INDUCTION OF RESISTANCE TO *RHIPICEPHALUS APPENDICULATUS* NEUMANN IN NEW ZEALAND WHITE RABBITS THROUGH IMMUNISATION WITH COMMERCIAL MOULTING HORMONES AND TICK-DERIVED HAEMOLYMPH

BY

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DECLARATION.

I, SAMUEL KAMAU MBOGO, hereby declare to the Senate of Kenyatta University that this thesis is a result of my own work except where acknowledged in the text. It has not been submitted nor is it being concurrently submitted for a similar qualification in any other University.

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DECLARATION BY SUPERVISOR.

This thesis has been submitted for examination with my approval as the supervisor

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DEDICATION.

TO MY FATHER

LAMECK MWANGI MBOGO

WHO PASSED AWAY BEFORE THIS WORK WAS ACCOMPLISHED.

MAY HIS SOUL REST IN ETERNAL PEACE

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ABSTRACT.

This study was conducted to assess the possibility of immunising rabbits against *Rhipicephalus appendicuatu* Neumann, the vector for East Coast fever and Corridor disease, the most important tick-borne diseases in East and Central Africa. The antigens whose efficacy in the control of this vector were assessed included commercially available insect moulting hormone, beta-ecdysone and tick haemolymph components. However, since antibodies play a major role in the acquisition of tick resistance, it was imperative before any immunisation attempts were made to study the passage of antibodies (specifically immunoglobulin G (IgG) across the tick midgut membrane since potentially protective antigens would be of little value if the antibodies whose production they elicit do not cross the midgut barrier. These studies were undertaken, using the enzyme linked immunosorbent assay (ELISA) and the results obtained indicated that IgG can cross the midgut barrier and retain its biological activity. In addition the study also showed that the quantities of the IgG that cross this barrier are positively correlated to the antibody titres in the host blood. Of prime importance was the finding that destruction of the midgut barrier with anti-tick midgut antibodies could facilitate the passage of IgG across the midgut by an eight fold concentration factor and these antibodies maybe directed against antigens entirely differemt from those of midgut origin. Based on this findings, it was found feasible to immunise hosts against ticks using antigens that are found in the haemolymph.

Rabbits were immunised against *R.appendiculatus* using commercially available moulting hormone (beta-ecdysone) that was conjugated to bovine serum albumin to render it immunogenic. The rabbits were also immunised with conjugated beta-ecdysone in combination with solubilised tick midgut membrane proteins (STGMP) to increase the titres of antibodies crossing the midgut. The immunised rabbits were infested with all the developmental instar stages of *R.appendiculatus*. The number of ticks that fed successfully to engorgement, their feeding durations and engorgement weights were recorded along with the percentage of the immature stages that moulted. In addition, the percentage of engorged females reaching the critical engorgement weight (300mg and above), the percentage that oviposited, the weights of the oviposited eggs and egg hatchabilities were recorded. The results obtained indicated that the immunisation had little effects on the immature stages. However, immunisation reduced the engorgement weights and fecundites of adult ticks. Adult ticks that had been applied onto rabbits immunised with a combination of beta-ecdysone and STGMP were the most adversely affected. The next developmental instars from these ticks when applied onto tick-naive

rabbits and the same parameters recorded showed that immunisation had no effect on the resultant larvae and nymphs. However, the percentage of the ticks that fed successfully to engorgement, the egg conversion ratio, the percentage of females that oviposited and the hatchabilities of the oviposited eggs were significantly reduced in the adults that fed as nymphs on rabbits immunised with either beta-ecdysone or beta-ecdysone in combination with STGMP. The adults that fed as nymphs on rabbits immunised with STGMP had reduced engorgement weights and hatchabilities.

The efficacy of haemolymph components in immunisation of hosts against ticks was assessed. To determine from which sex of tick haemolymph was to be used for immunisation, haemolymph was obtained from adult male and female *R.appendiculatus* at different days of feeding (0-5), was characterised using sodium dodecyle sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The results obtained demonstrated that the haemolymph contained sex specific proteins. Since it was easier to bleed partially fed ticks compared to engorged ones, haemolymph from 5 day fed male and female ticks was used to inoculate rabbits at a dosage rate of 1mg/kg intramuscularly. To facilitate the passage of antibodies across the tick midgut membrane, haemolymph components were used alone or in combination with STGMP along with STGMP control. After the last booster dose the rabbits were infested with all the developmental instar stages of *R.appendiculatus* and the same parameters indicated above recorded. The results obtained showed that the rabbits had been rendered resistant to all the developmental stages of. *R appendiculatus*. This resistance was more pronounced in adults where the number of ticks that fed successfully to engorgement were drastically reduced. In addition, the females had reduced engorgement weights and fecundities.

Rechallenging the resistant experimental rabbits with all the developmental instar stages of *R.appendiculatus* showed that resistance was even more pronounced during subsequent infestations. These results indicated that these immunogens could be of practical use in tick control in the field as protection conferred to the hosts was maintained in subsequent infestations.

CHAPTER ONE.

1.0 General introduction.

1.1 General biology of ticks.

Ticks are obligate haematophagous arthropods of the class Arachnida and order Acarina. They are important ectoparasites with a wide host range encompassing both homiotherms and poikilotherms. They show general (sometimes very definite) host specificity. Some ticks feed exclusively on mammals while others like the *Aponoma* species feed exclusively on reptiles. Other ticks are quite specific and will feed only on a particular host species. For example, *Amblyomma tholloni* feeds on elephants while *A.rhinocerotis* feeds exclusively on rhinoceros (Walker, 1974). From their morphological and physiological characteristics, ticks have been grouped into three families, the hard ticks (Ixodidae), the soft ticks (Argasidae) and the Nuttalliellidae which lies in between the two (Hoogstrall, 1976).

The family Ixodidae comprises of 650 different species belonging to 11 genera. Seven of these genera namely *Haemaphysalis*, *Rhipicephalus*, *Dermacentor*, *Hyalomma*, *Boophilus*, *Ixodes* and *Amblyomma* are vectors of economically important diseases (Arthur, 1962; FAO, 1984). Ixodid ticks have a scutum that extends over the entire dorsal surface in males and covers only a small area behind the head in the larva, nymphs and females while the argasids are covered by a leathery integument.

The feeding process of ixodid ticks can be divided into two phases (FAO, 1984). During the first phase, which is slow and gradual, integumental growth and mating occur. Mating is essential for female ticks to complete normal engorgement (Diehl *et al.*, 1982). The second phase is rapid and is accompanied by consumption of huge amounts of blood which they concentrate by injecting saliva into the host (Kaufman and Sauer, 1982). This saliva contains tick antigens (Krolack *et al.*, 1983) which on introduction into the host induce immunological responses which may have adverse effects on subsequent tick infestations. The argasids are

multi-host ticks and undergo irregular feedings for each developmental stage whenever their hosts are available.

Engorged females drop off from the host and oviposit. The oviposited eggs hatch into larvae which unlike other arachnids are six-legged. These larvae feed and moult into nymphs which after feeding moult into adults. Unlike the ixodid ticks which have only one nymphal moult, argasids have 2 to 6 nymphal moults before an adult emerges. The adult females feed, drop off the host, oviposit and the cycle is repeated. From the number of hosts required to complete their life cycles, Ixodid ticks can be classified into three groups, that is 3 host ticks (such as *Amblyomma variegatum*), 2 host ticks (such as *Rhipicephalus evertsi*) and one host ticks (such as *Boophilus decoloratus*). Six hundred out of the 650 Ixodid species have a 3 host life cycle. For the 3 host ticks, all instars feed on different hosts. In 2 host ticks, larvae and nymphs feed on the same host after which they drop off and moult into adults which feed on another host. In one host ticks, all the instars feed on the same host. The three-host life cycles are the most primitive in evolutionary terms and the one-host ticks the most advanced (Hoogstrall, 1976). The main advantages of the one and two host cycles over the three-host cycle are that the time required for completion of the cycle is reduced (as moulting on the host is faster), losses during a longer free-living phase are reduced and so is the necessity for repeatedly looking for a host. However, the abundance of these one-host ticks is rapidly reduced under unfavourable environmental conditions due to the vulnerability of the eggs and the un-fed larvae to heat and dry stresses (Norval *et al.*, 1992).

1.2 The life cycle of *R.appendiculatus*.

Rhipicephalus appendiculatus is a three host tick. An engorged female drops off the host and starts ovipositing 5 to 6 days later. It continues ovipositing for 14 days and lays between 3000 and 5000 eggs. The eggs hatch into larvae after 21 days. The larvae climb onto vegetation and await a host. When a host passes by, the larvae climb onto it and feed to engorgement within 5 to 7 days. The engorged larvae then drops off and moult into nymphs within 18 to 21 days. The

nymphs in turn climb onto vegetation, attach onto a host and feed to engorgement within 5 to 7 days. The nymphs drop off and moult into adults within 21 days. The adults like the larvae and nymphs look for a host and attach onto it. They prefer feeding along the edges and the inside of ears but they may also feed around the eyelids and on the base of the horn. The adults feed and mate on the host. After engorgement, which takes between 6 and 8 days, the engorged females drop off the host, oviposit and die. The oviposited eggs hatch into larvae and the cycle is repeated. The duration taken by *R.appendiculatus* to complete its life cycle depends on temperature, humidity and the availability of hosts.

1.3 The distribution of *R.appendiculatus*.

The distribution of *R.appendiculatus* is influenced mainly by climate, vegetation and availability of hosts. The absence of this tick in places where the climatic factors and vegetation are quite suitable such as Central Tanzania and West Africa may be attributed to the low densities of cattle and other herbivore hosts that have existed in the past (Norval *et al.*, 1992). This low densities may have been caused by trypanosomiasis and the presence of large forested areas with little grazing for ground dwelling herbivores. It must therefore be borne in mind that the ongoing deforestation, introduction of cattle and trypanosomiasis control are increasing the risks of the tick spreading to these areas. Indeed in Zimbabwe, *R.appendiculatus* was not found in the hot, dry southern lowveld in the 1960s but the tick was introduced in 1973 by a herd of saple antelopes (*Hippotagus niger*) translocated from a wildlife reserve to a small game park on a cattle ranch during a wet season. The tick became well established, spread rapidly within the following 7 years when the area had above average rainfall and had infested over a million hectares by 1980. However, in 1983/84, the tick disappeared after the area experienced drought for 4 continuous years (Norval *et al.*, 1992). One must therefore bear in mind that the distribution of this tick is not static. However, currently this tick is restricted in its distribution to East, Central and Southern Africa and has been reported in 15 countries. These include Central

African Republic, Sudan, Zaire, Uganda, Kenya, Rwanda, Burundi, Tanzania, Zambia, Malawi, Mozambique, Botswana, Swaziland and South Africa (Norval *et al.*, 1992 ; Figure 1).

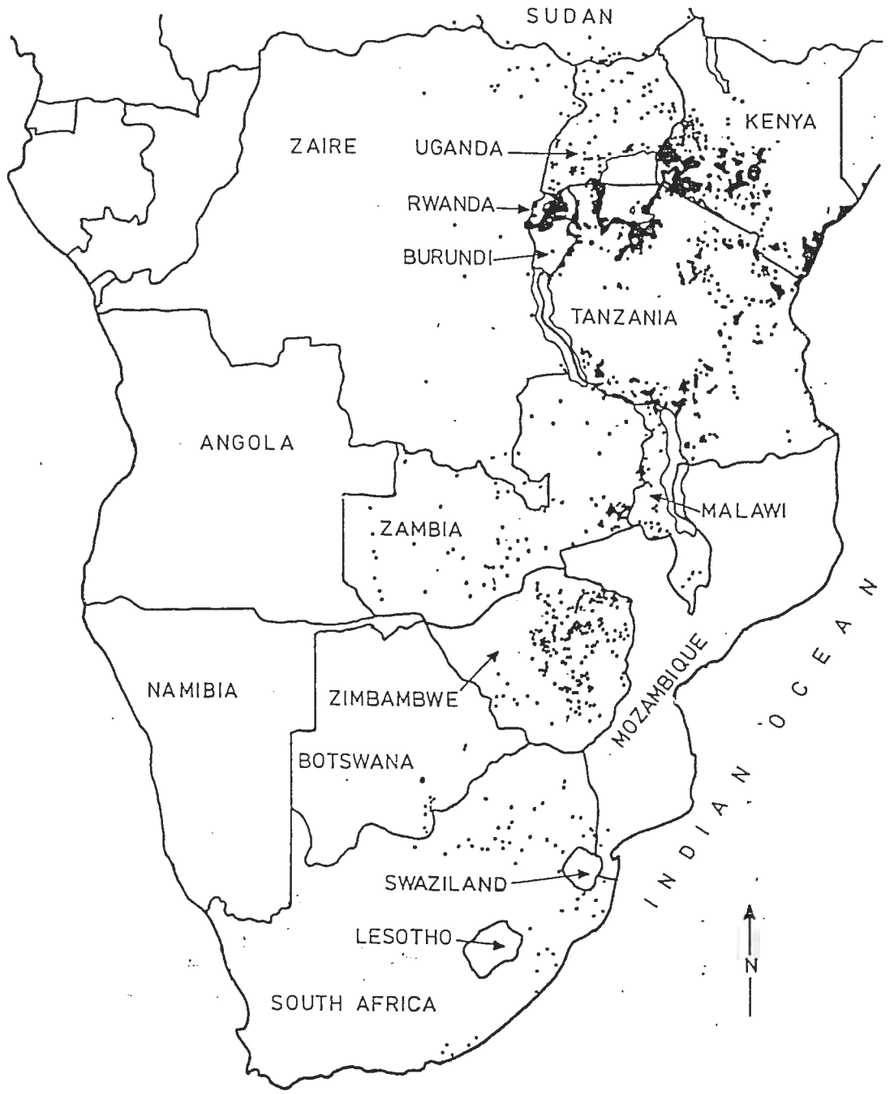

FIGURE 1.

A map of East, Central and Southern Africa showing the distribution of *R.appendiculatus* drawn from data assembled by Lessard *et al.*, (1990).

In Kenya, *R.appendiculatus* occurs in the south-western corner in areas lying between 0 and 2400 metres above sea level and with a rainfall of not less than 600 mm per annum (Figure 2).

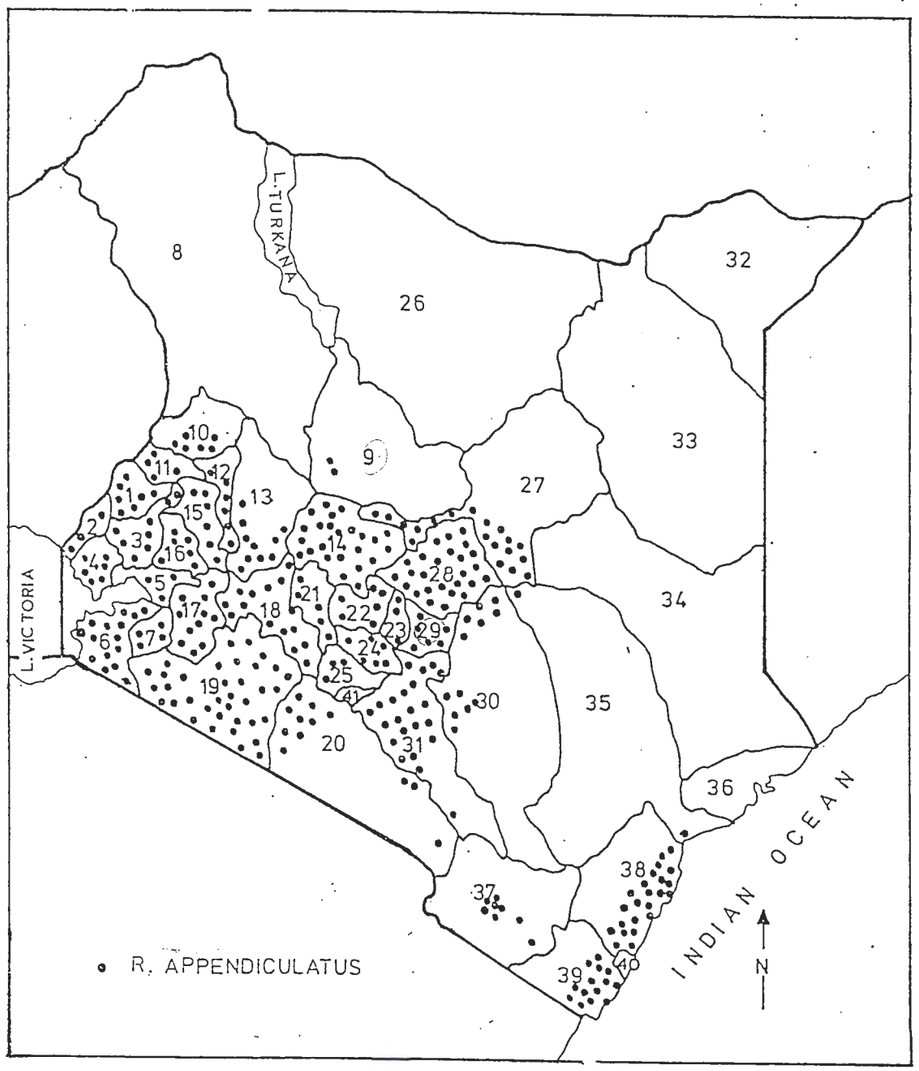

FIGURE 2.

A map showing the distribution of *R.appendiculatus* in Kenya (Adapted from Kariuki, 1988).

The numbers show the different Districts in Kenya which are named in the legend on the opposite page.

1.4 The economic importance of ticks.

The importance of ticks as a menace to livestock production was realised many years ago and "tick fever" has been referred to in an Egyptian papyrus scroll dated 1550 BC. (cited by Obenchain and Galun, 1982). Ticks and the diseases associated with them are an important global problem (Balashov, 1972; Bram, 1975; Steelman, 1976) since they result in diminished productivity and death of livestock thereby reducing the amounts of animal protein needed by man. Ticks were first shown to be disease vectors when it was discovered that *Boophilus* ticks were the vectors of the Texas fever (Babesiosis) pathogen, *Babesia bigemina* (Smith and Kilbourne, 1893). Since then, ticks have been shown to transmit numerous pathogens which include protozoa, rickettsia, viruses, bacteria and filaria and are the most important vectors of livestock diseases and rank second to mosquitoes as vectors of human disease (Obenchain and Galun, 1982).

1.4.1 Ticks as disease vectors.

Ticks are very efficient disease vectors that transmit, amplify the infection and serve as reservoirs of the diseases they transmit (Hoogstrall, 1985). In Livestock, ticks transmit economically important diseases which include theileriosis, babesiosis, anaplasmosis, cowdriosis, Nairobi sheep disease and spirochaetosis. Theileriosis, particularly East coast fever (ECF) which is transmitted between cattle and the equally serious corridor disease which is transmitted from buffalo to cattle is by far the most important tick borne diseases of cattle in East and Central Africa (Young and Leitch, 1981; Mukhebi *et al.*, 1992, 1994). Both corridor disease and ECF are transmitted transtadially by *R.appendiculatus* and they are endemic where the vector is present. Unfortunately, the most productive cattle are kept where the vector is readily available. These diseases have high morbidity and mortality rates and as much as their distribution is limited almost exclusively to the East coast of Africa, they kill a cow every minute (Osogo, 1981). Losses due to theileriosis in terms of reduced productivity, mortalities and the cost of

control are in millions of dollars (Mukhebi *et al.*, 1994). In addition a lot of money is spent on research aimed at finding ways of controlling both the disease and the vector.

Apart from transmission of diseases, massive tick infestations can cause anaemia and severe damage to skins and udders (FAO, 1984). Due to the irritation caused by tick bites, the affected host spends a lot of time scratching, rubbing and licking the tick bite lesions thus reducing its feeding time which results in reduced productivity (FAO, 1984). Norval *et al.*, (1988b) estimated that every adult *R.appendiculatus* female that completes feeding causes a loss of 4g in live weight gain of cattle. Tick bite lesions may be invaded by secondary pathogens such as bacteria and by screw worms causing myiasis (Maranga, 1988). Ticks can also introduce toxins into the host which may cause fatal toxic disorders such as tick paralysis (Doube and Kemp, 1975) and sweating sickness (Bezuidenhout and Malherbe, 1981).

1.5 Tick control.

The control of ticks is very important since over 75% of the world cattle population is located in regions where ticks are enzootic (Maranga, 1988). It is estimated that by eliminating ticks from Argentina's national beef herd of 13 million head, beef production would increase by 300,000 tons annually (Maranga, 1988). This means that ticks in Argentina cause a reduction of 23.07 Kg of beef per animal per annum. This is a great loss by any standard although even higher losses have been recorded in Mexico where tick infestations are responsible for loss of 30 Kg of beef per animal per annum (Maranga 1988). In Australia, the damage due to B.microplus infestation in the 1972-73 season alone was estimated to be 60 million US dollars (FAO, 1977). In Kenya up-to 70,000 mature cattle (which would cost millions of dollars) die of ECF annually ((Mukhebi *et al.*, 1994). Several methods employed in the management of ticks and tick-borne diseases have been discussed in a recent review (Young et al., 1988). According to these authors, application of acaricides either topically by immersion in dip tanks or by running animals through spray races is the commonest method used in the management of tick populations. However, acaricides are very expensive. In Kenya, 6 to 10 million US dollars are required for the

purchase of acaricides annually (Kariuki, 1990). To control the transmission of some tick-borne diseases such as theileriosis, acaricides need to be applied onto cattle twice a week, an effective but rigorous procedure that soon renders ticks resistant to the acaricides (Wharton and Rouston, 1970; Wharton, 1976). The cost of developing new acaricides in relation to the economic returns expected from their use before resistance develops might discourage research leading into the development of new ones (Durand, 1976). Furthermore, rigorous application of acaricides prevents the establishment of an endemic stability between ticks and their hosts and any breakdown in acaricide application becomes catastrophic (Norval, 1979). In addition, most acaricides are poisonous to man and domestic animals and acaricide application is expensive in terms of time consumed, cost of acaricide, manpower used, dip or spray race construction and reduction in feeding time since most dips are far and communal. To reduce these costs, attempts have been made at controlling ticks using ear tags impregnated with acaricides (Ahrens *et al.*, 1977; Davey *et al.*, 1980; Young *et al.*, 1985 a,b). It has also been demonstrated that some chemicals of low mammalian toxicity if given parenterally have anti-tick effects and can be used as systemic acaricides. Some of the systemic acaricides tested and shown to be effective against ticks include ivermectins (Drummond *et al.*, 1981; Kaufman *et al.*, 1986) and closantel (Kariuki and Mbogo, 1988). Recently, pour-ons (Duncan, 1991) and intraruminal ivermectin slow release devices providing protection for 90 days have been developed (Pegram *et al.*, 1993).

1.5.1 Possible alternative methods of tick control.

1.5.1.1 The use of pasture spelling.

Pasture spelling has been used to control ticks. The principle behind this method is to keep pastures free of hosts and thus deny ticks the opportunity to feed. It has been used successfully to control *Boophilus* species of ticks (Ellenberger and Chapin, 1919; Wilkinson, 1955). Since *Boophilus* are one host ticks and their larvae survive only for short periods while off the host (maximum of 9 months under optimum conditions), if no hosts are available in the pastures for 9 months or more the ticks will die out. Unfortunately this method is almost

impractical to nomadic pastoralists since they use communal land which is usually not fenced. In addition the method does not allow for maximum utilisation of the already scarce pastures. Also, the most important tick in East Africa, *R.appendiculatus*, is a 3 host tick with very long survival periods (12 months, 18 months and over 2 years under optimum conditions for larvae, nymphs and adults respectively) (Cunningham, 1981) and the adults can transmit fatal ECF after fasting for 554 days (Newson *et al.*, 1984). Furthermore, all instars of *R.appendiculatus* can feed on many alternative hosts including rodents which are quite difficult to fence off.

1.5.1.2 Destruction of vegetation to control ticks.

Destruction of tick breeding sites by burning pastures has been used successfully to control ticks (Milne, 1944). However, the resultant destruction of the environment in terms of indiscriminate destruction of all organisms in the area and the soil erosion that would result is a major drawback.

1.5.1.3 The sterile male release technique.

The sterile male release technique has been used to control various arthropods. It's basic requirements are that the sterile males must disperse for long distances, be able to mate and that the females must be mated only once. It is therefore of no practical use in reducing tick populations since apart from their limited mobility, irradiation inhibits the male's spermatogenic cycle making them aspermic and females can mate with many males (Mathewson, 1984).

