

AN INVESTIGATION INTO THE POTENTIAL IMMUNOGENICITY OF VARIOUS
EXTRACTS OF THE SOUTH AFRICAN BONT TICK *AMBLYOMMA HEBRAEUM*

THESIS

Submitted in fulfilment of the requirements
for the degree

MASTERS OF SCIENCE (MICROBIOLOGY)

in the Faculty of Science, Rhodes University,
Grahamstown, South Africa.

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November 1992

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ABSTRACT

Rabbits and goats were inoculated with crude, membrane-associated and soluble components extracted from unengorged adult females and nymphs of the bont tick *Amblyomma hebraeum*. Inoculation provided some protection against nymphal infestation, however it had little effect on adult feeding. Histological examination of adults fed on inoculated hosts showed evidence of gut damage.

Skin provocation testing with tick extracts elicited a Type I immediate hypersensitivity which was influenced by antihistamine. A delayed skin reaction was also evident. Whether this was attributable to Type III Arthus reaction or Type IV cell-mediated hypersensitivity was not determined. A comparative histological study of sites of tick extract injection, on inoculated and naive hosts, demonstrated the role of eosinophils in the hosts response to tick feeding.

Serological examination revealed elevated anti-*A. hebraeum* IgG titres following inoculation. These titres were found to decrease in the ten weeks after inoculation, despite the hosts being repeatedly infested with *A. hebraeum*. Although the IgG titres of naive control hosts increased after each tick infestation, they failed to reach the titres achieved through inoculation. Western blot analysis of serum from inoculated hosts recognized most of the *A. hebraeum* proteins against which it was screened.

CHAPTER ONE:

INTRODUCTION AND LITERATURE REVIEW

Ticks are obligate haematophagous ectoparasites, found on a wide range of warm- and cold-blooded vertebrate hosts. They belong to the Suborder Ixodida, Order Parasitiformes, Subclass Acari of the Class Arachnida. The Ixodida is comprised of three families, namely the Nuttalliellidae, Argasidae and Ixodidae. The South African bont tick *Amblyomma hebraeum* (Koch) belongs to the Ixodidae family (Krañtz, 1978).

The Ixodidae (hard ticks) comprise approximately 650 species and are essentially world wide in their distribution, although they occur more frequently in temperate regions (Hoogstraal, 1985). The ixodid life cycle is one of incomplete metamorphosis, with one larval and one nymphal stage. The majority of ixodid ticks, including *A. hebraeum*, follow a three-host cycle. Some, however have a two-host cycle, whilst others, such as the Pantropic blue tick *Boophilus microplus* undergo their entire life cycle on a single host.

1.1 THE HOSTS AND LIFE-CYCLE OF *AMBLYOMMA HEBRAEUM*

The preferred hosts of adult *Amblyomma hebraeum* appear to be ungulates. The most important domestic hosts of adults are cattle, although they are also found on other domestic livestock such as goats, sheep, horses, donkeys and pigs (Petney *et al.*, 1987). Adults also infest a wide range of wild animal hosts including reptiles, birds and mammals, however the main wild hosts are large ungulates (greater than 100 kg) such as white rhino, giraffe and buffalo (Norval, 1983a; Petney *et al.*, 1987).

Nymphs and larvae utilize similar hosts to adults, but can also be found on smaller mammals such as hares and ground-feeding birds (Howell *et al.*, 1978). Tortoises may also harbour large numbers of immature *A. hebraeum* (Walker & Schultz, 1984).

A. hebraeum has a long life-cycle, requiring 169-238 days for completion under laboratory conditions. This long life-cycle may be attributed to the three-host nature of the tick. The female lays approximately 15 000 eggs (Norval, 1974). After hatching, the six-legged larvae ascend vegetation to await passing hosts (Rechav, 1979), and are alerted to the presence of a potential host by stimuli such as, movement and microclimatic increases in heat and carbon dioxide (Norval *et al.*, 1987). Upon finding a suitable host, the larvae climb to their predilection site, usually on the head and limbs (Ducasse, 1969; Norval, 1974). After feeding for 4-15 days the fully engorged larvae drop from the host to seek shelter in, and under ground foliage, where they undergo the 14-25 day nymphal premoult period (Norval, 1974).

The eight-legged nymphs, shelter beneath the debris on the soil surface and only become active in response to host stimuli. They usually attach to the feet of ruminant hosts but are also found on the axillae, sternum, belly, groin and legs (Ducasse, 1969; Norval, 1974). After feeding for 5-13 days, the engorged nymphs drop to the ground and undergo a premoult period of 20-29 days (Norval 1974).

After a period of approximately seven days of relative inactivity, the adults search for suitable hosts. The males attach immediately, usually on the underside of the body (Howell *et al.*, 1978). The females move around on the host until they encounter a sexually mature male whereupon they attach and copulation occurs. Female engorgement occurs over the next 6-12 days (Norval, 1974). *A. hebraeum* males engorge for 149-244 days and have been reported to mate successfully 4-42 times (Jordaan & Baker, 1981). Ixodid females ingest significantly larger blood meals than males. After feeding the female falls to the ground and seeks shelter where she lays eggs and dies.

It is now known that in addition to stimuli such as increases in carbon dioxide, an aggregation-attachment pheromone (AAP) produced by attached males plays an important role in host selection and location (Rechav *et al.*, 1976). This pheromone-regulated behaviour plays an important role in the survival of ticks as they are attracted to suitable hosts, thereby largely eliminating the chances of attaching to a resistant or acaricide treated host (Norval *et al.*, 1989a).

1.2 THE ECONOMIC IMPORTANCE OF TICKS

It has been estimated that as much as 80% of the world's cattle population is exposed to the risk of tick infestation (M^cCosker, 1979). The economic losses associated with tick infestations are considerable in high tick-challenge areas. In 1976, Steelman estimated that ticks caused an annual world-wide loss of approximately eight billion U.S. dollars to the cattle industry alone. The costs of control and loss in production attributable to the Australian cattle tick *Boophilus microplus* alone, amount to approximately 100 to 150 million Australian dollars per annum (Willadsen & Kemp, 1988).

In Africa, where ticks infest livestock in all climatic and vegetative zones, no recent reliable data on economic losses is available. In 1977, the South African Bureau of Standards estimated that mortality due to tick-borne diseases together with the purchase of acaricides and vaccines, cost the South African livestock producers R70 million per annum (S.A.B.S Bulletin No. 6, 1977). It is widely believed that the total loss in livestock production attributable to ticks is greater in Africa than anywhere else in the world (Dipeolu, 1989).

Economic losses may be attributable to any of a number of factors, namely various tick-borne diseases, blood loss and general unthriftiness, hide damage and the development of secondary bacterial infections that often lead to abscessation at attachment sites. Abscesses in dairy cattle frequently infect the udder, resulting in loss in milk production (M^cCosker, 1979).

Tick-borne diseases are caused by a variety of pathogens (Bram, 1975). These include viruses affecting both man and a variety of wild and domestic animals (e.g. Crimean-Congo haemorrhagic fever), bacteria such as the spirochaete *Borrelia burgdorferi* (Lyme disease), rickettsias including *Rickettsia conori* (African tick typhus or tick-bite fever) and *Cowdria ruminantium* (heartwater), protozoa (e.g. babesiosis and theileriosis), and toxins (e.g. sweating sickness and Karoo paralysis). The occurrence and distribution of these diseases is dependant upon the distribution of their vectors, which in turn is dependant upon climatic and host factors.

1.3 THE ECONOMIC IMPORTANCE OF *AMBLYOMMA HEBRAEUM*

Lounsbury (1900) was the first to demonstrate that the bont tick *Amblyomma hebraeum* is an efficient vector of heartwater (cowdriosis), an acute frequently fatal disease of ruminants (Van de Pypekamp & Prozesky, 1987). The disease was first recognized in the 1830's (Provost & Bezuidenhout, 1987), however it was only in 1925 that the rickettsia, *Cowdria ruminantium* was recognised by E.V. Cowdry as being the causative organism (Cowdry, 1925).

In southern Africa the main heartwater vector is *A. hebraeum*. Infection rates of field populations of *A. hebraeum* vary from 0 to 44.9% depending on the instar concerned and the time of year (Norval *et al.*, 1990). In the rest of sub-Saharan Africa the tropical bont tick *Amblyomma variegatum* assumes the role of main heartwater vector (Norval *et al.*, 1990). Heartwater has been transmitted experimentally by a further eight African *Amblyomma* species (Table 1). It is not known how the epidemiology of the disease is affected by the different vectors, however it is possible that factors such as host specificity, infection rates and seasonal occurrence may play a role.

Heartwater is known to occur throughout most of sub-Saharan Africa, however it appears to be most serious in southern Africa, possibly due to the large number of exotic, more susceptible ruminants in the subcontinent (Uilenberg, 1983). In 1968, Nietz estimated the endemic area to be approximately 13 million square kilometres. With the advent of international livestock trade, the disease is now known to have spread to other areas, including many islands near Africa such as Madagascar, La Réunion and Mauritius. The disease has also been diagnosed in the Caribbean, and there are unconfirmed reports of the disease from Turkey, Iran, Yugoslavia and Malaysia. In many of these areas the tick vectors are unknown (Uilenberg, 1983).

Although heartwater was only diagnosed on Guadeloupe in the Caribbean by Perreau *et al.* in 1980, *A. variegatum*, the main vector of the disease in the area, was introduced from Senegal in the 1830's (Mebus & Logan, 1988) and the disease may have been present since then. Although none of the *Amblyomma* species serving as vectors of heartwater in Africa are naturally present on the north America mainland, the United States Department of

Agriculture have reported finding *A. variegatum* on imported exotic animals (Uilenberg, 1982). *A. variegatum* could establish itself in large areas of north and south America. Furthermore experimental transmission studies indicate that at least two north American *Amblyomma* species, namely *A. cajennense* and more importantly *A. maculatum* are efficient vectors of the disease (Uilenberg, 1982; Barré *et al.*, 1987) (Table 1). There is thus a justifiable fear of heartwater spreading to extensive regions of north, central and south America (Barré *et al.*, 1987).

Table 1: Proven experimental vectors of *Cowdria ruminantium* (modified from Uilenberg, 1983).

<i>Amblyomma</i> species	Reference
<u>African vectors:-</u>	
<i>A. hebraeum</i>	Lounsbury (1900)
<i>A. variegatum</i>	Daubney (1930)
<i>A. pomposum</i>	Neitz (1947)
<i>A. gemma</i>	Lewis (1949)*
<i>A. lepidum</i>	Karrar (1966)*
<i>A. tholloni</i>	MacKenzie & Norval (1980)
<i>A. sparsum</i>	Norval & MacKenzie (1981)
<i>A. astrion</i>	Uilenberg & Niewold (1981)*
<i>A. cohaerens</i>	Uilenberg (1983b)**
<i>A. marmoreum</i>	Bezuidenhout (1987)
<u>American vectors:-</u>	
<i>A. maculatum</i>	Uilenberg (1982)
<i>A. cajennense</i>	Uilenberg (1983b)**

* cited in Uilenberg (1983)

** cited in Bezuidenhout (1987)

Heartwater is regarded as the most important tick-transmitted disease in South Africa (Bezuidenhout, 1985). Neitz (1968) reported that mortalities due to heartwater in South Africa were three times as great as those due to either babesiosis or anaplasmosis. Furthermore, Neitz (1968) noted that the disease was extremely virulent in goats and sheep. The problem was so great that farming with small exotic ruminants was virtually

impossible in some parts of South Africa until acaricidal dipping was introduced (Spreull, 1922). More recently, it has been reported that 10% of the losses in the Angora goat industry in the Eastern Cape Province of South Africa, are attributable to heartwater (Du Plessis *et al.*, 1983). Furthermore, as exotic hosts are more susceptible to heartwater than indigenous breeds, the disease is a major obstacle to the introduction of exotic stock to Africa (Uilenberg, 1982).

In addition to losses attributable to heartwater, adult *Amblyomma* can have a severe effect on the liveweight-gain (L.W.G.) and milk production of domestic livestock. A single engorging *A. hebraeum* female may cause a loss in L.W.G. in cattle of approximately 10g (Norval *et al.*, 1989b), and a loss in milk production of 7g (Norval *et al.*, 1990, cited in Norval *et al.*, 1992). The large, robust mouth parts of *Amblyomma* ticks can also cause severe abscessation. Asselbergs & Lopes Pereira (1990), cited in Uilenberg (1992), reported that in 1984 only 53% of Zebu cattle on farms in Mozambique where dipping schemes had broken down had four functional teats. They attributed the damage to *A. hebraeum*, which were frequently found in large clusters on the udder. The screw-worm larvae of the blowfly, *Chrysomya bezziana*, which strike infested hosts can further affect productivity (Norval *et al.*, 1989b). Finally *Amblyomma* ticks act as vectors for many other pathogens, for example *A. hebraeum* can transmit *Theileria mutans*, causing benign bovine theileriosis, and *Rickettsia conori*, causing tick-bite fever in man (Howell *et al.*, 1978).

1.4 TICK AND TICK-BORNE DISEASE CONTROL

The control of a tick-borne disease is dependant upon the rupture of at least one link in the chain of cyclic development of the disease. Possibilities include a) the elimination of the tick vector, b) the elimination of the reservoir of infection of those vectors, c) immunization against tick-borne disease, or d) replacement of tick-sensitive hosts with tick-resistant hosts (Neitz & Alexander, 1945).

1.4.1 Control of ticks

Although improving management practices, such as rotational grazing, weed and bush control, and the selection of appropriate stock (in terms of both their suitability to a particular environment and their resistance to disease), will go a long way towards controlling ticks and tick-borne diseases, there are many situations where more specific control methods are required.

The control of ticks, and consequently tick-borne diseases, in the commercial sector is traditionally dependant upon the use of chemical acaricides that are applied at regular intervals, irrespective of tick challenge (M^cCosker, 1979). However, the realization that enzootic stability to both ticks and tick-borne diseases can be achieved by allowing ticks to feed on their hosts has resulted in a change of thinking (Norval, 1981, 1983b). To allow enzootic stability to develop, acaricidal control should be used strategically at times of high tick or disease challenge (Norval *et al.*, 1989b). Obvious advantages of strategic tick control include reduced dipping costs and the fact that many hosts may acquire natural resistance to ticks and tick-borne diseases (including heartwater). Disadvantages include initial losses in production due to increased tick burdens, and limited losses due to tick-borne disease, and higher vaccine costs (vaccination against disease is often not necessary with intensive tick control) (Bezuidenhout & Bigalke, 1987).

In the Third world, the traditional subsistence farmer does not have the financial resources required for the purchase of chemicals and maintenance of dipping programmes (Young *et al.*, 1988). Furthermore, it is well recognized that ticks may develop resistance to acaricides within four to six years of their introduction (Wharton, 1976; Sutherst, 1981; Solomon, 1983). Where chemicals have similar modes of action, cross-resistance and multi-resistance may often occur, thereby rendering large groups of chemicals ineffectual before they can be used in the field (Solomon, 1983). Schröder (1987) showed that many of the available acaricides failed to control *A. hebraeum* when used at their recommended concentrations. Durand (1976) warned that the projected economic returns prior to the development of resistance are frequently insufficient to cover the high costs of producing a new acaricide.

Several suggestions have been proposed to prolong the life of existing acaricides. These approaches include the manipulation of factors such as dose concentration, strategic timing of application, choice of acaricide for the particular pest, rotation or alternation of compounds, and using mixtures of the available toxicants (Georghiou, 1983).

As the costs of acaricides escalate and the problems of resistance and environmental pollution increase, increasing attention is being paid to the need to find alternative methods of tick control. These have been summarized by Mathewson (1984) and include novel strategies such as the use of tick predators or pathogens, sterile male release and tick repelling plants, as well as the more generally accepted methods of selectively breeding tick-resistant cattle (Seifert, 1971) or vaccinating animals against ticks (Kemp *et al.*, 1989).

1.4.2 Control of disease reservoirs

The control of disease reservoirs is impractical in Africa because of the wide host range of our economically important ticks (Petney *et al.*, 1987).

1.4.3 Immunization against tick-borne disease (heartwater)

Calves under the age of three weeks, irrespective of the immune status of the dams, possess an innate resistance to heartwater (Neitz & Alexander, 1945; Du Plessis & Malan, 1988). However, if infected during this period an inapparent disease develops and in 93% of cases a durable immunity develops (Neitz & Alexander, 1945). Lambs younger than eight days and kids younger than six weeks are also usually resistant to heartwater (Neitz & Alexander, 1941). This led to the idea of intra-venous immunization of young livestock with virulent blood. With the discovery of specific chemotherapeutic drugs such as sulphonamides (Neitz, 1940) and tetracyclines (Provost & Bezuidenhout, 1987) immunization has been extended to include older animals. Treatment does not appear to affect the resultant immunity (Neitz & Alexander, 1945).

Today, besides the use of chemical acaricides, the only prevention available against heartwater is a vaccine comprising a virulent strain of *Cowdria ruminantium* from sheep blood (Neitz & Alexander, 1941, 1945), or from homogenized *C. ruminantium*-infected

A. hebraeum nymphs (Bezuidenhout, 1981). Disadvantages of these vaccines are that they are expensive to produce (Oberem & Bezuidenhout, 1987), labile and highly pathogenic, they have to be intravenously administered and other diseases may be transmitted in the sheep blood or nymph suspension (Bezuidenhout, 1985). Furthermore, as the vaccines available are virulent, vaccinated animals must be kept under close observation (monitoring individuals temperatures) in order that the course of the infection can be controlled through the use of drugs (Van der Merwe, 1987). An alternative to the close monitoring of individuals is to 'block' treat animals irrespective of whether or not they have developed a febrile reaction to the vaccine. Although this latter method is not recommended for valuable, susceptible animals, it is useful for the treatment of large numbers of commercial stock (Du Plessis & Malan, 1987).

Finally, vaccination of livestock with a 'live' vaccine is definitely not suitable for countries where potential vectors are present but where the disease organism is absent. Heartwater will therefore remain a disease of major global importance until such time as either an effective and safe method of vaccination becomes available, or an alternative to acaricidal chemicals is found for the control of the disease vectors.

1.4.4 Resistance of domestic hosts to ticks

Host resistance to ticks is frequently acquired both naturally and artificially through repetitive infestations, and expressed by significant reductions in the tick yield, engorgement weight (Utech *et al.*, 1978), moulting success and reproductive capacity (Roberts & Kerr, 1976). This would not only cause a reduction in the number of ticks carried over to the next generation (and consequently a decrease in the number of vectors available for disease transmission), but may also directly interfere with the transmission of pathogenic organisms by the vector (Wikel, 1982; Jones & Nuttall, 1988; Fivaz *et al.*, 1989).

Johnston and Bancroft (1918) were the first to observe that cattle exhibited natural resistance to ticks. Since then it has been established that numerous laboratory animals and some species and breeds of domestic stock acquire resistance to various tick species, such as *Boophilus microplus* (Wilkinson, 1955; Riek, 1962; Roberts, 1968a; Hewetson,

1971; Wagland, 1978), *Dermacentor andersoni* (Allen, 1973), *Rhipicephalus evertsi evertsi* (Clarke *et al.*, 1989), *Rhipicephalus appendiculatus* (Fivaz, 1990), *Rhipicephalus zambeziensis* (Fivaz *et al.*, 1991b), *Amblyomma americanum* (Brown, 1982; Brown *et al.*, 1984a; George *et al.*, 1985; Barriga *et al.* 1991a), *Amblyomma variegatum* (Latif *et al.*, 1988) and *Amblyomma hebraeum* (Heller-Haupt *et al.*, 1981; Adamson *et al.*, 1991; Fivaz *et al.*, 1991a).

Hosts differ in their ability to acquire resistance to ticks following repetitive infestations (Reik, 1962; Seifert, 1971; Rechav & Kostrzewski, 1991), with certain species and breeds of hosts acquiring a stronger resistance than others. For example, Utech *et al.* (1978) demonstrated that Zebu *Bos indicus* (Brahman) cattle were more resistant than European *Bos taurus* (Jersey) cattle to infestation with *Boophilus microplus* larvae. George *et al.* (1985), Scholtz *et al.* (1991) and Fivaz *et al.* (1992) further demonstrated the capacity of indigenous (i.e. *Bos indicus*) cattle to develop a relatively stronger resistance to one-, two- and three-host African tick species than *Bos taurus* cattle. *Bos indicus* X *Bos taurus* cross-bred cattle developed an intermediate resistance (George *et al.*, 1985; Scholtz *et al.*, 1991).

There are conflicting reports on the ability of various hosts to acquire resistance following exposure to feeding instars of *A. hebraeum*. Norval (1978) showed that sheep and rabbits failed to acquire immunity to either larvae or nymphs following multiple infestations. Similar results were obtained following infestations, of Merino sheep with adult ticks (Norval *et al.*, 1988). In contrast, Adamson *et al.* (1991) demonstrated the ability of indigenous Boer goats to develop resistance following three infestations of *A. hebraeum* larvae. Kraidy (1991) found that although repetitive infestations of rabbits with *A. hebraeum* nymphs led to a significant reduction in the engorgement weights and moulting success of ticks, many nymphs were still able to engorge successfully during the fifth infestation. Recent field observations by Spickett *et al.* (1989) have demonstrated that indigenous cattle were able to develop resistance to *A. hebraeum*, exotic cattle were less resistant, while cross-breeds were intermediate in their resistance. These differing results may be attributable to any of the following factors, genetic difference in the ability of the different hosts to respond immunologically to tick challenge (Reik, 1962), differences in

the immunogenicity of the tick instars (Fivaz & Norval, 1990), or differences in the tick challenge sizes used in experiments (Norval, 1978; Adamson *et al.*, 1991).

Martinez *et al.* (1992) suggested that *A. hebraeum* may be one of the few tick species displaying immunosuppressive capabilities, and proposed that this might explain the relatively poor response of some hosts to feeding instars. Furthermore, it has been speculated that ticks with long, deeply penetrating mouthparts (e.g. *Hyalomma* and *Amblyomma* spp.) are generally less susceptible to the host's resistance mechanisms (i.e. grooming), than ticks with short mouthparts (e.g. *Boophilus* and *Rhipicephalus* spp.) (Norval, 1975).

There have been numerous reports of both intrageneric and intergeneric cross-resistance to ticks. Brown and Askenase (1981) reported that challenging hosts with *Rhipicephalus sanguineus* conferred significant levels of resistance to *Amblyomma americanum*. Heller-Haupt *et al.* (1981) reported low levels of cross-resistance in both rabbits and guinea-pigs between *A. hebraeum* and *A. variegatum*, but not between *A. hebraeum* and *Rhipicephalus appendiculatus*. These reports may indicate that there are common antigens between both species and certain genera.

