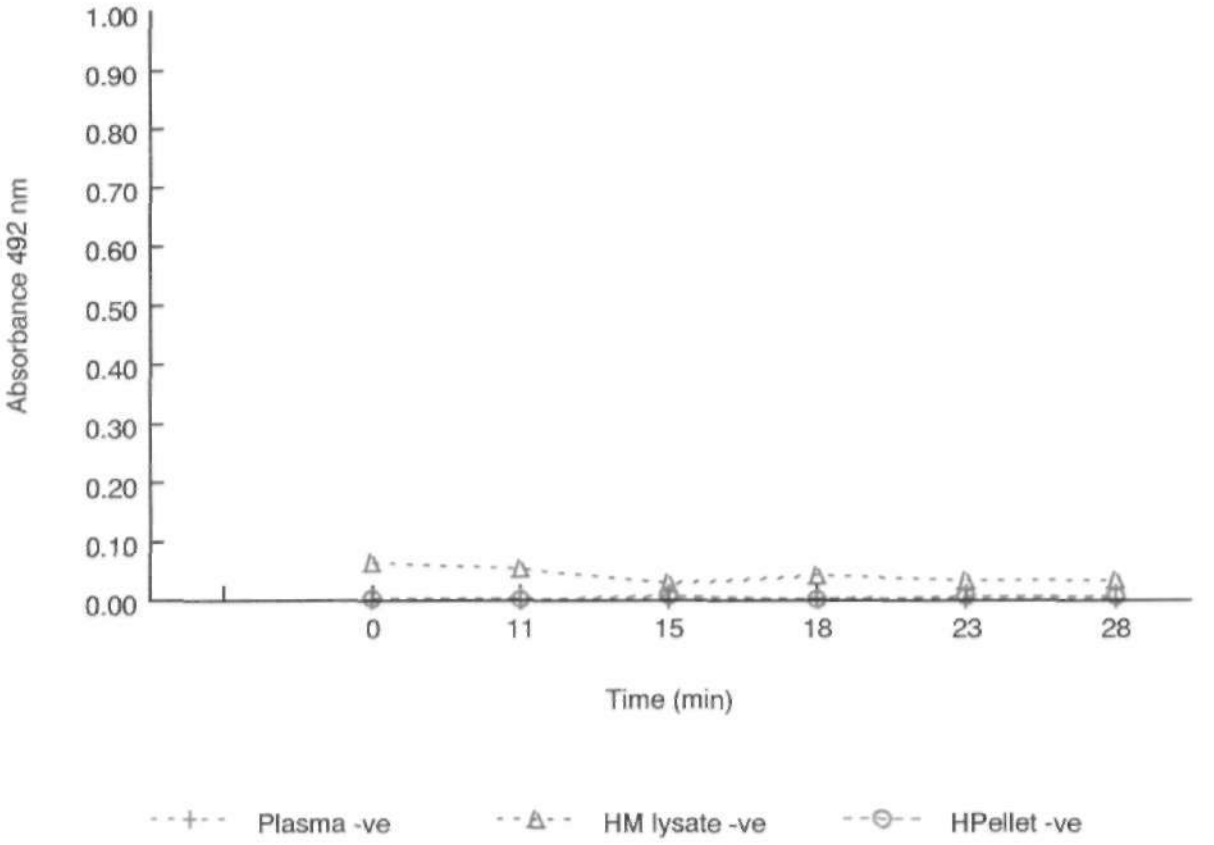


Figure 8.11 Phenoloxidase activity among clean (uninfected) *R. appendiculatus* ticks

CHAPTER NINE

9.0 INVASION OF *R. APPENDICULATUS* SALIVARY GLANDS BY *THEILERIA PARVA* PARASITES

9.1 Introduction

The life cycle stages of *T. parva* in its vector *R. appendiculatus* as described (Norval *et al.*, 1992) has been clearly illustrated in chapter 4. Briefly, in the gut, the red blood cells ingested by the tick are lysed releasing piroplasms. These transform into gametes that fuse into zygotes that later penetrate through the gut wall and transform into spindle shaped kinetes which are also motile. The kinetes leaves the gut through the haemolymph at the time of tick moulting to the next instar and invade the salivary glands. In the tick salivary glands, there are three types of acini, type I, type II and type III. Most infections of *T. parva* have been detected only in the type III acini (Fawcet *et. al* 1982b). The mechanism for this specialised selection are not well understood. There is a great possibility that in the tick vector, a comparable degree of selectivity occurs and has not been elucidated due to limited resolution of the light microscope, lack of evidence from the scanning microscope and the lack of dependable cytological criteria for the cell types. In the present study, a new exploration using a combination of scanning, light and electron microscopy for distinguishing and localising the parasite in the tick salivary glands, with the view to explaining why type III acini are selectively invaded, is described.

9.2 Materials and methods

9.2.1 Source of tissues

The tissues for transmission and scanning electron microscopy comprised of salivary glands of *R. appendiculatus*. For comparative purposes, ticks of the following categories were used:

- low and highly *T. parva* infected female salivary glands
- low and highly *T. parva* infected male salivary glands
- clean or un infected male and female salivary glands

9.2.2 Scanning electron microscopy

9.2.2.1 Fixation of tissues

For preparation of soft animal tissues like the tick salivary glands tissues for scanning electron microscopy, the method of double fixation was used (there are three methods of fixation: single fixation, combined fixation and double fixation). As opposed to the single fixation method whereby oxidation or reduction are used, or the combined method in which simultaneous oxidation and reduction are carried out, double fixation is by oxidisation after reduction (Glauert, 1975). Briefly, the tissues were prefixed with aldehydic fixative (Fixatives were available in two types: metallic fixative (OsO_4) based on oxidisation reaction and aldehyde fixative (glutaraldehyde) based on the reduction reaction).

The tissues were dissected and washed in Dulbeccos' PBS pH 7.4 and pre-fixed with 2.5 % glutaldehyde for 4 days for maximum fixation. The fixative was changed twice during this prefixation process. The fixative was then washed off the tissues using Dulbeccos' PBS pH 7.4 (on a shaker 10 min each) and transferred into 1% osmium tetraoxide solution for post fixation (2hr).

9.2.2.2 Dehydration

The fixative sticking to the tissues was again washed off as described above followed by dehydration in a series of increasing concentration of ethanol (30, 50, 70, 90, 96 % and three times in absolute) for 10 min each. The tissues were transferred on to a filter paper under the atmosphere to be air dried. The dried tissue thus obtained were stuck on metallic blocks for observation.

9.2.2.3 Preparation of metallic blocks

The metallic stabs, carefully numbered, were covered with double adhesive tape (Slotch) and tissues adhered. For further dehydration, the stabs were transferred into petridishes containing silica gel, which was previously heated at 100 ^{0} C to dry (silica gel appeared white at first but changed to blue on drying). The whole apparatus was transferred into a desiccation (Fisher Scientific; Brekel Phikla, Penna) for 24 hr.

9.2.2.4 Coating the tissues by vacuum evaporation

Since biological specimens usually have low conductivity, it was necessary to coat the specimen surface with a vacuum evaporated metal to prevent charge up and damage by heat. In this case, gold was used. These are highly effective in generating secondary electrons and the film made on the surface is not easily oxidised. In this process, the thickness of the film was kept uniform by rotating and tilting the specimen. Using a JEOL ion sputter machine, gold metal was coated on to the tissues by placing the metal stab in the sputter with a flame provided by Argon gas for 3 min within a vacuum. The ionising current used was 10mA, which vaporised the gold and coated the specimen. The stabs with the coated specimens were stored in silica gel until examined under a scanning electron microscope (JEOL Ltd. Japan) with an accelerating voltage of 10kv. The photographs were taken using a special camera and film (AGFA:Apx).

9.2.3 Transmission microscopy

9.2.3.1 Electron microscopy

To study the factors that influence kinete invasion of the salivary glands acini types, three different batches of ticks described above of both sexes were examined. In low infected ticks from both sexes, only type III acini usually get infected while among high infections, type II may be infected (Young pers.

comm). Highly infected ticks and clean (non infected) ticks were compared with the above.

9.2.3.1.1 Tissue preparation: Fixation

Salivary glands of ticks from each of the above three categories were dissected in Dulbeccos' PBS pH 7.4 with Ca^{2+} and Mg^{2+}, and immediately transferred into 2.5% glutaldehyde in 0.1M Sodium Cacodylate fixative overnight, whose composition is given below:

Glutaldehyde fixative composition

- 5.0 ml Glutaldehyde (25%; Agar)
- 2.5 gm Sucrose (or saccharose)
- 12.5ml Sodium cacodylate buffer (0.2M; pH 7.4)
- 5.0 ml $Ca\ CL_2$ (0.1M)

The mixture was made up to 50 ml with distilled water, giving a final concentration of 0.05M glutaldehyde; pH 7.2. Direct dissection into the fixative was difficult because the tissues stack on to the forceps.

9.2.3.1.2 Post fixation

Following the initial fixation, the tissues were washed 3 times in 0.1M Sodium cacodylate buffer, pH 7.4 on a shaker (Titertek, Flow laboratories; 10 min each). They were then post fixed into 10% Osmium tetraoxide (30-45 min) prepared in the following ratio:

- 1ml 0.1M Cacodylate buffer
- 1ml 4% Osmium tetraoxide

- 2ml distilled water.

To remove the Osmium tetraoxide, tissues were washed in 0.05M Sodium cacodylate buffer pH 7.4 and transferred into half saturated aqueous Uranyl diacetate ($UO_2(CH_3.COO)_2 2H_2O$; BDH, Analar) for block staining for a minimum of 2 hr. or over night at room temperature, in the dark. Half saturated Osmium tetraoxide was prepared by dissolving the Uranyl-diacetate (which had 5% solubility) into distilled water, then diluting the mixture into half (50 %). The Osmium tetraoxide fixes lipids and the reaction changes the tissue colours to black/dark brown. The glutaldehyde fixes proteins and glycoproteins. The Uranyl acetate does block staining.

9.2.3.1.3 Dehydration

The tissues were rinsed in sodium cacodylate buffer 3 times for 10 min each on a shaker to remove the Uranyl acetate and then transferred into a series of alcohol dilution starting with 30 % for 10 min followed by 50, 70, 80, 90 and 96 % for 10 min each. They were further dehydrated in absolute ethanol 3 times (10 min each) before being put into a mixture of absolute alcohol and Propylene oxide in a 1:1 ratio for 10 min and finally into pure propylene oxide for 10 min.

9.2.3.1.4 Infiltration of tissues

9.2.3.1.4.1 Araldite infiltration

The epoxy resins, which are polyaryl ethers of glycerol bearing terminal epoxy groups, were used as embedding media for electron microscopy because

they have the advantage polymerising uniformly with very little changes in volume and are relatively stable in the electron beam (Glauert *et al.*,1975). Their main disadvantage is their high viscosity which necessitated a lengthy infiltration procedure described below.

All tissues were transferred into propylene araldite overnight. The araldites used were mixtures of three basic components consisting of an epoxy resin, a hardener and an accelerator, sometimes with an additional plasticizer or epoxy flexibilizer (Glauert, 1975).

9.2.3.1.4.2 Preparation of Araldite

The Araldite in form of Epoxy aradite (Agar, Scientific Ltd) was provided in three epoxy resins which had to be mixed in the following proportions in order to ensure proper tissue blocking:

- 10 ml Aradite cy 212
- 10 ml Dodecyenyl succinic Anhydride ($C_{10}H_{26}O_3$;DOSA; a hardener)
- 0.4 ml Benzldimethy lamine ($C_9H_{13}N$;BDMA; a catalysit)
- 0.3 ml Dibutylphthalate ($C_6H_4(COO.C_4Hg)_2$; a stabiliser)

The above mixture was added to propylene in the ratio 1:1. The propylene acted as a carrier to the araldite into the tissues. All tissues were transferred into propylene araldite (ratio 1:1) overnight. To ensure slow and even evaporation, the tissues were left over night in the araldite/ propylene mixture covered with foil paper into which small perforations were made. This allowed slow

evaporation of the propylene, leaving the araldite inside the tissues. A fresh change of araldite was made the following day and the tissues again left overnight. The tissues were embedded after a further fresh change of araldite for 6 hr for further infiltration.

9.2.3.1.4.3 Embedding tissues

The tissues were embedded in araldite in Epo-Araldite in the embedding mould, (Agar Aids Ltd) under a microscope to ensure proper orientation and left at 60 ^{0}C for 3 days. The moulds were labelled with pencil labelled cards which were allowed to set simultaneously making proper tissue identification.

9.2.3.1.5 Trimming

The tissues were trimmed with the trimming machine (Ultrotome 8800 LKB, Bromma) and cut a in a microtome (Coreichert, Autria TM 60). Glass knives were prepared by first cleaning the glass bars with 100% ethanol and cutting them in triangular wedges, rough side facing downwards, with a Knifemaker (7800 LKB, Broma).

9.2.3.1.6 Sectioning

Sections were cut on to a "boat" made from black masking tape (Electrical tape, Scotch, U.K) fastened on to a glass knife. Dissolved wax was used to seal the boat on to the glass wedge. Water was put in the boat and section viewed through diffused light. A macrofeed that moved a distance of µl was used (LKB, Bromma). The sections floated on to the water and depending on their

thickness, showed different colours. They were flatted with chloroform. Thick sections (>1000 Å) appeared pink, while gold were thin 400-800 Å and used for EM. The grey ones, on the other hand, ranged between 300-500Å. Golden tissues for electron microscopy were picked on to copper grid (Agar) and stored in acetone until required.

9.2.3.1.7 Staining sections for electron microscopy

The tissues were stained in Uranyl acetate for 30 min after which they were washed with 50% ethanol. They were then counter stained in lead citrate (10 min) following which the specimens were washed in 0.02M Sodium hydroxide (NaOH) and finally rinsed in distilled water. The lead citrate stain was prepared according to Reynolds' (1963). It gave a clear delicate rendering of cellular structures. The citrate ions present in the staining solution helped prevent precipitation by acting as chelating agents. The reaction between lead citrate and sodium hydroxide resulted into a complex cation containing two lead atoms ($Pb(OH)_2Pb^{2+}$) which bound to reduced Osmium already present in the specimens, to hydroxy groups in carbohydrates and to the ribonucleic acid.

Reynolds' lead citrate stain composition

- 1.33g Lead citrate
- 1.76g Sodium citrate ($NaC_6H_5O_2$).2 H_2O
- 30 ml Distilled water

The mixture was put in a 50 ml flask and allowed to stand for 30 min with intermittent shaking to ensure complete conversion of lead nitrate to lead citrate.

Eight (8 ml) of 1 ml NaOH was added and the whole mixture made up to 50 ml with distilled water.

9.2.3.2. Light electron microscopy

9.2.3.2.1 Sections for light microscopy

Light microscopy sections were picked, with a tooth pick, on to a clean glass slide in a drop of water. The slide was heated on a heater (Gechardt, heater, Heizung) to flatten the tissue.

9.2.3.2.2 Toluidine blue Staining

Sections were stained in Toluidine blue (1 % Toluidine in 1% Borax; a metachromatic stain that stains the nucleus and cytoplasm blue) by putting a drop of the stain on to the section and heating the slide for a few seconds to help the Toluidine penetrate the araldite and enter the tissues. The stain was washed off with 70% ethanol followed by distilled water. The sections were examined under a microscope (Nikon microphot-FX).

9.3 RESULTS

Using scanning and transmission electron microscopy, a comparative study of the salivary glands in the adults of *R. appendiculatus* was made. The salivary glands represented different degrees of infections; highly infected; low infected and clean or non infected. The highly infected had many acini containing the parasites. They also had a high intensity (number of infected acini per tick

)compared to the low infected. The results below include only the highly infected and clean ticks.

9.3.1 Scanning electron microscopy

9.3.1.1 Structure of Salivary glands

The external structure of the salivary glands in *R. appendiculatus* revealed three morphologically distinct acini (Fig 9.1-9.6) on the basis of their characteristic surface structures. The shape of the salivary glands in general ranged from round or oval shaped and appeared much more like grapes. On the outside, the surfaces differed considerably with some acini smooth and others having sculpted surface patterns. The three acini were also different in size. Below is a brief description of each of the acini.

9.3.1.1.1 Type I acini in male and female *R. appendiculatus*

Type I acini in clean adults were the smallest of all and were located adjacent to the main salivary duct (Fig 9.1A). Their external surface was smooth as opposed to the type III acini and were all round to oval shaped. They were uniformly shaped and sized (Fig.9.1A) and closely packed forming a "grape" like appearance (Fig.9.1B).

9.3.1.1.2 Type II acini in male negative ticks

The type II acini were also round to oval but were more in the middle part of the salivary duct (Fig.9.2A & B). They were medium in size, slightly bigger than type I acini but smaller than type III and had sculpted surface or

indentations on the surface (Fig 9.2C). They occurred as grape-like structures along the secondary branches.

9.3.1.1.3 Type III acini in male negative *R. appendiculatus*

Type III acini were located to the posterior end of the salivary duct. They were more to the outside of the "*Grape batch*". In terms of size, they were bigger than the above two types. They were characterised by patterns on the surface which appeared rough. The whole structure was covered by fibrous material. At higher magnification pits occurred (Fig 9.2D).

9.3.1.1.3 Salivary glands acini from negative female ticks

The type I and type II acini in negative female ticks appeared like the ones in males (Fig 9.3 A&B).

9.3.1.1.4 Infected acini

No infected type II and I acini were observed among the infected ticks examined. Among the males, infected type III acini had a rough surface (Fig. 9.4 A), compared to the negative acini (Fig. 9.2 B). They had a "dusty" appearance (Fig. 9.4B). The Type III acini from highly infected females appeared bigger, more diffuse on the surface compared to the negative counterparts, that had showed compact indentations (Fig 9.4B).

9.3.1.1.5 Male and female acini

There were no major visible structural differences between the male and the female salivary glands although the females are believed to have more acini than the males (Young and Letich, 1981). Type I had smooth surfaces, Type II was patterned and Type III had surface sculptures. Among the males, the acini located at the periphery were bigger and rough surfaced. The only difference noted between males and females was that in males the acini type III had a "dusty" almost "woolly" appearance (Fig 9.4) at higher magnifications.

9.3.2 Transmission electron microscopy

9.3.2.1 Acini and cell types

The salivary glands of the adult male and female ticks consisted of over 500 acini comprising three morphologically distinct types (Fig 9.6-9.9). Cross sections of the salivary glands under light microscopy demonstrated the Type I acini to be situated adjacent to major salivary gland duct (9.6). This acini lacked many of the electron dense granules (agranular) and is concerned with osmoregulation (Soneshine, 1991). Each acini comprises a single large cell surrounded by peripheral cells. The granular acini, on the other hand, showed distinct cell types under the light microscope (Fig. 9.7). Note the secretory granules, stained light, medium and electron dense.

9.3.2.1.1 Cell organelles

Under the electron microscope, Type I acini consisted of a large cell type specialised for fluid and electron transport (Fig 9.8A&B) unlike type II and Type III that had several cell types. Besides being agranular, it was located at the anterior end of the salivary gland duct. Type I acini had a wedge shaped central nucleus (Fig 9.8B). It also had plenty of clear vacuoles (Fig. 9.8B). Its structure is suitable for osmoregulation and for fluid and electrolyte transport (Soneshine, 1991). The cell organelles such as rough endoplasimic reticulum, smooth endoplasmic reticulum and ribosomes could not be seen in this acini Type, but vacuoles were plenty together with peritubular cells having extensive basal in foldings. The basal labyrinth was well developed with lamellae. The cytoplasm was peripheral. Several mitochondria were observed (Fig 9.9A&B). There were abundant lipid constrictor cells or other osmoregulatory cells (Fig 9.9C).

9.3.2.2. Type II

Type II acini were observed to be concentrated along the lobular ducts in the anterior half of the gland (Fig 9.9). They contained cells whose granules were smaller than those observed in type III acini (Fig.9.10).

9.3.2.2.1Cell organelles

Type II acini were observed to have distinct cell types with a clear nucleus (Fig 9.10) and distinguishable secretory granules. Some of the cell types were smaller but had large secretory granules (Fig 9.11-9.12) . The mitochondria were

big with cristae (Fig 9.13 A). Other cell organelles observed included endoplasmic reticulum distributed at the cell periphery (Fig 9.13B). The nucleus was characterised by a coarse chromatin with uniform karyosomes finely distributed along the nuclear envelope. The nucleoplasma exhibited differentiation into areas of condensed and dispersed chromatin of a lighter stain. Large intercellular spaces were also observed. The cytoplasm was seen with endoplasmic reticulum without any anastomosis and branching (Fig 9.13 B). The mitochondria were very prominent in the cytoplasm. They varied from oval to elongate and spindle shaped with clear tubular invaginations of the inner membrane (cristae Fig. 9.13A).

9.3.2.3 Type III

Type III acini were most numerous at the periphery of the salivary gland in its posterior half associated with terminal branches of the salivary duct system and had three visible cell types D, E and F as described by Fawcett *et al.*, (1982). The acini were granular (Fig 9.14A-D). The D cells contained heterogeneous granules in subunits of varying electron density (Fig 9.14A) which function as cement secretors to ensure firm attachment to the host. The e cell had numerous homogeneous moderately dense membrane bound granules composed of densely subunits (Fig.9.14B). There were no golgi complex observed in both cells probably because in these cells the granules are formed in the cistae of the endoplasmic reticulum (Fig. 9.14C). The F cells lacked the granules and are specialised for water and fluid balance (Fig. 9.15).

9. 3.3 *T. parva* infected acini

9.3.3.1 Type I acini

No *T. parva* sporozoites were observed in type I acini.

9.3.3.2 Type II acini

Type II acini similarly showed no infections. The cell organelles were clearly distinguishable. Mitochondria, rough and smooth endoplasmic reticulum were defined.

9.3.3.3 Type III acini

Although in the clean ticks the mitochondria, smooth and rough endoplasmic reticulum and the nucleus of e cells were all well defined and clear and the glycogen granules dark and clear, in the positive acini, these organelles were more diffuse in nature. The cell organelles were diminished in some cases such as the nucleus being pushed to the side. Sporoblasts were observed in this acini type. The granules which had been observed in the negative ticks were no longer visible. The cell organelles are treated separately below:

Nucleus

In the uninfected acini, the nucleus was observed to have characteristic coarse chromatin in which karyosomes were uniform in size and uniformly distributed (Fig 9.16A). In the parasitised cell, however, the nucleus appeared enlarged (Fig.9.16B). Among the cytoplasm, there was autolysis of the

endoplasmic reticulum and excretory granules which created a decrease in the electron density of the host cells (Fig 9.16B).

Mitochondria

The infected e cell contained mitochondria in the apparently increased cytoplasm volume (Fig.9.17A). Such an organelle occurrence was indicative that the infected cells were metabolically active and that they required energy.

Endoplasmic reticulum

Well developed endoplasmic reticulum was observed in uninfected e cells of the Type III acini signifying their role in secreting large amounts of protein. In the infected cells, however, a reduction in the endoplasmic reticulum was observed probably due to dispersion in the enlarged volume of the cytoplasm as a consequence of the hypertrophied cell (Fig.9.17B).

Glycogen deposits

The uninfected e cells did not reveal the presence of glycogen deposits in the e cells, a confirmation to earlier observations that glycogen is not normally observed in the glandular cells (Fawcett *et al.*, 1982) . In the parasitised cells, however, deposits of particulate glycogen were observed dark granules (Fig.9.18). The presence of glycogen deposits implied that the invasion of the parasite induces an alternation in the carbohydrate metabolism resulting in the accumulation of glycogen in this cell. As the parasites develops, the deposits decrease, until the cell is full of mature sporozoites (Fig 9. 18) and no more deposits were visible.

