THE RELATIONSHIP BETWEEN THE FEEDING OF *AMBLYOMMA VARIEGATUM* TICKS AND THE SKIN DISEASE DERMATOPHILOSIS

by

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TABLE OF CONTENTS

DECLARATION

This investigation was part of a three year laboratory study sponsored by the Overseas Development Association of the United Kingdom. The experiments described herein, and the composition of this thesis were completed by myself.

Carolyn Marie Lloyd

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DEDICATION

To Jeff for his love, patience
and encouragement

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ABSTRACT

The relationship between the feeding of *Amblyomma variegatum* ticks and *Dermatophilus congolensis* infections has been studied under laboratory conditions.

The local effects of hypersensitive or inflammatory reactions to larval and nymphal *A.variegatum* on subsequent *D.congolensis* infections were investigated using rabbits and sheep respectively. Multiple or single infestations of ticks were used to produce hypersensitivity or inflammatory reactions respectively. These reactions were confirmed by histological assessment of the tick attachment sites. Identical titrated doses of *D.congolensis* were applied to the tick attachment sites after the ticks had detached, a control titration was set up on skin with no previous exposure to ticks. The progression of the resulting lesions was assessed using a non-parametric ranking system. There was no significant difference ($P > 0.05$) between the severity or duration of the three groups of dermatophilosis lesions, either on the sheep or the rabbits. Therefore it was concluded that the local effects of the feeding of immature instars of this tick do not affect the pathogenesis of subsequent *D.congolensis* infections.

The local effect of hypersensitive or inflammatory reactions to *A.variegatum* nymphs on simultaneous *D.congolensis* infections on rabbits was also studied. There was an increase in the initial severity of the dermatophilosis lesions and a positive correlation between inflammatory tick attachment sites and dermatophilosis foci. However, the local effects of the feeding of nymphal *A.variegatum* did not result in the development of chronic dermatophilosis lesions.

The systemic effect of adult and nymphal *A.variegatum* on simultaneous dermatophilosis lesions was compared, using sheep as the experimental hosts. *Dermatophilus congolensis* infections on sheep infested with adult *A.variegatum*

developed into chronic lesions which persisted for several months. Serological and skin tests revealed significantly ($P < 0.01$) reduced humoral and cellular immune responses in sheep infested with adult *A.variegatum* compared with sheep infested with nymphs or control sheep not exposed to ticks.

Comparative studies of whole salivary glands from all three instars of *A.variegatum* revealed significantly greater ($P < 0.01$) proportions of type-2 acini filled with *c1* secretory granules in the salivary glands from the adult ticks compared with the salivary glands from the immature ticks. There were also significantly ($P < 0.05$) greater proportions of individual type-3 acini filled with *e* secretory granules in the salivary glands from the adult ticks compared with the salivary glands from the larval ticks. Gel electrophoresis revealed an 11% dissimilarity between salivary glands from adult *A.variegatum* compared with both of the immature instars. Similar analysis of artificially induced saliva revealed a 48% dissimilarity between the adult and immature ticks.

ABBREVIATIONS

ANOVA	Analysis of variance
BHI	Brain-heart-infusion
BPO	Benzol-peroxide
CTVM	Centre for Tropical Veterinary Medicine, University of Edinburgh, Scotland
ELISA	Enzyme linked immunosorbent assay
HBSS	Hank's balanced salt solution
H/G	Hank's balanced salt solution with pig gelatin at 0.5% w/v
kDa	Kilodaltons
LTT	Lymphocyte transformation test
NSP	Neutralised soya peptone
RH	Relative humidity
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate - polyacrylamide gel electrophoresis

INTRODUCTION

Dermatophilosis is an exudative dermatitis caused by the actinomycete *Dermatophilus congolensis*. The disease has a world-wide distribution but it is usually in the wet tropics that it progresses into a severe, chronic form. Dermatophilosis is mainly a disease of ruminants, especially cattle and sheep. However, it also occurs in a wide variety of other animals including horses, gazelles, giraffe, lizards and even man.

In the humid tropics, where the disease in cattle and small ruminants becomes chronic, dermatophilosis can cause major economic losses in the livestock industry. In western and central Africa and the Caribbean the disease can cause losses due to damage to hides, deaths due to culling or infection and reduction in meat and milk production. The disease is also a major obstacle to the introduction of exotic cattle used to increase beef and milk production. In some areas of the Caribbean dermatophilosis is so severe that attempts at cattle farming have been abandoned.

Various mechanisms are thought to be associated with the development of chronic dermatophilosis; high humidity, wetting of skin, physical damage to the skin caused by ticks, insects and thorny plants, exposure to sunlight, hypersensitive reactions in the host's skin in response to tick or insect bites and immunosuppression caused by ticks or malnutrition. However, there is much conflicting evidence in both field observations and laboratory work. In Nigeria, the incidence and severity of the disease in cattle has been found markedly to increase during the wet season. On the other hand, severe outbreaks of dermatophilosis in cattle in St. Lucia have been recorded in the driest parts of the island.

In the United Kingdom, South Africa and Australia, the disease causes economic losses in the wool trade where it appears in a clinical form called lumpy wool. In the United Kingdom the incidence of dermatophilosis infection increases when damage to the epidermis caused by shearing coincides with heavy rain.

However, in Australia the disease in cattle occurs in dry conditions and appears to be associated with stress of poor feed.

Other workers observing the disease in more controlled conditions have found that severe lesions regress, even at 95% humidity, as long as the animals are protected from the attacks of insects and ticks. Although the physical damage to the epidermis caused by the bites of ticks and insects appears to be important in the formation of chronic lesions, many workers have found that physical damage alone is not enough to produce chronic lesions. Scarification and pricking of the epidermis at the site of inoculation with *D.congolensis* has not produced chronic lesions.

There has been a long association between ticks, especially *Amblyomma variegatum*, and chronic dermatophilosis. Some workers report initial dermatophilosis lesions at the predilection sites of adult *A.variegatum*, whilst others describe severe lesions forming along the dorsal midline. Immature ticks and biting flies feed in very large numbers on the dorsal surface of cattle. These bites can cause severe physical damage to the epidermis and can result in the formation of delayed hypersensitive reactions in the skin of the host. These delayed hypersensitive reactions may predispose to dermatophilosis.

Several aspects of the formation of chronic dermatophilosis have been investigated at the Centre for Tropical Veterinary Medicine (CTVM), University of Edinburgh, Scotland. These investigations have included both laboratory work and field work through collaborative projects in Nigeria, Ghana and the Caribbean.

Laboratory investigations at the CTVM have examined the effects of climatic factors such as high humidity and sunlight, along with the effect of hypersensitive reactions in the host's skin on local experimental *D.congolensis* infections. Artificial simulations of hypersensitive reactions to insect bites were found

to delay the resolution for as long as the hypersensitive reaction was maintained in the skin. High humidity and spraying of experimental infection sites produced more widely spread lesions but did not delay the healing.

Although it has been possible to produce acute experimental dermatophilosis lesions on guinea-pigs, rats, rabbits, sheep, goats and cattle it has been very difficult to produce chronic experimental lesions. Only recently has it been demonstrated, through the work at the CTVM, that it is possible to produce chronic experimental dermatophilosis lesions on sheep with simultaneous infestations of adult *A.variegatum*.

Many times in the field the occurrence of severe chronic dermatophilosis has been associated with the presence of *A.variegatum* ticks, but prior to recent work at the CTVM there had been no controlled work investigating this association.

The aim of this study was to carry out a broad parasitological and entomological study of the association between ticks and clinical infections. It was not intended to be a detailed immunological or dermatological study, but techniques from these areas were used to demonstrate the effects of the ticks.

The experimental hosts used for this study were rabbits and sheep. All of the rabbits used in the study were female New Zealand White rabbits obtained from one Home Office approved supplier. The experimental sheep were obtained from various sources, wherever possible female Blackface x Suffolk sheep were used. In some cases castrated males were used and for one experiment pure Blackface sheep were used when the crossbred sheep were not available. For experimental purposes both rabbits and sheep were matched by weight and size. Obviously, more closely matched pairs obtained from the same source or even the use of twins would have reduced individual variation. For the purposes of these experiments it was not

considered essential to work with perfectly matched pairs. The experiments were designed as preliminary experiments to investigate various scenarios involving *A.variegatum* ticks and dermatophilosis. For a more detailed study of one aspect of tick feeding and the development of dermatophilosis it would be necessary to use more closely matched pairs.

Although various haematophagous insects may also be involved in the aggravation of dermatophilosis, this study was confined to an investigation of the effects of *A.variegatum* ticks. Several colonies of *A.variegatum* have been maintained at the CTVM since 1976. The latest colony was produced from ticks originating from Kenya and maintained in the laboratory for several years; the colony was established for heartwater and dermatophilosis research carried out at the CTVM. Tsetse flies would have been the only insect readily available. These flies are not really applicable to the problem of the formation of chronic dermatophilosis due to the low population densities in the field compared with other biting insects.

The history of dermatophilosis research at the CTVM, which is still continuing, and the availability of *A.variegatum* makes this an ideal situation to carry out a controlled investigation into the possible relationship between this tick and the development of chronic dermatophilosis. Many factors are thought to be involved in the formation of chronic dermatophilosis lesions including climatic factors, immunosuppression, physical damage and hypersensitive reactions to ticks and insects. This study is designed to investigate only one of these factors: whether *A.variegatum* ticks have any effect on the progression of dermatophilosis and, if so, what is the relationship between the feeding of this tick and the formation of chronic dermatophilosis lesions.

CHAPTER ONE
LITERATURE REVIEW

1.1 INTRODUCTION

Dermatophilosis is a bacterial skin disease caused by a gram-positive actinomycete, *Dermatophilus congolensis*. The causal agent, *D.congolensis* was first described in a mycotic dermatitis on cattle in the Belgian Congo by Van Saceghem, (1915). Dermatophilosis has a world-wide distribution and has been recorded in South America (Moreiro and Barbosa, 1976), United States (Kelley, 1976), Israel (Nobel *et al.*, 1971), United Kingdom (Hart, 1976), Australia (Egerton, 1964), Western and Central Africa (Plowright, 1956), South Africa (Bekker, 1928) and the West Indies (Butler, 1975). However, it is usually in the wet tropics that the disease develops into a severe, chronic form. Dermatophilosis is also a problem with sheep rearing in Australia (Bull, 1929; Zaria, 1993) where the conditions are hot and the animals suffer stress from poor feed. Moule and Sutherland (1947) record chronic dermatophilosis on cattle in arid conditions in Australia, where chronic dermatophilosis is associated with physical damage.

In areas where dermatophilosis progresses into severe chronic lesions, it can have disastrous effects on the local livestock industry. Although dermatophilosis is most severe in the wet tropics, it has economic effects on the livestock industry wherever it occurs. Moreiro and Barbosa (1976) report economic losses in South America due to dermatophilosis. In the United Kingdom, lumpy wool causes economic losses due to damage to the fleece. The sheep may even die as a result of ruminal tympany when rolling to alleviate the irritation (Hart, 1967; Hart, 1976). Bull (1929) reports that lumpy wool in Australia is of great economic importance due to damage to the wool and general deterioration in the health of the infected animals. He reports situations in which 20% or more of the animals were infected in an outbreak. Dermatophilosis causes economic losses due to the damage to hides in Kenya

(Ainsworth and Austwick, 1959; Bwangamoi, 1976), Tanzania and Uganda (Bwangamoi, 1976).

In the wet tropics, dermatophilosis causes economic losses due to death of livestock, culling of severely affected animals, damage to hides, progressive emaciation, weight loss, and general decrease in beef and milk production (Plowright, 1956; Oduye, 1975a; Oduye, 1975b; Lloyd, 1976; Obeid, 1976; Oduye, 1976a; Pullan, 1980; Zaria, 1993).

In Nigeria, dermatophilosis is a major cause of economic loss due to damage to hides (Kelley and Bida, 1970; Bida and Dennis, 1976; Lloyd, 1976; Zaria, 1993). Oduye (1975b) states that dermatophilosis is the second most important disease in the livestock industry in Nigeria. In 1975 it was estimated that a total of 66,000 animals were lost through deaths and culling as a result of dermatophilosis in Nigeria alone (Oduye, 1975b). The economic losses due to damage to hides are very serious; hides are consumed as delicacies, exported, and used for local goods (Oduye, 1975b). Damaged hides can only be sold locally, at a reduced price.

The effect on milk production can also be very severe. Lloyd (1976) cites an outbreak of dermatophilosis in which 48% of the calving heifers and 64% of the bulling heifers were affected. In this outbreak, 28% of the animals had to be culled. The disease often affects the udder and teats, which can severely restrict suckling and milking (Lloyd, 1976); in some cases, milk yield can be reduced by as much as 20% (Nobel *et al.*, 1976).

Pullan (1980) reported dermatophilosis as one of the most important diseases limiting the productivity of cattle farming on the Jos plateau in Nigeria. In West and Central Africa, the introduction of exotic breeds to increase production is severely limited because of dermatophilosis (Lloyd, 1976; Zaria, 1993). In some areas

of the tropics, the disease totally destroys the viability of cattle farming (Stewart, 1972; Uilenberg *et al.*, 1984). In areas of St. Lucia, where dermatophilosis is associated with *Amblyomma variegatum* ticks, the problem is so severe that attempts at cattle farming have been futile (Butler, 1975). Severe cases of dermatophilosis cannot be cured and the animals have to be destroyed (Zlotnik, 1955; Bida and Dennis, 1976).

Due to the varied clinical signs, numerous names have been given to the disease in its different forms. On the Jos plateau, Nigeria, it is called kirchi (Oduye, 1975b; Pullan, 1980; Zaria, 1993); other names include senkobo skin disease (Zlotnik, 1955; Oduye, 1975b), savi (Zaria, 1993), ambarr-madow (Zaria, 1993), contagious impetigo (Van Saceghem, 1915), and dro-dro boka (Oduye, 1975b; Zaria, 1993). In cattle, the disease is widely known as streptothricosis (Bida and Dennis, 1976) whilst in sheep, it manifests in different forms such as mycotic dermatitis (Van Saceghem, 1915; Hart, 1967) or lumpy wool (Bull, 1929), and strawberry foot-rot (Thompson, 1954). The name dermatophilosis, derived from the genus name *Dermatophilus,* is used to refer to the disease in various animals, not just cattle (Zaria, 1993).

Dermatophilosis is a skin disease of ruminants, especially cattle and sheep, but infections have been reported in many species including horses (Macadam, 1964a) and donkeys (Oduye, 1975a), deer (Kelley, 1976), owl monkeys *Aotus trivirgatus* and woolly monkeys *Lagothrix lagotrichia* (Kaplan, 1976; Kelley, 1976), lizards (Simmons *et al.*, 1972), and even man (Stewart, 1972; Kaplan, 1976; Kelley, 1976). Dermatophilosis infections in man are not serious and do not affect the overall condition of the individual involved (Kaplan, 1976).

Many workers describe *D.congolensis* as a saprophyte (Bull, 1929; Zlotnik, 1955; McEwan Jenkinson, 1976) and an opportunist which infects the host under favourable conditions. Zlotnik (1955) considered *D.congolensis* to be

saprophytic on either cattle or overhanging branches of trees and, therefore, present to take advantage of physical damage caused to the epidermis. Macadam (1970) found that an isolated herd of cattle did not become infected, although conditions were favourable; he concludes, therefore, that the organism is not saprophytic.

Infective zoospores are thought to survive in soil and to provide a reservoir for infection (Roberts, 1967; Bida and Dennis, 1976; Kaminjolo and Karua, 1981; Zaria, 1993). The importance of soil as a source of infective zoospores is restricted to dry conditions (Roberts, 1967; Bida and Dennis, 1976), retaining infectivity for up to four months (Roberts, 1963). Wet soils show no signs of infectivity (Roberts, 1963; Roberts, 1967; Bida and Dennis, 1976).

Dermatophilosis is a superficial infection of the epidermis (Bull, 1929; Zlotnik, 1955; Egerton, 1964; Roberts, 1964; Nobel *et al.*, 1971; Amakiri, 1976; Zaria, 1993). Three barriers are thought to be involved in the initial infection of the epidermis; these are the hair or wool, sebaceous film (Roberts, 1964; Amakiri, 1976), and the *stratum corneum* (Roberts, 1964). The effectiveness of the sebaceous film is thought to be due to its completeness as a physical barrier rather than through any bacteriostatic effects (Roberts, 1964). Roberts (1967) found that removal of sebaceous film on calves, using ether, resulted in a larger number of infection foci compared with areas not swabbed with ether. Amakiri (1976) found *D.congolensis* in the sebaceous glands of infected animals, indicating that the sebum does not have any bacteriostatic properties.

Once *D.congolensis* has penetrated the epidermis, it does not normally extend into the dermis. This extension may be prevented by the basement membrane of the epidermis (Amakiri, 1976) or a layer of cellular exudate consisting mainly of neutrophils, macrophages and lymphocytes (Amakiri, 1976). Amakiri (1976) found that *D.congolensis* penetrated into the dermis only where the basement membrane

was disrupted. The greatest penetration of the *D.congolensis* hyphae occurs at the hair follicle sheaths (Egerton, 1964; Roberts, 1964; Amakiri, 1976; Oduye, 1976b) with a penetration of up to several millimetres a day (Roberts, 1964).

Infiltrating granulocytes separate the infected epidermis from the underlying matrix and a new, uninfected, epidermis is formed under the infiltrating cells by lateral branching from adjacent hair follicles (Roberts, 1964). This new epidermis becomes infected by lateral spread of *D.congolensis* filaments from the hair sheaths (Roberts, 1964).

Roberts (1964) found large numbers of plasma cells in chronic dermatophilosis infections but not in acute experimental infections, he concluded, therefore, that plasma cells are not involved in the defence against *D.congolensis*. Bull (1929) also reports large numbers of neutrophils, lymphocytes, and plasma cells at the site of chronic lesions. Suppression of the infiltration of granulocytes in rabbits resulted in a more rapid spread of *D.congolensis* in the epidermis and the penetration of the organism through the basement membrane into the dermis (Roberts, 1964). The possible involvement of granulocytes was reinforced by their ability to phagocytize *D.congolensis in vitro* (Roberts, 1964). Roberts (1964) concludes that the more rapid healing of lesions on sheep with previous exposure to *D.congolensis* is caused by the accelerated granulocyte infiltration associated with delayed hypersensitivity.

1.2 EPIZOOTIOLOGY OF DERMATOPHILOSIS

Much work has been carried out in the field and the laboratory to investigate the possible factors associated with the development of the severe, chronic, lesions. Several factors appear to be involved, with no one factor being solely responsible for the progression of the disease (Oduye, 1976a; Zaria, 1993). The

factors associated with chronic dermatophilosis are thought to be prolonged wetting of the skin (Hart, 1976; Oduye, 1976a; Zaria, 1993) and physical damage of the epidermis (Stewart, 1972; Oduye, 1975a; Macadam, 1976; Nobel *et al.*, 1971; Obeid, 1976; Oduye, 1976a; Oppong, 1976). Oppong (1976) suggests that even the dermatophilosis scabs can be abrasive, causing damage to the skin either on the same animal or other individuals. These scabs can also be a source of infective zoospores which are released when the scabs become wet (Roberts, 1967; Macadam, 1970; Oppong, 1976).

1.2.1 CLIMATIC FACTORS

The disease has long been associated with the onset of the rainy season in the tropics (Zlotnik, 1955; Plowright, 1956; Macadam, 1964b; Macadam, 1964c; Hart, 1967; Stewart, 1972; Bida, 1975; Oduye, 1975a; Bida and Dennis, 1976; Obeid, 1976; Oduye, 1976a; Oppong, 1976; Pullan, 1980; Morrow *et al.*, 1989; Zaria, 1993) or with periods of rainy weather in the temperate regions (Austwick, 1976; Kelley, 1976). Obeid (1976) recorded an incidence of 1.5% in the Kordofan province in Sudan in the dry season, whereas in the wet season, there was 15-20% rate of infection (Soltys, 1964, Cited by Obeid (1976)). Kelley and Bida (1970) found that 12% of the cattle in the Fulani herds in Northern Nigeria had dermatophilosis lesions in the wet season. In Ghana, dermatophilosis is also more prevalent during the wet season, with an incidence of 5% in the dry season compared with 13% in the wet season (Oduye, 1975b). It appears to be only the mild infections which regress in the dry season, with no regression of the severe generalised lesions (Zlotnik, 1955; Plowright, 1956; Coleman, 1967).

The increased severity of the disease is manifested by an increase in the incidence of the disease as well as increased severity and spread on individual animals (Oduye, 1976a; Oppong, 1976). Macadam (1962) concludes that the increase in the

number of lesions observed in the wet season is due to fresh infections caused by the bites of ectoparasites, rather than a direct effect of the increased humidity. Davis and Philpott (1980) found that the spraying of experimental dermatophilosis lesions on goats had no affect on the development of the lesions. However, spraying the inoculation sites did result in dispersal of the lesions which occurred in the areas of run-off. Roberts (1967) reports the spread of natural lesions on sheep when exposed to rain; fresh lesions developed on the flanks of the animals following the line of run-off from the original lesions.

Davis and Philpott (1980) suggest that moisture may form a film through which infective zoospores can migrate from the scabs to areas of damaged skin, such as arthropod bites. Carbon dioxide would also be able to diffuse through the film of moisture and zoospores would respond with a positive chemotactic response (Roberts, 1967). The diffusion of carbon dioxide would be greatest from sites where the sebaceous film or *stratum corneum* is incomplete; therefore, zoospores would be attracted to sites least resistant to their penetration (Roberts, 1967).

Different management systems also have an effect on the incidence of the disease. Animals protected from rainfall by shelter showed a lower incidence of infection (Koney and Morrow, 1990), whilst the practice of daily spraying of dairy cattle to clean and cool the animals has been associated with a higher incidence of infection (Nobel *et al.*, 1976). Housing of animals to protect them from rain has been suggested as a method to control dermatophilosis (Hart, 1967; Stewart, 1972).

Various reasons for the association of wet weather and severe chronic dermatophilosis have been suggested. Prolonged wetting of the skin may damage the barriers of the epidermis by emulsifying the sebaceous film (Oppong, 1976; Philpott and Ezeh, 1978) and the *stratum corneum* (Roberts and Graham, 1966). High rainfall may leach or dilute substances produced by microflora present on the skin which

inhibit *D.congolensis* (Kingali *et al.*, 1990). During the dry season many dermatophilosis scabs dry up and fall off, but on areas on the lower parts of the body not exposed to direct sunlight, some scabs may remain attached (Oppong, 1976; Zaria, 1993). At the start of the rainy season these scabs become wet and this allows the release of infective zoospores which reinfect the animal (Oppong, 1976). Oppong (1976) showed that zoospores in dry scabs could remain viable for up to 42 months.

Macadam (1961; 1964b; 1964c) found that dermatophilosis lesions on White Fulani bulls regressed even though the animals were kept in conditions of high humidity. He suggests that the increased prevalence of dermatophilosis observed at the onset of the rainy season is not due to the increase in humidity but to an increase in the insect population.

Other work indicates that humidity alone does not cause the increase in the prevalence of dermatophilosis in the rainy season (Plowright, 1956; Macadam, 1970). Macadam (1961; 1964b; 1964c) found that dermatophilosis lesions on cattle regressed, even at 95% humidity, as long as the animals were kept free of ticks. He suggests that the association of dermatophilosis with the rainy season is not due to a direct effect of the humidity, but the accompanying increase in the population of ectoparasites.

Morrow and Compton (1991) in their study of dermatophilosis on St. Lucia found that rainfall had no effect on the prevalence of the disease; it was the presence of *A.variegatum* which had a much greater effect on the prevalence of the disease. Another study of the severe dermatophilosis on St. Lucia, by Butler (1975), found that the cases of severe dermatophilosis occurred on one of the driest parts of the island. Observations by Moule and Sutherland (1947) of dermatophilosis on cattle in Australia indicated that there was no correlation between rainfall and the incidence

of lesions. Kaminjolo and Karua (1981) reported severe generalised dermatophilosis lesions on cattle in Malawi, three months after the last rains.

1.2.2 TICKS AND INSECTS

The onset of the rainy season is also the time when the tick and insect populations increase (Plowright, 1956; Macadam, 1961; Macadam, 1964b; Macadam, 1970; Bida, 1975; Oduye, 1975b; Oppong, 1976; Philpott and Ezeh, 1978; Davis and Philpott, 1980). Ticks, especially *A.variegatum*, have long been associated with the development of chronic dermatophilosis lesions in the field (Henderson, 1928; Macadam, 1970; Obeid, 1976; Oduye, 1976a; Pullan, 1980; Garris and Scotland, 1985; Martinez *et al.*, 1992; Zaria, 1993). Herd owners on the Jos plateau, Nigeria, remove ticks by hand because they consider that the ticks are the cause of severe dermatophilosis lesions (Pullan, 1980).

Wilson (1946) carried out a survey of ticks in Malawi and found that adult *A.variegatum* ticks were found on the cattle only in the wet season. The immature stages of this tick were found mainly in the dry season, with the exception of small numbers of nymphs. Garris and Scotland (1985) report similar changes in the fluctuation of *A.variegatum* in St. Lucia. Norval (1986) points out that dermatophilosis is only associated with *A.variegatum*, not even other species of the same genus. In Zimbabwe, dermatophilosis is only found in the areas where *A.variegatum* occurs, and is not found in the more widespread areas where only *Amblyomma hebraeum* occurs (Norval, 1986).

In the Caribbean, severe dermatophilosis is found only on the islands with *A.variegatum* ticks, and then only in those areas where the tick occurs (Butler, 1975; Burridge *et al.*, 1984; Uilenberg *et al.*, 1984; Morrow *et al.*, 1989; Morrow and Compton, 1991). Morrow *et al.* (1989) found that the prevalence of severe

dermatophilosis lesions increased as the number of adult *A.variegatum* ticks increased. In St. Lucia, severe dermatophilosis is confined to the areas with *A.variegatum* infestations (Butler, 1975; Morrow and Compton, 1991). Morrow and Compton (1991) found that the overall prevalence of dermatophilosis in the cattle population was 4.2%, but in areas where they found *A.variegatum*, the prevalence of the disease increased to 24%. The cattle tick *Boophilus microplus* is widespread on St. Lucia, but severe dermatophilosis only occurs where *A.variegatum* is present (Butler, 1975; Morrow *et al.*, 1989).

Koney and Morrow (1990) found that dipping to control ticks, including *A.variegatum*, resulted in a decreased incidence of dermatophilosis from 24.7%, in traditionally managed herds, to 3.6%. Dipping to control ticks has often been suggested as an effective method to control dermatophilosis (Henderson, 1928; Plowright, 1956; Macadam, 1970; Stewart, 1972; Oduye, 1975a; Oduye, 1975b; Koney and Morrow, 1990; Zaria, 1993). Oduye (1975b) reports that in a survey of dermatophilosis in Nigeria the incidence of the disease was lower on government farms where there was efficient tick control. In a study of the association between *A.variegatum* ticks and severe dermatophilosis, a 1½ year tick control program using flumethrin pour-on acaracide was accompanied by a decrease in the prevalence of the disease from 14% to 6% (Morrow and Compton, 1991). Butler (1975) recommends control of ticks to control dermatophilosis, but he found this resulted in the regression of only mild cases, and did not affect severe infections that were already established.

However, there are also reports that the use of acaricides does not affect the incidence of dermatophilosis (Oduye, 1976a; Zaria, 1993). In South Africa, Bekker (1928) states that dipping, rather than resulting in a decrease, was often associated with an increase in the prevalence of dermatophilosis.

It has also been suggested that the control of dermatophilosis by dipping to control ticks is due to a direct effect of the acaricides on *D.congolensis* (Zlotnik, 1955). Heron and Morrow (1989) tested the bacteriostatic effects of 13 different acaricidal preparations *in vitro*. They found that most acaricide preparations, when not contaminated with other materials, reduced the number of *D.congolensis* zoospores below a level required to produce infection. In a field situation, acaricides in dip tanks and spray races would be contaminated with other materials such as wool, faeces, and mud. To simulate conditions in the field, they added newborn calf serum to the preparations and found that only lindane ('cattle washing detergent', Denka International) and toxaphene, (Cooper's Animal Health) retained any ability to reduce infection. Flumethrin ('Bayticol pour-on', Bayer) was shown to have no antibacterial effects at all. In fact, *D.congolensis* not only survived, but remained infective in the majority of the acaricides tested. This might explain why dipping is sometimes associated with an increased incidence of dermatophilosis (Zaria, 1993).

Le Riche (1967) tested various dipping fluids for their effectiveness in preventing or curing dermatophilosis infections on sheep. None of the fluids had any therapeutic effect on established lesions, but he found that 0.1% magnesium fluosilicate and micronised sulphur was effective in preventing infection. He concluded that transmission of infections could occur via dipping, but that 0.5% zinc sulphate or 0.1% magnesium fluosilicate could prevent this.

In some situations, where other factors are also involved, it appears that ticks are not always necessary to produce chronic and severe dermatophilosis. Bull (1929) states that biting insects were not associated with dermatophilosis infections on sheep in Australia. Zlotnik (1955) considered from observations in Malawi that *A.variegatum* were not important in the development of severe dermatophilosis. Egerton (1964) observed dermatophilosis on cattle in Papua-New Guinea in areas

where there was no cattle tick, *B.microplus*. Nobel *et al.* (1976) found that although dairy cattle were dipped to control ticks and flies, the animals still developed dermatophilosis. In this case, the epidermis was being subjected to physical trauma by housing the cattle on abrasive concrete, and this was combined with 30 minutes of spraying, to cool and clean the cattle, at least once a day.

The mechanism by which ticks are thought to be involved in the formation of chronic dermatophilosis lesions is unclear. Martinez *et al.* (1992) investigated the possibility of transmission of *D.congolensis* by *A.variegatum*. They fed all three instars of this tick on *D.congolensis* inoculation sites on goats. Engorged ticks from infected animals were washed three times in sterile distilled water and then sectioned behind the first pair of legs. The anterior parts of the ticks were then incubated in broth: *D.congolensis* cultures were produced if it was present in the ticks. *D.congolensis* cultures were produced only by incubation of the adult male ticks. Since *D.congolensis* was found only in the adult males, and transstadial transmission does not occur, these findings indicate that it would be impossible for the tick to transmit this disease. On the other hand, Macadam (1962) succeeded in experimentally transmitting dermatophilosis from infected cattle on to rabbits by the feeding of *A.variegatum* ticks.

Dermatophilosis lesions have been found at the predilection sites for adult *A.variegatum* feeding on severely affected cattle with heavy tick burdens (Oduye, 1975a; Bwangamoi, 1976). Macadam (1964b) observed *A.variegatum* associated with severe scrotal lesions. During the rainy season in Nigeria, Plowright (1956) found that 70% of the cattle in the survey had dermatophilosis lesions, but only 15% had dorsal or lateral lesions. All of the cattle had lesions on the ventral aspect, which is the predilection site for adult *A.variegatum*. However, although initial lesions were often

observed on the groin, scrotum, axilla and teats, the ticks were not often associated with the primary lesions.

Although dermatophilosis lesions have been associated with the predilection sites for *A.variegatum* (Oduye, 1975a; Oduye, 1975b; Bwangamoi, 1976), there are many reports of lesions being widespread over the dorsal areas of infected animals, remote from the attachment sites of adult *A.variegatum* (Zlotnik, 1955; Macadam, 1964b; Macadam, 1964c; Morrow *et al.*, 1989). Oduye (1976a) noted that even on cattle with high tick burdens associated with severe dermatophilosis, the lesions were generalised and not associated just with the tick attachment sites. In St. Lucia, dermatophilosis lesions are generalised and occur along the back as well as on the axilla, dewlap and perineal region; the predilection sites of adult *A.variegatum* (Butler, 1975). Plowright (1956) observed that dermatophilosis lesions developed on the ventral surface, often associated with *A.variegatum*, but he also noted cases in which there was an obvious spread of initial lesions along the dorsal mid-line.

Immature *A.variegatum* and biting flies, such as muscid flies of *Haematobia* species, feed in large numbers on the dorsal surfaces of the animals and may influence the severity or distribution of the dermatophilosis lesions (Plowright, 1956; Macadam, 1964b; Macadam, 1964c; Stewart, 1972; Zaria, 1993). Stewart (1972) observed large numbers of the tabanid flies *Haematopota albihirta* and *Tabanus taeniola* feeding on the backs, flanks, and rumps of cattle associated with the majority of the dermatophilosis lesions. He demonstrated that *D.congolensis* was present in the puncture wounds produced by the flies. However, it is not clear whether the flies had transmitted *D.congolensis*, or if they just facilitated the penetration of the organism. The population of *Haematobia* flies can become very

dense, especially in the wet season, with as many as 10,000 flies seen on one cow at any one time (Lancaster and Meisch, 1986).

Biting flies have been seen to gather at lesions and then move around, feeding repeatedly either at different parts of the same animal or different individuals (Oduye, 1976a). This repeated feeding and moving could explain the more generalized dorsal lesions. The strong odour of the dermatophilosis lesions also attracts muscid flies (*Musca* and similar species) and stable flies (*Stomoxys* species), (Oppong, 1976). Hadrill *et al.* (1991) have shown that the control of *Haematobia*, using insecticides as permethrin pour-on, or cypermethrin impregnated ear-tags on cattle, had no effect on the severity or distribution of dermatophilosis lesions.

Biting flies, such as *Stomoxys* species, appear to be the main vectors of dermatophilosis on sheep in the Gambia (Macadam, 1976), although isolated sheep exposed to biting flies did not develop infections (Macadam, 1976). Macadam (1964b; 1964c) thought that the dorsal lesions which appeared on cattle were associated with the bites of ticks, flies and lice. Richard and Pier (1966) found that feeding *Stomoxys calcitrans* on rabbits infected with *D.congolensis* and then uninfected rabbits resulted in the formation of dermatophilosis lesions at the feeding sites on the uninfected rabbits. On the other hand, Martinez *et al.* (1992) found that even though goats became seropositive to *D.congolensis*, probably due to transmission by flies, they did not develop dermatophilosis unless they were exposed to the feeding of *A.variegatum*.