1.5.1.4 The use of natural enemies.

Parasitoids have been used to control arthropod pests (Laird, 1980). However, tick parasitoids like *Hunterellus hookeri* (Cheong and Rajamanikam, 1978) are obligatory parasites and would disappear if the ticks were greatly reduced. In addition, to have any impact on tick populations, very many parasitoids need to be cultured and released, an almost impossible task.

Ticks are eaten by fire ants (Butler *et al.*, 1979; Oliver *et al.*, 1979), ox-peckers (Bezuidenhout and Stutterheim, 1980; Stutterheim and Stutterheim, 1980), Lizards (Norval,

1976) and Chicken (Hassan *et al.*, 1991). It is however quite impractical to rear these predators in sufficient numbers for the purpose of reducing tick populations.

1.5.1.5 The use of "anti-tick" vegetation.

Some grasses have been shown to reduce tick survival (Thomson *et al.*, 1978) while some legumes have been shown to immobilise tick larvae (Sutherst *et al.*, 1982). However, their overall effectiveness in tick control is yet to be established.

1.5.1.6 The use of host resistance in tick control.

Bos indicus cattle have been shown to be more tick resistant than *Bos taurus* cattle (Johnson and Bancrofti, 1918; Siefert, 1971; Wharton, *et al* 1973). This resistance appears to be inheritable (Hewetson, 1968,1972) and therefore by selecting and culling tick susceptible animals, genetically tick resistant herds can be produced (Johnson and Bancrofti, 1918). Indeed in Australia, host resistance forms the basis of an integrated control programme for *B.microplus* on cattle (Powell, 1977). However, to achieve this, very high levels of farm management, farmers are required to ensure that all animals mate as planned and proper records of sires, dams and siblings are kept. This is a major drawback since most cattle in Africa are kept by nomadic tribes with high illiteracy rates. In addition, since the most tick susceptible animal may also be the most productive, this method of tick control may not be readily applicable.

Goats have been rendered resistant to *R.appendiculatus* (Maranga, 1983,1988). These resistant goats were shown to reduce tick numbers in the field drastically and their use in reducing populations of *R. appendiculatus* in pastures suggested (Maranga, 1988). Since red Maasai sheep have also been rendered resistant to *R.appendiculatus* (Wishitemi, 1983), introduction of these tick resistant sheep and goats into tick infested paddocks may play a role in tick control.

1.5.1.6.1 Induction of tick resistance.

It is well established that both domestic and laboratory animals can acquire tick resistance after a single tick infestation, which is maintained during subsequent infestations (Wikel, 1982, 1984; Wikel and Whelen, 1986). In addition hosts can be immunised against ticks with varying degrees of success as indicated in recent reviews (Willadsen, 1980; Wikel and Allen, 1982; Brown, 1985; Willadsen *et al.*, 1989). This acquired resistance to ticks may play an important role in the management of tick associated problems.

1.5.2 Integrated tick management package.

All the methods of managing tick populations discussed above have some limitations. The best approach would be one that would combine all or some of these methods to form a cost effective integrated tick management package. The benefits to the livestock owner, from tick control, should exceed the cost of the control. Many tick-borne diseases can be effectively controlled by either chemotherapy or chemoprophylaxis (Morrison, 1989). Therefore, if tick worry from excessive tick numbers and not tick-borne disease transmission is the main problem, the use of economic thresholds for tick control (daily numbers of engorged ticks below which control is not profitable) would be preferred especially in places where ticks reach high numbers only in particular seasons (FAO, 1984).

1.6 Host resistance to ticks.

The main features of the responses found in different host-tick systems can enable us to understand the principles underlying the immunological basis of tick resistance.

1.6.1 Tick resistance in the bovines.

As early as 1918, Johnson and Bancrofti observed that some cattle were persistently carrying more ticks than others. Since then, cattle have been shown to acquire resistance to numerous tick genera and species. These include *B.microplus* (Riek, 1956; 1962; Schleger *et al.*, 1976), *I.holocyclus* (Allen *et al.*, 1977), *H. asiaticum asiaticum* (Berdyer and Khudainazarova,

1976), *R.appendiculatus* (Dipeolu *et al.*, 1992; de Castro *et al.*, 1989; Fivaz and Norval, 1990), *R. evertsi* (Latif, 1984), *A.americanum* (Strother *et al.*, 1974), *H.anatolicum anatolicum* (Latif, 1984) and *D.andersoni* (Wikel, 1982). Indeed resistance of cattle to tick infestation has been well documented and reported to consist of both innate and acquired components (Wikel and Allen, 1982). Several workers have reported that *B.indicus* cattle have higher levels of innate tick resistance than *B.taurus* cattle (Riek, 1962; Francis and Little, 1964; Brown, 1985) but a few workers strongly refute the presence of any innate resistance (Hewetson, 1971; Wagland, 1975; 1978). Marked variabilities occur within and between breeds (Wilkinson,1955; Seifert, 1971) and between and within sexes (Seifert, 1971). It is however generally accepted that *B. indicus* cattle acquire high levels of resistance more readily than *B.taurus* cattle (Utech *et al.*, 1978; George *et al.*, 1985). This difference might be caused by differences in immune response genes between the two bovine species (Wikel and Whelen, 1986).

1.6.2 Tick resistance in laboratory animals.

The ability of different hosts to acquire resistance to different tick genera and species has been reported by several authors. Guinea pigs have been shown to acquire immunity to *D.variabilis* (Trager 1939a,b; Allen, 1973), *D.andersoni* (Wikel and Allen, 1976a), *A.americanum* (Brown, 1982) and *I.holocyclus* (Bagnall, 1985). Rabbits have been shown to acquire resistance to *R.appendiculatus* (Branagan, 1974; Rubaire-Akiki and Mutinga, 1980 a,b; Mongi, 1982), *Haemophysalis leporipalustris* (Boese, 1974), *I.ricinus* (Brossard, 1977) and *R.sanguineus* (Garin and Grabarev, 1972). Resistance to *D.variabilis* has been reported in rats (Ackerman *et al.*, 1980) and in BALB/c mice (Den Hollander and Allen, 1985). Resistance to *R.sanguineus* by dogs (Tatchell and Moorhouse, 1970) and even to *A.testudinis* , an amphibian (Schneider *et al.*, 1971) has also been reported. The possible mechanisms of acquiring this resistance may be similar in the different hosts.

1.6.3 Tick resistance in the ovines and caprines.

Acquisition of resistance to *R.appendiculatus* after successive infestations has been reported in red Maasai sheep (Wishitemi, 1983, Orago *et al.*, 1983) and in goats (Maranga, 1983,1988). In goats, Maranga (1983) suggested that resistance interfered with the attachment and feeding phases of the tick. Goats acquired resistance to *R.appendiculatus* after the third infestation as compared to guinea pigs which acquired resistance to *D.variabilis* after one infestation (Trager, 1939a). Orago *et al.*, (1983) observed a similar phenomenon in sheep and rabbits using *R.appendiculatus*. He suggested that these may have happened due to the sheep being the natural hosts of the ticks as compared to rabbits. Norval (1978) and Norval *et al.*, (1988a) was able to feed *A.hebraeum* repeatedly on sheep without acquisition of resistance. This may have been due to evasive tactics of the tick and not the host since cattle were also unable to acquire resistance to this tick (Norval *et al.*, 1988a). A possible evasive mechanism may be the tick's ability to attach on strategic areas like the scrotum and the udder where grooming is difficult since grooming has been shown to be important in removal of ticks (Snowball, 1956; Bennett, 1969).

1.6.4 Manifestations of tick resistance.

Tick resistance can be measured using several parameters which include the number of ticks attaching, duration taken to feed to engorgement, engorgement weight, moulting percentages for larvae and nymphs, fecundity of adult females and hatchability of the oviposited eggs. Classically, if ticks were applied onto a resistant host, only a few would attach and these would have prolonged feeding durations, increased mortalities while feeding, low engorgement weights, few larvae and nymphs would moult and females would lay few eggs most of which would not hatch. Conflicting results have been observed with some of these parameters. Some tick species achieve higher engorgement weights while feeding on resistant hosts than while feeding on susceptible ones (Den-Hollander and Allen, 1985) while others take shorter duration's to feed to

engorgement while feeding on resistant hosts than while feeding on susceptible ones (de Castro *et al.*, 1985).

1.6.5 Mechanisms of acquired resistance to tick infestations.

Numerous studies have been done on tick-host relationships using many different tick-host systems as is indicated in recent reviews (Wikel and Allen, 1982; Willadsen, 1980). Despite all these studies, no single host-tick interaction is quite clearly understood (Willadsen, 1980) but although the finer details may differ in different host-tick systems, all studies undertaken indicate that acquisition of tick resistance does occur in all the host-tick systems investigated. Trager (1939a) demonstrated that tick resistance was generalised and was not limited to the site of previous tick attachment. In addition, Allen (1973) greatly reduced the expression of tick resistance in tick-resistant guinea pigs by administering them with methotrexate. Unfortunately methotrexate, is a broad spectrum immunosuppressant which affects both antibody responsiveness (Friedman *et al.,* 1962) and contact hypersensitivity reactions (Pritchard and Haynes, 1961) in guinea pigs and therefore the actual immunological mechanism interfered with could not be ascertained. However, recent studies have shown clearly that acquired resistance to tick infestations is an immunological phenomenon consisting of humoral, complement dependent and cellular components (Willadsen, 1980; Wikel and Allen, 1982). Bagnall (1975) showed that stronger resistance was passively transferred from immune to naive syngeneic guinea pigs using a combination of viable lymphocytes and serum as compared to using either component alone. This observation suggested a synergistic effect for humoral and cellular effector mechanisms in the expression of resistance.

1.6.5.1 The role of humoral immunity in acquisition of resistance.

Resistance to ticks has been passively transferred using serum (Trager, 1939 b; Brossard, 1977; Brossard and Girardin, 1979; Roberts and Kerr, 1976; Maranga, 1988). By treating guinea pigs with just enough cyclophosphamide to specifically block B cells,Wikel and Allen (1976b), were able to block acquisition of resistance. Brossard (1976) demonstrated an

increase in scrum gammaglobulin concentration in cattle following infestation with *B.microplus*. The presence of specific antibodies in serum has also been demonstrated in guinea pigs infested with *D.variabilis* (Ackerman *et al.*, 1981) and in cattle following infestation with *B.microplus* (Brossard, 1976; Willadsen *et al.*, 1978). Reich and Zorzopulous (1980) demonstrated the presence of antibodies specific to tick digestive enzymes in cattle infested with *B.microplus* while Tracey-Patte (1979) indicated that the activity of an enzyme secreted by *B.microplus* into the skin of cattle within an hour of attachment can be removed by a host previously exposed to the tick. Maranga (1988) demonstrated maternal transfer of resistance to *R.appendiculatus* in goats and attributed it to the antibodies passed on to kids through colostrum. Passive transfer of resistance to *R.appendiculatus* by serum from goats immunised with midgut antigens has been reported (Maranga, 1988). It has been shown that Fc receptors are required for acquisition of tick resistance (Brown and Askenase, 1985). All these observations indicate that antibodies play a role in acquisition of resistance to ticks. However, the correlation between antibody titres and the degree of resistance is controversial. Brossard (1976) and Fujisaki (1978) found a positive correlation while Willadsen *et al.*, (1978), demonstrated negative correlation and Riek (1962) observed no correlation at all. These differences may have been observed since the total antibody titres towards a whole organ might not reflect the concentration of a critical antigen or the effectiveness of an antibody in blocking a particular mechanism such as inhibition of an enzyme or blocking the effects of a hormone.

1.6.5.2 The role of complement in acquisition of resistance.

The accumulation of basophils in tick-bite lesions suggests that tick resistance is due to cutaneous basophil-hypersensitivity (Wikel and Allen, 1976). Indeed expression of resistance in *A.americanum* was blocked by destruction of host basophils by administering them with anti-basophil serum (Brown *et al.*, 1982). Depletion of complement levels using cobra venom factor was shown to cause a great reduction in the number of basophils accumulating at the site of tick attachment and to block acquisition of resistance to *D.andersoni* in guinea pigs (Wikel and Allen,

1977). It therefore appears that, the functions of basophils in expression of resistance require complement. However, the actual pathway of complement activation necessary for acquisition of resistance was not ascertained since cobra venom factor depletes the C3 component (Cochrane *et al.*, 1970) which is involved in both the classical and alternate pathways of complement fixation (Osler, 1976). However, the development and expression of tick resistance was similar in guinea pigs totally lacking in complement component C4 and immunologically competent controls (Wikel, 1979). Since the alternate pathway of complement activation does not require the participation of C1, C2 and C4 (Osler, 1976) these observations indicate that activation of complement, through the alternate pathway (Santoro *et al.*, 1979) was required for the acquisition of tick resistance.

1.6.5.3 The role of delayed hypersensitivity reactions in acquisition of resistance.

Delayed type hypersensitivity reactions have been elicited by intradermal inoculation of antigenic material derived from salivary glands of partially fed adult female *D.andersoni* ticks into tick resistant guinea pigs (Wikel *et al.*, 1978). Using the same antigenic material, these investigators were able to stimulate lymphocyte blastogenesis using lymphocytes from immune donors but not from non immune controls (Wikel *et al.*, 1978). This means that introduction of these antigens by ticks during feeding, may cause similar reactions which would cause an unfavourable tick feeding site. In addition an inflammatory reaction would occur prompting the host to groom and thereby detach the ticks.

Langerhans cells have been shown to trap salivary gland antigens at tick attachment sites on guinea pigs resistant to *D.andersoni* (Allen *et al.*, 1979) and to present tick salivary gland antigens to syngeneic lymphocytes (Nithiuthai and Allen, 1985). They have also been demonstrated on tick feeding sites and shown to increase in subsequent infestations (Nithiuthai and Allen, 1984a). Destruction of these Langerhans cells by ultraviolet irradiation was shown to impair acquisition of resistance (Nithiuthai and Allen, 1984 b,c). In addition tick resistance has been adoptively transferred in guinea pigs using peritoneal exudate cells (Brown and Askenase,

1981; Askenase *et al.,* 1982) and lymphnode cells (Wikel and Allen, 1976 a). Since these mechanisms are the ones required for delayed type hypersensitivity reactions, these studies indicate that these delayed type hypersensitivity reactions play a role in the acquisition of tick resistance.

1.6.5.4 The role of immediate hypersensitivity reactions in acquisition of resistance.

While studying the association between cattle and *B.microplus* infestations, Riek (1956, 1962) reported that cattle exposed to the tick were intensely irritated by larvae. Riek (1962) also observed papular reactions around nymphs and adults feeding on resistant cattle and a transient increase in blood histamine levels in previously exposed cattle during tick infestations. He suggested that immediate type hypersensitivity reactions were occurring and were responsible for the resistance. Reports indicating that oedema resulting from hypersensitivity reactions was advantageous to the tick (Tatchell, 1969; Tatchell and Moorhouse, 1968) raised some doubts on the importance of immediate type hypersensitivity reactions in the acquisition of tick resistance. However, it was later found out that ticks have alternative ways of causing oedema (Tatchell and Binnington, 1973) by using mediators like prostaglandins from saliva (Dickinson *et al.*, 1976; Higgs *et al.*, 1976). These observations indicated that immediate hypersensitivity reactions were not necessary for successful tick feeding but may play a role in acquisition of resistance. This was proved when immediate hypersensitivity reactions were induced using tick derived allergens (Willadsen *et al.*, 1978; Willadsen and Riding, 1979) and found to be positively correlated with the level of resistance (Willadsen *et al.,* 1978). Histamine, the main mediator of immediate hypersensitivity reactions, has been shown to cause detachment of *B.microplus in vivo* and *in vitro* (Kemp and Bourne, 1980). Similarly, histological examination of tick bite lesions in the skin of resistant cattle have shown the presence of eosinophils, mast cells (Schleger *et al.,* 1976) and basophils (Allen *et al.,* 1977). The bite lesions of *R.appendiculatus* on goats was shown to be infiltrated by mononuclear cells, mast cells, eosinophils and neutrophils (Maranga, 1988). The number of cosinophils was shown to increase in subsequent infestations (Maranga, 1988). These

cells are associated with immediate hypersensitivity reactions (Schwartz and Austen, 1982). Furthermore, tick bite lesions on resistant guinea pigs showed intense cutaneous hypersensitivity reactions when infested with either *D.andersoni* (Allen, 1973) or *A.americanum* (Brown, 1985). Skin sensitising antibodies have been demonstrated by passive cutaneous anaphylaxis on guinea pigs infested with *I.holocyclus* (Bagnall, 1976) and on rabbits infested with *H.leporipalustis* (Boese, 1974) and shown to be dependent on the level of resistance (Boese, 1974). Indeed, destruction of basophils in guinea pigs by administering them with anti-basophil serum was shown to affect expression of resistance to *A.americanum* (Brown *et al.*, 1982). All these reports indicate that immediate hypersensitivity reactions are important in acquisition of tick resistance. Stebblings (1974) suggested that hosts rejection of arthropod infestations result from immediate hypersensitivity reactions to the bite lesions which leads to localised cutaneous irritation and subsequent removal of the arthropod by grooming.

1.6.5.5 An overview of the likely sequence of events in eliciting tick resistance.

From the above reports, it is evident that a variety of host-effector mechanisms are involved in acquisition of resistance. Rejection of ticks feeding on a resistant host is likely to take the following sequence. Antigens are injected with saliva into the host (Krolack *et al.*, 1983). These antigens are processed by Langerhans cells and presented to T lymphocytes. The T lymphocytes release soluble mediators which attract basophils to the attachment site. They also cooperate with B cells resulting in the production of homocytotropic and skin sensitising antibodies (Allen, 1973; Wikel and Allen, 1976 a,b). The introduced antigens and the resulting homocytotropic antibodies activate the complement pathway resulting in the release of products with chemotactic, anaphylatoxin and tissue damaging properties which play a role in the development of the tick bite lesion (Wikel, 1988). It has been shown that Fc receptors are required for acquisition of tick resistance (Brown and Askenase, 1985). Antibodies occupy Fc receptors on basophils and mast cells and further introduction of antigen results in their degranulation and the release of hypersensitivity mediators resulting in a classical

hypersensitivity reaction (Schwartz and Austen, 1982). This hypersensitivity reaction irritates the hosts skin stimulating grooming of the infested area. Grooming activity results in physical removal or destruction of ticks and thereby reduces their numbers (Snowball, 1956; Bennett, 1969; Hewetson, 1971; Koudstaal *et al.,* 1978). Indeed, increased tick burdens have been reported in immune animals restricted from grooming (Bennett, 1969).

Since ticks feeding on resistant hosts may either die *in situ* (without physical damage) or have reduced engorgement weights, reduced fecundity, prolonged engorgement duration's and the immature instars may not even moult, other mechanisms play a big role in maintaining reduced tick burdens. Anti-tick antibodies imbibed with the blood meal may cause direct damage to tick tissues (Wikel, 1988). Since the released hypersensitivity mediators are chemotactic to polymorphonuclear cells and increase vascular permeability, they increase the concentration of cells and tick reactive circulating antibodies at the attachment site which may have deleterious effects on the feeding ticks (Wikel, 1988).

1.7 Immunisation against ticks.

Ticks have adopted strategies to thwart and subvert host immune protective mechanisms enabling them to co-exist for millions of years. One such mechanism is the ability to immunosuppress infested hosts (Wikel and Osburn, 1982; Fivaz, 1989), a phenomenon which many other parasites show (Callow and Stewart, 1978; Cohen, 1982; Mbogo, 1983). This tick induced immunosuppression ensures that under natural conditions, the tick, its host and infectious agent will continue to coexist. Since ticks are a major constraint to the livestock industry, these co-existence must be destroyed. To achieve this, attempts have been made at immunising hosts with anti-tick antigens (Wikel and Allen, 1982; Willadsen, 1980).

1.7.1 The choice of antigen.

Most immunisation attempts to-date have used crude preparations like homogenates from larvae (Trager, 1939a; Opdebeeck *et al.,* 1989) nymphs and adults (Ackerman *et al.*, 1980;

Mongi, 1982). Other workers have immunised hosts using midgut antigens (Ackerman *et al.*, 1980; Opdebeeck *et al.*, 1988 b; Maranga, 1988; Essuman *et al.*, 1991), internal organs (Allen and Humphreys, 1979), tissue culture cells (Wikel, 1985) synganglion extracts (Opdebeeck *et al.*, 1988 a) and Haemolymph (Ben-Yakir and Barker, 1987) with varying degrees of success. Antigens from tick salivary glands have been tried most extensively and the many interactions between them described (Wikel and Allen, 1982). Resistant hosts usually exhibit cutaneous hypersensitivity reactions to tick saliva which is the mechanism that prevent ticks from feeding on them.

Immunisations using crude tick antigen preparations have not improved on the resistance attained naturally in the field following tick infestations (Willadsen, 1980). The degree of resistance acquired by goats to *R.appendiculatus* through natural infestation was similar to that acquired through a combination of natural infestation and immunisation with crude midgut antigens from the tick (Maranga, 1988). It has been suggested that proteins present in the crude homogenates may hinder the immunological response perhaps by antigenic competition with the many antigens present in the homogenate (Mongi *et al.*, 1986,b). In addition, the protective antigens may be present in very minute concentrations in the homogenates resulting in the reduction of their full potential effect (Mongi *et al.*, 1986,b). To improve on the resistance attained after immunisation, attempts have been made to identify and isolate the tick antigens that are important in inducing tick resistance with the hope of using pure concentrated tick antigens for the induction of artificial resistance to ticks (Mongi *et al.*, 1986a,b).

Characterisation of tick saliva has shown that it contains enzymes (Geczy *et al.*, 1971) and prostaglandins (Dickinson *et al.*, 1976; Higgs *et al.*, 1976) which increase vascular permeabilty and increase vasodilation and hence blood flow to the tick during feeding. Destruction of these substances immunologically or otherwise may interfere with tick feeding and therefore help reduce tick population. Brown *et al.*, (1984) induced resistance to *Amblyomma* ticks by immunising rabbits with a 20 kilodalton protein derived from the salivary glands of the

tick. Recently very good protection was achieved in cattle immunised against *B.microplus* using a purified antigen derived from its midgut (Willadsen *et al.*, 1989). Rand *et al.*, (1989) immunised cattle and got good protection using *B.microplus* antigens that had been sequenced and cloned in *Escherichia coli*. It is therefore possible to produce a good anti-tick vaccine by purification and characterisation of crude antigenic preparations.

1.7.1.2 The use of concealed antigens.

Concealed antigens have been defined as antigens that are not encountered by ticks during feeding (Willadsen and Kemp, 1988) as opposed to non concealed antigens which are injected into the host during feeding. Although these non concealed antigens are responsible for acquisition of natural immunity (Willadsen, 1980), they donot exhaust the repertoire of immunological possibilities that can be used to induce tick resistance (Willadsen and Kemp, 1988). Indeed, hyperimmunization with salivary gland antigens may result in hypersensitivity reactions and cause intense cutaneous inflammation and skin lesions that are prone to infection and would reduce hide quality (Wikel, 1981). Furthermore, hosts may become immunologically tolerant to salivary gland antigens following repeated exposure (Berdyer and Khudainazarova, 1976). There is therefore need to derive antigens from other tick tissues apart from the salivary glands and to test their potential as anti-tick immunogens to avoid the aforesaid complications.

1.7.1.3 Passage of host antibodies across the midgut.

Antibodies cross the tick midgut into the haemolymph and retain their biological activity (Ackerman *et al.*, 1981; Fujisaki *et al* 1984; Brossard and Rais 1984; Ben-Yakir *et al* 1986). However, only small amounts of antibodies cross the midgut into the haemolymph. It may therefore be possible to immunologically block a hormone or any other protein found in very minute concentrations in the haemolymph. Kemp *et al.*, (1986) demonstrated massive destruction of the midgut in *B.microplus* ticks that were fed on rabbits immunised with midgut antigens. The ability to destroy the midgut of *R.appendiculatus* with antibodies to solubilised tick midgut membrane proteins (STGMP) and the effect of this destruction on the passage of antibodies

across the midgut into the haemolymph will be determined. If destruction of the midgut increases the passage of antibodies across it then the antigens to be evaluated as potential anti-tick immunogens will be used in combination with STGMP.