***T.parva* sporozoites**

The parasite mature sporozoites were clearly visible (Fig. 9.19) in the type III acini.

9.4 Discussion

In this study, scanning and electron microscopy has been used to illustrate the external surface structures of salivary glands of *R. appendiculatus*. In addition, transmission electron microscopy at light and electron level have demonstrated the internal structure of the salivary glands. Three acini types have been observed. Structurally, the position of these acini in relation to the salivary duct is such that the type I acini are most proximal to the duct followed by type II and the distal end is type III acini. The study has also confirmed earlier observations in the cell organelles that all types are structurally different.

Among the infected ticks, *T. parva* infections were only observed in one particular cell the type III e cell. Similar results were observed by several authors (Fawcett *et al.*, 1982b; Binnington *et al.*, 1983). This high degree of selectivity implies special characteristics peculiar to the e cell that favour parasite invasion and subsequent development.

Changes in the cell organelles were observed in the infected cells implying that the parasite influences the type III e cells in organelles structure on infection. The cytoplasmic volume increased and the nucleus and secretory

granules were reduced in number. Similar reports were obtained by Fawcett *et al.*(1982b; 1985) and Young *et al.*(1983). Deposits of particulate glycogen were observed in the infected type III acini. This observation indicates that the invasion of the parasite induces a change in the carbohydrate metabolism resulting in the accumulation of glycogen in type III acini e cells. There was later a decrease in these deposits indicating that the parasite presence changes the metabolism of that cell. This would perhaps mean that there could be a chemical attraction to the particular type III acini e cell at the time of kinete penetration which is then complemented by the specialised cell structure. At this point it is possible that the kinete is aided both by the receptor and the chemo attraction. Furthermore, the endoplasmic reticulum were diffuse among infected cells implying that a chemical signal may be involved in attracting the kinete to the e cells.

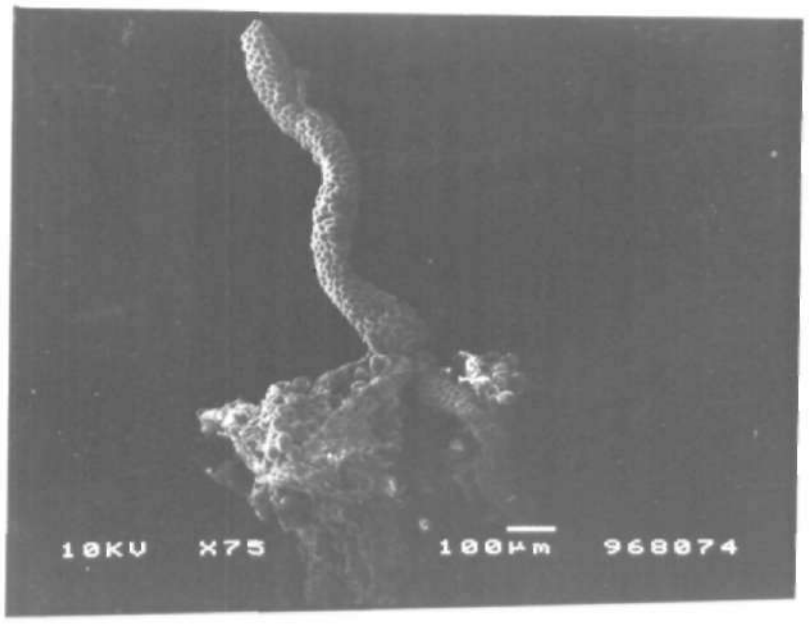
Looking at the scanning micrographs, infected cells had pits on the surface implying that the kinetes penetrate using a selective mechanism. The pits were all uniform indicating that this is a specialised process. There is most probably a receptor on the type III e cell surface that the kinete recognises and utilises in its penetration.

The mechanism for this specialised selection of type III acini has been a subject of debate and is not well understood. According to Norval *et al.* (1992), the invasion is by chance since the type III acini are located on the outside of the salivary glands and are located to the gut so that when the kinetes migrating from the gut, through the haemolymph gain easy access to these acini. On the

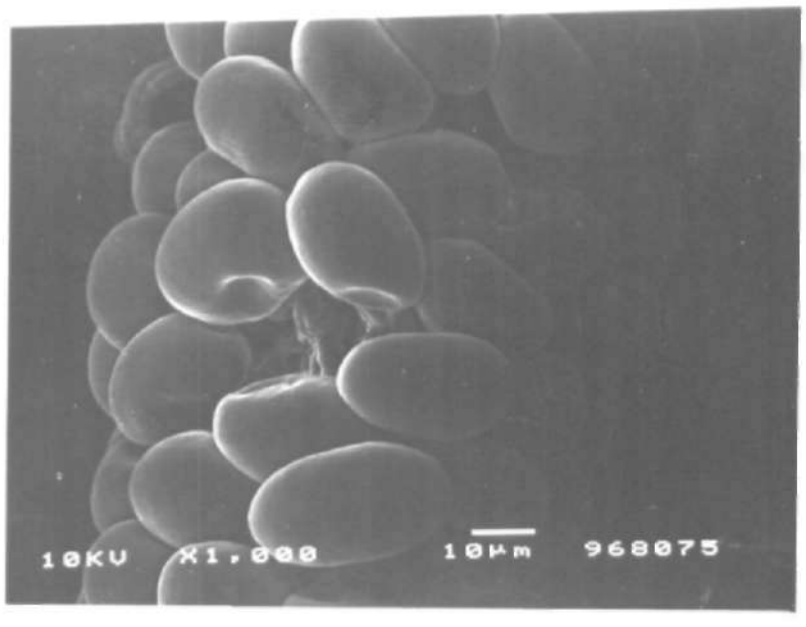
other hand, Bell (1980) found no chemo-attraction to the kinetes *in vitro*. Judging from these studies, no other cell, apart from the type III have been observed to be infected. Similar results were observed by several authors (Fawcett *et al.*, 1982a&b; 1985; Binnington *et al.*, (1983) although Type II acini D cells were claimed to be infected by Mehlhorn and Schein (1984). Similar studies that related basic physiology of the salivary glands to the function and susceptibility of trypanosome infection of *T.brucei* in *Glossina* have been reported (Okolo *et al.*, 1990). The hypothesis investigated here was that there may be a structural adaptation as envisaged by the electron microscopy studies that there may be a receptor on the type III acini. These differences in morphology relating to physiology of the salivary gland acini have been elucidated using ultrastructure and scanning microscopy.

Furthermore, the fact that infected type III acini was observed to have several cell types suggests that the mechanism of sporozoite entry into the type III acini most likely involves specific receptors in order to target the right cell. Looking at the same parasite in the cattle host, *T. parva* demonstrates cell type selectivity in the mammalian host at the sporozoite stage, infecting lymphoid cells preferentially at the sporozoite stage and erythrocytes at the merozoite stage (Norval *et al.*, 1992). The parasite does not utilise rhoptries for entry but uses them to destroy host cell. This prevents the host cell from being able to use lysosomal activity against it (Norval *et al.*, 1992). Similarly, in the tick the salivary glands infected e cells become hyper plastic with great enlargement of the cell and nucleus (Binnington *et al.*, 1983; Fawcett *et al.*, 1982b). A similar

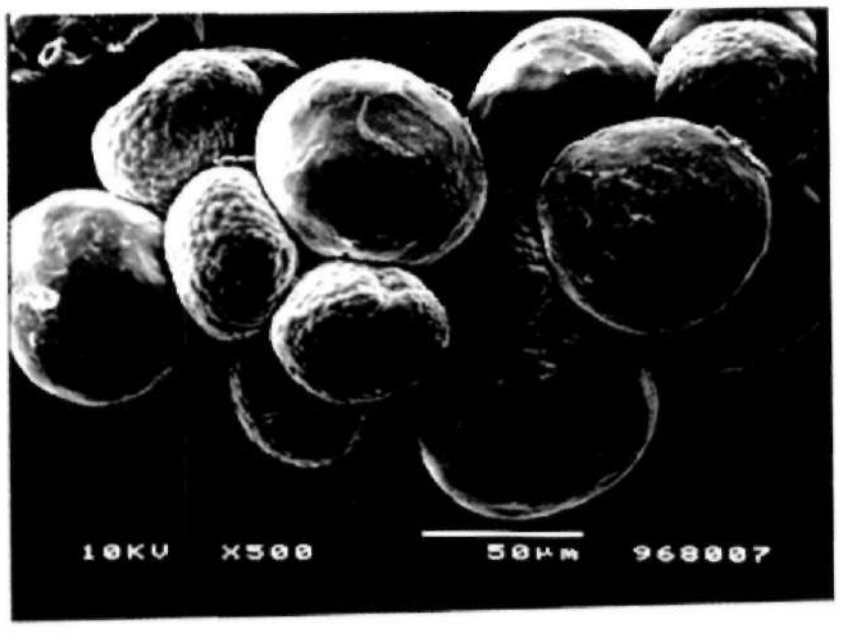
mechanism occurs when the parasite enters the lymphoid cell which become modified by the parasite to proliferate into schizonts. The parasite seems to take over the cell for its reproduction whenever it develops and the likelihood of there being a receptor to enter a particular cell seems the best option. In related *Plasmodium* parasites, malarial sporozoites invade only the medial lobe and the distal portions of the lateral lobes of the salivary glands of the vector mosquitoes (Perrone *et al.*, 1986). Surface characteristics were also shown to mark the identity of such sites. The basal laminar over the various regions of *Aedes aegypti* salivary glands was characterised by unique carbohydrates. It is unlikely, therefore, that the kinetes enter the type III acini by chance as proposed by Young *et al.*, (1980) and Norval *et al.* (1992). It seems that the parasite has already got evolutional advantage to invade, develop and overtake the invaded cells for its own survival and reproduction, and to do this, it must have special receptors and chemo attraction to these cells. This suggests that surface receptors may provide the specificity that limits invasions by kinetes to particular salivary gland acini in particular the e cells. Further studies using fluorescein labelled lectins to label the salivary glands of the tick vectors are recommended.



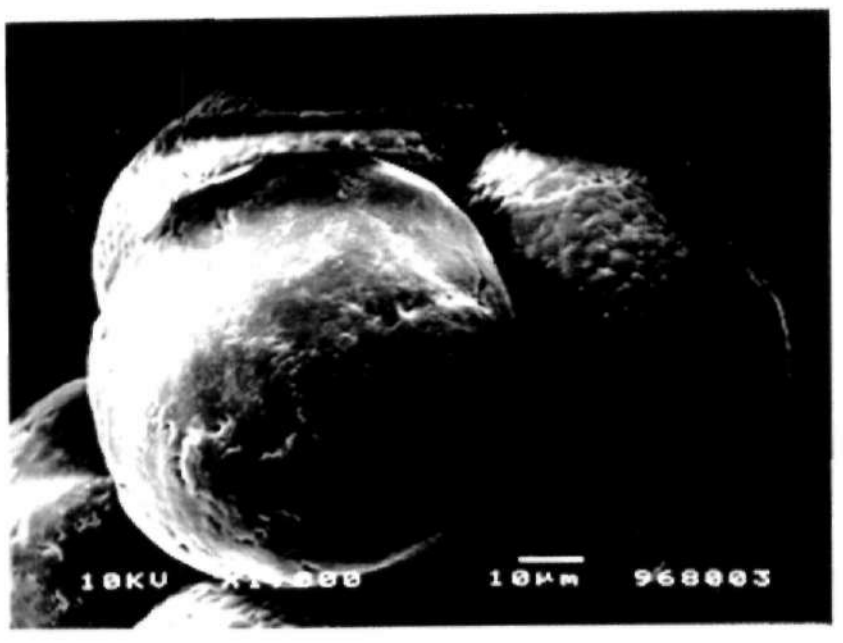
A



B



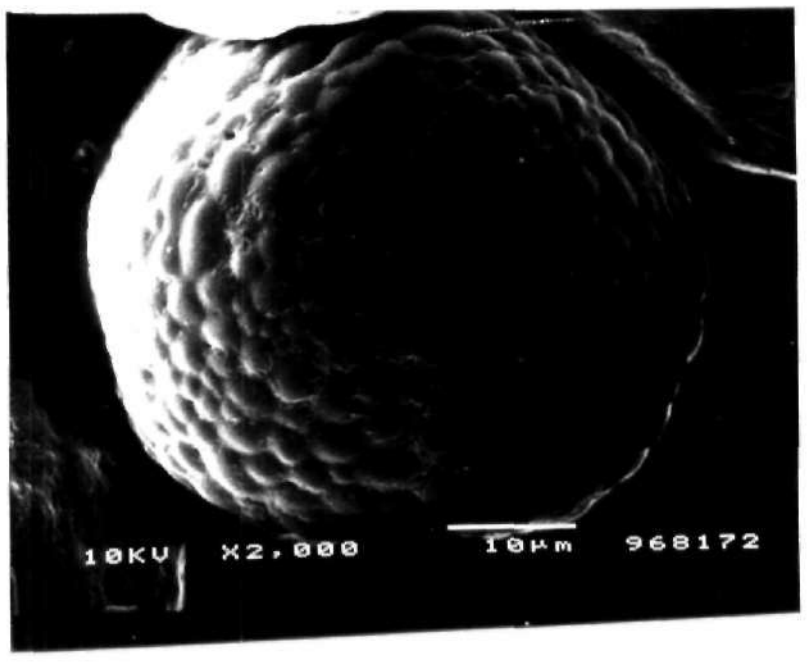
A



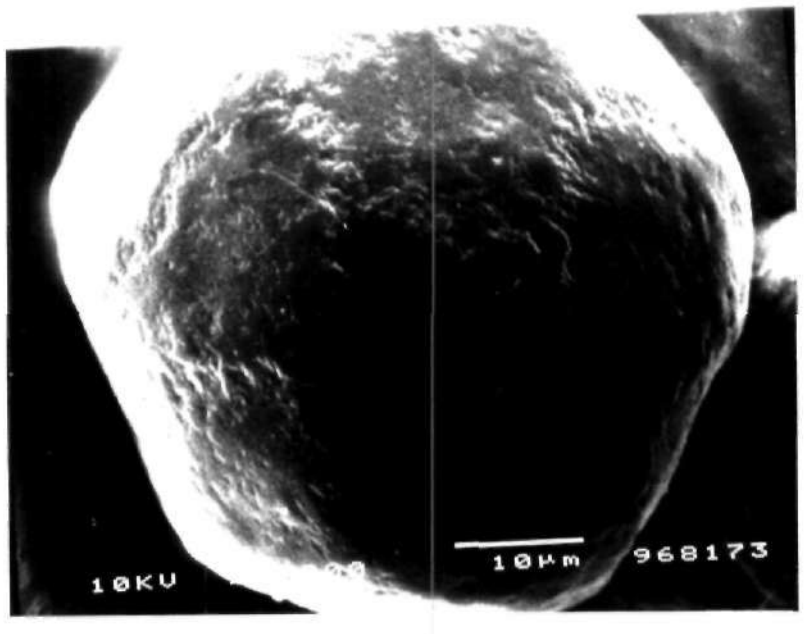
B

Figure 9.2C Scanning micropgraph illustrating male *R. appendiculatus* negative type I I acini surface patterns (x 2000).

Figure 9.2D Male negative salivary gland type II acini.

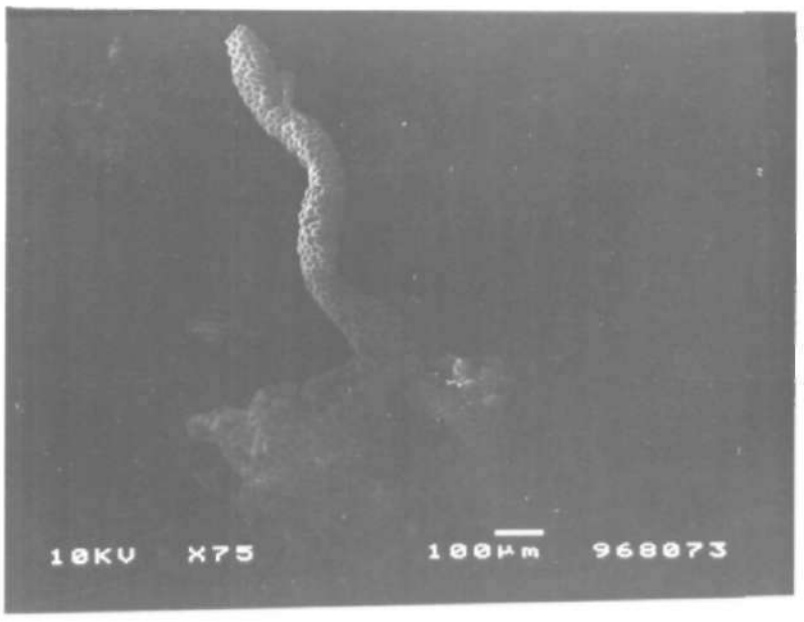


C

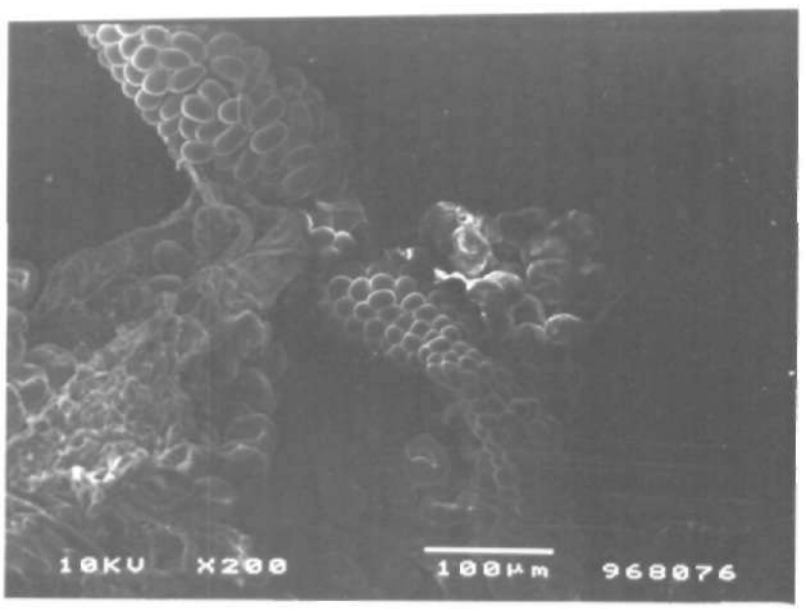


D

Figure 9.3 A&B. Scanning micrograph illustrating type I and II acini from a negative female tick



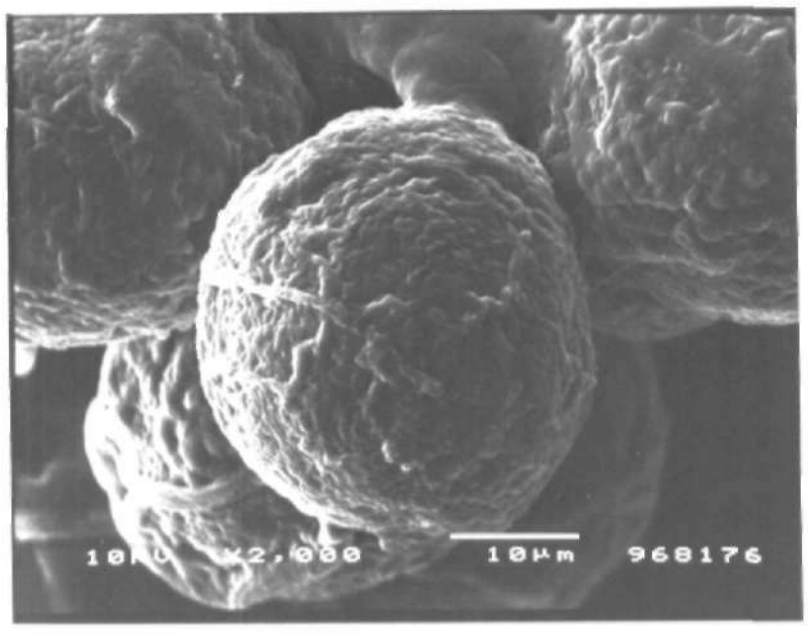
A



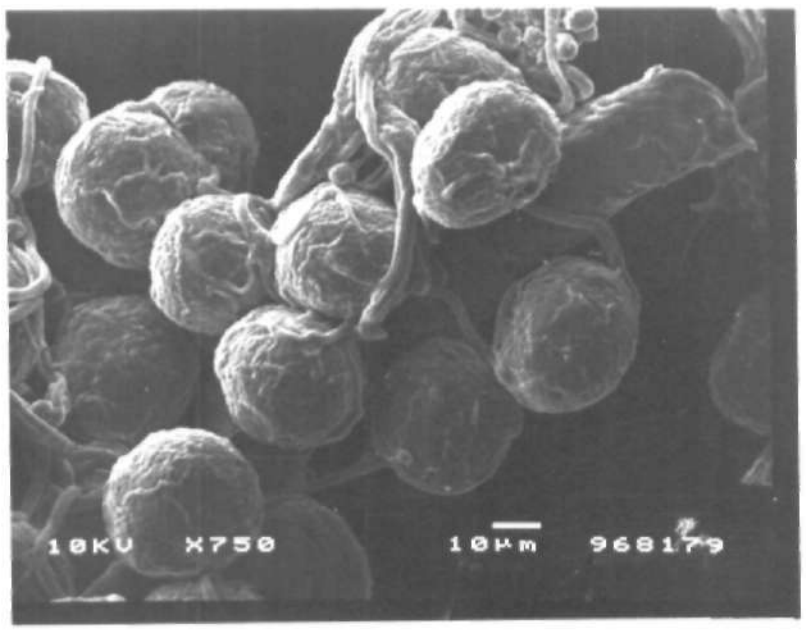
B

Figure 9.4A Scanning micrograph of infected type III acini from male adult *R.appendiculatus* (x 2000).

Figure 9.4 B A group of infected type III acini from male *R. appendiculatus* (x 750)



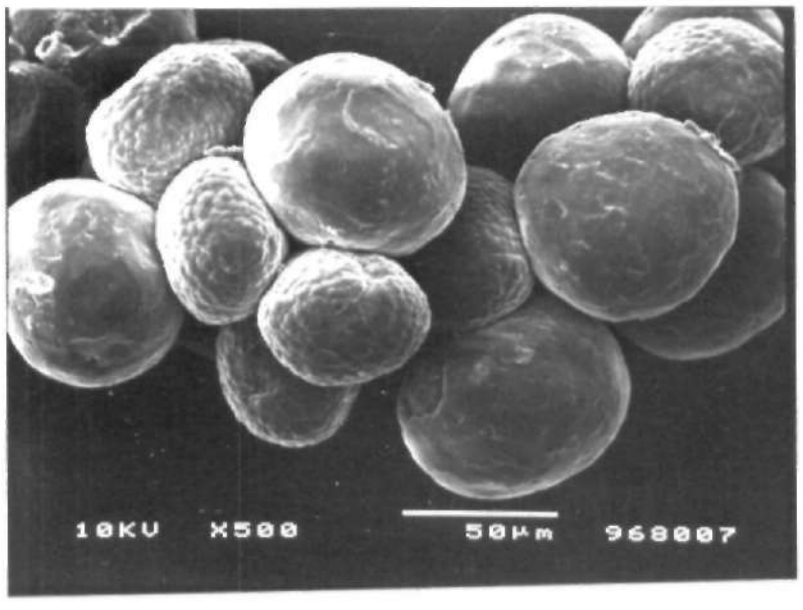
A



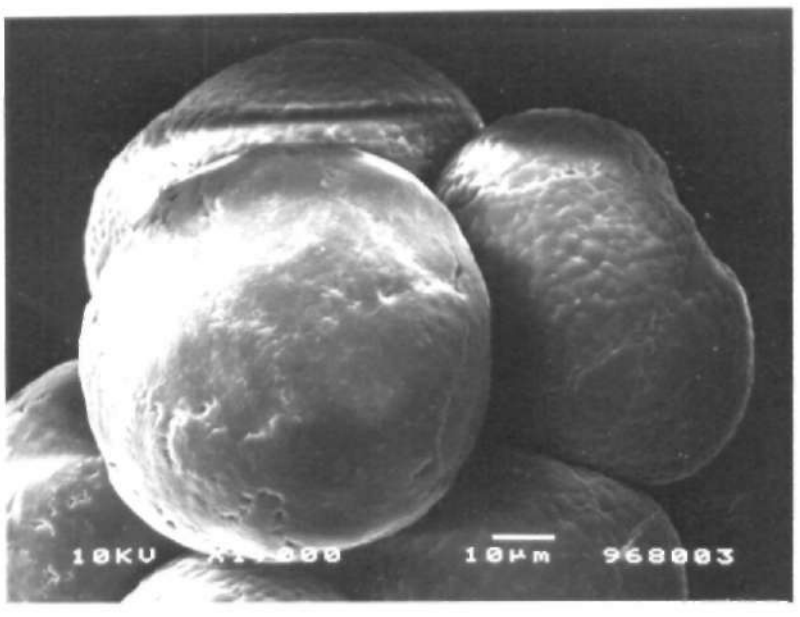
B

Figure 9.5A Scanning micrograph illustrating granular acini from infected adult female *R. appendiculatus*. The acini are bigger and more diffuse (500).