Large numbers of non-biting muscid flies, with labellar mouthparts, have been observed feeding on cattle (Lloyd and Dipeolu, 1974). *D.congolensis* infections have been experimentally transmitted to uninfected cattle (Philpott and Ezeh, 1978) and uninfected rabbits by the feeding of *Musca domestica* (Richard and Pier, 1966)

which had previously fed on infected animals. This transmission was facilitated by wetting of scabs on the infected animals (Richard and Pier, 1966).

1.2.3 PHYSICAL DAMAGE

Physical damage to the epidermis is thought to be necessary to allow initiation of dermatophilosis (Ainsworth and Austwick, 1959; Stewart, 1972; Oduye, 1976a; Oppong, 1976; Zaria, 1993). Bull (1929) managed to produce dermatophilosis lesions on rabbits by inoculating with *D.congolensis* at sites from which the fur had been plucked; inoculations on undamaged skin failed to produce lesions. Physical damage may be caused by thorns (Zlotnik, 1955; Oduye, 1975b; Oduye, 1976a), shearing (Ainsworth and Austwick, 1959), cattle egrets (Oduye, 1976a), ox-pecker birds (*Boophagus africanus africanus*) (Oduye 1975b), hooves of other animals (Moule and Sutherland, 1947), abrasive concrete in animal housing (Nobel *et al.*, 1976), branding (Stewart, 1972), dermatophilosis scabs (Oppong, 1976), ticks (Oduye, 1975a; Obeid, 1976; Oduye, 1976a; Zaria, 1993), and insects (Macadam, 1976; Oduye, 1976a; Oppong, 1976; Zaria, 1993).

However, mechanical disruption of the epidermis is not enough by itself to produce dermatophilosis lesions. Martinez *et al.* (1992) found that scarification of infected sites did not produce dermatophilosis lesions on goats. The inoculation of sheep at sites of trauma, where the wool had been plucked, produced dermatophilosis lesions that were no different from lesions on normal skin (Austwick, 1976). Macadam (1964b) found that the physical damage caused by pricking the skin with a needle did not produce lesions, even at sites inoculated with *D.congolensis*. A combination of physical damage and high rainfall has been associated with the distribution (Oppong, 1976) and severity (Hart, 1976) of dermatophilosis lesions. Removal of thorny trees and bushes from pasture has been shown to produce a decrease in the incidence of lesions (Zlotnik, 1955; Macadam, 1964b).

1.2.4 STRESS

The development of severe chronic dermatophilosis is widely associated with stress (Nobel *et al.*, 1971; Kelley, 1976; McEwan Jenkinson, 1976; Munz, 1976; Nobel *et al.*, 1976) caused by transportation of animals (Nobel *et al.*, 1976), cold weather (Kelley, 1976), poor feed (Egerton, 1964), pregnancy (Nobel *et al.*, 1971), and high tick and insect burdens. McEwan Jenkinson (1976) suggests that stress may result in physiological changes which could alter the composition of sebum or sweat, causing the skin surface environment to be more favourable to pathogens.

Sanders *et al.* (1990) found that dermatophilosis lesions persisted longer on lambs suffering from energy malnutrition. Lesions on control lambs, fed on a normal diet, resolved by day 35 whereas on test lambs the lesions persisted until day 50 when the experiment was terminated. They found that malnutrition resulted in reduced activity in the hair follicles and sebaceous glands which indicated that there would also be a slower proliferation of the epidermal cells. Sanders *et al.* (1990) suggest the reduction in epidermal turnover causes a prolonged course of infection because of the slow generation of new epidermis, thinner scabs, due to fewer layers of *stratum corneum*, and increased penetration of the organism, because the epidermal cells do not form new epidermis under the infected areas. They also found that malnutrition did not appear to affect the susceptibility of the skin to infection by *D.congolensis* as there was no significant difference in the minimum dose required to cause infection on the test and control animals.

1.2.5 IMMUNOSUPPRESSION

Immunosuppression of the host by natural causes or experimental procedures results in severe generalised lesions. Munz (1976) found that removal of the spleen resulted in severe dermatophilosis in both sheep and goats. Many of the

experimental animals developed the infection simultaneously, implying that the animals had latent infections which only developed when there was immunosuppression induced by the splenectomies (Munz, 1976). Animals in poor health or suffering from malnutrition appear to be more susceptible to the disease. Oduye (1976a) states that animals in good health and body condition were affected with *D.congolensis*, and it was the infection itself which caused the poor condition of the infected animals.

1.2.6 INTERCURRENT INFECTIONS

Severe dermatophilosis can be associated with other infections (Plowright, 1956; Stewart, 1972; Munz, 1976; Oppong, 1976; Lloyd, 1984; Zaria, 1993), with dermatophilosis aggravating other pathogenic infections or *vice versa* (Munz, 1976). Plowright (1956) states that rinderpest and trypanosomiasis may aid the development of dermatophilosis by lowering the resistance of the animal to *D.congolensis* which is a saprophyte on the skin of normal animals. Bull (1929) states that *D.congolensis* appears to be saprophytic, causing lesions whenever conditions become favourable.

Simultaneous orf and dermatophilosis infections on goats and sheep in Kenya resulted in very severe infections and several deaths (Munz, 1976). Yeruham *et al.* (1991) also report simultaneous dermatophilosis and orf infections occurring on a flock of Yaez. Over 80% of the kids died, and this high morbidity and mortality was attributed to the combined effect of the two infections. Orf infection by itself is not considered a problem in Kenya as it is usually benign, but the combined effects of the two infections were extremely severe (Munz, 1976). Oduye (1976a) and Zlotnik (1955) report that demodectic mange on the necks, flanks and backs was very common on cattle with dermatophilosis lesions. Stewart (1972) observed that mange mites were often associated with dermatophilosis. The damage caused to the epidermis by the demodectic mange may have allowed the establishment of

D.congolensis. Dermatophilosis is also associated with lumpy skin disease, globidiosis and trypanosomiasis (Stewart, 1972).

Coleman (1967) reported that the number of cattle dying from dermatophilosis during the wet season in West Africa was probably underestimated because of deaths being attributed to secondary infections such as anaemia, enteritis and pneumonia.

1.2.7 ANIMAL BEHAVIOUR

The behaviour of the infected animals may also affect the spread of the disease. Oppong (1976) observed the spread of facial lesions on calves onto the limbs caused by repeated rubbing to relieve the irritation. Austwick (1976) states that suckling lambs and the flocking behaviour of sheep aided the spread of the infection from one individual to another. Plowright (1956) did not observe any spread of infection from infected to uninfected cattle, even when kept in small paddocks, although this does not exclude the possibility of animals becoming carriers without showing clinical signs of infection.

1.2.8 VARIATION IN INDIVIDUAL AND BREED SUSCEPTIBILITY

Susceptibility to infection is very variable between breeds and individuals of the same breed, even when kept under the same management conditions (Oduye, 1976a). In Nigeria, Oduye (1975b) reports that some breeds are more resistant to infection, with local breeds such as Muturu, N'dama and Baole being resistant and other breeds such as Chad brahman and Arab Zebu being susceptible. Even 50% crossbred cattle can be susceptible to dermatophilosis such as Aberdeen Angus x N'dama cattle killed by dermatophilosis (Lloyd, 1976). Some crossbred cattle are more susceptible than others: out of four N'Dama x Hereford, none developed lesions,

whereas nine out of thirteen N'Dama x Angus died from dermatophilosis (Coleman, 1967).

Coleman (1967) found that N'Dama cattle were extremely resistant to dermatophilosis. He kept two N'Dama cattle together with two N'Dama x Angus cattle. These cattle were exposed to rain, fly attack (*Stomoxys calcitrans* and *Musca domestica*) and ticks (species not stated). Both of the crossbred cattle developed severe dermatophilosis and eventually died whilst the N'Dama cattle showed no signs of lesions. Artificial inoculation at scarified sites did not produce any lesions on the N'Dama even though the animals were exposed to rain and insect attack. It was only when one of the N'Dama became fatally infested with internal parasites that it developed a few mild dermatophilosis lesions.

Hart (1967; 1976) suggests various factors which might produce different breed susceptibilities in sheep. Breeds with finer wool, such as Suffolk sheep, are more susceptible than hill breeds with coarser wool. There is a lot of variation in the sebum barrier on different breeds of sheep, and another possible factor is the difference in intensity of the inflammatory reactions in the different breeds.

Kingali *et al.* (1990) note that differences in the microflora present on the skin of various breeds of cattle may partially account for differences in breed susceptibility. Nwufoh and Amakiri (1981) studied the microflora on the skin of three breeds of cattle: White Fulani, N'Dama and Friesian. They did not find *D.congolensis* on any of the breeds. *Staphylococcus* species were abundant on the White Fulani and Friesian cattle but *Bacillus* species predominated on the N'Dama. Some *Bacillus* species, such as *Bacillus subtilis*, are known to produce antibacterial substances. N'Dama cattle are resistant to various skin diseases including dermatophilosis, and Nwufoh and Amakiri (1981) suggest that the microflora may be an important factor in this resistance.

Biological control of dermatophilosis by exploiting the production of the antibacterial substances produced by skin microflora is being investigated (Zaria, 1993).

1.2.9 IMMUNITY TO DERMATOPHILOSIS

Susceptible breeds are repeatedly reinfected and appear to develop no immunity to dermatophilosis (Macadam, 1964b; Oduye, 1975b). Macadam (1970) found that when cattle, sheep, goats, horses and rabbits were infected with three consecutive *D.congolensis* infections, at the same site, none of the species developed any immunity to the infections. Although circulating antibodies are produced in response to *D.congolensis*, these antibodies do not appear to provide any protection against reinfection (Bida and Kelley, 1976; Lloyd, 1984; Zaria, 1993). Despite such evidence, Provost *et al.* (1976) succeeded in protecting Zebu cattle by vaccination. By inoculation of a young culture of *D.congolensis* into Zebu cattle in Chad they reduced the prevalence of dermatophilosis to 1.96% compared to 30% in the surrounding areas.

How and Lloyd (1990) protected rabbits from challenge infections of *D.congolensis* by inoculation with a mixture of zoospores and filaments. The protection consisted of increasing the minimum dose required to produce infections on the inoculated rabbits. Increased antibody titres were observed in the inoculated animals but there was no evidence that the antibodies were providing the protection from infection.

1.3 THE POSSIBLE ROLE OF TICKS

1.3.1 FEEDING MECHANISMS IN TICKS AND INSECTS

Figure 1.1 compares the mouthparts of various arthropods which have been associated with the aggravation, and spread, of dermatophilosis lesions. The different feeding mechanisms result in varying degrees of physical damage to the epidermis.

Ticks are pool feeders. They use their chelicerae to cut through the epidermis and then disrupt the underlying dermis and venules; this is called telmophagy (Sonenshine, 1991). They cause intense localised trauma at the attachment site by the formation of the feeding lesion. The lesion is formed also by proteolytic enzymes in saliva (Gill and Walker, 1988). During attachment, ticks secrete attachment cement to glue onto collagen and keratinised cells around the hypostome and to secure the mouthparts throughout the long period of feeding, which may be several days or weeks. The hypostome embeds deeply in the dermis, and there is much induration and oedema. The ticks tend to feed in clusters and cause immense damage to the skin. Abcesses often form at the sites of tick attachment.

The biting flies, which are also thought to be involved in the development of the severe, chronic, dermatophilosis lesions, are *Haematobia* and *Stomoxys* species (Oppong, 1976). The feeding mechanisms of the biting flies are very different from that of the ticks. None of the flies feeds for prolonged periods at one attachment site: *Stomoxys* species feed repeatedly, moving from one area of the individual to another (Oppong, 1976; Kettle, 1990). *Haematobia* species also move around the host taking repeated small blood meals. The populations of biting flies increase with the onset of the rainy season and up to 10,000 *Haematobia* flies have been observed feeding on

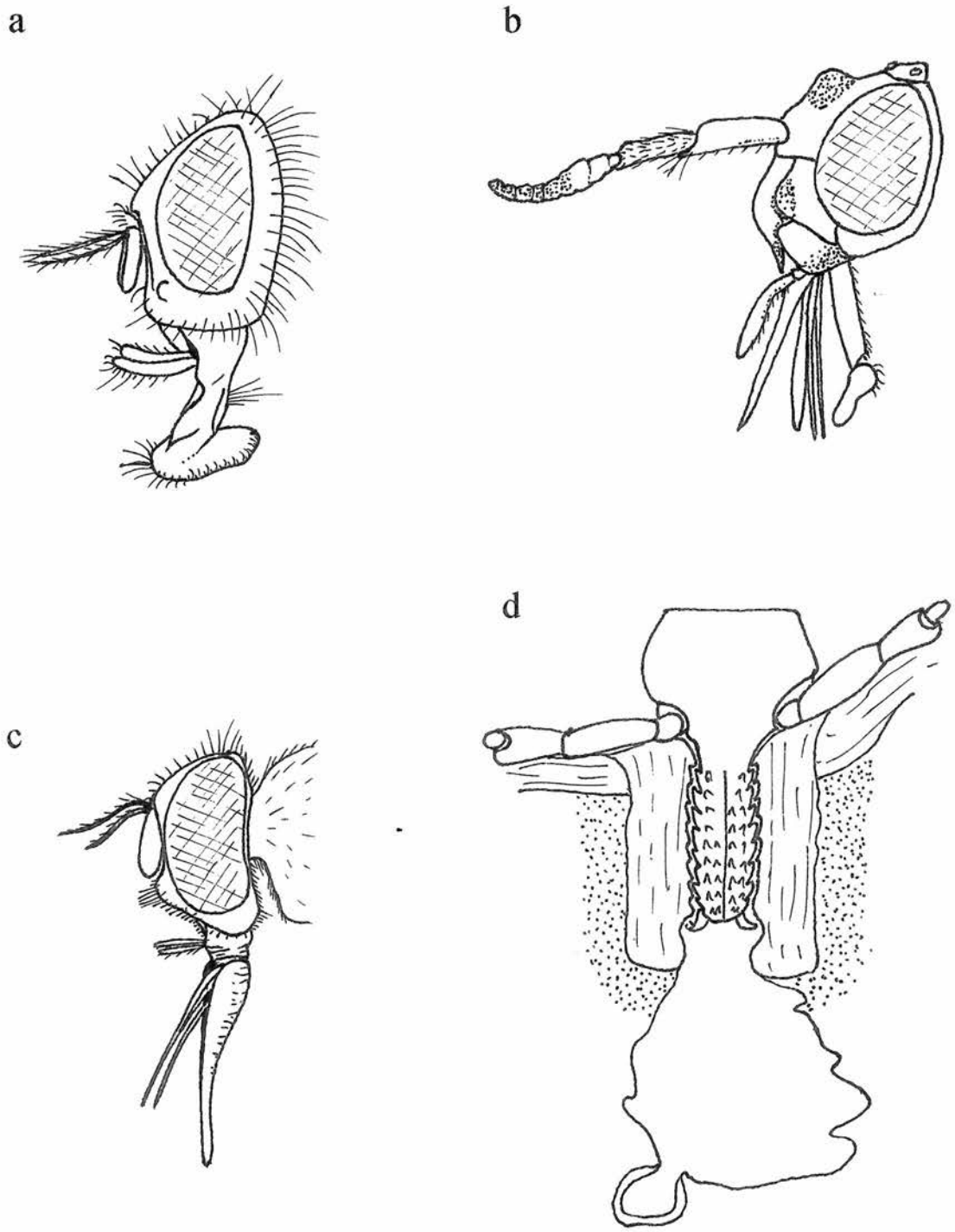


Figure 1.1 a, *Musca domestica* (Harwood and James, 1979), an example of a fly with labellar mouthparts, causing no penetration of the epidermis; b, *Chrysops* species (Richards and Davies, 1977), these flies have mouthparts adapted for a combination of piercing and sponging; c, *Stomoxys* species (Harwood and James, 1979), an example of a fly with piercing mouthparts; d, Tick attachment site (Morel, 1989), the hypostome pierces the epidermis and the tick feeds from a lesion formed in the dermis.

one animal (Lancaster and Meisch, 1986). There are numerous sites of small scale damage to the epidermis due to the large numbers of flies and repeated feeds.

1.3.2 PHYSICAL DAMAGE

Amblyomma variegatum ticks have long mouthparts (1-2 mm), and the hypostome of the mouthparts penetrates through the epidermis and embeds in the papillary dermis. The rasping chelicerae at the tip of the hypostome cut at the epidermis and the underlying dermis to form a feeding lesion. The physical damage to the epidermis allows penetration of pathogenic organisms such as *D.congolensis*. The feeding of the ticks is prolonged for several days or weeks allowing ample time for the penetration of microorganisms into the breached epidermis. Butler (1975) states that *A.variegatum* ticks cause severe damage due to their long mouthparts and that these ticks play an important role in the spread of dermatophilosis.

1.3.3 HYPERSENSITIVITY OR INFLAMMATORY DAMAGE

The feeding of *A.variegatum* ticks produces delayed (type IV) hypersensitive reactions, with the formation of intra-epidermal pustules and increased proportions of infiltrating granulocytes (Latif *et al.*, 1991a). Bida and Dennis (1976) suggest that endogenous moisture, in the form of serous exudate from a tick bite or other injury, may have the same effect as rain on the release of infective *D.congolensis* zoospores.

The development of cellular reactions in guinea-pigs in response to repeated tick infestations are characterized by a cutaneous basophil hypersensitivity (Brown *et al.*, 1983). This type of cellular reaction has also been demonstrated by repeated infestations of *Amblyomma americanum* on bovine hosts (Brown *et al.*, 1984). However, with *A.variegatum* feeding on rabbit hosts, the initial cellular

reactions are predominantly neutrophil and mononuclear infiltration, with eosinophils also infiltrating attachment sites after repeated infestations (Latif *et al.*, 1990).

The production of hypersensitive reactions has been associated with increased resistance of hosts to various tick species, with pustulation reducing the attachment of the ticks and increased infiltration reducing their engorgement (Walker and Fletcher, 1987). The development of resistance to *A.variegatum* has been observed to be limited compared to host resistance to other tick species (Jongejan *et al.*, 1989). Resistance to immature *A.variegatum* ticks in Zebu cattle has been demonstrated in studies of natural tick burdens (Latif *et al.*, 1991b). Host resistance to experimental infestations of adult and immature stages of *A.variegatum* on cattle is accompanied with increased hypersensitive reactions at the tick attachment sites (Latif *et al.*, 1991a).

Work by Davis and Philpott (1980) has indicated that delayed type hypersensitive reactions in the host's skin at the site of infection with *D.congolensis* can produce dermatophilosis lesions similar to natural, chronic infections. The delayed type hypersensitive reactions in their experiments were produced artificially by the application of the hapten dinitrochlorobenzene, and were simulations of the reactions which may occur at an arthropod feeding site. Immature ticks and *Haematobia* biting flies will produce inflammatory and hypersensitive lesions scattered widely on the host. These localised reactions may predispose to dermatophilosis (Macadam, 1962; Bida and Dennis, 1976; Davis and Philpott, 1980; Lloyd, 1984).

Davis (1984) found that *D.congolensis* zoospores persisted longer at sites of delayed hypersensitivity reactions on guinea-pigs than on unsensitized skin. The delayed hypersensitive reaction was produced by sensitization to dinitrochlorobenzene, and was intended as a simulation of an arthropod feeding site. He postulates that the immune reactions at an arthropod feeding site interfere with the

elimination of *D.congolensis* and allows the development of chronic dermatophilosis. Although the zoospores persisted longer on the skin, application of *D.congolensis* to delayed hypersensitive reactions alone was not enough to produce chronic lesions.

Resistance may be directed at a variety of tick antigens; sources of known antigens are saliva and attachment cement (Gregson, 1973). Gregson (1970) showed that hosts sensitized to the feeding of *Dermacentor andersoni* Stiles responded equally to saliva and tick attachment cement when challenged by skin testing. He also demonstrated a lymphocyte response to both materials.

1.3.4 SALIVARY ACTIVATED TRANSMISSION

Until recently, the transmission of arboviruses has been thought to be dependent on a sufficiently high virus titer in the host to overcome the threshold barrier of infection in the vector (Hardy *et al.*, 1983). Jones *et al.* (1987) have studied the transmission of Thogoto virus in guinea-pigs and have discovered that a viraemic host is not essential for the transmission of the virus to uninfected *Rhipicephalus appendiculatus* nymphs cofeeding with infected adult ticks. None of the hosts developed viraemia throughout the engorgement of the ticks. In fact, when the experiment was repeated on hamsters which developed high virus titers, there was significantly less transmission ($P < 0.01$) to the uninfected ticks.

The mechanism by which this nonviraemic transmission occurs is unclear, but it has been demonstrated to be associated with a factor or factors from the salivary glands of *R.appendiculatus* and *A.variegatum* (Jones *et al.*, 1989). Using guinea-pigs as the hosts, test animals were infected with Thogoto virus and inoculated with homogenised salivary glands from adult female *R.appendiculatus* or *A.variegatum*. Control animals were infected with the virus with no salivary gland extract. All of the animals were infested with uninfected *R.appendiculatus* nymphs.

There was a ten-fold increase in the mean number of nymphs becoming infected on the animals inoculated with salivary gland extracts. This enhancement in transmission was not observed with homogenates of other organs from *R.appendiculatus* or salivary glands from *Anopheles stephensi*.

Salivary glands from the sandfly *Lutzomyia longipalpis* have been shown to enhance *Leishmania major* infections in mice (Titus and Ribeiro, 1990). Mice injected with a mixture of salivary glands and *L.major* developed larger lesions and were found to contain 5,000-fold more parasites than the control mice injected with the parasite only.

Salivary activated transmission demonstrates the ability of small numbers of *A.variegatum* to trigger a systemic effect in the host. However, salivary activated transmission cannot be applied to the relationship between *A.variegatum* and dermatophilosis because these ticks do not transmit *D.congolensis*.

1.3.5 IMMUNOSUPPRESSION

Ticks appear to be generally toxic in their nature. It has been demonstrated that tick toxins may be responsible for making hosts susceptible to disease. Thomas and Neitz (1958) found that cattle that were originally immune to parasitic tick-borne diseases became susceptible when infested with *R.appendiculatus*. This type of immunosuppression does not appear to be beneficial to the tick if the host dies of the disease before engorgement is complete.

However, this susceptibility to disease may be a result of a functional immunosuppression designed to decrease host resistance to the tick feeding (Wikel and Whelen, 1986). Tick feeding may be inhibited by resistance of the host: therefore, functional immunosuppression by the tick may be essential to allow the tick to complete its engorgement (Wikel and Whelen, 1986). The long feeding periods of

ixodid ticks exposes them to the immunologic responses of the host (Tatchell, 1969a). These immunological reactions to the tick feeding could damage the ticks (Ribeiro *et al.*, 1985).

The saliva of ixodid ticks has been shown to have several physiological effects on the host including anticoagulant, antiinflammatory and immunosuppressive (Ribeiro *et al.*, 1985; Ribeiro, 1987a; Ribeiro *et al.*, 1990). Inflammatory reactions at tick attachment sites cause pain and irritation resulting in increased grooming and removal of the ticks by the host (Tatchell, 1969a; Ribeiro *et al.*, 1985). However, erythema associated with the inflammatory reaction would increase blood flow to the attachment site; therefore, ticks may regulate the level of inflammatory reactions for optimum conditions (Ribeiro *et al.*, 1985).

Infestations of adult *A.variegatum* result in a delayed antibody response to *D.congolensis* until nine to ten weeks after infection, whereas in uninfested goats, the same level of antibody response would be obtained three to four weeks after infection (Martinez *et al.*, 1992). The feeding of adult *R.appendiculatus* on rabbits has been shown to cause humoral and cellular immunosuppression in the host (Fivaz, 1989). Cattle infested with *R.appendiculatus* have become susceptible to infection with *Babesia bigemina* and *Anaplasma marginale* (Thomas and Neitz, 1958). The feeding of the cattle tick *B.microplus* has also been shown to reduce T and B-cell responses in bovine hosts (Inokuma, 1993).

1.3.6 TICK PARALYSIS AND TOXAEMIA

It has long been known that the feeding of some ticks under certain conditions can result in paralysis of the host. The first report of an association with ticks and paralysis was in Australia in 1824 (Scott, 1921, cited by Gregson (1973)). Although paralysis of livestock associated with the feeding of ticks was reported at

various times and places since then, it was not until 1905 that the association was confirmed. Borthwick (1905, cited by Gregson (1973)) was able to prevent paralysis by dipping to control ticks.

Tick paralysis is caused by a wide variety of ticks (up to 31 species have been associated with it; Murnaghan and O'Rourke, 1978), with different species being responsible for paralysis in different geographical regions. *D.andersoni*, *Ixodes holocyclus* and *Ixodes rubicundus* are the main species responsible for tick paralysis in North America, Australia and South Africa, respectively (Gregson, 1973). Other species of ticks in these genera as well as from other genera such as *Amblyomma, Haemaphysalis, Boophilus, Rhipicentor, Rhipicephalus,* and *Hyalomma* (Gregson, 1973; Murnaghan and O'Rourke, 1978) have occasionally caused tick paralysis.

The nature of paralysis caused by different species of ticks varies. *D.andersoni* causes an ascending paralysis which is easily reversed by removal of the tick, with complete recovery usually occurring within days and sometimes hours (Gregson, 1973). Paralysis caused by *I.holocyclus* is more often a regional paralysis, preceded by inflammation of the eyes and loss of appetite (Gregson, 1973). Removal of *I.holocyclus* is not always accompanied by regression of the paralysis (Murnaghan and O'Rourke, 1978). *I.rubicundus* causes a more acute form of paralysis accompanied by vomiting. The paralysis occurs simultaneously in all the limbs and removal of the tick does not always result in a regression of the symptoms. Even when the paralysis is reversed, recovery is slow and may take weeks (Gregson, 1973).

The variation in the symptoms of paralysis caused by different species of ticks suggests that more than one toxin is involved in this phenomenon. Kaire (1966) isolated a toxin (a protein) from homogenised *I.holocyclus* which produced symptoms of natural tick paralysis in experimental animals. Gregson (1973) found that it was

impossible to isolate a similar toxin from *D.andersoni* by using the same methods of Kaire (1966).

Paralysis caused by ixodid ticks is usually caused by adult ticks, but there are occasional reports of paralysis caused by nymphs (Gregson, 1973). Ascending paralysis is confined to the feeding of female ticks, but male ticks sometimes cause regional paralysis (Gregson, 1973). Paralysis usually occurs after the female tick has fed for about five days, which is the time during which long periods of salivation occur, as opposed to the alternation between sucking and salivation (Gregson, 1973). It is widely believed that the salivary glands are the most obvious source of the paralysing toxin, but Eaton (1913, cited by Murnaghan and O'Rourke (1978)) reports that incomplete removal of the mouthparts can prevent regression of paralysis.

There is much conflicting work on the mechanism of the toxins involved in causing tick paralysis. This is probably complicated by the fact that different toxins are probably responsible for paralysis caused by different species of ticks. Gregson (1973) suggests a few origins of the toxin: it is produced by tick tissues; it is a toxic metabolite produced by tick secretions reacting with the host tissue; or it is a product of an organism living in the tick. It is widely accepted that the toxin is a normal metabolite of tick tissues, either for use or excretion (Gregson, 1973).

Various toxins are associated with the feeding of ixodid ticks. Tick toxins produced by *Rhipicephalus evertsi evertsi* are found in the salivary glands (Viljoen *et al.*, 1986). Sweating sickness is a toxaemic disease associated with the feeding of *Hyalomma truncatum* (Burger *et al.*, 1991)

Salivary glands are not the only possible source of tick toxins. Hosts also demonstrate antigenic responses to haemolymph proteins transported to salivary

glands in *B.microplus* (Binnington and Kemp, 1980), and materials found in the gut of *B.microplus* (Gregson, 1960) and *D.andersoni* (Allen and Humphreys, 1979).

1.4 IMMUNE MECHANISMS OF RABBITS AND SHEEP IN RESPONSE TO EPIDERMAL PATHOGENS

Rabbits and sheep both have high levels of natural somatic agglutinin, with rabbits having levels of 100-400 and sheep 200-3000 (Roberts, 1965a). These agglutinins appear to provide no protection against infection with *D.congolensis*. Circulating antibodies to *D.congolensis* do not seem to be the cause of accelerated healing seen in animals previously infected (Roberts, 1965a). Roberts (1966) found that in animals which were hypersensitized to *D.congolensis* by injection with antiserum, there was no reduction in the penetration of the hyphae. This indicates that the reduced penetration in animals hypersensitized by previous infection is not due to circulating antibodies.

However, Sutherland *et al.* (1987) have concluded that skin surface antibodies may be involved in immunity to *D.congolensis*. Roberts (1964) found large numbers of plasma cells in chronic dermatophilosis infections but not in acute experimental infections, he concluded, therefore, that plasma cells are not involved in the defence against *D.congolensis*. Large numbers of plasma cells accumulate at chronic dermatophilosis lesions on sheep infested with adult *A.variegatum* (Walker and Lloyd, 1993). The ticks may be suppressing antibody production by these cells, resulting in the accumulation of large numbers of ineffective plasma cells. Immunosuppression of B-lymphocytes has been demonstrated in *R.appendiculatus* (Fivaz, 1989) and *B.microplus* (Inokuma, 1993).

Previous *D.congolensis* infections result in accelerated cellular reactions to subsequent infections. In sheep the proportion of hair sheaths penetrated by *D.congolensis* hyphae was reduced in subsequent infections (Roberts, 1965a). In rabbits, the penetration of sheaths is always limited (Roberts, 1965a).

Roberts (1965a) suggests that the inhibition of *D.congolensis* in rabbits is dependent on circulating granulocytes. The emigration of granulocytes to the infection site may be increased by the hyperaemia associated with the delayed reaction. The association between granulocytes and reduced hyphal penetration was demonstrated in rabbits depleted of granulocytes. With depletion of granulocytes, with delayed reaction and mononuclear infiltration still occurring, the hyphae freely invaded the epidermis and dermis (Roberts, 1965a).

The association between the immune response and the healing of *D.congolensis* appears to vary in different host species. In sheep, penetration of the hyphae into hair sheaths stops at the same time that delayed hypersensitivity is developed. In rabbits, the penetration of the *D.congolensis* cocci is inhibited before the onset of the delayed hypersensitivity (Roberts, 1965a).

Hypersensitive reactions do not appear to affect chronic lesions; they only seem to be able to affect acute lesions or protect healthy skin on chronically affected animals (Roberts, 1965a). Delayed hypersensitive reaction to infection was associated with accelerated granulocyte infiltration of the lesions, accompanied by decreased follicular penetration and accelerated healing (Roberts, 1965a).

CHAPTER TWO
EXPERIMENTAL FORMAT

There are obviously many factors involved in the formation of chronic dermatophilosis lesions. A review of the relevant literature reveals much conflicting evidence on the relative importance of the various factors involved in the formation of chronic infections, and these in turn appear to vary according to the geographical area and host species. It is also apparent that no one single factor causes the progression of the disease but that several factors act together to produce favourable conditions for the formation of chronic lesions. Figures 2.1 and 2.2 provide a summary of the various factors thought to be involved.

The association between *Amblyomma variegatum* ticks and chronic dermatophilosis has been clearly documented (Butler, 1975; Norval, 1986; Morrow *et al.*, 1989; Martinez *et al.*, 1992); however, the mechanisms by which this association occurs are unclear. Some workers associate severe dermatophilosis lesions with the predilection sites of adult *A.variegatum* (Plowright, 1956; Macadam, 1964b) but in other cases the initial lesions occur on the dorsal surface where immature ticks and biting flies attack (Zlotnik, 1955; Macadam, 1964b and c; Morrow *et al.*, 1989). The purpose of this study is to investigate the possible role of *A.variegatum* in the formation of chronic lesions.

There are various mechanisms by which a supposed association between *A.variegatum* ticks and chronic dermatophilosis may occur. Macadam (1962) succeeded in experimentally transmitting *Dermatophilus congolensis* from infected cattle onto rabbits by the feeding of adult *A.variegatum* ticks. This demonstrates the possible role of *A.variegatum* ticks in the mechanical transmission of *D.congolensis*. Martinez *et al.*, (1992) found *D.congolensis* only in adult males, demonstrated by the culture of *D.congolensis* from anterior sections of washed ticks. Since transstadial transmission of *D.congolensis* does not occur in these ticks the results of Martinez *et al.*, (1992) indicate that transmission by this tick is not possible.