1.5 MECHANISMS OF HOST RESISTANCE

The immunology of the responses of domestic stock to ticks has received little attention due to the prohibitive costs involved and unavailability of inbred strains of these hosts. The need for basic information concerning the mechanisms involved in the immune response to ticks has resulted in the use of model systems employing laboratory animals such as guinea-pigs (Allen, 1973; Brown, 1982; Clarke *et al.*, 1989) and rabbits (Brown, 1988c; Fivaz, 1990) as hosts. The use of these laboratory hosts may be justified by the fact that the immatures of many tick species, including *Amblyomma hebraeum*, often feed on rodents (Brown, 1988b). The results obtained in laboratory models do not unfortunately always accurately represent the field situation in terms of the host/tick relationship (Allen, 1989).

The immune system provides the most sophisticated host defence against foreign intrusions. However, there are a number of non-immunological barriers that constitute the first line of defence. These non-immunological defences operate against almost any substance that is exposed to the body and are referred to collectively as innate or natural immunity. They include body surfaces that act as physical barriers to environmental agents, chemical components such as pH, and various internal elements including phagocytic cells (Benjamini & Leskowitz, 1991; Clark, 1991).

There are two immunological ways in which the host expresses its resistance to ticks. Firstly, humoral immunity which centres around the production of antibodies in the form of various immunoglobins (eg. IgG, IgM, IgE) by the B lymphocytes (or B-cells) in response to an initial contact with the antigen/s presented by the tick (Clark, 1991). The second way is via a cell-mediated immune response involving specific lymphocytes (T-cells) and activated macrophages which recognize and bind to specific antigens (Clark, 1991). Although these divisions of the immune response can be considered as theoretically distinct, the response to any antigen/s is likely to involve a complex interaction between both components and innate immunity.

Riek (1956) recorded raised blood histamine levels in tick-resistant *Bos taurus* cattle and proposed that their resistance is due to the development of a Type I immediate IgE-mediated skin hypersensitivity to the tick's salivary gland secretions. The IgE antibodies are cytophilic for mast cells and basophils, binding to these cells at the Fc region, leaving the antigen-binding sites of the molecule facing outwards. Once the IgE molecule attaches to an antigen, it triggers biochemical reactions leading to the release of pharmacological agents (such as histamine) which mediate the inflammatory symptoms associated with hypersensitivity (Clark, 1991).

Riek (1962) demonstrated that infestation of resistant cattle with *Boophilus microplus* led to the appearance of a conspicuous number of mast cells and an intense infiltration of the dermis by eosinophils. The latter could support the theory of a Type I immediate hypersensitivity, since an eosinophil chemotactic factor (ECF-A) is known to be released by basophils, mast cells and activated complement during IgE-mediated hypersensitivity

reactions. The accumulation of both mast cells and eosinophils has however also been associated with delayed hypersensitivity initiated by T-cells (Clark, 1991).

Dvorak *et al.* (1970) was the first to report the involvement of basophils in hypersensitivity reactions. This type of sensitivity, termed cutaneous basophil hypersensitivity (CBH), was distinguished from the classical delayed hypersensitivity by both the histology and the immunogenic requirements of the reaction (Richerson *et al.*, 1970; Askenase, 1973). Allen (1973), working on guinea-pigs, was the first to record the occurrence of basophils in skin reactions to tick feeding. Subsequently, basophil accumulations have been shown in immune response to ticks in cattle (Brown *et al.*, 1984a), rabbits (Brown, 1988c) and guinea-pigs (Brown, 1982, 1988a). Ticks appear to be the most potent inducers of cutaneous basophilia known (Brown, 1988a).

Askenase *et al.* (1975) demonstrated that serum factors contribute to CBH reactions while Wikel & Allen (1976) presented evidence that resistance to ticks could be passively transferred with viable lymph node cells but not with serum, suggesting a delayed hypersensitivity mechanism (cellular immune response). Wikel & Allen (1982) conducted experiments to assay the relative effectiveness of T-cells and/or B-cells in the transfer of tick resistance, and found that although resistance was more readily transferred with T-cell enriched populations, neither transferred resistance as effectively as an unfractionated population of viable lymph node cells. This is in keeping with the view of Askenase (1977), that basophils accumulate in the tissue in response to immunological recruitment involving both T-cells and B-cells.

Despite numerous studies, the exact mechanisms involved in the expression of immunity by hosts to ixodid tick infestations are still not known. It may be that mediators released by basophils and/or eosinophils inhibit normal enzyme function within the gut, thereby preventing feeding and leading to death in some, while only hindering feeding and resulting in reduced tick weights in others (Brown, 1982). Paine *et al.* (1983) showed that histamine and serotonin directly affected tick feeding.

Irritation of the host as a result of hypersensitivity reactions leads to increased grooming which may reduce the tick burden (Riek, 1962; Norval, 1978). Despite this, Riek (1962)

found no correlation between the intensity of the skin reaction and the level of resistance to ticks displayed by the host. The mechanisms of resistance appear to vary with the host (Brown, 1988a, 1988c) and tick species involved (Jongejan *et al.*, 1989).

1.6 ARTIFICIAL INDUCTION OF HOST IMMUNITY TO TICKS WITH TICK ANTIGENS (CONCEALED ANTIGENS)

A logical outcome of research on host resistance to ticks has been the attempt to artificially induce an immunologically-based resistance to infestation by direct immunization with extracts of various tick species. A detailed knowledge of the hosts immune response to tick feeding, and the molecules involved, is essential for development of immunization as a tick control strategy.

Trager (1939) immunized guinea-pigs with crude larval extracts of *Dermacentor variabilis*, and demonstrated that the immunization had a detrimental effect on tick feeding. It was however 40 years later before Allen and Humphreys (1979) expanded on Trager's work and suggested that vaccination had the potential to develop into an alternative tick control method. Their work demonstrated that less than 10% of larvae feeding on guinea pigs immunized with extracts of *Dermacentor andersoni* were able to engorge fully, while ticks fed on immunized cattle showed reduced engorgement weights.

Artificial immunity has been induced in various hosts against several tick species, namely *Amblyomma maculatum* (M^cGowan *et al.*, 1980), *Amblyomma americanum* (Brown *et al.*, 1984b; Heller-Haupt *et al.*, 1989), *Amblyomma variegatum* (Heller-Haupt *et al.*, 1987; Jorgejan *et al.*, 1989), *Dermacentor variabilis* (Ackerman *et al.*, 1980), *Boophilus microplus* (Agdebe & Kemp, 1986; Johnston *et al.*, 1986; Kemp *et al.*, 1986; Opdebeeck *et al.*, 1988c, 1989), *Rhipicephalus zambeziensis* (Fivaz *et al.*, 1991b) and *Rhipicephalus appendiculatus* (Jongejan *et al.*, 1989; Dhadialla *et al.*, 1990). Few attempts have been made to artificially immunize against *Amblyomma hebraeum* (Heller-Haupt *et al.*, 1989, Adamson *et al.*, 1991)

As the primary objective of vaccination is to guide the hosts immune system to those antigens that elicit a protective response and to ignore the irrelevant proteins, the first step in the development of a vaccine against ticks must involve the identification and isolation of protective antigens. Preliminary work on vaccination against ticks, frequently involves crude tick extracts as the removal of irrelevant proteins, non-immunogenic material, and potentially immunosuppressant molecules is a difficult task (Wikel & Whelan, 1986). In some instances, immunization of host animals with crude tick extracts has been reported to induce a significant antibody response to only two or three of the many proteins present (Brown *et al.*, 1984b). If this were the case isolation of the protective antigens would presumably be a relatively simple task. It has however been demonstrated that immunization with crude extracts frequently induces the production of many antibodies, some of which are directed against minor proteins (Mongi *et al.*, 1986; Jongejan *et al.*, 1989). Thus the identification and purification of protective antigens becomes a challenging task.

Various sources of antigen have been used in attempts to vaccinate animals against ticks. These have ranged from crude extracts of fed or unfed whole ticks (M^cGowan *et al.*, 1980; Kemp *et al.*, 1986, 1989; Heller-Haupt *et al.*, 1988; Varma *et al.*, 1990), to salivary glands (Brown *et al.*, 1984b; Jongejan *et al.*, 1989), reproductive organs (Allen & Humphreys, 1979) and gut tissue (Opdebeeck *et al.*, 1988b; Willadsen *et al.*, 1989; Jackson & Opdebeeck, 1990).

Initially, tick salivary glands were used as a potential antigen source on the basis that hosts are exposed to salivary gland secretions during the normal feeding process, and consequently may have a role in the development of naturally acquired immunity (Jongejan *et al.*, 1989). Hosts have been found to display a variety of immune responses to these substances (Willadsen, 1980). Partially engorged female *D. andersoni*-derived salivary glands induced a level of resistance to tick challenge similar to that expressed by animals displaying acquired immunity (Wikel, 1981). Brown *et al.* (1984b) reported that salivary gland antigens of *A. americanum* induced resistance in guinea-pigs, however the minimum effective dose of salivary gland antigen was between 100 and 120 μ g per animal. Furthermore immunization with salivary glands often results in a host response characterized by intense cutaneous hypersensitivity at the tick attachment site, an

undesirable consequence of vaccination against ticks due to the resultant hide damage (Wikel, 1988).

The concept of "concealed" antigens, or antigens not normally exposed to the host during feeding, was proposed by Willadsen & Kemp (1988). It was hypothesized that if these antigens could be exposed to the host, then ingested immune components could react with the "concealed" antigens and cause some damage. In order to achieve this, the "concealed" molecules would have to be identified, isolated and used to prime the host first (Willadsen & Kemp, 1988). The concept of incorporating "concealed" antigens in vaccines is of particular interest since the host and parasite will not have evolved immunological interactions associated with these antigens (Opdebeeck *et al.*, 1988a).

Major practical advances in the development of a vaccine against ticks have been made in Australia using antigens extracted from *Boophilus microplus* (Johnston *et al.*, 1986; Kemp *et al.*, 1986, 1989; Lee & Opdebeeck, 1991; Opdebeeck *et al.*, 1988a, 1988b, 1989; Rand *et al.*, 1989 and Willadsen *et al.*, 1988, 1989). Immunization of cattle with extracts from adult female ticks induces partial immunity to *B. microplus* (Johnston *et al.*, 1986; Kemp *et al.*, 1986, 1989). Ticks which dropped from immunized cattle showed some degree of damage to the gut and reduced engorgement weights. Host antibody levels were not however found to correlate with their immunity to ticks. This may have been due to the crude nature of the vaccine, in which many of the antigens present were irrelevant to protection (Johnston *et al.*, 1986).

Opdebeeck *et al.* (1988a) showed that cattle vaccinated with gut and synganglion tissue from *B. microplus* were almost totally protected against tick challenge. The protective antigens were subsequently found to be associated with the membrane-bound component of the tick gut (Opdebeeck *et al.*, 1988b). Kemp *et al.* (1989) showed that the protective antigens were located on the plasma membrane of the tick gut cells. Willadsen *et al.* (1988), used differential centrifugation and detergent extraction to split the crude *B. microplus* extract into three fractions, viz. cell debris, a soluble fraction and a membrane-associated fraction. In keeping with the findings of Opdebeeck *et al.* (1988b), the latter fraction was found to be the most protective.

The isolation of sufficient tick material for vaccine production is a major problem (e.g. 1.2kg of semi-engorged *B. microplus* ticks only yielded about 100 μ g of the protective antigen) (Willadsen & Kemp, 1988). A protective *B. microplus* glycoprotein (89 kDa) has been isolated and partially synthesized through recombinant DNA technology using *Escherichia coli* to express the engineered protein (Rand *et al.*, 1989). The synthesised antigen was found to induced an immune response in cattle that damaged but did not kill the ticks, and Rand *et al.* (1989) speculated that a single antigen may be insufficient to kill ticks.

Another method of isolating sufficient tick material for vaccine production involves the use of monoclonal antibodies (mAb). Lee & Opdebeeck (1991) demonstrated that more than 99% protection was provided to cattle immunized with gut membrane antigens of *Boophilus microplus* precipitated by the monoclonal antibody mAb QU13. Monoclonal antibodies (such as QU13) might be used for the production of anti-idiotypic antibodies, which could then be used as surrogate antigens in a tick vaccine. This would be especially effective if the epitopes recognised by the mAb are repeated on a variety of protective glycoproteins (Lee & Opdebeeck, 1991).

Various adjuvants have been used during attempts to artificially induce immunity to ticks. The most widely used adjuvants in tick vaccines are Freund's complete (FCA) and Freund's incomplete (FIA) adjuvants (Heller-Haupt *et al.*, 1988). Both of these are however toxic due to the fact that the mineral oil used in them is non-metabolisable and that the mycobacterial elements in FCA produce severe granulomatous reactions. A third adjuvant, Quil-A, which is a semi-purified product from a crude saponin extract of the bark of the South American Molina tree (*Quillaia saponaria*), has also been used with success for the immunization of laboratory animals with tick homogenates (Heller-Haupt *et al.*, 1988, 1989).

1.7 PROPOSED STUDY

Apart from the work cited above, very little has been done to determine the efficacy of immunization against *A. hebraeum*, and what has been reported has not been detailed.

Work done to date has been restricted to crude *A. hebraeum* extracts. Adult *A. hebraeum* fed on guinea-pigs immunized with a crude extract of unfed nymphs showed an insignificant reduction in engorgement weight (Heller-Haupt *et al.*, 1989). Goats immunized with a crude larval extract were found to develop antibodies against larval proteins, although no detrimental effect to tick feeding occurred (Adamson *et al.*, 1991). As Willadsen *et al.* (1988) showed that results of vaccination against *Boophilus microplus* improved as antigen purification proceeded, it was hypothesized that purification of *A. hebraeum* extracts might lead to improved results.

It was therefore decided to partially purify various extracts of *A. hebraeum* nymphs and adults and screen them for any protective immunological capacity in rabbits and Boer goats on the basis of established protocols used for *Boophilus microplus*. The work was intended to be expanded should encouraging results be forthcoming. Studies were based on immunization and tick challenge experiments coupled with skin provocation testing and histological and serological analysis to confirm the immunogenicity of the *A. hebraeum* extracts.

CHAPTER TWO:

MATERIALS AND METHODS

2.1 HOST ANIMALS

2.1.1 Rabbits

Adult New Zealand Giant White rabbits obtained from the University of Durban-Westville, were kept in individual cages in a tick-free environment at the Rhodes University Animal House, Grahamstown, South Africa. Rabbits were fed standard commercially available rabbit pellets containing 14% protein. Food and water were given *ad libitum*.

2.1.2 Goats

Recently weaned two-month-old Boer goats were acquired from the University of Fort Hare Experimental Farm, Alice, Ciskei, where the tick challenge is low (Fivaz, pers. comm.). Goats were reared and maintained in tick-free stables until the final tick challenge was completed. Goats were fed a commercial ration containing whole maize seed, urea and molasses, to which was added maize meal and milled lucerne. Food and water was given *ad libitum*.

2.1.3 Cattle

Recently weaned heartwater-susceptible Friesland calves were housed in a tick-free, concrete-floored stable. Calves were fed a calf rearing ration containing 14% protein. Wheat hay and water were supplied *ad libitum*.

2.2 TICKS

Pathogen-free *A. hebraeum* adults were acquired from the South African Bureau of Standards, East London, South Africa. Ticks were fed in plastic chambers attached with contact glue to the shaved flanks of the Friesland calves. The plastic chambers were approximately 12cm in diameter with a ventilated screw-top lid. Calves were prevented from grooming using a neck clamp. Twenty male *A. hebraeum* were placed in each chamber and allowed five days to attach and start releasing pheromones. Twenty female *A. hebraeum* were then placed in each chamber. Engorged female ticks were collected daily and placed individually in ventilated vials. These vials were kept in a rearing chamber maintained at 85% relative humidity in a controlled environment (CE) room at 25°C, with a 12-hour photoperiod (Brown *et al.*, 1984b). Relative humidity was maintained by placing a saturated solution of potassium chloride in the base of the rearing chamber (Fivaz, 1990). Following hatching of the eggs, larvae were placed in ear-bags on the Friesland calves to allow them to feed. Ear-bags were attached to the base of the ear with adhesive tape, as described by Fivaz & Norval (1990). Engorged larvae were collected daily, placed in conical flasks stoppered with cotton wool bungs, and maintained in the rearing chambers until they moulted into nymphs.

2.3 EXTRACTS OF *AMBLYOMMA HEBRAEUM*

The protocol followed for the preparation of tick extracts (Figure 1) was modified from that of Willadsen *et al.* (1988).

2.3.1 Preparation of Crude Extracts

Unfed *A. hebraeum* adult females and nymphs were killed by chilling at -20°C for 20 minutes. They were then surface sterilized by immersion in 95% ethanol for five minutes, and washed twice in 0.1M phosphate buffered saline pH 7.2 (PBS). Washed adult and nymphal ticks were weighed separately, and ground in a mortar and pestle with sterile sand, using 2.5ml of chilled TAS blender buffer (0.05M Tris, 0.025M acetic acid, 0.1M sodium chloride) per gram of ticks. One volume of an antibiotic mixture (200 i.u./ml Penicillin,

100 μ g/ml Streptomycin, 100 μ g/ml Neomycin and 1mM EDTA) was added to nine volumes of each of the tick homogenates (adult and nymphal). These solutions were then centrifuged at 15 000g for 30 minutes at 4°C (using a SS34 rotor in a DuPont Sorval RC-5 centrifuge). The resultant pellets containing sterile sand, tick cuticles and other debris were discarded, while the supernatants of adult and nymphal crude extracts (ACE and NCE respectively) were retained. The protein concentrations of these two extracts were determined using Bradford's protein assay (Bradford, 1976). The remaining ACE and NCE was divided into 1ml aliquots for inoculation, and 20ml aliquots for isolation of soluble and membrane-bound proteins. All aliquots were stored at -20°C.

2.3.2 Preparation of Soluble and Membrane-Associated Extracts

Soluble and membrane- or particle-bound material was isolated by centrifuging the adult and nymphal crude extracts (ACE and NCE respectively) at 105 000g for 60 minutes at 4°C (using a Type 65 rotor in a Beckman L-70 ultracentrifuge). The supernatants, henceforth referred to as the adult and nymphal soluble extracts (ASE and NSE respectively), were removed and frozen at -20°C in 1ml aliquots until required for inoculation. The precipitates containing the membrane or particulate matter were resuspended in blender buffer and protein concentrations were determined using Bradford's protein assay (Bradford, 1976). Membrane- or particle-bound proteins were then extracted with Nonidet P-40 at 36°C for 90 minutes, using a detergent concentration of 5% and a protein : detergent ratio of 1 : 2 (w/w). The suspensions were centrifuged at 15 000g for 20 minutes (using a bench-top microfuge), to yield "P-40 precipitates" and supernatants containing the extracted membrane- or particle-bound proteins. The latter, henceforth referred to as the adult and nymphal membrane-associated extracts (AME and NME respectively), were frozen in 1ml aliquots until required for inoculation.

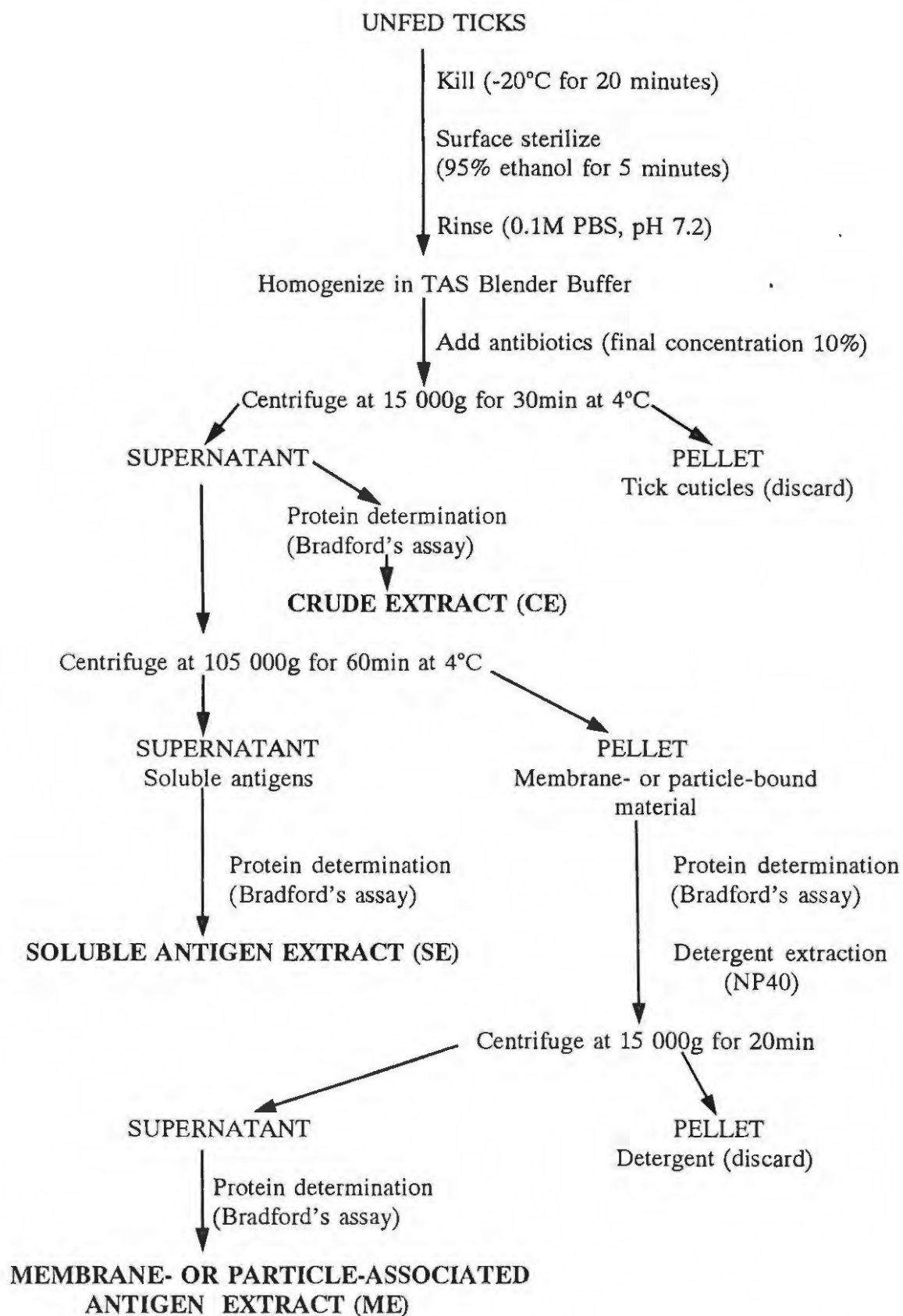


Figure 1: Protocol for purification of antigens from *Amblyomma hebraeum*

2.4 HOST INOCULATION WITH *AMBLYOMMA HEBRAEUM* EXTRACTS

Rabbits were divided up into eight groups of two. Six groups of rabbits were inoculated, with both rabbits within a group being inoculated with one of the 6 extracts, namely adult crude (ACE), adult soluble (ASE), adult membrane-associated (AME), nymphal crude (NCE), nymphal soluble (NSE), or nymphal membrane-associated (NME) extracts. The animals within the remaining two groups were maintained as tick-naïve controls.