Figure 9.5B Higher magnification of acini (x 1000).



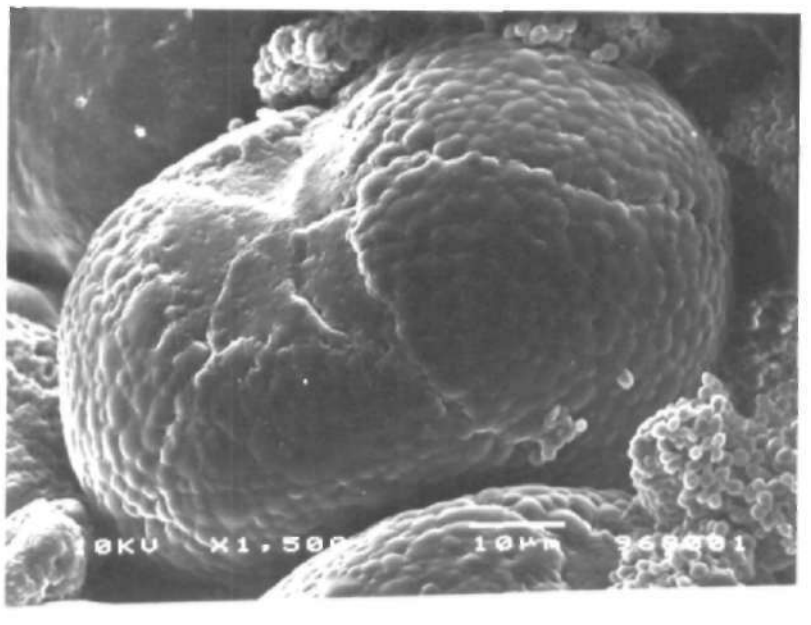
A



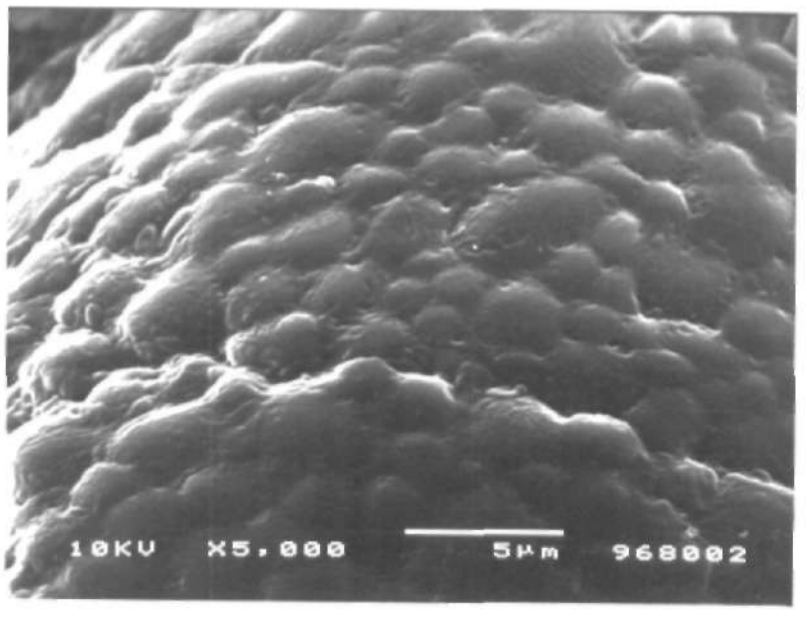
B

Figure 9.5C Surface structure of infected granular acini from female *R. appendiculatus* showing undulations (x 1500)

Figure 9.5D Surface structures of type II acini from infected female *R .appendiculatus* (x 5000)



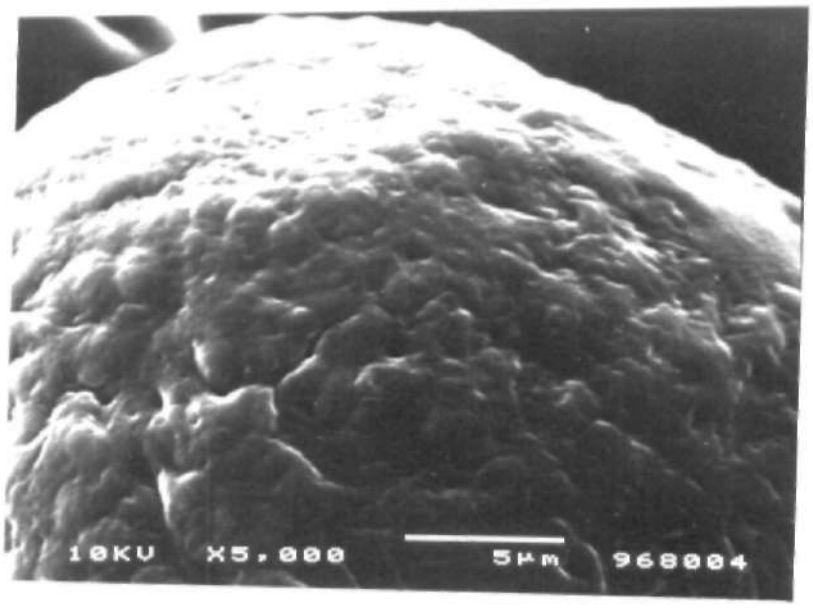
C



D

Figure 9.5E Surface structure of type III acini from a negative female *R. appendiculatus* (x 5,000). Note the protrusions of the negative tick are not as prominent as those of the positive ones.

E



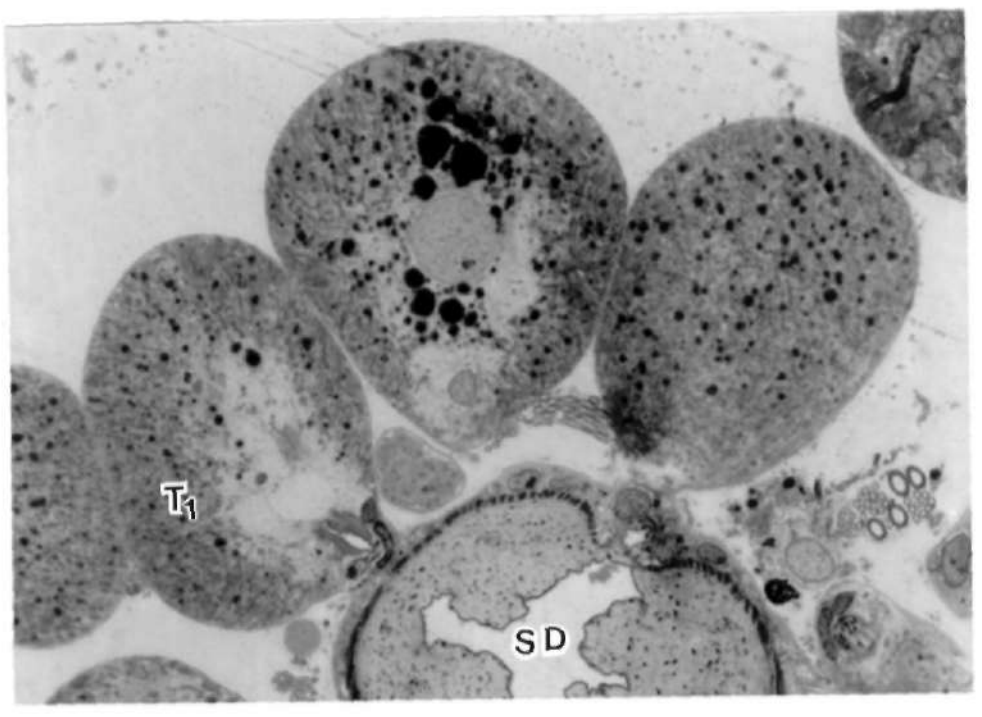


Figure 9.6 Light micrograph illustrating type I acini situated adjacent to the main salivary duct. Note these acini open directly into the duct *(x600).*

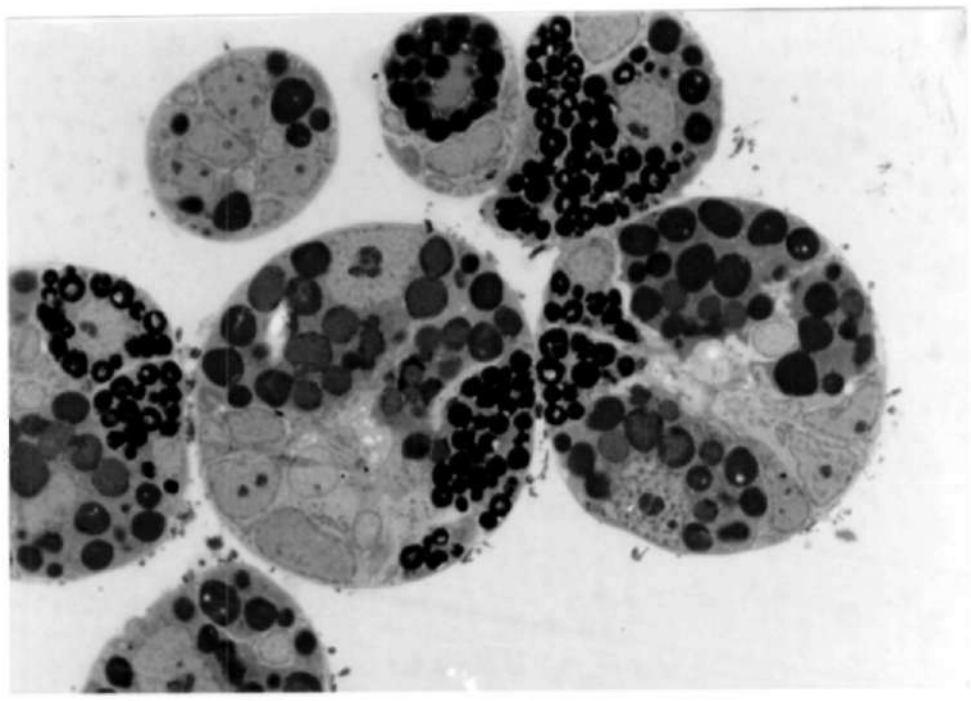
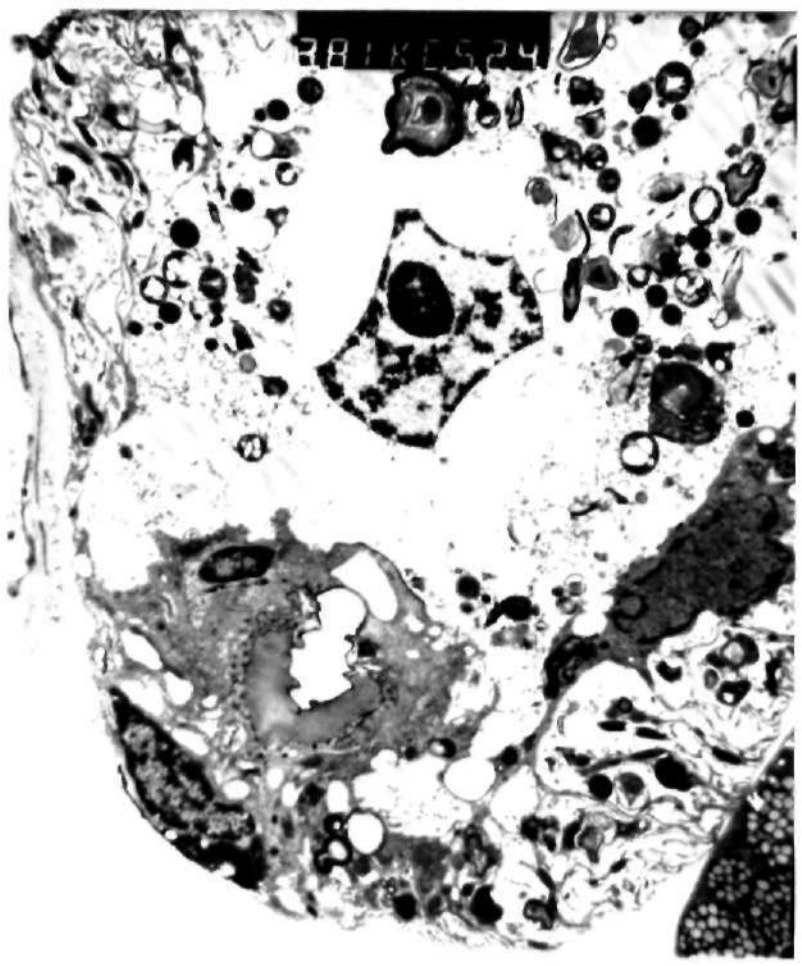


Plate 9.7 Light micrograph illustrating the appearance of granular type II and type III acini secretory granules *(x 600)*.

Figure 9.8A&B Transmission electron micrograph illustrating the ultrastructure of type I acini of a negative adult *R. appendiculatus* male. A large part of the cell is vacuolated and has a wedge like nucleus surrounded by peritubular cells. The acini also has other peripheral cells. (x 3800).



A

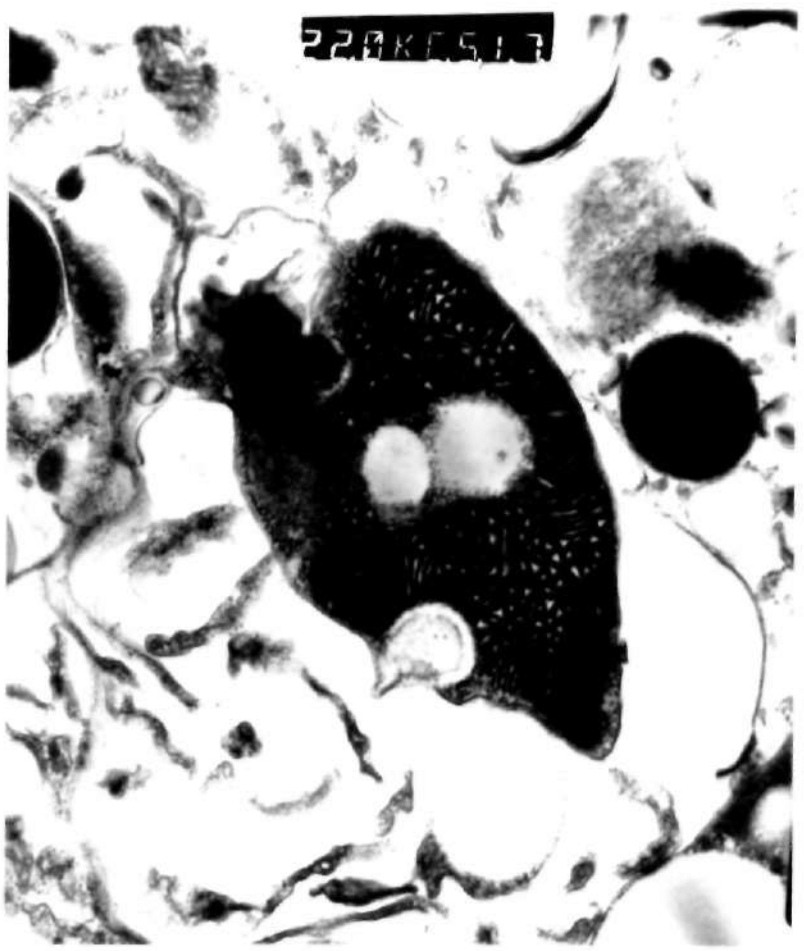


B

Figure 9.9A &B. Transmission electron micrograph of type I acini salivary glands of male *R. appendiculatus* illustrating mitochondria.



A



B

Figure 9.9 C Electron micrograph illustrating some of the different cells in type I acini probably involved in osmoregulation.

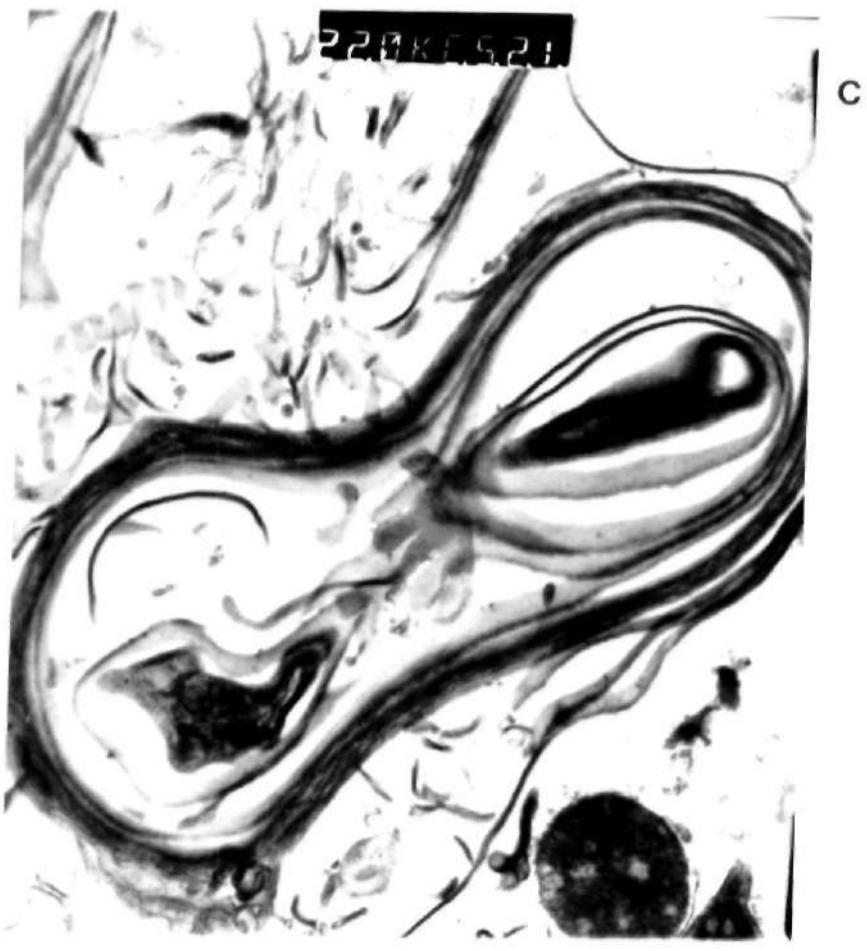

C

Figure 9.10 Electron micrograph showing type II acini of adult male uninfected *R. appendiculatus* with distinct secretory granules, nucleus and glandular cell types (x 3,000).

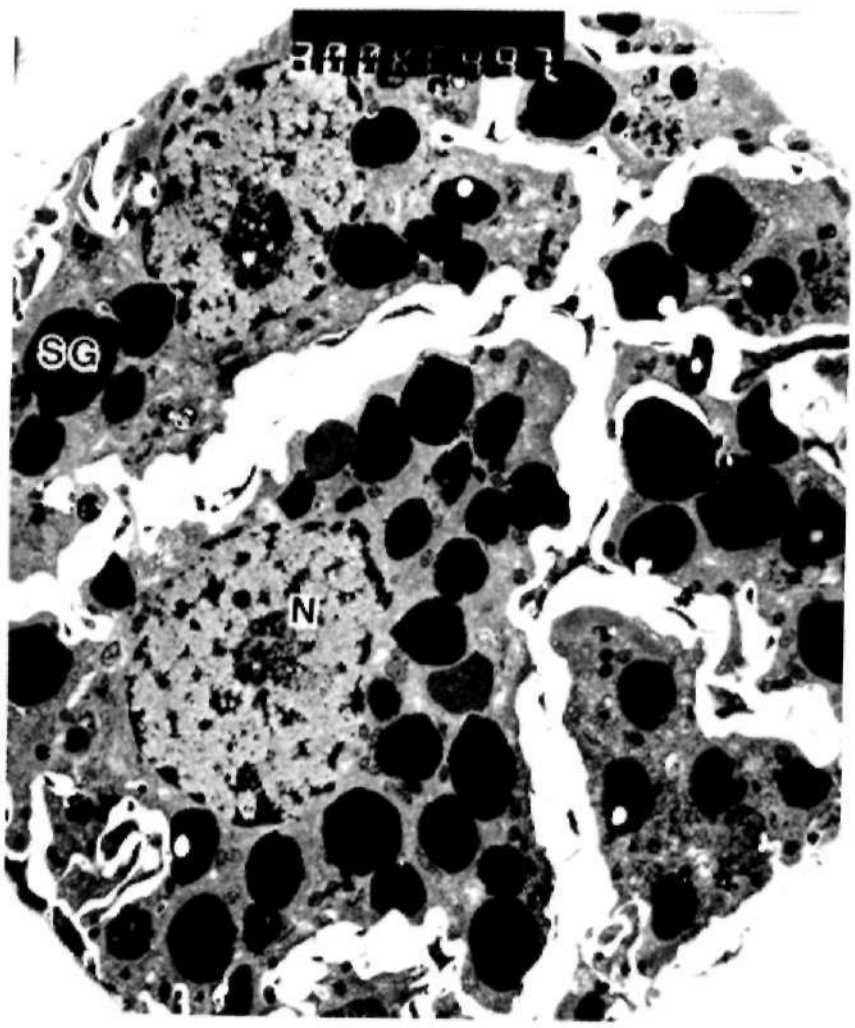
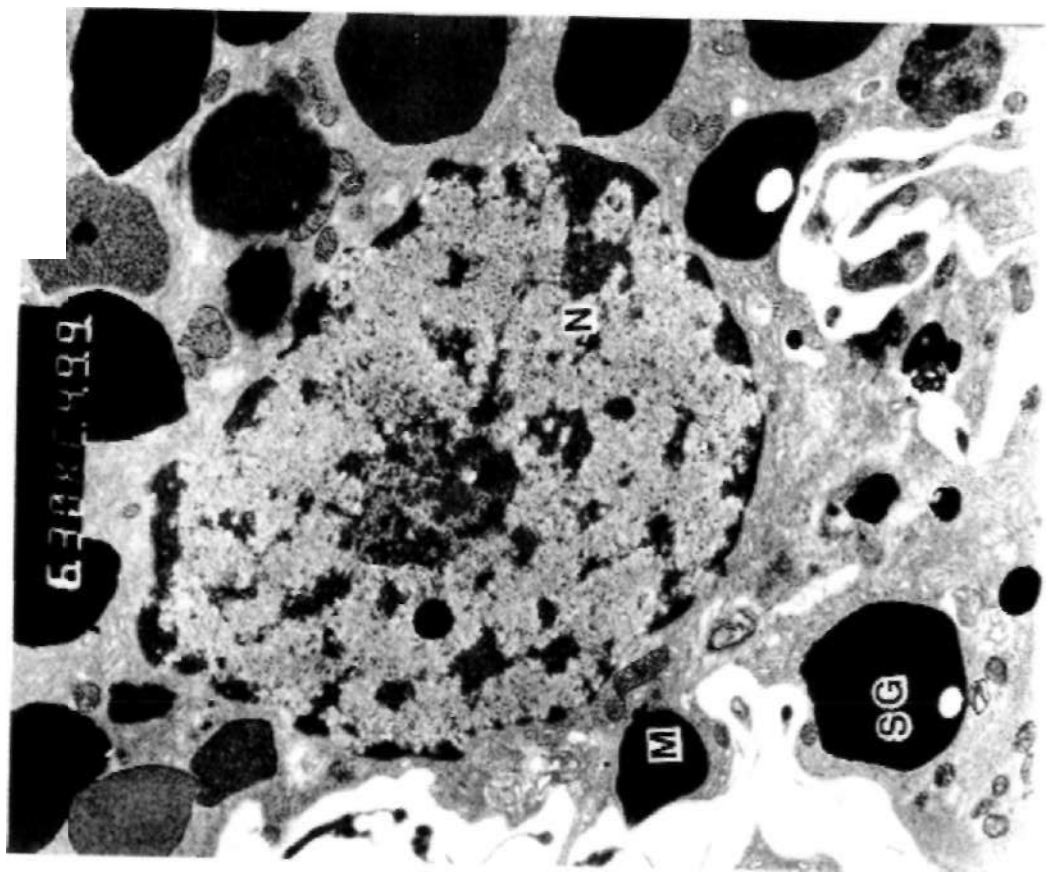

SG
N

Figure 9.11 Cell organelles in type II acini *R. appendiculatus* salivary glands (uninfected). The secretory granules are large with distinctive electron density. Mitochondria are clearly visible with horizontal invaginations (cristae; x 6300).