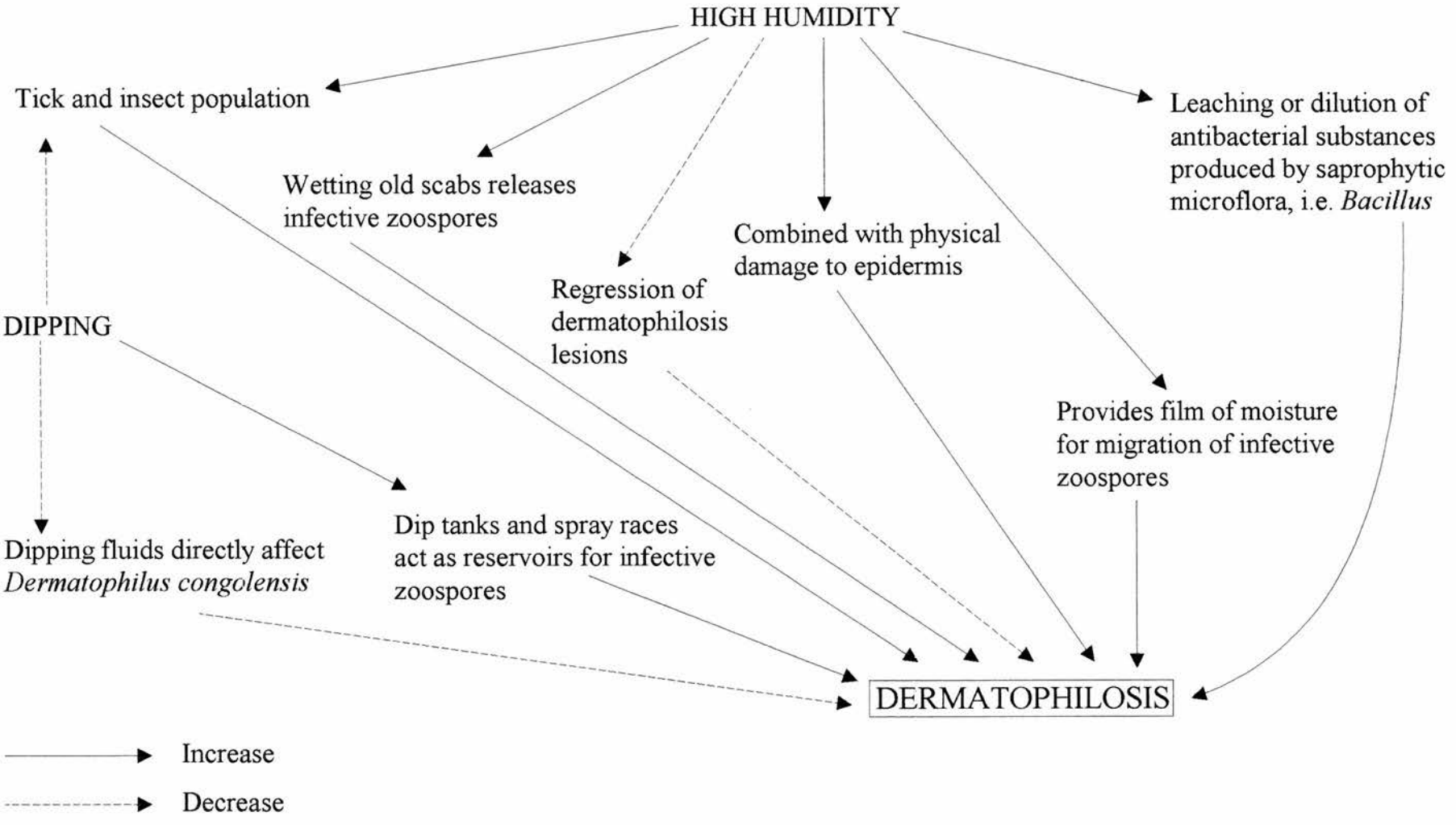


Figure 2.1 Diagrammatic scheme of the direct and indirect effect of high humidity on the development of chronic dermatophilosis

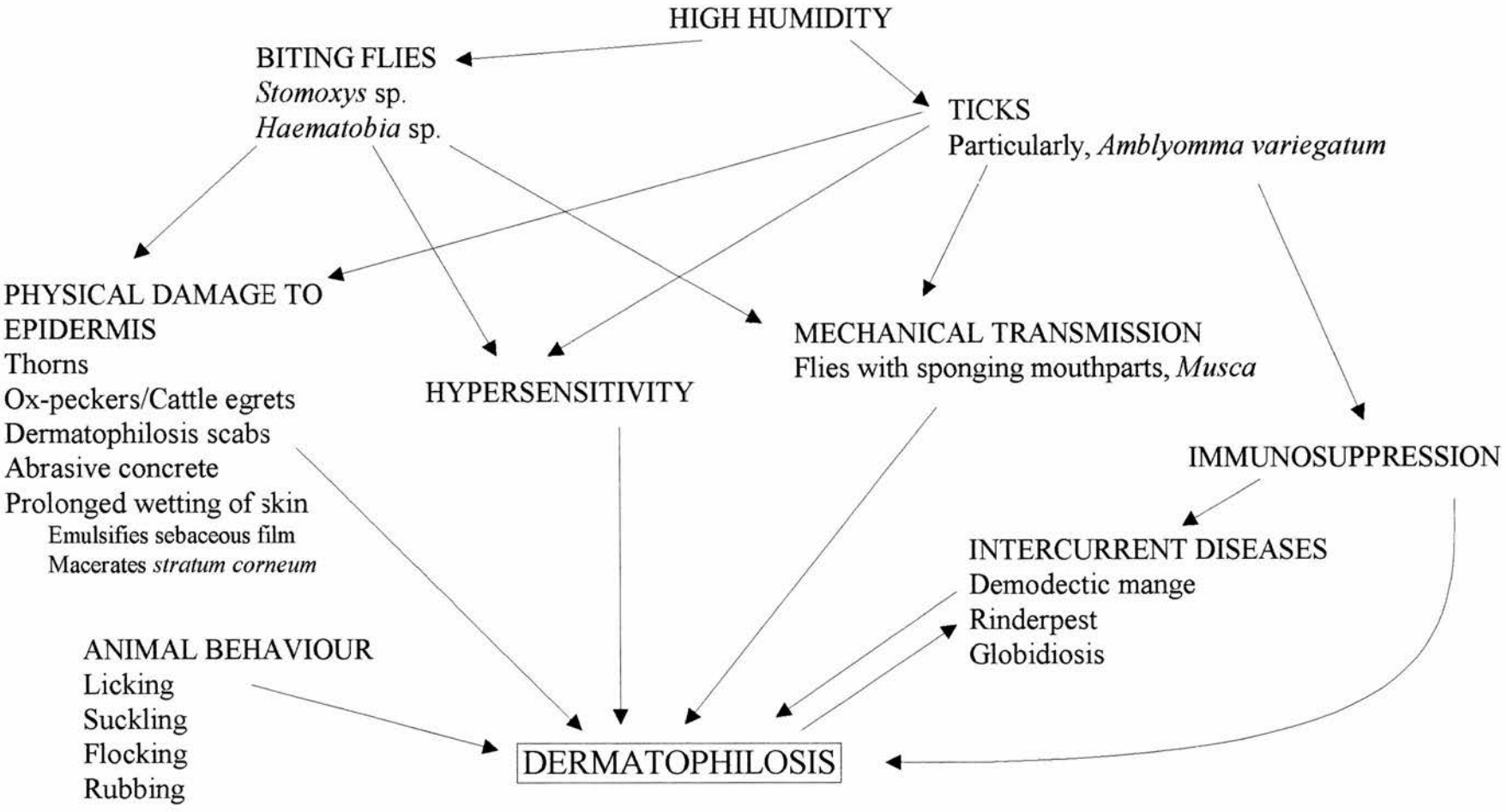


Figure 2.2 Diagrammatic scheme of the association between ticks and biting flies and the development of chronic dermatophilosis, including other related factors.

Other mechanisms by which the feeding of *A.variegatum* may aggravate dermatophilosis include local effects caused by physical damage to the epidermis, local immunosuppression caused by the feeding of the tick, or the development of delayed hypersensitivity in the host which may interfere with the inhibition of *D.congolensis*. Adult ticks may have a systemic effect, either due to the physical trauma caused by the large mouthparts and prolonged feeding or by the introduction of a substance via the salivary glands during the process of feeding.

The initial experiments are designed to investigate the claims that hypersensitive reactions to ticks and insects will predispose to dermatophilosis (Davis and Philpott, 1980). The effects of the development of delayed hypersensitivity in the host's skin in response to larval and nymphal infestations of *A.variegatum* will be studied in rabbits and sheep respectively. Inflammatory and hypersensitive reactions to the feeding of the immature ticks will be produced by single and multiple infestations on the experimental hosts. Infective *D.congolensis* cocci will be applied to tick attachment sites after removal of the ticks and the resulting infections will be compared with identical infections applied to skin not exposed to tick feeding. From the results of the work by Davis and Philpott (1980) it is expected that infections at the site of delayed hypersensitive reactions to the ticks will progress and become more severe and prolonged than infections at sites with inflammatory reactions to the ticks, and infections at sites not exposed to ticks.

Davis and Philpott (1980) found that the dermatophilosis lesions were only prolonged as long as the delayed hypersensitive reactions in the host's skin were maintained. Also in field conditions, feeding of ticks occur prior to and simultaneously with *D.congolensis* infections. Therefore, the effect of inflammatory and hypersensitive reactions to ongoing infestations of nymphal *A.variegatum* on simultaneous *D.congolensis* infections will also be investigated. If hypersensitive

reactions to the feeding of immature *A.variegatum* do predispose to dermatophilosis, the lesions on those hosts repeatedly infested with ticks will be more severe than lesions on hosts infested once or not at all. If the physical damage caused by the tick mouthparts is important in the formation of initial lesions there may be a correlation between the tick attachment sites and initial foci of infection.

In the field, chronic dermatophilosis is associated with the feeding of adult *A.variegatum* both with initial lesions at the predilection sites (Plowright, 1956; Macadam, 1964b) and with initial lesions remote from the ticks (Zlotnik, 1955; Morrow *et al.*, 1989). This suggests that the adult ticks may have a systemic effect on the formation of dermatophilosis lesions. This hypothesis has been reinforced by recent findings of Walker and Lloyd (1993) who produced chronic lesions on sheep at sites remote from simultaneous infestations of *A.variegatum*. This systemic effect may or may not extend to the immature stages of *A.variegatum*. Therefore, the systemic effect of adult and nymphal *A.variegatum* will be compared on simultaneous *D.congolensis* infections on sheep. The possible mechanisms of the systemic effect on cellular and humoral immunity of the host will be investigated using skin testing and enzyme linked immunosorbent assay (ELISA), respectively.

Finally it is proposed to carry out a histological study of salivary glands and electrophoretic studies of whole salivary glands and saliva from adult *A.variegatum*. The results of these studies will be compared with equivalent material from another tick not demonstrating the systemic effect. If the systemic effect of adult *A.variegatum* is not produced by the nymphal ticks then salivary material from the two instars will be compared to see if there are any obvious differences.

CHAPTER THREE
GENERAL METHODS

3.1 INTRODUCTION

The following materials and methods were used throughout the investigation into the relationship between the feeding of *A.variegatum* and the progression of dermatophilosis. Where experimental details differ from these methods it is stated in the relevant sections of the individual chapters.

3.2 EXPERIMENTAL HOSTS

3.2.1 RABBITS

All of the rabbits used during this study were female New Zealand White rabbits obtained from a commercial, Home Office approved supplier. Rabbits were used when they were two to three kilograms and were matched for age and size for experimental use. None of the rabbits had prior exposure to ticks or experimental infections of *D.congolensis*.

All of the rabbits were kept in constant environmental conditions at 20-22°C, with 12 daylight hours/12 dark; these conditions remained the same prior to and during the experimental procedures. The rabbits were fed on a maintenance diet of rabbit pellets and hay.

3.2.2 SHEEP

Sheep used for this study were obtained from several sources. Where possible female Blackface x Suffolk sheep were used; when there were not enough females available castrated males were used. Although there were differences in source, sex and in one case breed (see Chapter Seven) between the individual hosts, sheep used in a single experiment were matched as closely as possible. Matching was

achieved primarily by size and weight. None of the sheep were experimentally exposed to ticks or *D.congolensis* prior to the experiments.

All of the sheep were kept in constant environmental conditions at 20-25°C with 12 daylight hours/12 dark. The sheep were fed on a maintenance diet of sheep nuts and hay.

All of the animals were used under the sanctions of the Animals (Scientific Procedures) Act 1986 of the U.K.

3.3 TICKS

3.3.1 ORIGIN OF THE TICKS

All of the ticks used during this study were from an uninfected laboratory colony of *A.variegatum* maintained at the Centre for Tropical Veterinary Medicine, University of Edinburgh. This colony was produced from ticks originating from Kenya.

3.3.2 MAINTENANCE OF TICKS

The laboratory colony of *A.variegatum* was stored at 18°C with 14 daylight hours/10 dark at 85% relative humidity (RH). The ticks were fed routinely on laboratory rabbits and sheep, moulted at 28°C and then stored at 18°C. Larvae were used two to five months after hatching and nymphs and adults were used two to six months after moulting.

3.3.3 INFESTATIONS

3.3.3.1 Batching of ticks

Adult and nymphal ticks were counted individually to obtain the correct number for experimental infestations. For larval infestations, fertile eggs were batched by weight prior to hatching. By calculating the weight of a known number of eggs, batches of 500 larvae were separated into individual tubes to be applied to the host animal after hatching.

3.3.3.2 Calculation of equivalent numbers of the three instars of *Amblyomma variegatum*

It was necessary to obtain a measure of equivalent numbers of the three instars for experiments comparing the effects of the feeding of the different instars and to enable some comparison between experiments. The number and average volume of type-2 and type-3 acini in whole salivary glands from unfed larvae, nymphs, and adults, were used as a measure of the effect of the feeding of the three instars.

Unfed ticks were secured in wax and whole salivary glands were dissected out under phosphate buffered saline. The salivary glands were stained in 1% methyl green (see Appendix 3.1 for details), the total number of type-2 and type-3 acini was recorded as well as the average diameter of each of the two acini types (see Appendix 3.2 for raw data). The average volume of each of the two secretory acini types was calculated and multiplied by the number of acini in a single gland to give a value for the total volume of secretory material in a single salivary gland. Comparing the calculated volume of secretory material found in a single salivary gland from each instar produced a ratio of one adult: 30 nymphs: 800 larvae.

3.3.4 APPLICATION OF TICKS

3.3.4.1 Rabbits

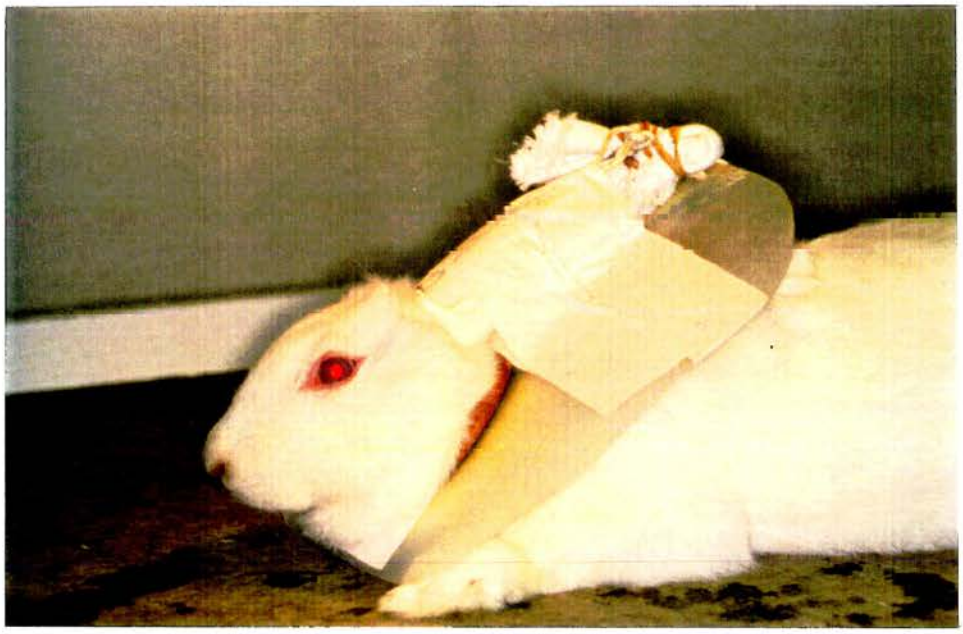
Infestations of ticks were applied either to the ears or bodies of rabbits. Infestations on the ears were enclosed by ear-bags with the top two-thirds of the ears shaved to facilitate tick feeding. Ear-bags were secured by tape to the lower part of the ear, and the bags were closed using elastic bands which were easily opened to check infestations, and for the removal of fully engorged ticks (see Plate 3.1).

Infestations on the body of the rabbits were applied to shaved areas on the back of the rabbit and the torso was completely enclosed using cloth body-bags. These bags were elasticated at both ends, to prevent ticks escaping, with an opening secured with 'Velcro' press fastener, to allow the removal of fully engorged ticks (see Plate 3.1).

3.3.4.2 Sheep

Infestations of ticks on sheep were enclosed by cloth bags glued to the wool on the shoulder of the sheep. The wool was clipped from the area to be enclosed by the bags, leaving enough wool on the surrounding area to apply the glue without wetting the skin. After clipping, the area was degreased using a 1:1 mixture of ethanol and ether. When the area was dry the bags were then glued to the wool using a water based latex rubber glue (Copydex). The tops of the bags were closed using elastic bands which could be opened to assess the infestations and to remove fully engorged ticks (Plate 3.2).

a



b

Plate 3.1 Infestation of *Amblyomma variegatum* ticks on New Zealand White rabbits. a. Infestations on the ears enclosed by ear-bags; b. Infestation on the torso enclosed by a body-bag.

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Plate 3.2 Infestation of *Amblyomma variegatum* ticks feeding on the shoulder of a Black-face x Suffolk sheep. The ticks are enclosed by a cloth bag glued to the wool.

3.3.4.3 Timing of applications

Infestations of larvae and nymphs were applied in either one large batch or several smaller batches at weekly intervals to prolong the infestation time. This regime varied between individual experiments and is stated in the text of the relevant sections. For adult infestations, males were applied two weeks before the females to allow the males time to attach and commence feeding before the application of the females. Work by Dipeolu and Ogunji (1980) has shown that the timing of the applications of the males and females affects the engorgement success and viability of the resulting eggs.

3.3.5 ASSESSMENT OF RESISTANCE

Three parameters were recorded to assess the development of host resistance to repeated infestations of *A.variegatum*. These were the mass of individual fully engorged ticks, the number of ticks successfully engorging and the number of ticks completing a successful moult. The resistance of individual hosts to *A.variegatum* was calculated using the methods of Walker *et al.*, (1990).

$$\left[1-\left(\frac{\text{resistant mass}}{\text{control mass}}\times\frac{\text{resistant numbers}}{\text{control numbers}}\times\frac{\text{resistant survival}}{\text{control survival}}\right)\right]$$

Changes in resistance were compared with the initial resistance of individual hosts to primary infestations and were expressed as percentage change in resistance.

3.4 *DERMATOPHILUS CONGOLENSIS* INFECTIONS

Dermatophilus congolensis, isolated from sheep, was used to culture infective cocci to produce the experimental infections. Large batches of stabilate of

infective *D.congolensis* cocci, at known concentrations, were produced and stored at -20°C. Single vials of the stabilate were thawed when required and diluted in Hank's balanced salt solution with 0.5% pig gelatin (H/G) to produce a suspension of infective cocci for application onto the experimental hosts. Section 4.2.1.2 contains details of the production of the stabilate and suspension of infective *D.congolensis* cocci.

Viability of the infective *D.congolensis* cocci was checked before application onto the hosts. Cocci diluted in Hank's balanced salt solution (HBSS) were checked for motility under a x100 lens of a microscope.

3.4.1 Applications onto rabbits

Before application of the infective cocci onto the host's skin the area was clipped and swabbed with a 1:1 mixture of ethanol and ether to defat the epidermis and to remove any competing bacteria from the skin surface (Lloyd, 1980). The infection areas were marked using an indelible felt-tip pen and template, and skin fold measurements were taken of each infection site prior to application of infective cocci. The cocci were then applied, without scarification, using a bent pipette tip.

3.4.2 Applications onto sheep

Each sheep was washed one day before the infections were applied. The flanks of all the sheep were clipped and washed with an anionic detergent Tween 80 (Sigma or BDH) followed by copious water. The following day, when the sheep were dry, the infection sites were cleaned and the cocci applied using the same technique as with the rabbits.

3.4.3 Statistical analysis of experimental infections

Dermatophilosis lesions produced by experimental infection of sheep and rabbits with infective *D.congolensis* cocci were assessed using a nonparametric ranking system (see Section 4.2.1.5). Various nonparametric tests were used to analyse the clinical ranked scores recorded for the dermatophilosis.

The Kruskal-Wallis test (Siegal and Castellan, 1988) was used to compare three or more groups, and the Mann-Whitney test (Siegal and Castellan, 1988) was used for the comparison of two groups. The Mann-Whitney test was also used to determine where the significant difference between three or more groups occurred, when the Kruskal-Wallis test was significant.

The Friedman's test (Siegal and Castellan, 1988) was used to compare three or more groups of data when each group contained results which varied due to titration effect or progression of the infection. When significant differences occurred using the Friedman's test, multiple comparisons with Friedman's test (Siegal and Castellan, 1988) were used to demonstrate where the significant differences occurred.

CHAPTER FOUR
PRELIMINARY EXPERIMENTS

4.1 INTRODUCTION

The experimental investigation into the relationship of the feeding of *Amblyomma variegatum* and the progression of *Dermatophilus congolensis* infections was carried out using two species of laboratory hosts; rabbits and sheep. A system of titrated doses of infective *D.congolensis* cocci was already in use in the laboratory for producing experimental infections on sheep. How and Lloyd (1990) had already shown that it was possible experimentally to infect rabbits with *D.congolensis* cocci, but it was necessary to establish a working system for the experimental infection of rabbits.

Preliminary experiments were on the culture and enumeration of *D.congolensis* cocci and to determine an optimum titration of, and suitable areas for, *D.congolensis* infections on rabbits.

When a suitable protocol had been established to produce *D.congolensis* infections on rabbits it was then necessary to establish a system to produce replicate titrations throughout a series of experiments. This was achieved by producing a large batch of stabilate, of known concentration. The viability of this stabilate was tested by comparing resulting infections with infections produced by freshly cultured cocci at the same concentration.

4.2 METHODS

4.2.1 PRELIMINARY EXPERIMENT FOR SITING AND TITRATION RANGE FOR *DERMATOPHILUS CONGOLENSIS* INFECTIONS ON RABBITS

4.2.1.1 Experimental hosts

Two female New Zealand White rabbits (rabbits 1 and 2), were used as the experimental hosts; both had been used for colony feeds of *A.variegatum*. Neither of the rabbits were previously exposed to *D.congolensis*.

4.2.1.2 *Dermatophilus congolensis* culture.

The stabilate used for these experiments was produced from an isolate of *D.congolensis* from sheep in the Pentland hills (near Edinburgh) which had been frozen and stored at -20°C. The stabilate used for freezing suspensions of *D.congolensis* cocci contained 10% v/v newborn or foetal calf serum and 7% v/v glycerol.

One vial of this stabilate was enough to produce a suspension of infective cocci for application onto the experimental hosts. The vial of frozen stabilate was thawed then plated out onto at least two blood agar plates. The blood agar was prepared with Columbia agar base at 44g/litre plus whole defribrinated sheep blood at 7-8% v/v. The stabilate was applied in 0.5ml lots to each plate and was spread, using a flamed loop, in three times three intersecting lines of cocci. The plates were then incubated in CO_2 at 37°C for 72 hours.

After 72 hours in CO_2, clusters of *D.congolensis* growths were growing on the blood agar. Several tubes of sterile broth of neutralized soya peptone and

brain-heart infusion (NSP/BHI) were inoculated with these growths which were removed from the agar using a sterile loop. The large clumps of *D.congolensis* were broken up by repeated pipetting of the broth. The inoculated broth was then incubated in plain air, at 37°C, for 72 hours.

After 72 hours the *D.congolensis* had developed into filaments forming a distinct layer at the bottom of the broth. These filaments were then subcultured to produce infective cocci. Before plating out the broth, the tube was shaken thoroughly to break up and mix the filaments into the broth. The broth was then applied to each of six plates in aliquots of 0.5ml; each aliquot being allowed to stabilize before the addition of the next. In this way it was possible to apply 2ml of the broth to each plate, allowing a larger number of *D.congolensis* filaments to be applied without the broth spreading to the edges of the plates. After plating out the filaments, they were incubated for a further 48 hours, at 37°C, in plain air.

After 48 hours, clusters of *D.congolensis* cocci were harvested into sterile Hank's balanced salt solution with pig gelatin at 0.5% w/v (H/G). Two millilitres of H/G were applied to each plate and, using a bent Pasteur pipette, the *D.congolensis* growths were disrupted and mixed into the H/G; this mixture was then collected into a sterile universal tube. The growths were collected from all six plates until a concentrated suspension of *D.congolensis* in H/G had been obtained. Large clumps of material were broken up by repeated pipetting and the suspension was left for 10 minutes for any remaining clumps to settle. The supernatant liquid was removed by suction to count the infective *D.congolensis* cocci prior to topical infection of the experimental hosts.

4.2.1.3 *Dermatophilus congolensis* cocci counts

Two methods of counting the cocci were used and the mean of the results was taken as the concentration of cocci.

Method and calculation for a smear count.

A sample of harvested cocci was diluted 1:10 and 1:100 in H/G and 12µl of each of these dilutions were quickly and evenly spread over a known area on a clean microscope slide. The slides were stained with Giemsa's stain and the number of cocci was counted over 20 randomly selected areas of the slide using an eyepiece graticule in a x100 lens. The concentration of cocci was estimated assuming that 10µl had been smeared on the slide with 2µl loss on the needle. The size of the graticule gave a ratio of area examined to total area of 1:61663. To express the results as millions of cocci per microlitre the total count for a 1:100 dilution was multiplied by 0.062.

Method and calculation using a Helber Counter.

A sample of cocci was diluted 1:10 and 1:100 in 2% formalin. Two Helber counters were used for the counts of each dilution and 2-3µl of a dilution was put on one Helber counter. The cocci were counted using light microscopy with a x100 lens and counting the total number of cocci over twenty of the smallest divisions on the Helber counter. To express the results as millions of cocci per microlitre the total count for a 1:100 dilution was multiplied by 0.1.

4.2.1.4 Titrations

It was necessary to establish a working titration for infection on the rabbits. Two titration ranges were tried as follows:

i). The first titration range was adapted from the titration used to produce experimental *D.congolensis* infections on sheep in previous experiments in this laboratory (Walker and Lloyd, 1993). The same concentrations of cocci were applied per unit area but the application sites were 2 x 2cm instead of 2 x 4cm as on the sheep.

Freshly harvested cocci at a concentration of 2.0×10^7 cocci/µl were diluted to a starting concentration of 5×10^6 cocci/µl with seven ten-fold dilutions for the titration. Each infection area was 2 x 2cm and received a dose of 100µl, with the concentration of cocci ranging from 1.25×10^8 cocci/cm^2 to 125 cocci/cm^2. This titration range was applied to rabbit 1.

ii). A wider range of concentrations of cocci were used for the second titration using more, smaller, areas.

The titration consisted of 50µl doses applied to areas 1 x 2cm diluted from freshly harvested cocci at a concentration of 3.2×10^7 cocci/µl with a starting concentration of 1.0×10^7 cocci/µl with ten five-fold dilutions. Using this titration the concentration of cocci ranged from 2.5×10^8 cocci/cm^2 to 128 cocci/cm^2. This titration was applied to rabbit 2.

4.2.1.5 Assessment of *Dermatophilus congolensis* infections

The skin on the experimental hosts was prepared as described in Section 3.4.1 with the *D.congolensis* cocci being applied to undamaged skin within marked areas. Care was taken not to spread the cocci outwith the marked areas.

The resulting infections were monitored every two or three days, for two weeks, to record the severity and duration of the lesions. Three parameters were recorded, at each assessment, using a nonparametric ranking system. A combined

score of the three parameters was recorded for each of the infection areas, giving a maximum possible score of 12.

i. Area of infection.

The percentage area of each infection site showing signs of infection was recorded by ranking from 0 to 4.

0 - 0%

1 - 1 - 25%

2 - 26 - 50%

3 - 51 - 75%

4 - 76 - 100%

ii. Scab.

The severity of the scabs ranging from erythema to thick layers of dead flaking epidermis was ranked from 0 to 4.

0 - No scab

1 - Erythema and slight flakiness at small foci

2 - Formation of discrete scabs

3 - Thick profuse scabs

4 - Very thick crusty scabs

iii. Exposed dermis.

As the infections progressed, the scabs began to break up and detach leaving areas of exposed dermis. The amount of exposed dermis was ranked from 0 to 4.

0 - No exposed dermis

1 - A few small areas of exposed dermis

2 - A few large areas of exposed dermis

3 - Several large areas of exposed dermis

4 - Extensive exposure of dermis over whole site

Callipers were used to obtain measurements of the skin thickness (mm) at each of the infection sites for each assessment during the course of the dermatophilosis infection.

4.2.2 PRODUCTION AND TESTING OF *DERMATOPHILUS CONGOLENSIS* STABILATE

4.2.2.1 Experimental hosts

The experimental hosts were two female New Zealand White rabbits. These two rabbits (rabbits 3 and 4) had been previously exposed to one infestation of *A.variegatum* larvae on their bodies.

4.2.2.2 *Dermatophilus congolensis* culture

The same protocol as in 4.2.1.2 was used for the culture of the infective *D.congolensis* cocci, with one slight change. Serum agar plates were used for the culture of the cocci with foetal calf serum replacing the defibrinated sheep blood. Serum agar plates produced better *D.congolensis* growths and could be stored for longer periods without deteriorating. Three different isolates of *D.congolensis* from sheep in the Pentland Hills were used for the culture.

4.2.2.3 *Dermatophilus congolensis* stabilate

The same procedure as in 4.2.1.2 was used for the plating out of *D.congolensis* up to the final stage of the production of infective cocci on the serum

agar. At this stage the cocci were harvested into NSP/BHI broth with 7.5% glycerol, not H/G, to form a stabilate suitable for cryopreservation prior to direct application onto hosts to produce experimental dermatophilosis infections.

A small amount of the stabilate was assessed using a Helber counter and smear count (see Section 4.2.1.3) to determine the concentration of the *D.congolensis* cocci. The mean of six counts determined the concentration to be approximately 1.2 x 10^7 cocci/µl.

Forty plates of the infective cocci were produced and a minimum of 2ml of the broth and glycerol was used to collect the cocci from each plate. The stabilate was divided into 2ml aliquots containing approximately 2.4 x 10^{10} cocci. Twenty-five vials of this stabilate were frozen at -20°C and the remainder at -70°C, for prolonged storage.

4.2.2.4 Titrations

One vial of the *D.congolensis* stabilate was used to produce an experimental infection after one week of storage at -20°C. When completely thawed a viability check was carried out by observing the motility of the cocci by microscopy. The stabilate was then diluted in H/G to produce the required titrations.

For the experimental infections the stabilate was diluted to a starting concentration of 1 x 10^7 cocci/µl with a titration of ten five-fold dilutions which were applied to areas 1 x 2cm on the backs of the rabbits. The dose size to each area was 50µl. This titration ranged from 2.5 x 10^8 cocci/cm^2 to 128 cocci/cm^2 over the ten infection areas.

4.2.2.5 Assessment of *Dermatophilus congolensis* infections

The skin on the experimental hosts was prepared as described in Section 3.4.1 with the *D.congolensis* cocci being applied to undamaged skin within marked areas. Care was taken not to spread the cocci outwith the marked areas.

The resulting infections were monitored, and the progress was recorded using the ranking system to measure the area infected, the severity of scab and amount of exposed dermis (see 4.2.1.5). As well as recording the parameters using the ranking system, the skin fold at each infection site was also recorded at each assessment.

The infections produced on the two rabbits were assessed at days four and six. These infections were then compared with the infections produced using identical titrations of freshly cultured *D.congolensis* cocci. Friedman's test was used to compare the ranked severity of the infections produced by freshly cultured or cryopreserved *D.congolensis* cocci, up to day six of the infections.

4.3 RESULTS

4.3.1 SITING AND TITRATION RANGE FOR *DERMATOPHILUS CONGOLENSIS* INFECTIONS

Dermatophilosis lesions developed at the infection sites on rabbits 1 and 2. No lesions were observed outwith the application areas. Both of the infection sites, 1 x 2cm and 2 x 2cm, produced readily analysed lesions.

By the first assessment, four or five days after infection, the reactions at the infection sites had progressed from erythema and slight swelling to discrete scabs.

The lesions on both of the rabbits continued to progress until approximately one week after infection.

After one week the dermatophilosis lesions began to regress. The scabs began to crack and drop off the rabbits; in some cases this exposed the dermis, but generally the scabs fell off revealing intact skin. By day 14 all of the scabs had detached from rabbit 1 and the only signs of the infection were a few patches of depilated but otherwise intact skin. On rabbit 2 all of the scabs had detached by day 13 but two small areas of exposed dermis were recorded. This exposed dermis was on the areas infected with 1×10^7 cocci/cm^2 and 8×10^4 cocci/cm^2 (Table 4.1).

Scores of 3 or 4 for individual parameters were recorded at the two highest concentration sites of the titration starting at 1.25×10^8 cocci/cm^2 (rabbit 1) and the three highest concentration sites of the titration starting at 2.5×10^8 cocci/cm^2 (rabbit 2). For the titration range starting at 2.5×10^8 cocci/cm^2 with ten five-fold dilutions, three of the last four dilutions produced no visible signs of infection, whilst the eighth dilution produced only a very low level infection (Table 4.1).

Figure 4.1 shows the ranked scores for the individual parameters used for the assessment of the dermatophilosis infections. From these graphs it can be seen that discrete dermatophilosis scabs had formed within the first four or five days after infection whereas exposed dermis was not recorded until approximately one week after infection. After two weeks of infection the scabs had detached from both rabbits but there was still damage to the skin in the form of exposed dermis.

As the dermatophilosis infections progressed, the scabs became thicker and harder, and measurement of the skin thickness became increasingly difficult. Skin fold measurements had to be abandoned for some of the areas infected with a high

Table 4.1 Ranked clinical scores for dermatophilus infections on rabbits infected with two different titrations of freshly cultured *Dermatophilus congolensis* cocci.