Goats were divided into four groups of two. Two groups of goats were inoculated, one with the adult crude extract (ACE) and the other with the nymphal crude extract (NCE). The remaining two groups were maintained as tick-naïve controls.

All hosts were inoculated with the tick extracts at a dose of 1.5mg.kg^{-1} body weight of host (McGowan *et al.*, 1980; Heller-Haupt *et al.*, 1989). Prior to inoculation, each extract was thoroughly mixed with $50\mu\text{g}$ Quil-A adjuvant in $100\mu\text{l}$ PBS using the method of Heller-Haupt *et al.* (1989). The suspended extracts were administered intramuscularly in the upper hind-leg using standard sterile techniques. All inoculated hosts (both rabbits and goats) received two booster inoculations on days 14 and 21.

2.5 TICK CHALLENGE

2.5.1 Rabbits

Four weeks after the third inoculation, all experimental rabbits and four tick-naïve (control) rabbits were challenged with 10 male and 10 female *A. hebraeum* adults. The ticks were applied to the animals' backs in body bags which were secured with drawstrings immediately posterior to the fore-legs and anterior to the hind-legs. In order to allow male ticks to attach and start releasing pheromones, they were applied five days before the females were included. Rubber collars were placed around the rabbits' necks to prevent them from removing or damaging the body bags.

Eight weeks after the third inoculation, rabbits inoculated with adult tick extracts (ACE, ASE or AME) and two of the four control rabbits were challenged with a further 10 male and 10 female adult *A. hebraeum*. Simultaneously, rabbits inoculated with nymphal tick extracts (NCE, NSE or NME) and the remaining two control rabbits were challenged with 50 *A. hebraeum* nymphs. Nymphs were placed in ear-bags attached to the base of the ear with adhesive tape. Rubber collars were used to prevent rabbits from removing or damaging the ear-bags.

Following each infestation, body or ear-bags were examined daily and fully engorged, detached ticks were collected. Engorged females were counted, weighed and maintained in individual vials at 25°C and 85% humidity to lay eggs. Total egg weights per female tick were recorded. Engorged nymphs were counted, weighed and kept at 25°C and 85% humidity to determine the percentage moulting success.

Finally, the site of tick attachment on inoculated rabbits was monitored. Macroscopic changes were noted and compared with those found at tick attachment sites on control rabbits.

2.5.2 Goats

Four weeks after the third inoculation, all inoculated goats and three tick-naïve (control) goats were placed in individual cages and challenged with 10 male and 10 female *A. hebraeum* adults on the right ear, and 50 *A. hebraeum* nymphs on the left ear. Male ticks were allowed five days to attach and start releasing pheromones before the females and nymphs were applied to the right and left ears respectively. All goats were rechallenged with a further 50 nymphs 8 and 12 weeks post-inoculation. Both *A. hebraeum* adults and nymphs were applied in bags placed over the goats' ears and secured with adhesive tape. The goats were unrestrained, but the bases of the ear-bags were taped to the adjacent horns in order to prevent them from hanging in the drinking water or being removed by grooming. Following each infestation, ticks were collected daily and processed in the same manner as discussed earlier for tick recovery from rabbits.

Grooming has been reported to be an important component of tick/host interactions (Bonsma, 1944; Bennett, 1969). The role of grooming in the response of Boer goats to infestation by *A. hebraeum*, and whether grooming was enhanced by inoculation of hosts with *A. hebraeum* adult or nymphal crude extracts was examined. Inoculated and control goats, all of which had previously been challenged once with *A. hebraeum* adults and three times with nymphs, were challenged with a further 100 nymphs (50 per ear). A further two goats, with no previous exposure to ticks, were similarly challenged. Nymphs were applied in ear-bags secured to the ears with adhesive tape and taped to the adjacent horns. After allowing 48 hours for tick attachment, one ear-bag was removed from each goat to allow for grooming while the other ear remained covered. Ear-bags, cages and the surrounding area were examined daily for fully engorged ticks dropping from the ungroomed and groomed ears respectively.

2.6 STATISTICAL ANALYSIS

One-way analysis of variance was used to compare differences between the mean engorgement weight, mean egg weight and mean engorgement time of ticks engorged on ACE-, ASE-, AME-, NCE-, NSE- and NME-inoculated hosts with those of ticks engorged on tick-naïve (control) hosts. Data was analyzed using Statgraphics Version 5.0.

2.7 SKIN PROVOCATION TESTING

The type and degree of hypersensitivity induced in rabbits and goats by inoculation with a crude extract of adult *A. hebraeum* females (ACE) was determined using intradermal skin reaction tests (Fivaz, 1989). Initially, different quantities of antigen were administered at adjacent sites on a single rabbit in order to determine the optimum quantity (30µg) required to obtain a skin reaction.

2.7.1 Rabbits

30 μ g (10 μ l) of ACE was injected intradermally into shaved sites on the lumbar region of each of the ACE-inoculated and control rabbits using a standard tuberculin syringe. An equal volume of TAS blender buffer was injected into adjacent sites on the ACE-inoculated rabbits as controls. Changes in skin thickness were determined 3, 6, 9, 12, 24, 48 and 72 hours after injection with the aid of measuring callipers. Results obtained with ACE-inoculated and tick-naïve (control) rabbits were compared with those obtained by Kraidy (1991) following the intradermal injection (with 30 μ g of ACE) of rabbits previously infested five times with approximately 50 *A. hebraeum* nymphs.

2.7.2 Goats

ACE-inoculated and tick-naïve (control) goats were intradermally injected with 30 μ g (10 μ l) of ACE at a shaved site on the lateral thorax using a standard tuberculin syringe. Skin thickness changes were measured at 3, 6, 9, 12, 24, 48 and 72 hours after injection using measuring callipers.

Riek (1956) reported raised blood histamine concentrations in tick-resistant cattle. Histamine is now known to be released during an immediate IgE-mediated skin hypersensitivity. In order to examine the role of histamine in skin reactions to ACE, ACE-inoculated goats were injected intravenously with the antihistamine promethazine hydrochloride ('Phenegan', Maybaker, England), at a dose rate of 2mg.kg⁻¹, one hour prior to the intradermal injection of 30 μ g ACE. Skin thickness increases were compared with those of ACE-inoculated goats injected with ACE but having had no prior treatment with an antihistamine.

2.8 HISTOLOGY

2.8.1 Cellular Changes in Rabbits in Response to Inoculation with Tick Antigens

Cellular changes associated with tick feeding on ACE-inoculated rabbits were studied histologically. After the third inoculation, rabbits were intradermally injected with 30 μ g (10 μ l) of ACE at four sites in a shaved area on the lumbar region. A fifth site was injected with an equal volume of TAS blender buffer as a control. Skin biopsy samples were taken from the four sites injected with ACE 3, 12, 24 and 48 hours after injection, and from the site injected with TAS blender buffer 24 hours after injection. Biopsies were carried out by infiltrating the skin with 2% xylocaine (Xylotox, RBM, UK) and taking punch biopsies in the conventional manner. Skin samples were fixed in 10% buffered formalin and submitted to a consultant pathologist for diagnostic examination. Sections were stained with Giemsa, PAS, and Haematoxylin and Eosin. Sections stained with Haematoxylin and Eosin were photographed at magnifications of 40 and 250x.

2.8.2 Histological Changes in the Tick Gut Following Feeding on Inoculated Hosts

Vaccination of hosts with various tick extracts has been reported to result in observable damage to the gut of engorging ticks (Agdebe & Kemp, 1986; Kemp *et al.*, 1986). In order to determine whether inoculation with *Amblyomma hebraeum* extracts resulted in damage to the gut of subsequently engorged ticks, transmission electron microscopy (TEM) of the gut of several adult ticks fed on rabbits inoculated with *A. hebraeum* extracts was carried out. The protocol followed for the preparation of gut tissue for TEM was that of Cross (1987).

Eight *Amblyomma hebraeum* adults engorged to repletion on rabbits previously inoculated with *A. hebraeum* adult extracts (ACE, ASE or AME), and two adults engorged to repletion on tick-naïve (control) rabbits were collected, killed by chilling at -20°C for 20 minutes, and embedded in wax in order that the gut could be dissected out. Dissected gut was rinsed three times in PBS (pH 7.2) to remove host blood or blood products. Gut was then cut into sections approximately 2mm long, placed in a 0.2M cacodylate-buffered 2.5% glutaraldehyde solution (pH 7.1) and refrigerated overnight. Gut sections then underwent

two 10 minute washes in PBS (pH 7.2) before being placed in fixative (1% osmium tetroxide in phosphate buffer) for 90 minutes. The fixative was removed with a further two PBS washes, and gut tissue was dehydrated through an ascending alcohol series from 30% - 100%, with two changes of absolute alcohol. Gut tissue was placed in propylene oxide for two 15 minutes periods (using fresh propylene oxide each time). Infiltration was carried out with a propylene oxide:resin (Taad/Araldite) series in the proportions 3:1, 1:1 and 1:3 for 60 minutes each, and then in pure resin overnight. The tissue was finally placed in pure resin in specimen moulds, and polymerised for 36 hours at 60°C. Blocks were removed from the moulds and trimmed to facilitate sectioning. Sections were cut to a silver/gold interface (60 - 70nm thick) using an 8800 Ultratome III (LKB Bromma) sectioner, and carefully mounted on 300nm mesh copper grids.

Sections were stained as follows. Parafilm was placed in the base of two petri-dishes. Sodium hydroxide pellets were placed around the parafilm in one petri-dish and left for at least 5 minutes in order to create a carbon-dioxide free environment. Six drop of freshly prepared 5% aqueous uranyl acetate were placed on the parafilm in the second petri-dish. One grid, with the sections facing downwards, was placed on each uranyl acetate droplet and left for 30 minutes. Six droplets of lead acetate were placed on the parafilm in the first petri-dish (containing the sodium hydroxide pellets). Lead acetate was prepared by adding 10ml of well shaken 4.4% lead nitrate and 5.9% sodium citrate to 2.6ml freshly prepared 1M sodium hydroxide and shaking gently until cloudiness disappeared, and finally adding 4.0ml distilled water (dH₂O). Grids were removed from the uranyl acetate, rinsed twice by pipetting approximately 5ml of dH₂O over them, dried by blotting on filter paper and carefully placed on the lead acetate droplets (allowing as little CO₂ as possible into the petri-dish). After five minutes, grids were removed, rinsed and blotted as described above. Sections were then viewed and photographed using a JOEL JEM 100 CX11 transmission electron-microscope operated at 80K.V..

2.9 SEROLOGY

All hosts were bled prior to inoculation, after the third vaccination and after each tick infestation. Rabbits were bled by 'nicking' the marginal ear vein with a sharp razor blade

and collecting the blood in a test tube. Due to the limited amount of sera obtained when bleeding rabbits, sera obtained from rabbits within the same treatment group was pooled at each bleed. Goats were bled from the jugular vein using regular Vac-U-Test tubes.

Blood was refrigerated overnight before being centrifuged at 2500rpm for 10 minutes (using a Searle-B400 bench-top centrifuge). Serum was drawn off, aliquoted and stored at -20°C until required for serological survey. Once thawed, sera could be stored for a further month at 4°C by adding 0.1% thiomersal to prevent bacterial growth (Garvey *et al.*, 1977).

2.9.1 Enzyme-Linked immunosorbent assays (ELISA)

Enzyme-linked immunosorbent assays were conducted to quantify the anti-*Amblyomma hebraeum* IgG titres raised in host animals following inoculation with extracts of *A. hebraeum*. The protocol followed was a modification of that used by Clark & Adams (1977).

2.9.1.1 ELISA - Rabbit Sera.

Optimal working dilutions of antigen and conjugate were determined by checkerboard titration. The wells of 96 well flat-bottomed microtitre plates (Sero-well) were coated with 100µl of either ACE or NCE diluted to 12ug.ml⁻¹ in antigen coating buffer (10mM PBS pH 7.4, 150mM NaCl). Plates were incubated overnight at 4°C. Coating solution was poured off and the wells were thoroughly washed by flooding them three times with washing buffer (10mM PBS pH 7.4, 150 mM NaCl, 0.05% Tween 20) and leaving for several minutes between the washes. Washing is vital for the removal of traces of soluble reactants which may cause non-specific binding (Clark & Adams, 1977).

100µl of blocking solution (10mM PBS pH 7.4, 150mM NaCl, 2% Elité milk powder) was placed in each well and plates were incubated at 37°C for 1 hour to prevent non-specific binding. Clarke & Adam (1977) used bovine serum albumin (BSA) as the blocking agent. However, Elité milk powder was found to inhibit non-specific binding as efficiently as BSA. Blocking solution was removed by washing three times with washing buffer.

100 μ l of four-fold dilutions of test and control sera, made up in antibody diluent (10mM PBS pH7.4, 150mM NaCl, 0.05% Tween 20, 2% Elité milk powder), were added to each well. Plates were incubated at room temperature for 30 minutes, after which wells were washed three times in washing buffer.

100 μ l of goat anti-rabbit IgG alkaline phosphatase conjugate (Bio-makor) diluted 1 : 2000 in washing buffer was then added to each well. The plates were incubated on a rocker at room temperature for two hours and subsequently washed three times in washing buffer and three times in distilled water.

200 μ l of freshly prepared alkaline phosphatase substrate (0.1% p- nitrophenyl phosphate, 10% diethanolamine, pH 9.8) was added to each well and plates were incubated on a rocker at room temperature. After 30 minutes the enzymatic reaction was stopped by adding 50 μ l 3.0M NaOH to each well. Results were obtained by reading the absorbance of each well at 400nm on a Dynatech ELISA minireader II.

Blanking of the ELISA minireader was done by reading the absorbance of wells a) lacking coating antigen, b) lacking test serum, and c) containing alkaline phosphatase substrate only.

2.9.1.2 ELISA - Goat Sera

The protocol used for titrating goat sera using the ELISA technique was essentially similar to that used for rabbit sera. However, in this case rabbit anti-goat IgG horseradish peroxidase conjugate (Bio-Rad) was used and a different substrate was required.

As with the anti-rabbit IgG alkaline phosphatase conjugate, the anti-goat IgG horseradish peroxidase conjugate was diluted 1:2000. The substrate consisted of 0.1mg 3'3'5'5'-tetramethylbenzidine (TMB), 0.1ml dimethylsulfoxide and 9.9ml 0.1M sodium acetate (pH 6.0) mixed and filtered through Whatman No. 1M filter paper, with 0.01% hydrogen peroxide (Harlow & Lane, 1988). 100 μ l of substrate was added to each well and incubated for 10-30 minutes. The enzymatic reaction was stopped by adding 100 μ l 1M H₂SO₄.

Absorbance was read at 400nm on a Dynatech minireader II. The procedure for blanking the minireader was essentially similar to that followed when determining the rabbit IgG titres, with the exception that the third blanking lane contained horseradish peroxidase substrate rather than alkaline phosphatase substrate.

2.9.2 Western Blotting

Crude tick extracts (ACE and NCE) were resolved by discontinuous SDS polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970) using stacking and resolving gels of 4% and 10% respectively. Gels and blots were analyzed with the aid of a UVP Gel Analyzing computer software package.

2.9.2.1 Western blot - Rabbit sera

Serum obtained from all rabbits inoculated with adult extracts (ACE, ASE or AME) was screened against ACE proteins, while serum obtained from rabbits inoculated with nymphal extracts (NCE, NSE or NME) was screened against NCE proteins. Each polyacrylamide gel used to resolve the extracts had 10 sample lanes, nine were loaded with the tick extract while the tenth lane was loaded with either Crombithek (Boehringer Mannheim) or Sigma molecular weight marker proteins.

Electrophoresis was performed for approximately 16 hours at 40V. At the end of electrophoresis, the marker lane and one sample lane were excised and stained with Coomassie brilliant blue in order to visualize the resolved proteins. This section of gel was preserved by vacuum drying on a slab gel drier for three hours, using heating for the first hour. The remaining section of gel was placed in prechilled transfer buffer (25mM Tris, 190mM glycine, 20% methanol) and left to equilibrate for 30 minutes.

The Bio-Rad transblotting cell was used for the transfer of proteins from the gel to nitrocellulose paper (Hoeffer Scientific). Fibre pads, pre-cut Whatman No. 3M filter paper and nitrocellulose strips (approximately 8mm wide) were pre-soaked in chilled transfer buffer. Nitrocellulose paper was handled with forceps to prevent unwanted foreign proteins attaching to the paper.

Once the gel had equilibrated, it was placed on a piece of filter paper on top of a fibre pad. Nitrocellulose strips were carefully placed over the lanes containing the proteins, ensuring that no air bubbles were trapped between the gel and nitrocellulose. A second piece of filter paper and fibre pad were then placed over the nitrocellulose. This 'sandwich' was held together in a plastic holder. The holder was then placed in the transfer tank containing transfer buffer with the gel closest to the anode and the nitrocellulose closest to the cathode. The transfer unit was placed on a magnetic stirrer to keep the buffer circulating, and water cooling was used. Transfer took place at 0.15A for 23 hours at approximately 22°C.

At the end of the transfer period, the nitrocellulose paper was placed in a 2% gelatine in TPBS (PBS pH 7.2, 5% Tween 20) solution and incubated at 37°C for 60 minutes. The gelatine solution was removed and nitrocellulose strips were washed 3 x 10 minutes with TPBS solution prior to being placed in a 5% gelatine in TPBS solution and incubated at 4°C overnight. Nitrocellulose was again washed three times in TPBS, before individual nitrocellulose strips were incubated in the test serum diluted 1:75 in TPBS for 2 hours. After washing three times in TPBS, nitrocellulose was then incubated for one hour in goat anti-rabbit IgG horseradish peroxidase conjugate (Bio-Rad) diluted (1:6000) in TPBS.

Following a final three washes in TPBS, nitrocellulose strips were rinsed for 20 minutes in freshly prepared colour solution consisting of 20ml of 0.3% HRP-colour development reagent (Bio-Rad) in chilled methanol and protected from light, mixed with 100ml of 0.06% chilled hydrogen peroxide in TPBS. The developed blot was then placed in chilled dH₂O for 5 minutes before being air dried and stored in the dark. As colour faded within a couple of hours photographs were taken as soon as the blots were dry.

2.9.2.2 Western blot - Goat sera

The protocol was essentially similar to that followed when testing the rabbit sera, except that two Sigma SDS-PAGE molecular weight marker protein kits were used, and the dilution used for the rabbit anti-goat IgG horseradish peroxidase conjugate (Sigma) was 1:4000.

CHAPTER THREE:

RESULTS:

IMMUNITY OF RABBITS TO *AMBLYOMMA HEBRAEUM* **FOLLOWING INOCULATION WITH *A. HEBRAEUM* EXTRACTS**

3.1 TICK CHALLENGE

Unfortunately one rabbit inoculated with *Amblyomma hebraeum* nymphal membrane-associated extract (NME) died from an unrelated cause, prior to tick challenge. Consequently, results presented for the response of ticks feeding on NME-inoculated rabbits, represent the response of ticks fed on a single host only.

3.1.1 Tick engorgement weights

Inoculation of rabbits with either *Amblyomma hebraeum* adult crude (ACE), adult soluble (ASE) or adult membrane-associated (AME) extracts did not result in a significant decline (at the 95% level of confidence) in mean engorgement weights of female *A. hebraeum* during a primary tick challenge (Table 2). Unfortunately no results were obtained for a second adult infestation of ACE-inoculated rabbits as both rabbits died from unrelated causes. However, adults ticks fed during a second infestations of the ASE- and AME-inoculated rabbits showed no significant decline in engorgement weight when compared with ticks fed on tick-naive controls (Table 2).

Adult *A. hebraeum* fed on rabbits inoculated with *A. hebraeum* nymphal crude extract (NCE) had significantly higher engorgement weights ($1.733 \pm 0.213\text{g}$) than those fed on tick-naive (control) rabbits ($1.099 \pm 0.180\text{g}$). Nymphs fed on NCE-inoculated hosts during a subsequent infestation had significantly lower engorgement weights ($0.040 \pm 0.004\text{g}$) than those fed on the control rabbits ($0.052 \pm 0.004\text{g}$). Inoculation with the nymphal soluble

extract (NSE) and nymphal membrane-associated extract (NME) had no significant effect on either adult or nymphal tick engorgement weights (Table 2).

Table 2: Mean engorgement weights of *Amblyomma hebraeum* engorged on rabbits inoculated with *A. hebraeum* adult crude (ACE), adult soluble (ASE), adult membrane-associated (AME), nymphal crude (NCE), nymphal soluble (NSE) or nymphal membrane-associated (NME) extract, and on tick-naïve (control) rabbits.

Treatment	Number of hosts	1° Adult Infestation		2° Adult Infestation		1° Nymphal Infestation	
		Mean weight of ticks* (g) ± SE	Significance p value**	Mean weight of ticks* (g) ± SE	Significance p value**	Mean weight of ticks* (g) ± SE	Significance p value**
ACE	2	0.723 ± 0.174	ns	-	-	-	-
ASE	2	0.512 ± 0.143	ns	0.831 ± 0.158	ns	-	-
AME	2	1.034 ± 0.379	ns	1.449 ± 0.184	ns	-	-
Control	2	0.723 ± 0.245	-	1.378 ± 0.088	-	-	-
NCE	2	1.733 ± 0.213	p < 0.05	-	-	0.040 ± 0.004	p < 0.05
NSE	2	1.421 ± 0.167	ns	-	-	0.053 ± 0.003	ns
NME	1	0.727 ± 0.148	ns	-	-	0.046 ± 0.003	ns
Control	2	1.099 ± 0.180	-	-	-	0.052 ± 0.004	-

* means of cumulative total dropped from rabbits

** significance when compared with controls

3.1.2 Duration of tick engorgement

Adult female *A. hebraeum* took considerably longer than the 6-12 days that is considered normal (Norval, 1974) to engorge on all rabbits during a primary tick challenge. However, ticks fed on rabbits inoculated with adult extracts (ACE, ASE or AME) showed no significant increase in engorgement time (period of attachment to repletion) when compared with those fed on tick-naïve (control) rabbits (Table 3). However, during a second challenge adult ticks on both the ASE- and AME-inoculated hosts took significantly longer to engorge (12.63 ± 0.29 and 14.07 ± 0.77 days respectively) than those fed on control rabbits (9.60 ± 0.68 days). No results were obtained for a second adult infestation of the ACE-inoculated rabbits, due to the death of both rabbits (Table 3).