N
M
SG

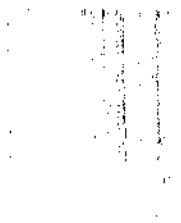


Fig.9.12 Secretory granules of type II acini of *R. appendiculatus*. Part of the nucleus, several mitochondria and secretory granules are visible here. (x1700).

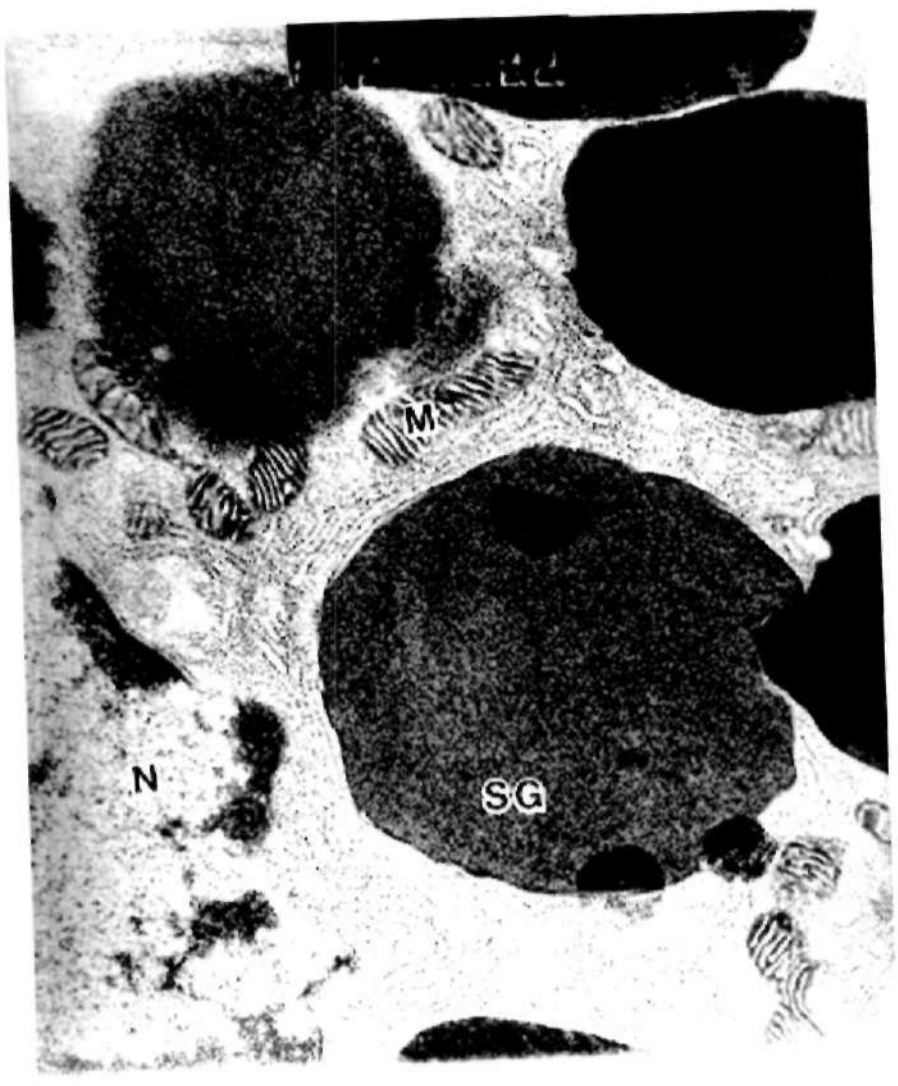

M
N
SG

Figure 9.13A An electron micrograph of cell organelles at higher magnification observed in type II acini of salivary gland of *R. appendiculatus*. The mitochondria have multiple cristae projecting into a patterned matrix (x 60,000).

Figure 9. 13B The endoplasmic reticulum appears as elongated profiles with heavily stained ribosomes (x 66,000).

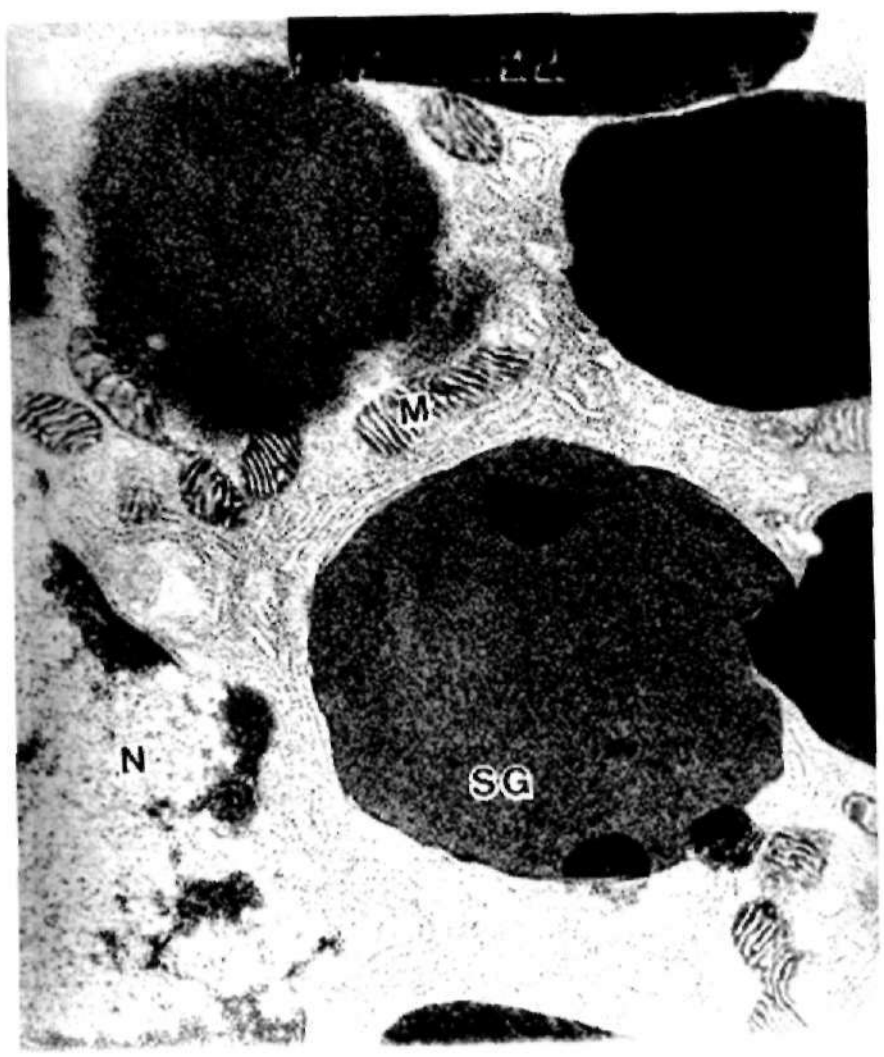
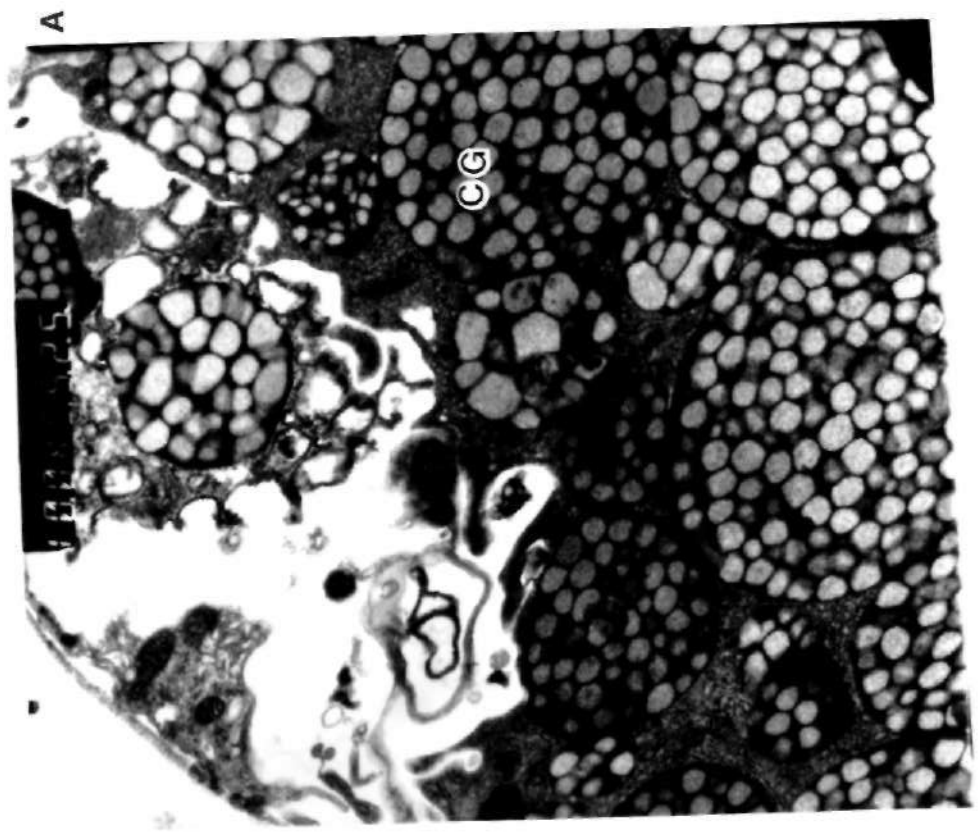

M
N
SG

Figure 9.14 Transmission electron micrographs of section through type III acini from adult male negative ticks illustrating different cell types and granules.

Fig. 9.14A D cell with compound granules composed of numerous subunits.

Fig.9.14B Two adjacent e cells. The granules appear to contain homogeneous moderately dense material.


A
CG

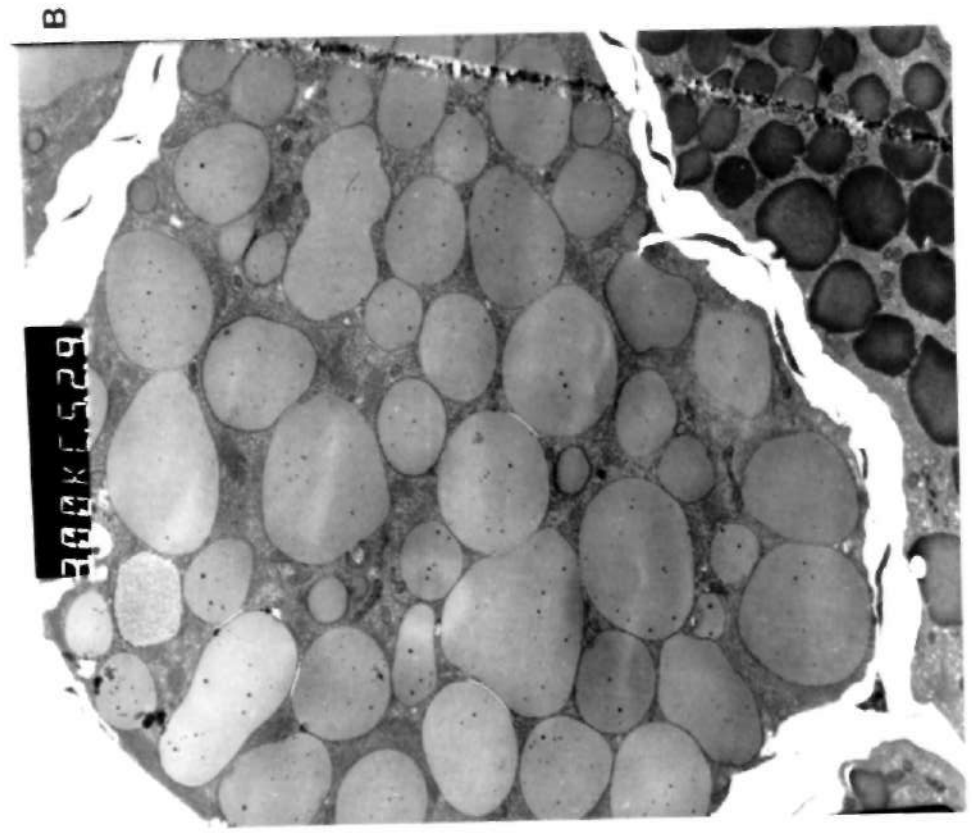
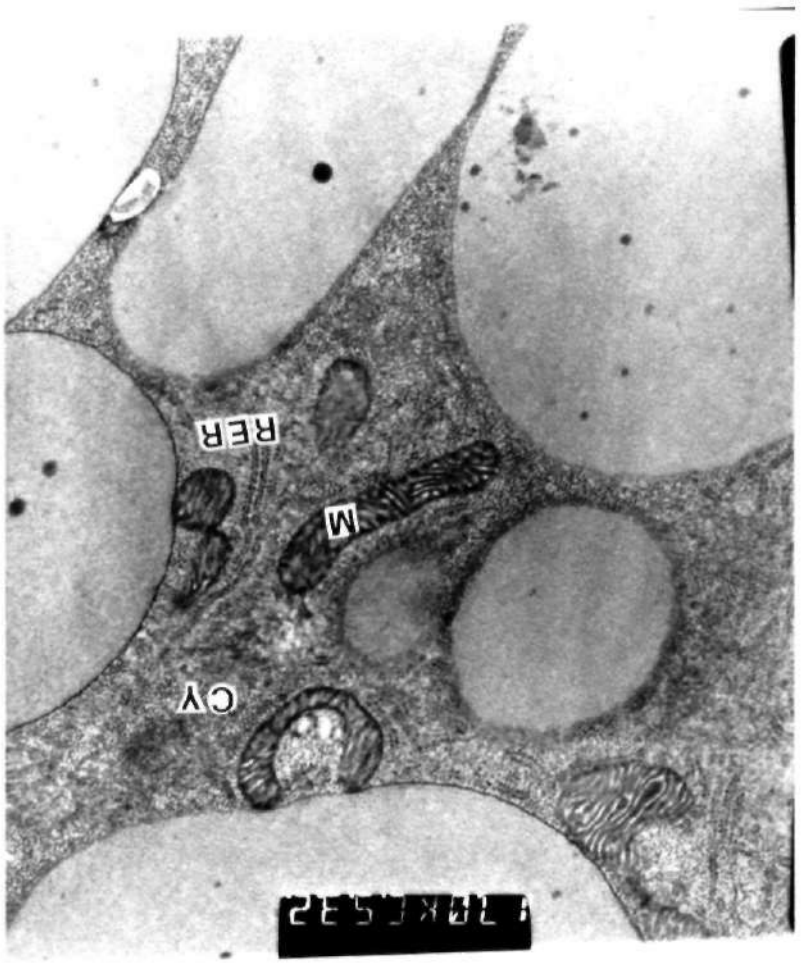

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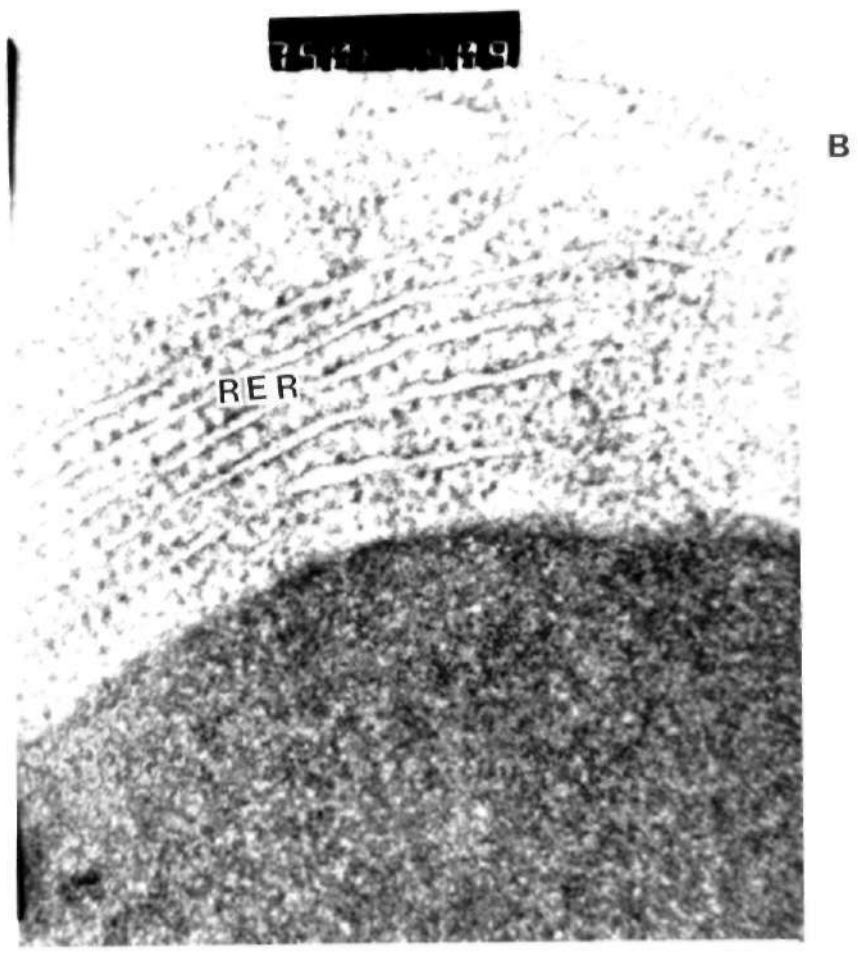
Figure 9.14C Granules of the e cell at higher magnification. Note the presence of rough endoplasmic reticulum and mitochondria in the cytoplasm between the granules. There are also dense staining bodies in the granules (x 17,000).



C

Figure 9.15 Transmission electron micrograph illustrating a salivary gland duct and F cell with adjacent D cell

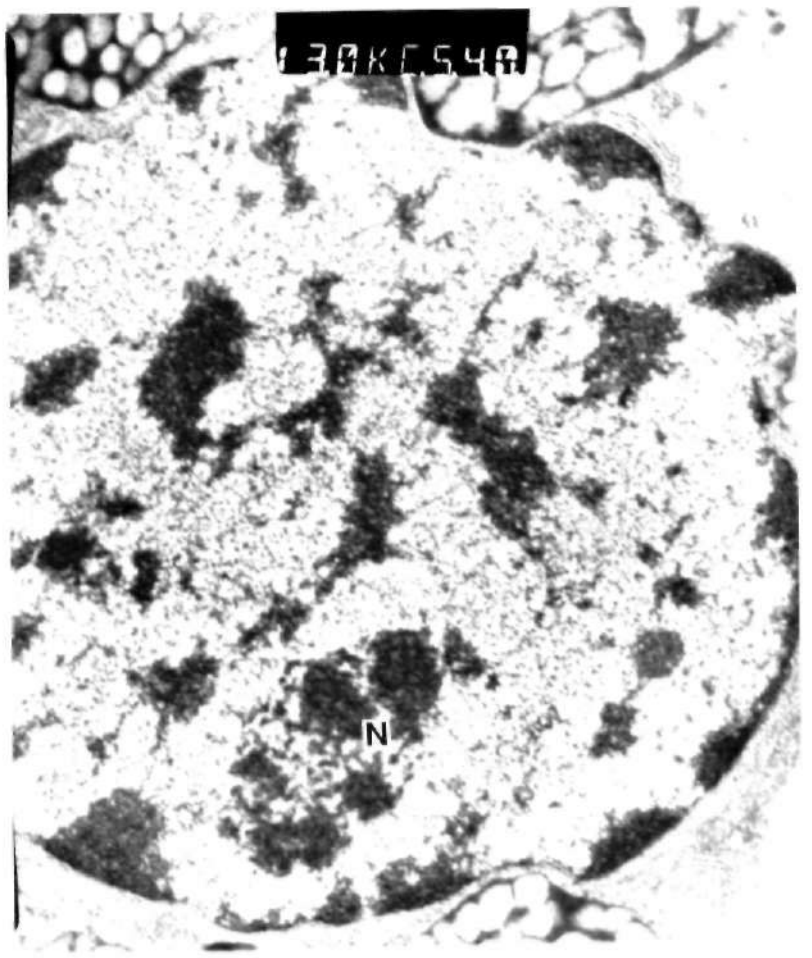

A
M


B
RER


SD

Figure 9.16A Transmission electron micrograph showing a nucleus of uninfected type III acini. Note the coarse uniformly distributed chromatin material (x 13,000).

Figure 9.16B Type III infected acini cell with enlarged nucleus. The cell has undergone hypertrophy and the parasite proliferation. Note dispersed chromatin (x 10,000) and the sporozoites.