Rabbit 1

	Ranked clinical scores							
Day of infection	1.25 x* 10^8	1.25 x 10^7	1.25 x 10^6	1.25 x 10^5	1.25 x 10^4	1.25 x 10^3	1.25 x 10^2	Total scores
5	7	6	4	3	2	3	2	27
7	10	7	5	4	4	4	3	37
9	9	8	5	3	4	5	4	38
12	5	3	3	2	3	3	2	21
14	0	0	0	0	0	0	0	0
Total scores	31	24	17	12	13	15	11	123

Rabbit 2

	Ranked clinical scores									
Day of infection	2.5 x* 10^8	5 x 10^7	1 x 10^7	2 x 10^6	4 x 10^5	8 x 10^4	1.6 x 10^4	3.2 x 10^3	6.4 x 10^2	1.28 x 10^2	Total scores
4	4	5	7	4	2	2	0	3	0	0	27
6	6	7	7	6	3	2	0	5	0	0	36
8	8	6	9	6	3	3	0	7	0	0	42
11	4	6	8	4	3	3	0	6	0	0	34
13	0	0	1	0	0	1	0	0	0	0	2
Total scores	22	24	32	20	11	11	0	21	0	0	141

* = cocci/cm^2

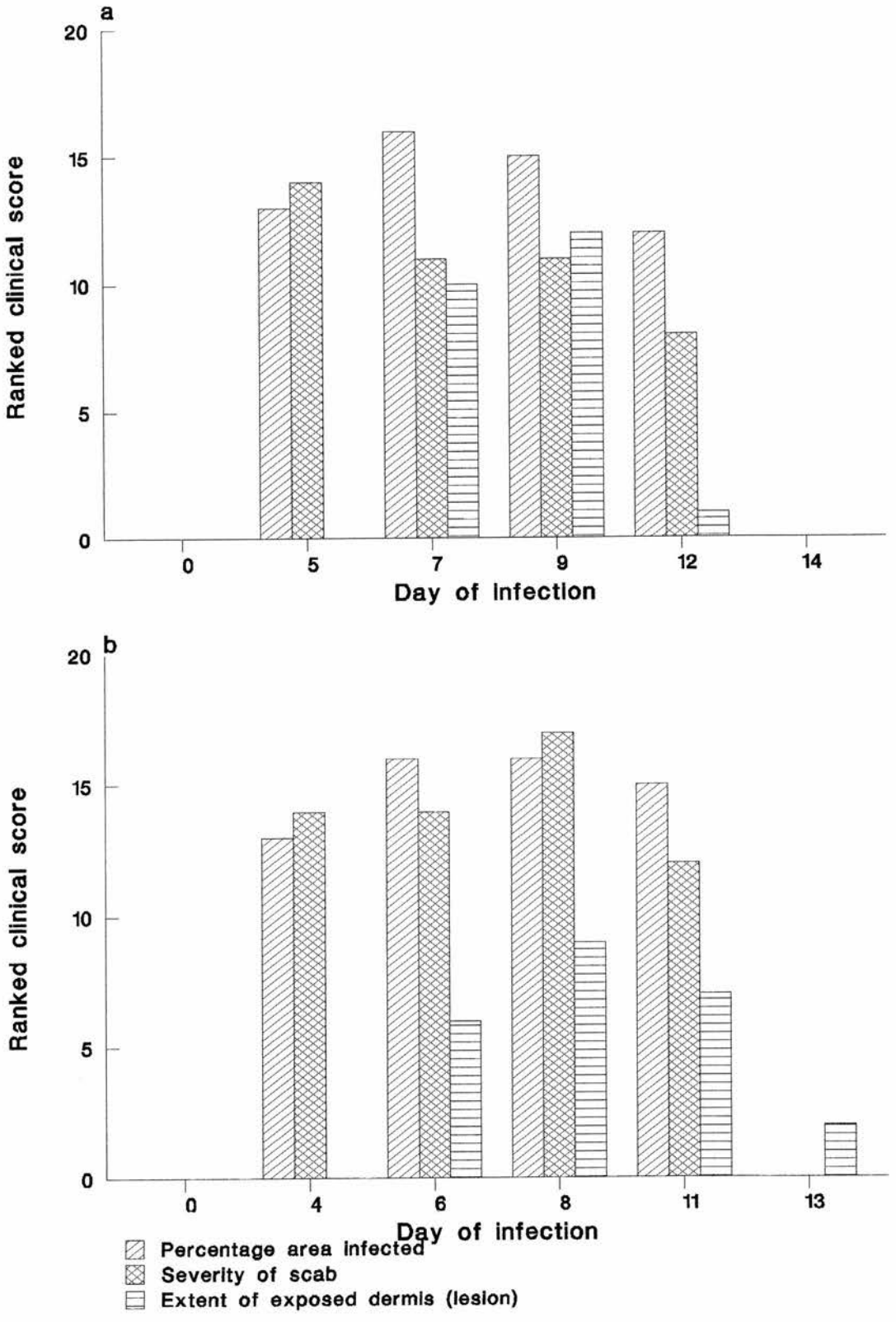


Figure 4.1 Changes in clinical ranked scores for percentage area infected, severity of scab and amount of exposed dermis during the course of *Dermatophilus congolensis* infections on rabbits. a. Rabbit 1 infected with seven ten-fold dilutions, b. Rabbit 2 infected with ten five-fold dilutions.

concentration of cocci. Care had to be taken not to aggravate the lesions when taking the measurements (Table 4.2).

4.3.2 COMPARISON OF FRESHLY CULTURED AND PREVIOUSLY CRYOPRESERVED COCCI FOR THE PRODUCTION OF *DERMATOPHILUS CONGOLENSIS* INFECTIONS

Both rabbits infected with cocci from the thawed stabilate developed lesions in the same way as the lesions produced by using freshly cultured cocci. Initial signs of infection were manifested as erythema and progressed to form discrete scabs. Although the rabbits 3 and 4 had been infected with identical titrated doses of *D.congolensis* cocci there was a marked difference in the severity of the lesions on them. By day six of the dermatophilosis infection the highest ranked score for one infection area on rabbit 4 was nine compared with the highest score of four on rabbit 3. Using the Mann-Whitney test on the scores given to the individual infection sites at day six of the infection there was found to be no significant difference between the severity of the dermatophilosis on these two rabbits ($P > 0.05$).

Using Friedman's test, the clinical ranked scores given to the ten dermatophilosis infection areas on these two rabbits were compared with the infections produced by freshly cultured cocci (Table 4.3). The infections were compared at four and six days after infection and in both cases there was no significant difference between the dermatophilosis infections on the three rabbits, $P > 0.05$ ($n = 10$ = number of infection sites, $k = 3$ = number of animals).

Table 4.2 Skin fold measurements (mm) of dermatophilosis lesions on rabbits infected with two different titrations of freshly cultured *Dermatophilus congolensis* cocci.

Rabbit 1

	Skin fold measurements (mm)						
Day of infection	1.25 x* 10^8	1.25 x 10^7	1.25 x 10^6	1.25 x 10^5	1.25 x 10^4	1.25 x 10^3	1.25 x 10^2
5	9.1	4.8	5.6	5.2	4.0	3.7	5.5
7	9.0	5.0	4.6	4.2	4.4	4.3	5.5
9	8.7	4.8	4.1	3.4	3.6	4.6	4.9
12	4.5	3.8	3.5	3.5	3.5	3.5	4.9
14	Lesions healed and no skin fold measurements taken						

Rabbit 2

	Skin fold measurements (mm)									
Day of infection	2.5 x* 10^8	5 x 10^7	1 x 10^7	2 x 10^6	4 x 10^5	8 x 10^4	1.6 x 10^4	3.2 x 10^3	6.4 x 10^2	1.28 x 10^2
4	5.4	5.8	5.3	5.0	5.5	5.7	4.7	5.0	5.5	5.1
6	6.4	7.5	8.3	5.4	4.5	3.9	3.9	3.7	4.0	3.8
8	5.4	7.4	N/A	5.2	4.5	4.6	3.4	4.2	3.7	4.4
11	4.5	7.9	N/A	4.7	4.3	3.6	3.5	3.2	4.1	4.0

* = cocci/cm^2

N/A = dermatophilosis scab too thick and hard to measure.

Table 4.3 Ranked clinical scores of *Dermatophilus congolensis* infections on rabbits infected with identical doses of freshly cultured or previously cryopreserved cocci.

	Ranked clinical scores					
	Day 4 of infection			Day 6 of infection		
Concentration of cocci/cm^2	Freshly cultured cocci Rabbit 2	Cryopreserved cocci Rabbit 3	Cryopreserved cocci Rabbit 4	Freshly cultured cocci Rabbit 2	Cryopreserved cocci Rabbit 3	Cryopreserved cocci Rabbit 4
2.5×10^8	4	3	6	6	3	8
5×10^7	5	2	6	7	4	8
1×10^7	7	3	7	7	4	9
2×10^6	4	1	5	6	2	7
4×10^5	2	0	2	3	0	2
8×10^4	2	2	2	2	3	3
1.6×10^4	0	0	0	0	0	2
3.2×10^3	3	0	0	5	0	0
6.4×10^2	0	0	0	0	0	0
1.28×10^2	0	0	0	0	0	0
Totals	27	11	28	36	16	39

4.4 DISCUSSION

Initial experiments were set up to produce a suitable working system for the production of experimental dermatophilosis infections on rabbits.

Two titration ranges were used for the initial infections which were produced from freshly cultured *D.congolensis* cocci. The first titration consisted of seven ten-fold dilutions ranging from 1.25×10^8 to 1.25×10^2 cocci/cm^2 which produced easily recorded infections over the entire range. The second titration consisted of ten five-fold dilutions ranging from 2.5×10^8 to 1.28×10^2 cocci/cm^2 which produced lesions on only seven of the ten infection sites.

Although both titrations covered similar concentration ranges, the titration using a lower dilution factor over more areas resulted in several areas infected with very low concentrations of cocci not producing any dermatophilosis lesions. For future *D.congolensis* infections on rabbits the best titration range would be seven ten-fold dilutions starting at 1.25×10^8 cocci/cm^2. Using a titration range with a higher dilution factor allows a wider range of concentrations of cocci to be applied over a smaller number of areas which is a useful conservation of the limited space available when using rabbits as experimental hosts. Also, the use of the higher dilution factor reduces the number of areas infected with very low concentration of cocci which may not produce any dermatophilosis lesions.

The progress of the disease was closely monitored to establish a ranking system to record the development of various characteristics of the infection. Four parameters were monitored; the skin fold thickness; the area of each infection site showing signs of infection; the severity of the scabs, ranging from erythema to thick layers of dead flaking epidermis; and the extent of exposed dermis at the infection sites. With the scab formation occurring at the beginning of the assessment and the

amount of exposed dermis increasing near the end of an infection, a combination of these two parameters allowed scoring of the infection throughout its course.

The measurement of skin fold thickness was only of limited use. As the infections progressed to form scabs, the skin fold thickness became greater; however, these changes were already recorded by scoring the area infected and severity of scab. As the lesions progressed and the scabs became thicker and harder the measurement of the skin fold thickness became increasingly difficult and in some cases impossible. Care had to be taken not to aggravate the lesions by cracking or knocking off the scabs when taking the skin fold measurements. At the sites infected with low concentrations of cocci, some slight swelling was recorded at the beginning of the assessment which then subsided without the formation of discrete scabs. Therefore, skin fold measurements may be of more use if measuring low level infections.

Once a suitable working system had been found for siting and titration ranges on rabbits it was necessary to establish a method to produce identical titrations for a series of experiments. In previous experiments fresh *D.congolensis* cocci were cultured when required for experimental infections. Each time the *D.congolensis* cocci were harvested the concentration of cocci in the suspension was calculated and then the suspension was diluted to the required concentration. The production of separate batches of infective *D.congolensis* cocci may have resulted in experimental variation. To allow direct comparison between infections produced at different times a large amount of stabilate was produced. This stabilate was stored in 2ml aliquots which could be thawed and diluted to the required concentration when needed.

Dermatophilosis infections were produced on rabbits by using titrations of *D.congolensis* cocci prepared from the thawed stabilate. The resulting infections were compared with infections produced by freshly cultured cocci over the same titration range. There was no significant difference between the infections produced by freshly

cultured cocci and those applied directly from a frozen stabilate ($P > 0.05$). It was therefore proposed that the frozen stabilate would be used for the production of subsequent experimental dermatophilosis infections.

Once suitable sites, titration ranges, and a method to produce constant dermatophilosis infections on rabbits had been determined, it was possible to begin the investigation into the relationship between the feeding of *A.variegatum* and the progression of dermatophilosis.

4.5 SUMMARY

1. Two different titration ranges of *D.congolensis* cocci were tested on rabbits. The titration starting at a concentration of 1.25×10^8 cocci/cm^2, with seven ten-fold dilutions, resulted in dermatophilosis lesions over the whole range. This titration covered a wide range of concentrations over fewer sites which is an advantage when producing infections on the limited space available on the body of a rabbit. This titration, or similar titrations, were used to produce experimental *D.congolensis* infections on rabbits.

2. Two different sizes of infection sites were tested, 1 x 2cm and 2 x 2cm, and both sizes produced easily analysed lesions. It was decided to use the smaller size infection sites for experimental infections due to economy of space and to prevent undue stress on the experimental animals.

3. A ranking system was established to assess the progression of the experimental infections. This system recorded the percentage area showing signs of infection, the severity of the scab, and the amount of exposed dermis. The combination of these parameters allowed assessment throughout the course of

the infection. Skin fold measurements were found to be of limited use, especially at the sites infected with high concentration of *D.congolensis* cocci.

4. A stabilate was produced containing infective *D.congolensis* cocci at a concentration of 1.2×10^7 to allow replicate infections. The viability of this stabilate was tested on rabbits. There was no significant difference between dermatophilosis infections produced by application of cocci from this stabilate and infections produced by freshly cultured cocci. Therefore, this stabilate was used to produce the titrations of infective *D.congolensis* cocci in the following experiments.

CHAPTER FIVE
THE LOCAL EFFECT OF HYPERSENSITIVE OR INFLAMMATORY REACTIONS TO LARVAL AND NYMPHAL *AMBLYOMMA VARIEGATUM* ON SUBSEQUENT *DERMATOPHILUS CONGOLENSIS* INFECTIONS

5.1 INTRODUCTION

Amblyomma variegatum ticks have long been associated with the development of chronic dermatophilosis lesions (Plowright, 1956; Macadam, 1964b; Macadam 1964c; Stewart, 1972; Morrow *et al.*, 1989; Martinez *et al.*, 1992). However, it is not clear by which mechanism the tick produces this effect or which of the instars are involved.

Primary dermatophilosis lesions have been associated with the attachment sites of adult *A.variegatum* (Plowright, 1956; Oduye, 1975a; Bwangamoi, 1976). There have also been many reports of chronic lesions occurring at sites remote from the predilection sites of adult *A.variegatum* (Zlotnik, 1955; Macadam, 1964b; Macadam, 1964c; Morrow *et al.*, 1989).

Adult *A.variegatum* have been shown to have a significant systemic effect on the progression of dermatophilosis on sheep (Walker and Lloyd, 1993) which could account for the occurrence of remote chronic lesions. However, immature ticks and *Haematobia* biting flies have been observed feeding in large numbers on the dorsal surfaces of cattle (Plowright, 1956; Macadam, 1964b; Macadam, 1964c; Stewart, 1972). Furthermore, Stewart (1972) observed large numbers of the tabanid flies *Haematopota albihirta* and *Tabanus taeniola* associated with the majority of dermatophilosis lesions on the dorsal surfaces of cattle. The feeding of immature ticks and biting flies produces large numbers of inflammatory and hypersensitive lesions distributed widely over the hosts; these localised reactions may predispose to dermatophilosis (Macadam, 1962; Bida and Dennis, 1976; Davis and Philpott, 1980; Lloyd, 1984).

Work by Davis and Philpott (1980) has shown that delayed type hypersensitivity reactions to the hapten dinitrochlorobenzene, in goat's skin at the site of infection with *Dermatophilus congolensis* can facilitate the development of severe dermatophilosis lesions similar to natural chronic infections. These dermatophilosis lesions remained as long as local delayed (type IV) hypersensitive reactions persisted.

The feeding of *A.variegatum* ticks has been shown to produce delayed (type IV) hypersensitive reactions with the formation of intra-epidermal pustules and increased proportions of infiltrating granulocytes (Latif *et al.*, 1991a). The development of cellular reactions in guinea-pigs in response to repeated tick infestations is characterised by a cutaneous basophil hypersensitivity (Brown *et al.*, 1983). This type of cellular reaction has also been demonstrated by repeated infestations of *Amblyomma americanum* on bovine hosts (Brown *et al.*, 1984). However, the inflammatory response to *A.variegatum* feeding on rabbits is predominantly neutrophilic and mononuclear with eosinophils also infiltrating attachment sites after repeated infestations (Latif *et al.*, 1990).

The production of hypersensitive reactions has been associated with increased resistance of hosts to various tick species, with the pustulation reducing the attachment of the ticks and the increased infiltration limiting the engorgement of the ticks (Walker and Fletcher, 1987). Resistance to immature *A.variegatum* ticks in Zebu cattle has been demonstrated in studies of natural tick burdens (Latif *et al.*, 1991b). Host resistance to experimental infestations of adult and immature stages of *A.variegatum* on cattle is accompanied with increased hypersensitive reactions at the tick attachment sites (Latif *et al.*, 1991a).

To test the hypothesis that hypersensitivity reactions in skin predispose to dermatophilosis, we studied the effects of the reactions to tick feeding on the progression of subsequent local and remote *D.congolensis* infections. Using rabbits

and sheep as the experimental hosts, inflammatory and hypersensitive reactions in response to the feeding of immature *A.variegatum* ticks were produced by single or multiple infestations respectively.

5.2 METHODS

5.2.1 THE LOCAL EFFECT OF REPEATED LARVAL TICK INFESTATIONS ON THE DEVELOPMENT OF DERMATOPHILOSIS ON RABBITS

5.2.1.1 Experimental hosts

Three groups of female New Zealand White rabbits (see Section 3.2.1) were used to compare the effect of inflammatory and delayed (type IV) hypersensitive reactions on the progression of local *D.congolensis* infections.

One of the three groups of rabbits (Group A) was infested with four consecutive infestations of larval *A.variegatum* to produce delayed (type IV) hypersensitive reactions in the host's skin. Three immunizing infestations of 500 larvae were applied to alternate ears. After three infestations visible signs of hypersensitive reactions were apparent at the tick attachment sites; a challenge infestation was then applied on the backs of the rabbits to produce hypersensitive reactions at the site of subsequent *D.congolensis* infections. A second group of rabbits (Group B) was infested once only, to produce inflammatory reactions at the site of the following *D.congolensis* infection. The third group of rabbits (Group C) was used as the control. Figure 5.1 shows the sequence of tick infestations and *D.congolensis* infections on the three groups of rabbits.

Figure 5.1 The sequence of larval *Amblyomma variegatum* infestations and *Dermatophilus congolensis* infections on rabbits used to test the effect of hypersensitive and inflammatory reactions to ticks on subsequent infections.

Day of experiment	Hypersensitive reactions to larval *A.variegatum* (Group A)	Inflammatory reactions to larval *A.variegatum* (Group B)	No exposure to ticks (Group C)
0	Immunizing infestation of 500 larvae applied to left ear	____	____
28	Immunizing infestation of 500 larvae applied to right ear	____	____
57	Immunizing infestation of 500 larvae applied to left ear	____	____
84	Challenge infestation of 500 larvae applied to area (10 x 10cm) on back	Challenge infestation of 500 larvae applied to area (10 x 10cm) on back	____
95	Day 11 of challenge infestation, delayed (Type IV) hypersensitive reactions in skin, all ticks removed	Day 11 of challenge infestation, inflammatory reactions in skin, all ticks removed	____
96	All three groups of rabbits infected with *D.congolensis*, at the site of previous tick attachment on Groups A and B		

5.2.1.2 Tick infestations

The ticks used in this study were larval *A.variegatum* from the laboratory colony (Section 3.3). These larvae were all of the same age, from eggs laid by a group of females fed at the same time. Each of the infestations consisted of approximately five hundred larvae; the eggs were batched by weight prior to hatching (Section 3.3.3.1). Immunizing infestations, to produce hypersensitive reactions in the host's skin, were applied to the ears of the test rabbits and confined within ear-bags. The final infestation on the test rabbits and the single infestation on the control rabbits were applied to the body (for details of tick application see Section 3.3.4.1). This protocol was used to produce either hypersensitive or inflammatory sites respectively, on the test and control rabbits at the site of the subsequent *D.congolensis* infections.

The challenge infestations of ticks were confined within a patch glued onto the backs of the rabbits. The areas covered by the patches were marginally larger than the areas to be infected with *D.congolensis*. After twenty-four hours the patches were removed, by this time the majority of the ticks had attached to the host. In this way the tick feeding was confined to a small area on the back at the same site as the subsequent *D.congolensis* applications. A body-bag was put on each rabbit to confine any unattached ticks and to collect the engorged ticks as they dropped off the host.

The test rabbits each received three immunizing infestations of ticks until hypersensitive reactions were visible; the visible reactions consisted of the production of exudate, sloughing of skin and formation of pustules. At the end of one infestation there was an interval of seven to 21 days before the next infestation. This timing allowed operational flexibility whilst remaining within an immunizing regime expected to produce hypersensitivity.

For each infestation the number of successfully engorging ticks, the average weight of engorged ticks and the moulting success were recorded. The combined results for the assessment of these parameters were used to measure changes in host resistance (Walker *et al.*, 1990), (see Section 3.3.5).

Each immunizing infestation of ticks was left on the host until the majority of the ticks had engorged; during this time the detached engorged larvae were collected daily. When the majority of ticks had engorged and detached, any ticks still feeding were detached manually. Some ticks would attach to the host without engorging successfully and to prolong infestations to allow every tick to engorge would have caused unnecessary stress for the experimental hosts.

For the challenge infestation only, on both groups of rabbits, the ticks were removed on the eleventh day of the infestation; therefore, the number of ticks still attached to the hosts was high. The stage of engorgement of these ticks was recorded and those in the final stages of engorgement were included in the number of successfully engorged ticks.

5.2.1.3 *Dermatophilus congolensis* infections

The *D.congolensis* used for this experiment was taken from the large batch of stabilate previously cultured and frozen at -20°C and -70°C, at a concentration of 1.2×10^7 cocci/µl (see Section 4.2.2).

All of the rabbits were infected with identical doses of *D.congolensis* cocci one day after the end of the final infestation of ticks. The *D.congolensis* was applied to ten test areas 1cm x 2cm on the torso, in the same area as the previous tick attachment sites and to a similar area on the control rabbits. Five of the areas were infected with a titrated dose of *D.congolensis*, the other five areas were infected with

a constant dose. A further three areas (1 x 1cm) were infected for biopsy sites. The infective cocci were applied to the skin using the procedure as described in Section 3.4.1.

The dose size for the ten test areas (1 x 2cm) was 50µl each and the dose for the biopsy sites was 25µl each. The concentration of cocci ranged from 2.5×10^8 cocci/cm^2 to 4.0×10^5 cocci/cm^2 over the five titrated areas and 5.0×10^7 cocci/cm^2 for the five constant areas and biopsy sites.

The lesions were scored using the ranking system which was set up during the preliminary experiments (see Section 4.2.1.5). Care was taken with obtaining skin fold measurements as the disease developed because it may have aggravated the scabs and facilitated the formation of lesions.

The progress of the resulting dermatophilosis lesions was recorded every three to four days for three weeks.

5.2.1.4 Skin biopsies

5.2.1.4.1 Biopsy material

When the rabbits were infected with *D.congolensis* at ten test areas, three extra areas (1cm^2) were also infected. Each of these areas received an application of *D.congolensis* from the middle of the titration range when the *D.congolensis* was applied to the rabbits (see Section 5.2.1.3).

Skin biopsies were obtained from these areas from each of the rabbits at intervals during the *D.congolensis* infection. Eight biopsies of the attachment sites of larval *A.variegatum* were obtained at day five of the infestations; four each of the first and fourth infestation on rabbits. Histological reactions of the skin at the site of

D.congolensis infections, on the test and control rabbits, were also recorded using light microscopy.

The skin around the proposed site of the biopsies was cleaned using ethanol, being careful not to wet the dermatophilosis scabs. Once the area surrounding the biopsy site had been cleaned a local anaesthetic (lignocaine hydrochloride, without adrenaline) was injected at several adjacent points. For the first set of biopsies the anaesthetic was administered using a needle. For all subsequent biopsies a high pressure jet injection apparatus, Dermojet (Etablissements Akra, France) was used to inject 0.1ml at each site.

Biopsies were taken, with a diameter of 3mm and depth of 5mm, using an electrically powered trephine and a depth limiting gauge of perspex block, ensuring that the biopsies did not penetrate through into the body cavity. Immediately upon removal, the biopsies were placed into Karnovsky's fixative held at 0°C, then left at room temperature on an agitator, for two hours.

5.2.1.4.2 Embedding and staining

Once fixation was complete the biopsies were washed three times in 0.1M phosphate buffer, each wash lasting 20 minutes (for the composition of Karnovsky's fixative and 0.1M phosphate buffer see Appendix 5.1). The biopsies were then embedded in hydroxyethylmethacrylate monomer (Historesin kit, Leica Cambridge Ltd, U.K.), according to the manufacturer's instructions.

Biopsies were infiltrated, on an agitator, for one hour with 75% Historesin and benzol-peroxide (BPO), diluted with distilled water and then with 100% Historesin and BPO for three days.

When thoroughly infiltrated the biopsies were embedded in 8mm diameter, flat bottom, polythene, embedding capsules. To make eight blocks, the

embedding medium consisted of 7ml Historesin and BPO and 0.5ml of the proprietary hardener. The mixture was made up over an ice bath, to delay polymerisation, and was thoroughly mixed by repeated pipetting.

Each capsule was filled with Historesin before the biopsies were placed in the capsules. The capsules were then sealed, to prevent oxygen from impairing the polymerisation process, left at 4°C overnight and then cured by storage for several days at 25°C.

Sections (1.5μm thick) were stained separately with 5% Giemsa's stain in acidic (pH 4.5) and alkali (pH 7.2) buffer, then differentiated in 20% methanol.

5.2.1.4.3 Histological assessment of the tick attachment sites

Biopsies of the tick attachment sites were evaluated for signs of delayed (type IV) cutaneous hypersensitive reactions, including the infiltration of basophils and mononuclear cells, the formation of intra-epidermal pustules and production of exudate and necrosis (Thomson, 1978; Roitt *et al.*, 1985). The biopsies were also assessed for acute inflammatory abscesses involving infiltration of neutrophils and mononuclear cells, degranulating mast cells and the development of oedema and haemorrhage (Turk, 1967; Roitt *et al.*, 1985).

Using an eyepiece graticule and x100 lens to study the dermis, the numbers of eosinophils, basophils, neutrophils, mast cells, capillaries and mononuclear cells were recorded. In rabbits, both eosinophils and neutrophils contain eosinophilic granules, these two granulocytes were distinguished by the paler staining nuclei and large, elongate granules in the eosinophils. Also the neutrophils had distinct, multilobed nuclei. The other two granulocytes, basophils and mast cells, contained basophilic granules. These two cells were distinguished by the large oval nuclei in mast cells compared with the irregular shape of the nuclei in basophils.

Two sections from each biopsy were stained with Giemsa at pH 4.5. The numbers of cells and capillaries over the whole of the graticule (112.5 x 112.5μm) were recorded for ten fields from each section, covering a total area of $0.13mm^2$ from each section. The degree of haemorrhage was also recorded for the same fields, but in this case the area infected was recorded by counting the number of squares over the whole graticule affected.

The degree of oedema and necrosis was recorded for separate sections from the same area of the biopsy, stained in Giemsa at pH 7.2. Observations were done using the eyepiece graticule and x50 lens. The area of the dermis affected was recorded by counting the number of squares affected over the whole graticule for ten fields. At x50 magnification the graticule covered an area 233.3 x 233.3μm and each square was $544.3\mu m^2$. Therefore, the total area assessed for each section was $0.54mm^2$.

Of the ten fields used for the assessment of the histological changes in the dermis, five fields were adjacent to the epidermis and five positioned with the centre of the graticule approximately 520μm beneath the epidermis.

5.2.1.4.4 Histological assessment of the dermatophilosis lesions

Two sections from each biopsy were used for the histological assessment of the epidermis at the site of the dermatophilosis lesions. Each section was viewed using a x10 objective lens to record the presence of intra-epidermal pustules, oedema and necrosis. The occurrence of these were recorded using the following ranking system

Observed in 0 sections = -

Observed in 1-25% of sections = +

Observed in 26-50% of sections = ++

Observed in 51-75% of sections = +++

Observed in 76-100% of sections = ++++

Using the x100 lens, ten fields covering a length of 1.1mm of epidermis were assessed for hyperplasia, diapedesis of granulocytes through the epidermis, presence of Langerhan's cells and haemorrhage.

The Langerhan's cells were distinguished as cells with dark staining nuclei and clear cytoplasm (Uno and Hanifin, 1980). They were distinguished from other, similar staining cells by dendritic processes extending into the intracellular spaces.

The hyperplasia was recorded by counting the number of layers at the centre of each field of view and obtaining a total number for each section. The average number of epidermal layers was calculated for each set of biopsies, taken from the same group of hosts at the same stage of infection, and then given a ranked score.

1-4 epidermal layers = -

5-8 epidermal layers = +

9-12 epidermal layers = ++

13-16 epidermal layers = +++

17+ epidermal layers = ++++

The presence of eosinophils, neutrophils, basophils, mast cells and Langerhan's cells and erythrocytes in the epidermis was also recorded using a ranking system. A ranking system was used instead of actual numbers because of the very large numbers of neutrophils in some sections.

0 cells present = -

1-15 cells/section = +

16-30 cells/section = ++

31+ cells/section = +++

5.2.2 THE LOCAL EFFECT OF REPEATED NYMPHAL TICK INFESTATIONS ON THE DEVELOPMENT OF DERMATOPHILOSIS ON SHEEP

5.2.2.1 Experimental hosts

Two pairs of Suffolk x Black-faced sheep were used to investigate the local and systemic effect of inflammatory and hypersensitive reactions in sheep, in response to nymphal tick feeding, on the progression of subsequent *D.congolensis* infections. These sheep originated from breeding stock at the Moredun Research Institute and had no previous experimental exposure to ticks or to *D.congolensis*.

Two females (sheep 3 and 4) and two castrated males (sheep 1 and 2) were used, with one of each sex in each of the test and control groups. The sheep were paired for weight and size, rather than sex.

All four sheep were infested once with 400 *A.variegatum* nymphs, to produce inflammatory reactions in the host's skin, followed by infection with *D.congolensis*. The infections on the two test sheep were at the same site as the previous tick infestations, but at sites remote from the previous tick attachment sites on the control sheep. After the dermatophilosis lesions had healed, four more consecutive infestations of *A.variegatum* were used to produce delayed (type IV) hypersensitive reactions in the host's skin. All four sheep were then infected a second time with *D.congolensis*, again with the infections on the test sheep at the same site as the tick attachment, but at remote sites on the control sheep. Figure 5.2 shows a

Figure 5.2 The sequence of nymphal *Amblyomma variegatum* infestations and *Dermatophilus congolensis* infections on sheep used to test the effect of hypersensitive and inflammatory reactions to ticks on subsequent infections.

Day of experiment	*D.congolensis* infections at same site as previous tick infestation	*D.congolensis* infections at sites remote from previous tick infestations
0	Challenge infestation of100 nymphs applied to shoulder and 300 nymphs divided between five patches on the flank	Challenge infestation of 400 nymphs applied to the shoulder
11	All ticks removed, leaving inflammatory reactions in the skin of the shoulder and flank	All ticks removed, leaving inflammatory reactions in the skin of the shoulder only
12	Titrated doses of *D.congolensis* applied to five areas on flank, at same site as previous tick attachment	Titrated doses of *D.congolensis* applied to five areas on flank, at site remote from previous tick attachment
39	Final assessment of first *D.congolensis* infection	
42	Immunizing infestation of 100 nymphs applied to shoulder	
63	Immunizing infestation of 100 nymphs applied to shoulder	
103	Immunizing infestation of 100 nymphs applied to shoulder	
140	Challenge infestation of100 nymphs applied to shoulder and 300 nymphs divided between five patches on the flank	Challenge infestation of 400 nymphs applied to the shoulder
151	All ticks removed, leaving hypersensitive reactions in the skin of the shoulder and flank	All ticks removed, leaving hypersensitive reactions in the skin of the shoulder only
152	Titrated doses of *D.congolensis* applied to five areas on flank, at same site as previous tick attachment	Titrated doses of *D.congolensis* applied to five areas on flank, at site remote from previous tick attachment

summary of the sequence of nymphal *A.variegatum* infestations and *D.congolensis* infections on the sheep.

5.2.2.2 Tick infestations

The ticks used for this experiment were *A.variegatum* nymphs from five different batches, aged two to six months, obtained from the laboratory colony (see Section 3.3).

All four of the sheep received five infestations of nymphs. The first infestation was a challenge infestation to produce inflammatory reactions in the host's skin, with the inflammatory reactions on the test sheep being produced at the sites of the following *D.congolensis* infections. All of the sheep were infested with 400 nymphs: the controls were infested at one area on the shoulder, whilst the infestations on the test sheep were divided between one area on the shoulder and five areas on the flank.

All of the infestations were enclosed by cloth patches or bags glued to the wool on the flanks and shoulders of the sheep (see Section 3.3.4.2). The infestations at the shoulder were enclosed with bags that could be opened throughout the infestation for the removal of engorged ticks and assessment of resistance. The infestations on the flanks were enclosed by five sealed patches which were opened at the end of the infestation and infective *D.congolensis* cocci were applied to these sites (Plate 5.1).

Three immunizing infestations of 100 nymphs followed, with the ticks being applied to one bag on the shoulders of all four sheep. Finally a second challenge infestation of 400 nymphs was applied to the sheep with a similar distribution as the first test infestation.

Plate 5.1 Test sheep, infested with *Amblyomma variegatum* nymphs. The infestation is divided between five patches on the flanks, each containing 60 nymphs, and one bag on the shoulder containing 100 nymphs. The patches remained sealed throughout the infestation but the bag could be opened to assess resistance and to remove engorged ticks.

The infestations applied to the shoulders of the sheep were used to record the engorgement success of the ticks using the same parameters and calculations as before (see Section 5.2.1.2).

Ticks detaching at the peak of the detachment period were used in the batches for the assessment of tick feeding success. By selecting these ticks, individuals at either end of the detachment period were avoided which allowed consistent comparisons between separate infestations.

5.2.2.3 *Dermatophilus congolensis* infections

All four of the sheep received two titrated *D.congolensis* infections. The first was applied to the sheep one day after the end of the first tick infestation, and the second was applied one day after the end of the fifth tick infestation.

Infective *D.congolensis* cocci were obtained from the large stock of *D.congolensis* stabilate stored at a concentration of 1.2×10^7 cocci/µl. Doses of 100µl/8cm^2 were applied to each of the titration sites; the concentration of cocci per cm^2 ranged from 1.25×10^6 cocci/cm^2 with five, ten fold dilutions to 1.25×10^2 cocci/cm^2.

Both the primary and secondary *D.congolensis* infections were applied using identical protocols. All of the infection sites were degreased and cleaned prior to application of the infective *D.congolensis* cocci (see Section 3.4.2). Then five separate areas (2 x 4 cm) were infected for the *D.congolensis* titration, plus two other areas of the same size, for histological studies.