1.7.1.4 Neutralisation of biologically active materials using specific antibodies

Biologically active molecules like toxins, enzymes and hormones can be neutralised by specific antibodies (Butler *et al.*, 1973). Indeed fatal neutralisation of an osmoregulatory protein in the haemolymph of tsetse flies using specific anti-sera has been reported (Nogge and Giannetti, 1980). It may therefore be possible to block the effects of a haemolymph protein or a tick hormone using specific antibodies. These would have deleterious effects onto the ticks and will be attempted.

1.8 The rationale and overall aims of the project.

The main objective of the present study is to destroy the endemic stability that occurs between ticks and their hosts by immunising hosts against tick infestation. Since better results have been achieved when purified or semi-purified materials were used for immunisation (Willadsen *et al.*, 1989, Rand *et al.*, 1989), the efficacy of commercially available beta-ecdysone of known purity as a potential anti-tick immunogen will be evaluated. This will be done by infesting rabbits immunised with beta-ecdysone with all the developmental instar stages of *R.appendiculatus* and assessing the rabbits for tick resistance using the parameters discussed in section 1.6.4. In addition the effects of injecting unfed adults, engorged females and engorged nymphs will also be studied.

The potential effects of the protective antigens in crude preparations may be reduced since out of all the antibodies produced very few may be directed towards target antigens. Therefore crude antigens whose physiological activities can be blocked by very minute amounts of antibodies may be useful. Consequently, the efficacy of haemolymph components as potential anti-tick immunogens will be evaluated. To achieve this, rabbits immunised with pooled whole

haemolymph from *R.appendiculatus* will be infested with all the developmental instar stages of *R.appendiculatus* and assessed for tick resistance using the parameters discussed in section 1.6.4. The effect of the immunisation on subsequent tick infestations will also be studied.

CHAPTER TWO.

2.0 Studies on the passage of host immunoglobulin G into the haemolymph of *Rhipicephalus appendiculatus*.

2.1 Introduction.

Immunisation of hosts against ticks is a research area that requires a lot of emphasis because of the immense economic losses associated with ticks and tick borne diseases globally (Bram, 1975; McCosker, 1979). Immunisation using antigens normally encountered by the hosts during feeding may result in either hypersensitivity reactions that lead to intense cutaneous inflammation and secondary infection (Wikel, 1981) or immunological tolerance following repeated exposure (Berdyer and Khudainazarova, 1976; Benjamini *et al.*, 1960). It may therefore, be more appropriate to immunise hosts against ticks using concealed antigens, that is antigens not normally encountered by the host. Most of these antigens may be obtained from tick internal organs and haemolymph.

To be able to immunologically attack these concealed antigens, host antibodies must cross the tick's midgut and retain their biological activity. This has been shown to occur in both hard ticks (Fujisaki *et al.*, 1984; Tracey-Patte *et al.*, 1987; Ben-Yakir *et al.*, 1986, 1987) and soft ticks (Minoura *et al.*, 1985, Chinzei and Minoura, 1987, Ben-Yakir, 1989). However, quantification studies indicate that only minute amounts of antibodies are able to cross the midgut barrier (Ben-Yakir *et al.*, 1986, 1987) with significant differences observed between and within species (Ben-Yakir, 1989). This, therefore, means that the amount of antibodies which may cross the midgut of the particular tick species to be immunised against must be established. The passage of antibodies across the midgut into the haemolymph of R.*appendiculatus*, the vector for the most economically important tick borne diseases in East and Central Africa (Young and Leitch, 1981) has not been studied. This needs to be established before any immunisation attempts against it using concealed antigens can be undertaken.

Damage of the midgut has been reported in *B. microplus* ticks that were fed on cattle vaccinated with extracts derived from whole adult ticks (Kemp *et al.*, 1986). Histopathological studies of these ticks demonstrated midgut damage (Agbede and Kemp, 1986), severe enough to allow erythrocytes to pass across the tick's midgut (Kemp *et al.*, 1986). It is, therefore, reasonable to postulate that the passage of antibodies across the tick midgut into the haemolymph may be increased if ticks feed on blood containing anti-tick midgut antibodies. However, no studies have been undertaken to elucidate this assumption.

It has been suggested that inhibition of tick enzymes and neutralisation of their hormones with specific antibodies can be used to reduce tick populations (Roberts, 1968; Galun, 1978; Ackerman *et al.*, 1980). The practical implications of these suggestions can be more readily recognised if passage of antibodies across the tick midgut barrier can be facilitated.

2.2 Overall objectives of this section of the study.

The objectives of this work were to study the passage of host IgG across the midgut barrier of *R.appendiculatus*, to assess whether these antibodies retain their biological activity and to determine whether feeding these ticks on blood containing anti-tick midgut antibodies would have any effect on the passage of these antibodies.

2.3 Materials and Methods.

2.3.1 Experimental animals.

Eight, three months old, male New Zealand White rabbits naive against ticks and weighing at least 2 Kg were used. On acquisition they were given therapeutic doses of coccidiostats and antibiotics (Sulphadimidine; Bimadine, Bimeda). They were caged in pairs and offered standard rabbit cubes and water *ad libitum* for the entire period of the experiment.

2.3.2 Ticks.

Adult parasite free *R.appendiculatus* ticks reared in the laboratory since 1952 were used in all the experiments. While off the host, the ticks were placed in tubes which were then

closed with cotton wool and placed in a desiccator containing a saturated solution of potassium chloride to give a relative humidity of 85% (Wiston and Bates, 1960). The desiccator was incubated at 28^0C. The tick colony was maintained as described by Bailey (1960) and Irvin and Brocklesby (1970). The tick colony was maintained using tick-naive rabbits. Before application of ticks, the rabbit ears were shaved, ear bags applied and collars attached to prevent the rabbits from grooming (Figure 3). Adult *R.appendiculatus* were applied onto the rabbits and allowed to feed to engorgement. They were then removed by opening the ear bags and incubated at 28^0C in desiccators with a relative humidity of 85% to oviposit. The oviposited eggs were incubated under similar conditions to hatch to larvae. The larvae were applied onto tick-rabbits where they fed to engorgement and were incubated at 28^0C and 85% relative humidity to moult to nymphs. The nymphs were applied and treated exactly like the larvae. The nymphs moulted to adults and the cycle was repeated.

2.3.3 Preparation of antigens.

The antigens used in this study were bovine serum albumin (BSA) and solubilized tick midgut membrane proteins (STGMP). One month old adult ticks were applied onto rabbits as described by Bailey (1960). After 5 days of feeding, the ticks were detached, washed 3 times using tap water and dried using a blotting paper. They were then sexed and the females embedded live on molten paraffin wax in petri-dishes with the dorsal scutum facing upwards. The ticks were then covered with 0.15M phosphate buffered saline pH 7.2 (PBS). Using a sharp scapel blade (size 11), an incision was made along the lateral sides of the ticks and the dorsal scutum removed. The exposed midguts were then removed with fine forceps, rinsed with PBS, placed in bijou bottles containing extraction buffer (PBS 0.15 M, pH 7.2 with protease inhibitors which included, 1 mM Phenyl methyl sulfonyl fluoride, 1 mM Iodoacetamide, 1 mM aprotinin, 5 mM glutathione and 1 mM Diisopropyl flourophosphate) and frozen at -20^0C. The midguts were then disrupted by freeze-thawing them four times as previously described (Mongi *et al.*, 1986a). They were then washed three times by centrifuging them at 10,000 X g for 20 minutes at 4^0C

using a Beckman L8-70 ultracentrifuge. After every wash, the supernatant was discarded and the pellet resuspended in extraction buffer. The pellet was homogenised five times using one minute bursts with a glass homogeniser placed on ice and then washed three times by centrifugation at 10,000 X g for 20 minutes at 4^0C. Thereafter it was resuspended in extraction buffer containing 1% Triton X-100. It was then homogenised as described earlier and centrifuged at 100,000 X g for 1 hour at 4^0C. The supernatant solution was collected and deoxycholic acid added to it to make a final concentration of 1%. The mixture was aliquoted in 1 ml fractions and stored at -20^0C until required for immunisation.

2.3.4 Determination of protein concentration.

The protein concentration of the antigen used was determined using the Bio-Rad automated protein assay procedure with no modifications (Bio-rad Protein Assay, 1985).

2.3.5 Immunisation of the experimental animals.

The experimental rabbits were inoculated intramuscularly around the popliteal lymph nodes with the antigens emulsified with Freund's adjuvants (Freund *et al.*, 1948). Antigens were emulsified in Freund's complete adjuvant (FCA, Difco, Detroit, MI) for the initial dose and Freund's incomplete adjuvant (FIA, Difco) for all the booster doses. The rabbits were randomly divided into four pairs. Each group was inoculated with 1mg of either BSA, STGMP, a mixture of 50% BSA and 50% STGMP or buffer. Before each inoculation, the antigens were emulsified in adjuvant at an antigen:adjuvant ratio of 1:2. The rabbits were given three booster doses. The first booster was given 2 weeks after the primary dose while the other 2 were given at monthly intervals.

2.3.6 Serum collection.

Rabbits were bled from the central ear vein two weeks before the initial dose (preimmune serum) and two weeks after each booster dose. The collected blood was kept for 4 hours at room temperature to clot. Serum was separated from the clotted blood by draining it into

centrifuge tubes and centrifuging it at 3,000 X g for 10 minutes. It was then aliquoted in 1 ml fractions and stored at -20^{0}C until required

2.3.7 Application and removal of ticks.

Sixty adult ticks (30 males, 30 females) were applied onto each of the experimental rabbits two weeks after the last booster dose and enclosed in previously attached ear bags. The ear bags were then sealed with paragon tape and collars attached to prevent the rabbits from removing them (Figure 3). To remove the ticks, ear bags were opened and the detached ticks collected into containers after which the ears were resealed with paragon tape.

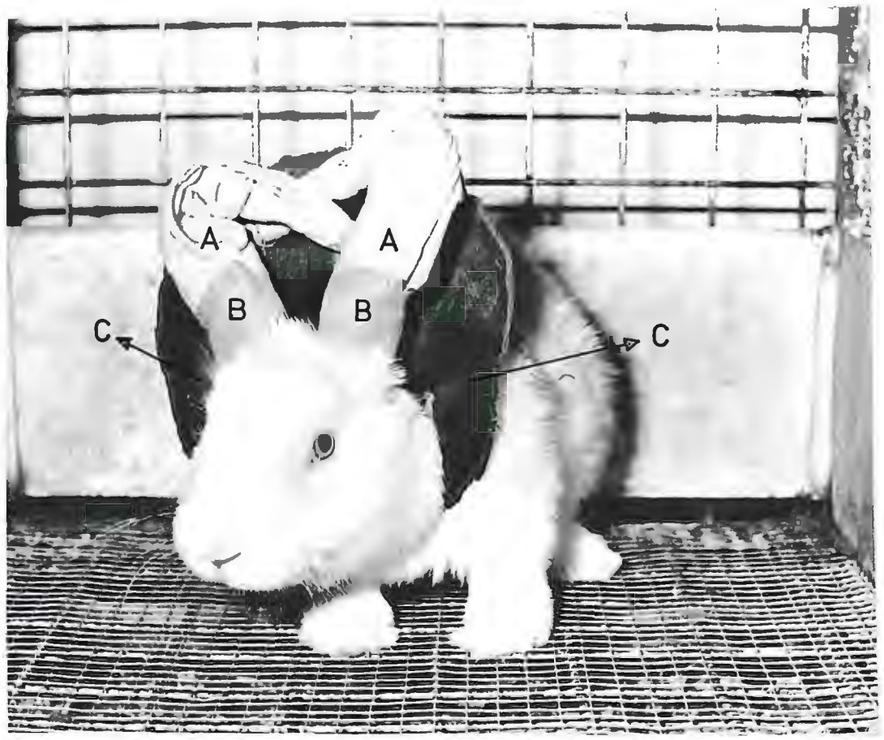

FIGURE 3.

A photograph showing the application of ticks on an experimental rabbit using ear bags

A. Shows the earbags used to enclose ticks.

B. Shows the paragon tape used to seal the rabbit ears.

C. Shows the collar attached to prevent the rabbits from either scratching the ears and removing the ear bags or detaching the ticks

2.3.8 Preparation of pure rabbit IgG.

Rabbit IgG was purified from rabbit serum using a method adapted from Hudson and Hay (1980a). Rabbit serum was obtained as described in section 2.3.6 and precipitated with 50% ammonium sulphate solution (pH 7, maintained with ammonia solution). The precipitate was cent rifuged at 3000 X g for 10 minutes at 4^0C and the supernatant solution discarded and the pellet resuspended in PBS. The pellet was then dialysed overnight twice, first in distilled water and then in 0.005M Phosphate buffer pH 8. The dialysate was passed through an ion exchange chromatography column packed with diethylaminoethyl (DEAE) cellulose (DE 51, micro granular, Whatman, pre swollen, UK) that had been previously equilibrated with 0.005M Phosphate buffer pH 8. After complete adsorption, the column was eluted first with 0.005M phosphate buffer pH 8 and then with 0.05M phosphate buffer pH 8. The eluate through the column was monitored at 280nm absorbance and 2 ml fractions were collected at a flow rate of 30 ml per hour. The fractions obtained were lyophilised and kept at 4^0C and their purity determined by immunoelectrophoresis.

2.3.9 Immunoelectrophoretic studies to determine the purity of the immunoglobulins.

This was performed as described by Hudson and Hay (1980b). Briefly agarose was added to barbitone buffer (0.05M, pH 8.6) to make a 1% solution and 0.05% sodium azide added to it. This solution was boiled and poured onto a glass plate (8cm X 8cm) previously coated with 0.5% agarose and allowed to harden at room temperature on a horizontal surface. Using a template, wells were cut out of the agarose and evacuated by suction. Twenty microlitres of the test samples at a concentration of 20 mg/ml were applied onto separate wells. To one of the wells, a crystal of Bromophenol blue was added to check whether the current was moving in the right direction and to indicate completion of the immunoelectrophoresis. The plates were placed in an electrophoresis tank containing the Barbitone buffer and a constant current of 80 volts applied across the plate. The plate was removed when the Bromophenol blue (tracking dye) had

moved three quarters towards the end of the plate. A trough was cut between the wells with the test samples and agarose removed from it. The trough was then filled with goat anti-whole rabbit serum available within the laboratory and incubated in a humid box at room temperature for 48 hours. When precipitin lines had formed, the plate was washed three times with PBS and then placed in distilled water overnight. The gel was then press-dried and stained with Coomassie brilliant blue (Weeke, 1973) for a permanent record.

2.3.10 Harvesting of tick haemolymph.

Haemolymph was obtained from female *R.appendiculatus* ticks which had fed to engorgement on either the immunised or the control rabbits. After collecting the ticks, they were washed with cold water to remove serum exudates, blood or tick faeces. Thereafter, haemolymph was collected into capillary tubes after amputating the distal portion of one or more legs with a sharp scapel blade (size 11). The harvested haemolymph was pooled and placed in capped containers containing extraction buffer and stored at -20^{0}C until required.

2.3.11 Quantification of total rabbit IgG concentration and rabbit anti-BSA titres in tick haemolymph and in sera of the rabbits.

This was done using the double antibody sandwich Enzyme Linked Immunosorbent Assay (ELISA) techniques (Voller *et al.,* 1979). Polyvinyl chloride microtitre plates (Cooke Microtitre plates MZa AR) were coated with either 50µl of goat anti-rabbit IgG (concentration 20 mg/ml) or 50 µl of a solution of 20 mg/ml of BSA in coating buffer (carbonate/bicarbonate buffer pH 9.6) to determine the concentration of total rabbit IgG and anti-BSA antibody titres respectively. The plates were then incubated in a humid box at 37 ^{0}C , overnight. After this, the plates were washed five times with washing buffer (0.1% PBS containing 0.05% polyoxyethylenesorbitan monolaurate (Tween 20, Sigma Chemical Co). Fifty microlitres of the samples and the purified rabbit IgG (standard) diluted in sample buffer (PBS containing 10% fat free milk (FFM) and 0.05% Tween 20) were added in duplicate to the wells. The samples were then serially diluted ranging from 1:25 to 1:3200, 1:250 to 1:32000 and 100 mg/ml to 0.09

mg/ml while assaying haemolymph samples, serum samples and for the purified standard rabbit IgG respectively. The plates were then incubated in a humid box at 37 ^{0}C centigrade for 1 hour. After incubation, the plates were washed as previously described. Fifty microlitres of goat anti-rabbit IgG conjugated to Horse radish peroxidase (HRP; Nordic Immunological Laboratories, Tilburg, Netherlands) diluted in conjugate buffer (PBS containing 1% FFM and 0.05% Tween-20) to form a 0.05% dilution was added to the plates. The plates were once more incubated in a humid box at 37^{0}C for 1 hour and washed as previously described. The chromogen (trimethyl benzidine) was diluted in substrate buffer (Citrate Ammonium Sulphate pH 5) to make a 1 : 100 dilution. Seventy microlitres of hydrogen peroxide, the substrate, were added to 100 ml of this solution and 50 ul of this final solution added to each well and the plates incubated for 15 minutes at room temperature. Thereafter, the optical densities of the different wells were read at 449nm using an automatic ELISA Titertek Multiskan M-C plate reader (Flow Laboratories, UK.). A reaction was considered positive when the optical density was >0.3.

2.4 Results.

2.4.1 Preparation of pure rabbit IgG.

Two fractions containing IgG were eluted from the ion exchange chromatography column. The first fraction was eluted using 0.005M phosphate buffer pH 8 while the second fraction was eluted using 0.05M phosphate buffer pH 8 (Figure 4).

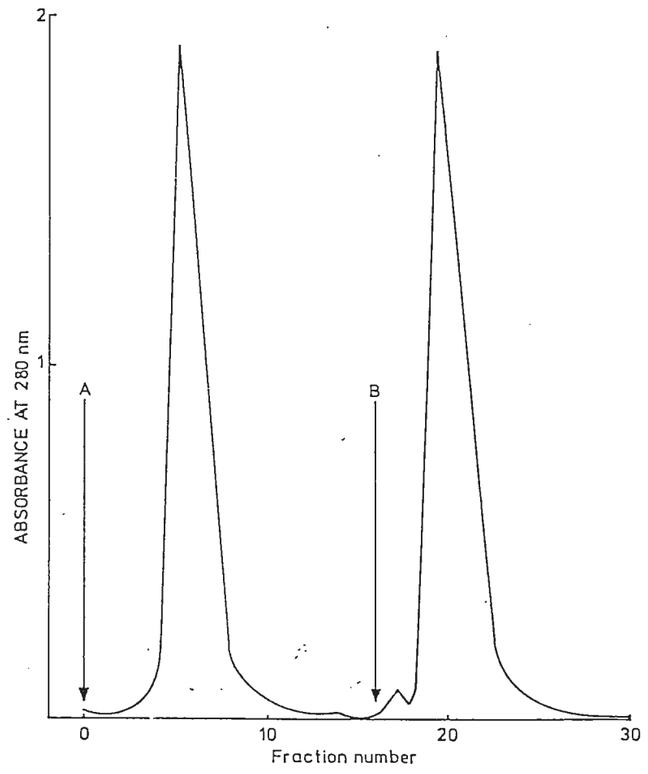

FIGURE 4.

The elution profiles of rabbit immunoglobulins after ionic exchange chromatograph using a DEAE cellulose column

A. indicates the point when 0.005 M phosphate buffer started being used for elution

B. indicates the point when 0.05 M phosphate buffer started being used for elution.

2.4.2 Immunoelectrophoretic studies to determine the purity of the immunoglobulins.

After the 2 eluted fractions were electrophoresed and diffused against goat anti-whole rabbit serum, the first fraction gave only one precipitin line while the second fraction gave six precipitin lines (Figure 5).

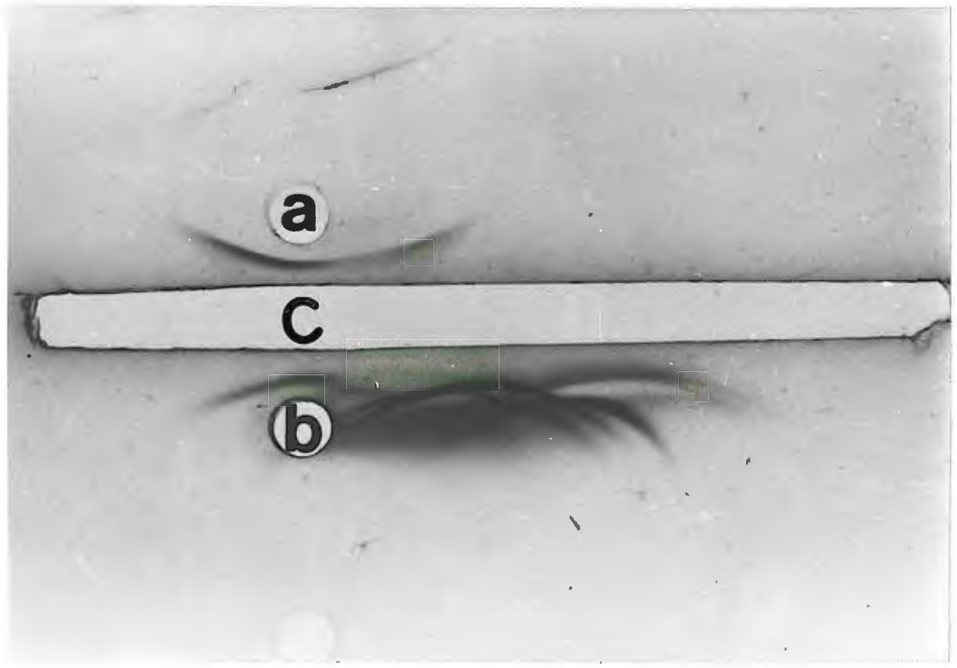

FIGURE 5.

The electrophoretic profiles of rabbit immunoglobulin fractions eluted from a DEAE cellulose ionic exchange column.

a. Well containing the first fraction to be eluted (pure rabbit IgG).
b. Well containing the second fraction to be eluted (a mixture of all whole rabbit serum proteins).
c. Trough containing Goat anti-whole rabbit serum.

2.4.3 ELISA results.

Optical densities read using known concentrations of pure rabbit IgG were used to draw standard curves from which the IgG amounts in the samples were determined. To avoid variation between plates a standard curve was made for each plate. The optical densities had a linear correlation (r = 0.98) with the IgG concentration. Table 1 shows the concentration of IgG in mg/ml in the serum of rabbits immunised with BSA, a combination of BSA and STGMP, STGMP and buffer respectively. Similarly, the concentration of IgG in mg/ml in the haemolymph of ticks that were fed on rabbits immunised with a combination of BSA and STGMP, STGMP, BSA and buffer respectively is shown (Table 1).

Table 1

Concentration of rabbit IgG in the serum of immunised rabbits and in the haemolymph of the ticks that fed on them.

Immunogens administered	n*	Serum IgG concentration (mg/ml ± SE)	Haemolymph IgG concentration (mg/ml ± SE)	Percent Serum IgG crossing into haemolymph
BSA	2	34.05(±7.08)	0.015 (±0.005)	0.044
STGMP	2	16.27 (±3.68)	0.073 (±0.019)	0.45
STGMP+BSA	2	33.18 (±10.53)	0.136 (±0.004)	0.41
BUFFER	2	12.08 (±4.23)	0.006 (±0.002)	0.05

n* Indicates the number of rabbits used. 60 ticks were applied onto each rabbit.

Haemolymph from ticks feeding on each group of rabbits was harvested and pooled

To calculate means 5 replicates were run

The amounts of specific anti-BSA antibodies in the samples were recorded as antibody titres. The titres were considered to be the highest dilutions giving positive reactions. Serum from rabbits immunised with either BSA or a combination of BSA and STGMP had anti-BSA titres of 1:16000. Haemolymph from ticks that were fed on rabbits immunised with either BSA

alone or BSA in combination with STGMP had anti-BSA antibodies titres of 1:100 and 1:800 respectively (Table 2).

Table 2.

Anti-BSA antibody titres in the serum of immunised rabbits and in the haemolymph of the ticks that fed on them.

Immunogens administered	n*	Serum anti-body titre.	Haemolymph anti-BSA antibody titre.
BSA	2	1:16000	1:100
STGMP + BSA	2	1:16000	1:800

n* Indicates the number of rabbits used. 60 ticks were applied onto each rabbit.