A
N

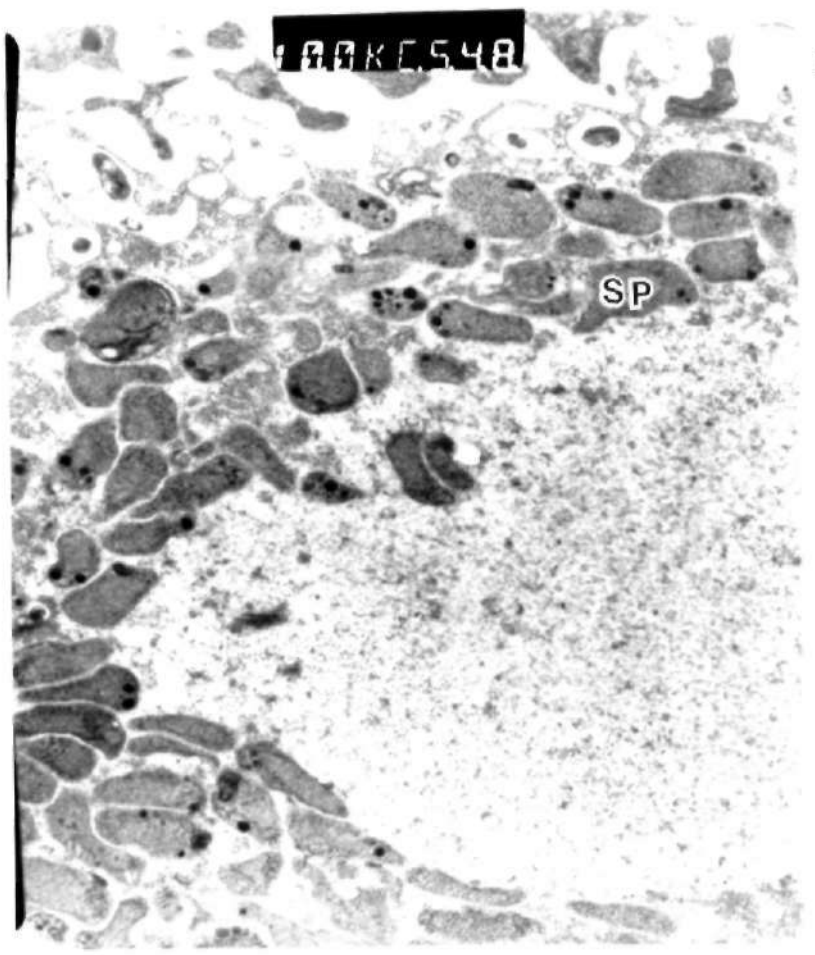
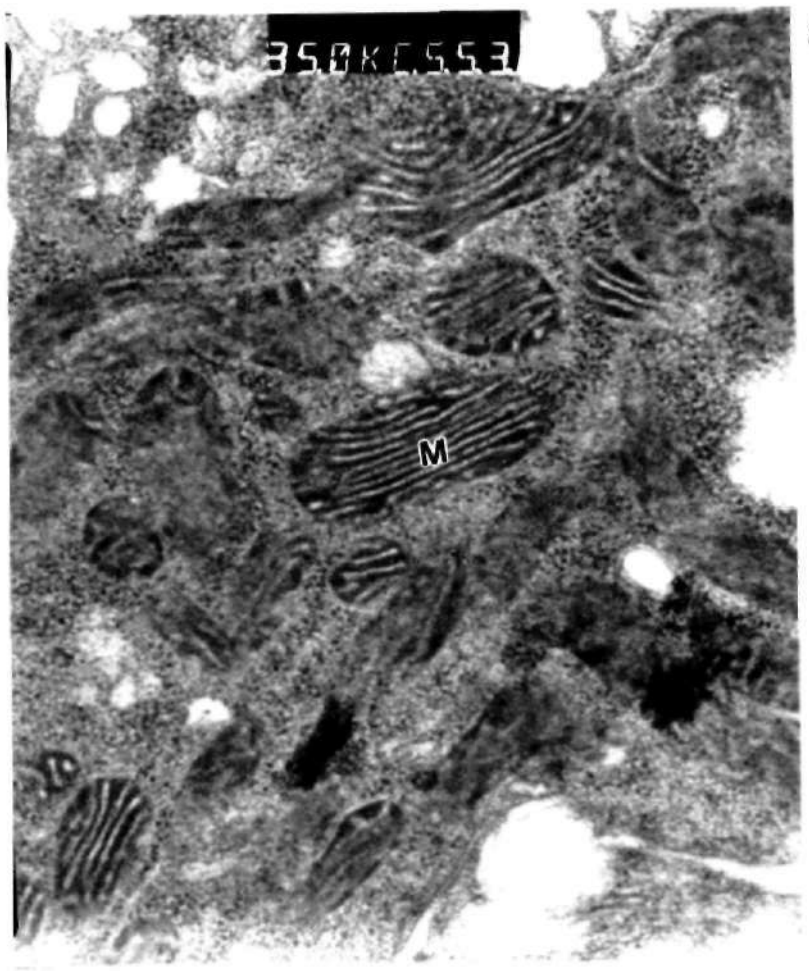
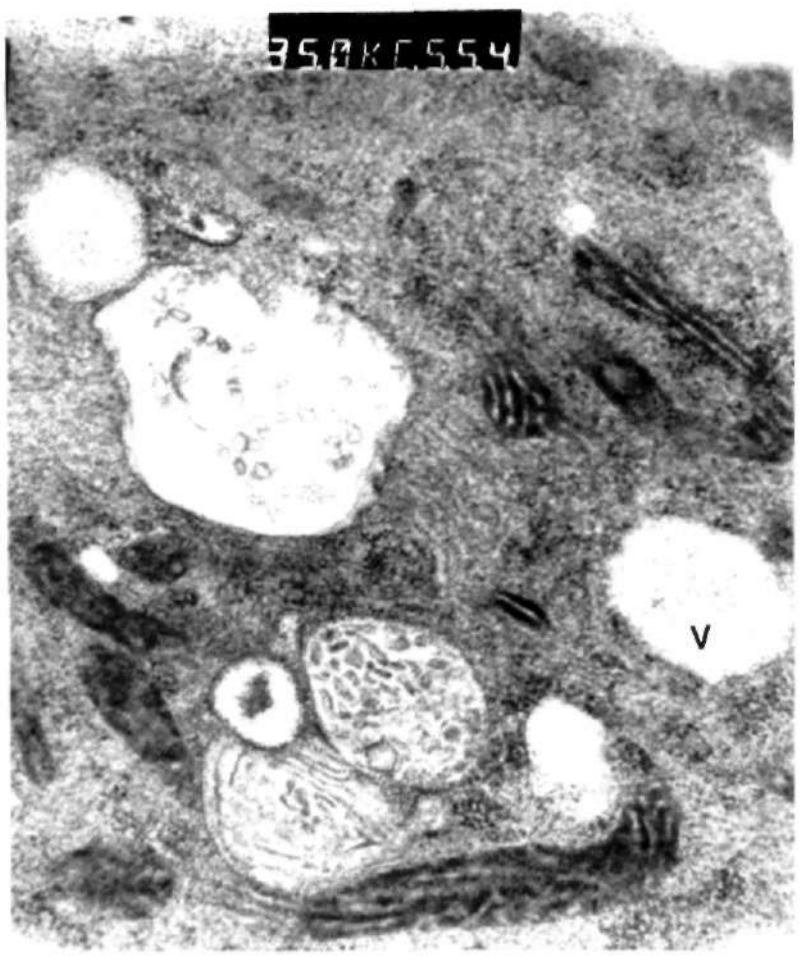

B
SP

Figure 9.17 A Electron micrograph showing the presence of mitochondria in an infected type III acini indicating that the cell remain metabolically active (x 35,000).

Figure 9.17B The endoplasmic reticulum of infected e cells appear reduced in amount. Numerous autophagic vacuoles containing membranous ribosomes were visible empty-appearing cytoplasmic matrix resulting from autolysis in the infected cells.



A



B

Figure 9.18 Type III acini e cell of *R. appendiculatus* crowded with sporozoites. Note glycogen is no longer detectable (x 11,500).

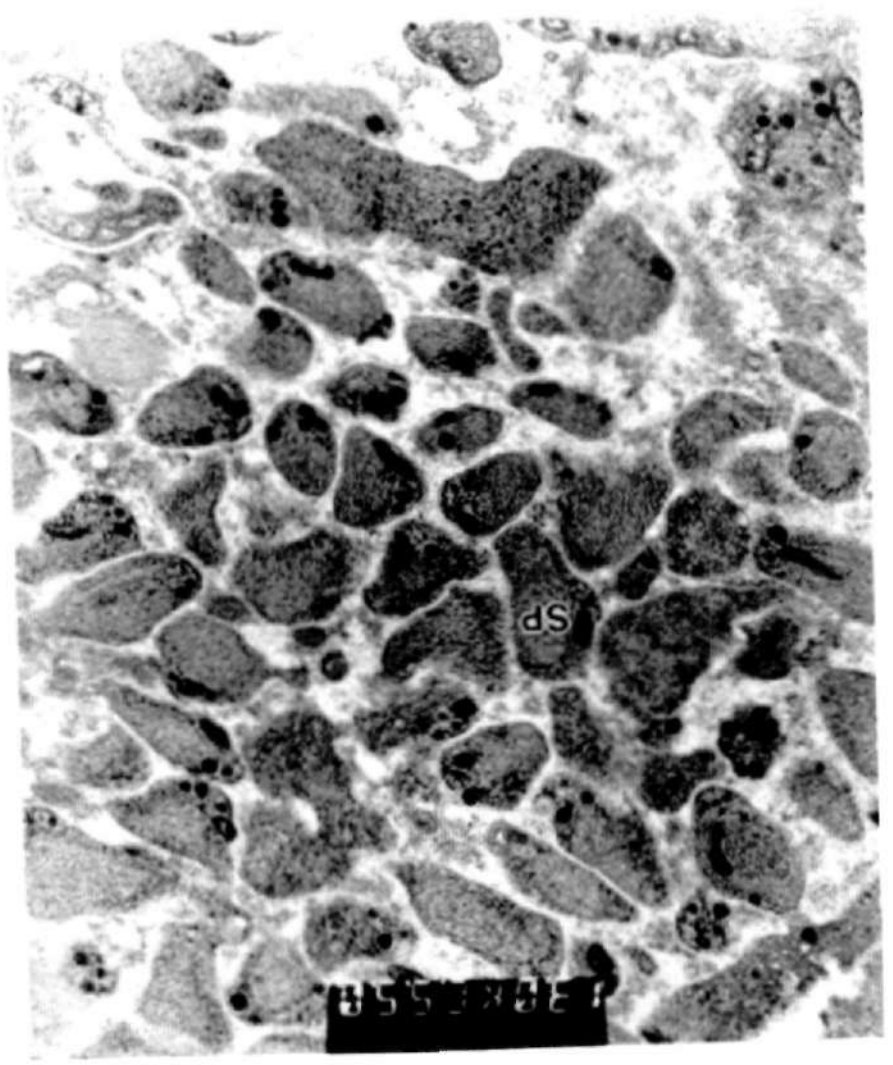

dS

Figure 9.19 ***Theileria parva*** **mature sporozoite in the type III acini e cell of** ***R. appendiculatus*** **female (x 75,000)**



CHAPTER TEN

GENERAL DISCUSSION

East coast fever is still a killer disease characterised by high mobidity and mortality. Several methods of control have been tried and now it is generally accepted that an integrated approach is best. Acaricides, for instance play a major role in controlling ticks. However, their use is limited by the high cost and development of resistance. Currently, the infection treatment method has been adopted on small scale diaries in several countries including Zambia, Malawi, Kenya, Rwanda, Uganda and Burundi. It is, however, disadvantaged in that it utilises live parasites making it difficult to adopt widely. The control is also curtailed by lack of proper understanding of the factors influencing disease transmission from the host, vector and the environment. Current research is aimed at improving the use of vector/parasite/host interrelationships in designing control strategies. The interaction between the vector and the parasite is complex characterised by high infections in small percentages of the population, moderate to low in others and none at all in a large part of the population. An understanding of this phenomena is an important prerequisite to evaluating the factors controlling the transmission of *T. parva* and the epidemiology of the disease. As far as the vector is concerned, the vector competence is determined by the parasite species, the host and the environment. The aim of this study was, therefore to identify the factors in the vector that influence transmission of *T. parva.* The objective was achieved by looking at how enzymes, for instance, trypsin, the blood meal provided *in vitro* and *in vivo*, hormones and lectins influence the development of the parasite. The transmission was assessed *in vitro* by sporozoite infection lympocytes while the development was evaluated by scoring infection rates in the adults fed upon immunised rabbits, lectin antibodies and lectin inhibitory carbohydrates.

Lectins were detected and isolated in the haemolypmh, salivary glands and gut of the brown ear tick using haemagglutination of erythrocytes from domestic and wild life vertebrates. The gut lectin was best inhibited by Fetuin and galactosamine while the

haemolymph lectin was best inhibited by raffinose and arabinose, which indicates that these lectins were different. Similar results have been found by several authors in the class insects (Diptera: Glossinidae (Ingram and Molyneux, 1988; Maudlin and Welburn, 1994) in sandflies (Wallbanks *et al.*, 1986), in mosquitoes (Mohammed and Ingram, 1994) and in reduviid bugs (Pereira *et al.*, 1981; Gomes *et al.*, 1988; Barrocco and Loch., 1988). The ability of lectins to agglutinate parasites plays a role in the immunity as well as self and non self recognition (Kaaya *et al.*, 1986; Ingram and Molyneux.,1991; Welburn *et al.*, 1989).

The chemical/physical characterisation of these lectins revealed that they were Ca^{2+} and Mg^{2+} dependant and pH dependant. The gut was heat resistant while the haemolymph was thermal labile as found in several insects (Ingram *et al.*, 1990) indicating that it is a protein or glycoprotein and further confirmed by the influence of trypsin, chymotrypsin and protease. The gut lectin was demonstrated to be from the integral membranes, which not only has implications on the role of this lectin in parasite development, but made the purification more complicated, achieved by affinity chromatography. Lectins were found to agglutinate purified piroplasms and their antibodies bound onto the piroplasms and schizonts. Furthermore, the ticks that fed upon inhibitory sugars and glyco proteins were found to develop no infections implying that lectins are required in the maturation of *T. parva*. This signifies the role of lectins in *T. parva* parasite development in the ticks and points out the possible targets of intervention in the disease. Recent reports indicate that an important role for lectins in the interaction between vectors and the pathogens they transmit (Ingram & Molyneux, 1991). Anti bodies to lectins fed *invitro* to ticks or *in vivo* on lectin immunised rabbits, revealed reduced infection rates. Electron microscopy showed hypertrophied cells in the salivary gland and gut, which could mean that transmission of the disease could be blocked using lectins. Similar reactions were noted by Kocan *et al.* (1996).

The study on lectins, therefore, demonstrated two important aspects, that different lectins exist in the tick, *R. appendiculatus*, they have an important role to play in the development of the parasite *T. parva*. They should be targeted as subunit vaccine

molecules in the control of Theileriosis. Further work should be carried out to characterise and clone the genes.

The blood meal has also been shown to be an important factor in the transmission of *T. parva*. *In vitro* sporozoite infection of lymphocytes revealed that the ticks fed upon whole blood produced higher infections than fed on cell or plasma. This is the first time sporozoites obtained *in vitro* have been used to infect lymphocytes *in vitro* and forms an important research avenue through which transmission studies can be made.

Hormones given *in vitro* or topically applied on to ticks revealed that they play an important role in the transmission of the disease. Ticks fed on ecdyson for instance moulted faster and had low infection rates. Normally the tick synchronise the moulting with the invasion of the kinetes to the salivary glands of the next developmental stage. If the moulting is hastened by the hormone, could it mean that some of the parasites were not given enough time to develop into the infective stages hence the reduction in infection rates? Such questions are pertinent when assessing the effects of hormones on the disease transmission. If this is true, then hormones could be used as a means of reducing transmission of *T. parva* and should be investigated further. In addition, those ticks that fed on hormones had significantly smaller engorgment weights which influences both the fecundity and survival of the ticks and therefore may mean a reduction in the population.

Enzyme studies have shown that trypsin and protease were blood meal induced and their activity could be detected at least 24 hr. following a blood meal. Since there was no significant difference between the infected and clean ticks, little conclusion could be drawn on the possible role of enzymes on the parasite development. However, there were significantly higher trypsin activity in the *R. pulchellus*, a non vector of *T. parva*. More studies are recommended in this area since the red blood cells are lysed to release piroplasms in the gut.

In arthropods the cuticle is a physical and chemical barrier to invading parasites (Soderhall *et al.*, 1986; Ratcliff *et al.*, 1985), but if the parasite traverses this

barrier the haemocoel blood cells or haemocyctes can phagocytose small particles while the bigger ones are encapsulated. It has been reported that the prophenoloxidase system plays a role in the intercommunication between the various haemocytes. The terminal component is the phenoloxidase an enzyme responsible for the melanization reaction observed as a response to parasites in the body cavity of arthropods. These studies have indicated that most phenoloxidase activity occurs in the plasma and infected ticks had higher activity than clean ones. The phenoloxidase system is normally activated in the presence of parasites hence the observed high activity in infected ticks. The role it plays is perhaps surpassed by the parasite which is able to develop in the ticks. In the non vector, *R. pulcellus*, there was significantly higher activity which may imply that the phenoloxidase may play a role in preventing establishment of the parasite. The higher phenoloxidase activity in the insects, locusts compared to ticks may be because of species differences. The class insects is above the Arachnids in the evolutionary hierarchy and it is not surprising that the enzyme activity may be higher. The arachnids are taken to be more primitive than the insects. The phenoloxidase system, therefore, plays an important role in the tick immune system.

Apart from the blood meal, lectins, hormones, enzymes and phenoloxidase, an important aspect that influences the parasite in the tick is the salivary gland where it develops to maturity. Ticks having three to four types of acini present a choice the parasite has to make when it is liberated from the haemolymph as a kinete. Since the gut lays in close proximity to the type III acini it is believed that these offer the best chance of being invaded, simply by chance. On the other hand, the type I and II acini structure as observed in these studies are adapted to fit specific functions such as water balance and osmoregulation as is type I acini. Type III surface characteristics, differed markedly and seem to specifically offer the best choice to the parasite in addition to having lots of granules of glycogen as food reserves before it is invaded. As the parasite develops, these get depleted and the cell becomes diffuse. The parasite takes over the cell division and the nuclear material is changed. There must be a chemical that attracts the kinete in the type III acini, in this case, glycogen and a specific receptor in the type III acini that enables the kinete penetrate the e cell. Scanning electron microscopy also revealed differences in the

infected and non infected that pointed to the presence of a receptor mechanism in the selection of the acini.

All in all, these studies have illustrated that the transmission of *T. parva* is modulated by several factors including the quality of blood meal, the presence of enzymes, the hormone status of the ticks as well as the lectins quantity and the availability of type III acini determine whether the parasite would develop and be transmitted to the host. Several possible interventions could be mapped out especially lectins and hormones targeted at the gut, haemolymph and salivary glands which could block transmission to be potential vaccine molecules. Future research should therefore concentrate on how to make these control mechanisms of theileriosis feasible.

REFERENCES

Ackerman, S., Floyd, M and Soneshine, D.E. (1981). Artificial immunity to *Dermacentor variabilis* (Acari:Ixodidae): Vaccination using tick antigens. *Journal of Medical Entomology*.17: 391-397.

Agbede, R.I.S and Kemp, D.H. (1986). Immunization of cattle against *Boophilus microplus* using extracts derived from adult female ticks: Histopathology of ticks feeding on vaccinated cattle. *International Journal for Parasitology*, 16: 35-41.

Akov, S. (1982). Blood digestion in ticks. In. *Physiology of ticks* (Obenchain,F.D. and Galun, R. eds). Pergamon Press. Oxford.pp.197-211.

Akov, A. (1972). Protein digestion in haematophagus insects. In *Insects and invertebrate nutrition* (ed) Rodriquez, J.G).pp.531-540. North-Holland, Amsterdam.

Allen, H.J and. Johnson, E.A.Z (1977). A simple procedure for the isolation of L-Fuctose-binding lectins from *Ulex europaeus* and *Lotus tetragonolobus*. *Carbohydrate Research,* 58:253-265.

Allison, A.C and Elsie, E.M. (1981). Approaches to vaccines against protozoan parasites of cattle. In *The ruminant immune systems*.J.E.Butler (ed).Plenum Publishing cooperation.

Amoo, A.O.J. (1984). Field and laboratory studies on the bionomics of *Boophilus decoloratus* (Koch, 1840) and *Boophilus geigyi* (Aeschlimann and Mornel, 1965). PhD Thesis, Un. of Ibadan, Nigeria.

Anderson, R.S., Day, N.K.B and Good, R.A. (1972). Specific haemagglutination and a modulator of complementin cockroach heamolymph. *Infection and Immunity*. 5:55-59.

Araman, S.E. (1979). Protein digestion and synthesis in ixodid females. In *Recent advances in acaralogy* vol.1. (Rodriguez, J.G. ed). Academic press, NNY.pp.385-395..

Arthur, D.R (1962). Ticks and disease; Pergamon press pp. 445.

Arthur, D.R. (1965). Feeding in ectoparasitic Acari with special reference to ticks. *Advances in parasitology*. 3:249-298.

Ashida, M. (1971). Purification and characterization of prophenoloxidase from haemolymph of the silkworm *Bombyx mori* Archives of *Biochemistry and Biophyics* .144.

Ashida, M. (1981). A cane sugar factor supressing activation of prophenoloxidase in haemolymph of the silk worm, *Bombyx mori. Insect Biochemistry*. 11: 57-65.

Ashida, M., Iwama, R., Iwahana, H and Yoshida, H. (1982). Control and function of the prophenoloxidase activating system. *In* proceedings of the 3rd International colloquium on invertebrate pathology (Edited by Payne C.C and Burges, H.D).pp.81-86. University of Sussex, Brighton.

Ashida, M and Ohnishi, E. (1967). Activation of prophenoloxidase in haemolymph of silk worm, *Bombyx mori Archives of Biochemisty and Biophysics*.122:411-416.

Aso, Y., Kramer, K.J., Hopkins, T.L. and Loohort, C. (1985). Characterization of haemolymhp protyrosinase and a cuticular activator from *Manduca texta. Insect Biochemistry* ,15:9.

Aspan, A., and Soderhall, K. (1991). Purification of the phenoloxidase from crayfish blood cells and its activation by an endogenous serine proteinase.*Insect Biochemistry.* 21(4):363-373.

Aspan, A., Hall, M. and Soderhall, K. (1990). The effect of endogenous proteinase inhibitor of the prophenoloxidase activating system, a serine protease from crayfish haemocytes.*Insect Biochemistry*.20:485-492.

Bailey, K.P. (1960). Notes on the rearing of *Rhipicephalus appendiculatus* and their infection with *Theileria parva* for experimental transmission. *Buletin of. Epizootic Diseases in Africa.* 8: 33-64.

Baker, J.A.F. (1978). Resistance to Ixodicides in Africa south of the Equator with some thoughts on tick control in this area. *In* Tick-borne Diseases and their Vectors. Proceedings of an International Conference Held in Edinburgh, 27 September-October, 1976. Editor J.K.H. Wilde, Centre for Tropical Veterinary Medicine, University of Edinburgh, pp.101-109.

Baker, J.A.F., Miles, J.O. and Robertson, W.D. (1978). Resistance to certain organophosphorus ixodicides in the bont tick *Amblyomma hebraeum,* in the Republic of South Africa and Swaziland. *Journal of South Africa Veterinary Association,* 49: 337-341.

Balashov, Y.S. (1972). Blood sucking ticks (Ixodidae). Vectors of disease of man and animals. Miscellaneous. Publication. *Entomology. Society.America.* 8: 161-362.

Barnett, S.F. (1968). Theileriosis. In *Infectious Blood Diseases of Man and Animals.* Editors, M. Ristic and D.Weinman. Vol. 2. Academic press, New York. pp. 269-328.

Barracco, M.A. and Loch, C.T. (1988). Naturally occuring lectins in the haemolymph of *Panstrogylus megistus* (Hemiptera: Reduviidae). *Memorias do Instituto Oswaldo* cruz, Rio de Janeiro, 83:525-527.

Beckage, N.E., Metcalf, J.S., Nesbit, D.J., Schleifer, K.W., Zetlan., S.R and Deburon,I. (1990). Host haemolymph monophenoloxidase activity in parasitised *M. sexta* larvae and evidence for inhibition by wasp polyDNA virus. *Insect Biochemistry*. 20(3): 285-294.

Bell, L.J. (1980). Organ culture of *Rhipicephalus appendiculatus* with maturation of *Theileria parva* in tick salivary glands *in vitro*. *Acta Tropica*. 37: 319-325.

Bell, L.J. and Reid, G.D.F (1981). *Theileria* in tick organ culture. *Transactions of the Royal Society of Tropical Medicine and Hygiene*. 7516:897-898.