Inoculation of rabbits with nymphal extracts (NCE, NSE or NME) had no significant effect on mean adult engorgement time. The mean engorgement times of nymphs fed on NCE- and NME-inoculated rabbits (10.06 ± 0.25 days and 9.05 ± 0.30 days respectively) were however both significantly longer than that of nymphs fed on the control rabbits (8.08 ± 0.25 days). In contrast, the mean engorgement time of nymphs fed on NSE-inoculated hosts (7.03 ± 0.19 days) was significantly shorter than that of nymphs fed on the control rabbits (Table 3).

Table 3: Mean times taken by *Amblyomma hebraeum* to engorge to repletion on rabbits inoculated with *A. hebraeum* adult crude (ACE), adult soluble (ASE), adult membrane-associated (AME), nymphal crude (NCE), nymphal soluble (NSE) or nymphal membrane-associated (NME) extract, and on tick-naïve (control) rabbits.

Treatment	Number of hosts	1° Adult Infestation		2° Adult Infestation		1° Nymphal Infestation	
		Mean engorgement time* (days) \pm SE	Significance p value**	Mean engorgement time* (days) \pm SE	Significance p value**	Mean engorgement time* (days) \pm SE	Significance p value**
ACE	2	35.40 ± 3.44	ns	-	-	-	-
ASE	2	39.00 ± 2.97	ns	12.63 ± 0.29	$p < 0.01$	-	-
AME	2	41.50 ± 1.15	ns	14.07 ± 0.77	$p < 0.05$	-	-
Control	2	38.60 ± 1.59	-	9.60 ± 0.68	-	-	-
NCE	2	10.94 ± 0.78	ns	-	-	10.06 ± 0.25	$p < 0.01$
NSE	2	8.79 ± 0.44	ns	-	-	7.03 ± 0.19	$p < 0.01$
NME	1	9.73 ± 0.83	ns	-	-	9.05 ± 0.30	$p < 0.05$
Control	2	10.56 ± 0.98	-	-	-	8.08 ± 0.25	-

* period of attachment to repletion

** significance when compared with controls

3.1.3 Percentage ticks engorged to repletion

Inoculation of rabbits with adult extracts (ACE, ASE or AME) did not appear to result in a significant decline in the number of ticks that successfully engorged to repletion during a primary or secondary tick challenge. Similarly, inoculation with nymphal extracts (NCE, NSE or NME) had little effect on the number of adults or nymphs engorging successfully (Table 4).

Table 4: Percentages of *Amblyomma hebraeum* that engorged to repletion on rabbits inoculated with *A. hebraeum* adult crude (ACE), adult soluble (ASE), adult membrane-associated (AME), nymphal crude (NCE), nymphal soluble (NSE) or nymphal membrane-associated (NME) extract, and on tick-naïve (control) rabbits.

Treatment	Number of hosts	1° Adult Infestation	2° Adult Infestation	1° Nymphal Infestation
		Adult engorgement (%)	Adult engorgement (%)	Nymphal engorgement (%)
ACE	2	25,0	-	-
ASE	2	50,0	80,0	-
AME	2	30,0	65,0	-
Control	2	45,0	40,0	-
NCE	2	85,0	-	48,0
NSE	2	95,0	-	58,0
NME	1	100,0	-	42,0
Control	2	90,0	-	60,0

3.1.4 Tick fecundity and moulting success

Numerous female ticks (engorged on both inoculated and control hosts) died prior to egg laying due to a suspected bacterial or fungal infection. These females were excluded from calculations of the mean egg weight produced by any one group. Analysis of variance showed no significant decline in mean egg weights produced by ticks fed during a primary challenge of hosts inoculated with adult extracts (ACE, ASE or AME). However, ticks fed during a secondary challenge of hosts inoculated with ASE were found to produce significantly reduced egg masses ($0.027 \pm 0.011\text{g}$) than ticks fed on the control rabbits ($0.503 \pm 0.151\text{g}$) (Table 5). No results were obtained for a second adult infestation of the ACE-inoculated rabbits as both rabbits died from unrelated causes prior to the second tick challenge.

Inoculation of rabbits with nymphal extracts (NCE, NSE or NME) had no significant effect on mean egg weights produced by engorged *A. hebraeum*. Percentage moult of nymphs off NCE- and NSE- inoculated rabbits were 62.0 and 69.9% respectively, which is

considerably lower than the 94.8% moulting success of nymphs fed on control rabbits. However, inoculation with NME had little observable effect on moulting success (Table 5).

Table 5: Mean egg weights from, and percentage moulting success of, *Amblyomma hebraeum* fed on rabbits inoculated with *A. hebraeum* adult crude (ACE), adult soluble (ASE), adult membrane-associated (AME), nymphal crude (NCE), nymphal soluble (NSE) or nymphal membrane-associated (NME) extract, and on tick-naïve (control) rabbits.

Treatment	Number of hosts	1° Adult Infestation		2° Adult Infestation		1° Nymphal Infestation
		Mean weight of eggs* (g) ± SE	Significance p value**	Mean weight of eggs* (g) ± SE	Significance p value**	Moulting success (%)
ACE	2	0.560 ± 0.174	ns	-	-	-
ASE	2	0.510 ± 0.142	ns	0.027 ± 0.011	p < 0.01	-
AME	2	0.743 ± 0.130	ns	0.491 ± 0.136	ns	-
Control	2	0.620 ± 0.162	-	0.502 ± 0.151	-	-
NCE	2	0.513 ± 0.136	ns	-	-	62.0
NSE	2	0.523 ± 0.109	ns	-	-	69.9
NME	1	0.353 ± 0.094	ns	-	-	100.0
Control	2	0.412 ± 0.120	-	-	-	94.8

* means of eggs laid by surviving female ticks

** significance when compared with controls

3.1.5 Macroscopic changes at the tick attachment site

Infestation of all inoculated rabbits and the tick-naïve (control) rabbits with *A. hebraeum* adults resulted in severe skin reactions, possibly due to the rabbit being an unnatural host for adult *A. hebraeum*. The reactions included severe oedema of the skin and the secretion of a purulent exudate from the sites of tick attachment within 72 hours of attachment. Despite the severity of the reaction, adult ticks were able to engorge to repletion.

Amblyomma hebraeum nymphal infestations of rabbits previously inoculated with the nymphal extracts (NCE, NSE or NME) resulted in visible oedema of the ear-pinnae and evidence of a moist exudate on the surface of the skin within 48-72 hours of nymphal attachment (Figure 2a). The wet exudate later dried, forming crusts (Figure 2b).

Occasionally attached nymphs were entrapped within the crusts and died. No skin reaction was evident in the tick-naïve (control) group (Figure 2c).



Figure 2a: Site of tick attachment 72 hours after nymphal infestation of a NCE-inoculated rabbit, showing evidence of a moist exudate (me).



Figure 2b: Site of tick attachment eight days after nymphal infestation of a NCE-inoculated rabbit, showing the dried exudate (de) forming a crust on the surface of the skin.



Figure 2c: Site of tick attachment 72 hours after nymphal infestation of a tick-naive (control) rabbit, with no evidence of a skin reaction.

3.2 SKIN PROVOCATION TESTS

Due to the high costs involved in purchasing and maintaining livestock, treatment groups consisted of two rabbits only. Consequently, statistical analysis of skin reactions (such as the calculation of standard error) was not feasible and results are presented as means only.

Rabbits previously inoculated with *Amblyomma hebraeum* adult crude extract (ACE) yielded mild reactions within three hours of an intradermal injection with ACE, and skin thickness increased to a maximum at 24 hours before declining. During an unrelated study, Kraidy (1991) recorded similar responses to the intradermal injection of ACE in repetitively infested rabbits. However, natural infestation appeared to stimulate a more intense delayed skin reaction (at 24 hours) than did inoculation (Figure 3).

The intradermal injection of tick-naive (control) hosts with ACE appeared to induce a very mild immediate reaction of a more transitory nature, with the peak reaction occurring 3-12

hours after injection. Responses of the ACE-inoculated rabbits to intradermal injections of TAS blender buffer were mild in comparison with those induced by the crude tick extract. Thereby proving that the response induced by injection with ACE was not attributable to irritation induced by the process of injection, or the buffer in which the tick extract was prepared (Figure 3).

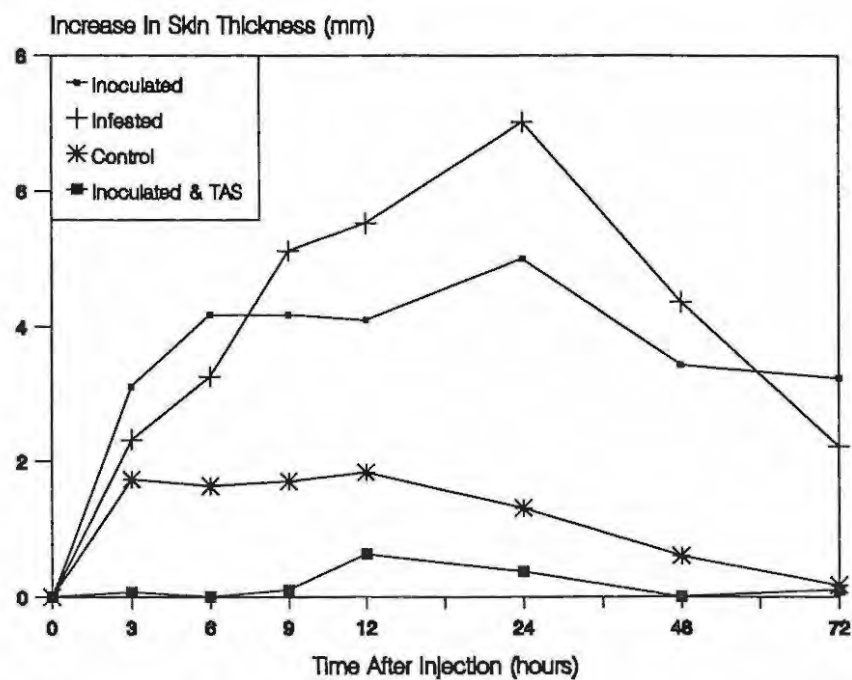


Figure 3: Skin responses of rabbits inoculated with *Amblyomma hebraeum* adult crude extract (ACE), repetitively infested rabbits (Kraidy, 1991) and tick-naïve (control) rabbits to the intradermal injection of 10 μ l of ACE. Results are compared with the skin response of ACE-immunized hosts to the intradermal injection of 10 μ l of TAS blender buffer.

3.3 HISTOLOGY

3.3.1 Cellular changes in response to tick antigens

The histological examination of the injection sites following the intradermal injection of rabbits that had previously been inoculated with *Amblyomma hebraeum* adult crude extract (ACE) with 10 μ l of either ACE or TAS blender buffer revealed significant differences in the type and intensity of cellular infiltration.

24 hours after injection with TAS Buffer.

A mild dermal oedema was evident. There were diffuse infiltrations of eosinophils, mast cells, lymphocytes, a few fibroblasts and the occasional heterophil (Figure 4a and b).

3 hours after injection with ACE.

A mild dermal oedema was noted, with diffuse, moderate infiltrations of eosinophils and an occasional mast cell and lymphocyte (Figures 5a and b).

12 hours after injection with ACE.

Similar to the above with eosinophils being the dominant cells in the reaction. However, a few more mast cells and heterophils were present.

24 hours after injection with ACE.

There was a focal area of necrosis with a massive eosinophil infiltration at the injection site (Figure 6a). Some macrophages and heterophils and a few peripheral mast cells were also present. The cell reaction in the rest of the dermis consisted of eosinophil infiltrations and the appearance of many more mast cells than in the earlier sections (Figure 6b and c).

48 hours after injection with ACE.

The focal area of necrosis was still evident. The cell reaction at the area of necrosis consisted predominantly of eosinophil and macrophage infiltrations. The rest of the dermis displayed a reduced eosinophil infiltration, while macrophages and lymphocytes were still present (Figures 7a and b).

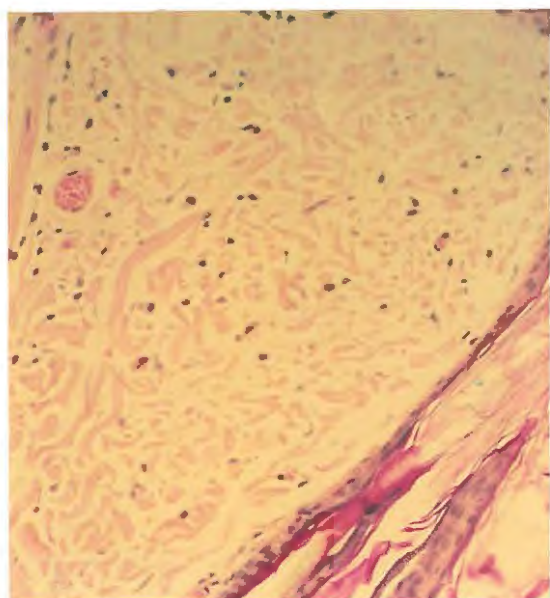


Figure 4a: 40x mag.

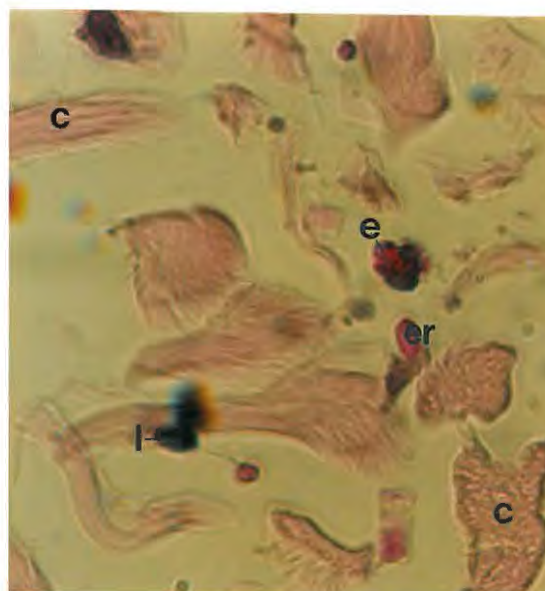


Figure 4b: 250x mag.

Figures 4a & b: Cellular changes at the site of injection of a rabbit immunized with *Amblyomma hebraeum* adult crude extract (ACE) 24 hours after an intradermal injection of 20 μ l TAS blender buffer. Collagen fibres (c), erythrocyte (er), eosinophil (e) and lymphocyte (l) are shown.

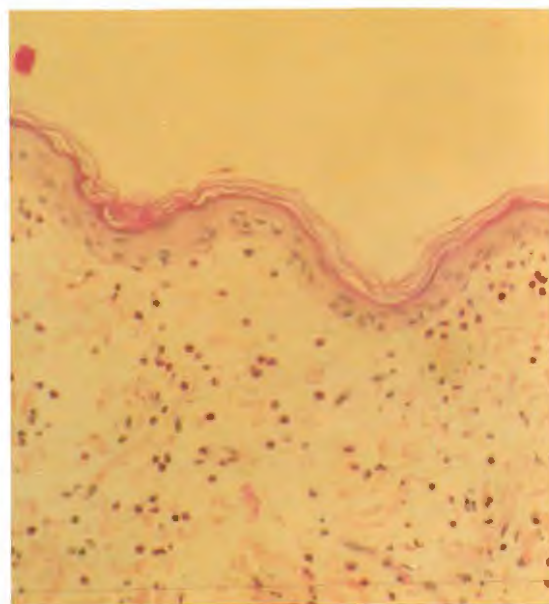


Figure 5a: 40x mag.

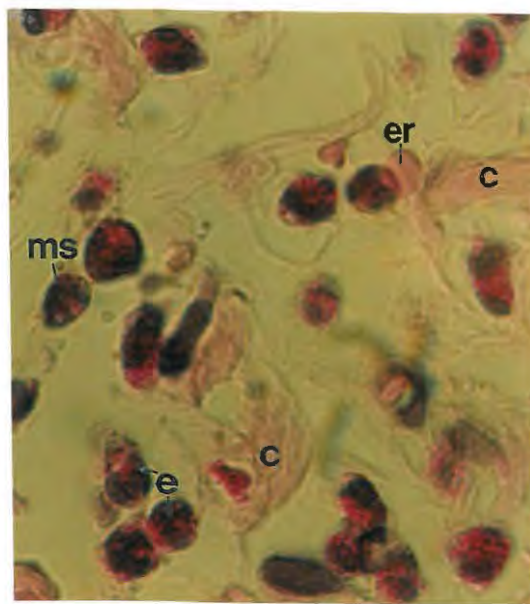


Figure 5b: 250x mag.

Figures 5a & b: Cellular changes at the site of injection of a rabbit immunized with *Amblyomma hebraeum* adult crude extract (ACE) 3 hours after an intradermal injection of 20 μ l ACE. Collagen fibres (c), erythrocyte (er), mast cells (ms) and eosinophils (e) are shown.

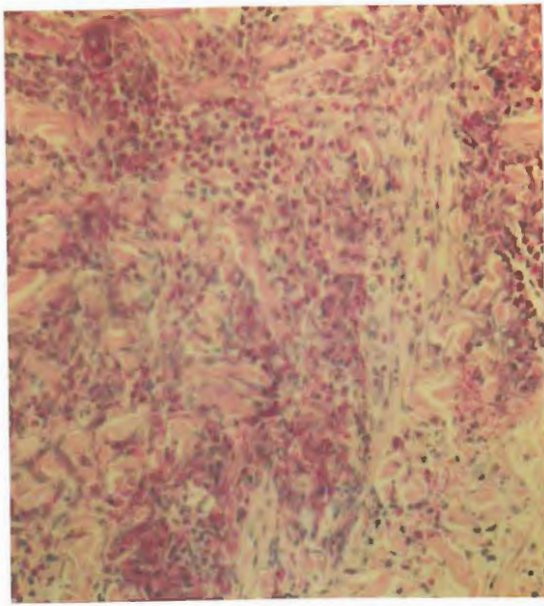


Figure 6a: Focal area of necrosis.
40x mag.

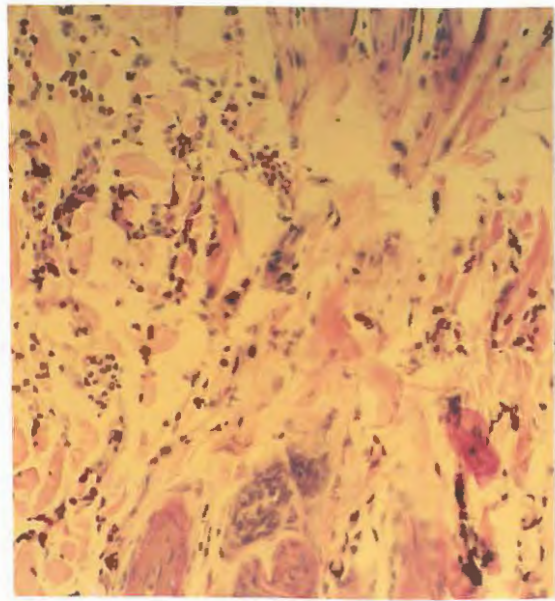


Figure 6b: 40x mag.

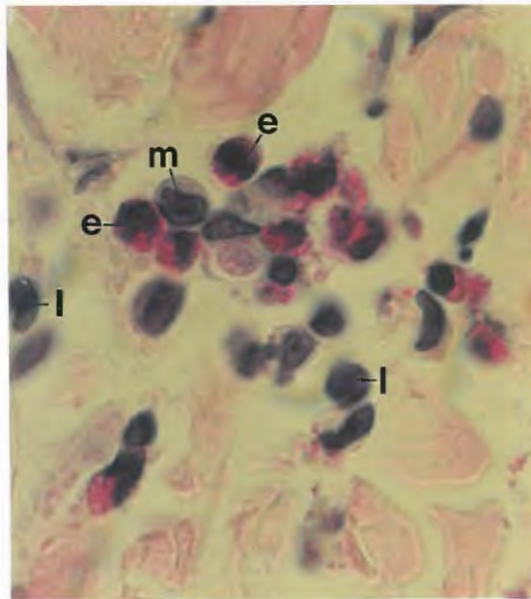


Figure 6c: 250x mag.

Figure 6a,b & c: Cellular changes at the site of injection of a rabbit immunized with *Amblyomma hebraeum* adult crude extract (ACE) 24 hours after an intradermal injection of 20 μ l ACE. Lymphocytes (l), eosinophils (e) and monocytes (m) are shown.

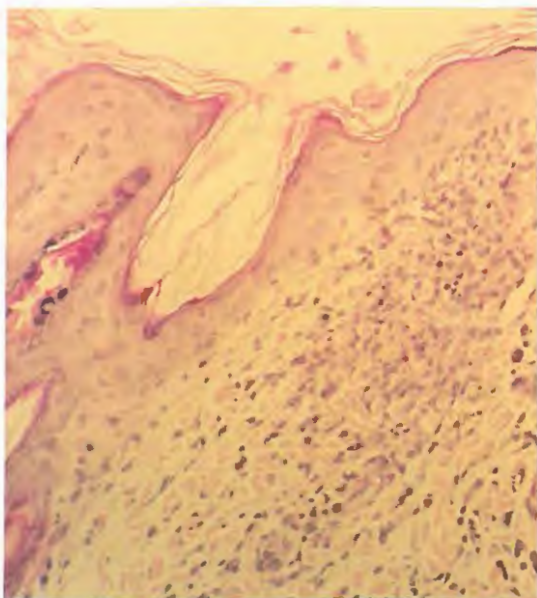


Figure 7a: 40x mag.

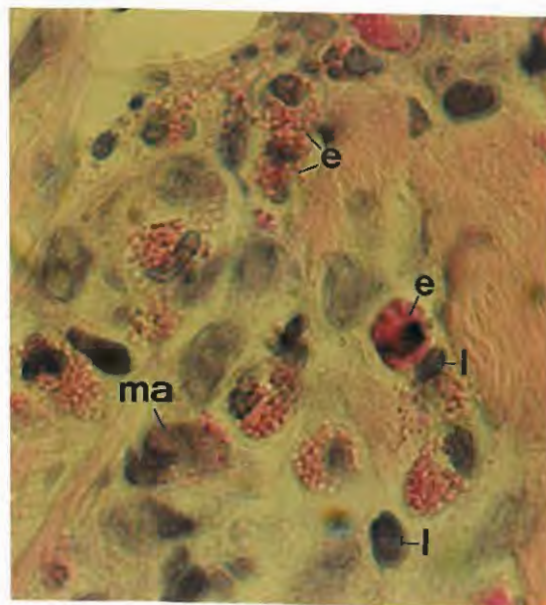


Figure 7b: 250x mag.