On the test sheep, the five areas for the titration were positioned at the bottom end of the area of tick infestation as this was the site of most attachments. The

ticks were removed the day before infection with *D.congolensis*. On the control sheep the areas were on the flank at sites with no previous infestation of ticks.

The two areas for histological studies on each of the test sheep were positioned at the site of the tick infestations on the shoulder. On the control sheep they were at sites with no exposure to ticks. Each area was $8cm^2$ and received a dose of 100µl at a concentration of 1.25×10^4 cocci/cm^2.

The resulting infections were assessed using the ranking system established in the preliminary experiments (see Section 4.2.1.5). The progression of the dermatophilosis was assessed at three or four day intervals from day three to twenty-seven of the infection.

The second infestation of ticks was applied on the sheep 30 days after the beginning of the first *D.congolensis* infection. By this time the infection was resolved, and there was no recurrence of the infection after the ticks were applied.

A second infection of *D.congolensis* was applied to all four sheep after the fifth infestation of ticks. The titration and assessment of the *D.congolensis* was exactly the same as above.

5.2.2.4 Skin biopsies

Biopsies were taken at day five of the first and last infestations of nymphal *A.variegatum* on the test sheep, and from all four sheep at days 3, 6, 13 and 27 of the primary and secondary *D.congolensis* infections. For details of preparation of biopsies and histological assessment of tick attachment sites see Section 5.2.1.4.

5.3 RESULTS

5.3.1 REACTIONS TO TICKS

5.3.1.1 Resistance

Resistance was calculated using the method of Walker *et al.*, (1990) using a combination of the change in mass, engorgement and moulting success of successive infestations of ticks. Appendices 5.2 and 5.3 contain the raw data for the changes in average mass, engorgement and moulting success of the repeated infestations of larvae and nymphs on rabbits and sheep respectively.

The development of resistance was extremely varied in rabbits repeatedly infested with larval *A.variegatum,* but all four of the sheep developed a marked resistance to repeated infestations of *A.variegatum* nymphs. There was a marked increase in resistance in only one of the rabbits (No. 4). In the other three rabbits the amount of resistance fluctuated, and at the final infestation two of the rabbits showed no marked change in resistance compared with the first infestation. In the remaining rabbit (No. 1) there was a marked decrease in resistance. In contrast, by the fifth infestation on the sheep, all four had developed a significant resistance to *A.variegatum* nymphs (Table 5.1).

5.3.1.2 Histology of larval and nymphal *Amblyomma variegatum* attachment sites

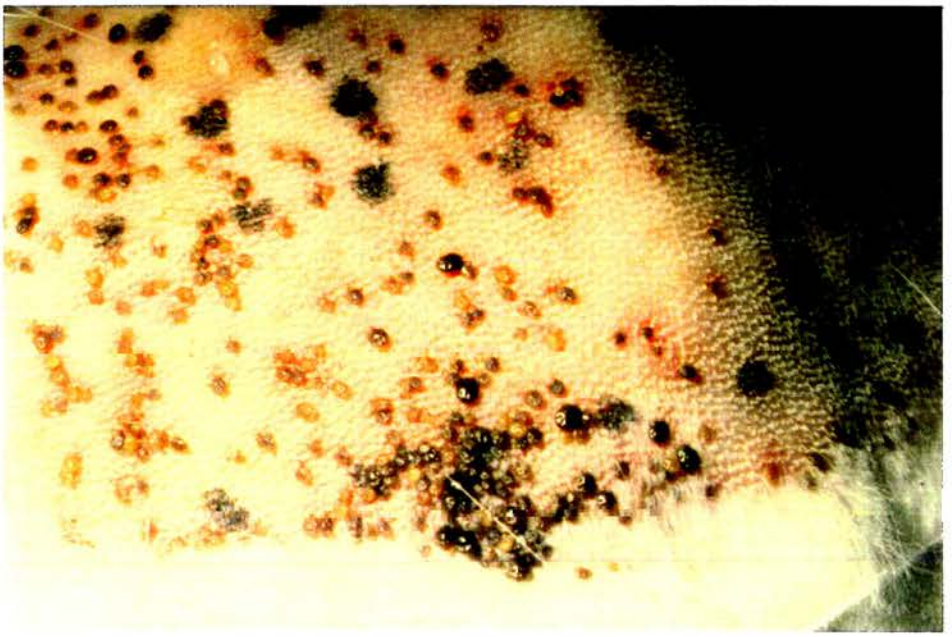
Inflammatory reactions in the host's skin in response to initial exposure to tick feeding increased in severity after repeated infestations, suggesting the development of hypersensitivity. Plate 5.2 shows day five of the first and fourth infestation of *A.variegatum* larvae, feeding on rabbits. The reduced engorgement of

Table 5.1 Changes in resistance to *Amblyomma variegatum* larvae and nymphs feeding on rabbits and sheep respectively, calculated by the method of Walker *et al.*, (1990). Changes in the resistance were compared with the initial resistance of the individual hosts; in some cases there was a decrease in resistance.

	RESISTANCE (%) AT EACH INFESTATION			
HOST	2nd	3rd	4th	5th
Rabbit 1	-36	28	-116	N/A
Rabbit 2	-49	47	7	N/A
Rabbit 3	9	-20	-1	N/A
Rabbit 4	74	N/A*	94	N/A
Sheep 1	16	82	100	97
Sheep 2	0	19	12	84
Sheep 3	21	37	30	70
Sheep 4	32	70	65	90

* = infestation discontinued due to ear infection

a



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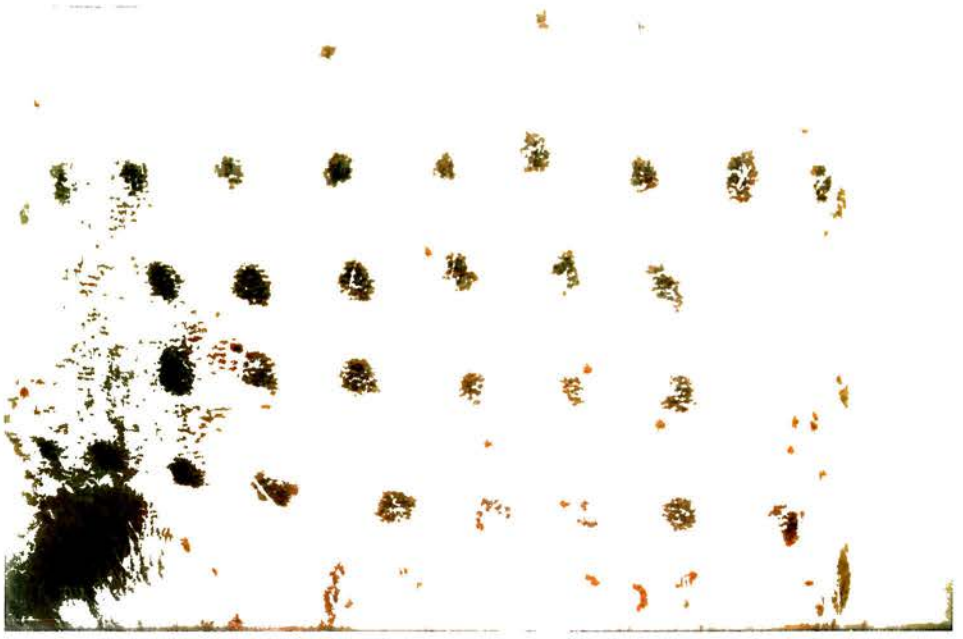


Plate 5.2 Day five of infestations of larval *Amblyomma variegatum* feeding on rabbits. a. First infestation; b Fourth infestation, with reduced engorgement of the larvae and extensive pustulation in the skin.

the ticks, production of exudate and development of erythema are clearly visible at the fourth infestation. Plate 5.3 shows day five of the fifth infestation of nymphs feeding on sheep. The engorgement of the nymphs was reduced, compared with initial infestations, and there was some erythema but the signs of hypersensitive reactions in the skin were not as visible as on the rabbits

Two biopsies were taken from each of the four rabbits that were repeatedly infested with *A.variegatum* larvae: the first biopsy was taken at day five of the first infestation and the second at day five of the fourth infestation. Two sections from each biopsy were used for the assessment of the larval attachment sites on the rabbits. Using the counts from individual sections allowed a comparison of eight counts from the first infestation and eight from the fourth.

Using separate Mann-Whitney tests for the counts of each of the cell types and pathological scores showed significant differences characterizing the distinction between initial inflammatory reactions and later hypersensitive reactions. There was significantly more eosinophils ($P < 0.01$), basophils ($0.01 < P < 0.05$), mononuclear cells ($P < 0.01$) and oedema ($P < 0.01$) at the attachment sites of the fourth infestation (Table 5.2). These results suggest that a delayed (type IV) hypersensitive reaction developed in the skin of the rabbits after repeated infestations. Plates 5.4 and 5.5 show larval *A.variegatum* feeding lesions at day five of the first and fourth infestations on rabbits.

Due to the small sample size for the nymphal tick attachment sites on the sheep it was not possible to use the Mann-Whitney test on the results for the individual cell counts and pathological scores. Instead, a chi-square test on the proportion of eosinophils, neutrophils, mononuclear cells and capillaries at the attachment sites of the first and fifth nymphal infestations showed significant differences in the proportions of these cells ($P < 0.01$). Relatively more eosinophils,

Plate 5.3 Day five of fifth infestation of nymphal *Amblyomma variegatum* feeding on a sheep. The engorgement of the nymphs was reduced compared with the initial infestations.

Table 5.2 Histological reactions at the tick attachment sites on rabbits and sheep in response to the feeding of larvae and nymphs of *Amblyomma variegatum*, respectively.

	RABBIT HOSTS (N=4)		SHEEP HOSTS[1]	
	1st	4th	1st	5th
Cell numbers[2]:				
Eosinophils	24	506*	7	16.5
Neutrophils	153	657	711	571.5
Mast cells	13	3	8	0.5
Basophils	62	181*	1	5.5
Mononuclear	1376	4523*	545	781.5
Pathological scores:				
Haemorrhage[3]	291	152	369	127*
Necrosis[4]	106	354	768	220*
Oedema[4]	326	1737*	173	78*
Capillaries[2]	54	34	9	7

[1] Total counts for one sheep at the first infestation and the median of the counts for two sheep at the fifth infestation.

[2] Total numbers from 20 fields, 112.5μm square, from each of four biopsies, using a x100 light microscope objective.

[3] These scores represent the number of areas (126.6μm^2) affected (Maximum = 100 x 20 fields) using x100 light microscope objective.

[4] These scores represent the number of areas (544.3μm^2) affected (Maximum = 100 x 20 fields) using x50 light microscope objective.

* Statistically significant differences at P = 0.05 or less, between 1st and 4th or 5th infestation.

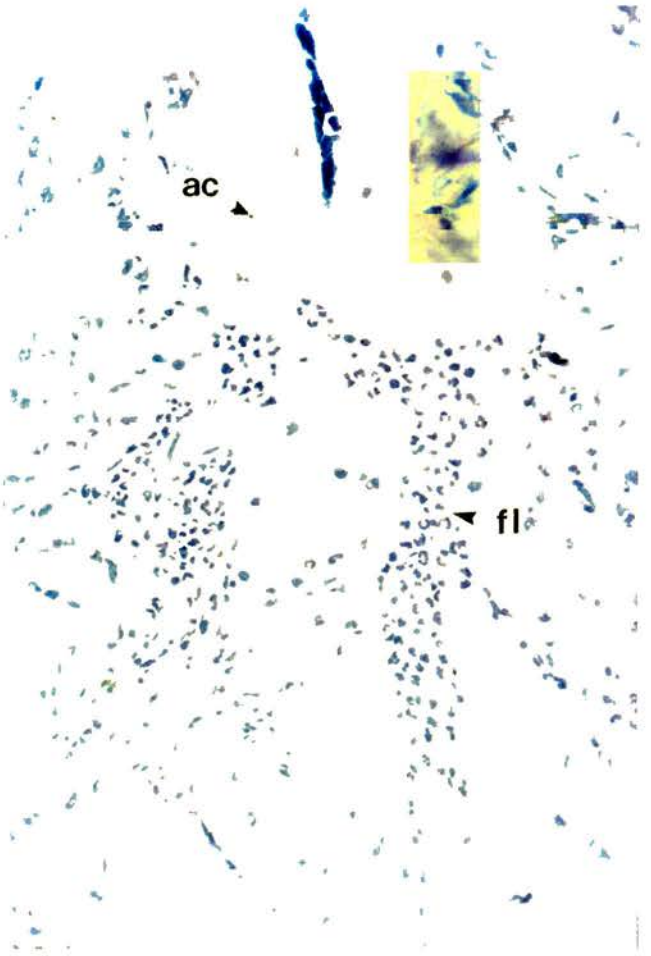


Plate 5.4 Day five of the first infestation of larval *Amblyomma variegatum* on a rabbit with inflammatory reactions to tick feeding. ac = attachment cement; fl = feeding lesion, packed with erythrocytes and granulocytes, mainly neutrophils with darkly stained, multilobed nuclei and eosinophilic granules. Stained with 5% Giemsa and acidic buffer (pH 4.5).

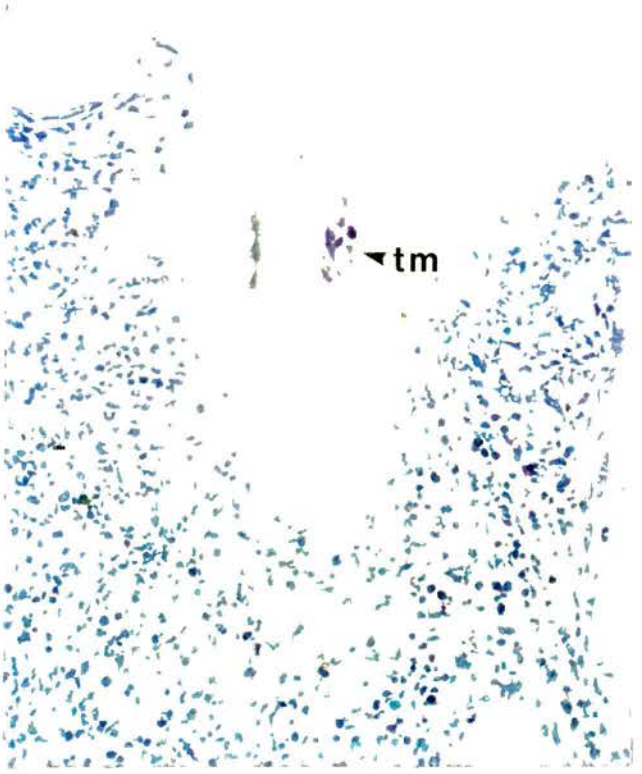


Plate 5.5 Day five of the fourth infestation of larval *Amblyomma variegatum* on a rabbit with delayed (type IV) reactions to tick feeding. The cellular reactions are much more extensive, and extend deeper into the dermis than the reactions at the inflammatory tick attachment sites. tm = tick mouthparts; the feeding lesion is packed with granulocytes, mainly neutrophils and eosinophils. Stained with 5% Giemsa and acidic buffer (pH 4.5).

basophils and mononuclear cells were observed at the final infestation with greater numbers of neutrophils and capillaries found at the attachment sites of the first infestation of nymphs (Table 5.2). Fisher's exact test on the number of basophils and mast cells also showed significant differences in the proportions of these cells at the first and final infestations of nymphs ($0.01 < P < 0.02$) (Table 5.2). Plates 5.6 and 5.7 show the histological reactions in the skin of sheep in response to the first and fifth infestations of nymphal *A.variegatum*.

5.3.1.3 Assessment of cellular reactions at granulomas after detachment of ticks

Assessment of the reactions in the skin of sheep at attachment sites of *A.variegatum* nymphs three days after the fifth infestation showed that there were large numbers of granulocytes still present in the dermis. Also, there were large numbers of mononuclear cells, with oedema and haemorrhage still affecting the tick attachment sites (Table 5.3 and Plate 5.8). Plate 5.8 also shows the visible signs of the hypersensitive reactions in the skin of the sheep one day after the fifth infestation of *A.variegatum* nymphs. The subsequent *D.congolensis* infections were applied to these areas on the test sheep and at sites remote from these reactions on the control sheep.

5.3.2 *Dermatophilus congolensis* infections on rabbits

5.3.2.1 Individual variation

Table 5.4 shows the total ranked clinical scores for the *D.congolensis* infections, for each of the assessment days, on the individual rabbits. Differences in the progression of the *D.congolensis* infections on the individual rabbits, within groups, were tested using Friedman's test. No significant difference ($P > 0.05$) was found between the *D.congolensis* infections on the four individual rabbits repeatedly infested with larvae prior to infection. However, for the rabbits infested with ticks

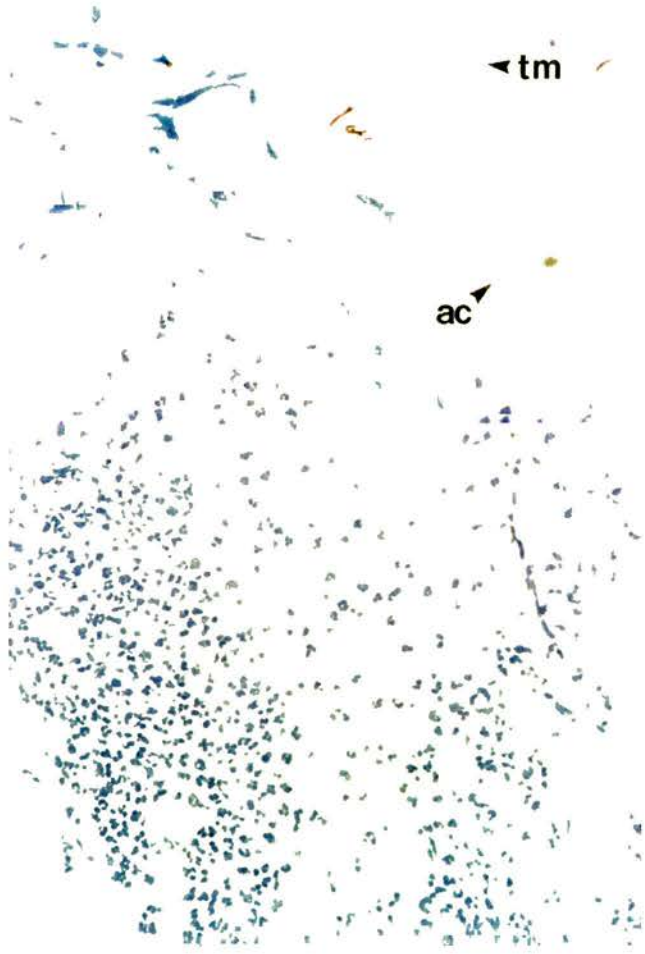


Plate 5.6 Day five of the first infestation of nymphal *Amblyomma variegatum* on a sheep with inflammatory reactions to tick feeding. ac = attachment cement; tm = tick mouthparts; the feeding lesion is packed with erythrocytes and neutrophils. In sheep the neutrophil granules are not eosinophilic and remain clear. Stained with 5% Giemsa and acidic buffer (pH 4.5).

Plate 5.7 Day five of the fifth infestation of nymphal *Amblyomma variegatum* on a sheep with delayed (type IV) hypersensitive reactions to tick feeding. The cellular reactions are much more extensive, and extend deeper into the dermis than in the inflammatory tick attachment sites. ac = attachment cement; the feeding lesion is packed with neutrophils. In sheep the neutrophil granules are not eosinophilic and remain clear. Stained with 5% Giemsa and acidic buffer (pH 4.5).

Table 5.3 Cell counts and pathological scores recorded in the dermis at tick attachment sites three days after detachment of fifth infestation of *Amblyomma variegatum* nymphs on the test sheep.

	Cell counts and pathological scores for 2 sections/biopsy	Mean (S.D.)
Cell counts[1]:		
Eosinophils	43, 33, 29, 36	35 (6)
Neutrophils	47, 44, 1, 1	23 (26)
Mast cells	4, 3, 4, 5	4 (1)
Basophils	2, 6, 12, 12	8 (5)
Mononuclear cells	333, 441, 483, 397	414 (64)
Pathological scores:		
Haemorrhage[2]	2, 2, 20, 8	8 (8)
Necrosis[3]	0, 0, 0, 0	0
Oedema[3]	359, 408, 651, 467	471 (128)
Capillaries[1]	8, 1, 8, 4	5 (3)

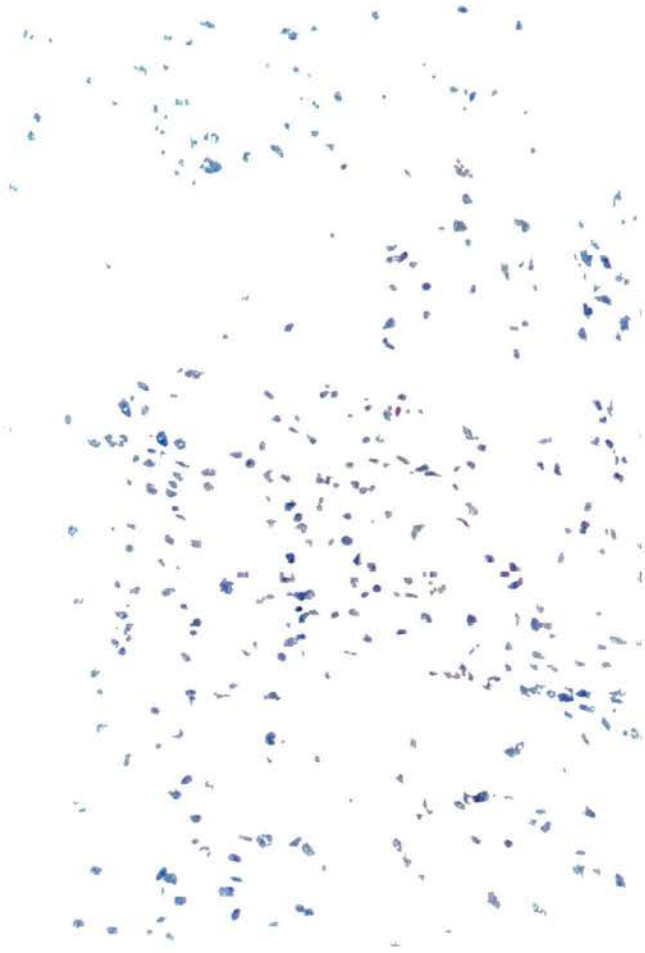
Oedema and hyperplasia recorded in the epidermis from all four sections

[1] Total numbers from 20 fields, 112.5μm square, from each of four biopsies, using a x100 light microscope objective.

[2] These scores represent the number of areas (126.6μm^2) affected (Maximum = 100 x 20 fields) using x100 light microscope objective.

[3] These scores represent the number of areas (544.3μm^2) affected (Maximum = 100 x 20 fields) using x50 light microscope objective.

a



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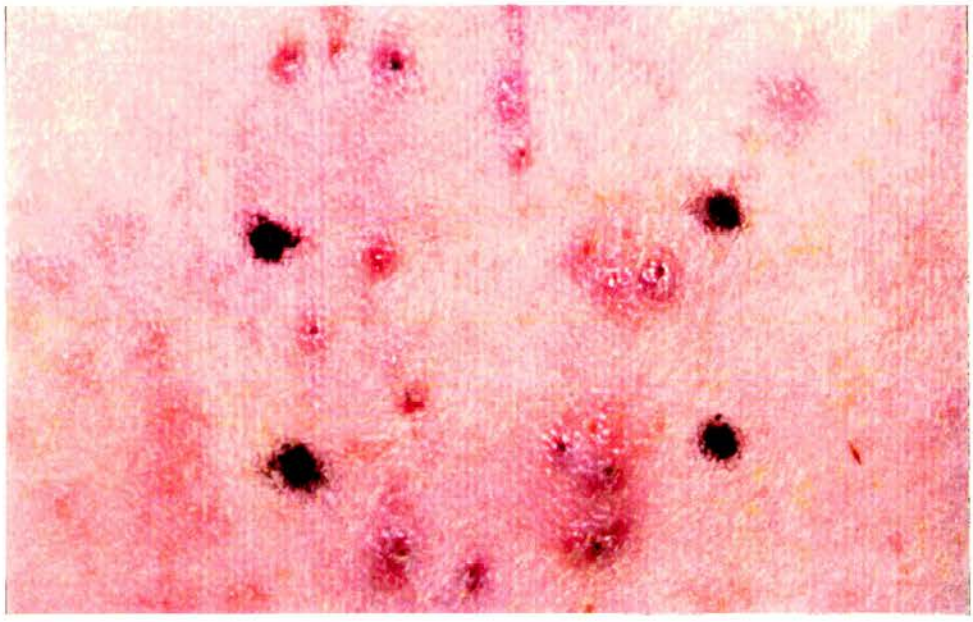


Plate 5.8 Hypersensitivity reactions in sheep skin after a fifth infestation of nymphal *Amblyomma variegatum*. a, One day after the removal of the ticks there are numerous granulocytes and mononuclear cells in the dermis. Stained with 5% Giemsa and acidic buffer (pH 4.5); b, Granulomas in the skin, three days after the removal of the ticks.

Table 5.4 The total ranked clinical scores for the *Dermatophilus congolensis* infections for each assessment day, on individual rabbits.

Day of infection	RANKED CLINICAL SCORES Infections at hypersensitive tick attachment sites Rabbit 1	Rabbit 2	Rabbit 3	Rabbit 4	Median
3	52	47	33	36	41.5
6	71	70	58	58	64
9	87	67	64	65	66
13	9	7	30	39	19.5
16	3	3	21	0	3
Day of infection	Infections at inflammatory tick attachment sites Rabbit 5	Rabbit 6	Rabbit 7	Rabbit 8	Median
3	46	31	19	31	31
6	69	54	46	49	51.5
9	66	67	52	58	62
13	17	0	19	52	18
16	13	0	0	31	6.5
Day of infection	Infections with no previous exposure to ticks Rabbit 9	Rabbit 10	Rabbit 11	Rabbit 12	Median
3	35	21	48	29	32
6	59	41	58	36	49.5
9	78	48	72	33	60
13	2	23	54	0	12.5
16	0	10	29	0	5

once, or not at all, there were significant differences between the dermatophilosis infections on individual rabbits ($0.01 < P < 0.05$ and $P < 0.01$, respectively).

5.3.2.2 The effect of inflammatory and hypersensitive reactions in rabbit's skin on subsequent *Dermatophilus congolensis* infections

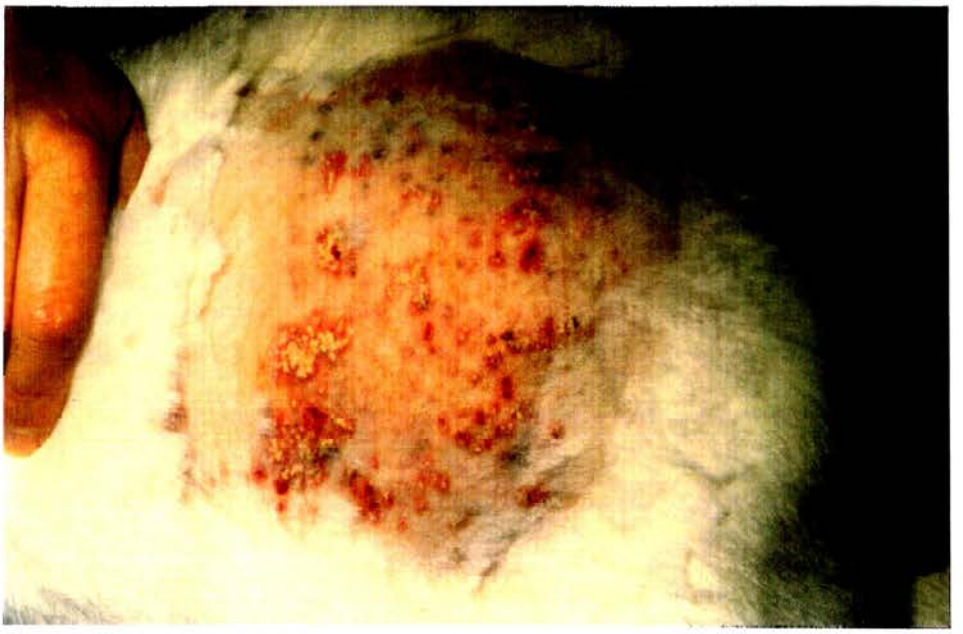
Plates 5.9 and 5.10 show examples of the dermatophilosis lesions on rabbits from each of the three groups. There were no obvious visible differences between the infections produced on skin with inflammatory or hypersensitive reactions to larvae, and on skin not exposed to infestations of larval *A. variegatum*.

Separate Kruskal-Wallis tests, using the total clinical scores for the individual rabbits for each of the assessment days, were used to compare the severity of the infections on all three groups. No significant difference was found between the three groups at any time during the course of the dermatophilosis infections ($P > 0.05$, $k = 3$, $n = 4$), (Table 5.5).

5.3.2.3 Duration of infections on rabbits

The peak of the dermatophilosis infections was recorded at day 9 on all 12 rabbits. After this time the lesions began to heal rapidly, with the scabs detaching and exposing intact but depilated skin. At the last assessment, on day 16, most of the lesions had healed completely (Figure 5.3). Three rabbits still had lesions with high clinical scores of 21, 31 and 29 but these were divided equally between the three groups (Table 5.4).

a



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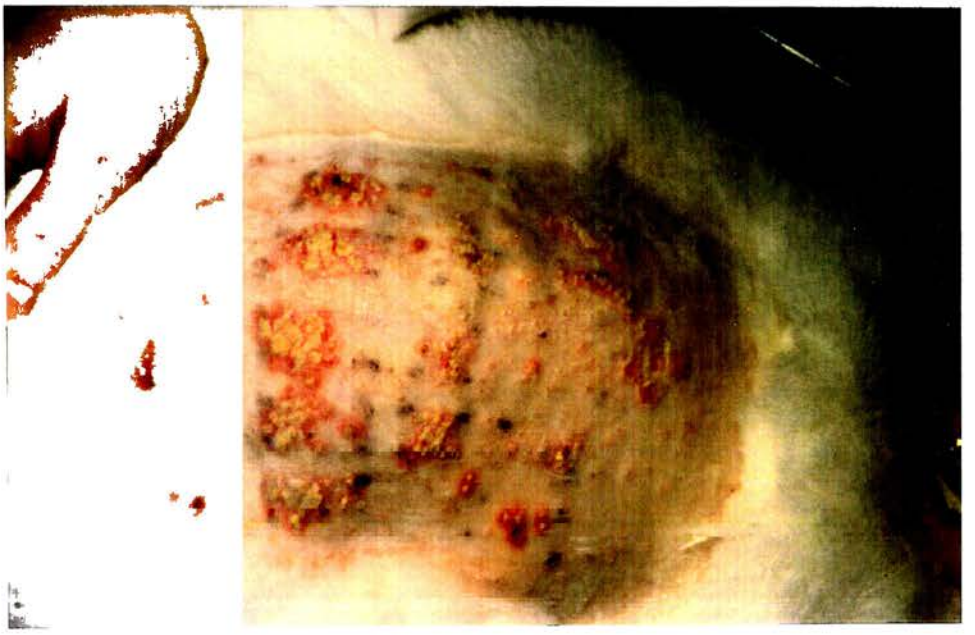


Plate 5.9 Day six of primary *Dermatophilus congolensis* infections on rabbits. a. Infections at the site of inflammatory reactions to a previous infestation of larval *Amblyomma variegatum*; b. Infections at the site of delayed (type IV) hypersensitive reactions to a previous infestation of larval *A. variegatum*.

Plate 5.10 Day seven of a primary *Dermatophilus congolensis* infection on a rabbit with no previous exposure to larval *Amblyomma variegatum*.

Table 5.5 Results of the Kruskal-Wallis test on the clinical scores for the dermatophilosis lesions on the individual rabbits for each assessment day comparing the effect of hypersensitive and inflammatory reactions in the host's skin; a third group, with no exposure to ticks has been included as a control.

Day of infection	Kruskal-wallis H	k	n	P	Interpretation
3	3.04	3	4	P > 0.05	ns
6	3.63	3	4	P > 0.05	ns
9	0.89	3	4	P > 0.05	ns
13	0.13	3	4	P > 0.05	ns
16	0.04	3	4	P > 0.05	ns

k = number of groups compared
n = number of individuals within groups
ns = not significant

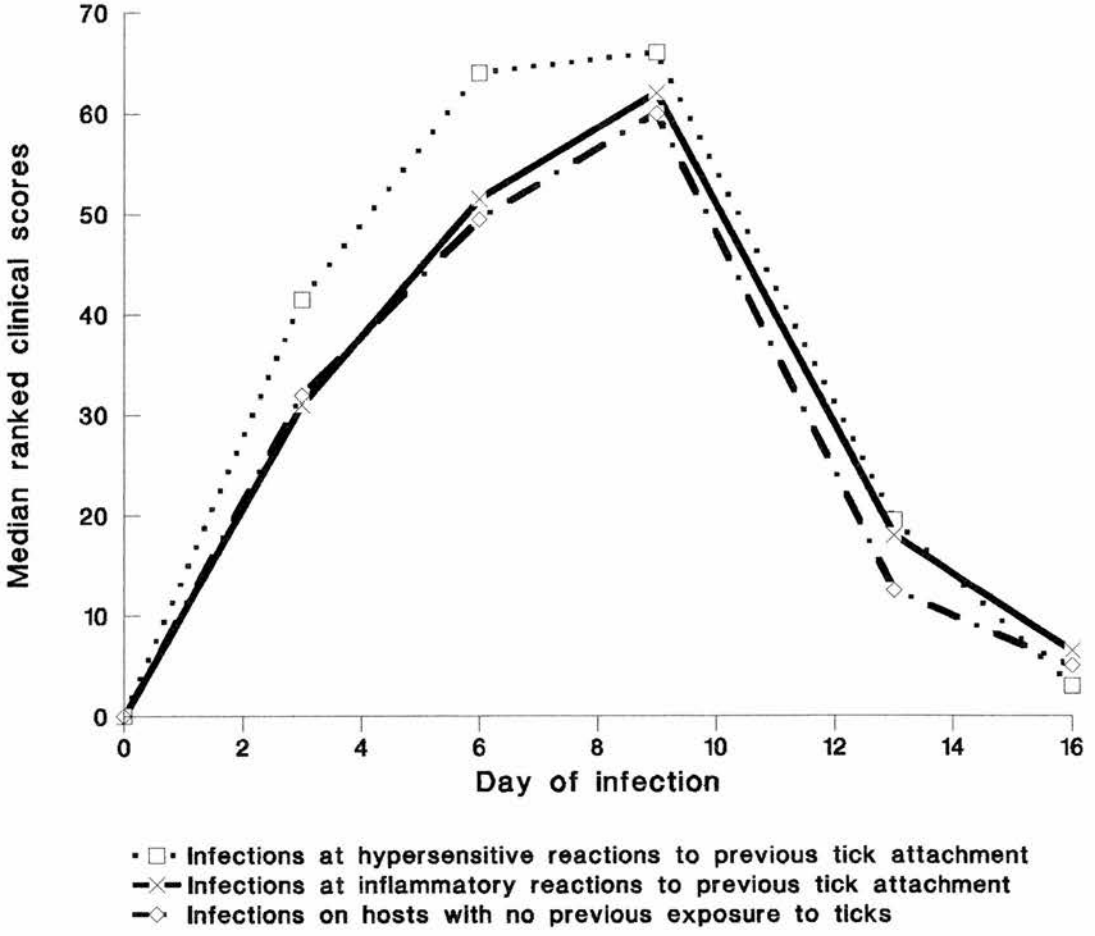


Figure 5.3. Median ranked clinical scores for primary *Dermatophilus congolensis* infections on rabbits. Comparison of the effect of hypersensitive or inflammatory reactions in the host's skin, in response to previous infestations of larval *Amblyomma variegatum*, on the progression of subsequent *D.congolensis* infections on rabbits.