Bezuidenhout, J.D. and Bigalle, R.B. (1987). The control of heart water by means of tick control. *Onderstepoort Journal of veterinary Research*. 54: 525-528.

Binnington, K.C., Young, A.S. and Obenchain, F.D. (1983). Morphology of normal and *Theileria*-infected salivary glands of *Rhipicephalus appendiculatus*. *Journal of Parasitology*, 69:421-442.

Binnington, K.C. (1972). The distribution and morphology of probable photoreceptors in eight species of ticks (Ixodoidea). *Zietschit fur Parasitekunde*. 40:321-332.

Boehn, P., Copper, K., Hidson, A.T., Elphick, J.P and McHardy, N. (1981). *In vitro* activity of 2-alkyl-3 hydroxy-1,4-naphthoquinones against *Theileria parva*. *Journal of medical Chemistry*.24:295-299.

Bogin, E and Hadani, A. (1973). Digestive enzymes in hard ticks (Ixodoidea, Ixodidae). 1. Proteolytic enzyme activity in the gut of *Hyalomma excavatum* female ticks. *Zeitschrit fur Paraitenkunde*. 41: 139-146.

Boman, H.G and Hultmark, G. (1987). Cell-free immunity in insects. *Annal Review of Microbiology*. 41:103-126.

Bowman, H.G., Nilsson, I. and Rasmuson, B. (1972). Inducible Antibacterial defence system in *Drosophila*. *Nature*: 237(5352):232-235.

Borovsky, D and Schlein,Y. (1987). Trypsin and Chymotrypsin-like enzymes of the sandfly *Phlebotomus papatasi* infected with *Leishmania* and their possible role in vector competence. *Medical and Veterinary Entomology*. 1:235-242.

Branagan, D. (1975). Observations on the development and survival of the ixodid tick *Rhipicephalus appendiculatus* Neumann 1901 under quasi-natural conditions. *Tropical Animal Health and Production*. 5: 153-165.

Brehelin, N.(1986). Immunity in invertebrates, cells molecules and defence reactions. Heidelberge:Springer.Verlag.233pp.

Bretting, H., Kabat, E.A., Liao, J and Pereira, M.E.A. (1976). Purification and characterization of the agglutinins from the sponge *Aaptos papillata* and a study of their combining sites. *Biochemistry*. 15: 5029.

Brookman, J.L. (1988). Studies on the prophenoloxidase system of insects. PhD.Thesis University of Wales.

Brown, C.G.D (1987). Theileridae. *In In vitro* methods for parasite cultivation. Editors. A.E.R. Taylor and H.R. Baker. Academic press, London. pp.230-253.

Brown, C.G.D. (1990). Control of Tropical Theileriosis in cattle (*Theleria annulata* infection of cattle. *Parassitologia*. 32:23-31.

Castro, V.M., Boman, H.G. and Hammerstrom, S.(1987). Isolation and characterization of isolectins with galactose/N-acetyl galactosamine specificity from hemolymph of the giant silkworm *Hyalophora cecropia*. *Insect Biochemistry*. 17, 513-523.

Chalk, R. Townson, H., Natori, S., Desmond, H and Ham, P.J. (1993). Purification of an insect defensin from the mosquito, *Aedes aegypti*. *Insect Biochemistry and Molecular Biology*. 24(4):403-410.

Chema, S. (1984). Evaluation of new acaricides in Kenya and views on selection for acaricide resistance in past and future. *In*: *Report of the Third FAO Expert Consultation on Tich-Borne diseases and their vectors*. FAO Italy, Rome.29

Chema, S., Chumo, R.S., Dolan, T.T., Gathuma, J.M. James, A.D., Irvin, A.D and Young, A.S. (1987). Clinical trials of halofuginone lactate for treatment of East coast fever in Kenya. *Veterinary Record*. 120: 575-577.

Chizyuka, H.G.B. and Mangani, M.P.C. (1986). Ticks and tick- borne diseases in Zambia. In *Ticks and tick- borne diseases* (Ed by Surtherst R.W) pp 24-25.

Connat, J.L., Ducommun J., Diehl, P.A and Aeschlimann, A. (1986). Some aspects of the control of the gonadotrophic cycle in the tick *Ornithodorus moubata* (Ixodoidea: Argasidae). *In*: *Morphology and behavioural biology of ticks* Editors. Sauer, J.R. and Hair, J.A). Pub. Ellis Horwood Ltd.pp. 194-210.

Comb, D.R. Ey.P.L. and Jenkin, C.R. (1984). Self and non- self recognition in invertebrates. *Quaterly Review of Biology* 59: 231.

Coons, L.B., Rosell- Davies, R. and Tarnowski, B.I. (1986). Bloodmeal digestion in ticks. *In Morphology, physiology and behavioural biology of ticks*. Editors Sauer J.R. and Hair J.A. Ellis Horwood Ltd., Chichester, pp. 248- 271.

Cornelissen, A.W.C.A and Schetters, T.P.M. (1996). Vaccines against protozoa diseases of veterinary importance. *Immunology and Medical microbiology*. 15: 61-72.

Cowdry, E.V. and Ham, A.W. (1932). Studies on East Coast fever. II. Behaviour of the parasite and development of distinctive lesions in susceptible animals. *Parasitology*, 25: 1-63.

Cox, B.L.(1960). Hormonal involvement in the moulting process in the soft tick *Ornithodoros turicata* Duges. PhD. Thesis. University of Oklahoma.

Cox, F.E.G (1983). How red blood cells and malaria parasites recognize each other. *Nature.* 30:654.

Cunningham, M.P., Brown, C.D.G.D., Burridge, M.J. amd Purnell, R.E. (1973). Cryopreservation of infective particles of *Theileria parva. International Journal for Parasitology.* 3: 583-587.

Cunningham, M.P., Brown,C.G.D., Burridge, M.J., Musoke, A.J., Purnell, R.E ., Radley, D.E and Sempebwa, C. (1974). East coast fever: titration in cattle of suspension of *Theileria parva* derived from ticks. *British veterinary Journal.* 13:336-345.

David Chao, Yun-An chen, Jong-Kang Liu and Tai-Chu Huang .(1990). Analyses of surgace membrane carbohydrates in parasitic flagellates of the order kinetoplastida using lectins. *Proc. Natl. Sci. Counc. B. Roc* 14(1): 54-59.

De Castro, J.J. and Newson, R.M. (1993). Host resistance in cattle tick control. *Parasitology today.* 9(1): 13-17.

De Castro, J.J., Dransfield, R.D., Cunningham, M.P., Dolan, T.T., Newson, RM and Young, A.S. (1985). Effects of natural tick infectstion on cattle immunized against theileriosis in an endemic area of Kenya. *Research in Veterinary Science.* 39: 279-288.

Delbesque, J.P., Diehl. P.A and O'Connor, J.D. (1978). Presence of ecdyson and ecdysterone in the tick *Amblyomma herbraeum.* Koch. *Experimentia.* 34: 1379-1380.

Dipeolu, O.O. (1984). Studies on ticks of veterinary importance in Nigeria. The size changes of adult ticks during engorgement and oviposition. *Bulletin Animal. Health. Production Africa.*22:171-179.

Dipeolu, O.O. (1989). Research on ticks in Africa: Review of the trends, advances and milestons in tick biology and ecology in the decade 1980-1990. *Insect Science and Application* 10: 723- 740.

Dolan, T.T (1981). Progress in the chemotherapy of theileriosis. *In*: Advances in the Control of Theileriosis: Proceedings of an International Conference ILRAD, Nairobi, 9-13. 1981. Editors. A.D. Irvin M.P. Cunningham and A.S. Young, Martinus Nijhoff Publishers. The Hague, pp. 186-208.

Dolan, T.T and McKeever, D.J (1992). Current and future vaccines against Theileriosis. In. *Veterinary Veccines*. Pandey (ed). Springer. Verlag. Ny.pp.318-337.

Dolan, T.T. (1986). Editor. Chemotherapy of east coast fever. Treatment of infection induced by isolates of *Theileria parva* with halofuginone. *Acta tropica*. 43: 165-173.

Dolan, T.T. (1989). Editor. Theileriosis in Eastern, Central and Southern Africa: proceedings of the workshop on East Coast fever Immunisation held in Lilongwe, malawi, 20-22, sep. 1988. ILRAD, Nairobi.pp.191.

Dolan, T.T., Young, A.S., Losos, G.J., McMillan, I., Minder, C.E. and Soulsby,K. (1984)a. Dose dependant responses of cattle to *Theileria parva* stabilate infection. *International Journal for Parasitology*. 14: 89-95.

Dolan, T.T., Young, A.S. Leitch, B.L. and Stagg, D.A. (1984)b. Chemotherapy of East coast fever: Parvaquone treatment of clinical disease induced by isolates of *Theileria. Veterinary parasitology*. 15:103-116.

Dolan, T.T. (1981). Progresss in the chemotherapy of theileriosis. *In Advances in the control of theileriosis. Proceedings of an international congress held at ILRAD,* Nairobi, 9-13, Feb, 1981. editors, A.D . Irvin,.M.P., Cunningham and A.S. Young, Martinus Nijhoff. Publishers, The Hague. pp.186-208.

Dolan, T.T., Teal, A.J., Stagg, D.A., Kemp, S.J. Cowan, K.N., Young, A.S., Groocock, C.M., Leitch, B.L., Spooner, R.L., and Brown, C.G.D. (1984)c. Histocompatibility barrier to immunisation against East Coast fever using *Theileria parva* infected lymphoblastoid cell lines.

Dorai, D.T., Bachhawat, B.K., Bishaye, S., Kannan, K. and Rao, D.R. (1981). Further characterization of the sialic acid-binding lectin from the horseshoe crab *Carcinoscorpius rotunda cauda. Archives of Biochemistry and Biophysics* 209:325-333.

Drifand, L and Brehelin, M. (1994). Purification and characterization of an agglutinin from the hemolymph of *Locusta migratoria* (Orthoptera). *Insect Biochemistry and Molecular Biology*. 24(3):283-289.

Dularay, B and Lackie, A.M. (1985). Haemocyte enccapsulation and the prophenoloxidase activation pathway in the locust *S.gregaria*. Forsk. *Insect Biochemistry*. 6:827-834.

Elbil, A. and Anastos, G. (1966). Ixodid ticks (Acarina, Ixodidae) of Central Africa. Volume III. Genus *Rhipicephalus* Koch, 1844. Musee Royal del'Africae Centrale, Tervuren, Belgique Annales Sciences Zoologiques, 147: 53-57.

Essuman, S., Dipeolu, O.O and Odhiambo, T.R. (1991). Immunization of cattle with semipurified fraction solubilised membrane-bound antigens from the midgut of the tick *Rhipicephalus appendiculatus*. *Experimental & Applied Acarology*. 13:65-73.

Ey, P.L. and Jenkin, C.R (1982). The Reticuloendothelial System. A comparative Treatise.VOl III (Edited by Cohen N. and Sigel M.M) pp. 321-391.Plenum Press.New York.

Fabricius, J.C. (1794). Entomologia systematica, emendata et aucta, secundum classes. ornides, genera, species adjectis synonimis, locis, observationibus, descriptionibus. 4:472pp. Hafniae.

FAO. (1983). Ticks and tick- borne disease control: The Sudan. Studies on Important Tick- borne diseases of cattle. Technical report no.2. AG: GCP/SUD/024/DEN. Food and Agriculture Organization, Rome.

FAO. (1984). Tick and Tick-Borne Disease Control: A practical Field Mannual. Volumes I and II. Food and Agriculture Organization, Rome, 621pp.

Fawcett, D.W., Doxey, S. and Buscher, G. (1982)a. Salivary gland of the tick vector of East Coast fever.III. Ultrastructure of sporogony in the *Theileria parva*. *Tissue and Cell*, 14: 183-206.

Fawcett, D.W., Doxsey, S and Buscher, G. (1981). Salivary gland of the tick vector of East Coast fever. II. Cellular basis for fluid secretion in the type III acinus. *Tissue and Cell*,13:231-251.

Fawcett, D.W., Young, A.S. and Leitch, B.L. (1985). Sporogony in *Theileria* (Apicomplexa: Piroplasmida). A comparative ultra study. *Journal of Submicroscopic Cytology*, 17: 299- 314.

Fawcett, D. W and Doxsey, S. (1982). The entry of sporozoites of *Theileria in vitro*. Electron microscopic observations. *European Journal of cell Biology*., 27: *10-21*.

Fawcett, D.W., Buscher, G and Doxsey, S. (1982)b. Salivary glands of the tick vector East coast fever. IV. Cell type selectivity and host reponsiveness to *Theileria parva*. *Tissue and cell*. 14: 397-444.

Ferrante, A and Allison, A.C. (1983). Alternative pathway activation of compliment by African trypanosomes lacking a glycoprotein coat. *Parasite immunology*., 5: 491-498.

Fornster, N. and Porath J. (1975). Characterization studies on a new lectin found in seeds of *vicia ervilis*. *FEBS Lett* 57:187-191.

Fries, C.R. (1984). Protein haemolymph factors and their roles in invertebrate mechanisms: a review. Comparative pathobiology (ed. by T.C. Cheng) vol. 6 pp. 49-109. Plum press. London.

García, E. S., Uhl, M. and Rembold, H. (1986). Azadirachtin, a chemical probe for the study of moulting processes in *Rhodnius prolixus*. Zeitschrift fur Naturforschung 41C: 771-5.

Gomes, Y.M., Furtaddo, A.F. and De Carvalho, A.B. (1988). Natural lectin activity in the haemolymph of *Panstrangylus megistus* (Hemiptera:Reduvidae). Memorias do Instituto Oswaldo Cruz, Rio de Janeiro 83: 509-512.

Gingrich, J, B., Ward, R.A., Macken, L.M and Schoenbecker, M.J.(1982). *Trypanosoma brucei rhodesiense* (trypanosomatidae):Factors influencing infection rates of a recent human isolate in the tsetse *Glossina morsitans* (Diptera: Glossinidae). *Journal Medical Entomology.*, 19: 268-274.

Glauert, A.M. (1975). Fixation, dehydration and Embedding of Biological specimens. North-Holland. Pub. Comp.pp.207.

Goddeeris, B.M and Morrison, W.I. (1988). Techniques for the generation, cloning and characterization fo Bovine cytotoxic T cells specific for the protozoan *Theileria parva. Journal of Tissue Culture methods.*.11(2) :101-109.

Goddeeris, B.M., Katende, J.M., Irvin, A.D. and Chumo, R.S.C. (1982). Indirect fluorescent antibody test for experimental and epizootiological studies on East coast fever (*Theileria parva*) infection in cattle. Evaluation of a cell culture schizont antigen fixed and stored in suspension. *Research in veterinary science 33: 360-365.*

Gold, E.R and Balding, P (eds) (1975). Receptor-specific proteins: Plant and animal lectins. *Exceprta Medica,* Amsterdam.

Goldstein, I.J., Hughes, R.C., Monsigny, M., Osawa, T and Sharon, N (1980). What should be called a lectin. *Nature*.285:66.

Gomes, Y.M., Furtaddo, A.F. and De Carvalho, A.B. (1988). Natural lectin activity in the haemolymph of *Panstrangylus megistus* (Hemiptera:Reduvidae). *Memorias do Instituto Oswaldo Cruz, Rio de Janeiro* 83: 509-512.

Gooding, R.J. (1972). Digestive processes of haematophagus insects. 1: *Literature review Quaest. Entomology.* 8: 5-60.

Gooding, R.H. (1947). Digestion of proteins by *Glossina morsitans*. 3rd International Congress parasitology. Proceedings. 2:946.

Gooding, R.J. (1974). Digestive processes of haematophagus insects. V. Inhibition of trypsin from *Glossina morsitans morsitans*: Diptera: Glossinidae. *Canadian., Entomologist.* 106:39-41.

Gooding, R.H. (1975). Digestive enzymes and their control in haematophagous arthropods. *Acta Tropica.*32: 96-111.

Gotz, P. and Boman, H.G. (1985). Insect immunity *In Comparative Insect Physiology, Biochem and Phamacology*. Kerkut G.A and Gibert L.L ed. Pegramon Press Oxford. Vol. 3 pp. 453-585.

Grandjean, O. (1984). Blood digestion in *Ornithodororus moubata* Murray *sensu stricto* Walton (Ixodoidea: Aragsidae) females.I. Biochemical changes in the midgut lumen and ultrastructure of the midgut cell related to intracellular digestion. *Acarology* 2:147-165.

Grandjean, O. and Aeshlimann, A. (1973). Contribution to the study of digestion in ticks: Histology and fine structure of the mid gut epithelium of *Ornithodorus moubata* Murray (Ixodoidea: Argasidae). *Acta Tropica*, 30: 193-212.

Grandjean, O. (1983). Blood digestion if *Ornithodoros moubata* Murray sensu stricto Walton females (Ixodidea: Argasidae).II. Modification of mid gut cells related to the digestin cycle and to the triggering action of mating. *Annales des Parasitologie Humaine et Comparatif.* 58: 493-514.

Grootenhuis, J. G., Young, A.S., Stagg, D.A., Leitch, B.L., Dolan, T.T and Conrad P.A. (1987). Infection of African buffalo (*Syncercus caffer*) and cattle with *Theileria parva* lawrencei after serial passage in cattle. *Research in Veterinary Science* , 42:326-350.

Grubhoffer, L Uhlir, J and Volf, P. (1993). Lectins as the molecular factors of recognition and defence reactions of ticks. *Modern Acarology.* 2:381-388.

Grubhoffer, l., Muska, M and Volf, P. (1994). Midgut haemagglutinin in five species of tsetse flies (*Glossina* spp). Two different lectin systems in the midgut of *Glossina tachnoides*. Folia Parasitologica. 41: 229-232.

Hall, F.R. (1988). Antigens and immunity in *Theileria annulata. Parasitology Today.*4:257-261.

Harris, R.A and Kaufman, W.R. (1981). Hormonal control of salivary galns degeneration in the ixodid tick *Amblyoma habraeum. Journal of Insect, Physiology.* 27:241-248.

Harris, R.A and Kaufman, W.R. (1985). Ecdysteroids: possible candidates for the hormone which triggers salivary gland degeneration inthe ixodid ticks *Amblyomma hebraeum. Experientia.* 41: 740-742.

Hoogstral, H. (1956). African Ixodidae. I. Ticks of the Sudan (with special reference to Equatorial Province and with preliminary reviews of the genera *Boophilus, Margaropus* and *Hyalomma*). Research Report NM 0055.050.29.27. Department of the Navy, Bureau of Medicine and Surgery, Washington, D.C.1.101pp.

Horridge, G.A. (1965). Arthropods: details of the group. In Structure and function of the nervous system of invertebrates. Vol II. Bullock, T.H. and Horridge, G.A (ed) Freeman, San Fransisco.pp. 1165-1270.

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. *In Tick Biology and Control: Proceedings of an International Conference Held in Grahamstown,* 27-29 January. 1981. Editors, G.B. Whitehead and J.D.Gibson, Tick Research Unit, Rhodes University, Grahamston, pp. 61-66.

Hudson, L. and Hay, F.C. (1989). Practical immunology third edition.Blackwell Scientific Publications.pp 507.

Hudson, L and Hay, F.C. (1976). Practical Immunology (3rd edition). Blackwell Scientific Publications.pp.507.

Hughes, T.E (1954). Some histological changes which occur in the gut epithelium of *Ixodes ricinus* females during gorging and up to oviposition. *Annals Tropical Medicine Parasitology,* 48: 397-404.

Hulliger, L, Wilde J.K., Brown, C.G.D and Turner, L. (1964). Mode of multiplication of *Theileria* in cultures of bovine cells. *Nature,* London 203:728-30.

Hulliger, L. (1965). Cultivation of three species of *Theileria* in lymphoid cells *in vitro. Journal of Protozoology.* 12:649-655.

Hultmark, D., Steiner, H., Rasmuson, T and Boman, H.G. (1980). Insect immunity. purification and properties of three inducible bacterial proteins from haemalymph of immunised pupae of *Hyalophora cercopia. European Journal of Biochemistry.* 106: 7-16.

Ibrahim, E.A., Ingram, G.A. and Molyneux, D.H. (1984). Haemagglutinins and parasite agglutinins in haemolymph and guts of *Glossina. Tropical Medicine and Parasitogy.* 35:151-156.

Imbuga, M.O., Osir, E.O and Labongo, V.C. (1992). Inhibitory effect of *Typanosoma brucei* on *Glossina morsitans* midgut trypsin *in vitro, Parasitology Research.*, 78: 273-276.

Ingram, G. A. and Molyneux , D.H (1990). Lectins (Haemagglutinins) in the haemolymph of *Glossina fuscipes fuscipes*: isolation, partial characterization, selected physico-chemical properties and carbohydrate-binding specificities. *Journal of Biochemisty.* 20(1):13-27.

Ingram, G.A and Molyneux, D.H. (1988). Sugar specificities of anti-humanABO(H) blood group erythrocyte agglutinins(letcins) and haemolytic activity in the haemolymph and gut extracts of three *Glossina* spp. *Insect Biochemistry,* 18:269-279.

Ingram, G.A. Molyleux D.H. (1991). Insect lectins: Role in parasite-vector interactions. *Lectins reviews.* 1: 103-127.

Ioffe, I.D. and Uspensky, I.V. (1979). Effect of insect juvenille hormone analogies on ixodid tick nymphs. *Medica Parasitenkunde Moskva.* 48:39-46.

Irvin, A.D and Morrison, W.I. (1987). Immunopathology, immunology and immunoprophylaxis of *Theileria* infections. *In* Immune responses in parasitic infections. *Immunology, immunopathology and immunoprophylaxis* (Ed. EJL Soulsby) pp. 223- 274. Boca Raton, Florida. CRC Press.

Irvin, A.D. (1987). Characterization of species and strains of *Theileria. Advances In Parasitology.* 26:45-197.

Irvin, A.D., Ocama, J.G.R and Spooner, P.R. (1982). Cycle of bovive lymphoblastoid cells parasitized by *Theileria parva. Research in veterinary Science.*33: 298-304.

Ishiyama, I., Mukaida, M and Takatsu, A. (1974). Haemagglutinins and enzyme inhibitions: comparative studies on the reactivity of anti-A agglutinins of *Helix pomatia* and *Euhadra callizona amaliae. Annals of New York Academy of Science.* 234:75.

Jacobson, R.L. and Doyle, R.J. (1996). Lectin-parasite interactions. *Parasitology today.* 12(2):55-61.

Jarret, W.F.H., Crighton, G.W and Pirie, H.W. (1969). *Theileria parva*: kinetics of replication. *Experimental Parasitology.* 24: 9-25.

Johnson, M.W and Soderhall, K. (1985). Exocytosis of the prophenoloxidase activating enzyme from crayfich haemocytes. *Journal of Comparative Physiology and Biochemistry.* 156:175-181.

Johnston, L.A.Y. Kemp, D.H. and Pearson, R.D. (1986). Immunization of cattle against *Boophilus microplus* using extracts derived from adult female ticks: Effects of induced immunity on tick populations. *International Journal for Parasitology* 16: 27-34.

Jungery, M. Boyle, D., Patel, T., Pasvol, G and Weatherallm, D.G. (1983). Lectin-like polypeptides of *P.faliparum* and the human red cell sialoglycoproteins. *Nature:* 301: 704-705.

Jura, W.G.Z.O., Brown, C.G.D and Kelly, B. (1983). Fine structure and invasive behaviour of the early development stages of *Theileria annulata invitro. Veterinary Parasitology* .,12: 31-37.

Kaaya, G.P. (1990). A review of the progress made in recent years on research and understanding of immunity in insect vectors of human and animal diseases. Insect. Science and Application. 10(6): 751-769.