Figure 7a & b: Cellular changes at the site of injection of a rabbit immunized with *Amblyomma hebraeum* adult crude extract (ACE) 48 hours after an intradermal injection of 20 μ l ACE (48 hours). Lymphocytes (l), eosinophils (e) and macrophages (ma) are shown.

3.3.2 Histological changes in the tick gut following feeding on inoculated hosts

Electron microscopic studies indicated that only one of the eight adult *Amblyomma hebraeum* engorged on rabbits inoculated with *A. hebraeum* adult extracts (ACE, ASE or AME) that were studied, had visible gut damage. The ticks engorged on tick-naïve (control) hosts (Figure 8) and seven of the eight ticks fed on inoculated rabbits (Figure 9) had intact guts with no evidence of cellular damage. The gut-damaged tick (Figure 10) had fed on a rabbit inoculated with adult crude extract (ACE). Gut damage was indicated by the presence of lysed digestive cells and a basal lamina denuded of cells.

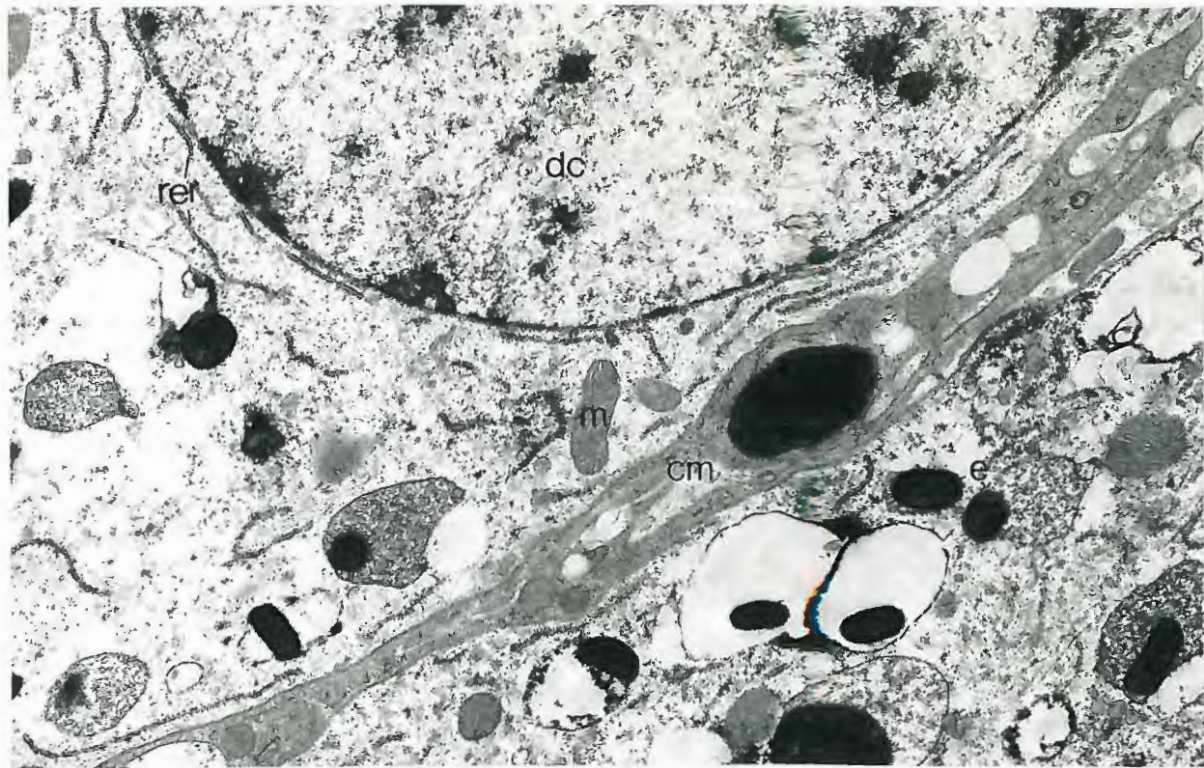


Figure 8: Section of mid-gut of an adult *Amblyomma hebraeum* engorged on a tick-naive (control) rabbit (7200 x mag). The digestive cell nucleus (dc); cytoplasmic membrane (cm); endosomes (e); mitochondrion (m) and rough endoplasmic reticulum (rer) are shown.

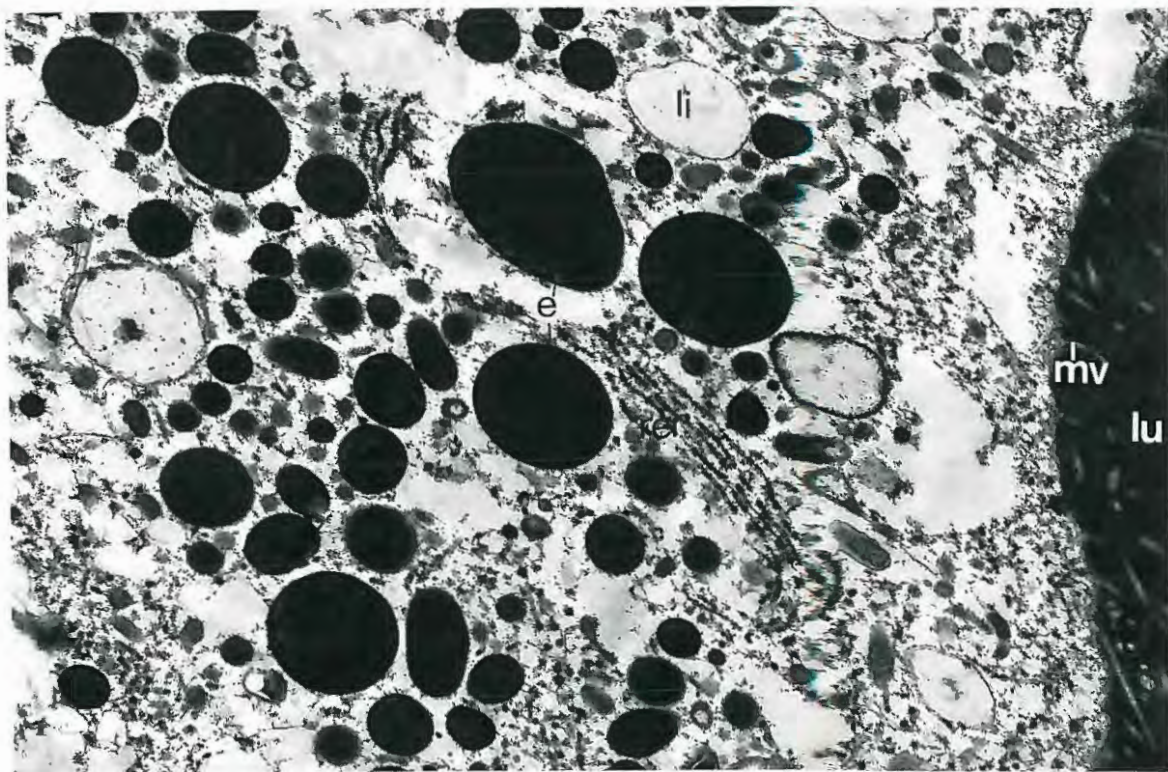


Figure 9: Section of midgut of an adult *Amblyomma hebraeum* engorged on a rabbit inoculated with *A. hebraeum* adult membrane-associated extract (AME) (7200 x mag). Endosomes in a digestive cell (e); rough endoplasmic reticulum (rer); lipid inclusions (li); gut lumen (lu) and microvilli (mv) are shown.

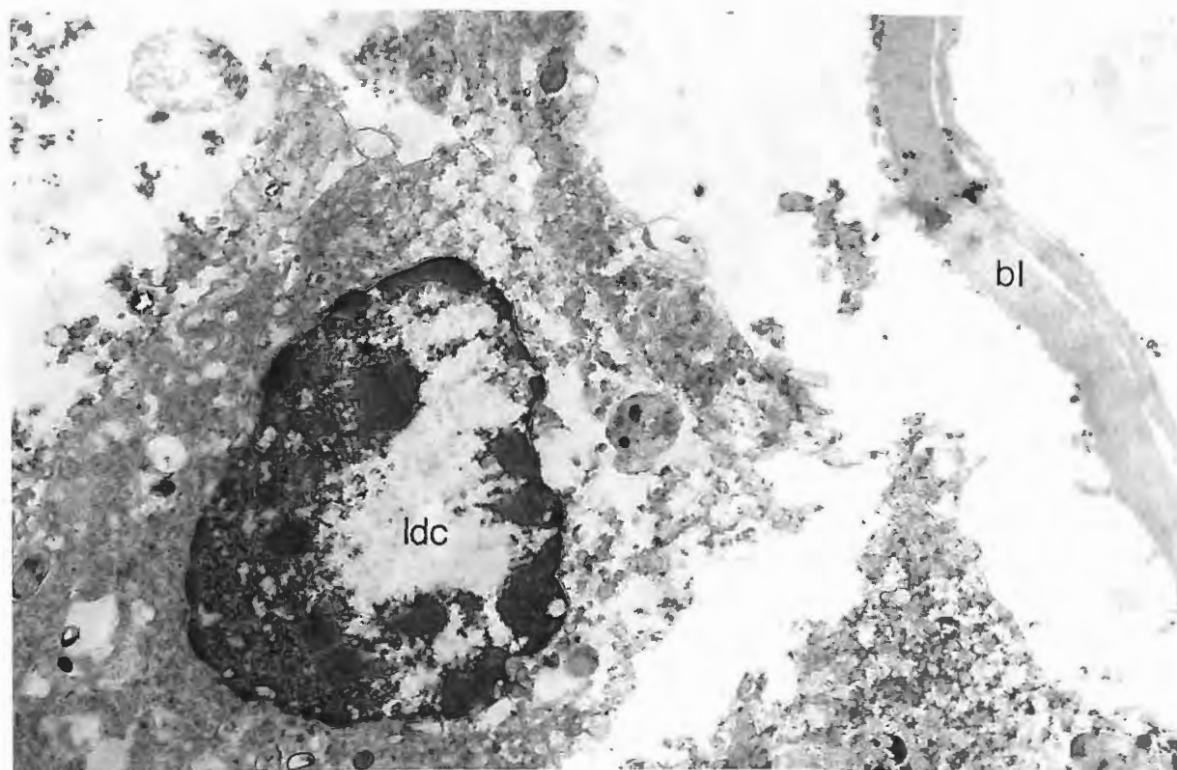


Figure 10: Section of midgut of an adult *Amblyomma hebraeum* engorged on a rabbit inoculated with *A. hebraeum* adult crude extract (ACE) (5800 x mag). A basal lamina denuded of cells (bl) and, a lysed digestive cell (ldc) are shown.

3.4 SEROLOGY

3.4.1 Enzyme-linked immunosorbent assay (ELISA)

Anti-*Amblyomma hebraeum* IgG titres were read off graphs (of absorbance versus antibody dilution) where absorbance at 400nm gave readings of 0.4 OD. The kinetics of the antibody responses of rabbits to inoculation with *A. hebraeum* extracts and subsequent infestations are shown in Figures 11 and 12.

In all cases, sera obtained from experimental and control rabbits, prior to inoculation or infestation, was found to have an antibody titre of lower than 1/5. Elevated IgG titres were detected in sera obtained from all the inoculated rabbits.

3.4.1.1 Rabbits inoculated with *Amblyomma hebraeum* adults extracts

Pooled sera of rabbits inoculated with the adult crude extract (ACE) developed a IgG titre of 1/2000, while the rabbits inoculated with adult soluble extract (ASE) and adult membrane-associated extract (AME) developed IgG titres of 1/1300 and 1/900 respectively (Figure 11). Subsequent infestations of inoculated hosts with adult *Amblyomma hebraeum* failed to further elevate the IgG titres produced by inoculation. Sera from all adult extract inoculated rabbits showed that anti-*A. hebraeum* IgG titres decreased in the 10 weeks after inoculation, despite exposure to the ticks.

The primary and secondary exposure of the uninoculated control rabbits to adult *A. hebraeum*, led to post-infestation IgG titres of 1/20 and 1/80 respectively (Figure 11).

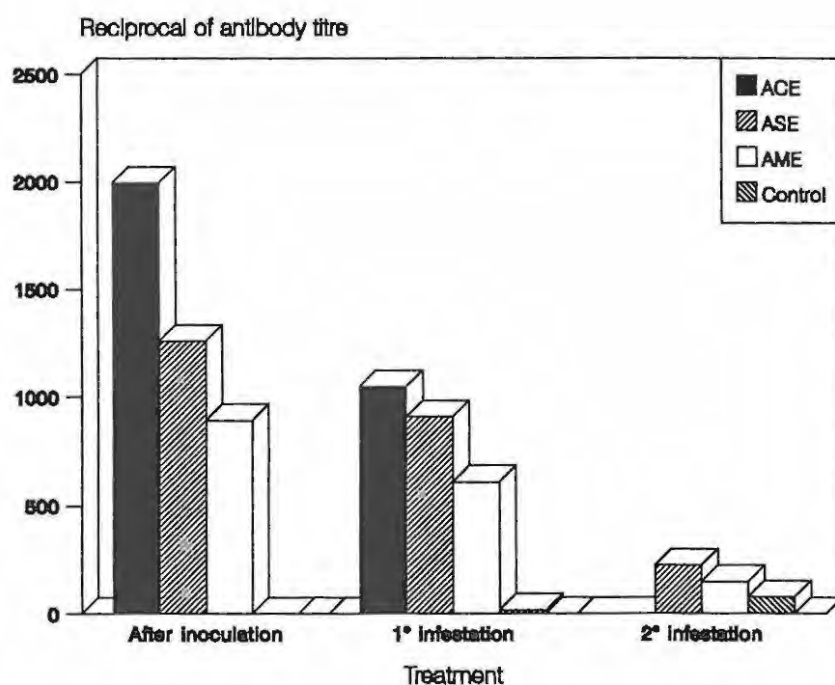


Figure 11: Specific anti-*Amblyomma hebraeum* IgG titres of rabbits inoculated with *A. hebraeum* adult crude (ACE), adult soluble (ASE) or adult membrane-associated (AME) extract are shown immediately after inoculation and after a primary and secondary adult *A. hebraeum* infestation. IgG titres are compared with those of tick-naïve (control) rabbits following each infestation.

3.4.1.2 Rabbits inoculated with *Amblyomma hebraeum* nymphal extracts

Pooled sera of rabbits inoculated with nymphal crude extract (NCE) displayed antibody titres of 1/400, while nymphal soluble extract (NSE) and nymphal membrane-associated extract (NME) inoculated rabbits displayed titres of 1/2800 and 1/3100 respectively (Figure 12). As with rabbits inoculated with adult extracts, infestation of rabbits with *A. hebraeum* failed to enhance the immune response induced by inoculation, and IgG titres declined in the 10 weeks after inoculation despite exposure to ticks.

Specific IgG titres of tick-naïve (control) rabbits increased after each infestation. Titres following the second tick challenge were found to be slightly higher (1/750) than those of *A. hebraeum* NCE- and NSE-inoculated hosts (1/200 and 1/650 respectively) (Figure 12).

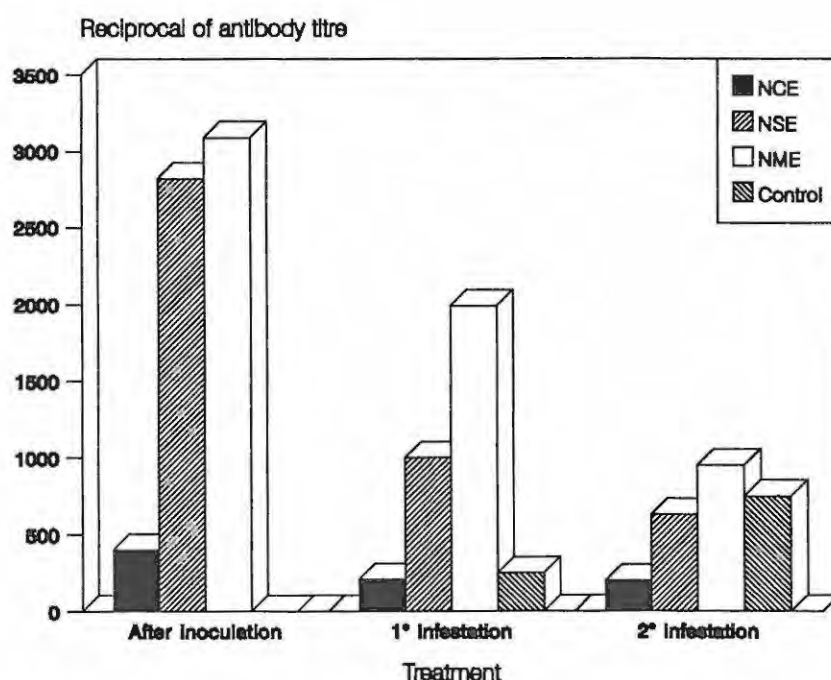


Figure 12: Specific anti-*Amblyomma hebraeum* IgG titres of rabbits inoculated with *A. hebraeum* nymphal crude (NCE), nymphal soluble (NSE) or nymphal membrane-associated (NME) extract and tick-naïve (control) rabbits are shown immediately after inoculation and after a primary and secondary adult *A. hebraeum* infestation.

3.4.2 Western blotting

Unfortunately polyacrylamide gels were found to expand slightly during the staining and destaining process and consequently the molecular weights of the bands on the blots can not be determined directly from the molecular weight marker proteins in the gel. Following staining of the gel, molecular weights of the resolved tick extract (ACE or NCE) proteins were determined with the aid of the UVP Gel Analysing computer software package. Where possible approximate molecular weights of the prominent bands on the blots have been determined by matching the banding pattern on the blot with the banding pattern of the resolved tick extract that was stained together with the marker proteins in the gel.

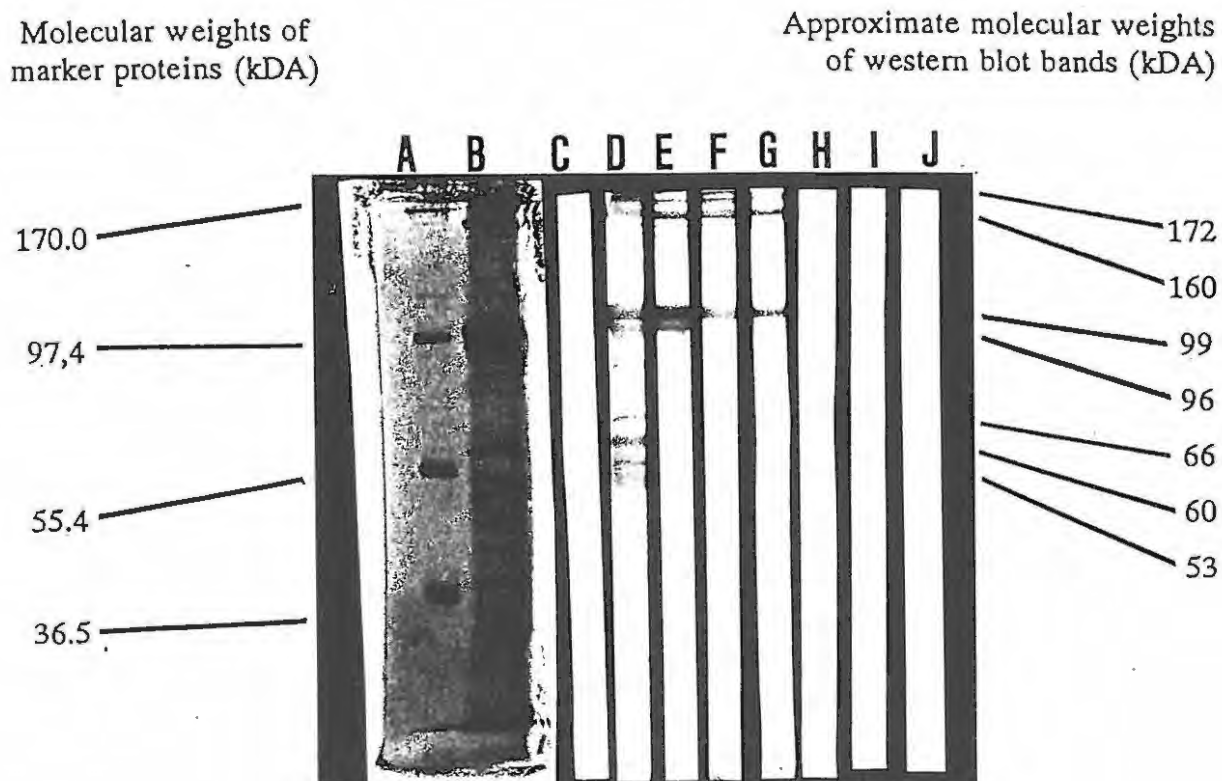
3.4.2.1 Rabbits inoculated with *Amblyomma hebraeum* adult extracts

Figure 13 shows the profile of the *A. hebraeum* adult crude extract (ACE) proteins recognized by sera from tick-naïve (control) rabbits, from rabbits inoculated with adult crude (ACE), adult soluble (ASE) and adult membrane-associated (AME) extract (both pre- and post-infestation), and finally from repetitively infested rabbits.

Serum from tick-naïve (control) rabbits did not recognise any of the *A. hebraeum* ACE proteins. Serum obtained from ACE-inoculated rabbits had antibodies which recognised many of the ACE proteins with varying affinity. The proteins most strongly recognised were those of approximately 172, 160, 99, 96, 66, 60 and 53kDa. Six weeks later, after a primary infestation with adult *A. hebraeum*, serum from the same rabbits recognized fewer proteins. The high molecular weight proteins, especially those of 172, 160, 99 and 96kDa were still recognized strongly, while the 66kDa protein was only weakly recognized, and the 60 and 53kDa proteins were no longer recognized.

Several proteins were recognized by sera from ASE-inoculated rabbits, with the protein of approximately 172, 160 and 99kDa giving the most intense bands. The lower molecular weight proteins (approximately 66, 60 and 53kDa) appeared as extremely faint smudges on the blots, indicating very low antibody titres or low levels of affinity. The banding pattern obtained using serum from the same hosts after two challenges with *A. hebraeum* adults

was similar to that obtained above, with the exception that the 172kDa protein was only weakly recognized, and the small proteins (66, 60 and 53kDa) were no longer detectable.



Lane A	-	Molecular weight markers / Coomassie Stained
Lane B	-	Adult crude extract (ACE) / Coomassie Stained
Lane C	-	Tick-naïve sera
Lane D	-	ACE-inoculated
Lane E	-	ACE-inoculated & 1°adult infestation
Lane F	-	ASE-inoculated
Lane G	-	ASE-inoculated & 2°adult infestation
Lane H	-	AME-inoculated
Lane I	-	AME-inoculated & 2° adult infestation
Lane J	-	2° adult infestation

Figure 13: Western Blot profile of adult *Amblyomma hebraeum* proteins recognized by sera from tick-naïve (control) rabbits, from rabbits inoculated with *A. hebraeum* adult crude (ACE), adult soluble (ASE) or adult membrane-associated (AME) extract (both pre- and post-infestation), and from infested rabbits.