5.3.2.4 Histology of *Dermatophilus congolensis* infections on rabbits

The actual counts and pathological scores are recorded in Appendix 5.4 and 5.5. This data was used to compare the histological reactions in the dermatophilosis lesions. The counts or scores, containing data with a normal distribution were compared using analysis of variance (ANOVA), and when significant differences were found the Duncan's range test was used to show where the significant difference occurred. In cases where ANOVA was not valid the Kruskal-Wallis test was used to compare the counts or scores for all three groups and when significant differences were found the Mann-Whitney test was used to show where the significant difference occurred. The results of the statistical analysis are incorporated in Table 5.6.

Table 5.6 shows the mean cell counts and pathological scores for the dermis at the sites of dermatophilosis lesions on rabbits with hypersensitive or inflammatory reactions in the skin to *A.variegatum* larvae or with no exposure to ticks.

Comparison of the histology of dermatophilosis lesions at hypersensitive or inflammatory reactions to larval *A.variegatum* demonstrated some significant differences. At days six to seven of the primary *D.congolensis* infections there were found to be significantly more capillaries and oedema at the lesions at the same site of hypersensitive reactions to the larval ticks, compared with lesions at inflammatory reactions to ticks. By day 13 these significant differences had disappeared; however, there were significantly more basophils in the lesions at the site of hypersensitive reactions to larval *A.variegatum*.

The assessment of lesions at days six to seven of the *D.congolensis* infections demonstrated that there were significantly more eosinophils, mast cells and

Table 5.6 Mean cell counts and pathological scores for the dermis at the site of *Dermatophilus congolensis* infections on rabbits; after single, multiple or no infestations with *Amblyomma variegatum* larvae.

	Mean cell counts and pathological scores (S.D.)						
	Hypersensitive attachment sites		Inflammatory attachment sites		No exposure to ticks		Statistical analysis
Day 6/7 of 1st *D.congolensis* infection							
Eosinophils[1]	5^{a}	(3)	2^{ab}	(3)	2^{b}	(2)	K-W + M-W
Neutrophils[1]	121^{a}	(87)	109^{a}	(106)	87^{a}	(93)	ANOVA
Mast cells[1]	5^{a}	(3)	3^{b}	(3)	2^{b}	(2)	ANOVA + DRT
Basophils[1]	47^{a}	(14)	27^{a}	(14)	31^{a}	(20)	ANOVA
Mononuclear[1]	298^{a}	(36)	228^{ab}	(88)	207^{b}	(27)	K-W + M-W
Capillaries[1]	13^{a}	(3)	9^{b}	(3)	11^{ab}	(7)	ANOVA + DRT
Haemorrhage[2]	19^{b}	(26)	66^{ab}	(68)	85^{a}	(49)	ANOVA + DRT
Necrosis[3]	36^{a}	(37)	14^{a}	(27)	12^{a}	(17)	K-W
Oedema[3]	315^{a}	(191)	96^{b}	(151)	201^{ab}	(217)	K-W + M-W
Day 13 of 1st *D.congolensis* infection							
Eosinophils[1]	4^{a}	(4)	2^{a}	(2)	2^{a}	(2)	K-W
Neutrophils[1]	6^{a}	(7)	60^{a}	(109)	12^{a}	(10)	K-W
Mast cells[1]	5^{a}	(5)	5^{a}	(4)	7^{a}	(3)	ANOVA
Basophils[1]	31^{a}	(7)	14^{b}	(4)	30^{ab}	(16)	K-W + M-W
Mononuclear[1]	264^{a}	(132)	320^{a}	(80)	238^{a}	(77)	ANOVA
Capillaries[1]	7^{a}	(5)	7^{a}	(2)	10^{a}	(4)	ANOVA
Haemorrhage[2]	8^{a}	(9)	9^{a}	(14)	12^{a}	(23)	K-W
Necrosis[3]	9^{a}	(13)	21^{a}	(33)	19^{a}	(19)	K-W
Oedema[3]	62^{b}	(67)	65^{b}	(60)	253^{a}	(210)	K-w + M-w

[1] mean of eight counts (0.13mm^2 each), using x100 light microscope objective; [2] mean number of graticule squares affected (126.6µm^2 each), using x100 light microscope objective; [3] mean number of graticule squares affected (544.3µm^2 each), using x50 light microscope objective. Maximum for [1] and [2] = 1000.

Different letters on the same row denote a significant difference with P=0.05 or less. [a] is significantly greater than [b].

ANOVA = Analysis of variance; DRT = Duncan's Range Test; K-W = Kruskal-Wallis; M-W = Mann-Whitney.

mononuclear cells recorded in dermatophilosis lesions at hypersensitive tick attachment sites compared with dermatophilosis lesions in skin with no previous exposure to ticks. However there was significantly more haemorrhage recorded in the dermatophilosis lesions on rabbits with no exposure to ticks compared with the lesions on rabbits showing hypersensitive reactions to larval *A.variegatum*. By day 13 the only significant difference occurred in the amount of oedema, with significantly more oedema recorded in the lesions on the rabbits with no exposure to ticks compared with the lesions at the hypersensitive reactions to ticks.

The histological comparison of the dermatophilosis lesions at inflammatory reactions to larval *A.variegatum* and lesions on rabbits with no exposure to ticks showed only one significant difference. At day 13 of the infection there was found to be significantly more oedema in the dermatophilosis lesions on the rabbits with no previous exposure to ticks.

Table 5.7 shows the ranked scores for the histological assessment of the epidermis of rabbits at the site of dermatophilosis lesions after multiple or single infestations of *A.variegatum* larvae or with no exposure to ticks. One week into the infection diapedesis of eosinophils, neutrophils and basophils into the epidermis was recorded in the dermatophilosis lesions on all three groups of rabbits. In the lesions at the sites of hypersensitive reactions to the larval ticks and on the rabbits with no exposure to ticks, the majority of the granulocytes were neutrophils. Small numbers of mast cells were recorded only in the epidermis at lesions with hypersensitive reactions in the skin.

By day 13 of the infection only small numbers of granulocytes were recorded in the epidermis, with a few neutrophils still found in both groups of lesions on skin with previous exposure to ticks. Small numbers of basophils were recorded in

Table 5.7 Ranked scores for diapedesis of granulocytes, numbers of Langerhan's cells, hyperplasia, haemorrhage, intra-epidermal pustules, oedema and necrosis in the epidermis at the site of *Dermatophilus congolensis* infections on rabbits; after single, multiple or no infestations with *Amblyomma variegatum* larvae.

	Eosinophils[a]	Neutrophils[a]	Mast cells[a]	Basophils[a]	Langerhan's cells[a]	Hyperplasia[b]	Haemorrhage[a]	Intra-epidermal pustules[c]	Oedema[c]	Necrosis[c]
Day 6/7 of 1st *D.congolensis* infection										
Hypersensitive reactions to ticks	+	+++	+	+	++	++	+	-	++++	-
Inflammatory reactions to ticks	+	+	-	+	+	++	+	-	+++	+
No exposure to ticks	+	+++	-	+	++	++	+	-	+++	+
Day 13 of 1st *D.congolensis* infection										
Hypersensitive reactions to ticks	-	+	-	-	++	++	-	-	+++	-
Inflammatory reactions to ticks	-	+	-	+	++	++	+	+	+++	-
No exposure to ticks	-	-	-	+	+++	++	+	-	++++	-

[a] = ranked score given to the number of cells/section (1.1mm length of epidermis), x100 light microscope objective; [b] = ranked score for average number of epidermal layers, x100 light microscope objective; [c] ranked score for number of sections affected, x100 light microscope objective.

the lesions in skin with inflammatory reactions to ticks and on the rabbits with no exposure to ticks.

Low to moderate numbers of Langerhan's cells were recorded in all of the lesions, with the greatest number found at day 13 in lesions on rabbits with no exposure to the larval *A.variegatum*. Moderate levels of hyperplasia were recorded in all of the dermatophilosis lesions. These scores for the degree of hyperplasia were probably underestimates due to loss of the outer layers of the dermatophilosis scabs during the processing of the biopsy material.

Of the other pathological changes in the epidermis, oedema was by far the most severe in all of the lesions. Small numbers of erythrocytes were recorded in the epidermis in all of the lesions one week into the infection. By day 13 small numbers of erythrocytes were only recorded in the lesions at the site of inflammatory reactions to the larval *A.variegatum* and on the rabbits not exposed to ticks. Intra-epidermal pustules were only recorded on day 13 of the *D.congolensis* infections in the dermatophilosis lesions at the inflammatory reactions to the ticks. Small areas of necrotic tissue were recorded only one week into the infection, at the site of inflammatory reactions to the ticks and in lesions on rabbits with no exposure to ticks.

Dermatophilus congolensis was not observed in the deeper layers of the epidermis in any of the biopsies. Hyphae were observed in the upper layers of the epidermis and scabs. The majority of the hyphae were observed around hair follicles, with some penetration into the hair follicle sheaths (Plate 5.11).

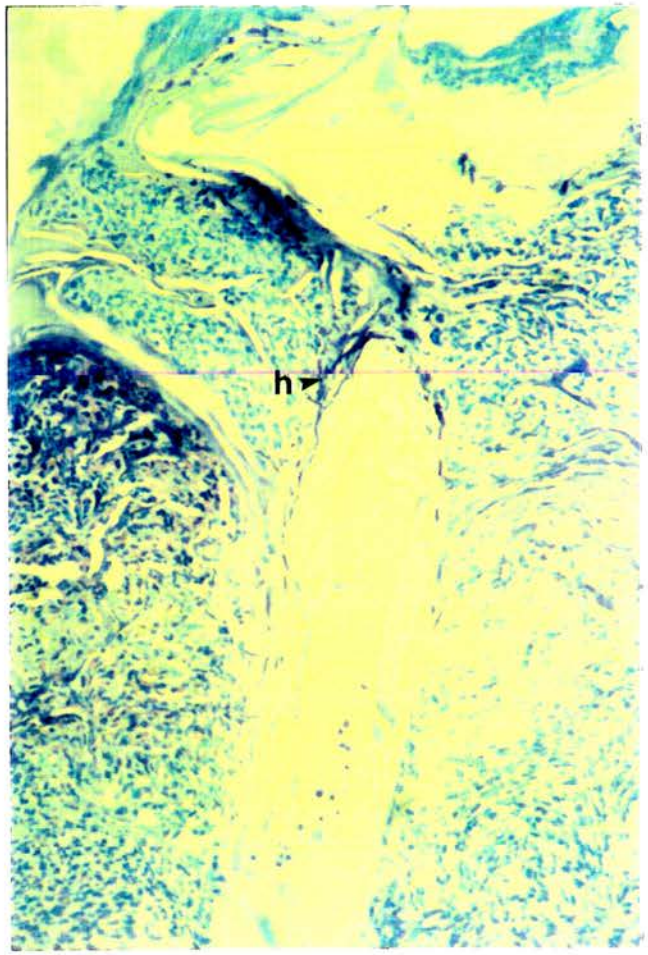


Plate 5.11 Day six of a primary *Dermatophilus congolensis* infection on a rabbit, stained with 5% Giemsa and alkali buffer (pH 7.2). Numerous hyphae are visible around the hair follicle and penetrating deeper into the follicle sheath. h = *D.congolensis* hyphae.

5.3.3 *Dermatophilus congolensis* infections on sheep

5.3.3.1 Comparison of local and remote *Dermatophilus congolensis* infections after single and multiple tick infestations

Due to the small numbers of experimental sheep, it was not possible to use the same statistical analysis for the assessment of the dermatophilosis lesions as for the rabbits. Table 5.8 shows the total ranked clinical scores given to each of the four sheep on the individual assessment days throughout the two *D.congolensis* infections. Differences between the infections on the four sheep were tested using Friedman's test on the ranked clinical scores for each assessment day for both the primary and secondary infections.

There was a significant difference between the infections on the four sheep ($P < 0.05$, $k = 4$, $n = 7$). However, further analysis, using multiple comparisons in the Friedman's test (see Section 3.4.3), revealed that one of the control sheep (No. 2) had a significantly lower infection than one of the test sheep (No. 1). However, there was no significant difference between the infections on the two test sheep (Nos. 1 and 3) and the other control sheep (No. 4). Plate 5.12 gives a comparison of dermatophilosis lesions at the same site as, and remote from inflammatory reactions to, previous tick attachment. It can be seen that there is no visible difference between the dermatophilosis lesions at the site of previous tick attachment compared with lesions remote from the previous tick attachment.

All four of the sheep were infested with another four infestations of *A.variegatum* nymphs and then infected with a secondary *D.congolensis* infection. Friedman's test was applied in the same way as above and again there was found to be a significant difference between the infections on the four sheep ($P < 0.05$, $k = 4$, $n = 7$).

Table 5.8 Total ranked clinical scores given to each of the sheep on individual assessment days during the primary and secondary *Dermatophilus congolensis* infections.

	RANKED CLINICAL SCORES					
	Infections at same sites as previous tick attachment sites			Infections at sites remote from previous tick attachment sites		
Day of infection	Sheep 1	Sheep 3	Median	Sheep 2	Sheep 4	Median
1st *D.congolensis* infection and inflammatory reactions to *A.variegatum*						
3	21	21	21	8	15	11.5
6	23	22	22.5	12	18	15
9	29	25	27	15	24	19.5
13	31	23	27	17	29	23
16	26	18	22	10	26	18
20	5	0	2.5	5	14	9.5
27	0	0	0	0	0	0
2nd *D.congolensis* infection and hypersensitive reactions to *A.variegatum*						
3	13	13	13	12	13	12.5
6	14	21	17.5	15	16	15.5
9	15	23	19	14	19	16.5
13	14	23	18.5	16	16	16
16	12	21	16.5	14	0	7
20	8	13	10.5	0	0	0
27	6	8	7	0	0	0

a

b

Plate 5.12 Day 13 of primary *Dermatophilus congolensis* infections on sheep with inflammatory reactions to nymphal *Amblyomma variegatum*. Close up of the infection area in the middle of the titration range, with a concentration of 1.25 x 10^4 cocci/cm^2. a. Infection at same site as previous tick attachment; b Infection at site remote from previous tick attachment.

The *D.congolensis* infection on the control sheep (No. 2) was again significantly less severe than on one of the test sheep (No. 3). The significant difference between the infections on the individual sheep was due to individual variation rather than differences between the infections being actually at the site of previous tick feeding compared with infections at sites remote from previous tick feeding.

5.3.3.2 The effect of inflammatory and hypersensitive reactions in sheep's skin on subsequent *Dermatophilus congolensis* infections

As there was no significant difference between the *D.congolensis* infections at sites local or remote from the previous tick attachment sites the median scores for all four sheep were used to compare the primary and secondary infections.

If the hypersensitive reactions in the host's skin, in response to repeated nymphal infestations, were having a significant effect, the secondary infections would be significantly more severe than the primary infections. However, using Mann-Whitney on the median scores for the primary and secondary infections, no significant difference was found between the infections on sheep with hypersensitive or inflammatory reactions in the skin in response to nymphal *A.variegatum* ($P > 0.05$, $n_1 = 7$, $n_2 = 7$), (Figure 5.4). The ranked clinical scores for the initial dermatophilosis lesions were greater on sheep with inflammatory reactions to larval *A.variegatum* than on the sheep with hypersensitive reactions to ticks. Despite the initial severity of the lesions on the sheep with inflammatory reactions at the previous tick attachment sites, these lesions were completely healed by day 27, whereas, slight lesions still persisted on the sheep with hypersensitive reactions at previous tick attachment sites.

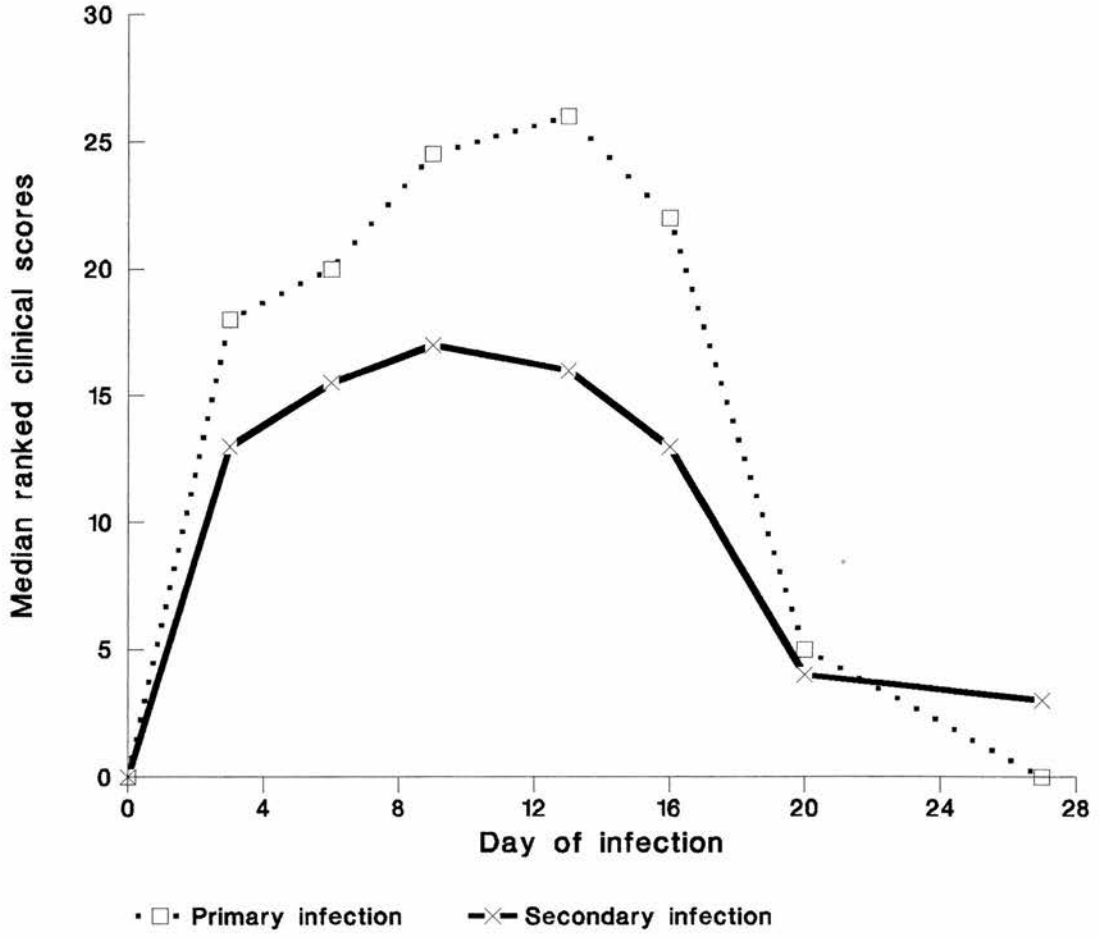


Figure 5.4. Median ranked clinical scores for primary and secondary *Dermatophilus congolensis* infections on sheep. Comparison of the inflammatory or hypersensitive reactions in the host's skin, in response to previous infestations of nymphal *Amblyomma variegatum*, on the progression of subsequent primary or secondary *D.congolensis* infections, respectively.

5.3.3.3 Duration of *Dermatophilus congolensis* infections on sheep

The infections persisted for longer on the sheep than on the rabbits; however, by day 16 all of the infections began to resolve. By day 27, apart from very slight lesions on the two test sheep at the second infection, no lesions were visible on any of the sheep (Figure 5.4). The lesions on the test sheep at this time may have been the remains of the reaction to tick attachment. In any case, the lesions at these sites were healing rapidly with the larger part of the dermatophilosis having detached leaving healthy, undamaged skin.

5.3.3.4 Histology of *Dermatophilus congolensis* infections on sheep

The actual cell counts for the dermis at the site of dermatophilosis lesions on sheep are in Appendices 5.6 and 5.7. Table 5.9 Shows mean cell counts and pathological scores for histological assessment of the dermis. Due to the small sample numbers it was not possible to use statistical analysis for comparison of the cell counts and pathological scores for the dermatophilosis lesions on the sheep.

The infiltration of granulocytes was predominantly neutrophilic, under all of the conditions. In the primary lesions, on sheep with inflammatory reactions to nymphal *A.variegatum*, the highest counts were 38 and 33 for lesions at the same site or remote from inflammatory reactions at previous tick attachment sites, respectively. These high counts were recorded in the first week of infection and in both cases the counts dropped to 1 by day 27. In the dermatophilosis lesions at the same site as hypersensitive reactions at previous tick attachment sites there was a higher count of 49, but the number dropped back down to 3 by day 27. In the secondary dermatophilosis lesions, remote from the hypersensitive reactions at the previous tick attachment sites, there was a very large infiltration of neutrophils in the initial lesions,

Table 5.9 Mean cell counts and pathological scores for the dermis at the site of *Dermatophilus congolensis* infections on sheep; after single, multiple or no infestations with *Amblyomma variegatum* nymphs.

	Mean cell counts and pathological scores (S.D.)															
	Eosinophils[a]		Neutrophils[a]		Mast cells[a]		Basophils[a]		Mononuclear cells[a]		Capillaries[a]		Haemorrhage[b]		Necrosis[c]		Oedema[c]	
1st *D.congolensis* infection at same site as inflammatory reactions to *A.variegatum* nymphs																		
Day 3	26	(31)	33	(40)	2	(1)	4	(3)	438	(100)	3	(1)	1	(1)	26	(30)	181	(70)
Day 6	12	(1)	38	(40)	3	(3)	4	(2)	504	(125)	7	(2)	3	(2)	1	(3)	259	(83)
Day 13	4	(4)	2	(2)	4	(2)	3	(2)	385	(110)	7	(3)	2	(3)	0	(0)	25	(15)
Day 27	2	(1)	1	(1)	4	(1)	2	(2)	235	(88)	5	(2)	2	(2)	37	(43)	119	(73)
1st *D.congolensis* infection at site remote from inflammatory reactions to *A.variegatum* nymphs																		
Day 3	6	(6)	33	(27)	2	(2)	3	(1)	179	(53)	6	(4)	67	(64)	0	(0)	90	(84)
Day 6	22	(2)	28	(28)	1	(1)	3	(2)	263	(28)	6	(3)	10	(6)	0	(0)	177	(183)
Day 13	3	(2)	0	(0)	1	(0)	2	(2)	192	(16)	5	(1)	0	(0)	0	(0)	72	(6)
Day 27	22	(25)	1	(1)	4	(2)	3	(2)	172	(62)	5	(2)	1	(2)	0	(0)	159	(148)
2nd *D.congolensis* infection at same site as inflammatory reactions to *A.variegatum* nymphs																		
Day 3	11	(6)	25	(28)	2	(1)	4	(1)	342	(96)	4	(3)	69	(70)	318	(88)	439	(172)
Day 6	3	(2)	49	(1)	3	(0)	6	(0)	717	(37)	5	(1)	17	(11)	0	(0)	323	(47)
Day 13	12	(8)	2	(2)	7	(1)	7	(2)	391	(126)	10	(1)	6	(7)	48	(57)	169	(152)
Day 27	3	(1)	3	(3)	4	(1)	2	(2)	154	(15)	9	(4)	17	(22)	74	(85)	216	(106)
2nd *D.congolensis* infection at site remote from inflammatory reactions to *A.variegatum* nymphs																		
Day 3	4	(2)	169	(37)	2	(2)	2	(2)	315	(74)	6	(5)	17	(12)	197	(102)	47	(13)
Day 6	19	(6)	72	(72)	1	(1)	3	(2)	299	(95)	6	(5)	27	(26)	0	(0)	28	(26)
Day 13	18	(9)	0	(1)	3	(2)	6	(2)	259	(125)	6	(3)	2	(4)	0	(0)	68	(38)
Day 27	9	(6)	2	(2)	1	(1)	5	(4)	280	(37)	11	(7)	10	(15)	155	(201)	445	(110)

[a] = mean cell counts/section (0.13mm^2 each), x100 light microscope objective; [b] = mean number of graticule squares affected (126.6µm^2 each), x100 light microscope objective; [c] = mean number of graticule squares affected (544.3µm^2 each), x50 light microscope objective.

with a mean count of 169 by day 3 of the infection. Despite the very large initial infiltration, the numbers of neutrophils decreased to a mean count of 2 by day 27.

Moderate numbers of eosinophils were recorded during the course of the primary and secondary infections, both at the same site and remote to the previous tick attachment sites. The largest mean count of 26 was recorded at day 3 of the primary *D.congolensis* infections at the same site as the inflammatory reactions to the nymphal *A.variegatum*, and 22 for the primary infection remote from the tick attachment sites. There were slightly lower numbers of eosinophils recorded in the dermis at the secondary lesions. The numbers of eosinophils recorded was very variable and there are no obvious differences between the four groups.

Small numbers of mast cells and basophils were recorded in all of the lesions, with the highest mean count being 7 for both, on day 13 of the secondary lesions at the same site as the hypersensitive reactions to the nymphal *A.variegatum*.

Large numbers of mononuclear cells were recorded in the dermis at the site of all the dermatophilosis lesions. The highest numbers were recorded in the lesions at the same site as the previous tick attachment, with mean counts of 504 and 717 being recorded in lesions at the same site as inflammatory and hypersensitive reactions, respectively. By day 27, the numbers of mononuclear cells were decreasing in all of the lesions and were lower in the lesions at the site of the previous tick attachment compared with the lesions remote from the previous tick attachment.

Some haemorrhage was recorded in the dermis at dermatophilosis lesions under all four conditions. The highest count was 64 for the primary lesions remote from the inflammatory reactions, and 69 for the secondary infections at the same site as the hypersensitive reactions to the ticks. There was much individual variation between the sections, as can be seen by the large standard deviations. Overall there

appeared to be more haemorrhage in the dermis at the site of the secondary infections compared with the primary infections. There appears to be no increase in haemorrhage when the lesions are at the same site as the previous tick attachment.

The degree of oedema and necrosis is greater at the secondary infection compared with the primary infection. The highest mean pathological score for necrosis during the primary infection was 37 compared with 318 at the secondary infection. Both of these highest mean scores occurred in the lesions at the same site as the previous tick attachment sites. Similarly the amount of oedema increased in the secondary infection with the highest mean score for the primary infection occurring in the lesions at the same site as the previous tick attachment sites. For the secondary infection, the highest mean score for oedema was recorded in the dermatophilosis lesions remote from the previous tick attachment site, but the mean scores were consistently high for the dermatophilosis lesions at the same site as the previous tick attachment sites.

Table 5.10 shows the ranked scores for the histological assessment of the epidermis during *D.congolensis* infections on sheep. The diapedesis of the granulocytes into the epidermis consisted mainly of neutrophils with the occasional eosinophil, mast cell and basophil. Large numbers of neutrophils were recorded in the epidermis at the site of primary and secondary lesions, both at the same site as and remote from previous tick attachment sites. These large numbers of neutrophils were recorded by day three of the infection in the lesions remote from the tick attachment sites, but large numbers of neutrophils were not recorded until day six in the lesions at the same site as both the inflammatory and hypersensitive reactions to the ticks.

Basophils were found only in the epidermis at dermatophilosis lesions at the site of previous tick attachment. More basophils were recorded in the lesions at the site of hypersensitive reactions to the nymphal *A.variegatum*.

Table 5.10 Ranked scores for diapedesis of granulocytes, numbers of Langerhan's cells, hyperplasia, haemorrhage, intra-epidermal pustules, oedema and necrosis in the epidermis at the site of *Dermatophilus congolensis* infections on sheep; after single or multiple infestations of *Amblyomma variegatum* nymphs at sites local to or remote from the previous tick infestations.

	Eosinophils[a]	Neutrophils[a]	Mast cells[a]	Basophils[a]	Langerhan's cells[a]	Hyperplasia[b]	Haemorrhage[a]	Intra-epidermal pustules[c]	Oedema[c]	Necrosis[c]
1st *D.congolensis* infection at same site as inflammatory reaction to *A.variegatum* nymphs										
Day 3	-	-	-	-	++	++	-	-	++	-
Day 6	+	+++	+	+	++	+++	-	-	+	+
Day 13	-	-	-	-	+	++	+	-	-	-
Day 27	-	-	-	-	+	+	-	-	-	-
1st *D.congolensis* infection at site remote from inflammatory reaction to *A.variegatum* nymphs										
Day 3	+	+++	-	-	+	++	-	+	++	+
Day 6	-	+	-	-	++	++	-	+	++	+
Day 13	-	-	-	-	+	++	-	-	++	-
Day 27	-	+	+	-	+	+	-	-	+	-
2nd *D.congolensis* infection at same site as hypersensitive reaction to *A.variegatum* nymphs										
Day 3	-	-	-	+	++	++++	-	+	++	++
Day 6	+	+++	-	-	+	++++	-	++	++	++
Day 13	-	-	-	+	++	++	+	-	++	-
Day 27	-	-	-	+	+	++	-	-	++	-
2nd *D.congolensis* infection at site remote from hypersensitive reaction to *A.variegatum* nymphs										
Day 3	-	+++	-	-	+	++	+	++	++	++
Day 6	+	-	-	-	+	++	+	-	+	-
Day 13	-	-	-	-	+	++	-	-	+	-
Day 27	-	-	+	-	+	++	-	-	++	-

[a] = ranked score given to the number of cells/section (1.1mm length of epidermis), x100 light microscope objective; [b] = ranked score for average number of epidermal layers, x100 light microscope objective; [c] ranked score for number of sections affected, x100 light microscope objective.

The numbers of Langerhan's cells were fairly consistent in all of the dermatophilosis lesions. Hyperplasia occurs in all of the lesions but to a greater extent in the lesions at the same site as the previous tick attachment. The hyperplasia was greatest in the lesions at the hypersensitive reactions at the previous tick attachment sites. The reduction in hyperplasia appeared to be slightly delayed in the secondary infections, with moderate scores still being recorded by day 27.

The number of erythrocytes recorded in the epidermis was very low, and none were recorded at all in the primary lesions remote from the previous tick attachment sites. The pathological scores for intra-epidermal pustules, oedema and necrosis were all marginally higher in the dermatophilosis lesions at the same site as the hypersensitive reactions at the previous tick attachment sites. These differences were not large and only occurred during the first week of the infection, with no necrosis or intra-epidermal pustules being recorded in any of the lesions after day six. The oedema persisted until the end of the assessment in three of the groups with no oedema being recorded after day 13 in the primary lesions at the same site as the inflammatory tick attachment sites.

5.4 DISCUSSION

Work by Davis and Philpott (1980) has indicated that delayed type hypersensitive reactions in goat's skin, at the site of infection with *D.congolensis* can produce dermatophilosis lesions similar to natural, chronic infections. They used the hapten, dinitrochlorobenzene to produce hypersensitive reactions in the skin, intended as an artificial simulation of the hypersensitive reaction at an arthropod feeding site. The experiments reported here were a study of the effect of inflammatory and

hypersensitive reactions to the feeding of immature *A.variegatum* on subsequent *D.congolensis* infections on rabbits and sheep.

Repeated infestations of *A.variegatum* larvae and nymphs on rabbits and sheep respectively produced increasing macroscopic reactions in the host's skin. Macroscopic signs of inflammatory reactions to the first infestations became more severe after repeated infestations, suggesting the development of hypersensitive reactions. This was confirmed by histological assessment of the tick attachment sites.

It was expected that the hypersensitive reactions of the hosts in response to the tick feeding would have adversely affected the tick feeding success, due to the ticks being literally pushed off the hosts by the formation of pustules, or drowning in exudate (Walker and Fletcher, 1986). Increased host resistance of rabbits, to the feeding of *A.variegatum* and *Rhipicephalus appendiculatus*, is associated with the development of hypersensitive reactions in the skin of the host (Walker and Fletcher, 1986; Latif *et al.*, 1990).

Analysis of *D.congolensis* infections on rabbits, during this investigation, revealed that individual variation resulted in significant differences in the severity of the dermatophilosis lesions, whereas no significant difference was recorded between dermatophilosis lesions at the sites of previous tick feeding, compared with lesions on rabbits with no previous exposure to ticks.

Oduye (1976a) reports a wide range of host susceptibility in a herd of cattle which were all exposed to the same risk of infection. The cattle that he observed were out in the open and were therefore exposed to numerous variables. It had been hoped that individual variation in the *D.congolensis* infections on rabbits and sheep would have been reduced when carrying out the experiments in controlled laboratory conditions. However, there were significant differences between the

severity of the dermatophilosis lesions on the individual rabbits and sheep. This indicates that the variation in individual host susceptibility to dermatophilosis is caused by some intrinsic factor.