Haemolymph from ticks feeding on each group of rabbits was harvested and pooled

To calculate means 5 replicates were run

2.5. Discussion.

The presence of host antibodies in the haemolymph of several tick species has been reported (Ben-Yakir, 1989). However, there has been no previous report on the passage of antibodies across the midgut of *R. appendiculatus*. This study reports this and also confirms earlier reports that the concentration of IgG in the haemolymph is influenced by the antibody titres of host blood (Ben-Yakir *et al.,* 1986). It also confirms earlier reports that antibodies retain their biological activity after crossing the midgut (Ackerman *et al.,* 1981; Fujisaki *et al.,* 1984) since the antibodies in the haemolymph were able to recognise and react with BSA.

Large molecules like albumin, transferrin and even immunoglobulins have been demonstrated in the haemolymph of ticks (Ackerman *et al.,* 1981) but the mechanisms they use to cross an intact midgut barrier remains a mystery. The fact that blood digestion by ticks is a slow intracellular process (Balashov, 1972) and that the tick digestive enzymes act rapidly on haemoglobin but slowly on other proteins (Akov, 1982) ensures that these proteins stay intact in the midgut for prolonged periods giving them enough time to cross the midgut barrier.

Antibodies have been shown to immunologically destroy the midgut barrier (Agbede and Kemp, 1986) sometimes to the extent of allowing huge substances like host erythrocytes to cross the midgut (Kemp *et al.*, 1986). This indicates that feeding ticks on blood containing anti-tick midgut antibodies might facilitate the passage of antibodies across the midgut. Indeed this study demonstrates this possibility. The antibodies crossing the midgut maybe directed against an entirely different protein since in this case feeding ticks on rabbits immunised with a combination of STGMP and BSA increased the passage of anti-BSA antibodies across the midgut eight times. The implications of these findings are of importance since it might now be possible to immunise hosts with STGMP (primary antigen) and a concealed antigen (secondary antigen) to facilitate passage of antibodies raised against the concealed antigen into the haemolymph.

The kind of massive destruction of the midgut epithelium that allows erythrocytes to cross the midgut (Kemp *et al.*, 1986) in *B.microplus* has not been observed in *R.appendiculatus*. Whether this is as a result of the short feeding durations of this tick species as compared to *Boophilus* ticks need to be investigated. However, if this massive destruction of the midgut was to happen readily in all tick species, for the purpose of tick control the need for a second antigen would not arise since ticks with damaged midgut will most likely die. However, when this does not happen, increasing the passage of antibodies across the midgut into the haemolymph would greatly increase the number of target antigens that can be used to immunise hosts against ticks.

These findings demonstrate that antibodies are able to cross the midgut of *R.appendiculatus* and retain their biological activity. They also show that the amounts of antibodies crossing the midgut barrier depend on the concentration of that specific antibody in the host's blood. Lastly, this study indicates that the passage of these antibodies can be increased by destroying the midgut with anti-midgut antibodies and that the antibodies whose passage across the midgut are facilitated may be directed against antigens entirely different from those of midgut origin since in this study the passage of anti-BSA antibodies across the midgut was increased eight times.

The importance of the ability to facilitate the passage of antibodies against a particular antigen across the midgut will be determined by attempting to increase the passage of anti-beta-ecdysone (Chapter 3) and anti-tick haemolymph (Chapter 4) antibodies across the tick midgut by immunising hosts with beta-ecdysone (conjugated to BSA) and tick haemolymph components in combination with STGMP.

CHAPTER THREE.

3.0 Evaluation of Beta-ecdysone as a potential tick immunogen.

3.1 Introduction.

Beta-ecdysone is one of the numerous polyhydroxylated steroids collectively referred to as ecdysteroids (Goodwin *et al.*, 1978). Ecdysteroids are common in invertebrates and have been demonstrated in arthropods (Hetru and Horn, 1981), nematodes (Koolman *et al.*, 1984; Rees and Mendis, 1984) trematodes (Koolman *et al.*, 1984; Nirde *et al.*, 1984) annelids (Porchet *et al.*, 1984) Molluscs (Romer, 1979) and coelenterates (Sturaro *et al.*, 1982). However, they are absent in normal vertebrates. Indeed the demonstration of ecdysteroids in mammalian sera has been used to diagnose parasitic infections (Koolman and Moeller, 1986; Nirde *et al.*, 1984).

The roles played by ecdysteroids in the physiology of insects are well studied and include regulating the moulting process (Riddiford, 1980) vitellogenesis (Hagedorn *et al.*, 1975) and termination of diapause (Koolman and Spindler, 1983). Ecdysteroids, including beta-ecdysone have been demonstrated in ticks (Delbeque *et al.*, 1978; Solomon *et al.*, 1982) and found to be necessary for termination of diapause, moulting, oogenesis, spermatogenesis, sex pheromone activity and ixodid salivary gland degeneration (Diehl *et al.*, 1986). These physiological processes are vital for tick survival and therefore blocking them would be precarious to the ticks. Indeed Williams (1967) suggested that interference with the functions of ecdysteroids could be used to develop "third generation pesticides". It is possible to block these processes using specific antibodies since antibodies against steroid hormones have been raised (Hirn and Delaage, 1981) and biologically active substances like enzymes and hormones have been rendered ineffective by neutralising them with specific antibodies (Butler *et al.*, 1973). Indeed beta-ecdysone is a very immunogenic hapten and high antibody titres have been elicited after the first booster injection (Hirn and Delaage, 1981).

Beta-ecdysone is commercially available and has been found not only to be non toxic to vertebrates (Ogawa *et al.*, 1974), but it is also able to reduce their parasite burdens. (Koolman *et al.*, 1984; Nirde *et al.*, 1984). Since antibodies can cross the tick midgut barrier and retain their biological activity (Ackerman *et al.*, 1981; Fujisaki *et al.*, 1984; Mbogo, *et al.*, 1992), it is therefore theoretically possible to block the physiological effects of beta-ecdysone in ticks by feeding them on host blood containing anti-beta-ecdysone antibodies. Consequently, the practical use of anti-beta-ecdysone antibodies in the control of ticks should be assessed.

For any immunological attempt at interfering with the functions of the tick internal organs or haemolymph components to be viable, reasonable amounts of antibodies must be able to cross the tick midgut barrier. Previous work indicates that only minute amounts of antibodies can cross the tick midgut barrier (Ben-Yakir *et al.*, 1986; 1987). However, this can be reasonably increased by feeding ticks on hosts immunised with solubilized tick midgut membrane proteins (STGMP) (Mbogo *et al.*, 1992). Therefore, to facilitate the passage of anti-beta-ecdysone antibodies across the midgut barrier, ticks were applied onto hosts immunised with a combination of conjugated beta-ecdysone and STGMP. Indeed to achieve optimum results, it may be necessary to inject the antibodies directly into the haemocoel of the tick. Consequently, the potential effects of feeding ticks on rabbits immunised with conjugated beta-ecdysone were evaluated by injecting anti-beta-ecdysone antibodies directly into the haemocoel of the tick.

3.2 The overall objectives of this section of the study.

The objectives of this section of the study were therefore to assess the efficacy of anti-beta-ecdysone antibodies in the control of *R.appendiculatus* by infesting ticks on rabbits immunised with beta-ecdysone alone and in combination with STGMP or by injecting anti-beta-ecdysone antibodies directly into the haemolymph of the ticks. In addition, the study also assessed the effects of the immunisations on the next generation of ticks.

3.3 Materials and methods.

The antigens used were Beta-ecdysone (20-Hydroxyecdysone, Insect moulting hormone, Sigma Chemical company, St Loius, Missouri) and STGMP (see chapter two). Beta-ecdysone is a low molecular weight molecule (480 daltons) and must therefore, be coupled to a protein carrier to render it immunogenic. This was achieved by derivatising one of the positions on it to create a carboxylic function by random succinylation of the secondary alcohols at position 2,3 and 22 (Lauer, *et al.*, 1974).

3.3.1 Succinylation of beta-ecdysone's secondary alcohols at positions 2,3 and 22.

To achieve this, 40 mg of beta-ecdysone was dissolved in 20 ml dry tetrahydrofuran containing 25 ul of dry pyridine and 25 ul of freshly distilled succinyl chloride and incubated for 16 hours at 0 degrees centigrade. Excess succinylchloride was decomposed using double distilled water. Formation of the required hemisuccinates was determined by thin layer chromatography (TLC) using methanol:chloroform 2:3 as the solvent system. The hemisuccinates formed were then purified by high performance liquid chromatography (HPLC) using a reverse phase column and 60% methanol in HPLC grade water. Fractions were collected at 1 minute intervals at a flow rate of 1 ml per minute and the different peaks monitored at 280nm absorbance. Similar peaks were pooled and their volume reduced by evaporation. The mass spectrum of all the fractions was determined and the desired fraction identified.

3.3.2 Conjugation of the hemisuccinates to a protein carrier.

The choice of the protein carrier is not critical since equally good results have been obtained using either BSA (Borst and O'Connor, 1974), human serum albumin (Lauer *et al.*, 1974) or thyroglobulin (Horn *et al.*, 1976). The hemisuccinates were coupled to purified BSA by activation of the carboxylic function using a carbodiimide (Lauer *et al.*, 1974; Spindler *et al.*, 1978).

3.3.3 Purification of BSA for conjugation.

To achieve optimum conjugation results pure homogeneous BSA was required. Commercial preparations are usually not pure enough for conjugation and require to be purified further before use. Bovine serum albumin (BDH Chemicals) was dissolved in fast performance liquid chromatography (FLPC) grade PBS and filtered using 0.2 micron filters. The BSA was then purified using an FPLC gel permeation column (Super-rose 12, Pharmacia fine chemicals) and eluted with FPLC grade PBS. The eluate was monitored at 280nm absorbance and 1 ml fractions collected at a flow rate of 0.5 ml per minute. Fractions with the same mobility were pooled. The fraction that had the highest peak was tested for purity.

3.3.4 Testing for the purity of the BSA.

This was done by passing the purified BSA through the same FPLC column under identical conditions and monitoring the number of different peaks at 280nm absorbance. Afterwards the BSA was dialysed overnight against distilled water and lyophilised.

3.3.5 Coupling of the beta-ecdysone derivative to BSA.

The beta-ecdysone derivatives were coupled to BSA according to the method of Lauer *et al.,* (1974). Briefly, the derivative, N-hydroxysuccinimide and dicyclohexycarbodiimide were mixed in equimolar ratios. They were dissolved in dry tetrahydrofuran and kept with constant stirring for 24 hours at 4^0C followed by 48 hours at room temperature. To avoid moisture the reaction was done under nitrogen. This resulted in formation of N-Hydroxysuccinimide ester. The excess dicyclohexyurea was removed by filtration and the N-Hydroxysuccinimide ester dried by evaporation. The ester was mixed with BSA at a molar ratio of 20:1. The mixture was dissolved in 50% aqueous tetrahydrofuran pH 8.5 (maintained using sodium carbonate) and kept for 24 hours at 4^0C with constant stirring. The solution was then dialysed against running water for 48 hours and lyophilised.

3.3.6 Testing for successful conjugation.

To test for successful conjugation, the purified BSA, beta-ecdysone and the lyophilised product were passed through a high performance liquid chromatography (HPLC) reverse phase column using 0.1% triflouroacetic acid in propanol as the solvent system and the resulting peaks studied.

3.3.7 Immunisation of rabbits with conjugated beta-ecdysone.

Twenty five male rabbits from the batch described in chapter two and similarly maintained were randomly selected and allocated to five groups of five rabbits each. Each group was inoculated with 1 mg of either conjugated beta-ecdysone, purified BSA, STGMP or a 50.50 mixture of conjugated beta-ecdysone and STGMP. One group was immunised with PBS and acted as the non immunised control. Protein concentration was estimated using the Bio-rad automated protein assay procedure (Bio-rad, 1985). The route of immunisation, the adjuvant system used and the booster regime were as previously described in chapter one. Fourteen days after administration of the last booster dose, the rabbits were challenged with all instars of *R. appendiculatus*.

3.3.8 Serology.

The sera collected were assayed for the presence of precipitating antibodies against the administered antigens using the double immunodiffusion test (Ouchterlony, 1958). For the rabbits immunised with a combination of conjugated beta-ecdysone and STGMP, the sera were tested for the presence of both anti-STGMP and anti-conjugated beta-ecdysone antibodies.

3.3.9 Infestation of the immunised rabbits with ticks.

All instars of parasite free *R.appendiculatus* ticks were applied onto the ears of the immunised rabbits and the controls as previously described (Bailey, 1960; Irvin and Brocklesby, 1970). One hundred larvae and 50 nymphs were applied onto one ear while 60 adults (30 males and 30 females) were applied onto the other ear. The ticks were closely observed and the number

of days taken by the ticks to feed to engorgement, the mortality, the engorgement weight and the number of larvae and nymphs that moulted recorded. In addition, for engorged females, the weight of the egg masses oviposited, the egg conversion ratio, the percent hatchability and the number of females attaining the 'critical engorgement weight', that is, an engorgement weight of at least 300 mg were also recorded.

3.3.10 Effects of the immunisation on the next developmental instars.

To determine this, larvae, nymphs and adults that were harvested after incubating eggs, larvae and nymphs respectively from ticks that had previously been fed on immunised and control rabbits were infested on tick naive rabbits. For each experimental group, 10 larvae were collected at random from the egg batches of every tick in the group and pooled. In addition, for each experimental group, all the nymphs and adults were collected and pooled. Tick naive rabbits were selected and grouped into 3 groups of 2 rabbits per group. Each group was infested with ticks harvested from a group of rabbits immunised with one of the three immunogens. Thirty nymphs, 100 larvae and 16 adults (8 males and 8 females) were applied onto the left ear of each experimental rabbit. Similar numbers of ticks harvested after incubating eggs, larvae and nymphs respectively from ticks that had previously been fed on control rabbits were applied onto the right ears and acted as the controls. The ticks were closely observed and the parameters described in section 3.3.9 recorded.

3.3.11 Injection of ticks with anti-beta-ecdysone antibodies.

Anti-beta-ecdysone antibodies were obtained from sera harvested from rabbits with high antibody titres detectable using the double immunodiffusion test (Ouchterlony, 1958). The immunoglobulins were precipitated by reacting them with 33% ammonium sulphate solution (Hudson and Hay, 1981a). Briefly, the serum was mixed with saturated ammonium sulphate solution (pH 7, adjusted with ammonia solution) at a ratio of 2:1 and stirred overnight at 4^0C. The mixture was then centrifuged at 3,000 X g for 10 minutes at 4^0C and the supernatant discarded. The pellet was resuspended in PBS (pH 7.2) and dialysed in PBS for 48 hours at 4^0C

after which it was lyophilised. Normal rabbit serum was treated in the same way and used as the control. The lyophilised products were tested for retention of activity using the double immunodiffusion test (Ouchterlony, 1958).

Adult unfed ticks were randomly selected and allocated to 6 groups namely A, B, C, D, E and F. Each had 40 ticks (20 males and 20 females). Groups A, B, C, and D were injected with 3 ul of neat (100 mg/ml), 1:5, 1:10, and 1:20 dilutions of lyophilised anti-conjugated beta-ecdysone immunoglobulins in PBS respectively. Ticks in group E and F were injected with 3 ul neat normal rabbit serum and PBS respectively and acted as the controls. These unfed ticks were infested on 10 tick naive rabbits (2 rabbits per test group). The ticks injected with antibodies were placed on the left ears while those injected with buffer (controls) were placed on the right ears. After application the ticks were monitored daily for the parameters described above.

Engorged nymphs with approximately similar engorgement weights (9-12 mg) and engorged females with engorgement weights ranging from 350-400 mg were allocated at random to 6 groups A, B, C, D, E and F with 20 ticks per group. The females and the nymphs were then injected with 10 ul and 2 ul respectively of the antibodies or buffer. Ticks in groups A, B, C and D were injected with neat (100 mg/ml), 1:5, 1:10, and 1:20 dilutions of lyophilised anti-conjugated beta-ecdysone immunoglobulins in PBS respectively. Ticks in group E and F were injected with neat normal rabbit serum and PBS respectively and acted as the controls. The ticks were then incubated at 28^0C and 85%. The number of nymphs that moulted and the duration they took to moult were recorded. The number of adults that oviposited, the weight of the oviposited eggs, the egg conversion ratios and the percentage of the eggs that hatched were recorded.

3.3.12 Statistical analysis.

Data was analysed using analysis of variance (ANOVA) and more specifically the general linear model to cater for unbalanced data. The Duncan multiple range test was used at the 5% level of significance to show whether means were significantly different.

3.4 Results.

3.4.1 Purification of the hemisuccinate by HPLC.

After passing the hemisuccinates through the HPLC column and monitoring their passage by absorbance at 280nM, 3 peaks, A, B, and C were identified (Figure 6).

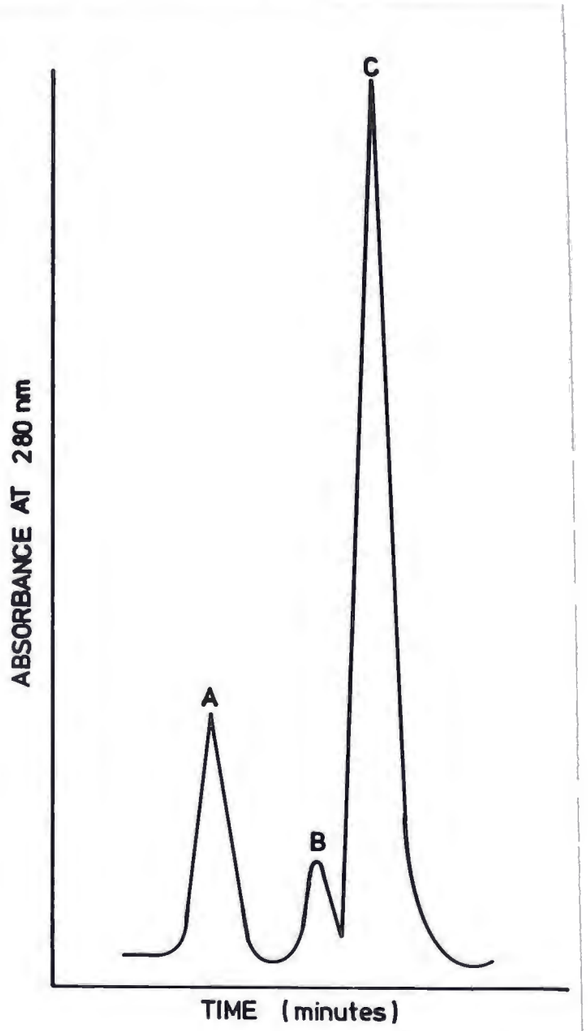

FIGURE 6.

A photograph showing the profiles obtained after passing the hemisuccinates through a reverse phase HPLC column.

Fraction C was pooled, concentrated by evaporation, lyophilised and used for conjugation after studying its mass spectrum.

3.4.2 Mass spectrum of the desired fraction.

The mass spectrum of the 3 fractions were determined. The mass spectrum of fraction C was shown to be closely related to that of beta-ecdysone indicating that successful succinylation had been achieved (Figure 7).

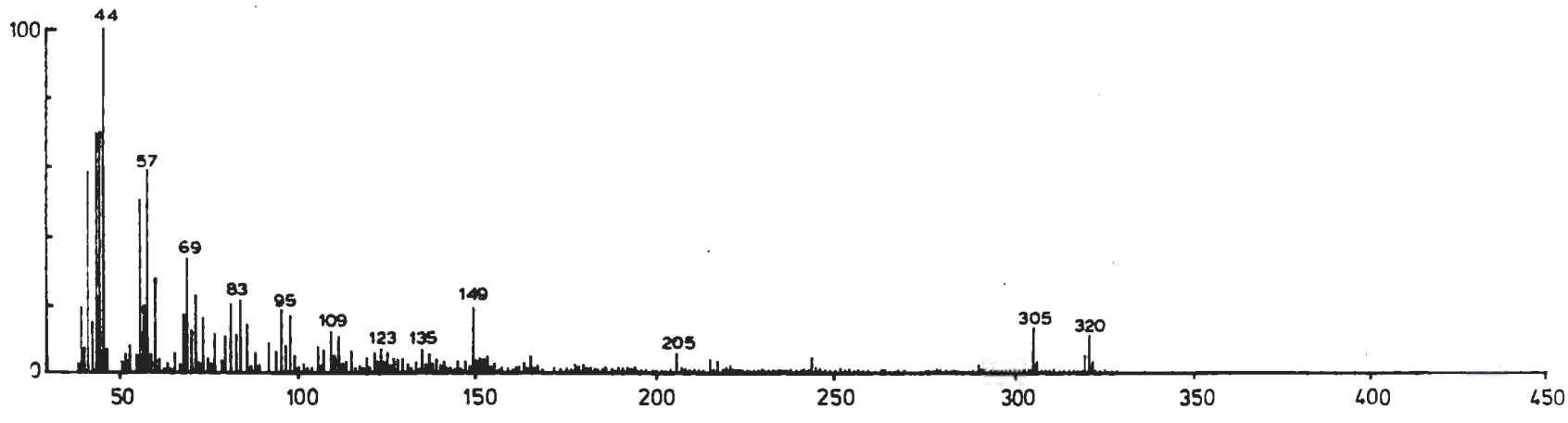

FIGURE 7

A photograph showing the mass spectrum of the hemisuccinate fraction used for conjugation.

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3.4.3 Purification of BSA.

After passing the BSA through an FPLC column, 3 separate peaks were identified (Figure 8). The highest peak was selected and tested for purity.

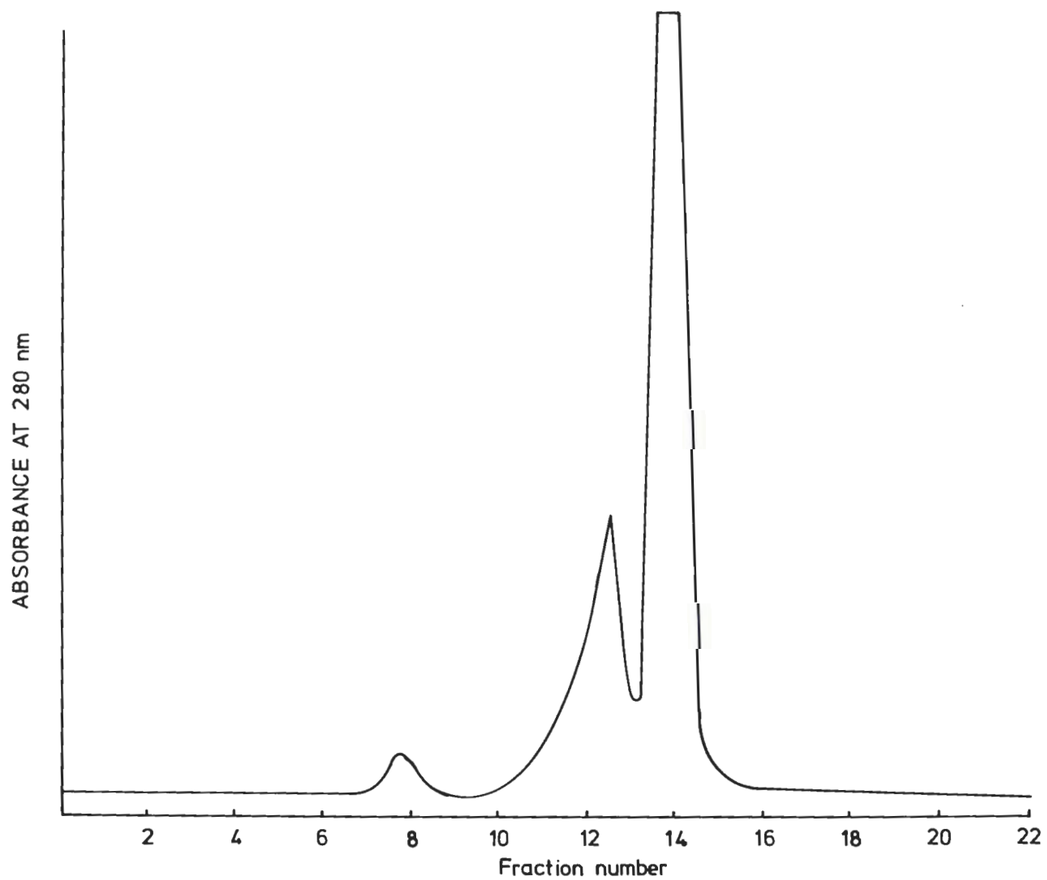

FIGURE 8

A photograph showing the different peaks obtained after passing commercial BSA through an FPLC (superose - 12) gel permeation column.

Note that 3 peaks were eluted showing how impure the BSA was.