Only the 172 and 160kDa proteins were weakly recognised by serum from AME-inoculated rabbits. The same two proteins were also weakly recognised by sera obtained after two challenges with *A. hebraeum* adults. In addition, after infestation, the AME-inoculated rabbits sera also recognized the 99 and 60kDa proteins, the latter only faintly.

Finally, serum obtained from rabbits following two infestations with adult *A. hebraeum* failed to recognize any proteins present in the crude extract.

3.4.2.2 Rabbits inoculated with *Amblyomma hebraeum* nymphal extracts

Figure 14 shows the profile of the *A. hebraeum* nymphal crude extract (NCE) proteins recognized by sera from tick-naive (control) rabbits, from rabbits inoculated with nymphal crude (NCE), nymphal soluble (NSE), and nymphal membrane-associated (NME) extract (both pre- and post-infestation), and from repetitively infested rabbits.

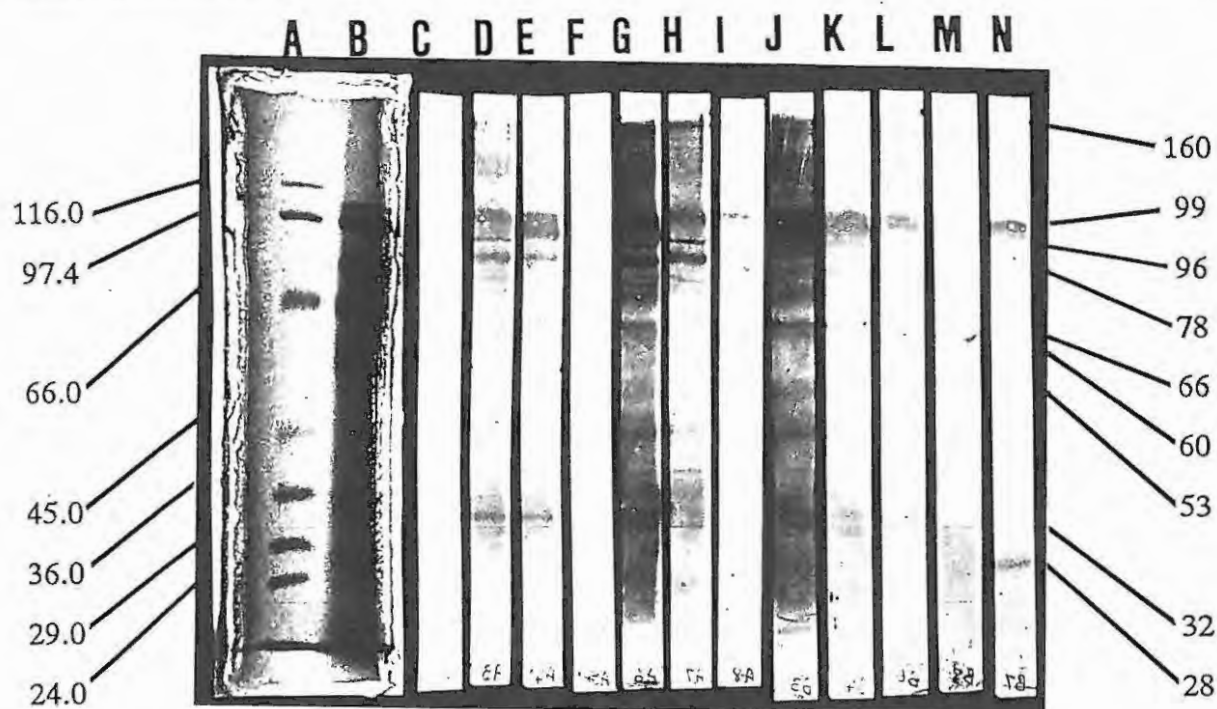
Sera from tick-naive (control) rabbits recognized numerous NCE proteins, with those of approximately 99, 96 and 32kDa being recognized most strongly. A faint smudge on the blot, at a molecular weight of approximately 38kDa, indicated that tick-naive sera may weakly recognize that protein too.

Sera obtained from NCE-inoculated rabbits appeared to recognize all the *A. hebraeum* ACE proteins. However, those most strongly recognized were the antigens of approximately 99, 96, 78, 66, 60 and 32kDa. Sera obtained from the same host following an infestation with ten adult *A. hebraeum* recognized a similar antigen profile, however some of the very high molecular weight proteins did not appear to be as strongly recognized as they were immediately after NCE-inoculation. Following a further infestation of 50 nymphs, the very high molecular weight proteins were no longer apparent and the 32kDa protein was only weakly recognized. All the other proteins recognised previously were still apparent but as somewhat weaker bands (Figure 14).

As with the sera from NCE-inoculated rabbits, sera from NSE-inoculated rabbits appeared to recognize all the NCE proteins against which it was screened. However, although all the bands were recognised more strongly than they were by the NCE-inoculated host's sera,

Molecular weights of
marker proteins (kDa)

Approximate molecular weights
of western blot bands (kDa)



- | | | |
|--------|---|--|
| Lane A | - | Molecular weight markers / Coomassie Stained |
| Lane B | - | Nymphal crude extract (NCE) / Coomassie Stained |
| Lane C | - | Tick-naïve sera |
| Lane D | - | NCE-inoculated |
| Lane E | - | NCE-inoculated & 1° adult infestation |
| Lane F | - | NCE-inoculated & 1° adult and 1° nymphal infestation |
| Lane G | - | NSE-inoculated |
| Lane H | - | NSE-inoculated & 1° adult infestation |
| Lane I | - | NSE-inoculated & 1° adult and 1° nymphal infestation |
| Lane J | - | NME-inoculated |
| Lane K | - | NME-inoculated & 1° adult infestation |
| Lane L | - | NME-inoculated & 1° adult and 1° nymphal infestation |
| Lane M | - | 1° adult infestation |
| Lane N | - | 1° adult and 1° nymphal infestation |

Figure 14: Western Blot profile of nymphal *Amblyomma hebraeum* proteins recognized by sera from tick-naïve (control) rabbits, from rabbits inoculated with *A. hebraeum* nymphal crude (NCE), nymphal soluble (NSE) or nymphal membrane-associated (NME) extracts (both pre- and post-infestation), and from infested rabbits.

the proteins of approximately 160, 78 and 32kDa appeared as relatively more intense bands than when screened with sera from NCE-inoculated hosts. Following infestations of the hosts with *A. hebraeum* adults and nymphs, the antigen profile recognized remained essentially the same, although somewhat weaker. After the nymphal infestation, however, a band of approximately 60kDa was strongly recognized.

As with the NCE- and NSE-inoculated hosts, sera from NME-inoculated rabbits recognized the vast majority of the tick proteins against which they were screened. Sera from NME-inoculated rabbits yielded a more intense response than sera from NCE- or NSE-inoculated animals, with the 99, 97 and 66kDa proteins being particularly intense. Furthermore, as with the NCE- and NSE-inoculated animals, subsequent adult and nymphal infestations did not appear to result any significant alteration in the antigen profile recognized.

Finally, sera obtained from rabbits after an initial infestation with adult *A. hebraeum* recognized many of the NCE proteins. Proteins of approximately 99, 66 and 53kDa were the most strongly recognized. Subsequent exposure to nymphs enhanced the response to the 99kDa protein, in addition protein of approximately 78 and 28kDa were recognized.

CHAPTER FOUR:

RESULTS:

IMMUNITY OF BOER GOAT TO *AMBLYOMMA HEBRAEUM* **FOLLOWING INOCULATION WITH *A. HEBRAEUM* EXTRACTS**

4.1 TICK CHALLENGE

Unfortunately one goat inoculated with *Amblyomma hebraeum* nymphal crude extract (NCE) died from an unrelated cause prior to tick challenge, consequently results presented represent the tick responses to NCE-inoculation on a single host only.

4.1.1 Tick engorgement weights

Inoculation of goats with either *Amblyomma hebraeum* adult crude extract (ACE) or nymphal crude extract (NCE) did not result in a significant decline in mean engorgement weights of either *A. hebraeum* adults, or nymphs during a primary challenge. Ticks from a second nymphal challenge of ACE-inoculated hosts had a significantly lower mean engorgement weight ($0.066 \pm 0.003\text{g}$) than those off control host ($0.073 \pm 0.002\text{g}$). Similarly, ticks from a second nymphal challenge of NCE-inoculated goats had a significantly lower mean engorgement weight ($0.062 \pm 0.002\text{g}$) than those fed on the controls ($0.073 \pm 0.001\text{g}$). However a third challenge with nymphs again revealed no significant difference between engorgement weights of ticks fed on inoculated (with either ACE or NCE) goats and control goats (Table 6).

4.1.2. Duration of tick engorgement

Inoculation of goats with either *A. hebraeum* ACE or NCE had no significant effect on the mean engorgement time of adult ticks. In a primary and secondary nymphal challenge

nymphs were found to take significantly longer to engorge on ACE-inoculated hosts (7.91 ± 0.16 days and 8.72 ± 0.33 days respectively) than on control hosts (7.26 ± 0.12 days and 7.08 ± 0.23 days respectively). However in the third infestation, ticks on ACE-inoculated hosts engorged significantly faster (6.79 ± 0.02 days) than those on the controls (7.75 ± 0.20 days) (Table 7).

Table 6: Mean engorgement weights of *Amblyomma hebraeum* engorged on goats inoculated with *A. hebraeum* adult crude extract (ACE) or nymphal crude extract (NCE), and on tick-naïve (control) goats.

Treatment	Number of hosts	1° Adult Infestation		1° Nymphal Infestation		2° Nymphal Infestation		3° Nymphal Infestation	
		Mean weight of ticks* (g) \pm SE	Significance p value**	Mean weight of ticks* (g) \pm SE	Significance p value**	Mean weight of ticks* (g) \pm SE	Significance p value**	Mean weight of ticks* (g) \pm SE	Significance p value**
ACE	2	2.146 ± 0.433	ns	0.069 ± 0.002	ns	0.066 ± 0.003	$p < 0.05$	0.062 ± 0.001	ns
Control	2	2.619 ± 0.150	-	0.062 ± 0.002	-	0.073 ± 0.002	-	0.061 ± 0.002	-
NCE	1	1.698 ± 0.470	ns	0.073 ± 0.003	ns	0.062 ± 0.002	$p < 0.01$	0.064 ± 0.003	ns
Control	2	2.328 ± 0.291	-	0.071 ± 0.002	-	0.073 ± 0.001	-	0.065 ± 0.001	-

* means of cumulative total dropped from goats

** significance when compared with controls

Table 7: Mean times taken by *Amblyomma hebraeum* to engorge to repletion on goats inoculated with adult crude extract (ACE) or nymphal crude extract (NCE), and on tick-naïve (control) goats.

Treatment	Number of hosts	1° Adult Infestation		1° Nymphal Infestation		2° Nymphal Infestation		3° Nymphal Infestation	
		Mean engorgement time* (days) \pm SE	Significance p value**	Mean engorgement time* (days) \pm SE	Significance p value**	Mean engorgement time* (days) \pm SE	Significance p value**	Mean engorgement time* (days) \pm SE	Significance p value**
ACE	2	12.17 ± 1.30	ns	7.91 ± 0.16	$p < 0.01$	8.72 ± 0.33	$p < 0.01$	6.97 ± 0.02	$p < 0.01$
Control	2	13.07 ± 0.45	-	7.26 ± 0.12	-	7.08 ± 0.23	-	7.75 ± 0.20	-
NCE	1	7.50 ± 0.33	ns	8.88 ± 0.30	$p < 0.01$	6.32 ± 0.12	$p < 0.01$	6.91 ± 0.17	$p < 0.01$
Control	2	8.06 ± 0.25	-	6.40 ± 0.09	-	5.56 ± 0.07	-	6.16 ± 0.09	-

* period of attachment to repletion

** significance when compared with controls

Mean nymphal engorgement times were significantly longer for nymphs fed on NCE-inoculated hosts than those fed on control goats during primary (8.88 ± 0.30 versus 6.40 ± 0.09 days), secondary (6.32 ± 0.12 versus 5.56 ± 0.07 days) and tertiary (6.91 ± 0.17 versus 6.16 ± 0.09 days) infestations. The differences in mean engorgement time were however found to decrease with each successive infestation, suggesting either that the effect of inoculation was diminishing, or that the control goats were developing some immunity due to being infested repeatedly (Table 7).

4.1.3 Percentage ticks engorged to repletion

It was notable that the number of adult *A. hebraeum* recovered from ACE-inoculated hosts was considerably lower than off the controls. It is pertinent to point out that adult ticks were placed on goats in ear-bags and tick mouthparts frequently penetrated directly into arteries and veins leading to excessive bleeding and this may have affected the results. Inoculation with ACE appeared to have little effect on the number of nymphs that successfully engorged to repletion (Table 8).

There was no difference between the percentage adult ticks recovered from goats inoculated with NCE and from the controls. Once again results may have been affected by adult ticks attaching directly into blood vessels. In all three nymphal infestations the number of ticks recovered from goats inoculated with NCE was lower than that recovered from controls, with the most marked difference occurring in the primary infestation (Table 8).

Table 8: Percentages of *Amblyomma hebraeum* that engorged to repletion on goats inoculated with *Amblyomma hebraeum* adult crude extract (ACE) or nymphal crude extract (NCE), and on tick-naïve (control) goats.

Treatment	Number of hosts	1° Adult Infestation	1° Nymphal Infestation	2° Nymphal Infestation	3° Nymphal Infestation
		Adult engorgement (%)	Nymphal engorgement (%)	Nymphal engorgement (%)	Nymphal engorgement (%)
ACE	2	30.0	21.0	21.5	38.0
Control	2	70.0	12.6	31.0	28.5
NCE	1	80.0	16.0	57.0	23.0
Control	2	80.0	94.0	69.5	40.0

4.1.4. Tick fecundity and moulting success

A suspected bacterial contamination resulted in the death of all adult female ticks fed on hosts inoculated with ACE, and consequently no egg weights were obtained. When comparing moulting success of nymphs engorging on ACE-inoculated and tick-naïve (control) hosts, it was found that the percentage moult following the initial nymphal challenge was higher for the inoculated animals than for the controls. Subsequent infestations led to a marked drop in the percentage moult of ticks engorged on inoculated hosts, in contrast no such effect was found in ticks fed on control. Following a third nymphal infestation the percentage moult of ticks off ACE-inoculated hosts was considerably lower than those off the controls (Table 9).

Inoculation with NCE was found to have no significant effect on mean egg weight. Although the percentage moulting success of nymphs off NCE-inoculated goats declined with each successive infestation, the decline in was comparable with a decline in the moulting success of ticks fed on the control goats (Table 9).

Table 9: Mean egg weights from, and percentage moulting success of, *Amblyomma hebraeum* fed on goats inoculated with *A. hebraeum* adult crude extract (ACE) or nymphal crude extract (NCE), and on tick-naïve (control) goats.

Treatment	Number of hosts	1° Adult Infestation		1° Nymphal Infestation	2° Nymphal Infestation	3° Nymphal Infestation
		Mean Weight of eggs* (g) ± SE	Significance P value**	Moulting success (%)	Moulting success (%)	Moulting success (%)
ACE	2	-	-	60.35	30.50	8.00
Control	2	-	-	20.73	39.50	74.00
NCE	1	0.872 ± 0.373	ns	56.25	53.20	25.00
Control	2	0.373 ± 0.216	-	66.00	75.70	28.50

* means of eggs laid by surviving female ticks

** significance when compared with controls

4.1.5 Grooming

As can be seen in Table 10, the number of nymphs successfully engorging to repletion on all goats was drastically reduced when grooming was allowed, thus indicating that grooming plays a significant role in the response of Boer goats to infestation with *A. hebraeum* nymphs. There was however little difference between inoculated, infested and tick-naïve (control) goats in the percentage reduction in tick yield attributable to grooming.

Table 10: Effect of grooming on *Amblyomma hebraeum* yield from goats inoculated with *A. hebraeum* adult crude extract (ACE) or nymphal crude extract (NCE), from repetitively infested goats, and from tick-naïve (control) goats.

Treatment	Mean number of ticks engorged to repletion		Reduction in ticks attributable to grooming (%)
	Restrained (ungroomed)	Unrestrained (groomed)	
ACE-inoculated & 4 Infestations	36	12	66.7
NCE-inoculated & 4 Infestations	23	5	78.3
4 Infestations	42	7	83.3
Controls	32	7	78.1

4.2 SKIN PROVOCATION TESTS

As with the rabbits, the high cost involved in the purchasing and maintenance of livestock limited the number of goat in each treatment group. Statistical analysis of skin responses to provocation by injection with tick extracts was therefore not feasible and results are presented as means only.

Goats previously inoculated with *Amblyomma hebraeum* adult crude extract (ACE) yielded an intense oedematous wheal and flare reaction within one hour of an intradermal injection with ACE. Skin thickness increases reached a maximum at three hours, before declining.

The administration of the antihistamine promethazine hydrochloride to ACE-inoculated goats prior to the intradermal injection of ACE caused a dramatic reduction in the severity of the skin reaction during the immediate hypersensitivity phase (three hours after

injection). The antihistamine appeared to have little effect on the later phase of the reaction, indicating that the later phase was not histamine mediated (Figure 15)

Tick-naïve (control) goats produced an immediate skin reaction to the intradermal injection of ACE, however it was considerably weaker than that found in the ACE-inoculated goats (Figure 15).

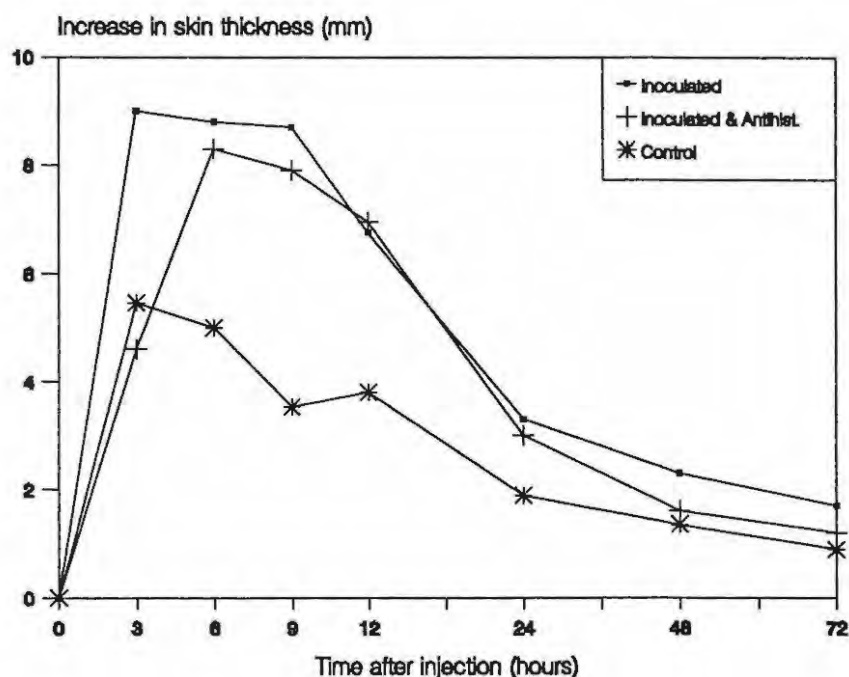


Figure 15: Skin responses of goats inoculated with *Amblyomma hebraeum* adult crude extract (ACE), and of tick-naïve (control) goats to intradermal injections of 10 μ l of ACE. Results are compared with the skin responses following the intradermal injection with 10 μ l ACE of ACE-inoculated goats that were previously treated with antihistamines.

4.3. SEROLOGY

4.3.1 Enzyme Linked immunosorbent assay (ELISA)

Anti-*Amblyomma hebraeum* IgG titres were read of graphs (of absorbance versus antibody dilution) where absorbance at 400nm gave readings of 0.4 OD. IgG titres were determined

for each individual goat within a treatment group. Unfortunately due to the small number of hosts (two) within a treatment group, statistical analysis (such as the calculation of standard errors) was not feasible and results are presented as means only. The kinetics of the antibody responses of goats to inoculation with *A. hebraeum* extracts and subsequent infestations are shown in Figure 16.

All serum obtained from goats prior to inoculation displayed anti-tick IgG titres of lower than 1/5. However sera obtained from the tick-naïve (control) goats gave a titre of 1/25 (Figure 16). These findings indicate that either a) there may have been cross-reactivity with antigens from another source, b) animals had been exposed to ticks before the trial commenced or c) that non-specific binding was occurring.

The anti-tick IgG titre of sera obtained from goats inoculated with *A. hebraeum* adult crude extracts (ACE) was 1/2500. The specific IgG titre dropped to 1/800 following the simultaneous infestation of inoculated hosts with 10 adult females and 50 nymphal ticks, and continued to decline despite a second nymphal challenge (1/160). A third nymphal challenge of this group appeared to enhance the immune response, leading to an antibody titre of 1/225.

Following a third inoculation with *A. hebraeum* nymphal crude extract (NCE) goats developed an anti-tick IgG titre of 1/1600. As with the goats inoculated with ACE, serum obtained from inoculated animals after they were infested simultaneously with *A. hebraeum* adult females and nymphs displayed a markedly reduced antibody titre (1/600). Furthermore, as with the ACE-inoculated goats specific IgG titres of NCE-inoculated goats were found to decrease further despite a second nymphal challenge (1/125), while a third nymphal challenge appeared to enhance the response slightly, resulting in a titre of 1/200.

The specific IgG titres of tick-naïve (control) goats increased from 1/25 prior to exposure to ticks to 1/40 after infestation with *A. hebraeum* adult females and nymphs. This titre was further increased to 1/60 and 1/100 after second and third nymphal challenge respectively.