Local reactions in the skin of rabbits and sheep, in response to the feeding of the immature stages of *A.variegatum* did not facilitate the progression of the disease to form chronic lesions; neither did differences in the type of host reaction to the ticks have any significant effect on the severity or duration of the dermatophilosis lesions formed.

Histological assessment of the dermis, at the site of dermatophilosis lesions recorded large numbers of neutrophils infiltrating the dermis at day six of the *D.congolensis* infections on all of the lesions on both rabbits and sheep. By day 13 the numbers had decreased markedly. No significant difference was recorded between the numbers of neutrophils infiltrating into the lesions on the three groups of rabbits.

Assessment of the lesions on rabbits recorded significantly more eosinophils, mast cells and mononuclear cells in the dermis in six day old dermatophilosis lesions at hypersensitive reactions to larval tick feeding compared with lesions on rabbits not exposed to ticks. All of these cells are involved in hypersensitive reactions (Turk, 1967; Roitt *et al.*, 1985), therefore these significant differences may be due to the remains of the hypersensitive reactions in response to tick feeding.

By day 13 of the *D.congolensis* infection on the rabbits there were no significant differences in the numbers of eosinophils, mast cells or mononuclear cells in any of the groups. However, there were significantly more basophils in the dermis, at day 13, in the lesions at the site of hypersensitive reactions to tick feeding compared with lesions at inflammatory reactions to ticks. There was also significantly

more oedema in the dermatophilosis lesions on the rabbits not exposed to tick feeding compared with both of the groups previously exposed to ticks. These significant differences recorded in the histology of the dermatophilosis lesions on the three groups of rabbits did not appear to have any effect on the severity or duration of the *D.congolensis* infections on the rabbits.

Large numbers of mononuclear cells and extensive oedema were also recorded in all of the dermatophilosis lesions. More mononuclear cells and oedema were recorded in the lesions at the same site as the previous tick attachment.

These differences in the histology of the dermis did not appear to have any effect on the severity or duration of the dermatophilosis lesions, on either of the host species. Slight increases in the ranked scores for the dermatophilosis lesions at the same site as the previous tick attachment on sheep were not statistically significant and the reactions to the ticks at the same sites could have been responsible for over-estimation of the severity of the lesions. In any case the extended time period for the infections to resolve, as recorded by Davis and Philpott, (1980), was not observed, and differences of a few days are not important when chronic experimental lesions on sheep can persist for months (Chapter Seven; Walker and Lloyd, 1993).

The conclusion from these results is that neither inflammatory nor delayed type hypersensitive reactions to the feeding of the immature stages of this tick affect the progression of dermatophilosis on rabbits or sheep. These results indicate that the local effects of the feeding of larval and nymphal *A.variegatum* are unlikely to facilitate the development of chronic *D.congolensis* once the ticks have been removed.

Davis (1984) found that *D.congolensis* zoospores persisted for longer periods at sites of artificially produced, delayed hypersensitivity reactions on guinea-

pigs than on unsensitized skin. He postulated that the immune reactions, at an arthropod feeding site interfered with the elimination of *D.congolensis*, allowing the development of chronic lesions. However, he found that although the zoospores persisted for longer, the presence of hypersensitive reactions alone in the host's skin was not enough to produce chronic lesions.

The experiments reported here have investigated the effect of inflammatory and hypersensitive reactions in response to tick feeding on dermatophilosis lesions after the removal of the ticks. The work by Davis and Philpott (1980) demonstrated that *D.congolensis* infections were prolonged as long as there was an active hypersensitive reaction in the skin. They achieved this by repeated applications of the hapten, dinitrochlorobenzene. This repeated stimulation of the hypersensitive reaction during the course of the infection is similar to the situation in the field where the infected animals would be exposed to repeated tick and insect attacks.

Further experiments are required to investigate the effect of inflammatory or hypersensitive reactions in the host's skin in response to the feeding of immature *A.variegatum* on simultaneous *D.congolensis* infections. It is important to know if local reactions to the feeding of immature *A.variegatum* aggravate dermatophilosis lesions. Adult *A.variegatum* have been associated with chronic dermatophilosis in the field (Morrow *et al.*, 1989) and dipping to control this tick has been used to successfully control dermatophilosis (Koney and Morrow, 1990). Effective control of the adult ticks can be achieved by dipping during the periods of the year when the population of adult *A.variegatum* is at its largest.

Wilson (1946) found that adult *A.variegatum* were only found during the wet season, whereas the majority of immature *A.variegatum* were found in the dry season; similar fluctuations in the relative abundance of the different instars have been

reported in the Caribbean (Garris and Scotland, 1985). If immature *A.variegatum* are found to aggravate dermatophilosis control measures would need to be extended over the times of peak numbers of immature ticks.

5.5 SUMMARY

1. Repeated infestations of larval and nymphal *A.variegatum* on rabbits and sheep, respectively, resulted in increasing signs of hypersensitivity in the host's skin. Inflammatory and hypersensitive reactions to the first and last infestations were confirmed by histological assessment of tick attachment sites on both host species.

2. Increasing hypersensitive reactions were accompanied by increased resistance to the feeding of nymphal *A.variegatum* on sheep. Repeated infestations of larval *A.variegatum* on rabbits did not result in increased host resistance to the tick feeding.

3. The severity and duration of dermatophilosis lesions at the site of inflammatory or hypersensitive reactions to immature *A.variegatum* was compared with dermatophilosis lesions at sites with no previous exposure to ticks. Histological assessment of the dermatophilosis lesions revealed some significant differences between the infections. These differences were considered to be associated with the host reactions to the ticks rather than differences in the progression of the dermatophilosis. These differences were recorded during the initial stages of the infections and became less as the infections progressed.

4. Macroscopic dermatophilosis lesions on rabbits with inflammatory or hypersensitive reactions to larval *A.variegatum* were compared with lesions on rabbits with no previous exposure to ticks. There was no significant difference between the severity or duration of the dermatophilosis lesions on the three groups.

5. Macroscopic dermatophilosis lesions on sheep with inflammatory reactions to nymphal *A.variegatum* were compared with lesions on sheep with hypersensitive reactions to ticks. There was no significant difference between the lesions on the two groups of sheep.

6. Individual variation, in both rabbits and sheep, resulted in significant differences in the severity of dermatophilosis lesions.

7. It is concluded that inflammatory or hypersensitive reactions, persisting in the skin of rabbits and sheep, after the detachment of immature *A.variegatum* do not have any significant effect on the progression of subsequent *D.congolensis* infections. Further experiments are required to investigate the local effects of inflammatory and hypersensitive reactions to immature *A.variegatum* on simultaneous *D.congolensis* infections.

CHAPTER SIX
THE LOCAL EFFECT OF HYPERSENSITIVE OR INFLAMMATORY REACTIONS TO NYMPHAL *AMBLYOMMA VARIEGATUM* ON SIMULTANEOUS *DERMATOPHILUS CONGOLENSIS* INFECTIONS

6.1 INTRODUCTION

Previous experiments in this laboratory have demonstrated that the feeding of adult *Amblyomma variegatum* has a significant systemic effect on the progression of simultaneous *Dermatophilus congolensis* infections (Walker and Lloyd, 1993). This systemic effect appears to be confined to the adults only (Chapter Seven). However, immature stages of this tick may have a local effect on dermatophilosis infections.

There are many observations of dermatophilosis lesions being widespread over the dorsal areas of infected animals, at sites remote from the predilection areas of adult *A.variegatum* (Zlotnik, 1955; Macadam, 1964b; Macadam, 1964c; Morrow *et al.*, 1989). These dorsal lesions may be aggravated by the systemic effect of adult *A.variegatum*; however, immature ticks and other biting insects feed in large numbers on the dorsal surfaces of the animals and may influence the severity or distribution of the dermatophilosis lesions (Plowright, 1956; Macadam, 1964b; Macadam, 1964c; Stewart, 1972).

Work by Davis and Philpott (1980) has shown that delayed (type IV) hypersensitive reactions in the skin significantly affect the progression of local, simultaneous *D.congolensis* infections. The work in Chapter Five of this thesis has shown that repeated infestations of larval and nymphal *A.variegatum* results in the formation of delayed (type IV) hypersensitive reactions in the skin of rabbits and sheep respectively. However, these immune reactions, which persisted after the ticks had detached, did not facilitate the progression of dermatophilosis lesions produced by application of infective cocci at the same site.

The work by Davis and Philpott (1980) demonstrated that *D.congolensis* infections were prolonged as long as there was an active hypersensitive reaction in the

skin. They achieved this by repeated applications of the hapten, dinitrochlorobenzene during the course of the *D.congolensis* infections. This repeated stimulation of the hypersensitive reaction is a better model of the situation in the field, where infected animals would be exposed to repeated tick and insect attacks.

Any investigation into the local effect of the feeding of immature *A.variegatum* on *D.congolensis* would be incomplete without studying the effect on simultaneous infections. Therefore the experiments reported here have involved an investigation into the local and systemic effect of hypersensitive and inflammatory reactions in the skin of rabbits in response to the feeding of nymphal *A.variegatum* on simultaneous *D.congolensis* infections.

6.2 METHODS

6.2.1 EXPERIMENTAL HOSTS

Two groups of four female New Zealand White rabbits 3-5 kg each were used. Four of the rabbits were infested with three immunizing infestations of *A.variegatum* nymphs applied to alternate ears, in order to produce hypersensitive reactions in the host's skin. After the third infestation all eight rabbits were infected, at two areas ($25cm^2$) on the back, with identical doses of *D.congolensis* cocci. Infestations of 20 nymphs were applied to one infection area on each of the eight rabbits, as soon as the suspension of cocci had dried. The severity of the resulting lesions and position in relation to the tick feeding sites was recorded over a three week period.

Figure 6.1 gives a summary of the sequence of infestations of nymphal *A.variegatum* and *D.congolensis* infections on the two groups of rabbits.

Figure 6.1 The sequence of nymphal *Amblyomma variegatum* infestations and *Dermatophilus congolensis* infections on rabbits used to test the effect of hypersensitive and inflammatory reactions to ticks on simultaneous infections.

Day of experiment	Hypersensitive reactions to nymphal *A.variegatum* (N = 4 rabbits)	Inflammatory reactions to nymphal *A.variegatum* (N = 4 rabbits)
0	Immunizing infestation of 20 nymphs applied to left ear	____
28	Immunizing infestation of 20 nymphs applied to right ear	____
56	Immunizing infestation of 20 nymphs applied to left ear	____
78	Two areas (5 x 5cm) infected with *D.congolensis*, on each rabbit	
78	Challenge infestation of 20 nymphs applied to one of the infection sites on each rabbit, resulting in delayed (type IV) hypersensitive reactions in the host's skin	Challenge infestation of 20 nymphs applied to one of the infection sites on each rabbit, resulting in inflammatory reactions in the host's skin

6.2.2 TICK INFESTATIONS

In previous experiments, using *A.variegatum* larvae on rabbits, hypersensitive reactions to the ticks were shown to develop at the tick attachment sites after repeated infestations (Chapter Five). In the previous chapter, three sensitizing infestations of five hundred larvae were applied followed by the challenge infestation, also consisting of five hundred larvae.

Using the ratio of whole salivary glands calculated using the size and number of type-2 and type-3 salivary gland acini, 500 larvae were equivalent to 20 nymphs (Section 3.3.3.2). Therefore, to produce an equivalent reaction in the skin of these rabbits they were infested with three sensitizing infestations of 20 nymphs.

The feeding success of the nymphs, for each infestation, was recorded to provide an indication of the degree of resistance and hypersensitivity developing in the rabbits. The engorgement success, average weight and moulting success were recorded and the resistance calculated using the method of Walker *et al.*, (1990) (Section 3.3.5).

Changes in the reactions in the skin of rabbits and sheep in response to repeated infestations of *A.variegatum* larvae and nymphs respectively, has already been adequately demonstrated by previous experiments in this laboratory (Chapter Five). Latif *et al.*, (1991a) found that increased resistance in cattle to the feeding of *A.variegatum* and *Rhipicephalus appendiculatus* was accompanied by the development of hypersensitive reactions in the skin. Therefore, it was not considered necessary to take biopsies of the tick attachment sites to demonstrate the development of delayed (type IV) hypersensitive reactions.

Challenge infestations of *A.variegatum* nymphs were applied to one of the two areas infected with *D.congolensis* on each rabbit. These infestations were

contained within cloth bags glued to the rabbit's fur. The bag could be opened to assess the attachment of the ticks and then removed when the ticks had attached, allowing easier assessment of the dermatophilosis infections.

6.2.3 *DERMATOPHILUS CONGOLENSIS* INFECTIONS

All of the rabbits were infected with identical doses of *D.congolensis* at two sites (5 x 5cm), one on either side of the torso. After application of the cocci the areas were allowed to dry, and then the ticks were applied to the infection site on the right of the torso.

Due to problems with the large stock of stabilate which had been prepared and frozen at a concentration of 1.2×10^7 cocci/µl, the cocci for these infections were obtained from another stabilate frozen at a concentration of 3.5×10^7 cocci/µl. The same method of production had been used for both stabilates and the cocci were applied onto the experimental hosts as before (Section 4.2.2).

Both infection sites on all eight rabbits were infected with 1×10^7 cocci/cm^2 at a concentration of 4×10^5 cocci/µl. In previous experiments, cocci applied at this concentration produced discrete lesions over the application areas. Higher concentrations would have produced stronger reactions which would have disguised any correlation between the position of the ticks and the establishment of infection foci.

The progress of the dermatophilosis was monitored using the nonparametric ranking system already established (Section 4.2.1.5). Each of the two areas on each rabbit was assessed separately. The scores for the area, severity and amount of exposed dermis were applied to the whole area. However, due to the large size of the areas, two readings of the skin fold thickness were taken and the mean skin

fold thickness was calculated for each area. The infections were assessed every three or four days until day 20 when the infections were virtually resolved.

Hand drawn diagrams of the position of the tick attachment sites and the foci of the *D.congolensis* infections were produced at days six and nine. The diagrams were drawn at a scale of 1:1. Gridlines, at 1cm intervals divided each diagram into 25 equal areas and the numbers of ticks and infection foci were recorded for each area. The relationship between the position of the tick attachment sites and the dermatophilosis lesions was then tested using a 2 x 2 contingency table.

6.3 RESULTS

6.3.1 RESISTANCE TO TICKS

Using the methods of Walker *et al.*, 1990 (Section 3.3.5) three out of the four rabbits were shown to develop a marked resistance to repeated infestations of *A.variegatum* nymphs (Table 6.1), (for changes in the individual parameters of the feeding success see Appendix 6.1).

The development of host resistance to the repeated infestations of nymphal *A.variegatum* was manifested in fewer, smaller ticks engorging at the fourth infestation compared with infestations on naive rabbits (Plate 6.1).

6.3.2 DEVELOPMENT OF HYPERSENSITIVE AND INFLAMMATORY REACTIONS IN RESPONSE TO *AMBLYOMMA VARIEGATUM* NYMPHS

Histological studies of the attachment sites were not carried out in this experiment because the development of hypersensitive reactions in response to repeated infestations of *A.variegatum* had already been demonstrated in previous experiments (Chapter Five). However, the increasing severity of macroscopic signs of

Table 6.1 Changes in resistance to repeated infestations of *Amblyomma variegatum* nymphs on rabbits. Resistance was calculated by the method of Walker *et al.*, (1990); changes in resistance were compared with the initial resistance of individual hosts, in some cases there was a decrease in resistance.

	RESISTANCE (%) AT EACH INFESTATION		
HOST	2nd infestation	3rd infestation	4th infestation
Rabbit 1	33	45	56
Rabbit 2	54	76	60
Rabbit 3	42	67	65
Rabbit 4	N/A	42*	-17*

* For rabbit No. 4 the results from the 1st infestation were not available, therefore subsequent infestations were compared with the 2nd infestation.

Plate 6.1 The effect of host resistance on the size and number of successfully engorging *Amblyomma variegatum* nymphs feeding on a rabbit. The ticks on the left were fed on a sensitized rabbit (with three previous infestations of *A.variegatum* nymphs) the ticks on the right were fed on a naive rabbit (with no previous exposure to *A.variegatum* nymphs).

hypersensitivity in response to the consecutive infestations was observed and photographed (Plate 6.2).

6.3.3 THE LOCAL EFFECT OF THE FEEDING OF *AMBLYOMMA VARIEGATUM* NYMPHS ON *DERMATOPHILUS CONGOLENSIS* INFECTIONS

All of the control (no ticks) and test (with ticks) infection sites developed moderate dermatophilosis lesions which persisted for approximately three weeks (Figure 6.2).

The ranked clinical scores for the dermatophilosis lesions on the individual rabbits on each assessment day are recorded in Table 6.2. Using the Kruskal-Wallis test on the scores given to individual rabbits throughout the assessment there was found to be no significant difference between the infections on the control sites on the individual rabbits within either of the two groups ($P > 0.05$, $k = 4$ = number of rabbits compared, $n = 6$ = number of assessments for each rabbit). There was also no significant difference between the severity of the test lesions (at the same sites as the ticks) on the four rabbits with hypersensitive reactions to the nymphs ($P > 0.05$, $k = 4$ and $n = 6$). However, there was a significant difference between the test infections on the individual rabbits with inflammatory reactions to the ticks ($P < 0.05$, $k = 4$ and $n = 6$). Therefore, there was some individual variation but it was only significant for the test infection sites with inflammatory reactions to the nymphs.

The median ranked clinical scores for the four groups of dermatophilosis infection sites were compared using a Kruskal-Wallis test. The dermatophilosis infections were shown to fall into two significant overlapping subsets. The infections on the control areas (remote from the tick attachment sites) on both groups of rabbits were significantly milder ($P < 0.05$) than the four test infections, at the site of inflammatory reactions to the ticks. There was no significant difference between the

a

b

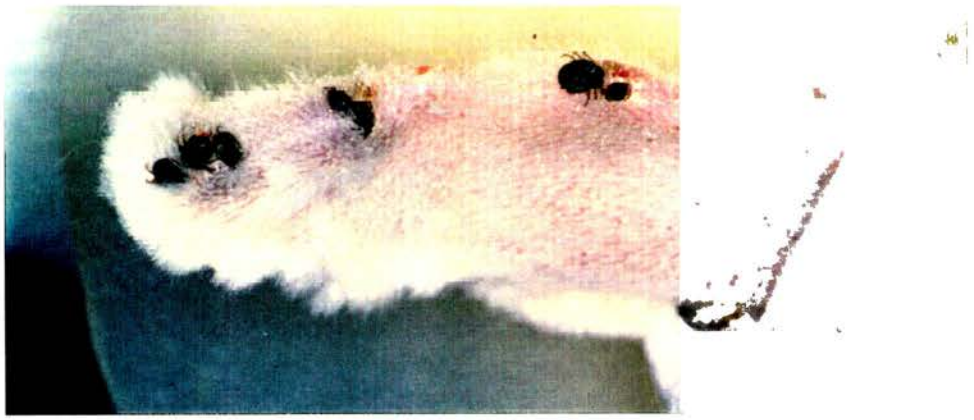


Plate 6.2 Day eight of infestations of nymphal *Amblyomma variegtum* feeding on the ears of rabbits. a, These nymphs are feeding on a rabbit which has had no previous exposure to tick feeding; b, A second infestation of *A. variegatum* nymphs on a rabbit, showing reduced engorgement of the ticks and increased pustulation around the tick feeding sites.

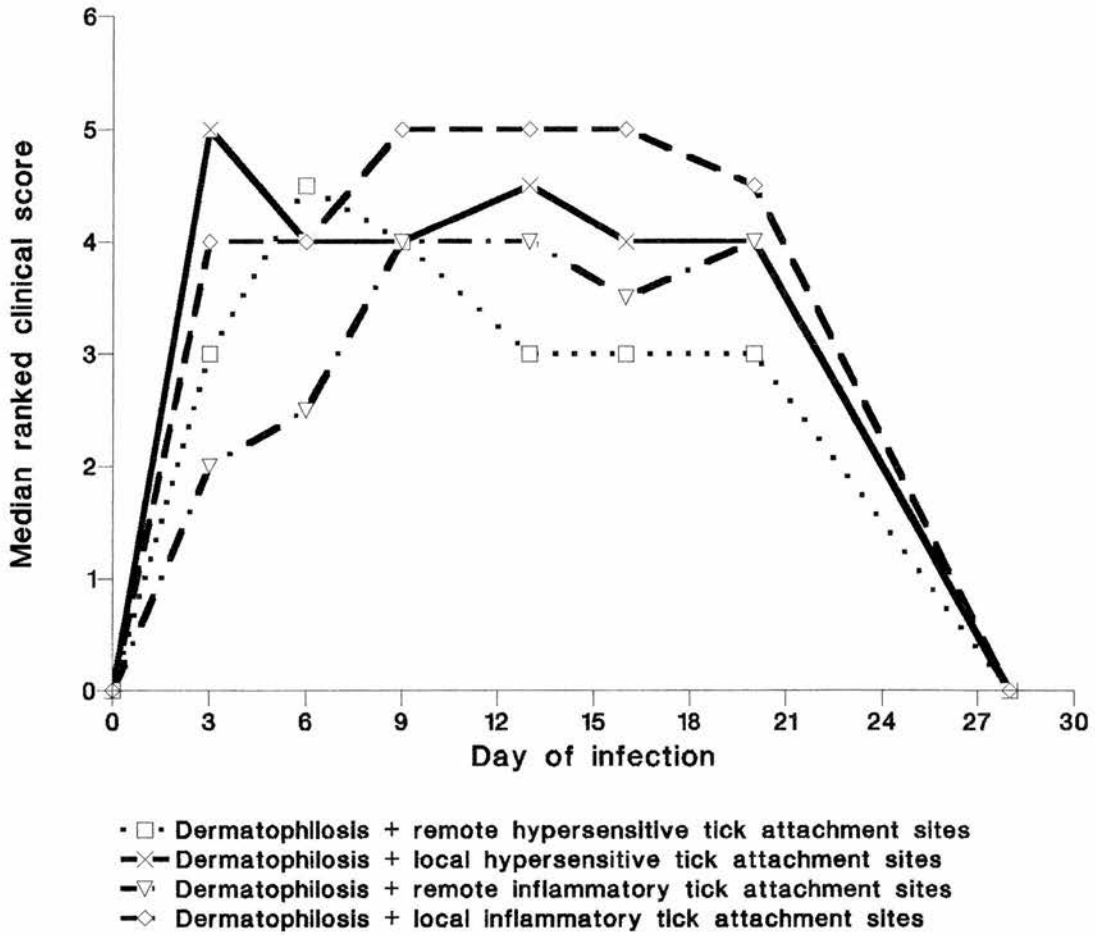


Figure 6.2 The effect of hypersensitive or inflammatory attachment sites of nymphal *Amblyomma variegatum* on the progression of local or remote *Dermatophilus congolensis* infections on rabbits.

Table 6.2 *Dermatophilus congolensis* infections on rabbits at sites local and remote from hypersensitive or inflammatory reactions to simultaneous infestations of nymphal *Amblyomma variegatum*.

	RANKED CLINICAL SCORES									
	Dermatophilosis remote from nymphal *A.variegatum* attachment sites					Dermatophilosis at the same site as nymphal *A.variegatum* attachment sites				
D.congolensis infections on rabbits with hypersensitive reactions to nymphs										
	Rabbit numbers					Rabbit numbers				
Day of infection	1	2	3	4	Median	1	2	3	4	Median
3	4	3	3	3	3	5	5	4	5	5
6	4	3	5	5	4.5	4	4	4	4	4
9	4	4	6	4	4	5	4	4	3	4
13	3	3	4	3	3	6	4	4	5	4.5
16	3	3	3	4	3	3	4	4	4	4
20	3	3	3	3	3	3	4	4	4	4
D.congolensis infections on rabbits with inflammatory reactions to nymphs										
	Rabbit numbers					Rabbit numbers				
Day of infection	5	6	7	8	Median	5	6	7	8	Median
3	2	3	2	2	2	4	4	4	4	4
6	2	5	3	2	2.5	4	5	4	3	4
9	3	5	4	4	4	5	6	5	3	5
13	3	4	4	4	4	5	5	6	4	5
16	3	3	4	5	3.5	5	5	5	4	5
20	4	4	0	5	4	5	5	3	4	4.5

four test areas, at hypersensitive tick attachment sites, and the four control areas remote from hypersensitive or inflammatory reactions to ticks ($P > 0.05$).

6.3.4 DIFFERENCES IN *DERMATOPHILUS CONGOLENSIS* INFECTIONS RECORDED AT EACH ASSESSMENT

The Kruskal-Wallis test was used to compare the four groups of infection sites, using the ranked clinical scores for the dermatophilosis lesions on the individual animals at each assessment. There were found to be significant differences between the four areas at days three and thirteen ($P < 0.01$ and $P < 0.05$ respectively, $k = 4$ and $n = 4$ Table 6.3).

6.3.5 THE EFFECT OF HYPERSENSITIVE AND INFLAMMATORY REACTIONS ON SKIN FOLD THICKNESS OF *DERMATOPHILUS CONGOLENSIS* INFECTIONS

At each assessment, skin fold measurements were recorded at both infection areas on all of the rabbits. The raw data is presented in Appendix 6.2. Skin fold measurements were taken from each infection site at day 0 of the infections; these measurements were used as controls. The following equation was used to calculate the percentage change in skin fold thickness for each assessment day, compared with the initial thickness.

$$\text{Percentage change} = \left[\left(\frac{\text{Skin fold thickness at assessment day}}{\text{Control skin fold thickness measured on day } 0}\right)\times 100\right] - 100$$

The mean percentage changes in skin fold thickness for the control and test infection areas on both groups of rabbits are shown in Appendix 6.3.

Figure 6.3 illustrates the mean percentage change in skin fold thickness, compared with the original skin fold thickness, throughout the course of the *D.congolensis* infections. On the infection sites at the same site as the tick feeding, the

Table 6.3 Difference in severity of dermatophilosis lesions on rabbits with hypersensitive or inflammatory reactions to nymphal *Amblyomma variegatum*.

Day of infection	Kruskal-Wallis H	k	n	P	Interpretation
3	12.94	4	4	P < 0.01	vs
6	2.84	4	4	P > 0.05	ns
9	1.71	4	4	P > 0.05	ns
13	9.22	4	4	P < 0.05	s
16	7.12	4	4	P > 0.05	ns
20	4.83	4	4	P < 0.05	ns

k = number of groups compared, 1. *Dermatophilus congolensis* infections remote from hypersensitive attachment sites of nymphal *A.variegatum*, 2. *D.congolensis* infections at the same site as hypersensitive attachment sites of nymphal *A.variegatum*, 3. *D.congolensis* infections remote from inflammatory attachment sites of nymphal *A.variegatum*, 4. *D.congolensis* infections at the same site as inflammatory attachment sites of *A.variegatum*.

n = number of individual areas within groups

vs = very significant; s = significant; ns = not significant

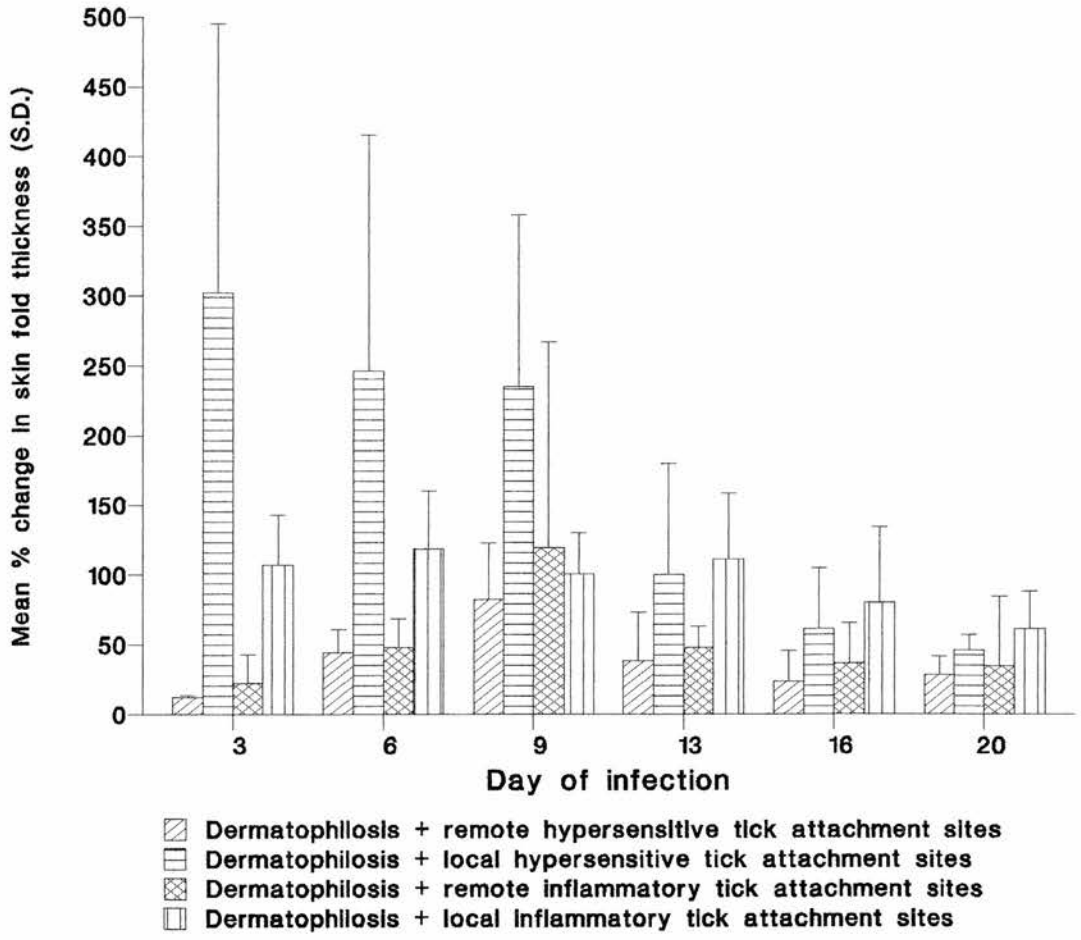


Figure 6.3 The effect of hypersensitive or inflammatory attachment sites of nymphal *Amblyomma variegatum* nymphs, on the skin fold thickness of local or remote *Dermatophilus congolensis* infections on rabbits.

largest skin fold thickness occurred at day three (302.3%) and day six (118.8%) at the hypersensitive and inflammatory attachment sites respectively. For both of the control areas remote from the tick attachment sites the largest skin fold thickness was not recorded until day nine of the infection. As well as the slower increase in the skin fold thickness, the maximum changes in skin fold measurements were 82.5% and 119.8% on the rabbits with hypersensitive and inflammatory reactions to ticks respectively.

The Kruskal-Wallis test and Mann-Whitney test were used to compare the changes in the skin fold thickness on the four groups of dermatophilosis lesions. In this way it was found that the percentage change in skin fold thickness at both groups of areas infested with ticks was significantly greater ($P = 0.01$) than the percentage change in skin fold thickness of the infection areas remote from the hypersensitive tick attachment sites. But there was no significant difference ($P > 0.05$) between the percentage change in skin fold thickness recorded on the areas remote from inflammatory tick attachment sites and the two groups at the same site as the tick attachment.

6.3.6 THE EFFECT OF HYPERSENSITIVE AND INFLAMMATORY TICK ATTACHMENT SITES ON THE DISTRIBUTION AND NUMBER OF *DERMATOPHILUS CONGOLENSIS* INFECTION FOCI

The relative positions of the tick attachment site and dermatophilosis infection foci are recorded in Table 6.4. Using the combined data from the four rabbits, in a 2x2 contingency table (Kershaw, 1973), revealed that there was no association between the dermatophilosis and hypersensitive tick attachment sites ($P > 0.05$). However, there was a very significant positive association between the inflammatory tick attachment sites and the dermatophilosis foci ($P < 0.01$).

Table 6.4 The association of dermatophilosis lesions and nymphal *Amblyomma variegatum* attachment sites on rabbits day 9 of a primary *Dermatophilus congolensis* infection. Each 25cm^2 area was divided into 1cm^2 plots and the presence or absence of ticks and dermatophilosis was recorded for each plot.

Host	Dermatophilosis only (c)	Ticks only (b)	Ticks and dermatophilosis (a)	Neither (d)
Dermatophilosis and hypersensitive tick attachment sites				
Rabbit 1	12	4	5	4
Rabbit 2	14	2	4	5
Rabbit 3	6	3	8	8
Rabbit 4	13	2	8	2
Total	45	11	25	19
Dermatophilosis and inflammatory tick attachment sites				
Rabbit 5	11	1	5	8
Rabbit 6	11	2	6	6
Rabbit 7	13	0	9	3
Rabbit 8	9	0	8	8
Total	44	3	28	25

6.4 DISCUSSION

Rabbits became resistant after repeated infestations of *A.variegatum* nymphs. This resistance was accompanied by macroscopic signs of hypersensitivity, such as pustulation and sloughing of skin. Biopsies of tick attachment sites after repeated infestations have previously confirmed the development of hypersensitive reactions in rabbits and sheep (Chapter Five). Other work with *R.appendiculatus* and *A.variegatum* has also demonstrated the development of hypersensitive reactions with increased host resistance (Walker and Fletcher, 1987; Latif *et al.*, 1991a).

The results of this experiment indicate that the local effect of the feeding of nymphal *A.variegatum* manifests itself in increased severity of the initial dermatophilosis lesions. However, when the severity of the four groups of infections was compared on individual assessment days, significant differences were found only at days three and thirteen. This shows that even though inflammatory reactions to the ticks had a significant effect on the overall ranked severity, the feeding of these ticks did not result in the progression of the dermatophilosis into severe, chronic lesions.

Skin thickness at the sites of dermatophilosis lesions was greatly increased with local tick feeding. This increase in skin fold thickness was probably a result of the strong host reaction to tick feeding. There was no significant difference between the dermatophilosis lesions on the two groups of infections infested with nymphs. However, there was a large difference in the skin thickness between the two groups, with the greatest increase in skin fold thickness occurring at the hypersensitive tick attachment sites. The hypersensitive host reactions to the tick feeding had resulted in oedematous swelling and the formation of intra-epidermal pustules which would produce large increases in skin fold thickness.