3.4.4 Testing for the purity of BSA.

After passing the BSA peak selected (the one with the highest peak in Figure 8), through an FPLC column similar to the one used for purification and under identical conditions, only one peak was formed indicating that the BSA in the selected peak was homogenous (Figure 9).

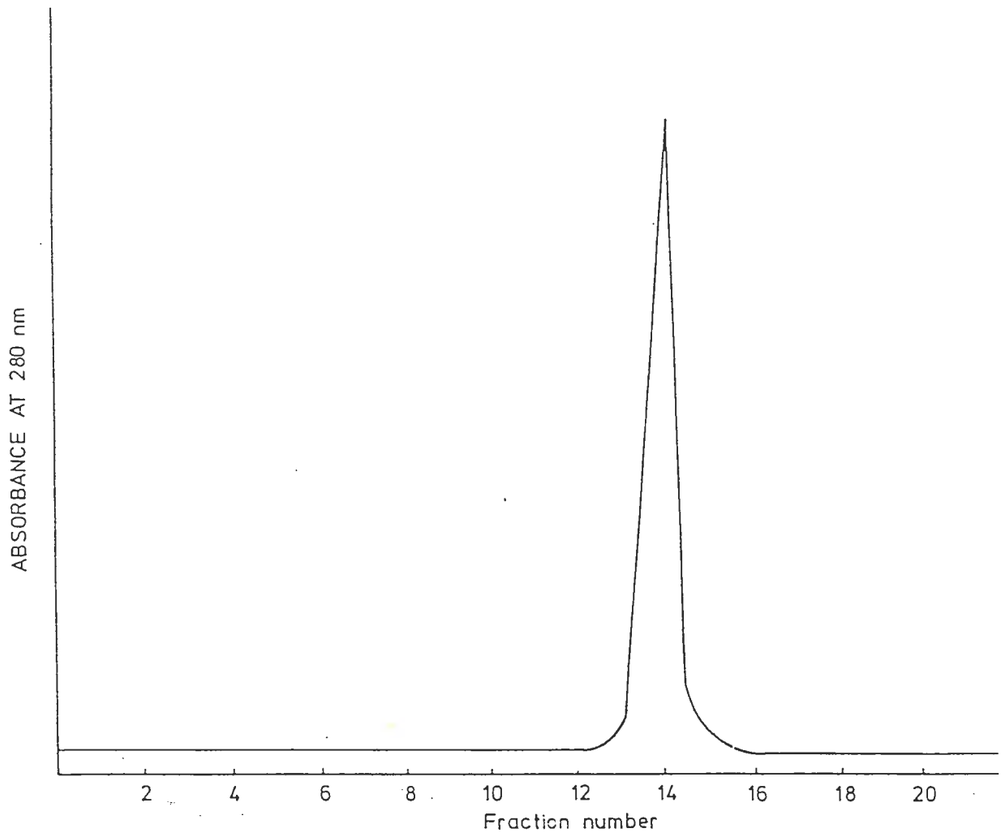

FIGURE 9.

A photograph showing the results obtained after passing the purified BSA through an FPLC (superose-12) gel permeation column

Note that only one peak was eluted showing that the BSA was successfully purified.

3.4.5 Testing for successful conjugation.

The different profiles observed after passing the purified BSA, beta-ecdysone and the conjugated product through the HPLC reverse phase column are shown in Figure 10. Beta-ecdysone was the first to be eluted followed by BSA. The conjugated material came last. This showed that it was heavier than BSA and therefore the conjugation was successful.

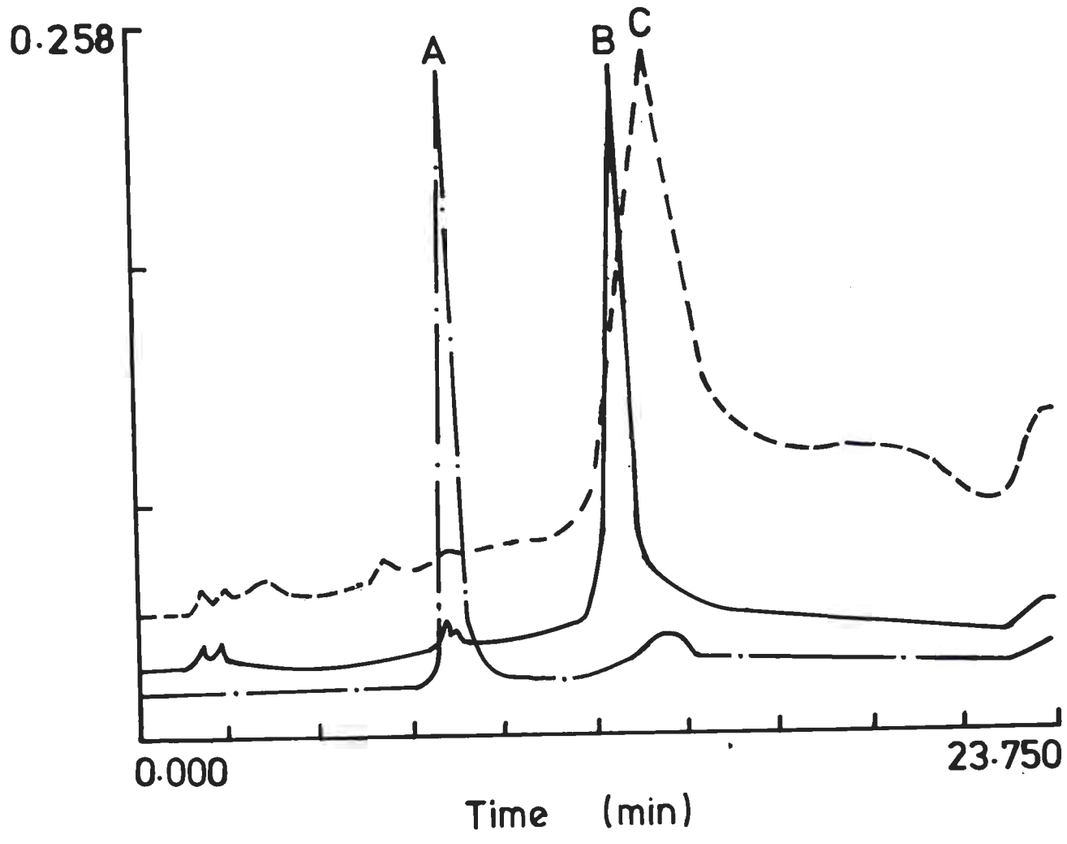

FIGURE 10.

A photograph showing the profiles obtained after passing Beta-ecdysone (A), BSA, (B) and the conjugated beta-ecdysone through a HPLC reverse phase column.

3.4.6 Serology.

The sera collected from the rabbits 2 weeks after the initial dose had precipitating antibodies against the immunising antigens. Serum from the controls did not develop precipitin lines. Figure 11 shows the results obtained using the group of rabbits immunised with conjugated beta-ecdysone. Rabbits immunised with other immunogens showed similar precipitin lines.

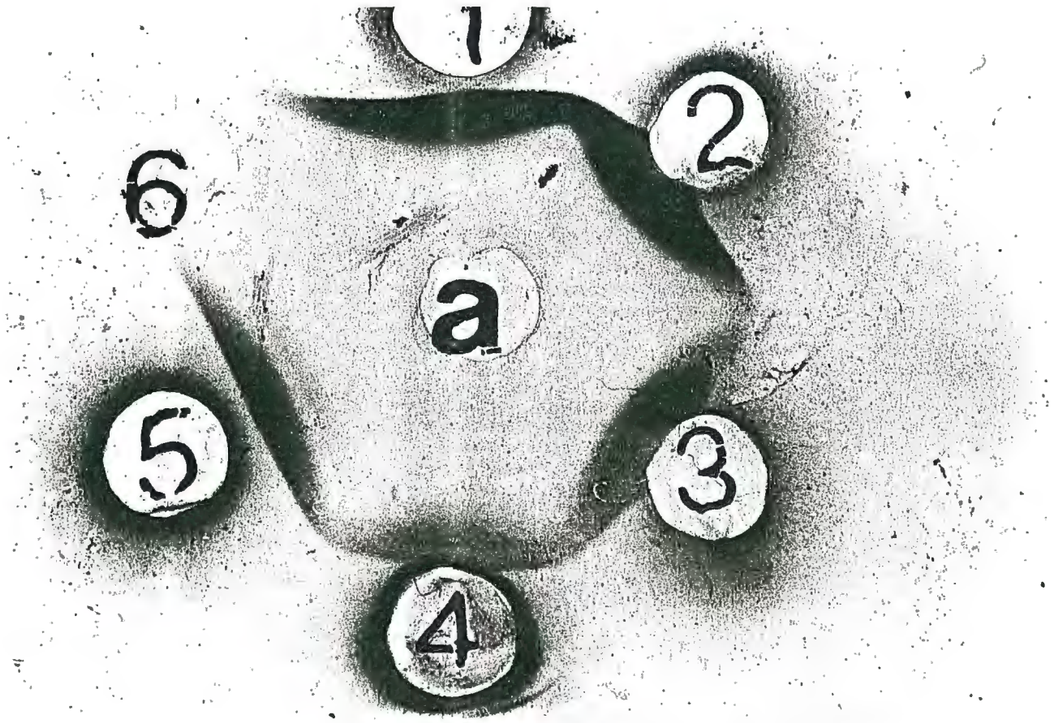

Figure 11

Photograph showing precipitin lines of complete identity after immunodiffusion using serum from rabbits immunised with conjugated beta-ecdysone.
Well a contained conjugated beta-ecdysone.
Wells 1-5 contained sera from the 5 rabbits immunised with beta-ecdysone.
Well 6 contained buffer (PBS).

3.4.7 Infestation results.

For all the parameters recorded in all the instars, the ticks that were infested on rabbits immunised with BSA were not significantly different from those that were infested on controls.

The results obtained after infesting larvae on the immunised and the control rabbits are presented in Table 3. There were no significant differences in the engorgement weights and the percentage that moulted between the larvae that were infested on immunised rabbits and those that were infested on the controls. However, the feeding duration and the percentage that fed successfully to engorgement were significantly lower in the larvae that were infested on immunised rabbits than those that were infested on the controls

Table 3.

The feeding performance and the moulting ability of *R.appendiculatus* larvae exposed to immunised rabbits.

Biological parameters	Immunogens used.				
	Beta-ecdysone	BSA	Beta-ecdysone + STGMP	STGMP	Control
Feeding duration (days, Mean ± SE)	3.87A (±0.17)	4.07A (±0.09)	4.10A (±0.07)	4.29A (±0.08)	5.20A (±0.24)
Engorgement weight (Mg, Mean ± SE)	0.53A (±0.02)	0.48A (±0.04)	0.48A (±0.03)	0.50A (±0.15)	0.51A (±0.01)
Percentge feeding (%, Mean ± SE)	64.60B (±9.24)	66.78A,B (±11.21)	74.00 A,B (±7.29)	68.00A,B (±4.98)	84.00A (±17.04)
Percentage moulting (%, Mean ± SE)	65.83A (±8.71)	63.14A (±7.60)	64.68A (±8.89)	55.44A (±9.14)	67.03A (±8.28)

Means followed by the same letter in rows are not significantly different at the 5% level.
The means are of 5 rabbits per immunogen challenged with 100 *R.appendiculatus* larvae.

3.4.7.1 Results obtained using Nymphs.

Table 4 summarises the effects of the immunisation on the feeding performance and the ability to moult in the nymphs that were infested on the experimental rabbits. Nymphs that were infested on rabbits immunised with either conjugated beta-ecdysone or STGMP fed for a

significantly shorter time than those that were infested on controls. However, there were no significant differences in all the other parameters recorded.

Table 4.

The feeding performance and the moulting ability of nymphal *R.appendiculatus* exposed to immunised rabbits.

Biological parameters	**Immunogens used.**				
	Beta-ecdysone	BSA	Beta-ecdysone + STGMP	STGMP	Control
Feeding duration (days, Mean ± SE)	4.93**B** (±0.06)	5.12**A** (±0.10)	4.89**B** (±0.04)	4.89**B** (±0.04)	5.20**A** (±0.10)
Engorgement weight (mg,Mean ± SE)	9.07**A** (±0.15)	9.10**A** (±0.17)	9.16**A** (±0.15)	9.29**A** (±0.15)	9.07**A** (±0.16)
Percentage feeding (%, Mean ± SE)	72.80**A** (±4.32)	71.40**A** (±3.26)	64.80**A** (±4.18)	74.00**A** (±4.98)	69.50**A** (±5.86)
Percent moulting (%, Mean ± SE)	96.65**A** (±2.48)	96.39**A** (±1.76)	96.71**A** (±2.10)	97.58**A** (±0.72)	98.70**A** (±0.78)

Means followed by the same letter in rows are not significantly different at the 5% level.
The means are of 5 rabbits per immunogen challenged with 50 *R.appendiculatus* nymphs.

3.4.7.3 Results obtained using adults.

The results on the feeding performance and fecundity of adult ticks that were infested on the experimental rabbits are presented in Tables 5a and b. There was no significant difference in the feeding duration between the adults that were infested on immunised rabbits and those that were infested on controls. However, the engorgement weights of the adult ticks that were infested on immunised rabbits were significantly lower than those of the ticks that were infested on the controls. The ticks that were infested on rabbits immunised with a combination of STGMP and conjugated beta-ecdysone had the lowest engorgement weights and egg masses. The percent reductions in the engorgement weights were 36.82, 30.18 and 20.67 for the ticks that were infested on rabbits immunised with a combination of STGMP and conjugated beta-ecdysone, beta-ecdysone and STGMP respectively. The weights of the eggs oviposited by the females that

were infested on immunised rabbits were significantly lower than those of the females that were infested on controls. The percent reduction in the weights of eggs oviposited were 40.91, 37.12 and 25.26 for the ticks that were infested on rabbits immunised with a combination of STGMP and conjugated beta-ecdysone, beta-ecdysone and STGMP respectively. The egg conversion ratios were significantly lower in ticks that were infested on immunised rabbits than those that were infested on controls. The percent reduction in the egg conversion ratios were 13.36, 9.19 and 6.91 for the ticks that were infested on rabbits immunised with conjugated beta-ecdysone, STGMP or a combination of STGMP and beta-ecdysone respectively. The hatchabilities of the oviposited eggs were not affected by the immunisation. However, the ticks that were infested on rabbits immunised with STGMP oviposited eggs with hatchabilities that were 25.60% higher than that of the eggs oviposited by the ticks infested on rabbits immunised with conjugated beta-ecdysone. The percentage of ticks that fed successfully to engorgement and the percentage that oviposited were not significantly different in ticks that were infested on immunised rabbits and those that were infested on controls. The percentage of females that attained the critical engorgement weight was lower by as much as 67.41% in ticks that were infested on immunised rabbits as compared to those that were infested on controls.

Table 5a

The feeding performance of adult *R.appendiculatus* exposed to immunised rabbits.

Biological parameters	**Immunogens used.**				
	Beta-ecdysone	BSA	Beta-ecdysone + STGMP	STGMP	Control
Feeding duration (days,Mean ± SE)	7.92**A** (±0.14)	7.82**A** (±0.14)	7.72**A** (±0.12)	7.91**A** (±0.13)	7.96**A** (±0.15)
Engorgement weight (mg, Mean ± SE)	303.29**B,C** (±12.28)	396.82**A** (±9.35)	288.57**C** (±8.65)	327.19**B** (±11.50)	394.81**A** (±9.95)
Percentage feeding (%, Mean ± SE)	74.67**A** (±9.46)	82.13**A** (±3.48)	86.67**A** (±5.68)	76.59**A** (±10.33)	83.33**A** (±8.92)
Percentage attaining CEW (%,Mean + SE)	58.72**B** (±11.05)	83.52**A** (±6.84)	53.61**B** (±8.74)	64.57**B** (±7.48)	89.75**A** (±4.10)

Means followed by the same letter in rows are not significantly different at the 5% level.

The means are of 5 rabbits per immunogen challenged with 60 (30 males, 30 females) *R.appendiculatus* adults. All parameters assessed were done using female ticks.

Table 5b

The fecundity of adult *R.appendiculatus* exposed to immunised rabbits.

Biological parameters	**Immunogens used.**				
	Beta-ecdysone	BSA	Beta-ecdysone + STGMP	STGMP	Control
Egg mass (mg, (Mean ± SE)	134.72**B,C** (±5.25)	180.01**A** (±5.69)	131.10**C** (±4.50)	147.48**B** (±5.19)	184.73**A** (±5.25)
Egg conversion ratio (Mean ± SE)	41.38**B** (±0.99)	45.34**A** (±1.32)	43.88**B** (±0.88)	42.96**B** (±0.92)	46.91**A** (±0.70)
Percentage hatchability (%, Mean ± SE)	54.56**B** (±4.37)	58.76**A, B** (±2.37)	59.77**A,B** (±3.79)	68.53**A** (±3.81)	61.96**A, B** (±4.13)
Percentage ovipositing (%, Mean ± SE)	83.01**A** (±5.95)	91.37**A** (±7.26)	91.86**A** (±4.56)	88.09**A** (±7.53)	87.38**A** (±5.24)

Means followed by the same letter in rows are not significantly different at the 5% level.

The means are of 5 rabbits per immunogen challenged with 60 (30 males, 30 females) *R.appendiculatus* adults. All parameters assessed were done using female ticks.

3.4.8 Effects of the immunisation on the next developmental instars.

There were no significant differences in all the biological parameters assessed between the larvae that had hatched from eggs oviposited by ticks that had been fed on immunised rabbits irrespective of the immunogens used and the larvae that had hatched from eggs oviposited by ticks that had been fed on controls (Tables 6, 7 and 8).

Table 6.

The feeding performance and moulting ability of larval *R.appendiculatus* which had hatched from eggs oviposited by females that had been infested on rabbits immunised with conjugated beta-ecdysone.

Biological parameters	Immunogens used.	
	Beta-ecdysone	Control
Feeding duration (days, Mean ± SE)	4.31A (±0.19)	4.08A (±0.06)
Engorgement weight (mg, Mean ± SE)	0.51A (±0.02)	0.72A (±0.25)
Percentage feeding (%, Mean ± SE)	51.00A (±39.00)	92.00A (±4.00)
Percentage moulting (% ,ean ± SE)	67.67A (±17.78)	78.79A (±12.88)

Means followed by the same letter in rows are not significantly different at the 5% level.
The means are of 2 rabbits per immunogen challenged with 100 *R.appendiculatus* larvae.

Table 7.

The biology of larval *R.appendiculatus* which had hatched from eggs oviposited by females that had been infested on rabbits immunised by STGMP.

Biological parameters	Immunogens used.	
	STGMP	Control
Feeding duration (days,Mean ± SE)	4.25A (±0.12)	4.68A (±0.32)
Engorgement weight (mg, Mean ± SE)	0.54A (±0.04)	0.49A (±0.02)
Percentage feeding (%, Mean ± SE)	70.00A (±9.00)	63.26A (±7.64)
Percentage moulting (%, Mean ± SE)	93.74A (±1.34)	94.24A (±3.58)

Means followed by the same letter in rows are not significantly different at the 5% level
The means are of 2 rabbits per immunogen challenged with 100 *R.appendiculatus* larvae.

Table 8.

The biology of larval *R.appendiculatus* which had hatched from eggs oviposited by females that had been infested on rabbits immunised with a combination of conjugated beta-ecdysone and STGMP.

Biological parameters	**Immunogens used.**	
	Beta-ecdysone+STGMP	Control
Feeding duration (days, Mean ± SE)	4.39**A** (±0.39)	4.08**A** (±0.08)
Engorgement weight (mg, Mean ± SE)	0.50**A** (±0.02)	0.58**A** (±0.08)
Percentage feeding (%, Mean ± SE)	84.36**A** (±7.26)	91.50**A** (±2.50)
Percentage moulting (%, Mean ± SE)	94.93**A** (±5.07)	97.22**A** (±1.72)

Means followed by the same letter in rows are not significantly different at the 5% level.

The means are of 2 rabbits per immunogen challenged with 100 *R.appendiculatus* larvae.

Similarly, there were no significant difference in the parameters monitored between the nymphs that had moulted from the larvae that had been fed on immunised rabbits irrespective of the immunogens used and the nymphs that had moulted from larvae that had been fed on controls (Tables 9, 10 and 11).

Table 9.

The biology of *R.appendiculatus* nymphs which moulted from larvae that had been infested on rabbits immunised with conjugated beta-ecdysone.

Biological parameters	**Immunogens used.**	
	Beta-ecdysone	Control
Feeding duration (days, Mean ± SE)	5.03**B** (±0.06)	4.65**A** (±0.07)
Engorgement weight (mg, Mean ± SE)	6.51**A** (±0.29)	6.30**A** (±0.29)
Percentage feeding (%, Mean ± SE)	54.00**A** (±6.00)	57.67**A** (±13.35)
Percentage moulting (%, Mean ± SE)	98.33**A** (±1.67)	100**A**

Means followed by the same letter in rows are not significantly different at the 5% level.

The means are of 2 rabbits per immunogen challenged with 30 *R.appendiculatus* nymphs.

Table 10.

The biology of *R.appendiculatus* nymphs which moulted from larvae that had been infested on rabbits immunised with conjugated STGMP.

Biological parameters	**Immunogens used.**	
	STGMP	Control
Feeding duration (days,Mean ± SE)	5.45**A** (±0.09)	5.56**A** (±0.13)
Engorgement weight (mg, Mean ± SE)	5.40**B** (±0.04)	6.81**A** (±0.57)
Percentage feeding (%, Mean ± SE)	73.00**A** (±9.00)	67.00**A** (±12.00)
Percentage moulting (%, Mean ± SE)	90.74**B** (±1.34)	100**A**

Means followed by the same letter in rows are not significantly different at the 5% level.

The means are of 2 rabbits per immunogen challenged with 30 *R.appendiculatus* nymphs.

Table 11.

The biology of *R.appendiculatus* nymphs which as larvae were infested on rabbits immunised with a combination of conjugated beta-ecdysone and STGMP.

Biological parameters	**Immunogens used.**	
	Beta-ecdysone + STGMP	Control
Feeding duration (days, Mean ± SE)	4.29**A** (±0.33)	4.18**A** (±0.08)
Engorgement weight (mg, Mean ± SE)	7.03**A** (±0.24)	7.82**A** (±0.82)
Percentage feeding (%, Mean ± SE)	88.38**A** (±7.26)	91.50**A** (±2.50)
Percentage moulting (%, Mean ± SE)	93.95**A** (±5.07)	97.22**A** (±1.72)

Means followed by the same letter in rows are not significantly different at the 5% level.

The means are of 2 rabbits per immunogen challenged with 30 *R.appendiculatus* nymphs.

3.4.8.1 Results obtained using the resultant adults.

The effects of the immunisation on the adults that were fed as nymphs on rabbits immunised with conjugated beta-ecdysone are summarised in Table 12. There was no significant difference in the feeding duration, engorgement weight, weights of the eggs that were oviposited and the percentage of the ticks that achieved the critical engorgement weight between the adults which fed as nymphs on rabbits immunised with conjugated beta-ecdysone and those that had fed

on the controls as nymphs. However, the percentage of the ticks that fed successfully to engorgement, the egg conversion ratio, the percentage of the females that oviposited and the hatchabilities of the oviposited eggs were significantly lower between the adults which fed as nymphs on rabbits immunised with conjugated beta-ecdysone and those that had fed on the controls. The percent reductions in the percentage of the ticks that fed successfully to engorgement, the egg conversion ratio, the percent of the females that oviposited and the hatchabilities of the oviposited eggs were 9.58, 14.71, 12.05 and 36.73 respectively.

Table 12.

The biology of adult *R.appendiculatus* ticks which moulted from nymphs that had been infested on rabbits immunised with conjugated beta-ecdysone.

Biological parameter	**Immunogens used.**	
	Beta-ecdysone	Control
Feeding duration (days, Mean ± SE)	7.55**A** (±0.09)	7.50**A** (±0.26)
Engorgement weight (mg, Mean ± SE)	432.06**A** (±10.56)	416.10**A** (±23.47)
Egg mass (mg, Mean ± SE)	220.45**A** (±36.20)	197.90**A** (±7.92)
Egg conversion ratio (Mean ± SE)	43.59**B** (±1.14)	50.00**A** (±1.28)
Percentage hatchability (%, Mean ± SE)	72.22**B** (±4.92)	98.75**A** (±1.25)
Percentage feeding (%, Mean ± SE)	84.36**A** (±7.26)	91.50**A** (±2.50)
Percentage attaining CEW (%,Mean ± SE)	85.71**A** (±14.29)	98.38**A** (±1.61)
Percentage ovipositing (%,Mean ± SE)	82.85**B** (±2.86)	92.83**A** (±3.94

Means followed by the same in rows are not significantly different at the 5% level.