Kaaya, G.P., Otieno, L.H, Darji, N and Alemu, P. (1986). Defence reactions of *Glossina morsitans morsitans* against different species of bacteria and *Trypanosoma brucei brucei. Acta tropica,* 43:31-42.

Kaiser, M.N, Sutherst R.W and Bourne A.S. (1982). Relationship between ticks and Zebu cattle in Southern Uganda. Tropical *Animal Health Production* ,14:63-74.

Kamel, M.Y. and Fahmy, A.S. (1982). Biochemical studies of tick embrogenesis. V. Purification and partial Characterization of isocitrate lyase from eggs of the tick *Hyalomma dromodarii. Comparative Biochemitry and Physiology.* 72:107-115.

Kamwendo, S.P., Musisi, F.L., Trees, A.J. and Molyneux, D.H. (1995). Effects of haemagglutination (lectin) inhibitory sugars on *Theileria parva* infections in *R. appendiculatus. International Journal of Parasitology*, 254:29-35.

Katende, J.M., Goddeeris, B.M., Morzaria, S.P. Nkonge, C.G. and Musoke A.J. (1990). Identification of a *Theileria mutans*-specific antigen for use in an antibody and antigen detection ELISA. *Parasite Immunology.*,12: 419-433.

Kaufman,W and Sauer, J. (1982). Ion and water balance in feeding ticks: Mechanisims of tick excretion. *In Physiology of ticks.* Editors Obenchain, F and R.Galun. Pub. Pergamon Press, Oxford. pp.197-211.

Kaufman, W.R and Nuttall, P.A. (1996). *Amblyomma variegatum* (Acari:Ixodidae): Mechanism and control of Arbovirus secretion in tick saliva. *Experimental Parasitology* 82: 316-323.

Kaufman, R.W. (1991). Further investigations on the action of ecdysteroids on the salivary glands of the female tick *Amblyomma americanum. Experintal & Applied Acarology.*10:259-265.

Kaufmann, R.W. (1989). Tick host infestations: A synthesis of current concepts. *Parasitology Today* 5: 47-56.

Kemp, D.H., Stone, B.F and Binnington, K.E (1982). Tick attachment and feeding: role of the mouthparts, feeding apparatus, salivary glands secretions and the host respomse. *In Physiology of ticks* (Editors Obenchain F. D. & Galun R.) pp. 119-168.

Kemp, D.H., Agbede, R.I.S., Johnston, L.A.Y. and Gough, J.M. (1986). Immunization of cattle against *Boophilus microplus* using extracts derived from adult female ticks: Feeding and survival of the parasite on vaccinated cattle. *International Journal for Parasitology.* 16:115- 120.

Keppi, E., Zachary, D., Robertson, M. and Hoffman, J.A. (1986). Induced antibacterial proteins in the haemolymph of *Phormia terranovae* (Diptera): *Insect Biochemistry.* 16:395-402.

Kettle, D.S (1984). Medical and veterinary entomology. John Wiley & Sons, New York.

Kitaoka, S. (1972). Effetcts of ecdyson on ticks, especially on *Ornithodoros moubata* (Acarina: Argasidae). Proceedings of 4th International Congress Entomology. p.272.

Letchworth, G.J and Appleton, J.A (1984). Methods of production of Monoclonal antibodies.US. Agriculture Research Service.

Levine,L., Cowan, K.M., Osler, A.G. and Mayer, M.M. (1953). Complement. *Journal of Immunology.*71:359.

Lindsay, P.J and Kaufman.W.P.(1988). Action of some steroids on salivary glands degeneration in the ixodid tick *A. americanum. Journal of Insect Physiology* 24:351-359.

Lis, H., Lotan, R and Sharon, N. (1974). Synthesis and use of affinity chromatography columns for the purification o f plant lectins. *Annals of New York Academy of Science.*234:232.

Lounsbury, C.P.(1904). Transmission of African Coast fever. *Agricultural Journal of the Cape of Good Hope.* 24:428- 432.

Lowe, H.J.(1933). Disease Control: Report of the development of veterinary Science and animal husbandry. Tanganyika.

Lowenberger, C.A., Ferdig, M.T., Bulet, P., Khalili, S., Hoffmann, J.A. and Christensen, B.M. (1996). *Aedes aegypti*: Induced antibacterial proteins reduce the establishment and development of *Brugia malayi. Experimental Parasitology*, 83: 191-201.

Lowenstein, H and Ingrid, A.C. (1976). A micro method for determination proteolytic enzymes in the pH range of 2.8-4.8. Radial enzyme diffusion into skimmed milk containing agarose gel. *Analytical Biochemistry.*71:204-208.

Lowry, O.H., Rosebrough, N.J., Farr, A.L and Randall, R.J (1951).*Journal of Biological Chemistry.* 193: 265-275.

Malmquist, W.A., Nyindo, M.B.A and Brown, C.G.D. (1970). East Coast fever.cultivation *in vitro* of bovine spleen cell lines infected and transformed by *Theileria parva. Tropical Animal Health and Production.*2:139-145.

Mango, C., Odhiambo, T.R and Galun, R. (1976). Ecdysone and the super tick. *Nature.* 260(5549):318-319.

Mango, C.K.A (1979). The effects of beta-ecdyson and popnastrone A on the soft tick *Ornithodoros porcinus porcinus* Walton 1962. PhD Thesis. University of. Nairobi.

Mansingh, A and Rawlins, S.C. (1977). Antigonadotrophic action of insect hormone analogues on the cattle tick *Boophilus microplus. Naturvissenschaften.*64:41.

Marcial, L.R and Fetterer, R.H (1994). Purification and characterization of surface-associated proteins from adult *Haemonchus contortus. Journal of Parasitology* 80(5). 756-763.

Mathewson, M.D.(1984). The future of tick control: A review of the chemical and non chemical options. In: *Impact of disease on livestock production in the tropics.* Editors.H.P.Reimann and M.P.Burridge, Elsevier, Amsterdam.pp.559-568.

Maudlin, I and Welburn, S. (1994). Maturation of trypanosome infections in tsetse. *Experimental Parasitology,* 79:202-205.

Maudlin, I and Welburn, S. (1987). Lectin-medicated establishment of midgut infections of *Trypanosoma congolense* and *Trypanosoma brucei* in *Glossina morsitans. Tropical Medicine and Parasitology* ,38: 167-170.

Maudlin, I. (1991). Transmission of African trypanosomiasis: Interactions among tsetse immune systems, symbionts and parasites. *Advances in Disease vector research.*, 7: 117- 149.

Maudlin, I.,. Kabayo, J.P., Flood, M.E.T and Evans, D. A. (1984). Serum factors and the maturation of *Trypanosome congolense* infections in *Glossina morsitans., Zeitschrift fur Parasitenkd., 70: 11-19.*

Maywald, G.F. and Sutherst R.W. (1986). Ecological models. 1 Assessory climate formagility with CLIMEX. *In* ticks and tick-borne diseases (ed Surtherst R.W).pp 68-71. ACIAR Proceedings. No.17.

Maywald, G.F and Surtherst,R.W (1997). Report of Tick Modelling workshop (ICIPE,Nairobi, October, 1997).

McDaniel, I. R.S and Oliver, J.H.J. (1978). Effects of two Juvenile hormone analogs and beta-ecdysone on nymphal development, supermatogenesis and embryogenesis in *Dermacentor variabilis* (Say):(Acari:Ixodidae).*Journal of Parasitology.* 64:571-57.

McHard, N. (1984). Recent advances in the chemotherapy of theileriosis. *In* Impact of Diseases on livestock Production in the Tropics. Editors. H.P.Reinmann and M.J. Burridge, Elsevier, Amsterdam,pp 179-192.

McHardy, N., Haigh, A.J. and Dolan, T.T. (1976). Chemotherapy of *Theileria parva* infection. *Nature.*261:698-699.

McHardy, N and Wekesa, L.S (1985). Buvaparvaquone (BW 720C), anew anti-theilerial napthoquinone, its role in therapy and prophylaxis. *In Immunization against Theileriosis in Africa: proceedingss of a joint workshop, Nairobi,1-5* Oct.1984. Editor.A.D.Irvin, ILRAD. Nairobi.p.88.

Mehlhorn, H. and Schein, E. (1976). Elektronenmikropische untesuchungen an entwicklungsstadien von *Theileria parva* im darm der ubertragezecke *Hyalomma anatolicum exvatum.* Zeitschrift *fur Tropenmedzin und Parasitologie.* 27:182-191.

Mehlhorn, H. and Shein, E. (1984). The Piroplasms: Lifecycle and sexual stages. Advances in Parasitology, 23:37-103.

Mehlhorn, H. Shein, E. and Warneke, M. (1978). Electron microscopic studies on the development of kinetes of *Theileria parva* in the gut of the vector tick *Rhipicephalus appendiculatus*. Acta Tropica, 35: 123-136.

Mehlhorn, H. Weber, G. and Shein, G. (1975). Elektronmikroskopische untersuchangen an entwicklungsstadin von *Theileria annulata* imm dam und in der Hamolymphe *Hyalomma anatolicum excavatum*. *Zeitschrift fur Parasitenkunde*, 48:137-150.

Mihok, S. Stiles, J. K. Mpanga, E and Olubayo, R.O. (1994). Relationships between protease activity, host blood and infection rates in *Glossina morsitans* sspp infected with *Trypanosoma congolense*, *T. brucei* and *T. simiae*. *Medical and veterinary Entomology*,8: 47-50.

Mihok, S., Olubayo, R.O., Darji, N and Zweygarth,E (1993). The influence of host blood on infection rates in *Glossina morsitans* spp. infected with *Trypanosoma congolense, T.brucei and T.simiae*. *Parasitology*. 107:41-48.

Miller, L.H., Pino J.A and McLevey, J.S. Ed.(1977). Immunity to blood parasites of animals and man. New York Plenum Press 1-132.

Miller, Van. H., Ballback, R.C., Pauley, G.B., Krassner, S.M. (1972). A preliminary physicochemical characterization of an agglutinin found in the hemolymph of the crayfish *Procambarus clarkii*. *J. Invertebr. Pathol.*, 19: 83-93.

Micon, G (1994). Drugs or vaccines? *Parasitol. Today*. 10: 402-403.

Minshull, J.I. and Norval, R.A.I. (1982). Factors influencing the spartial distribution of *Rhipicephalus appendiculatus* in Kyle recreational park, Zimbabwe.*South African Journal of Wild life research*.12:118-123.

Mohamed, H.A, Ingram, G.A and Molyneux, H. (1992). Carbohydrate-binding specificities of anti-erythrocyte lectins (haemagglutinins) in *Anopheles gambie* gut and haemolymph. *Medical and veterinary entomology*, 6: 217-224.

Mohamed, H., Ingram, G and Molyneux, D.H. (1991). Use of fluorescein-labelled lectin binding of salivary glands to distinguish between *Anopheles stephensi* and *An.albumanus* species and strains. *Insect Biochemistry*. 21 (7):767-773.

Mohamed, H.A and Ingram, G.A (1994). Effects of physico-chemical treatments on haemagglutination of *Anopheles gambiae* haemolymph and midgut extract. *Medical and veterinary Entomology*, 8:8-14.

Mongi, A.O., Shapiro, S.Z., Doyle, J.J and Cunningham. (1986). Characterization of antigens from extracts of fed ticks using sera from rabbits immunised with extracted tick antigen and by successive tick infectsation. *Insect. Sci. Applic*. 7: (4). 497-487.

Moolo, S.K. (1981). Effects of maintaining *Glossina morsitans morstitans* on different hosts upon the vectors' subsequent infection rates with pathogenic trypanosomes. *Acta tropica.*, 38: 125-136.

Morrison, W.I., Buscher, G., Emery, D.L. Nelson, R.T and Murray, M. (1981). The kinetics of infection with *Theileria parva* in cattle and the relevance to the development of immunity. In *Advances in the Control of Theileriosis*. Proceedings of an International conference. ILRAD, Nairobi, Kenya,9-13, Feb.1981. Aditors.A.D.Irvin., M.P. Cunningham and A.S. Young, Martinus Nijhoff Publishers, The Hague.pp.311-326.

Morrison,W.I., Goddeeris, B.M., Brown, W.C., Baldwin, C.L. and Teal, A.J. (1989) b.*Theileria parva* in cattle.Characterization of infected lymphocytes and the immune responce they provoke.*Veterinary Immunology and Immunothology*.20:213-217.

Morzaria, S.P.(1989). Identification of *Theileria* species and characterization of *Theileria parva* stocks. In. Theileriosis in Eastern, central and Southern Africa:Proceedings of a workshop on East Coast fever Immunisation .Lilongwe, Malawi. 20-22, Sept. 1988. Editor.T.T.Dolan.ILRAD,Nairobi.pp.102-110.

Morzaria, S.P., Irvin, A.D., Wathanga, J., D'Souza, D., Katende, J., Young, A.S., Scott, J. and Gettinby, G. (1988). The effects of East coast fever immunization and different acaricidal treatments on the productivity of beef cattle. *Veterinary Record*. 123:313-320.

Morzaria, S.P., Young, J.R., Spooner, P.R., Dolan, T.T., Young, A.S. and Bishop, R.R. (1992). Evidence of a sexual cycle in *Theileria parva* characterisation of the recombinants in proceedings and abstracts of the International conference on ticks borne pathogens S.t.Paul. Minnesota, 1992. Sept, 1992. U.G. Munderrloh and T.J.Kurtti (Eds).

Mukhebi, A.W (1992). Economic Impact of Theileriosis and its Control in Africa. In *The Epidemiology of Theileriosis in Africa* pages 379-403; (editors. Norval, R.A. I., Perry, B.D and A.S. Young) Pub. Academ Press. Britain.

Mulla, A. F and Richman, L.R. (1988). How do African game animals control trypanosome infections? *Parasitol Today*, 4: 352-354.

Musisi, F.L. (1990). Methods currently used for the control of East coast fever: Their validity and proposals for future control strategies. *Parassitologia*.32:15-22.

Musoke, A.J., Nantulya, V.M., Rurangirwa, F.R. and Busher, G. (1984). Evidence for a common protective antigenic determinations on sporozoites of several different *Theileria parva* strains. *Immnology*, 52:231-238.

Musoke, A.J and Barbet, A.F. (1977). Activation of complement by variant-specific surface antigen of *Trypanosoma brucei*. *Nature.*, 270: 438.

Nolan, J. (1981). Current developments in resistance to amidine and pyrethroid tickicides in Australia. *In Tick Biology and Control*: Proceedings of an international Conference Held in Grahamstomn, 27-29, January, 1981. Editors. G.B. Whitehead and J.D. Gibson, Tick Research Unit, Rhodes University, Gramstown,pp 109-114.

Norval, R.A I. (1989). Tick control in relation to the epidemiology of theileriosis. *In Theileriosis in Eastern, Central and Southern Africa*: Proceedings of a workshop on East Coast fever Immunization held in Lilongwe, Malawi, 20-22. September, 1988. Editro, T.T.Dolan, ILRAD, Nairobi, pp 111-120.

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

Norval, R.A.I., Sutherst, R.W., Kurki, J., Gibson, J.D and Kerr, J. D. (1988). The effect of the brown ear tick *Rhipicephalus appendiculatus* on the growth of sanga and European breed cattle. *Veterinary Parsitology*, 30: 149-164.

Norval, R.A.I, Perry, B.D and Young, A.S (1992). The epidemiology of Theileriosis in Africa. Academic Press.pp.481.

Obenchain, D.F and Galun, R. (1982). *Physiology of ticks*. Pergamon Press. Oxford.pp. 509.

Obenchain, D.F. (1974). The diversity of cells in the nerosecretory system of the American dog tick *Dermacentor variabilis* Say (Acari;Ixodidae). PhD Thesis, Ohio State University.

Okolo, C.J., Jenni, R, Molyneux, D.H. and Wallbanks, K.R (1990). Surface carbohydrate differncies of *Glossina* salivary glands and infectivity of *Trypanosoma brucei gambiense* to *Glossina. Annals. Societe Belge Medical Tropicale.* 70: 39-47.

Ole-Muyoyi, O. (1989). *Theileria parva:* An intracellular parsite that induces revercible lymphocyte transformation. *Experimental Parasitology.* 69: 204-210.

Pappas, P and Oliver, J. (1972). Reproduction in ticks (Acari: Ixodoidea). II. Analysis of the stimulus for rapid and complete feeding of female *Dermacenter variabilis* (Say). *Journal of Medical Entomology*, 9: 47-50.

Pathak, J.P.N. (1993). *Insect immunity.* Mohan Primlani, New Delhi, India.pp.192.

Pegram, R.G., Perry B.D., Musisi F.L. and Mwanaumo B. (1986). Ecology and phenology of ticks in Zambia:seasonal dynamics on cattle. *Experimental and Applied Acarol*ogy.2.25-45.

Pegram, R.G., Lemche, J., Chizyuka, H.G.B., Sutherst, R.W., Floyd, R.B., Kerr, J.D and McCosker, P.J. (1989). Effects of tick control of liveweight gain of cattle in central Zambia. *Veterinary and Medical Entomology.* 3: 313-320.

Pereira, M.E.A, Andrade, A.F.B and Ribeiro, J.M.C. (1981). Lectins of distinct specificity in *Rhodnius prolixus* interact selectively with *Trypanosoma cruzi. Science.* N.Y 211: 597-600.

Perrone, J.B., Demario, J and Spielman, A. (1986). Regions of mosquito salivary glands distinguished by surface lectin-binding characterisctics. *Insect Biochemistry.* 16:313-318.

Pinder, M., Kar,S.K., Mayor-Withey., E.S., Lundin,L.B. and Roelants,G.E. (1981). Proliferation and lymphocyte stimulation capacity of *Theileria*-infected lymphoblastoid cells before and after elimination of intracellular parasites. *Immunology.* 44:51-60.

Potgieter, F.T., Stoltsz, W.H., Blouin, E.F and Roos, J.A. (1988). Corridor disease in South Africa: A review of the current status. *Journal of South African veterinary Association.* 59:155-160.

Powning, R.F., Davidson, W.J. (1976). Studies on insect bacterialytic enzymes:II.Some physical and enzymatic properties of lysosome from haemolymph of *Galleria mellonella. Comparative Biochmistry and.Physiology.*55:221-228.

Preston, J.W. and Taylor,R.L. (1970). Observations on the phenoloxidase system in the haemolymph of the cockroach Leucophaea maderae. *Journal of Insect Physiology* 16:1729-1744.

Preston, P.M., Brown, C.D and Richardson,W. (1992). Cytokines inhibit the development of Trophozoite-infected cells of *Theileria annulata* and *Theileria parva* but enhance proliferation of macroschizont-infected cell lines. *Parasite Immunology* 14: 125- 141.

Punyua, D and Newson, R.M. (1985). The brown ear tick *Rhipicephalus appendiculatus* Neumann (Acarina:Ixodidae) and associated tick species on wild and domestic hosts at Muguga, Kenya. *Journal of Parasitology*, 71:248-252.

Purnell, R.E. (1977). East coast fever: some recent research in East Africa. *Advances in Parasitology.* 15: 83-132.

Radley, D.E. (1981). Infection and treatment against theileriosis. *In Advances in the control of Theileriosis: proceedings of an International conference. ILARD, Nairobi. 9-13. Feb,* 1981.pp.227-236.

Radley, D.E.(1978). Immunization against Theileriosis by Chemoprophylaxis. FAO technical report.A.G. DP/RAF/67/077. Rome, FAO.

Radley, D.E., Brown, C.G.D., Burridge, M.J. Cunningham,M.P., Pierce,M.A. and Purnell,R.E. (1974). East coast fever:Quantitative studies of *Theileria parva* in cattle. *Experimental Parasitology.*36:278-287.

Radley, D.E., Brown,C.G.D., Cunningham,M.P. , Dolan,T.T. and Morzaria, S.P. (1975). East coast fever 3: Chemoprophylactic immunization of cattle using oxyttracycline and a combination of theilerial strains. *Veterinary Parasitology*.1:51-60.

Ratcliffe, N.A (1986). Insect cellular immunity and recognition for foreignness. Immune mechanisms in Invertebrate vectors (ed. by A.M.Lackie) pp. 21-43. Clarendon press, Oxford.

Ratcliffe, N.A. and Rowley, A.F. (1983). Recgonition factors in insect haemolymph. *Developmental and Comparative Immunology*. 7:653-656.

Ratcliffe, N.A., Leonard C. and Rowley, A.F. (1984). Prophenoloxidase activation: Non-self recognition and cell cooperation in insect immunity. *Science*. 226:557-559.

Ratcliffe, N.A., Rowley, A.F., Fitzgerald,S.W and Rhodes,C.P.(1985). Invertebrate Immunity: basic concepts and recent advances. *International Review of Cytology*. .97:183-350.

Rechav, Y. (1987). Use of impregnated ear tags for controling the brown ear tick (Acari:Ixodidae). *In* South Africa. *Journal of Economic Entomology*. 80:822-825.

Reduth, D., Grootenhuis, J. G., Olubayo, R.O., Muranjan, M., Otieno-Omomdi, F, P. Morgan G.A. Brun R. Williams, D.J.L and Black, S.J. (1992). African Buffalo serum contains novel trypanocidal proteins. *The Journal of Eukaryotic microbiology.*, 41: 95-103.

Rembold, H. (1989). Azadirachtins: their structure and mode of action. *In* Insecticides of plant origin (ed. Arnason J. T.Philogene B.J.R and Morand P.J. pp. 150-163. American Chemical Society Symposium Series 387: Washington D.C.

Renwrantz, L and Stahmer, A. (1983). Opsonizing properties of an isolated hemolymph agglutinin and demonstration of lectin-like recognition molecules at the surface of hemocytes from *Mytilus edulis*. *Journal of Comparative Physiol*ogy. 149:535-546.

Richards, E.H and Ratcliffe, N.A (1990). Direct binding and lectin-mediated binding of erythrocytes to haemocytes of the insect *Extatosoma tiaratum*. *Development and Comparative Immunology*.14: 269-281.

Richards, E.H., Ratcliffe, N.A and Renwrantz, L. (1988). Isolation and characterization of a serum lectin from the giant stick insect *Extatosoma tiaratum*. *Insect Biochemistry*. 18(7):691-700.

Richards, A.G. (1951). The intergument of Arthropods. University of Minnesota. Press. Minneapolis.

Riding, G.A, Jarmey, J and McKenna,R.V., Pearson.R., Dobson,C.S and Willadsen,P. (1994). A protective "concealed" antigen from *Boophilus microplus*. Purification, localisation and possible fuction.

Roshdy, M.A., Shoukrey, N.M and Coons, L.B (1973). The subgenus Persicargas (Ixodidea:Argasidae:Argas).17: A neurohemal organ. *Journal of Parasitology* 59:540-544.

Rudith, W and Hecker, H. (1989). Lectin-binding sites in the midgut of the mosquitoes *Anopheles stephensi* liston and *Aedes aegypti* L. (Diptera:Culicidae) .*Parasitology Research.* 75: 268-279.

Ruscoe, CN.E. (1974). Insect of hormones. *In* Biology in pests and disease control. Jones,D.P and Solomon,M.E (eds). Blackwell. Scientific Pub. Oxford.pp.147-161.

Sannasi, A and Subramoniam, T. (1972). Hormonal rupture of a larval diapause in the ticks *Rhippicephalus sanguines* .(Lat). *Experintia.*28:666-667.

Sauer, J.R., McSwain, J.l. and Essenberg, R.C. (1994). Cell membrane receptors and regulation of cell function in ticks and blood sucking insects. *International Journal of Parasitology*.24: 33-52.

Saul, J. and Sugumaran, M. (1988). Prophenoloxidase activation in the haemolymph of *Sarcophaga bullata* larvae. *Archives of Insect Biochemistry and Physiology* 7: 91-103.