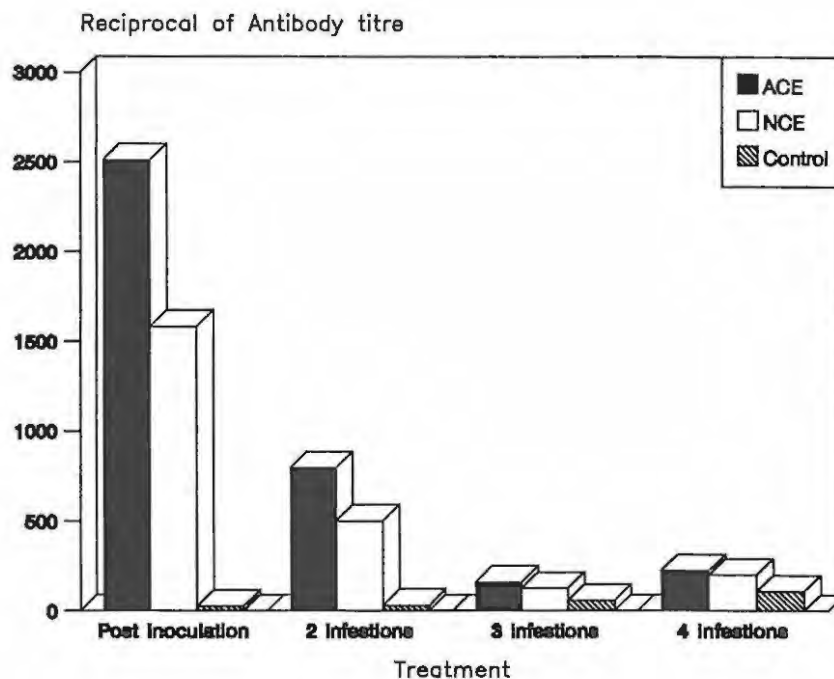


Figure 16: Specific anti-*Amblyomma hebraeum* IgG titres of goats inoculated with *A. hebraeum* adult crude extract (ACE) or nymphal crude extract are shown immediately after inoculation and after each of four *A. hebraeum* infestations. IgG titres are compared with those of tick-naïve (control) goats prior to, and following each of the four infestations.

4.3.2 Western Blotting

Staining and destaining of the polyacrylamide gels once again resulted in the gels expanding slightly, and consequently the molecular weights of the bands on the blots could not be determined directly from the molecular weights of the marker proteins in the gel. Approximate molecular weights of the bands on the blots have been determined by matching banding patterns with those of the tick extracts (ACE or NCE) stained alongside the molecular weight markers.

Although individual goat sera was screened against tick proteins, results for different hosts within a treatment group were practically identical. Sera from the different hosts within a treatment group was therefore pooled to give the results presented below.

4.3.2.1 Goats inoculated with *Amblyomma hebraeum* adult extracts

Figure 17 shows the profile of the *A. hebraeum* adult crude extract (ACE) proteins recognized by sera from tick-naïve (control) goats, goats inoculated with ACE (both pre- and post-infestation), and from repetitively infested goats.

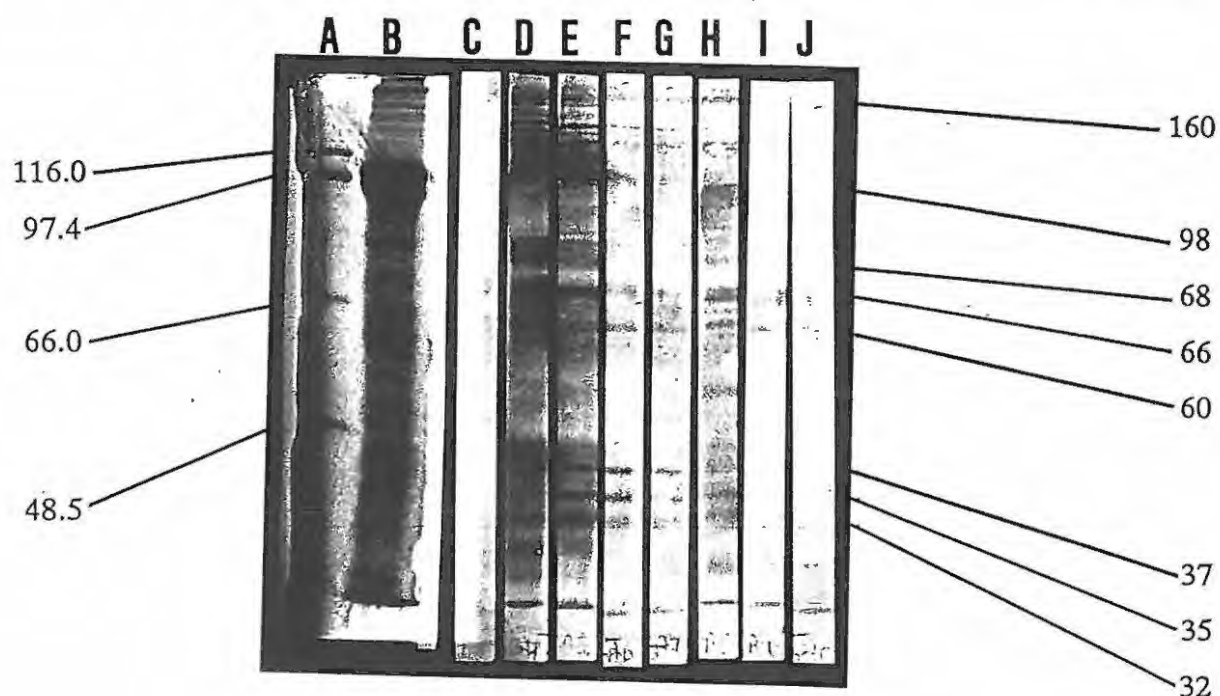
Serum obtained from tick-naïve (control) goats appeared to react weakly with numerous proteins in the adult crude extract, as evident by the presence of many smudged lines on the blot. Of the numerous antigens recognised, those of 68, 66 and 60kDa appeared to give the strongest response.

Serum obtained from the ACE-inoculated goats appeared to recognize many, if not all of the *A. hebraeum* ACE proteins. Serum obtained from the same host following a primary infestation with both adult and nymphal *A. hebraeum* recognized a similar antigen profile, however the response to some of the lower molecular weight proteins (e.g. those of approximately 37, 35 and 32kDa) was stronger. A further two nymphal infestations failed to further enhance the response to any particular proteins, to the contrary, the blots indicate that although the majority of the proteins were still recognized, the response was weaker.

Sera obtained from tick-naïve (control) goats after an initial infestation with *A. hebraeum* adults and nymphs recognized many of the clarified proteins, with the proteins of approximately 160, 98, 68, 66, 60, 35 and 32kDa giving the strongest bands. Following subsequent exposure of the same hosts to nymphs weaker bands were noted, possibly indicating that 50 nymphs was not sufficient numbers to sustain a significant response, however this contradicts the ELISA results which indicate rising antibody titres following each successive infestation of the control goats.

Molecular weights of
marker proteins (kDa)

Approximate molecular weights
of western blot bands (kDa)



Lane A	-	Molecular weight markers / Coomassie Stained
Lane B	-	Adult crude extract (ACE) / Coomassie Stained
Lane C	-	Tick-naive sera
Lane D	-	ACE-inoculated
Lane E	-	ACE-inoculated & 1° adult and 1° nymphal infestation
Lane F	-	ACE-inoculated & 1° adult and 2° nymphal infestations
Lane G	-	ACE-inoculated & 1° adult and 3° nymphal infestations
Lane H	-	1° adult and 1° nymphal infestation
Lane I	-	1° adult and 2° nymphal infestation
Lane J	-	1° adult and 3° nymphal infestations

Figure 17: Western Blot profile of adult *Amblyomma hebraeum* proteins recognized by sera from tick-naive (control) goats, from goats inoculated with *A. hebraeum* adult crude extract (ACE) (both pre- and post-infestation), and from infested rabbits.

4.3.2.2 Goats inoculated with *Amblyomma hebraeum* nymphal extracts

Figure 18 shows the profile of the *A. hebraeum* nymphal crude extract (NCE) proteins recognized by sera from tick-naïve (control) goats, from goats inoculated with NCE (both pre- and post-infestation), and from repetitively infested goats.

Serum obtained from tick-naïve (control) goats failed to recognize any of the *A. hebraeum* NCE proteins. Although sera obtained from NCE-inoculated goats appeared to recognize many of the clarified proteins, the bands on the blot were blurred possibly due to the presence of air between the gel and the nitrocellulose paper during protein transfer. Sera obtained from NCE-inoculated goats after infestation with adults and nymphs appeared to recognize many of the NCE proteins, with the bands of approximately 160, 99, 96, 68 and 60kDa giving the strongest response. However, as with the ACE-inoculated goats, bands became weaker despite a second nymphal infestation. A third nymphal infestation of the NCE-inoculated goats did however appear to enhance the response as bands became more distinct. This is in keeping with the ELISA results which indicate increased anti-*A. hebraeum* IgG titre following this last nymphal infestation.

Sera from repetitively infested goats faintly recognised the *A. hebraeum* NCE proteins of approximately 99,96 and 68kDa, with the response becoming slightly stronger after each infestation.

Molecular weights of
marker proteins (kDa)

Approximate molecular weights
of western blot bands (kDa)



Lane A	-	Molecular weights markers / Coomassie Stained
Lane B	-	Nymphal crude extract (NCE) / Coomassie Stained
Lane C	-	Tick-naive sera
Lane D	-	NCE-inoculated
Lane E	-	NCE-inoculated & 1° adult and 1° nymphal infestation
Lane F	-	NCE-inoculated & 1° adult and 2° nymphal infestations
Lane G	-	NCE-inoculated & 1° adult and 3° nymphal infestations
Lane H	-	1° adult and 1° nymphal infestation
Lane I	-	1° adult and 2° nymphal infestations
Lane J	-	1° adult and 3° nymphal infestations

Figure 18: Western Blot profile of nymphal *Amblyomma hebraeum* proteins recognized by sera from tick-naive (control) goats, goats inoculated with *A. hebraeum* nymphal crude extract (NCE) (both pre- and post-infestation), and from infested goats.

CHAPTER FIVE:

DISCUSSION

Whilst ticks and tick-borne diseases are known to be of considerable importance throughout the world, it is widely believed that economic losses in livestock production attributable to ticks are greater in Africa than anywhere else (Dipeolu, 1989).

The bont tick, *Amblyomma hebraeum*, is possibly the most economically important tick in southern Africa. In addition to causing severe losses in liveweight gain (Norval *et al.*, 1989b), reductions in milk production (Norval *et al.*, 1990 cited in Norval *et al.*, 1992) and hide damage (Norval *et al.*, 1989b), *A. hebraeum* is the principal vector in southern Africa of *Cowdria ruminantium*, the rickettsial organism responsible for heartwater (Bezuidenhout, 1984; Petney *et al.*, 1987). Although the information available on the economic impact of tick-borne diseases on livestock production is limited, it is generally accepted that heartwater is one of the most important tick-borne diseases of Africa (Norval *et al.*, 1992). The control of the disease, either directly or through the control of its vectors, is consequently of considerable economic importance.

In heartwater endemic areas of Africa, where the *C. ruminantium* infection rates of both the host and vector population are high, heartwater may be controlled by ensuring that livestock are moderately infested with the disease vectors, thus allowing enzootic stability to develop (Norval *et al.*, 1992). However, as the transmission rate necessary to maintain enzootic stability to heartwater is unknown, and the infection rate of the tick population is highly variable (Bezuidenhout, 1985), maintaining enzootic stability could be problematical. Furthermore, the advantages of this method of control have to be weighed against the losses attributable to direct damage caused by tick infestations.

Where heartwater vector numbers or infection rates are too low to maintain enzootic stability, immunization against the disease is recommended. To date, immunization involves the risky procedure of injection with virulent stocks of *C. ruminantium*, and

treatment of the resultant reaction with drugs. Understandably, there are considerable limitations to the use of such a vaccine and, although attempts are being made to develop a safe, non-living vaccine (Yunker, 1988), other means of controlling the disease, such as vector control, warrant attention.

For the past 75 years acaricide treatment of livestock has been widely practised in southern Africa, and has played an important role in the control of numerous tick-borne diseases and the prevention of direct tick damage and associated production losses (Norval *et al.*, 1992). Today however, it is known that total reliance on acaricides can lead to serious problems, such as the development of acaricide resistance (Wharton, 1976) and the loss of immunity of animals to tick-borne diseases due to the lack of natural challenge (Bezuidenhout, 1985). Furthermore, the costs involved in acaricide purchase and application are extremely high (Young *et al.*, 1988). Alternative approaches to tick control that are cost effective and do not involve substantial risks of tick-borne disease outbreaks, would therefore be welcomed by both the commercial and subsistence farming communities.

The pioneering work of Trager (1939), indicating that inoculation of guinea-pigs with crude larval extracts of *Dermacentor variabilis* had a detrimental effect on tick feeding, laid the foundation for the concept of immunomanipulating the potential host to make it resistant to tick infestations. Research on immunological approaches to the control of various tick species is being carried out in a number of laboratories throughout the world. Preliminary studies have indicated that artificial immunization can be used to at least partially protect against infestation by several tick species (Ackerman *et al.*, 1980; M^cGowan *et al.*, 1981; Varma *et al.*, 1990). The leading work in this field is that done on the Australian cattle tick, *Boophilus microplus*. This research is likely to culminate in a vaccine against *B. microplus* becoming commercially available in the foreseeable future (Norval *et al.*, 1992). Little work has been done on producing vaccines against economically important African tick species.

Where artificial immunization has been successful in the past, it has frequently been with tick-host systems in which a strong immunity is acquired following natural tick challenge, or when unnatural hosts (e.g. laboratory host models) are used. There is some controversy as to the ability of hosts to naturally develop immunity to *Amblyomma hebraeum* (Norval,

1978, 1991; Norval *et al.*, 1988; Adamson *et al.*, 1991), and very few attempts have been made to artificially immunize against this three-host tick. Heller-Haupt *et al.* (1989) attempted to immunize guinea-pigs against adult *A. hebraeum* using homogenates of unfed nymphal ticks, while Adamson *et al.* (1991) attempted to immunize goats against *A. hebraeum* larvae using homogenates of unfed larvae. In this study, further attempts were made to induce host resistance to *A. hebraeum* infestation through inoculation with extracts of unfed *A. hebraeum* adults and nymphs.

5.1 EFFECT OF INOCULATION ON TICK FEEDING SUCCESS

Several workers have reported that artificially induced immunity is quite different from that which is acquired naturally. Roberts (1968b) reported that, with naturally acquired resistance to *B. microplus*, rejection occurred mainly at the time of tick attachment, and manifested predominantly against the larval stage, having little or no effect on tick viability, engorgement weight or egg mass laid by the surviving ticks. Artificially induced immunity against the same tick species is, however, directed against the adult life stage, causing direct damage to the tick, frequently resulting in a large reduction in tick numbers, significantly reduced tick engorgement weights and reduced egg laying (Kemp *et al.*, 1986; Willadsen *et al.*, 1988).

Inoculation of animals with tick extracts resulting in host resistance to tick infestations is measured by factors such as decreased tick engorgement weights, decreased numbers of ticks engorging to repletion, prolonged duration of engorgement, and decreased egg numbers and moulting success (Wikel & Whelan, 1986).

5.1.1 Inoculation with *Amblyomma hebraeum* adult extracts

In this study, fewer adult ticks were found to engorge to repletion on goats inoculated with a crude extract of unfed *Amblyomma hebraeum* (ACE) than on tick-naïve (control) goats. However, besides this single factor, inoculation of rabbits and goats with ACE did not appear to induce any significant protection against a primary *A. hebraeum* adult infestation.

Willadsen *et al.* (1988) noted that results of vaccination against *Boophilus microplus* improved as antigen purification proceeded. Although, results of this study suggest that while ACE-inoculation had little detrimental effect on tick feeding, inoculation of rabbits with the slightly purified *A. hebraeum* soluble (ASE) or membrane-associated (AME) extracts may have resulted in some degree of protective immunity against adult infestations, the detrimental effect on tick feeding was only noted during a second adult tick challenge. As both ACE-inoculated rabbits died from unrelated causes prior to a second *A. hebraeum* challenge, it is unknown whether these hosts would have displayed some degree of resistance to a second tick challenge. Results presented suggest that contact with the tick is possibly required to enhance any immune response produced by vaccination.

It should be noted that results of infestations with adult *A. hebraeum* may be misrepresentative for a number of reasons. Firstly, the small number of hosts available (both rabbits and goats), and the limited number of adult ticks used per infestation, limit the usefulness of the available data. Secondly, rabbits are not a natural host for *A. hebraeum* adults (Petney *et al.*, 1987) and infestation resulted in very severe reactions at the site of tick attachment, which may have influenced feeding success. Thirdly, due to the difficulties encountered with preventing the goats from removing ticks through grooming, adult *A. hebraeum* were placed on the goats ears, where they would not normally attach (Howell *et al.*, 1978). The large mouthparts of the adult ticks frequently penetrated blood vessels in goats ears, leading to excessive bleeding. Whilst some female ticks attached and engorged successfully, others may have either been unable to locate a male due to the excessive bleeding resulting in large volumes of blood accumulating within the ear-bags, and therefore failed to attach (Norval, 1974), or, they may have penetrated a vessel and 'drowned' in the blood before engorging.

It is important, especially when dealing with three-host ticks, to determine whether immunity generated by one tick instar provides protection against other instars. Adamson *et al.* (1991) have shown that repetitive infestations of goats with larval *A. hebraeum* detrimentally affected nymphal feeding, and suggested that the different instars shared common antigens. There are, however, few reports of inoculation with adult tick extracts having a detrimental affect on another instar. M^cGowan *et al.* (1980) failed to induce resistance to *Amblyomma maculatum* nymphs by inoculating rabbits with crude extracts of

A. maculatum adults. Results obtained in this study indicate that inoculation of goats with *A. hebraeum* ACE may have provided some protection against infestation by nymphs.

5.1.2 Inoculation with *Amblyomma hebraeum* nymphal extracts

Previous papers have shown that rabbits and guinea-pigs could be inoculated against adult *Amblyomma variegatum* using homogenates of unfed nymphs (Heller-Haupt *et al.*, 1989) and unfed larvae (Heller-Haupt *et al.*, 1987). Although the present study was hampered by the problem of placing adult *A. hebraeum* on unnatural hosts and/or attachment sites, results suggest that inoculation of both rabbits and goats with crude extracts of unfed *A. hebraeum* nymphs (NCE) failed to provide any protective immunity to adult *A. hebraeum*. These results correlate with those of Heller-Haupt *et al.* (1989), where inoculation of guinea-pigs with an unfed *A. hebraeum* nymphal extract failed to cause a significant reduction in female engorgement weights. In this study, rabbits also failed to develop resistance to adult ticks following inoculation with the slightly purified *A. hebraeum* adult soluble (ASE) and membrane-associated (AME) extracts.

Despite the apparent lack of effect on adult feeding, inoculation with *A. hebraeum* nymphal extracts may be effective against nymphal infestations. Inoculation of rabbits with the nymphal crude extract (NCE) resulted in a significant reduction in mean nymphal engorgement weight, prolonged engorgement time, and a reduction in moulting success. Inoculation of goats with the same extract resulted in fewer nymphs engorging to repletion, and those that engorged successfully took longer to do so and had reduced moulting success. In contrast, Adamson *et al.* (1991) showed that inoculation of goats with crude *A. hebraeum* larval extracts had no observable affect on larval feeding. Inoculation of rabbits with nymphal soluble (NSE) and membrane-associated (NME) extracts had little detrimental effect on nymphal feeding.

5.1.3 Histology of tick gut

There are no reports of damage to the tick gut in those ticks rejected by hosts that have naturally acquired resistance, presumably due to the fact that the gut antigens do not come

into contact with the host's immune system during natural feeding. However, there have been numerous reports of the immunological response of vaccinated hosts causing severe damage to tick gut cells (Agdebe & Kemp, 1986; Kemp *et al.*, 1986, 1989). The vaccinated host's immunological response to tick gut antigens is therefore different from the response achieved through natural exposure to the ticks. Exploitation of this immune mechanism may result in the development of a greater level of host resistance to ticks than that achieved through repetitive infestation. It is, however, first necessary to investigate the exact site of gut damage, and the nature of the immune reaction.

Since digestion in ticks is largely an intracellular process, occurring within the digestive cells (Agdebe & Kemp, 1985), it is primarily these cells that are subjected to damage by components of the ingested blood (Agdebe & Kemp, 1986). Kemp *et al.* (1989) provided evidence to suggest that vaccination leads to the production of antibodies against antigens on the plasma membrane of the gut cells of adult ticks, and that these antibodies are sufficient to damage the gut cells. Complement may assist in cell damage, although it is not essential as both complement-inactivated serum, and isolated immunoglobulins reconstituted in normal serum have been shown to cause damage in an *in vitro* feeding system (Kemp unpublished data, cited in Willadsen & Kemp, 1988). Agdebe & Kemp (1986) proposed that host leucocytes may also contribute towards tick damage, however as Kemp *et al.* (1986) have reported damage occurring in the absence of leucocytes, they are not an imperative component of the response resulting in tick gut damage.

Once lesions have occurred in the gut, antibodies will leak into the haemocoel and immunological reactions against other tick tissues, such as muscles and Malpighian tubules have been reported (Agdebe & Kemp, 1986). Furthermore, in addition to the direct damage caused, gut damage could affect oviposition in females by either stopping digestion or preventing the synthesis of vitellogenins by the gut cells (Agdebe & Kemp, 1986).

Many of the reports of damage to tick guts following vaccination with crude tick extracts involve the Australian cattle tick *Boophilus microplus*. There have been no previous reports of gut damage in *Amblyomma hebraeum* fed on vaccinated hosts.

In this study, one of eight female *A. hebraeum* engorged on vaccinated rabbits showed evidence of gut damage, with lysed digestive cells and basal laminae denuded of cells. Not all ticks feeding on vaccinated hosts are damaged, as Kemp *et al.* (1986) report that only 12%, 86% and 73% of the females that engorged on three *Boophilus microplus*-vaccinated cattle, had gut damage. Therefore, although the occurrence of gut damage in a single tick fails to prove anything, it is indicative that vaccination may cause gut damage in this tick/hosts association. Before damage to the tick gut can be discounted as an important aspect of the vaccinated host's response to *A. hebraeum*, a larger number of ticks recovered from several vaccinated and tick-naïve control hosts should be examined.

The extensive research on gut damage of *Boophilus microplus* ticks fed on vaccinated hosts has culminated in the production of a vaccine against a specific glycoprotein on the plasma membrane of the tick gut digestive cells (Opdebeeck *et al.*, 1988b; Jackson & Opdebeeck, 1990). The finding that feeding on *A. hebraeum* extract inoculated hosts may result in gut damage lends hope to the prospect of developing a vaccine against *A. hebraeum* similar to that being produced against *B. microplus*. Whether such a vaccine would be as effective against a three-host tick, where the feeding periods of the individual instars are shorter than the feeding period of the one-host tick, *B. microplus*, remains to be determined. However the finding of Fivaz *et al.* (1991b) that adults of the three-host tick *Rhipicephalus zambeziensis* fed on tick extract inoculated rabbits had damage to the gut, suggests that developing a vaccine against the gut proteins of three-host tick species may be possible.