The means are of 2 rabbits per immunogen challenged with 16 (8 males and 8 females) *R.appendiculatus* adults. All parameters assessed were done using female ticks.

The effects of the immunisation on the adults that were fed as nymphs on rabbits immunised with STGMP are shown in Table 13. The engorgement weights and the hatchabilities of the oviposited eggs were significantly lower in the adults which fed as nymphs on rabbits immunised with STGMP and those that had fed on the controls as nymphs. There were no significant differences in all the other parameters that were recorded between the adults which fed

as nymphs on rabbits immunised with STGMP and those that had fed on the controls as nymphs. The percent reductions in the engorgement weights and the hatchabilities of the oviposited eggs were 19.41 and 24.46 respectively.

Table 13.

The feeding and fecundity of adult *R.appendiculatus* ticks which moulted from nymphs that had been infested on rabbits immunised with STGMP.

Biological parameter	**Immunogens used.**	
	STGMP	Control
Feeding duration (days, Mean ± SE)	7.26**A** (±0.11)	7.09**A** (±0.21)
Engorgement weight (mg, Mean ± SE))	396.18**B** (±13.89)	473.09**A** (±23.19)
Egg mass (mg, Mean ± SE)	192.89**A** (±22.34)	204.44**A** (±9.97)
Egg conversion ratio (Mean ± SE)	43.30**A** (±1.46)	46.02**A** (±2.84)
Percentage hatchability (Mean ± SE)	73.92**B** (±5.53)	92.00**A** (±8.00)
Percentage feeding (Mean ± SE)	56.25**A** (±6.25)	73.96**A** (±15.10)
Percentage attaining CEW (Mean ± SE)	92.68**A** (±4.57)	85.61**A** (±2.85)
Percentage ovipositing (Mean ± SE)	90.00**A** (±10.00)	91.38**A** (±8.62)

Means followed by the same letter in rows are not significantly different at the 5% level

The means are of 2 rabbits per immunogen challenged with 16 (8 males and 8 females) *R.appendiculatus* adults. All parameters assessed were done using female ticks.

The effects of the immunisation on the adults that were fed as nymphs on rabbits immunised with conjugated beta-ecdysone in combination with STGMP are shown in Table 14. The feeding duration, engorgement weight, the percentage of the adults that fed successfully to engorgement and the percentage of the females that oviposited were not significantly different. However, the weights and the hatchabilities of the oviposited eggs, the egg conversion ratio and the percentage of the ticks that attained the critical engorgement weight were significantly lower in the ticks which fed as nymphs on rabbits immunised with a combination of conjugated beta-ecdysone and STGMP as compared to those that had fed on the controls. The percent reductions in the weights and the hatchabilities of the oviposited eggs, the egg conversion ratio and the percentage of the ticks that attained the critical engorgement weight were 14.78, 28.68, 12.03 and 11.86 respectively.

Table 14.

The biology of adult *R.appendiculatus* ticks which moulted from nymphs that had been infested on rabbits immunised with a combination of conjugated beta-ecdysone and STGMP.

Biological parameters	Immunogens used.	
	Beta-ecdysone + STGMP	Control
Feeding duration (days, Mean ± SE)	7.09**A** (±0.08)	7.33**A** (±0.16)
Engorgement weight (mg, Mean ± SE)	423.54**A** (±11.73)	441.57**A** (±23.47)
Egg mass (mg, Mean ± SE)	183.50**B** (±8.83)	210.63**A** (±11.51)
Egg conversion ratio (Mean ± SE)	43.99**B** (±1.14)	49.28**A** (±1.02)
Percentage hatchability (%,Mean ± SE)	77.71**B** (±5.55)	100.00**A**
Percentage feeding (%,Mean ± SE)	75.00**A** (±25.00)	73.33**A** (±3.33)
Percentage attaining CEW (%,Mean ± SE)	93.75**A** (±6.25)	93.27**A** (±1.97)
Percentage ovipositing (%,Mean ± SE)	81.25**B** (±6.25)	90.89 (±0.41)

Means followed by the same letter in rows are not significantly different at the 5% level

The means are of 2 rabbits per immunogen challenged with 16 (8 males and 8 females) *R.appendiculatus* adults. All parameters assessed were done using female ticks.

3.4.9 Results obtained after injecting ticks with anti-beta-ecdysone antibodies.

After reacting the lyophilised serum with conjugated beta-ecdysone using the double immunodiffusion test, a single precipitin line was formed indicating that the antibodies were still active. A similar test indicated that the normal rabbit serum was also active.

3.4.9.1 Results obtained after injecting engorged nymphs with anti-beta-ecdysone antibodies.

The results obtained after injecting engorged nymphs with anti-beta-ecdysone antibodies are summarised in Table 15. There was no significant difference in the duration taken by the nymphs to moult between the nymphs that were injected with anti-beta-ecdysone antibodies and those that were injected with either buffer or the normal rabbit serum. However, the percentage of the nymphs that moulted was drastically reduced to 0, 15, 30 and 40 for the nymphs that were injected with neat, 1:5, 1:10 and 1:20 dilutions of the antibody respectively.

Table 15

The moulting ability of *R.appendiculatus* nymphs injected with anti-beta-ecdysone antibodies.

Biological parameter	n*	**Antibody dilution used.**					
	20	Neat	1:5	1:10	1:20	NRS*	Control
Moulting duration (days)	20	**	14	14	14	14	14
Percentage moulting. (%)	20	0	15	30	40	80	85

*NRS is an abbreviation for Normal rabbit serum.

** Indicates that the activity (moulting) did not occur

n* indicates the number of nymphs injected

3.4.9.2 Results obtained after injecting engorged females with anti-beta-ecdysone antibodies.

The fecundities of the female ticks that were injected with anti-beta-ecdysone antibodies are presented in Table 16. There was no significant difference in the weights of the oviposited eggs, the egg conversion ratios and the percentage of the eggs that hatched between the females that were injected with anti-beta-ecdysone antibodies and those that were injected with either buffer or the normal rabbit serum. However, the percentage of the females that oviposited was reduced to 0, 15, 20 and 35 for the females that were injected with neat, 1:5, 1:10 and 1:20 dilutions of the antibody respectively.

Table 16

The fecundity of *R.appendiculatus* females injected with anti-beta-ecdysone antibodies.

Biological Parameters	**Antibody dilution used.**					
	Neat	1:5	1:10	1:20	NRS*	Control
Percentage ovipositing (%, Mean ± SE)	0	15	20	35	100	95
Egg mass (mg, Mean ± SE)	*	148.17A (±25.99)	172.68A (±15.88)	192.80A (±18.51)	182.63A (±14.42)	165.01A (±10.96)
Egg conversion ratio (Mean ± SE)	*	42.84A (±6.84)	45.73A (±3.18)	43.76A (±4.62)	45.70A (±2.89)	44.86A (±2.76)
Percentage hatching (%, Mean ± SE)	*	64.17A (±18.19)	67.85A (±15.81)	52.50A (±20.56)	67.50A (±11.30)	65.79A (±9.68)

Means followed by the same letter in rows are not significantly different at the 5% level

* Indicates that the activity (oviposition) did not occur

The means are of 20 female *R.appendiculatus* adults injected with anti-beta-ecdysone antibodies.

3.4.9.3 Results obtained after injecting unfed adult ticks with anti-beta-ecdysone antibodies.

The effects of injecting unfed ticks with the anti-beta-ecdysone antibodies are summarised in Table 17. There was no significant difference in the feeding durations, the percentage of the ticks that fed successfully to engorgement and the percentage that oviposited between the ticks that were injected with anti-beta-ecdysone antibodies and those that were injected with either buffer or the normal rabbit serum. However, the engorgement weights of the females, the weights of the oviposited eggs, the percentage of the females that attained the critical engorgement weight and the egg conversion ratios were reduced. The percent reduction in the engorgement weights were 21.28 and 13.27 for the ticks that were injected with neat and 1:5 dilutions of the antibody respectively. The percent reduction in the weights of the eggs that were oviposited were 25.87 and 25.47 for the ticks that were injected with neat and 1:5 dilutions of the antibody respectively.

Table 17a.

The feeding performance of adult *R.appendiculatus* ticks injected with anti-beta-ecdysone antibodies.

Biological parameter	**Antibody dilution used.**					
	Neat	1:5	1:10	1:20	NRS*	Control
Feeding duration (days, Mean ± SE)	8.00**A** (±0.33)	8.06**A** (±0.23)	8.10**A** (±0.14)	8:29**A** (±0.20)	7.98A (±0.15)	7.93A (±0.07)
Engorgement weight (mg, Mean ± SE)	310.03**B** (±40.08)	331.97**B** (±19.98)	382.77**B** (±27.07)	399.13**A** (±22.47)	386.13**A** (±8.42)	376.01A (±7.81)
Percentage feeding (%, Mean ± SE)	42.50**A** (±2.50)	62.50**A** (±37.50)	47.50**A** (±7.50)	50.00**A** (±20.00)	55.00**A** (±20.00)	52.50A (±8.71)
Percentage attaining CEW (%, Mean ± SE)	25.17**C** (±24.83)	70.90**B** (±19.91)	63.06**B** (±0.57)	70.63**B** (±10.63)	91.73**A** (±2.83)	90.44A (±2.91)

Means followed by the same letter in rows are not significantly different at the 5% level.

The means are of 40 (20 male and 20 female) *R.appendiculatus* adults injected with anti-beta-ecdysone antibodies. All parameters assessed were done using female ticks.

Table 17b

The fecundity of adult *R.appendiculatus* ticks injected with anti-beta-ecdysone antibodies.

Biological parameters	**Antibody dilution used.**					
	Neat	1:5	1:10	1:20	NRS*	Control
Egg mass (mg, Mean ± SE)	126.38**B** (±20.13)	126.79**B** (±14.66)	143.67**B A** (±16.68)	146.56**B A** (±13.14)	164.72**A** (±6.37)	159.08A(±5.20)
Egg conversion ratio (Mean ± SE)	40.35**B** (±0.65)	38.49**B** (±1.32)	37.86**B** (±1.90)	36.72**B** (±3.65)	42.64**A** (±0.75)	42.34A (±0.95)
Percentage feeding (%, Mean ± SE)	42.50**A** (±2.50)	62.50**A** (±37.50)	47.50**A** (±7.50)	50.00**A** (±20.00)	55.00**A** (±20.00)	52.50A (±8.71)
Percentage ovipositing (%, Mean ± SE)	66.67**A** (±33.33)	80.00**A** (±20.00)	69.32**A** (±5.68)	66.67**A** (±20.00)	73.33**A** (±20.00)	83.33A (±10.33)
Percentage hatching (%, Mean ± SE)	73.46A (±8.763)	81.42A (±5.83)	82.46A (±6.73)	77.89A (±7.24)	84.60**A** (±5.76)	89.95A (±9.12)

Means followed by the same letter in rows are not significantly different at the 5% level.

The means are of 40 (20 male and 20 female) *R.appendiculatus* adults injected with anti-beta-ecdysone antibodies. All parameters assessed were done using female ticks.

4.5 Discussion.

Induction of resistance to ticks by immunising their hosts with various extracts derived from either whole ticks or tick organs is well documented (Morrison, 1989; Wikel, 1988; Allen and Humphreys, 1979). However, immunisation using antigens not derived from the ticks has not been reported. Apart from the antigens that have been sequenced and cloned in *Escherichia coli* (Rand *et al.*, 1989) this is the first study in which hosts have been immunised against ticks using commercially prepared extracts that have not been derived from ticks.

In this study, conjugated beta-ecdysone had some deleterious effects on all the instars of *R.appendiculatus*. However, the resistance induced was more pronounced in adults. The only effects observed in the immature stages were a slight reduction in the feeding duration of the larvae infested on rabbits immunised with either beta-ecdysone or STGMP and a reduction in the percentage of nymphs that fed successfully to engorgement. Since beta-ecdysone is the hormone responsible for moulting, it would have been expected that the immunisation would affect the moulting process. However this did not happen. It is possible that neutralisation of the circulating beta-ecdysone in the haemolymph by specific antibodies, stimulated production of more beta-ecdysone that was able to stimulate moulting. Alternatively, adequate amounts of antibodies did not cross the midgut barrier.

In adults, the resistance resulted in reduction in the engorgement weights, egg masses, egg conversion ratio and the percentage of the ticks that attained the critical engorgement weight. Maranga (1988) reported similar findings on goats rendered resistant to *R.appendiculatus* either by infestation or by immunisation with crude midgut antigens. These findings are of great importance since the fecundity of an engorged female is dependent on its engorgement weight (Diehl *et al.*, 1982). Indeed below a critical weight, the tick will not even oviposit (Diehl *et al.*, 1982). The heavier the tick, the more eggs it will oviposit (Diehl, *et al* 1982; Balashov, 1972). However, recent studies (Dipeolu 1991) have shown that after attaining a critical engorgement

weight of 300 mg, an increase in engorgement weight does not necessarily result in an increase in egg production.

The engorgement weight and consequently the weights of the eggs that were oviposited were most drastically reduced in ticks that were infested with a combination of conjugated beta-ecdysone and STGMP. This may have been as a result of either additive but independent effects since both conjugated beta-ecdysone and STGMP used independently were shown to be protective or STGMP could have facilitated the passage of anti-beta-ecdysone antibodies across the tick midgut barrier.

The egg conversion ratio can be defined as the fraction of the blood meal converted to eggs. The ticks that were immunised with beta-ecdysone had the lowest egg conversion ratios and hatchabilities. A reduction in any or both of these parameters will result in a reduction in the number of the resultant larvae and therefore, an antigen that can cause a reduction in any or both of these parameters (such as conjugated beta-ecdysone) will have alot of promise in the control of ticks. Beta-ecdysone is necessary for vitellogenesis (Diehl *et al.*, 1986). Neutralisation of the circulating beta-ecdysone antibodies in tick haemolymph with specific antibodies may have impaired vitellogenesis and result in low egg conversion ratios. It may also have interfered with egg quality and result in low hatchabilities. Since beta-ecdysone is required for enlargement of testes and spermatogenesis in males (Oliver, 1982), lack of it may have rendered the males aspermic. This may have been the cause of the low hatchabilities observed.

The effects any immunisation against ticks will have on the next developmental instars is important in reducing tick populations. In this study, the immature stages were not affected by having infested their progenitors on immunised rabbits. However, the adults which as nymphs had been infested on rabbits immunised with conjugated beta-ecdysone had reduced egg conversion ratios, reduced engorgement weights and the percentage of females that oviposited. In

addition, the eggs had low hatchabilities. This may have resulted from the inability of the nymphs to develop into "normal" adults due to inadequate ecdysteroid hormones.

The adults which as nymphs had been infested on rabbits immunised with STGMP were not affected as drastically as those that had been infested on rabbits immunised with conjugated beta-ecdysone. Only their hatchabilities were reduced. The adults which as nymphs had been infested on rabbits immunised with a combination of conjugated beta-ecdysone and STGMP had reduced egg conversion ratios, hatchabilities, egg masses and the percentage that attained the critical engorgement weight. Reduction in the egg conversion ratio and hatchability may have been caused by anti-beta-ecdysone antibodies while a reduction in the egg masses oviposited may have been as a result of a reduction in the number of ticks attaining the critical engorgement weight.

The number of nymphs that were able to moult into adults was reduced by injecting engorged nymphs with anti-beta-ecdysone antibodies. The reduction in the moulting ability was positively correlated to the amounts of antibodies injected. However, the moulting duration was not affected by the injection. It is interesting to note that the moulting ability was not affected when unfed nymphs were fed to engorgement on rabbits immunised with conjugated beta-ecdysone. This may be due to inadequate amounts of anti-beta-ecdysone antibodies crossing the midgut barrier to the haemolymph of the nymphs since 40% of the nymphs injected with 1:20 dilution of anti-beta-ecdysone antibodies moulted normally and none moulted when injected with the neat preparation.

Injecting engorged females with anti-beta-ecdysone antibodies did not affect their fecundity. The anti-beta-ecdysone antibodies may have been injected when the female no longer required beta-ecdysone for reproduction. This is because oogenesis begins very early in the life cycle of the tick (reviewed by Diehl *et al.*, 1982, Balashov 1972). The previtellogenic cycle starts with the initiation of the blood meal and if the tick is mated, complete vitellogenesis occurs just

after engorgement. Therefore, the timing of injection of the tick with anti-beta-ecdysone antibodies is important and should be right after mating.

It is important to note that the engorgement weight, the percentage of the ticks attaining the critical engorgement weight and the fecundity of the female ticks injected with anti-beta-ecdysone antibodies before feeding were adversely affected.

CHAPTER FOUR

4.0 Evaluation of haemolymph proteins as potential tick anti-immunogens.

4.1 Introduction.

Tick internal organs are surrounded by the haemocoel which contains the fluid (haemolymph) necessary for transportation of the components required for their normal physiological processes (Binnington and Obenchain, 1982). The haemocoel has no epithelial lining and tick internal organs are separated from the haemolymph bathing them by thin basement membranes only (Binnington and Obenchain, 1982). This greatly facilitates exchange of components between the haemolymph and the internal organs.

It has been proposed that interfering with the normal physiological activities of the haemolymph using specific antibodies might have deleterious effects on ticks (Roberts, 1968; Galun, 1978; McGowan *et al.,* 1980). This may be achieved by feeding ticks on hosts immunised with haemolymph components since antibodies have been shown to cross the gut epithelium of haematophagous arthropods and to attach to the components that serve as the sensitising antigens (Schlein and Lewis, 1976). In addition, specific antibodies can neutralise the effects of biologically active molecules like enzymes and hormones (Butler *et al.,* 1973). Indeed fatal neutralisation of some arthropod haemolymph proteins using specific antibodies has been reported (Nogge and Gianneti, 1980).

Throughout its feeding period, haemolymph comprises 23% of the ticks body weight (Kaufman and Phillips, 1973), which is quite a big proportion. In addition, haemolymph components are not secreted through saliva (Dolp and Hamdy, 1971) and are therefore not encountered during feeding. Consequently, the hypersensitivity lesions (Wikel, 1981) and the immunological tolerance (Berdyer and Khudainazarova, 1976) associated with immunisation of hosts with the antigens they normally encounter during feeding would not occur after immunising hosts with haemolymph components. In addition, haemolymph components are not toxic to

mammals (Tatchell, 1971) and immunisation with them results in production of high antibody titres (Ben-Yakir and Barker, 1987). It is therefore worthwhile to evaluate the possibility of immunising hosts against ticks using tick haemolymph components.

4.2 The Overall objectives of this section of the study.

The objectives of this section of the study were therefore to characterise the haemolymph components of both male and female ticks at different days of feeding and to determine the best to use for immunisation depending on the ease of harvesting of the haemolymph and its complexity. The study will also assessthe efficacy of haemolymph components in the control of *R.appendiculatus*.

Since immunisation against any parasite may only be of practical use if the immunised animal remains more resistant to the parasite during subsequent infestations than the non immunised ones, the experimental animals were reinfested with all instars of parasite free *R.appendiculatus* ticks and their levels of resistance compared.

4.3 Materials and methods.

4.3.1 Experimental animals.

Tick naive rabbits similarly maintained as those described earlier in section 2.3.1 and all instars of *R.appendiculatus* reared as previously described in section 2.3.2 were used for these experiments.

4.3.2 Harvesting and characterization of tick haemolymph.

Haemolymph was harvested as described in chapter two section 2.3.10 from batches of male and female *R. appendiculatus* ticks partially fed on tick naive rabbits for 3-5 days.

Haemolymph was characterised to assess for the presence of sex specific proteins using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemli (1970). Briefly SDS-PAGE was done using a stacking gel containing 1% acrylamide in stacking buffer (0.5M Tris-Hcl pH 6.8). The samples were run in a gradient gel of 5 - 20% acrylamide

concentration in running buffer (1.5M Tris-Hcl pH 8.8 containing 0.1% SDS (Maizel, 1971). Ammonium persulfate solution was used to initiate polymerisation and TEMED (N,N,N'-N'-tetramethylethylnediamine) used as a catalyst. The gels were run using an LKB power pack at 20mA until the samples passed the stacking gel and reached the running gel. Thereafter the samples were run at 30mA to completion. The gels were stained using Coommassie brilliant blue (Weeke, 1973) and the silver staining technique (Merril *et al.*, 1981).

4.3.4 Immunisation of the experimental rabbits.

Experimental rabbits were randomly divided into four groups of five rabbits each. Each group was inoculated intramuscularly with one mg of either haemolymph proteins, solubilised tick midgut proteins (STGMP), a mixture of 50% haemolymph and 50% STGMP or buffer. The protein concentration of the antigens, the adjuvants used and the booster regime were as previously described in sections 2.3.4 and 2.3.5.

4.3.5 Serology.

The experimental animals were bled for serum 2 weeks before immunisation (preimmune serum) and before each booster was given. The serum collected was tested for the presence of precipitating antibodies using the double immunodiffusion technique (Ouchterlony, 1958).

4.3.6 Challenge with ticks.

Two weeks after the last booster dose, all the experimental rabbits were infested with all the instars of *R.appendiculatus*. The rabbits were reinfested with similar ticks one month after all the ticks in the first infestation had dropped. The number of ticks applied, the method of application and the parameters monitored were as previously described in section 3.3.10.

4.3.7 Statistical analysis.

Data was analysed using ANOVA and the general linear model as described in section 3.3 13.

4.4. RESULTS.

4.4.1. Characterisation of tick haemolymph

After running SDS-PAGE on the haemolymph proteins and staining the gels with Coomassie brilliant blue, it was observed that both male and female ticks have specific haemolymph proteins unique to them (Figure 12).

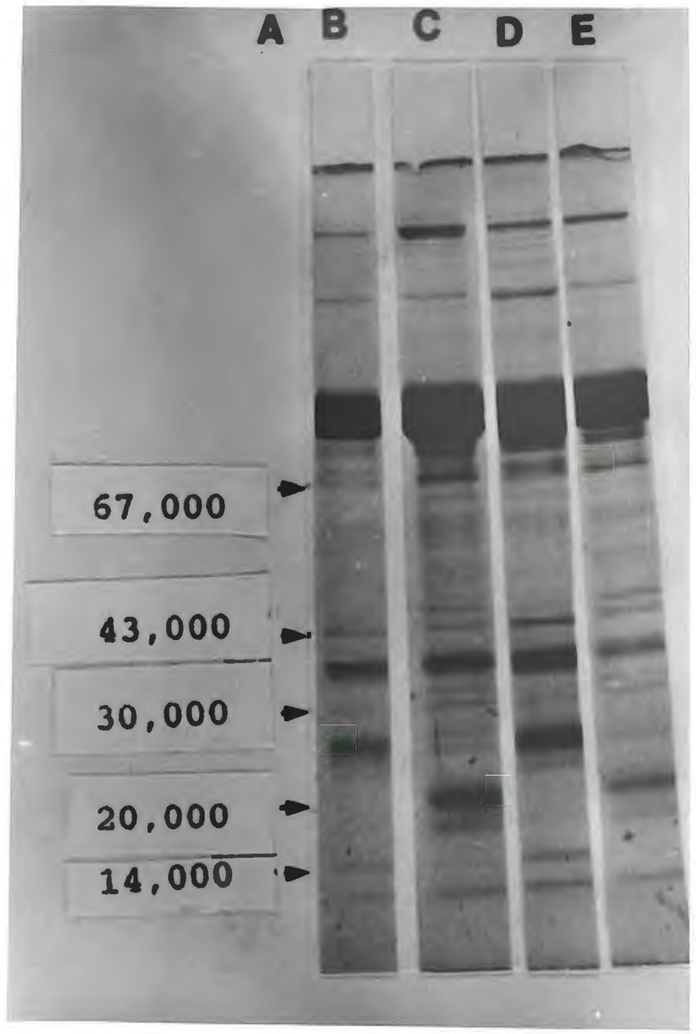

FIGURE 12.
Coommassie brilliant blue stained SDS-PAGE profiles of male and female haemolymph of *R.appendiculatus* at different days of feeding.

The differences between male and female haemolymph protein profiles were also demonstrated using the more sensitive silver staining technique (Figure 13).