Saul S.J. and Sugumaran, M. (1986)a. Phenoloxidase activation, proteases and protease inhbition in insect haemolymph. *Fed Proceedings.* 45: 1865.

Saul, S.J. and Sugumaran,M. (1986)b. Protease inhibitor controls prophenoloxidase activation in *Manduca sexta. FEBS. Lett.* 208(1):113-116.

Saul, S.J. and Sugumaran, M. (1987). Protease mediated prophenoloxidase activation in the hemolymph of the tobacco worm, *Manduca sexta. Archives of Insect Biochemistry and Physiology.*5:1-12.

Schein, E and Voigt, W.P.(1979). Chemotherapy of bovine theileeriosis with halofuginone. *Acta tropica.* 36:391-394.

Schein, E., Melhorn, H and Warnecke, M. (1978). Light electron microscopic studies on the schizogony of 4 *Theileria* species of cattle (*T. parva, T.lawrencei. T.mutans and T.annulata).Protistologica. 14:337-348.*

Schroder, J. and Van Schalkwyk, P.C. (1989). Die doeltreffenheid van alfametrien-deurweekte oorplaatjies teen beesbosluise. *Journal of the South African Veterinary Association.* 60:79-82.

Schumacher,E.F.B and Schill, W. (1972). Radial diffusion in gel for micro determination of enzymes II. Plasmingen activator, elastase and non specific proteasses. *Analytical Biochemistry.* 48:9-26.

Sergent, E, Donatien, A.L., Parrot, L.M., Lestoquard, F, Plantureux, E and Rougenief, H. (1924). Les piroplasmoses bovines d'Algerie:Premiere Memoire. *Archives de L'Institut Pasteur, d' Algerie.* 2: 1-46.

Service, M.W. (1988). New tools for old taxonomic problems in blood sucking insects. In *Biosystematics of Haematophagous insects* (Edited by Service, M.W).pp. 325-345. Clarendon Press, Oxford.

Service, M.W (ed) (1986). Lecture notes on medical Entomology. Blackwell Scientific Publications, Oxford.

Sevier, F.D. (1976). Sensitive solid -phase assay of proteolytic activity. *Analytical Biochemistry* 74: 592-596.

Sharon, N and Lis, H. (1989). Lectins as recognition molecules. *Science*, 246:227-247.

Sharon, N and Lis, H. (1972). Lectins: cell-agglutinating and sugar-specific proteins. *Science.* 177:949.

Shaw, M.K., Tinley, L.G.and MacKeever, D.J. (1993). Tick salivary gland extract and interleukin-2 stimulation enhance susceptibility of lymphocytes to infection by *Theileria parva* sporozoites.*Infection and Immunity.*61:1486-1495.

Shaw, M.K., Lewis, G., Tinley, Musoke,T.J. and Teal, A.J. (1995). MHC class I molecules are essential cell surface component involved in *Theileria parva* sporozoite binding to bovine lymphocytes. *Journal of Cell Science.*108:1587-1596.

Shaw, M.K., Tilney, L.G and Musoke, A.J. (1991). The entry of *Theileria parva* sporozoites into blood lymphocytes: Evidence for MHC class I involvement. *Journal of Cell Biology,* 113: 87-101.

Short, N.J., FLoyd R., Norval, R.A.I and Sutherst, R.W. (1989). Development rates fecundity and survival of developmental stages of the ticks *Rhipicephalus appendiculatus, Boophilus decoloratus* and *B. microplus* under field conditions in Zimbabwe. *Experimental and Applied Acarology,* 6:123-141.

Snow, K.R. (1967). Some aspects of the biology of *Hyalomma anatolicum* (Ixodoidea:Ixodidae). PhD Thesis, University of London.

Soderhall, K and Ajaxon, R. (1984). Lipopolysaccharide induce activation of prophenoloxidase-activating system in cray fish *Astacus astacus* hemocyte lysate. *Biochemistry Biophysics Acta.* 7:229-239.

Soderhall, K and Smith, V.J. (1986). The prophenoloxidase system: the biochemistry of its activation and role in Arthropod cellular immunity with special references to crustaceans. In: *Immunity in Invertebrates* (M. Brehelin, Ed)pp. 208-223. Springer-Verlag, Berlin.

Soderhall, K., Hall, L., Unestam, T and Nyhlem, L. (1979). Attachment of phenoloxidase to fungal cell walls in arthropods immunity. *Journal of Invertebrate Pathology.* 34:285-294.

Solomon, K.R. (1983). Acaricide resistence in ticks. *Advances in veterinary Science and Comparative Medicine.* 27: 273- 296.

Solomon, K.R., Mango, C.K.A and Obencahin, F.D (1983). Endocrine mechanism inticks. Effects of insect hormones and their mimics on development and reproduction. in Physiology of ticks (eds) Obenchain, F.D and Galun, R. vol I.pp.399-438. Prgamon Press. oxford.

Soneshine, D.E (1992). Biology of ticks Volume 1. Oxford University press. pp447.

Spooner, P.R. (1990). The effects of oxytetracycline on *Theileria parva in vitro. Parasitlogy*, 100: 11-17.

Stagg, D.A., Kanhai, G.K., Young, A.S. and Brown, C.G.D. (1976). The establishment of *Theileria* infected cell lines from an eland (*Tauretragus oryx*,lydekker, 1906). *Research in Veterinary Science.* 20: 122-126.

Stiles, J.K., Molyneux, D.H. and Wallbanks, K.R. (1989). Effects of gamma-irradiation on the midgut ultrastructure of *G. palpalis* spp. *Radiology Research..*118:353-363.

Stiles, J.K., Wallbanks, K.R. and Molyneux, D.H. (1991). The use of casein substrate gels for determinimg trypsin-like activity in the mid gut of *Glossina palpalis* spp (Diptera: Glossinidae). *Journal of Insect Physiology.* 37(4):247-254.

Sugumaran, M., Saul. S and Ramesh, N. (1985). Endogenous protease inhibitors prevent undesired activation of prophenoloxidase in insect haemolymph. *Biochemistry Biophsics Communication..*132:1124.

Sutherst, R.W and Maywald, G.F. (1985). A computerised system for matching climates in ecology. *Agriculture, Ecosystems and Environment.* 13:281-299.

Suzuki, T and Mori, K. (1990). Hemolymph lectin of the pearl oyster *Pinctada fucata martensii*: A possible non-self recognition system: *Developmental and Comparative Immunology* 14: 161-173.

Tatchell, R.J., Araman S.F. and Boctor F.N. (1972). Biochemical and physiological studies of certain ticks (Ixodoidea). Protease activity cycles in *Argus (Persicargas) persicus* (Ok(Oken) and A. (p). arboreus Kaiser, Hoogstraal, and Kohls (Argasidae). *Zeitschrift fur Parasitenkunde.* 39: 345-350.

Tatchell, R. J (1967)a. Salivary secretion in the cattle tick as a means of water elimination. *Nature*, Lond. 213: 940-941.

Tatchell, R.J. (1967)b. The ionic regulatory role of salivary secretion of the cattle tick *Boophilus microplus*. *Journal of Insect Physiology*. .15:1421-1430.

Tanner, M and Evans, D. (1994). Vaccines or drugs:Complementary crucial. *Parasitology Today*.10:406-407.

Tatchell, R.J and Easton E. (1986). Tick (Acari:Ixodidae) Ecological studies in Tanzania. *Bulletin of Entomological Research. 76: 229-246.*

Tatchell, R.J. (1964). Digestion in the tick, *Argas persicus* Oken. *Parasitology*. 54: 423-440.

Theiler, A. (1911). Some observations concerning the transmission of East Coast fever by ticks : in Report of Directorate of Veterinary Research, dept. of Agric. Union of S.A. Gov. Printing and Stationary Office Pretoria.pp. 208-222.

Tsukamoto, T., Ishiguro, M and Funatsu, M. (1986). Isolation of latent phenoloxidase from prepupae of the housefly, *Musca domestica. Insect Biochemistry*. 16:573-581.

Tsur, I and Pipano, E. (1966). Attenuation of virulence of strains of *Theleria annulata* by growth and passage through tissue culture. *Journal of Protozoology*. 13. Suppliment 33.

Uilenberg, G. (1981). Theilerial species of domestic livestock. *In* Advances in the control of theileriosis (ed A.D Irvin. M.P Cunningham and A.S Young) pp. 118-122. Nairobi, ILRAD.

Uilenberg, G., Silayo, R.A, Mpangala, C. Tondeur,W. Tatchell, R.J. and Sanga, H.J.N. (1977). Studies on Theilerridae (Sporozoa) in Tanzania. X. A large-scale field trial on immunization of cattle against theileriosis. *Tropenmedizin und parasitologie*. 28: 499-506.

Van Driesche, E., Fischer, J., Beeckmans, S and Bog-Hanser, J.C (1994). *Lectins: Biology, Biochemistry, Clinical Biochemistry*. Vo. 10. Pub. Texttop, Lemchesvej, Belgium.pp.374.

Vasta, G.R and Marchalonis, J.J (1987). Invertebrate agglitinins and the evolution of humoral and cellular recognition factors. invertebrate odels:*Cell receptors and Cell communication* (ed. by A.H. Greenberg).pp 104- 117. Karger, Basel.

Veres, J and Grubhoffer, L. (1990). Detection and partial characterization of a new plasma lectin in the hemolymph of the tick *Ornithodoros tartakovskyy. Microbios letters.* 45 (178): 61-64.

Volf, P., Killick-kendrick, R and Bates, R.A (1994). Comparison of the haemagglutination activities in gut and head extracts of various species and geographical populations of *Phlebotomus* sandflies. *Annals of Tropical Medicine and Parasitology.* 88(3):337-340.

Vundla, W.R.M. (1990). The digestive aspartic proteinases of the brown ear tick *Rhipicephalus appendiculatus* Neuman. PhD Thesis. University of Neachatel.

Vundla, W.R.M., Brossard, M., Pearson, D.J. and Labongo, V. (1992). Characterisation of aspartic proteinases from the gut of the tick *Rhipicephalus appendiculatus* Neuman. *Insect Biochemistry and Molecular Biology.* 22(4): 405-410.

Waladde, S.M. and Rice, M.J. (1982). The sensory basis of tick feeding behavoiur. 1*n. Physiology of ticks* (Editors Obenchain F.D & Galumn R).pp 71-118. Oxford: Pergamon Press.

Waladde, S.M. *et al.*, (1993). Transmission of *Theileria parva* to cattle by *Rhipicephalus appendiculatus* adults fed on nymphae *in vitro* on infected blood through an artificial membrane. *Parasitology.* 107:249-256.

Waladde, S.M. Young, A.S. and Morzaria, S.P.(1996). Artificial feeding of Ixodid ticks. *Parasitology today.* 12(7):272-278.

Walker, A.R. (1990). Parasitic adaptations in the transmission of *Theileria* by ticks. A review. *Tropical Animal Health and Production.* 22:23-33.

Walker, J.B. (1974). The ixodid ticks of Kenya: A review of present knowledge of their hosts and distribution. Commonwealth Insitute of entomology. The Eastern Press. Ltd.London and Reading.pp.220.

Wallbanks K., Ingram G.A and Molyneux, D.H. (1986). The agglutinins of erythrocytes and *Leishmania* parasites by sandfly gut extracts:evidence for lectin activity. *Tropical Medicine and Parasitology.* 37:409-413.

Wang, H and Nuttal, P.A. (1994). Excretion of host immunoglobulin in tick saliva and detection of TgG-binding proteins in tick haemolymph and salivary glands. *Parasitology.* 109:525-530.

Welburn, S., Maudlin, I and Molyneux, D.H (1994). Midgut lectin activity and sugar specificity in teneral and fed tsetse. *Medical and Veterinary Entomology* 8:81-87.

Welburn, S.C, Maudlin, I and Ellis, D.S. (1989). Rate of trypanosome killing by lectins in midguts of different species and strains of *Glossina*. *Medical and Veterinary Entomology*.3:77-82.

Welburn, S.C. and Maudlin, I. (1990). Heamolymph lectin and the maturation of trypanosome infections in tsetse. *Medical and Veterinary Entomology* .4: 43-48.

Welburn, S.C and Maudlin, I. (1989). Lectin signallinmg of maturation of *T.congolense* infections in tsetse. *Medical and Veterinary Entomology.* 3:141-145.

Wigglesworth, V.B. (1943). The fate of haemoglobin in *Rhodinius prolixus* and other blood sucking arthropods. *Biological Sciences.* 131: 313-339.

Wigglesworth, V.B. (1972). *The principles of Insect Physiology.* 7th edition.Chapman and Hall. Lond.

Wilde, J.K.H. (1967). East coast fever. *Advances in Veterinary Science.*11: 207-259.

Wilde, J.K.H., Brown, C.G.D., Hulliger, L., Gall. D and Macleod, W.G. (1968). East coast fever: Experiments with tissues of infected ticks. *British Veterinary Journal.* 124:196-208.

Willadsen, P. (1990). Perspectives for subunit vaccines for the control of ticks. *Parasitologia,*. 32:195-200.

Willadsen, P. and Kemp, D.H. (1988). Vaccination with 'concealed' antigens for tick control. *Parasitology Today*. 4:196-198.

Willadsen, P., Eisemann, C.H and Tellam, R.L. (1993). "Concealed" antigens: Expanding the range of immunological targets. *Parasitol Today*. 9:132.

Willadsen, P., McKenna, R.V, Riding, G.A. (1987). Purification and Characterisation of an esterase from the tick *Boophilus microplus*. *Insect Biochemistry*.. 17:659-664.

Willadsen, P., Riding, G.A., McKenna,R.V., Kemp, D.H., Tellam, R.L., Nielsen, J.N., Lahnstein, J., Cobon, G.S and Gouch,J.M. (1989). Immunological control of a parasitic arthropod: Identification of a protective antigen from *Boophilus microplus*. *Journal of Immunology*. 143:1346-1351.

Williams, C.M. (1968). Ecdysones and ecdysone analogues, their assay on diapausing pupae of the *Cynthia* silk worm. *Biological Bulletin*. 134: 344-355.

Williamson, S.M., Tait, A., Brown, C.G.D., Walker, A.R., Bech, P., Sheils, B., Fletcher, J and Hall, F.R. (1989). *Theileria annulata* sporozoites surface antigens expressed in *Escherichia coli* elicits neutralizing antibody. *Proceedings of the National Academy of Sceince. USA*. 86: 4639-4643.

Wilson, S.G. (1950). A check-list of Ixodidae on cattle found in Nyasaland with descriptions and biological notes of the rhipicephalids. *Bulletin of Entomological Research.*41: 415-428.

Wright, J.E (1969). Hormonal termination of larval diapause in *Dermacentor albipictus*. *Science.* 163:390-391.

Yeaton, R.W. (1982). Are invertebrate lectins primordials receptors? in:developmental Immunology: Clinical problems and aging. EL. Cooper & M.A. Brazier (eds). Academic press, New York 1982. 73-83.

Yeaton,W.R. (1981). Invertebrate lectins.1. Occurrence. *Experimental and Comparative Immunology.*5:391-402.

Yeoman, G.H. (1964). Cattle ticks in East Africa. *Outlook in Agriculture.* 4:126-135.

Yeoman, G.H. and Walker, J.B. (1967). The ixodid ticks of Tanzania. A study of the Zoogeography of the Ixodidae of an African Country. Common wealth Insitute of Entomology. Lond. pp.215.

Young, A.S and Leitch, B.C (1981). Epidemiology of East Coast Fever: Some effects of temperature on the development of *Theileria parva* in the tick vector, *Rhipicephalus appendiculatus.Parasitology.* 83: 199-211.

Young, A.S., Leitch, B.L. (1983). Identification of *Theileria* infections in living salivary glands of ticks. P*arasitology.* 86: 519-528.

Young, A.S. (1985). Immunization of cattle aginst theileriosis in the Trans-mara division of Kenya: Comparison of a trial under a ranch development and traditional Masai management. In *Immunization against Theileriosis in Africa. Proceedings of a joint workshop, 1-5 October, 1984.Nairobi.*pp.64-68.

Young, A.S. and Leitch B.L. (1980). A probable relationship between the development of *Theileria* species and the ecdysis of their host. *Journal of Parasitology.* 66:356-359.

Young, A.S. and Morzaria, S.P.(1986) Biology of *Babesia. Parasitology Today*, 2 (8):211-219.

Young, A.S., Brown, C.G.D, Cunningham, M.P and Radley, D.E. (1978). Evaluation of methods of immunizing cattle aginst Theileria lawrencei. In Tick-borne diseases and their vectors: Proceedings of a workshop Edinburgh, 27 Sept-Oct, 1976. Editor, J.K.H. Wilde, Centre for Tropical Vreetrinary Medicine. University of Edinburgh, 293-295.

Young, A.S., De Castro J.J., Burns C. and Murphy D.L. (1985). Potential of ear tags impregnated with acaricide for the control of the brown ear tick *Rhipicephalus appendiculatus* infesting cattle. *Parasitology.* 90:391- 399.

Appendix

General buffers:

1. piroplasm purification

Alsevers' Solution

72 mM Sodium Chloride, 27mM Sodium citrate, 3 mM Citric acid, 100mM dextrose.

TEN, pH 8.0

20 mM Tris-HCL, 10mM EDTA, 100 mM NaCL

Dulbeccos' Phosphate Buffered Saline (DPBS) pH 7.4

137 mM NaCL, 8.1 mM Na_2HPO_4, 2.7 mM KCL, 1.5mMKH_2PO_4, 1.2 mM$CaCL_2$, 0.5mM $MgCL_2$.

2.Tissue Culture -Monoclonal development

RPMI 1640, pH 7.2 with 25mM HEPES, 2 mM $CaCL_2$, 0.5mM $MgCL_2$

3.ELISA

Casein Blocking buffer pH 7.4

0.25% casein in Dulbeccos PBS.

Serum diluting buffer pH 7.4

5% Skimmed milk in Dulbeccos PBS, 0.1 % Tween-20.

Washing buffer, pH 7.4

0.1% Tween- 20 in Dulbeccos PBS

Substrate buffer, pH 4.0

200mM Na 2 HPO4, 160 mM Citric acid

Coating buffer (Dulbecco's phosphate buffered saline pH 7.4)
The above buffer was prepared as follows in 10 litre quantities:
Solution

KCL	2.0g
KH_2PO_4	2.0g
NaCL	80.0g
Na_2HPO_4	11.5g

The above chemicals were added separately to 6-7 litres distilled water while stirring, and sufficient water was added to make 8 litres. The (800ml) solution was aliquoted to 1 litre bottles and autoclaved for 30 min at 15 PSI (pressure per square inch).
Solution B

$CaCL_2$	1.0g

The above was dissolved in 800-900 ml distilled water and made up to 1 litre. The solution was aliquoted into 100 mls and autoclaved as above.

Solution C

$MgCl_2$ *0.47g*

A 1 litre solution was prepared, aliquoted and autoclaved. Under a laminar flow hood, the solutions, at room temperature, were combined aseptically, avoiding precipitates. This was done by creating a vortex in solution A by rotation in circular motion and adding solution B. Finally C was added following the same procedure. The pH was estimated and 100ml aliquotes stored at 4^0C.

Diluting buffer

This was made by removing 1 ml of coating Dulbecco's buffer pH 7.4 and replacing it with 1 ml Tween-20.

Blocking buffer (0.25% casein in Dulbecco's PBS)

0.25g caseing (sodium salt from bovine milk) was layered gently into 100 ml of Dulbecco's and gently stirred until it dissolved.

Substrate buffer

This buffer contained the following chemicals:

$Na_2HPO_4.2H_2O$ 35.6g
or Na_2HPO_4 28.4g
Citric acid (monohydrate) 33.6g

The salts were dissolved in one litre distilled water and pH adjusted to 4.0 with either 1M Na_2HPO_4 solution (142g Na_2HPO_4 anhydrous in a litre Distilled water) or 0.8M citric acid (168g in 1 litre) and finally made to 2.0 litres distilled water. The solution was then filtered through 5µm filter and stored at 4^0 C and warmed by incubation at 37^0 C for 15 min before use in ELISA.

Washing solution (2M NaCL)

This consisted of 116.88g NaCL in 1 litre of deionized water left to stand overnight at room temperature. Seven hundred fifty (750) ml of the solution was taken and 10 ml of tween- 20 added. The mixture was made up to 10.0 litres with deionized water and stirred until the detergent was dissolved.

Preparation of substrates
Hydrogen peroxide

30% Hydrogen peroxide 500µl
Distilled water 7.5ml
The above was mixed and stored in a dark bottle at 4^0 C.

Chromogen (40mM 2,2'-Azino-bis(3-Ethylbenzthiazoline-6-Sulfinic acid (ABTS) Diammonium salt (Sigma) $C_{18}N_40_6S_4.(NH)_{12}$

ABTS powder 0.135g
Distilled water 6.25ml

The powder was dissolved and stored at 4^0 C in a dark bottle, where it could keep for two weeks.

Working substrate/ chromogen solution:

Substrate buffer 25ml
1% substrate H_2O_2 100µl
40mM BTS (chromogen) 125µl

4.SDS-PAGE and Immunoblotting

4 x gel running buffer, pH 8.8

1.5 M Tris-HCL

4 x stacking gel buffer, pH 6.8

500 mM Tris HCL

Monomer solution

30 % acrylamide, 1 % bisacrylamide

7.5-17.5 % Gradient gel mix

	7.5%	17.5%
Monomer solution	3.75ml	8.75ml
4 x gel running buffer	3.75ml	3.75ml
Water	7.32ml	2.275ml
10% SDS	300µl	300µl
10% Ammonium persulphate (APS)	25µl	25µl
TEMED (N,N,N'N'-tetramethylethylenediamine	5µl	5µl

3% Stacking gel mixture

600µl monomer solution, 1.5 4 x stacking gel buffer, 3.8 ml distilled water, 60µl 10 % SDS, 20µl, 10 % APS, 3 µl TEMED.

Tank buffer pH 8.3

25mM Tris, 380 mM glycine, 0.1% SDS

SDS-PAGE Sample buffer, pH 6.8

50mM Tris-HCl, 0.1% SDS, 1.0% ß-mercaptoethanol, 0.002% Bromophenol blue, 10% glycerol.

Transfer buffer, pH 8.4

25mM Tris, 192 mM glycine, 20% methanol, 0.1% SDS.

Milk buffer pH 8.1

20mM Tris , 50 mM NaCL, 0.02% NaN_3

Milk blocking buffer

Milk buffer containing 5% (W/v) skimmed milk.

Antibody dilution buffer

Milk buffer 2.5% (W/V) skimmed milk.

Milk washing buffer

Milk buffer containing 2.5% (W/V) skimmed milk, 0.1% Triton x 100

Substrate/ Chromogen Solution

50mg diaminobenzidine (AB) 50µl 30% H_2O_2 in 100 mls Dulbeccos PBS.

5. Coomassie Blue Staining

Staining

0.5% Coomassie blue; 30% methanol; 9% acetic acid.

Destaining solution

10% acetic acid, 30 % methanol

6. Silver staining

Prefixation (50% methanol ;10% Acetic acid);Fix (10% glutaldehyde) followed by 5ug/ml Dithiothreitol (DTT); stain (0.1%) silver nitrate.
Developer (37% formaldehyde in 3% Na_2Co_3); followed by citric acid.
Gel stored in 0.3%Na_2Co_3

Milk buffer containing 2.5% (W/V) skimmed milk, 0.1% Triton x 100

Substrate/ Chromogen Solution

50mg diaminobenzidine (AB) 50µl 30% H_2O_2 in 100 mls Dulbeccos PBS.

5. Coomassie Blue Staining

Staining

0.5% Coomassie blue; 30% methanol; 9% acetic acid.

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