Just as ticks of *B. microplus* developed resistance to several acaricides, it has been postulated that resistance may develop against gut protein vaccine. Although no evidence of resistance to inoculation with tick proteins has been found to date, Kemp *et al.* (1989) suggest several ways in which such resistance may arise. Firstly, tick strains may occur in which the antigen is immunologically different from that which is incorporated in the vaccine. Secondly, attempts to repair damaged gut have been noted in female ticks surviving on vaccinated hosts (Binnington & Obenchain, 1982), and ticks may be able to improve this ability.

5.2 EFFECT OF INOCULATION ON HOST RESPONSE TO TICK FEEDING

5.2.1 Macroscopic changes at tick attachment site and the effect of grooming

Grooming is an important component in the expression of resistance to ticks (Bennett, 1969). Norval (1978) reported that the tick yield among rabbits is largely determined by the grooming behaviour of the individual animal, and that rabbits on which tick bites caused most irritation were found to groom most efficiently. In this study, examination of macroscopic changes at the tick attachment site on inoculated and tick-naïve (control) rabbits, all of which were prevented from grooming, revealed that infestation with adult *A. hebraeum* resulted in extremely severe reactions at the tick attachment sites. This may be attributable to rabbits being an unnatural host for the adult life-stage of *A. hebraeum* (Petney *et al.*, 1987). Nymphal infestations of inoculated rabbits resulted in a severe host response at the site of tick attachment, while no such response was found on the tick-naïve control hosts. If the intensity of grooming is directly related to the severity of skin irritation, as Norval (1978) suggests, it would appear that inoculation with *A. hebraeum* extracts may lead to enhanced grooming and a reduction in the number of nymphs that engorged successfully on inoculated rabbits.

When investigating the role that grooming played in the removal of ticks from Boer goats, it was found that, as in cattle (Norval *et al.*, 1988), grooming does play a significant role in the host's response to infestation with *A. hebraeum*. However, grooming was not found to be further enhanced in goats that had previously been inoculated with unfed *A. hebraeum* adult or nymphal extracts.

Although enhanced grooming is an important characteristic of a resistant host's response to tick infestation (Bennett, 1969), losses attributable to grooming may not be as high for *A. hebraeum* as they are for other tick species. Firstly, the large, robust mouthparts of the *Amblyomma* ticks are thought to make them more resilient to grooming (Norval, 1975). Secondly, the aggregation-attachment pheromone (AAP) produced by adult *A. hebraeum* males, minimises losses due to grooming as ticks are attracted to areas where the host has failed to remove those ticks already attached (Rechav *et al.*, 1976). Further evaluations

would therefore have to be undertaken in order to ascertain the role of grooming in the control of *A. hebraeum* under normal field conditions.

5.2.2 Nature of hypersensitivity induced by inoculation

Naturally acquired resistance to ticks has been shown to be associated with the development of a hypersensitivity to the salivary secretions of the tick (Riek, 1962). Today, the profile of the local skin reaction, following the intradermal injection of tick extracts, is frequently used to assess the nature of the hypersensitivity induced in terms of the classification of Coombs *et al.* (1975). This provides clues as to the mechanisms that might be involved in the expression of artificially acquired resistance.

Kraidy (1991) reported that the intradermal injection of crude adult *A. hebraeum* extracts (ACE) into repetitively infested rabbits resulted in a Type I (IgE-mediated) hypersensitivity reaction with a delayed time phase, and attributed the latter to the Type III Arthus reaction. The skin reaction profile obtained in this study following injection of ACE-inoculated rabbits with ACE was essentially the same as that obtained by Kraidy (1991). However, whether the delayed reaction is due to the Type III Arthus reaction or Type IV cell-mediated hypersensitivity requires further investigation.

According to Kimball (1983) the inflammation and tissue damage resulting from the Arthus reaction (complement being fixed by antigen-antibody complexes and causing increased vascular permeability and the release of lysosomal enzymes) reaches a peak 4-8 hours after the reaction has been initiated. In this study the reaction in rabbits peaked at 24 hours. Whether the Arthus reaction could have been delayed by factors such as immunosuppressive compounds within the tick extract to give a peak at 24 hours is unknown. The typical Type IV cell-mediated hypersensitivity reaction peaks at 24-48 hours (Roitt, 1975), fitting the profile found in this study.

There are several ways whereby the nature of the delayed reaction can be clarified. Firstly, the histology of the skin lesions differs in the two reactions. With the Arthus reaction, the site becomes infiltrated with neutrophils (heterophils), whereas cell-mediated hypersensitivity is characterized by heavy infiltrations of lymphocytes and macrophages

(Kimball, 1983). The second factor which Kimball (1983) maintains can be used to differentiate between the Arthus reaction and cell-mediated hypersensitivity, is that with the latter it is possible to transfer immunity to a naive animal by an injection of cells (T-lymphocytes), but not by injections of serum. However, in some instances delayed-in-onset skin reactivity has been passively transferred with serum (Askenase *et al.*, 1975). Therefore, before the transfer of sensitivity can be attributed to a cell-mediated immune response, the cells responsible must be shown to be T-cells.

The skin reaction in inoculated goats in this study manifested a Type I (IgE-mediated) hypersensitivity reaction to *A. hebraeum* adult crude extract (ACE), with the prolonged response (12 hours) possibly being attributable to the Type III Arthus reaction. In related studies, the intradermal inoculation of *A. hebraeum*-sensitized dairy bulls with extracts of salivary gland and eggs (Fivaz *et al.*, 1991a), and the intradermal inoculation of *A. hebraeum*-sensitized goats with egg extract (Adamson *et al.*, 1991) yielded similar skin reaction profiles.

The reaction of inoculated goats to an intradermal injection of ACE was characterised by the wheal and flare reaction typical of Type I hypersensitivity (Benjamini & Leskowitz, 1991). During Type I hypersensitivity, the IgE binds to receptors on mast cells and basophils, triggering the release of granules containing pharmacological agents (e.g. histamine). Fivaz (1990) showed that antihistamine had a dramatic effect on the severity of the skin reaction of rabbits repetitively infested with *Rhipicephalus appendiculatus*, thereby indicating that histamine plays an important role in that tick/host relationship.

In this study, the administration of antihistamine caused a severe reduction in the skin reaction during the immediate hypersensitivity phase, however it had little effect after 6 hours. The reduced influence of antihistamine during the latter stages of the reaction, may be due to the reaction becoming attributable to the Arthus reaction as opposed to Type I hypersensitivity and its associated histamine release. Histamine is a mediator of pain and therefore its accumulation at the site of tick attachment may promote grooming (Schleger *et al.*, 1981).

5.2.3 Cellular changes in response to inoculation with tick antigens

Many investigators have studied the histology of the cutaneous reaction at the sites of tick attachment, or of injection with tick antigens, in order to obtain information about the hosts response to tick feeding (Allen, 1973; Askenase 1973, 1977; Brown, 1988b). Riek (1956, 1962) postulated a mast-cell dependant eosinophilia as the basis for resistance to *Boophilus* ticks. However, without the histological techniques developed in 1970 (Dvorak *et al.*, 1970) which enabled the thin sectioning, fixation and specific staining of tissues, basophils were easily confused with eosinophils (Askenase, 1977). More recent studies reviewed by Brown (1988b) have revealed that basophils, and to a lesser extent eosinophils, make up a significant proportion of the cellular infiltrate at tick attachment sites in resistant hosts.

In this study, the histological reaction of rabbits inoculated with *A. hebraeum* adult crude extract (ACE) to an intradermal injection of ACE was dominated by eosinophils. Similarly, the histological reaction to feeding larvae on cattle repetitively infested with *A. hebraeum* has been reported to be dominated by eosinophils (Fivaz *et al.*, 1991a). The presence of eosinophils can support the theory of a Type I hypersensitivity with its associated degranulation of mast-cells and basophils and release of an eosinophil chemotactic factor (ECF-A). However, eosinophils have also been associated with Type IV cell-mediated delayed hypersensitivity (Clark, 1991).

Although the role of eosinophils in tick resistance is not clear, Ackerman *et al.* (1982) report that eosinophils contain substances that are toxic to schistosomes, nematodes and trypanosomes. It is unknown if eosinophils exert a direct toxic effect on feeding tick instars. However, they have been shown to play a role in concentrating histamine at tick attachment sites (Schleger *et al.*, 1981), thereby possibly stimulating grooming.

Brown *et al.* (1982) postulated that eosinophils have a secondary role in tick resistance, with basophils fulfilling the primary role. This postulate was based on the findings that treatment of resistant hosts with anti-basophil serum resulted in animals being depleted of bone marrow, blood and tissue basophils, and losing all resistance to ticks. While treatment with anti-eosinophil serum resulted in the expression of resistance being reduced but still apparent.

The apparent total absence of basophils in the rabbit tissues examined in this study, and in that of Fivaz *et al.* (1991a), may be a reflection of the staining method used. Askenase (1977) reported that basophils in formalin-fixed tissues are relatively ineffectively stained with Haematoxylin and Eosin or Giemsa stains. Ideally, these tissues should have been stained using the technique of Dvorak *et al.* (1970) devised specifically for staining basophils.

Neutrophils (heterophils) are commonly found at the site of tick feeding in several tick-host associations (Fivaz *et al.*, 1991a) and are characteristic of the Arthus reaction (Kimball, 1983). This study shows only occasional heterophils at the site of injection 12-24 hours after injection. Although this is the time-frame in which it is proposed that the Arthus reaction occurred, the limited number of heterophils present casts some uncertainty on the role of the Arthus reaction in the hypersensitivity of rabbits to *A. hebraeum*. Furthermore, as tick saliva is capable of cleaving the complement component C5 to produce C5a, which is chemotactic for neutrophils (Berenberg *et al.*, 1972), the role of neutrophils in tick-resistance is difficult to interpret, and has possibly frequently been exaggerated.

In this study, lymphocytes are found at the site of injection over the entire study period (3-48 hours). Macrophages are, however, only noted in the biopsies taken 24 and 48 hours after injection. Heavy infiltrations of both these cells are indicative of a cell-mediated hypersensitivity (Kimball, 1983). However, as with the neutrophil infiltrations, infiltrations of these two cell types are light and therefore no definite conclusions about the role of cell-mediated hypersensitivity in the response of rabbits to *A. hebraeum* can be drawn.

5.3 SEROLOGY

5.3.1 Serum antibody levels

Antibodies are known to be an important component of the inoculated host's immune response to ticks, being at least partially responsible for the damage caused to the gut (Kemp *et al.*, 1989). High titres of circulating antibodies to tick antigens have been

reported following inoculation with various tick extracts (M^cGowan *et al.*, 1980; Jongejan *et al.*, 1989).

In this study, *A. hebraeum* specific IgG antibody titres were found to be elevated in rabbits and goats following three inoculations with *A. hebraeum* extracts. Higher IgG titres were elicited by adult crude extracts (ACE) than by either the soluble (ASE) or membrane-associated (AME) extracts. This may be attributable to the fact that the less purified ACE would have presented a greater array of antigenic molecules to the host's immune system. However, in contrast, inoculation of rabbits with nymphal crude extract (NCE) elicited a considerably weaker IgG response than did the nymphal soluble (NSE) and membrane-associated (NME) extracts.

Goats inoculated with NCE produced a considerably stronger anti-*A. hebraeum* IgG response than rabbits inoculated with the same extract. However, as with the rabbits, inoculation of goats with NCE elicited a considerably weaker response than inoculation with ACE. Differences in the immunogenicity of tick instars has been reported previously (Fivaz & Norval, 1990), and may account for the stronger antibody response induced by inoculation with adult extracts.

In all inoculated rabbits and goats, IgG titres were found to decline rapidly in the period after the final inoculation, despite repeated exposure to ticks. This may be attributed to the fact that inoculation would result in the production of antibodies to a large range of tick proteins that are not exposed to the host's immune system during normal tick feeding ("concealed antigens"). Once inoculation has ceased, the titres of those antibodies raised against the "concealed" antigens would decrease. Thus it would be expected that antibody titres would decrease in the period after inoculation, regardless of tick infestation, until such time as they reach an antibody titre that can be maintained by antibodies raised against those antigens encountered during normal tick feeding (e.g. salivary gland antigens).

The decline in IgG titres following infestations of inoculated hosts may also be attributable to immunosuppression. Tick saliva of several species, namely *Ixodes dammini* (Ribeiro *et al.*, 1985), *Dermacentor variabilis* (Wikel & Osburn 1982, cited in Martinez *et al.*, 1992), and *Rhipicephalus appendiculatus* (Fivaz, 1989) has been demonstrated to have

some immunosuppressive activity. Although no studies have shown that *A. hebraeum* saliva has immunosuppressive activity, the possibility of this being the case has previously been used to explain the absence of resistance to *A. hebraeum* (Norval *et al.*, 1988). The possibility of *A. hebraeum* saliva having some immunosuppressive activity, and the nature of the immunosuppression, needs to be investigated further.

Although IgG titres of tick-naïve (control) rabbits and goats were found to rise following successive tick infestations, they failed to reach the titres achieved following inoculation. Antibody titres of the control rabbits following a second *A. hebraeum* challenge, (1/80 - 1/7500, as measured by ELISA) were remarkably lower than those found by Kraidy (1991) following a third challenge of rabbits by nymphs of the same tick species (1/1000 - 1/10 000, as measured by ELISA). This discrepancy may be attributable to variations in the immunological responses of individual hosts to the same treatment. Larger sample sizes should be tested in order to obtain more representative data.

No correlation was found when comparing antibody titre with resistance to tick infestations. Similarly Jongejan *et al.* (1989) found that there was no correlation between the titre of circulating antibodies to the salivary glands of *Rhipicephalus appendiculatus* in rabbits following repetitive infestations, and the engorgement weights of subsequently fed female ticks. Furthermore, Adamson *et al.* (1991) showed by immunodiffusion testing, that goats inoculated with a crude extract of unfed *A. hebraeum* larvae developed antibodies to the larval proteins, but failed to develop any protective immunity to subsequent larval infestations.

Although the ELISA technique provides an effective method of measuring the antibody response of hosts to inoculated with homogenized tick extracts, there are probably antibodies raised against many antigens or epitopes which have no detrimental effect on tick success. Barriga *et al.* (1991b) observed an inverse relationship between antibody responses and manifestations of resistance, and suggested a phenomenon of competition between irrelevant and protective antigens. In order for ELISA's to be more meaningful, it would be necessary to isolate those antigens that induce a protective immune response and assay for antibodies raised against those particular antigens. Wong & Opdebeeck (1989) have used the ELISA technique to show that the level of antibodies raised against

a *Boophilus microplus* gut membrane vaccine correlates with the level of protection achieved in cattle.

5.3.1 Western Blot Analysis

Several reports indicate that inoculation with crude tick extracts, seldom improves upon the limited resistance attained naturally in the field following tick infestation (Adamson *et al.*, 1991; Fivaz *et al.*, 1991a). Consequently, inoculation would not be a practical approach to the development of tick-resistant livestock. It is however possible that antigenic competition amongst the many antigens present in the crude extract may reduce the effectiveness of vaccination (Willadsen *et al.*, 1988), or that substances present within the crude extract may hinder the antibody response (Mongi *et al.*, 1986). The identification and purification of the protective antigen(s) is therefore a crucial step in the production of vaccines against ticks.

Several studies (Barriga, 1991a; Brown, 1988a; Brown *et al.*, 1984b; Wikel & Whelan, 1986) have analyzed the tick antigens recognised by the humoral immune reactions of resistant hosts, in the hope that one or more of the identified antigens could be successfully used in the production of an anti-tick vaccine. In this study, serum from naive, inoculated and repetitively infested rabbits and goats was screened against *A. hebraeum* adult and nymphal crude extracts, using the western blotting technique, in order to determine the tick antigens recognised.

Results obtained in this study suggest that *A. hebraeum* inoculated rabbits and goats frequently produce antibodies to the entire range of antigens presented in the inoculation. Furthermore, despite subsequent tick infestations of inoculated hosts, the banding patterns on the blots became progressively weaker in the weeks after the final inoculation, suggesting that the antibody response was becoming weaker. These results correlate with the progressively lower antibody titres recorded during ELISA testing of sera obtained from inoculated hosts during the weeks after inoculation.

Despite western blotting being a long and somewhat tedious technique it can prove extremely rewarding. There are, however, several factors which may limit the effectiveness

of this technique in the identification of antigenic components that may be important in the development of tick resistance. In order for western blot results to be meaningful, it is necessary that the possibility of non-specific binding of antibody to antigen be excluded. Although 'blocking' is undertaken in order to minimise non-specific binding, it can still readily occur, especially when the concentrations of serum and/or conjugate are too high. The use of tick-naïve serum as a control can serve as an indicator as to whether non-specific binding is occurring. However, it is not conclusive since, although animals are thought of as being naïve when they have had no prior contact with ticks, they are frequently found to have other parasites that may result in the production of antibodies that cross-react with tick antigens. It has been shown that calves infested with the ear-mite, *Psoroptes cuniculi*, had antibodies that cross-reacted with 30 antigens in an *Amblyomma variegatum* salivary gland extract (Jongejan *et al.*, 1989).

Therefore in order to identify those tick antigens involved in eliciting a protective immunological response, it is necessary to obtain experimental hosts that have had no prior contact with parasites. This may be achieved by breeding and rearing potential experimental animals in a parasite-free environment, and undertaking weekly examinations of the animals for ecto-parasites. If sera from these parasite-naïve hosts still recognizes tick antigens, non-specific binding should be suspected and eliminated by manipulating serum and/or conjugate concentration.

Serum from the control (uninoculated) hosts after tick infestations gave inconsistent results, either failing to recognise any tick proteins or recognising a vast array of proteins. Whether these differences were due to differing degrees of non-specific binding, differences in the host's immunological competence, or to some of the hosts having cross-reactive antibodies from previous contact with other parasites, was not determined.

5.4 CONCLUSION

Due to the small number of experimental animals used in these experiments it is not feasible to reach any firm conclusions regarding the effect of inoculation of goats and rabbits with *Amblyomma hebraeum* extracts on tick success. Individual hosts have

frequently been shown to differ in their immunological response to tick infestations and/or inoculations with tick extracts (Allen & Humphreys, 1979; Fivaz & Norval, 1990; Kraidy, 1991). Therefore, experimental groups should have been considerably larger (no smaller than five animals per group) in order to obtain credible data on the effect of inoculation with *A. hebraeum* extracts on tick success. Furthermore, results obtained from infestations where ticks were fed on unnatural hosts and/or placed on parts of the body where they would not normally attach, may not be the same as results obtained under natural conditions.

Despite the limitations imposed on this research by the limited finances available, results suggest that contrary to the findings of Willadsen *et al.* (1988) in *Boophilus microplus*, immunity produced by vaccination with *A. hebraeum* antigens is directed against the immature life-stages rather than against adults.

Evidence suggests that the immune response of hosts to inoculation with *A. hebraeum* extracts can result in damage to the gut of feeding ticks. However, whether this damage is sufficient to have any significant effect on tick success, or whether the extent of damage could be expanded through the isolation of, and inoculation with, the specific gut proteins involved, has yet to be determined.

Although inoculated animals can be readily identified using skin provocation testing, too few animals were used in this study to make any definite correlation between tick resistance and the profile of the skin reaction. The response of rabbits and goats previously inoculated with *A. hebraeum* adult crude extract (ACE) to intradermal injection with ACE (and presumably to subsequent tick feeding) is characterized by a typical Type 1 (IgE-mediated) hypersensitivity reaction, resulting in the release of ECF-A and histamine (together with other vasoactive amines). Eosinophils dominate the reaction site and although they are reported to have some effect on tick feeding, the exact nature of this has yet to be determined. It is, however, possible that eosinophils further concentrate histamine at the site of tick attachment, thereby stimulating grooming. The role of basophils should be examined further using more specific staining techniques. The specific roles that Type III and/or Type IV hypersensitivities play in the response to tick feeding are uncertain, although it would appear that they may both be involved.

Some workers take the fact that antibody titres don't correlate with immunity as an indication of the important role of cell-mediated immunity in anti-tick protection (Barriga *et al.*, 1991b). However, as western blot results suggest that inoculation with *A. hebraeum* extracts results in the production of antibodies to all antigens presented in the inoculum, it is possible that inoculation with a more purified extract (without all the irrelevant antigens) may result in humoral antibody levels correlating with the degree of tick resistance.

Although western blotting has frequently used to characterize tick antigens which induce host resistance to subsequent tick feeding (Brown 1998a, 1988c; Barriga, 1991b), in order for results to be meaningful it is essential that cross-reactivity and non-specific binding be excluded. The exclusion of these two factors is difficult when working with crude tick extracts and whole serum, and consequently results presented in the literature may frequently be subjective and misrepresentative.

Inoculation with *Amblyomma hebraeum* extracts resulted in enhanced hypersensitivity reactions, high anti-*A. hebraeum* IgG titres and, cellular immune responses at the site of injection. However despite all this, inoculation failed to improve upon the resistance to ticks which is acquired naturally, following repetitive infestations. Whether these results could be improved following the identification and purification of critical antigens, remains to be determined. The finding that feeding on inoculated hosts can result in gut damage lends hope to the prospect of developing a vaccine similar to that being developed against a gut protein of *Boophilus microplus*. Before this can be achieved the specific gut protein involved must be identified and isolated. If host resistance to *A. hebraeum* could be achieved, it would then be important to determine if the resistance produced interfered with the transmission of pathogenic organisms such as *Cowdria ruminantium*.

ACKNOWLEDGEMENTS

I would like to thank my supervisors Professors Bruce Fivaz and Don Hendry, for their guidance, encouragement and support. Professor Fivaz is also thanked for providing me with employment when the bursaries dried up.

Most sincere thanks go to the people who assisted with various technical aspects of this work. In this regard, particular thanks go to Mr Edward Williams and Mr Charles Willemse, for their unfailing helpfulness with handling the animals. Mr Robin Cross, Mr Neil Cannon and Mrs Shirley Pinchuck, of the Electron Microscopy Unit, Rhodes University, are thanked for the taking of photographs and their endless patience when demonstrating the techniques of TEM. Thanks also to Mrs Sally Terry and Miss Moira Pogrand for their support and assistance.

Penn Lloyd is thanked for the many hours that he spent reading and correcting this text, often to the detriment of his own research. William Froneman, Jane Nurton and Tania Rossouw are also thanked for proof-reading parts of the text. A special word of thanks go to John Goetsch who spent numerous hours formatting and printing.

All my friends and colleagues in Microbiology and Zoology deserve a special word of thanks for helping me through the times of demotivation, depression and anxiety.

Lastly, I would like to thank my parents Muir and Jane Adamson for providing me with the opportunity of an excellent education and for continually being a source of inspiration.

I am grateful for financial support from the following sources:-

Rhodes University Funding (through the Tick Research Unit)

Foundation for Research and Development (FRD) Masters Bursary

Rhodes University Graduate-assistant Bursary

Kathleen Abbott Bursary

Laura Stark Bursary

Sasol Bursary

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