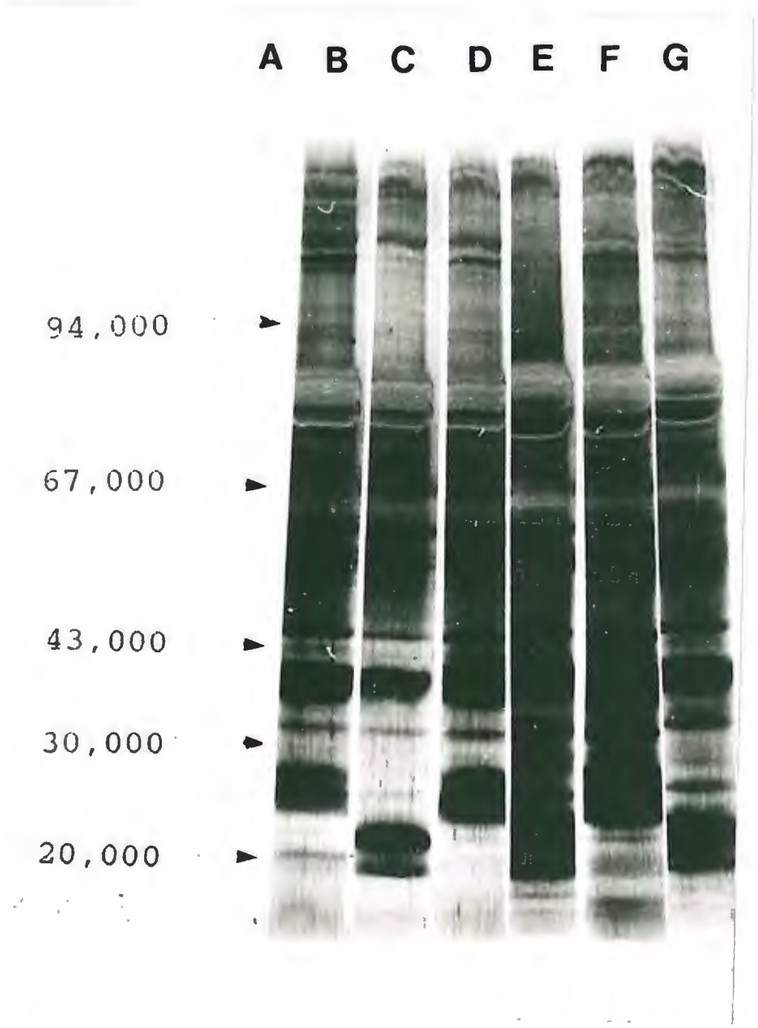

FIGURE 13.

Silver stained SDS-PAGE profiles of male and female haemolymph of *R.appendiculatus* at different days of feeding days of feeding.

4.4.2 Serology.

All the immunised rabbits raised precipitating antibodies against the antigens they were immunised with. Rabbits immunised with buffer did not raise any antibodies.

4.4.3 Results obtained during the first Infestation.

The results obtained after infesting larvae on the experimental animals are presented in Table 18. The larvae that were infested on rabbits immunised with either haemolymph components alone or in combination with STGMP had significantly shorter feeding durations than those that were infested on controls. The engorgement weights, the percentage that fed successfully to engorgement and the percentage that moulted were not significantly different between those larvae that fed on immunised rabbits and those that fed on controls.

Table 18.

The feeding performance and moulting ability of *R.appendiculatus* larvae exposed to immunised rabbits.

Biological parameter	Immunogens used.			
	Haemolymph	Haemolymph +STGMP	STGMP	Control
Feeding duration (days, Mean ± SE)	4.06B (±0.14)	4.16B (±0.06)	4.75A (±0.03)	4.62A (±0.03)
Engorgement weight (mg, Mean ± SE)	0.48A (±0.03)	0.44A (±0.01)	0.46A (±0.02)	0.49A (±0.03)
Percentage feeding (%, Mean ± SE)	53A (±3.39)	53.67A (±13.48)	45.75A (±6.42)	48.4A (±10.05)
Percentage moulting (%, Mean ± SE)	35.84A (±9.73)	56.7A (±12.57)	47.32A (±12.54)	65.16A (±9.58)

Means followed by the same letter in rows are not significantly different at the 5% level.

The means are of 5 rabbits per immunogen challenged with 100 *R.appendiculatus* larvae.

4..3.1 Results obtained using nymphs.

The results obtained after infesting nymphs on immunised and non immunised rabbits (controls) are summarised in Table 19. There was no significant difference in the feeding duration of the nymphs that were infested on rabbits immunised with STGMP and those that

were infested on controls. However, the feeding duration was reduced by 20.41% and 12.17% for nymphs that were infested on rabbits immunised with haemolymph components alone and a combination of haemolymph components and STGMP respectively. The engorgement weight of the nymphs that fed on immunised rabbits and those that fed on the controls were not significantly different. The percentage of nymphs that fed successfully to engorgement were reduced by 31.65% in ticks that were fed on rabbits immunised with STGMP alone but were not significantly different between the ticks that were fed on rabbits immunised with either haemolymph components alone or in combination with STGMP. The percentage of nymphs that moulted were not significantly different between the ticks that fed on rabbits immunised with either haemolymph components alone or in combination with STGMP.

Table 19.

The feeding performance and moulting ability of nymphal *R.appendiculatus* nymphs exposed to immunised rabbits.

Biological parameter	Immunogens used.			
	Haemolymph	Haemolymph +STGMP	STGMP	Control
Feeding duration (days, Mean ± SE)	4.25C (±0.07)	4.69**B** (±0.08)	5.74**A** (±0.09)	5.34**A** (±0.06)
Engorgement weight (mg, Mean ± SE)	9.48**A** (±0.26)	9.5**A** (±0.24)	9.1**A** (±0.19)	9.7**A** (±0.16)
Percentage feeding (%, Mean ± SE)	77**A,B** (±9.00)	80.67**A** (±6.96)	63.5**B** (±6.18)	83.6**A** (±2.38)
Percentage moulting (%, Mean ± SE)	97.29**A** (±1.73)	93.76**A** (±3.52)	96.39**A** (±1.76)	97.62**A** (±2.38)

Means followed by the same letter in rows are not significantly different at the 5% level.
The means are of 5 rabbits per immunogen challenged with 50 *R.appendiculatus* nymphs.

4.4.3.1 Results obtained using adults.

The results obtained after feeding adult ticks on immunised and non immunised rabbits are summarised in Tables 20a and b. The feeding duration of the ticks that were infested on immunised rabbits was shorter by 7.18%, 4.49% and 2.56% for the adults that were infested on

rabbits immunised with haemolymph components, a mixture of haemolymph components and STGMP and STGMP respectively.

The engorgement weights of the ticks that fed on immunised rabbits were significantly lower than those of the ticks that were fed on controls. The percent reduction in engorgement weights was 33.02, 24.37 and 23.89 for rabbits immunised with haemolymph components, a mixture of haemolymph components and STGMP and STGMP respectively. The engorgement weights of the ticks that fed on rabbits immunised with haemolymph components and those that fed on rabbits immunised with STGMP were not significantly different. The weight of the eggs oviposited (eggmass) by the ticks that fed on immunised rabbits were significantly lower than those of the ticks that were fed on controls. The percent reduction in the egg masses oviposited was 34.62, 28.60 and 25.10 for rabbits immunised with a mixture of haemolymph components, STGMP and haemolymph components respectively. The egg masses oviposited by the ticks that were fed on rabbits immunised with haemolymph components and those that were infested on rabbits immunised with STGMP were not significantly different.

The egg conversion ratio (ECR) was not significantly different in ticks that were fed on control rabbits and those that were fed on rabbits immunised with either haemolymph components or STGMP. However, the ECR of the ticks that were fed on rabbits immunised with STGMP was reduced by 7.38%.

The hatchability of the oviposited eggs was not significantly different in ticks that fed on control rabbits and those that fed on rabbits immunised with either a mixture of haemolymph components and STGMP or haemolymph components alone. However, ticks that were fed on rabbits immunised with STGMP oviposited eggs with 13.14% lower hatchabilities than those that fed on rabbits immunised with haemolymph components.

The percentage of the ticks that fed successfully to engorgement and the percentage that oviposited were not significantly different ($p > 0.05$) between ticks that were fed on immunised rabbits and those that were fed on the controls.

The number of ticks that attained the critical engorgement weight was lower in ticks that were fed on immunised rabbits than those that were fed on controls. The percent reduction in the number of ticks attaining the critical engorgement weight was 69.52, 42.33 and 12.74 for rabbits immunised with a mixture of haemolymph components and STGMP, STGMP and haemolymph components respectively.

Table 20a

The feeding performance of adult *R.appendiculatus* exposed to immunised rabbits.

Biological parameters	Immunogens used.			
	Haemolymph	Haemolymph + STGMP	STGMP	Control
Feeding duration (days, Mean ± SE)	7.24B (±0.07)	7.45B (±0.08)	7.60A (±0.11)	7.80A (±0.11)
Engorgement weight (mg, (Mean ± SE)	341.3B (±9.5)	302.3C (±8.7)	343.5B (±11.4)	451.3A (±8.1)
Percentage feeding (% , Mean ± SE)	91.67A (±8.24)	93.33A (±5.43)	90A (±4.91)	84A (±6.27)
Percentage attaining CEW (%, Mean ± SE)	82.34B (±6.84)	56.76C (±3.68)	65.22C (±9.36)	92.83A (±2.78)

Means followed by the same letter in rows are not significantly different at the 5% level.

The means are of 5 rabbits per immunogen challenged with 60 (30 males, 30 females) *R.appendiculatus* adults. All parameters assessed were done using female ticks.

Table 20b.

The fecundity of adult *R.appendiculatus* exposed to immunised rabbits.

Biological parameters	Immunogens used.			
	Haemolymph	Haemolymph + STGMP	STGMP	Control
Egg mass (mg, Mean ± SE)	173.1**B** (±7.5)	151.1**C** (±5.6)	165**BC** (±6.6)	231.1**A** (±5.8)
Egg conversion ratio (Mean ± SE)	51.69**B,A** (±1.37)	48.79**A** (±1.12)	49.59**B** (±1.25)	52.39**A** (±0.99)
Percentage hatchability (%, Mean ± SE)	68.15**A** (±3.37)	64.44**A,B** (±3.43)	57.32**B** (±3.34)	64.85**A,B** (±2.86)
Percentage ovipositing (%, Mean ± SE)	92.47**A** (±1.76)	86.90**A** (±3.38)	88.86**A** (±5.77)	85.22**A** (±7.67)

Means followed by the same letter in rows are not significantly different at the 5% level.

The means are of 5 rabbits per immunogen challenged with 60 (30 males, 30 females) *R.appendiculatus* adults. All parameters assessed were done using female ticks.

4.4.4 Results obtained after reinfestation.

The results obtained after reinfesting larvae on immunised and control rabbits are summarised in Table 21. The larvae that were reinfested on immunised rabbits had significantly shorter feeding durations than those that were fed on controls. The engorgement weights were not significantly different between those larvae that fed on immunised rabbits and those that fed on controls. The percentage of the larvae that fed successfully to engorgement were only significantly reduced in the larvae that fed on rabbits immunised with STGMP. The immunisation had no significant effect on the moulting percentages.

Table 21.

The feeding performance and the moulting ability of *R.appendiculatus* larvae that were reinfested on immunised rabbits.

Biological parameter	Immunogens used.			
	Haemolymph	Haemolymph + STGMP	STGMP	Control
Feeding duration (days, Mean ± SE)	4.26**B** (±0.04)	4.16**B** (±0.04)	4.75**A** (±0.12)	4.62**A** (±0.04)
Engorgement weight (mg, Mean ± SE)	0.44**B** (±0.26)	0.42**B** (±0.24)	0.34**A** (±0.19)	0.39**A** (±0.16)
Percentage feeding (%, Mean ± SE)	37.50**A** (±9.00)	29.67**A** (±6.96)	9**B** (±6.18)	22.4**A** (±2.38)
Percentage moulting (%, Mean ± SE)	61.79**A** (±15.64)	82.48**B** (±3.02)	46.87**A** (±13.96)	67.78**A** (±4.24)

Means followed by the same letter in rows are not significantly different at the 5% level.
The means are of 5 rabbits per immunogen challenged with 100 *R.appendiculatus* larvae.

4.4.4.2 Results obtained using nymphs.

The results obtained after reinfesting nymphs on immunised and control rabbits are shown in Table 22. The feeding duration, the percentage of nymphs that fed successfully to engorgement and the percentage of nymphs that moulted were not significantly different between nymphs that were fed on immunised rabbits and those that were fed on controls.

The engorgement weights of the nymphs that were fed on immunised rabbits were significantly lower than those of the nymphs that were fed on controls. The percent reduction in engorgement weight was 25.43, 12.72 and 6.47 for nymphs that were fed on rabbits immunised with a combination of haemolymph components and STGMP, STGMP alone and haemolymph components alone respectively.

Table 22.

The feeding performance and the moulting ability of *R.appendiculatus* nymphs exposed to immunised rabbits.

Biological parameter	Immunogens used.			
	Haemolymph	Haemolymph + STGMP	STGMP	Control
Feeding duration (days, Mean ± SE)	5.71**A** (±0.17)	5.63**A** (±0.08)	5.74**A** (±0.12)	5.34**A** (±0.11)
Engorgement weight (mg, Mean ± SE)	4.34**B** (±0.31)	3.46**C** (±0.22)	4.05**B** (±0.18)	4.64**A** (±0.14)
Percentage feeding (%, Mean ± SE)	52**A** (± 12)	56.67**A** (±8.67)	44**A** (±8.98)	46.8**A** (±10.61)
Percentage moulting (%, Mean ± SE)	85.59**A** (±8.17)	85.89**A** (±2.77)	92.39**B** (±2.76)	86.1**A** (±2.44)

Means followed by the same letter in rows are not significantly different at the 5% level.

The means are of 5 rabbits per immunogen challenged with 50 *R.appendiculatus* nymphs.

4.4.4.2 Results obtained using adults.

Tables 23 a and b show the results obtained after reinfesting the immunised and the control rabbits with adult ticks. The duration taken by the ticks to feed to engorgement while feeding on the control and on the immunised rabbits was not significantly different. Adults that fed on immunised rabbits had significantly lower engorgement weights than those that fed on controls. The percent reduction in engorgement weight was 78.84, 73.80 and 49.45 for rabbits immunised with a mixture of haemolymph components and STGMP, haemolymph components and STGMP respectively.

The weights of the eggs oviposited by the ticks that were fed on immunised rabbits were significantly lower than those from the ticks that were fed on controls. The percent reduction in the weight of the egg masses oviposited was 79.25, 58.37 and 44.42 for rabbits immunised with a mixture of haemolymph components and STGMP, haemolymph components and STGMP respectively. The weight of the eggs oviposited by the ticks that fed on rabbits immunised with either haemolymph components or STGMP were not significantly different.

The ECR and the hatchabilities of the oviposited eggs were not significantly different in ticks that fed on immunised rabbits and those that fed on the controls. The percentage of the ticks that fed successfully to engorgement, the percentage of those that oviposited and the percentage of those ticks that attained the critical engorgement weight was significantly different in ticks that were fed on immunised rabbits and those that were fed on controls. However, for these three parameters there was no significant difference in ticks that were fed on immunised rabbits irrespective of the immunogen(s) used.

Table 23a.

The feeding performance of adult *R.appendiculatus* that were reinfested on immunised rabbits.

Biological parameter	Immunogens used.			
	Haemolymph	Haemolymph + STGMP	STGMP	Control
Feeding duration (days, Mean ± SE)	7.25**A** (±0.12)	7.12**A** (±0.12)	7.73**A** (±0.12)	7.76**A** (±0.11)
Engorgement weight (mg, Mean ± SE)	32.34**C** (±5.3)	28.59**C** (±3.1)	62.4**B** (±6.3)	123.43**A** (±8.1)
Percentage feeding (%, Mean ± SE)	40**B** (±8.24)	55.55**B** (±5.43)	5917**B** (±4.91)	91.33**A** (±6.27)
Percentage attaining CEW (%, Mean ± SE)	OB	OB	1.39**B** (±1.39)	6.57**A** (±3.4)

Means followed by the same letter in rows are not significantly different at the 5% level.

The means are of 5 rabbits per immunogen challenged with 60 (30 males, 30 females) *R.appendiculatus* adults. All parameters assessed were done using female ticks.

Table 23b.

The feeding performance of adult R.appendiculatus that were reinfested on immunised rabbits.

Biological parameter	Immunogens used.			
	Haemolymph	Haemolymph + STGMP	STGMP	Control
Egg mass (mg, Mean ± SE)	12.24B (±7.5)	6.1C (±5.6)	16.34B (±6.6)	29.4A (±5.8)
Egg conversion ratio (Mean ± SE)	48.90A (±4.96)	46.52A (±3.74)	44.04A (±4.16)	49.24A (±6.76)
Percentage hatchability (%, Mean ± SE)	72A (±4.42)	69.33A (±3.83)	54.42A (±5.25)	61.13A (±2.98)
Percentage ovipositing (%, Mean ± SE)	55.79B (±1.76)	31.35B (±3.38)	50.26B (±5.77)	76.86A (±7.67)

Means followed by the same letter in rows are not significantly different at the 5% level.

The means are of 5 rabbits per immunogen challenged with 60 (30 males, 30 females) *R.appendiculatus* adults. All parameters assessed were done using female ticks.

4.5 Discussion.

The haemolymph of several Ixodid and Argasid ticks have been characterised by SDS-PAGE (Dolp and Hamdy, 1971; Tatchell, 1971; Araman, 1979). While working with *H.excavatum*, *H.dromedarii* (ixodid ticks) and *Argus persicus* and *A.arboreus* (argasid ticks), Dolp and Hamdy (1971) found that haemolymph protein patterns were more complex in the ixodid ticks than in the argasid ticks. However, on the basis of electophoretic mobilities haemolymph proteins of *D.variabilis* and *A.americanum* (Ben-Yakir, 1985), *B.microplus* (Tatchell, 1971) and *R. sanguineus* (Araman, 1979) were found to be similar. The findings reported so far were recorded using female ticks. This is therefore the first study on the electrophoretic mobilities of haemolymph from *R.appendiculatus* and from any male tick. This study shows that haemolymph components are sex specific. Whereas we would expect vitellins in females and not males, it was observed that males also have proteins unique to them. However, the identity of these proteins was not established. Based on these results it was therefore

important to immunise rabbits against *R.appendiculatus* using haemolymph harvested from both male and female ticks.

Resistance to all instars of *R.appendiculatus* was induced by immunising rabbits with the aforesaid haemolymph components alone and in combination with STGMP and by using STGMP alone. Whereas resistance to ticks has been induced by immunising their hosts with various tick antigens (Mongi, 1982; Wikel, 1988; Willadsen, 1988), few immunisations have been done using antigens derived from tick haemolymph. Indeed this is the first report on induction of resistance in rabbits to *R.appendiculatus* by immunising them with whole haemolymph components harvested from this tick. Attempts to induce resistance to *Dermacentar variabilis* and *Amblyomma americanum* by immunising rabbits with the acellular fraction of haemolymph harvested from these ticks was unsuccessful (Ben-Yakir and Barker, 1987). This may be explained by the fact that the ticks which haemolymph was harvested from were fed on sheep and since host serum proteins can cross the midgut barrier (Ackerman *et al.*, 1981) these workers may have immunised their experimental rabbits with alot of sheep protein. The protective antigens may be occurring in the cellular fraction they discarded and not the acellular fraction they used. Alternatively, their results may be explained in terms of differences between different tick species since resistance to *A. variegatum* after repeated infestations in sheep did not occur (Jongejan *et al.*, 1989; Norval *et al.*, 1988a) but resistance to *R.appendiculatus* in sheep was readily achieved after they were repeatedly exposed to this tick (Wishitemi, 1983).

The resistance induced was more pronounced in adult ticks with only little effects on the immature stages. This observation is in agreement with previous reports that resistance to ticks as a result of immunisation using antigens that are not normally exposed to hosts during feeding (concealed antigens) unlike naturally acquired immunity, is directed towards adults and only little effects are observed on the immature stages (Willadsen, 1988; Willadsen and Kemp, 1988). This may have been observed since the experimental animals were prevented from grooming and it has been reported that resistance to the immature stages is mainly due to

hypersensitivity reactions that result in grooming and physical removal of the ticks (Snowball, 1956; Bennett, 1969). In addition the concealed antigens used were derived from adults and may therefore be more protective against adults than to the immature stages. Furthermore, unlike reports that anti-STGMP antibodies facilitate passage of IgG across the midgut barrier of adult ticks, (Mbogo *et al.*, 1992) no such work has been carried out with the immature stages. Reports that larval membrane antigens can protect against adult *B.microplus* ticks (Wong and Opdebeeck, 1990) suggest that larvae and adults may be sharing some common antigens that are important in acquisition of resistance. It must be pointed out however, that, *B.microplus* is a one host tick and effects seen on larvae can be passed on readily to adults.

The effects on larvae and nymphs were manifested by reduction in the engorgement weights. This may be significant in the long run since small larvae and nymphs moult into small nymphs and adults respectively (Chiera *et al.*. 1985a) and their survival is positively related to their size (Chiera *et al.*, 1985a,b). The maximum engorgement weight that a fully fed female can attain depends on its size before feeding (Diehl *et al.*, 1982). Since small nymphs moult into small adults and small adults have reduced fecundity (Diehl *et al.*, 1982) this finding is of great practical importance.

Resistance to adults was manifested by a reduction in the number of ticks that fed successfully to engorgement, reduced feeding durations, reduced engorgement weights and reduced fecundity. Reduction in the feeding duration is important in disease transmission since ticks must feed for a minimum number of days before they can transmit disease. For example, *R.appendiculatus* ticks must feed for a minimum of 3-5 days before they can transmit *Theileria* sporozoites to mammalian hosts (Young *et al.*, 1975; Purnell *et al.*, 1973). The feeding duration was slightly reduced in all the instars that fed on immunised rabbits during the primary infestation. Indeed, it was reduced by as much as 20.41% in the nymphs that were fed on rabbits immunised with haemolymph components. Unfortunately, except for the larvae, the feeding

duration during the subsequent infestation was not significantly different between the ticks that were fed on immunised rabbits and those that were fed on controls.

The engorgement weights and the number of ticks that attained the critical engorgement weight among the females that were fed on immunised rabbits were reduced during the primary infestation and even more drastically during the subsequent infestation. Consequently, ticks that were fed on immunised rabbits oviposited fewer eggs than those that were fed on controls during the primary and more so during the subsequent infestation. Indeed, during the subsequent infestation the weights of the eggs oviposited were reduced by as much as 79.25%, a very drastic reduction.

The egg conversion ratio and the hatchabilities were similar in ticks that were fed on the controls and those that were fed on rabbits immunised with either haemolymph components alone or in combination with STGMP. However, the ticks that were fed on rabbits immunised with STGMP had lower egg conversion ratios and hatchabilities than the controls. Similar findings have been reported when ticks are fed on hosts immunised with antigens derived from the midgut (Essuman *et al.*, 1991).

Unlike most immunisations which do not improve on the resistance acquired through natural infestation (Willadsen, 1980), this immunisation improved on naturally acquired resistance since it was maintained in subsequent infestations. Infact the degree of resistance during subsequent infestations was more pronounced than during the primary infestation making the practical use of these immunogens for immunisation more feasible. This increase in the degree of resistance acquired may be explained by the fact that passage of antibodies across

the midgut improves during subsequent infestations (Brossard and Rais, 1974; Tracey-Patte *et al.*, 1987). This is a very significant finding since any immunisation against ticks may only be useful if the immunised hosts remain more resistant than those acquiring resistance as a result of repeated natural infestations.

The highest degree of resistance was recorded in ticks that were fed on rabbits immunised with a combination of haemolymph components and STGMP. This may be due to additive, but independent effects or STGMP may have facilitated the passage of anti-haemolymph antibodies across the gut into the haemolymph thereby improving on the overall effect of immunisation.

In summary, characterisation of haemolymph from *R. appendiculatus* indicated clearly that the ticks had sex specific proteins. Rabbits were rendered resistant to all instars of *R.appendiculatus* by immunising them with haemolymph components alone and in combination with STGMP. Resistance was more pronounced in adults where the number of ticks that were able to feed successfully to engorgement was drastically reduced. In addition the ticks that fed had reduced feeding durations, reduced engorgement weights and reduced fecundities. In larvae and nymphs, ticks that fed on immunised rabbits had reduced feeding durations during the primary infestation and reduced engorgement weights during subsequent infestations.

CHAPTER FIVE.

General discussion and conclusions.

These studies have demonstrated that passage of antibodies across the midgut of *R.appendiculatus* occurs and can be facilitated by feeding the ticks on anti-STGMP antibodies. Indeed this is the first report on the passage of antibodies across the midgut of this particular tick. These antibodies retained their biological activity and specificity, an observation that was in agreement with those of other researchers (Ackerman *et al.*, 1981, Mbogo *et al.*, 1992).

Better results were obtained by injecting ticks with antibodies than on feeding them on blood containing these antibodies (section 3.4.9). This may have been due to the presence of more antibodies in the haemolymph. A lot of work needs to be done to increase the passage of antibodies across the midgut. Some studies that need to be undertaken may include identifying and purifying the midgut antigens that are responsible for the increased passage of antibodies across the midgut. Attempts to purify these antigens by gel filtration chromatography have been undertaken (Essuman *et al.*, 1991). However, these workers were undertaking these purification studies to improve the abilities of these antigens in protecting cattle against *R.appendiculatus* and did not study the effect(s) that these purified antigens may have on the passage of antibodies across the midgut barrier. These antigens may also be identified and purified by determining their immunogenic components using immunoblotting techniques (Gershoni and Palade, 1983), transferring them onto nitro-cellulose paper (Towbin *et al.*, 1979), eluting them using dimethylsulphoxide and using specific fractions for immunisation (Knudsen, 1985).

Deleterious effects were observed after infesting ticks on rabbits immunised with conjugated beta-ecdysone. These effects were observed on the larvae, nymphs and adults that resulted from either the eggs oviposited by these ticks, or moulted from the larvae and nymphs that had been infested on these immunised rabbits.

Most of the reports on the immunisation of hosts against ticks where very good results have been achieved have utilised *B.microplus*, a one host tick (Kemp *et al.*, 1986). For the results obtained by researchers working on 3 host ticks to be comparable, these workers should infest larvae on hosts and observe them for any anti-tick effects. After the larvae moult, the nymphs should be attached onto the same host and the same process repeated until all the instars have fed to ensure they all feed on the same host. The studies reported in this thesis show that deleterious effects of the immunisation can be passed on to the resultant instars. Since these effects were observed using tick naive rabbits, even better results would have been observed had these instars been infested on the same host as their parents because rabbits can acquire natural resistance to *R.appendiculatus* after a single infestation. After reinfesting immunised rabbits with ticks, it was observed that the rabbits maintained their immunity and were indeed even more resistant to infestation using ticks that had been maintained on susceptible rabbits. These observations indicate that were all the instars of the tick allowed to feed on the same host, more dramatic results would probably have been obtained.

Immature stages of ticks have been shown to stimulate hypersensitivity reactions (Riek, 1956; 1962) which result in increased dermal irritation (Essuman *et al.*, 1991) and grooming. Grooming has been shown to reduce tick burdens significantly (Bennette, 1969). In these studies as well and those by other workers (Mongi *et al.*, 1986 a,b; Maranga, 1988; Essuman *et al.*, 1991), ticks were placed in well secured ear bags and the hosts prevented from grooming. This meant that the hosts could not groom themselves and could therefore not remove the ticks. Therefore to get more accurate results, hosts should not be prevented from grooming and procedures that will allow for this should be developed.

Deleterious effects to ticks were observed after injecting them with anti-beta-ecdysone antibodies. From this study, it appears that potential immunogens can be evaluated by injecting them directly into the haemolymph of ticks. This will be cheaper and may give better results because there will be no variations between different hosts. Where injections may be difficult to

give such as when using immature stages, feeding the ticks on artificial membranes may be useful (Wallade *et al.*, 1979).

Lastly, it must be remembered that the greatest economic importance ticks have is their ability to transmit diseases. It is therefore extremely important to study the effects any successful immunisation may have on disease transmission - an area that has been pathetically ignored.

CHAPTER SIX.

Suggested research priorities for the future.

The ability of host IgG to cross the midgut barrier of *R.appendiculatus* was discussed in Chapter 2 of this thesis. However the study undertaken did not indicate which class of IgG was crossing the midgut barrier. In addition, no work has been done to indicate whether other immunoglobulins like IgA, IgM. or IgE cross the midgut barrier. Since different antibody classes and indeed even IgG subclasses have different functions (Goodman, 1982) their ability to cross the midgut barrier into the haemolymph should be studied in detail. The finding that IgG1 antibodies were the subclass responsible for the ability to transfer cutaneous basophil associated immune resistance against *A.americunum* in guinea pigs (Brown et al., 1982) stresses further the importance of undertaking these studies.

Destruction of the midgut using anti - STGMP antibodies was shown to facilitate passage of IgG across the midgut barrier (Chapter 2, Mbogo *et al.*, 1992). Identification and purification of the actual STGMP antigens causing increased passage of antibodies across the midgut was not done. This needs to be done because these antigens maybe used as secondary antigens to facilitate passage of primary antigens targeted at tick internal organs or haemolymph components.

The possibility of immunologically blocking the effects of beta-ecdysone was discussed in chapter three. The effects were not as pronounced as expected. This may have been due to compensatory mechanisms allowing the ticks to make more antibodies after the hormone titres were reduced by neutralisation with the anti-beta-ecdysone antibodies. To-date, the organ responsible for production of beta-ecdysone in ticks has not been identified (Solomon *et al.*, 1982). This organ needs to be identified. After identification, attempts should be made to immunologically destroy it by immunising hosts against ticks using it. This approach has been utilised to immunise rabbits against haematophagus flies (Schlein and Lewis, 1976).

In this report, the effects of the immunisation were more pronounced in adults than in the immature stages. This may have been due to the ticks being in well protected ear bags which did not allow the host to scratch them off. As explained earlier, gromming is the method used by hosts to reduce their larval and nymphal tick burdens. In future, the parameters recorded to assess induction of resistance to ticks need to be changed for larvae and nymphs. Daily tick counts may be used to indicate whether resistant hosts were able to scratch their ticks off. Indeed this maybe a very important parameter since it simulates the natural system.

Haemolymph components were shown to protect rabbits against infestation with *R.appendiculatus* in the primary and successive infestations. For the practical use of immunisation against ticks, it would be important to know the duration of resistance offered by a single immunisation in order to plan how often the immunisation should be undertaken.

As already discussed, purified antigens preparations have been shown to offer better protection than crude ones. There is therefore need to purify the haemolymph components to improve their effectiveness.

Maybe the single most important need for controlling ticks is their ability to transmit diseases. Studies should be undertaken to determine the effects of immunisation against ticks on their ability to transmit diseases. These studies may include a determination of the lowest volume of infected blood a tick needs to have to transmit a disease. In addition, since it is well known that parasites (especially protozoan) multiply in particular cells of the midgut and salivary glands, studies should be undertaken to destroy these cells immunologically. This may prevent parasite development and therefore block disease transmission.

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APPENDIX ONE.

1.1.0 Buffers and reagents used.

1.1.1 Phosphate buffered saline (PBS).

To make 4 litres, mix the folllowing ingredients

a) 24.8 gm sodium phosphate (Diabasic)

b) 31.2 gm sodium phosphate (Monobasic)

c) 35 gm Sodium Chloride

d) 0.8 gm Sodium azide (optional)

The PH is adjusted using either Hydrochloric acid or Sodium Hydroxide.

1.1.2 Sodium docedyl Sulphate—Polyacrylamide gel Electrophoresis Buffers.

1.1.2.1 Running gel buffer. This is 1.5M Tris (hydroxymethyl) methylamine PH 8.8

1.1.2.2 Stacking gel buffer. This is 0.5M Tris (hydroxymethyl) methylamine PH 6.8

1.1.2.3 Tank buffer. To make 4 litres , mix the following ingredients.

a) 12.1 gm Tris (hydroxymethyl) methylamine (25 mm Tris-base)

b) 57.6 gm Glycine (192 mm Glysine)

c) 40 ml of 10% SDS (0.1% SDS)

The PH should be 8.3.

1.1.2.4 Sample buffer. This is mixed with the samples to be run in the gel. It is composed of 0.13 M Tris (hydroxymethyl) methylamine, 4% SDS, 20% glycerol, 1% Mercaptoethanol and 0.002% Bromophenol blue

1.1.3 Staining and Destaining solutions.

1.1.3.1 Coomassie brilliant blue stain.

To make up the solution, mix 2.5 gm Coomassie brilliant blue with, 450 ml methanol, 100 ml acetic acid and 450 ml distilled water.

1.1.3.2 Silver staining reagents.

1.1.3.2.1 Fixative. This is 40% methanol mixed with 10% acetic acid

1.1.3.2.2 Oxidiser. This is 1 gm per litre of Potassium dichromate containing 0.2 ml of 80% nitric acid.

1.1.3.2.3 Silver reagent. This is made by dissolving 2 gm of silver nitrate in a litre of double distilled water.

1.1.3.2.4 Developer. This is made by dissolving Sodium bicarbonate in a litre of double distilled water containing 0.5 ml of 37% (v/v) formalin per litre.

1.1.3.2.5 Stop bath. This is 5% acetic acid in double distilled water.

1.1.3.4 Destaining solution for Coomassie brilliant blue stained gels.

This is made up of 20% methanol and 10% acetic acid.

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APPENDIX TWO

GENERAL METHODOLOGIES.

2.1. Determination of protein concentration..

Known quantities of Bovine serum albumin (BSA) were used to make standard curves from which the concentration of the other proteins was determined. Dilutions of BSA standards containing 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2.0 mg per ml in PBS were prepared. The test samples were diluted in PBS to make 1:10, 1:50, 1:100 and 1:200 dilutions. One hundred microlitres of each of the BSA samples and of each of the dilutions of the test samples were placed in clean test tubes. Five microlitres of the Bio-rad reagent dye diluted 1:4 (v/v) in PBS was added to each test tube. The solutions were then mixed, kept at room temperature for 30 minutes and their optical densities read at 595 nm (OD 595). A graph of the optical density versus BSA concentration was drawn and used to estimate the protein concentrations of the test samples.

2.2. Emulsification of antigens with Freund's adjuvants.

Complete Friend's adjuvant was mixed vigorously to re suspend mycobacteria before use. After that the adjuvants (either complete or incomplete) were mixed with the antigens at a ration of 2:1 in a beaker. The antigen and the adjuvant were mixed thoroughly by drawing up the mixture in a 20 ml syringe and expelling it back into the beaker. This was repeated several times until the emulsion became thick creamy and difficult to draw-up. To test whether the antigens were well emulsified, a few drops of the mixture were allowed to fall into a beaker of cold water. Inability of the drop to disperse over the surface of the water indicated successful emulsification. The entire preparation was carried out over ice.

2.3. Preparation of DE 51 (Pre swollen DEAE Cellulose) for IgG purification.

This preparation can be undertaken using the 6 steps included below

a) Stir the DE 51 with 0.25M phosphate buffer for 2-3 minutes at room temperature (mix 10g DE 51 with 60 ml buffer)

b) Decant the fines

c) Adjust PH to 8 using either hydrochloric acid or sodium hydroxide.

Note : PH is very critical and MUST be 8.

d) Decant the fines

e) Wash three times with distilled water each time decanting the fines.

f) Add initial buffer (0.005M phosphate buffer PH 8) and adjust PH to 8.

The above preparation is then poured into a column and used for IgG purification

2.4. Running of a SDS-PAGE 5-20% gradient gel.

To run the above gel, you prepare a running gel by adding the ingredients indicated below in the amounts shown.

		Solution A (5%)	Solution B (20%)
a)	30% Acrylaride	2.5 ml	10ml
b)	1% Bis-acrylamide	3.9 ml	1 ml
c)	Running buffer	3.75 ml	3.75 ml
d)	Distilled water	4.65 ml	0.05 ml
e)	10% Ammonium per sulphate	0.05 ml	0.05 ml
f)	10% SDS	150 Microlitres	150 microlitres
g)	TEMED	10 Microlitres	10 Microlitres.

Degas the solution for 5 minutes before adding SDS and temed (Temed is a catalyst and MUST be added last). Put solution B in the inside section of the gradient former and solution A in the outside section. Mix the 2 and pour your gel between previously prepared glass plates.

Add distilled water (or butanol) on top of the running gel to ensure that a uniform line is maintained and leave it at room temperature for 1 hour. Then pour out the water (or butanol) and add the stacking gel. The following ingredients are mixed to make the stacking gel

a)	30% Acrylaride	1 ml
b)	1% Bis-acrylamide	1 ml
c)	Stacking gel buffer	2.5 ml
d)	Distilled water	5.35 ml
e)	10% Ammonium per sulphate	0.05 ml
f)	10% SDS	0.1 ml
g)	TEMED	0.005 ml

Pour the stacking gel on top of the running gel immediately after preparing it. Place a comb immediately after and leave it at room temperature for 1 hour. Remove the comb, add your samples and run the gel.

2.5. Silver staining technique.

The following steps are followed when staining a gel using the silver staining technique.

a) Fix the gel in the fixative for 1 hour

b) Rehydrate the gel in 10% ethanol/5% acetic acid for 30 minutes.

c) Repeat by using a fresh solution 2 times

d) Add 200ml oxidiser and leave for 10 minutes with constant agitation.

e) Remove the oxidiser by sunction

f) Rinse the gel in de ionised distilled water for 10 minutes.

g) Repeat (f) twice using fresh distilled water each time

h) Add 200 ml, silver reagent and leave for 30 minutes with constant agitation

i) Remove silver reagent by sunction

j) Rinse gel with 400 ml double distilled water and suck the water off.

k) Add 200 ml developer and suck it off after 2 minutes.

l) Repeat (k), but leave developer for 5 minutes or until lines appear.

m) Repeat (l) for another 5 minutes with fresh developer.

n) Suck off the developer and stop the reaction by adding 400 ml of 5% acetic acid.

APPENDIX THREE.

SELECTED RAW DATA AND THE STATISTICAL PROGRAMME USED.

3.1 STATISTICAL PROGRAMME USED.

```
TITLE'TEST ON ANTIGENIC EFFECTS';
OPTIONS LS=78 PS=21 NODATE NONUMBER;
DATA TICK1;
INFILE'ECDYSONE.PRN';
INPUT ANTIGEN $ RABBIT $ TICKNO FEEDUR ENGWHT EGGM ECR HATCH;
DROP TICKNO;
PROC SORT;
BY ANTIGEN RABBIT; RUN;
PROC GLM;
CLASS ANTIGEN RABBIT;
MODEL FEEDUR ENGWHT EGGM ECR HATCH=ANTIGEN RABBIT(ANTIGEN);
MEANS ANTIGEN RABBIT(ANTIGEN) /DUNCAN; RUN;
PROC SORT;
BY ANTIGEN; RUN;
PROC MEANS  MEAN STDERR; BY ANTIGEN;
VAR FEEDUR ENGWHT EGGM ECR HATCH;
RUN;
```

NOTE.

TICKNO = TICK NUMBER, FEEDUR = FEEDING DURATION,

EGGM= EGGMASS, ECR = EGG CONVERSION RATIO

d HATCH = PERCENTAGE OF THE EGGS WHICH HATCHED.

3.2 SELECTED RAW DATA.

The data set was very big and would occupy a lot of space if all of it was to be reproduced. The data got using conjugated beta-ecdysone, BSA, a combination of conjugated beta-ecdysone and solublised tick midgut protein membrane proteins (STGMP), STGMP and the controls would occupy five times the space occupied by the data given below. Therefore only a sample of the data collected using one antigen (Conjugated beta-ecdysone (BE). However, the data got using the other antigens was arranged in exactly the same way and analysed using "SAS" statistical package using the programme written above. All the data in a particular experiment was analysed together after arranging it as shown below for all the antigens to determine the effectiveness of the various antigens.

Abbreviations used.

BE ------- Conjugated beta-ecdysone.

AG ------- Antigen used

RAB ------ Rabbit used

T.No ----- Tick number. Ticks were assessed individually for the parameters shown below. They were numbered at the time of assessing their engorgement weight.

EWT-------Engorgement weight of the individual tick

EGGM---- The egg mass (weight of the eggs)oviposited by a single female tick

ECR------- The egg conversion ratio

HATCH—The percentage of ticks that hatched

. A dot was put to indicate missing data. This was to avoid missing data being read as zero.

AG	RAB	T.No	F-D	EWT	EGGM	ECR	HATCH
BE	A1	1	7	0.5332	0.3187	59.8	80
BE	A1	2	7	0.5175	0.2557	49.4	80
BE	A1	3	7	0.5802	0.302	52.1	80

BE	A1	4	7	0.4699	0.2617	55.7	80
BE	A1	5	7	0.5284	0.2871	54.3	80
BE	A1	6	7	0.414	0.1812	43.8	80
BE	A1	7	7	0.5304	0.2399	45.2	60
BE	A1	8	7	0.4383	0.2432	55.5	80
BE	A1	9	7	0.4682	0.2801	59.8	60
BE	A1	10	7	0.3793	0.2014	53.1	80
BE	A1	11	7	0.4635	0.2617	56.5	80
BE	A1	12	7	0.4667	0.2868	61.5	80
BE	A1	13	7	0.4139	0.2354	56.9	80
BE	A1	14	8	0.3611	0.1338	37.1	60
BE	A1	15	8	0.3965	0.2179	55	80
BE	A1	16	9	0.4721	0.2709	57.4	80
BE	A1	17	9	0.5115	0.2925	57.2	80
BE	A1	18	9	0.3716	0.1966	52.9	80
BE	A1	19	9	0.3663	0.1737	47.4	80
BE	A1	20	9	0.4516	0.2119	46.9	80
BE	A1	21	10	0.0242	.	.	.
BE	A2	1	7	0.4438	0.2442	55.02	80
BE	A2	2	7	0.4566	0.2561	56.09	80
BE	A2	3	7	0.5025	0.2657	52.88	80
BE	A2	4	7	0.5371	0.2883	53.68	80
BE	A2	5	7	0.3931	0.2357	59.96	80
BE	A2	6	7	0.4456	0.2553	57.29	80
BE	A2	7	7	0.5245	0.2566	48.92	80
BE	A2	8	7	0.4503	0.2488	55.25	80

BE	A2	9	7	0.5291	0.3134	59.23	80
BE	A2	10	8	0.4255	0.2353	55.3	80
BE	A2	11	8	0.4756	0.2694	56.64	80
BE	A2	12	8	0.498	0.2656	53.33	80
BE	A2	13	8	0.4239	0.2521	59.47	80
BE	A2	14	8	0.4601	0.1879	.	.
BE	A2	15	8	0.5399	0.2179	34.8	20
BE	A2	16	8	0.3971	0.1899	54.87	80
BE	A2	17	8	0.2997	0.2525	63.36	80
BE	A2	18	9	0.5964	0.2237	42.34	0
BE	A2	19	9	0.4103	0.213	54.52	80
BE	A2	20	9	0.5	0.1829	42.6	0
BE	A2	21	9	0.3507	0.1602	52.15	0
BE	A2	22	9	0.3381	0.089	.	.
BE	A2	23	9	0.3107	.	51.56	0
BE	A2	24	9	0.2436	.	36.54	60
BE	A3	1	6	0.3874	0.2357	60.84	80
BE	A3	2	6	0.3811	0.224	58.78	80
BE	A3	3	6	0.4603	0.2457	53.38	80
BE	A3	4	6	0.2791	0.146	52.31	80
BE	A3	5	6	0.4851	0.2855	58.85	80
BE	A3	6	6	0.4468	0.2198	49.19	80
BE	A3	7	6	0.2258	0.125	55.36	80
BE	A3	8	6	0.2305	0.1136	49.28	80
BE	A3	9	6	0.3872	0.2333	60.25	80
BE	A3	10	6	0.1592	0.0613	38.51	80

BE	A3	11	6	0.36	0.1893	52.58	0
BE	A3	12	7	0.2425	0.1391	57.36	0
BE	A3	13	7	0.2931	0.1793	61.17	80
BE	A3	14	7	0.526	0.2739	52.07	80
BE	A3	15	7	0.314	0.1955	62.26	80
BE	A3	16	7	0.4498	0.2676	59.49	80
BE	A3	17	7	0.3776	0.2101	55.64	80
BE	A3	18	7	0.3516	0.2083	59.24	80
BE	A3	19	7	0.2266	0.133	58.69	80
BE	A3	20	7	0.3381	0.2356	69.68	80
BE	A3	21	7	0.3387	0.1695	50.04	80
BE	A3	22	7	0.0593	0.014	23.61	0
BE	A3	23	9	0.4851	0.2623	54.07	60
BE	A3	24	9	0.3765	0.1198	31.82	0
BE	A3	25	9	0.3521	0.2201	62.51	0
BE	A3	26	9	0.117	0.1222	.	.
BE	A3	27	10	0.2981	0.1598	.	.
BE	A3	28	10	0.2448	0.0963	49.92	80
BE	A3	29	10	0.2767	.	57.75	80
BE	A3	30	10	0.2227	.	43.24	60
BE	A4	1	6	0.4967	0.2705	54.9	80
BE	A4	2	6	0.4991	0.2491	49.91	80
BE	A4	3	6	0.4162	0.2276	54.69	80
BE	A4	4	6	0.444	0.2623	59.08	80
BE	A4	5	6	0.3639	0.2033	55.87	80
BE	A4	6	6	0.4398	0.2541	57.78	60

BE	A4	7	6	0.3293	0.1672	50.77	80
BE	A4	8	7	0.4131	0.2206	53.4	80
BE	A4	9	7	0.4057	0.0881	21.72	80
BE	A4	10	7	0.4855	0.275	56.64	80
BE	A4	11	7	0.576	0.1694	29.41	80
BE	A4	12	7	0.2991	0.1672	55.9	80
BE	A4	13	7	0.3536	0.1885	53.31	0
BE	A4	14	7	0.4916	0.2902	59.03	60
BE	A4	15	7	0.4303	0.247	57.4	80
BE	A4	16	7	0.4326	0.1595	36.87	80
BE	A4	17	7	0.383	0.2056	53.68	60
BE	A4	18	7	0.2926	0.225	.	.
BE	A4	19	8	0.4236	0.25	53.12	80
BE	A4	20	8	0.4352	0.2097	57.44	0
BE	A4	21	8	0.3436	0.2277	61.03	60
BE	A4	22	8	0.4989	0.2582	45.64	20
BE	A4	23	8	0.4457	0.2634	57.93	80
BE	A4	24	8	0.2881	0.2402	.	.
BE	A4	25	9	0.4829	0.2572	54.55	40
BE	A4	26	9	0.4328	0.0431	55.5	0
BE	A4	27	9	0.4495	.	57.22	0
BE	A4	28	9	0.3355	.	.	.
BE	A4	29	10	0.1144	.	37.67	0
BE	A5	1	6	0.3178	0.1944	61.17	0
BE	A5	2	6	0.4052	0.2394	59.08	80
BE	A5	3	6	0.4763	0.2648	55.6	40

BE	A5	4	6	0.4438	0.277	62.42	80
BE	A5	5	6	0.4215	0.2424	57.51	80
BE	A5	6	6	0.4178	0.2314	55.39	80
BE	A5	7	6	0.4825	0.2586	53.6	80
BE	A5	8	6	0.3748	0.2093	55.84	0
BE	A5	9	6	0.4627	0.2459	53.14	80
BE	A5	10	6	0.2861	0.1689	59.04	80
BE	A5	11	6	0.4555	0.2439	53.55	40
BE	A5	12	7	0.391	0.2096	53.61	80
BE	A5	13	7	0.5349	0.1562	28.67	80
BE	A5	14	7	0.434	0.2191	50.48	80
BE	A5	15	7	0.3748	0.1779	47.47	20
BE	A5	16	7	0.4925	0.2776	56.37	80
BE	A5	17	7	0.3958	0.2203	55.66	80
BE	A5	18	7	0.3849	0.2	51.96	80
BE	A5	19	7	0.5191	0.2872	55.33	60
BE	A5	20	7	0.4624	0.2429	52.53	80
BE	A5	21	8	0.531	0.2636	49.64	0
BE	A5	22	8	0.3319	0.1424	42.9	80
BE	A5	23	8	0.3131	0.1895	60.52	80
BE	A5	24	8	0.39	0.2242	57.49	80
BE	A5	25	8	0.1724	0.0965	55.97	0
BE	A5	26	8	0.1502	0.0627	41.74	0
BE	A5	27	9	0.3555	0.0494	13.9	0