A strong association has been demonstrated between inflammatory tick feeding sites and dermatophilosis foci. Further studies are needed to determine if there is a similar association between inflammatory reactions to other species of ticks or biting insects and dermatophilosis lesions. No association has been demonstrated between hypersensitive *A.variegatum* feeding sites and dermatophilosis lesions. However, it is possible that any association with hypersensitive sites has been disguised due the host's resistance causing the ticks to repeatedly detach and reattach (Latif *et al.*, 1990).

These results indicate that the feeding of nymphal *A.variegatum* has only a limited effect on local, simultaneous *D.congolensis* infections on rabbits. The correlation between the tick feeding sites and the initial foci of dermatophilosis lesions indicates that the physical damage to the epidermis may facilitate initial lesion formation. The penetration of *D.congolensis* into the epidermis via puncture wounds caused by the biting of *Haematopota albihirta* and *Tabanus taeniola* has already been demonstrated by Stewart (1972).

Although the feeding of these ticks appears to influence the distribution of dermatophilosis lesions, there is no evidence that *A.variegatum* nymphs facilitate the formation of severe, chronic lesions. However, care must be taken in interpreting these results when considering the effects of these ticks on severe, chronic lesions on cattle and small ruminants. Attempts to produce chronic dermatophilosis lesions on rabbits by simultaneously infesting the rabbits with adult *A.variegatum* have been unsuccessful (Chapter Seven). In similar experiments, using sheep as the experimental hosts, simultaneous infestations of adult *A.variegatum* and *D.congolensis* infections have resulted in the development of chronic lesions, persisting for several months (Chapter Seven; Walker and Lloyd, 1993). It may not be possible to produce chronic dermatophilosis lesions on rabbits since they are not natural hosts for *D.congolensis*

(Macadam, 1962). Further work is required to investigate the local effect of immature *A.variegatum* on simultaneous *D.congolensis* infections on sheep or cattle.

6.5 SUMMARY

1. Repeated infestations of *A.variegatum* nymphs on rabbits resulted in increased host resistance to the tick feeding. This increased resistance was accompanied by the development of macroscopic signs of hypersensitivity in the host's skin.

2. There was a significant positive association between inflammatory feeding sites of *A.variegatum* nymphs and initial dermatophilosis foci on rabbits, but there was no association between hypersensitive feeding sites of *A.variegatum* nymphs and infection foci. The lack of association between the hypersensitive feeding sites and the dermatophilosis lesions may have been due to the host's resistance causing the ticks to repeatedly detach and reattach (Latif *et al.*, 1990).

3. Increased severity of some of the initial dermatophilosis lesions, at the same site as the tick feeding, was not consistent or prolonged. The simultaneous feeding of *A.variegatum* nymphs and *D.congolensis* infections did not result in the development of chronic dermatophilosis lesions on rabbits.

4. There is now some doubt whether it is possible to produce chronic dermatophilosis lesions on rabbits. Further investigations into the local effect of *A.variegatum* nymphs on simultaneous *D.congolensis* infections are required, using sheep or cattle as the experimental hosts.

CHAPTER SEVEN
THE SYSTEMIC EFFECT OF *AMBLYOMMA VARIEGATUM* TICKS ON THE PROGRESSION OF EXPERIMENTAL *DERMATOPHILUS CONGOLENSIS* INFECTIONS: A COMPARISON OF ADULT AND NYMPHAL TICKS

7.1 INTRODUCTION

Field observations suggest that it is adult *Amblyomma variegatum* that have a significant effect on the progression of dermatophilosis lesions (Plowright, 1956). Morrow *et al.*, (1989) found that the prevalence of severe dermatophilosis lesions increased with the population of adult *A.variegatum*.

Until recent experiments in this laboratory, the association between adult *A.variegatum* and dermatophilosis had not been investigated under controlled conditions. These experiments demonstrated that adult *A.variegatum* have a significant systemic effect on simultaneous *D.congolensis* infections on sheep (Walker and Lloyd, 1993). This systemic effect may also be caused by the immature stages of the tick which have already been shown to have a significant effect on the initial severity and distribution of local *D.congolensis* infections (Chapter Six). Therefore, the following laboratory experiments were conducted to compare the effect of adult and immature *A.variegatum* on the progression of simultaneous *D.congolensis* infections.

The comparison of the systemic effect of adult and nymphal *A.variegatum* was potentially useful for field applications and for laboratory studies. If the immature stages were found to be involved in the aggravation of the disease into its chronic form, control of *A.variegatum* to control dermatophilosis would have to extend to the immatures. If the systemic effect did not extend to the immatures, the experiments would have provided the closest possible comparison of a tick that does aggravate dermatophilosis with one that does not.

Initial experiments comparing the systemic effect of adults and nymphs were set up using rabbits as the experimental hosts. The initial results indicated that the systemic effect of the adults did not extend to the nymphs. To confirm these

results in a more natural scenario the same experiment was then repeated using sheep, natural hosts of *D.congolensis* and *A.variegatum*.

Three groups of sheep were used; the first group was infested with adult *A.variegatum*, the second group with nymphal *A.variegatum* and the third group was not exposed to ticks, and used as a control. The humoral and cellular immune responses were compared in the three groups, using ELISA and skin testing respectively. Previous attempts at using lymphocyte transformation tests in similar experiments in this laboratory have been unsuccessful and it was hoped that skin testing would be a good substitute.

7.2 METHODS

7.2.1 THE SYSTEMIC EFFECT OF ADULT AND NYMPHAL *AMBLYOMMA VARIEGATUM* ON SIMULTANEOUS *DERMATOPHILUS CONGOLENSIS* INFECTIONS ON RABBITS

7.2.1.1 Experimental hosts

Six female New Zealand White rabbits were available for the initial experiment, none of which had been previously exposed to ticks or *D.congolensis*. All the rabbits were approximately six months old at the start of the experiment. The rabbits were arbitrarily assigned to two groups of three, for experimental purposes. One rabbit from each group was infested with adult *A.variegatum* and simultaneously infected with *D.congolensis*. The second rabbit from each group was infested with *A.variegatum* nymphs and simultaneously infected with *D.congolensis*. The third rabbit from each group was used as a control, and was infected with *D.congolensis* but had no exposure to ticks (Figure 7.1).

Figure 7.1 The sequence of *Amblyomma variegatum* infestations and *Dermatophilus congolensis* infections on rabbits.

	Group A N=4	Group B N=4	Group C N=4
Day 0	14[1] adult males on each rabbit	____	____
Day 7	____	150 nymphs on each rabbit	____
Day 14	14 adult females on each rabbit	150 nymphs on each rabbit	____
Day 21	____	150 nymphs on each rabbit	____
Day 22	All 12 rabbits infected with the first *D.congolensis* infection		
Day 37	Last ticks detached from rabbits[2]		
Day 42	Day 20 of the *D.congolensis* infection and the last assessment.		

[1] Fourteen males and fourteen females were applied: once the ticks had attached, excess ticks were removed to leave seven feeding pairs.

[2] Nearly all of the nymphs had detached by this time; therefore, the adult ticks were detached manually to keep the amount of tick feeding on the two groups of infested rabbits as equal as possible.

The whole procedure was repeated on two rabbits in each group; the remaining two rabbits in each group were infested and infected only once.

Six more rabbits were obtained for the purpose of repeating the experiment; these were also of a similar age and kept in the same constant environmental conditions (Section 3.2.1).

7.2.1.2 Tick infestations

The adult infestations consisted of seven male and seven female *A.variegatum* ticks and nymphal infestations consisted of 450 ticks (equivalent numbers of adult and nymphal ticks were calculated using the ratio determined in Section 3.3.3.2). Fourteen male ticks were applied to each rabbit, after two weeks fourteen female ticks were applied. Once the ticks had started to feed excess ticks were removed, leaving seven feeding pairs. The *D.congolensis* infections were applied one week after the application of the female ticks.

Four hundred and fifty nymphs, instead of 420 were used to allow for the small number of ticks which were unsuccessful in attaching to the host. All of the infestations were on the backs of rabbits and enclosed in cloth bags (Section 3.3.4.1). The infestations of nymphs were applied in three batches, each batch consisting of 150 nymphs the second batch were applied approximately one week after the first, by which time the first batch had started to complete their engorgement. The final infestation was then applied one week after the second infestation and one day before the start of the *D.congolensis* infection.

The infestations were left on the host until all the nymphs and females had engorged and detached.

7.2.1.3 *Dermatophilus congolensis* infections

Both the test and control rabbits were infected with identical doses of *D.congolensis* three weeks after the first application of ticks. The *D.congolensis* was

applied to six test areas 1 x 2cm on the torso, at sites remote from the tick attachment sites.

The *D.congolensis* used for these infections was taken from a large batch of *D.congolensis* previously cultured and cryopreserved at -20°C at a concentration of 1.2 x 10^7 cocci/µl (Section 4.2.2). Six areas were each infected with a titrated dose of 50µl of *D.congolensis*, starting at a concentration of 2.5 x 10^8 cocci/cm^2 with five-fold dilutions to 8 x 10^4 cocci/cm^2.

Before the application of the *D.congolensis* the skin was cleaned and the infection areas were marked (Section 3.4.1). The progress of the dermatophilosis was recorded every three or four days for three weeks, using the ranking system developed in Chapter Four (Section 4.2.1).

7.2.2 THE SYSTEMIC EFFECT OF ADULT AND NYMPHAL *AMBLYOMMA VARIEGATUM* ON SIMULTANEOUS *DERMATOPHILUS CONGOLENSIS* INFECTIONS ON SHEEP

7.2.2.1 Experimental hosts

A group of six sheep, Black-faced x Suffolk females and castrated males, approximately two years old, were used for the initial experiment. The entire procedure was then repeated using a second group of six female Black-faced sheep. The experimental protocol for the second group of sheep was the same as for the first group, except where indicated in the text.

Three of the twelve sheep were castrated males; two were part of the group infested with adult ticks, and the third was used as a control. Ideally, the males should have been equally divided into each of the three test groups, but as they were castrated males any difference in reponse to the *D.congolensis* infections due to the

difference in sex would have been minimal (Section 3.2.2). All 12 sheep were kept in constant environmental conditions (Section 3.2.2).

None of the sheep had prior experimental exposure to ticks or *D.congolensis*; however, it was found that some of the sheep were seropositive to *D.congolensis* but with no clinical signs of the disease (Section 7.3.2.1.1). Each group of six sheep was divided into three pairs, by size. The first pair of sheep were infested with *A.variegatum* adults with a simultaneous infection of *D.congolensis* (Group A). The second pair was infested with nymphs with a simultaneous infection of *D.congolensis* (Group B) and the third pair were the controls, being infected with *D.congolensis* with no exposure to ticks (Group C), (Figure 7.2).

In previous experiments to investigate the effect of adult *A.variegatum* on simultaneous *D.congolensis* infections, the differences between the test and control infections were enhanced by repeating the infestations and infections using the same hosts (Walker and Lloyd, 1993). Therefore, the whole procedure of adult *A.variegatum* infestations and *D.congolensis* infections was repeated, using the same experimental animals (Figure 7.2).

7.2.2.2 Tick infestations

In order to investigate the systemic effect of adult *A.variegatum* on *D.congolensis* infections on sheep the infestations consisted of ten male and ten female ticks. Infestations of this size have been shown to have a significant effect on simultaneous *D.congolensis* infections (Walker and Lloyd, 1993). To produce infestations of ten males and ten females, 20 males were applied followed two weeks later by 20 females; then excess ticks were removed to leave ten feeding pairs. The equivalent size of nymphal infestations was calculated using the ratio of one adult to 30 nymphs (Section 3.3.3.2).

Figure 7.2 The sequence of *Amblyomma variegatum* infestations and *Dermatophilus congolensis* infections on sheep.

	Group A	Group B	Group C
Day 0	20[1] adult males applied to each sheep	____	____
Day 7	____	200 nymphs applied to each sheep	____
Day 14	20 adult females applied to each sheep	200 nymphs applied to each sheep	____
Day 21	____	200 nymphs applied to each sheep	____
Day 22	All twelve sheep were infected with a first *D.congolensis* infection		
Day 34-38	The last ticks detached from sheep one to six[2]		
Day 52	The last ticks detached from sheep seven to twelve		
Day 63	Day 41 of the first *D.congolensis,* last assessment		
Day 77	20 adult males applied to each sheep	____	____
Day 84	____	200 nymphs applied to each sheep	____
Day 91	20 adult females applied to each sheep	200 nymphs applied to each sheep	____
Day 98	____	200 nymphs applied to each sheep	____
Day 99	All twelve of the sheep were infected with a second *D.congolensis* infection		
Day 119	Last ticks detached from sheep one to six		
Day 135	Last ticks detached from sheep seven to twelve		
Day 140	Day 41 of second *D.congolensis* infection, last assessment		

[1] 20 ticks of each sex were applied to get an infestation of ten males and ten females.

[2] The tick infestations had to be removed due to severe abscess formation at the tick feeding sites.

Ten males and ten females were used for the adult infestations and six hundred ticks were used for the nymphal infestations. One day after the application of each batch of nymphs any ticks that had not successfully attached were removed and replaced with fresh ticks; this ensured that approximately 600 nymphs attached and fed on each sheep. The ticks were enclosed by cloth bags glued to the wool on the shoulders of the four test sheep (Section 3.3.4.2). The *D.congolensis* titrations were then applied to the sheep one week after the female ticks and one day after the final batch of nymphs (see Figure 7.2 for sequence of infestations and infections)

Due to problems of severe reactions occurring at the feeding sites of the first infestations on the sheep, the second infestation of ticks on the first group of sheep, and both infestations on the second group of sheep, were divided into several separate patches on each sheep. The adults were divided into two patches each containing ten males and ten females. Excess ticks were taken from clusters to try and reduce problems due to large numbers of ticks feeding from one area. The infestations of nymphs were divided into three patches and each of the batches of 200 ticks was applied to uninfested areas.

7.2.2.3 *Dermatophilus congolensis* infections

All twelve sheep were infected with identical doses of *D.congolensis*, three weeks after the first infestation of ticks. The stabilate previously cultured and cryopreserved (Section 4.2.2) was used to produce the first infection on all twelve sheep, and the second infections on sheep one to six. Due to doubts about the viability of the stabilate, freshly cultured *D.congolensis* cocci were used for the second infection on sheep seven to twelve.

All the *D.congolensis* infections were applied as 100µl doses to seven areas (2 x 4cm) along the flanks of each sheep at sites remote from the tick feeding.

The infections were applied as seven titrated doses, with ten-fold dilutions, starting at a concentration of 1.25×10^8 cocci/cm^2 (see Section 3.4.2 for details of application).

The progress of the dermatophilosis was recorded at three and four day intervals for four weeks, followed by two more assessments at weekly intervals using the ranking system developed in Section 4.2.1.5.

7.2.2.4 Skin testing

All twelve sheep were sensitized with identical doses of two foreign antigens, using a protocol previously devised in this laboratory. Skin tests were carried out to investigate the effect of adult or nymphal *A.variegatum* on the host response to a T-cell activator (ovalbumin) and a B-cell activator (polyamino acid or *Brucella abortus*).

All twelve sheep were inoculated with the same T-cell activator, chicken egg ovalbumin (Sigma grade V, approximately 99% by agarose electrophoresis, crystallized and lyophilized, essentially salt free).

A polyamino acid, Poly-d-glutamate-d-lysine (Sigma grade) was used initially for the sensitizing of B-lymphocytes. However, this antigen did not produce any response with the first group of six sheep; therefore, it was replaced with *Brucella abortus* bacteria when the experiment was repeated on the second group of sheep.

A source of killed *B.abortus* (S99), freeze dried in 0.5% phenol saline was obtained from the Central Veterinary Laboratory, Weybridge. Before use as a sensitizing antigen, the *B.abortus* was washed using three centrifugation washes with distilled water, to remove the phenol. After the third wash, the pellet was resuspended in distilled water and freeze dried.

7.2.2.4.1 Inoculation with foreign antigens

All 12 sheep were sensitized with two antigens, one B-cell and one T-cell activator, together in a mixed dose. All twelve sheep were sensitized to ovalbumin; sheep one to six were sensitized to the polyamino acid and sheep seven to twelve were sensitized to *B.abortus*. The antigens were injected into the host as anhydrous suspensions in Freund's incomplete adjuvant. This method of application was used to facilitate the intradermal injection of the antigens by means of a pressure jet apparatus 'Dermojet' (Etablissements Akra, France).

20mg of ovalbumin was ground into a paste in 1ml of Freund's incomplete adjuvant and was transferred to a glass vial. Any remaining sediment was rinsed into the vial using 1ml of the adjuvant to produce a mixture of 20mg ovalbumin in 2ml of adjuvant. The above procedure was then repeated with 20mg of the B-cell activator, either polyamino acid or *B.abortus*. The paste formed by the second antigen was added to the vial containing the ovalbumin, producing 4ml of adjuvant containing equal amounts of the two antigens.

The 4ml mixture was then sonicated in the adjuvant, for 10 minutes at room temperature. The mixture of antigens was injected intramuscularly above the right foreleg, using the 'Dermojet'. Each sheep had five injections of a nominal dose of 100µl, giving a total dose of 2.5mg of each antigen.

All of the sheep, test and controls, received the sensitizing doses at the same time as the application of the female ticks and the second batch of nymphs.

7.2.2.4.2 Booster doses

The booster sensitizations were applied at the time of the second infestation of female ticks, which was also the same time as the middle batch of the second infestation of nymphs. For the booster each sheep received two injections of

the antigen mixture, into the muscle above the left fore-leg, at the same concentration as the sensitizing dose containing 1mg of each antigen.

For sheep seven to twelve, being sensitized to ovalbumin and *B.abortus*, half the concentration of antigen was used for the booster dose to reduce the risk of abscess formation. Each sheep received approximately 1mg of each antigen in a total of five injections, using the 'Dermojet'.

7.2.2.4.3 Challenge injections

Each host received challenge applications of two antigens at the time of peak engorgement of the female ticks, during the first week of the second *D.congolensis* infections. The antigens were applied intradermally by 26 gauge needle in doses of 100µl, each antigen was applied separately dissolved in PBS or sonicated in PBS in the case of the *B.abortus*. Plate 7.1 shows the positioning of the challenge injections of ovalbumin, on a sheep with a simultaneous infestation of *A.variegatum* and *D.congolensis* infection. Five injections of each antigen were applied in the following titrations.

OVALBUMIN (left back leg) - This was applied in 100µl doses to five areas on each sheep. The doses were titrated, with a starting concentration of 2.5mg/100µl and five-fold dilutions.

Sheep 1-12

2.5mg/100µl = 50mg in 2ml PBS

500µg/100µl = 0.4ml above + 1.6ml PBS

100µg/100µl = 0.4ml above + 1.6ml PBS

20µg/100µl = 0.4ml above + 1.6ml PBS

4µg/100µl = 0.4ml above + 1.6ml PBS

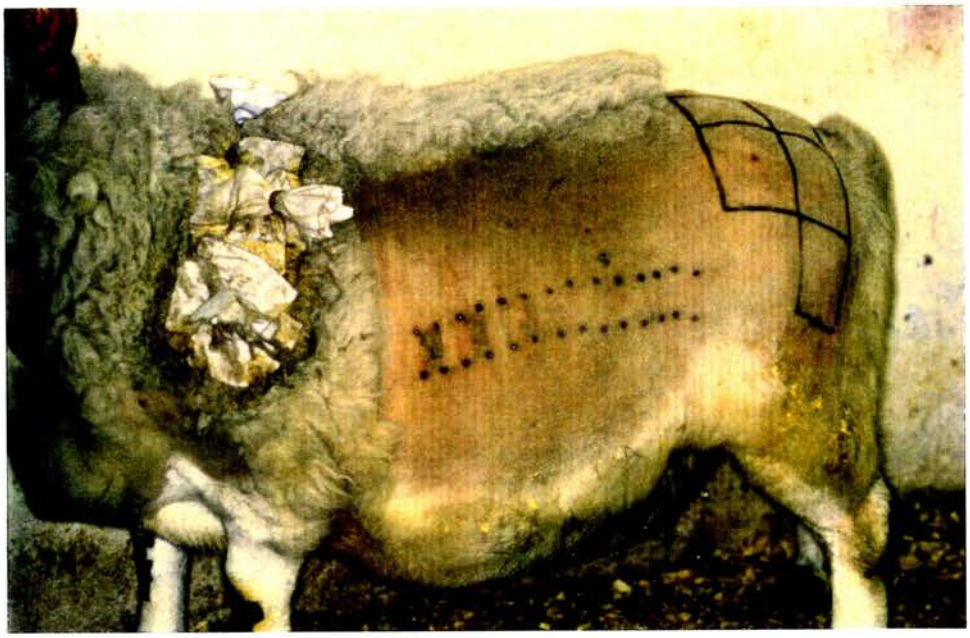


Plate 7.1 Positioning of challenge injections of ovalbumin above left rear leg, one injection was applied near the centre of each of the squares marked out in indelible pen. The challenge injections of *Brucella abortus* or polyamino acid were applied in a similar pattern, above the right rear leg. This sheep was also infested with *Amblyomma variegatum* ticks on the shoulder and infected with *Dermatophilus congolensis* along the flank.

POLYAMINO ACID (right back leg) - This was also applied as a titrated dose to five areas on each sheep. The dose size 100µl/area, with a starting concentration of 1mg/100µl and five-fold dilutions.

Sheep 1-6

1mg/100µl = 10mg in 1ml PBS

200µg/100µl = 200µl above + 800µl

40µg/100µl = 200µl above + 800µl

8µg/100µl = 200µl above + 800µl

1.6µg/100µl = 200µl above + 800µl

BRUCELLA ABORTUS (right back leg) - This was applied in 100µl doses to five areas on each sheep. The doses were titrated, with a starting concentration of 2.5mg/100µl and five-fold dilutions.

Sheep 7-12

2.5mg/100µl = 50mg in 2ml PBS

500µg/100µl = 0.4ml above + 1.6ml PBS

100µg/100µl = 0.4ml above + 1.6ml PBS

20µg/100µl = 0.4ml above + 1.6ml PBS

4µg/100µl = 0.4ml above + 1.6ml PBS

7.2.2.4.4 Analysis of skin test reactions

The response to the two antigens was assessed 24 and 48 hours after the challenge. The degree of response was measured as a product of the skin fold thickness and diameter of any visible response at each of the five challenge sites.

7.2.2.5 Indirect enzyme linked immunoassay (Indirect ELISA)

7.2.2.5.1 Serum samples

Blood samples were collected into sterile vacutainer tubes (without additives); immediately on collection the tubes were immersed in warm water then incubated at 37°C for one hour before being left at 4°C overnight. The following morning the serum was dispensed in 1ml aliquots into sterile Eppendorf tubes, under aseptic conditions. The serum was then stored at -20°C until used.

Control serum was collected from all twelve sheep prior to exposure to ticks, *D.congolensis* and the foreign antigens. Serum was then collected at regular intervals, fortnightly when possible, throughout the course of the two *D.congolensis* infections and tick infestations (for details of serum collected see Appendix 7.1).

7.2.2.5.2 Preparation of antigens

Both ovalbumin and the polyamino acid are water soluble, therefore stock solutions of both these antigens were made up at a concentration of 1mg antigen per 1ml of distilled water. Enough stock solution of each antigen for all the ELISAs was prepared at the beginning and this stock solution was stored at -20°C.

The bacterial antigens, *B.abortus* and *D.congolensis* were prepared in the following way. The bacterial cells were suspended in PBS and washed three times by repeated centrifugation. After the third wash the PBS was removed and the pellet was resuspended in TRIS-HCl buffer at pH 8.5. The washed antigenic material was then mixed with an equal volume of very small glass beads (ballotini, Grade 14, Jencons Scientific Ltd) and sonicated at 0°C for 20 minutes at maximum power. The sonicated material was then centrifuged once for 15 minutes at 2500g to remove particles of 96 Kilodaltons and larger.

The above method produced solubilised bacterial antigen suitable for use in an ELISA. The protein concentration of the two bacterial antigen preparations was measured using the Bio-Rad DC Protein Assay. This assay was used because it was compatible with the TRIS-HCl buffer. The concentration of the bacterial antigen preparations were 0.51mg/ml and 0.34mg/ml, for *B.abortus* and *D.congolensis* respectively.

7.2.2.5.3 Indirect ELISA using ovalbumin as the test antigen

All of the tests were carried out using 96 well, flat-bottomed ELISA plates (Immulon I, Dynatech Laboratories). The plates were coated overnight, at 4°C, with antigen diluted to the required concentration in 0.05M carbonate-bicarbonate buffer. The following day the antigen solution was removed and the plates were washed four times with 250µl of 0.05% PBS/Tween 20 (see Appendix 7.2) in each well.

Once the excess antigen was removed from the plates 100µl of serum diluted in 0.05% PBS/Tween 20 was placed in the wells. The serum was then incubated, with continuous agitation, at 37°C for 30 minutes. After the incubation the plates were washed three times with 0.05% PBS/Tween 20.

The serum dilutions were then replaced with rabbit anti-sheep immunoglobulin conjugate (RASh/ IgG(H+L)/ PO; Nordic Immunology, Immunoconjugate) diluted in 0.05% PBS/Tween 20. This was than incubated, with agitation, at 37°C for 30 minutes. After this incubation all of the wells were washed four times, with each rinse remaining on the plate for one minute. This was to ensure the plates were thoroughly washed to remove any excess conjugate.

The final stage of the ELISA was completed using the chromogen 3,3',5,5'-tetramethyl-benzidine dihydrochloride (Sigma) (Bos *et al.*, 1981). This

chromogen was dissolved in a phosphate-citrate buffer (Sigma) immediately prior to application to the plates. One hundred microlitres of the chromogen was put into all of the wells and the plate incubated for ten minutes at 37°C. The reaction was stopped by the addition of 50µl of 2M H_2SO_4 to each of the wells.

The reading of the plates was done automatically using an optical density scanner, at 450nm.

Before carrying out the test ELISAs, optimum concentrations of antigen, antibody and conjugate were ascertained using the chequerboard technique. For these preliminary ELISAs, each plate was divided into two with one half being used for the positive control serum and the other half for the negative control serum. In this case the negative control serum was a mixture of sera taken from six sheep prior to sensitisation with ovalbumin. A mixture of sera collected from two of the control sheep at day 35 after sensitisation with ovalbumin was used as the positive control serum.

Doubling dilutions of the test antigen were set up across the plate, from column one to six, and a second titration from column seven to twelve (Figure 7.3). Serum titrations were set up with doubling dilutions down the plate.

The stock antigen was diluted 1:50 in the bicarbonate-carbonate buffer and 200µl was put in each of the wells 1A to 1H and 7A to 7H. One hundred microlitres of coating buffer was then put into all of the empty wells. The titration was produced by pipetting 100µl of the antigen solution from each of the wells in column 1 to the corresponding wells in column 2, mixing and then pipetting onto the next column. Once the antigen and coating buffer had been mixed in column 5, the excess 100µl was disposed of instead of transferring to column 6; this column was left with

Figure 7.3 Antigen and serum titrations used to determine optimum titrations for the test ELISAs.

	Positive control						Negative control					
	1	2	3	4	5	6	7	8	9	10	11	12
A	ANT	AC	AC	AC	AC	CB	SE	SE	SE	SE	SE	SE
B	ANT	AC	AC	AC	AC	CB	SP	SP	SP	SP	SP	SP
C	ANT	AC	AC	AC	AC	CB	SP	SP	SP	SP	SP	SP
D	ANT	AC	AC	AC	AC	CB	SP	SP	SP	SP	SP	SP
E	ANT	AC	AC	AC	AC	CB	SP	SP	SP	SP	SP	SP
F	ANT	AC	AC	AC	AC	CB	SP	SP	SP	SP	SP	SP
G	ANT	AC	AC	AC	AC	CB	SP	SP	SP	SP	SP	SP
H	ANT	AC	AC	AC	AC	CB	PBS	PBS	PBS	PBS	PBS	PBS

ANT = 200µl antigen/well, 100µl per well transferred to column 2 and mixed with 100µl coating buffer then transferred to column 3 and so on until column 5. Column 6 contains 100µl coating buffer per well, no antigen control (CB). Identical titration set up in columns 7A-7H to 12A 12H.

AC = doubling dilutions of antigen in coating buffer, from columns one to five.

SE = 200µl serum/well, 100µl per well transferred to row B and mixed with 0.05% PBS/Tween 20 then 100µl transferred to row C and so on until row G. Row H contains 100µl 0.05% PBS/Tween 20 per well, no serum control (PBS). Identical titration set up in A1-A6 to H1-H6.

SP = doubling dilutions of serum in 0.05% PBS/Tween 20 from rows B to G.

100µl of coating buffer in each well as a control. An identical titration was produced over the other half of the plate using the same method as above.

The serum titrations were produced in the same way, but with the dilutions going down the plate (Figure 7.3). The serum was diluted in 0.05% PBS/Tween 20. The serum titrations started in row A with 200µl of serum in each well and ended in row G, row H was used as a control and contained 100µl 0.05% PBS/Tween 20 in each well.

Constant amounts of conjugate were applied to the entire plate when the antigen and serum were titrated. To find the optimum concentration of conjugate, plates were set up with constant serum dilutions over the entire plate and the conjugate was titrated as described for the serum.

Using this method the optimum concentrations were found to be a 1:400 dilution of the stock antigen to coat each well with 0.25µg of ovalbumin and a dilution of 1:5000 for the conjugate. For statistical purposes the test sera were titrated starting at a dilution of 1:1000.

Three test sera (one from each group of sheep) and positive and negative control sera were plated out, in duplicate, on each ELISA plate. The serum titrations started in row B and five doubling dilutions were produced down the plate (Figure 7.4). Row G was used as a control with 100µl of 0.05% PBS/Tween 20 in each well. The outer wells on the plates were not used to reduce the edge-effect (Tijssen, 1985).

7.2.2.5.4 Indirect ELISA using polyamino acid as the test antigen

Efforts to devise a technique for ELISAs using the polyamino acid as the test antigen were unsuccessful. This antigen was also unsuccessful as a skin test antigen and it was decided to concentrate on the other B-cell activator, *B.abortus*.

Figure 7.4 Test plate for ELISA using ovalbumin as the antigen. The titrations for the other antigens were also laid out in the same way but using different starting concentrations.

	1	2	3	4	5	6	7	8	9	10	11	12	Serum dilutions
A													
B		+ve	-ve	A	B	C	A	B	C	+ve	-ve		1:1000
C													1:2000
D													1:4000
E													1:8000
F													1:16000
G		PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS		
H													

+ve = positive control serum

-ve = negative control serum

A = test serum from a sheep infested with adult *Amblyomma variegatum*

B = test serum from a sheep infested with nymphal *Amblyomma variegatum*

C = test serum from a sheep not exposed to *Amblyomma variegatum*

PBS = No antibody (0.05% PBS/Tween 20 only)

7.2.2.5.5 Indirect ELISA using *Brucella abortus* as the test antigen

The optimum concentrations of *B.abortus*, antibody and conjugate were calculated using the same methods as described in Section 7.2.2.5.3.

The test and control sera on the test plates were set up as above (Figure 7.4). In this case each well was coated with 0.051μg of *B.abortus*, the conjugate was diluted 1:2000 and the serum titrations started at 1:2000. Apart from the differences in the working concentrations, the ELISA method used was identical to the ELISA using ovalbumin.

7.2.2.5.6 Indirect ELISA using *Dermatophilus congolensis* as the test antigen

A mixture of hyphae and cocci were used as the experimental antigen. The *D.congolensis* was cultured using the method described in Section 4.2.1.2 up to the point of production of hyphae in broth. At this point the hyphae and cocci were washed and sonicated as described in Section 7.2.2.5.2.

Control serum taken from all twelve sheep before the start of the experiment was tested for antibodies to *D.congolensis*. The same ELISA technique was used for this antigen as for the other antigens (Section 7.2.2.5.3). Serum from a previously gnotobiotic sheep infected with rotavirus (donated by Dr. D.R. Snodgrass, Moredun Research Institute) was used as a negative control. This sheep should not be positive to *D.congolensis* but it would have IgG antibodies because of the rotavirus infection. An equal mixture of serum taken from the four control sheep at day 27 of the first *D.congolensis* was used for the positive control.

Initial ELISAs produced optical densities of the positive serum only two to three times higher than the negative serum. The high optical densities of the negative serum were thought to be due to natural agglutinins in sheep (Lloyd, 1981; Roberts, 1966). To try and reduce the effect of these natural agglutinins on the

ELISA results all of the sera was heat inactivated by incubation at 60°C for 30 minutes. This heat inactivation should have affected only the IgM antibodies (natural agglutinins) without affecting the IgG antibodies. Heat inactivated negative control sera was also diluted 1:1000 in conjugate 30 minutes before application to the plates.

Apart from the heat inactivation and the addition of negative control serum to the conjugate, the ELISA was the same as used for the other antigens. The antigen was applied at a constant dose over the plate with 0.34μg coating each well. The serum titration started at 1:500 and the conjugate was diluted 1:1000 for application over the whole plate.

7.3 RESULTS

7.3.1 THE SYSTEMIC EFFECT OF ADULT AND NYMPHAL *AMBLYOMMA VARIEGATUM* ON SIMULTANEOUS *DERMATOPHILUS CONGOLENSIS* INFECTIONS ON RABBITS

The total ranked clinical scores for the primary *D.congolensis* infections on the individual rabbits are shown in Table 7.1. The severity of the dermatophilosis lesions on the three groups of rabbits was compared using the Kruskal-Wallis test on the total ranked scores given to the individual rabbits at each assessment day. Separate tests were done to compare the severity of the infections on each of the assessment days. At no point during the course of the primary infection on the rabbits was there found to be any significant difference between the infections on the three groups of rabbits ($P > 0.05$, $k = 3$, $n = 4$), (Table 7.2).

There was a lot of variation observed in the severity of the dermatophilosis lesions which developed on the individual rabbits. For the group of

Table 7.1 The systemic effect of adult or nymphal *Amblyomma variegatum* infestations on primary *Dermatophilus congolensis* infections on rabbits.

	Total ranked clinical scores				
Infested with adults Day of infection	Rabbit 1	Rabbit 2	Rabbit 7	Rabbit 8	Median
3	24	25	12	12	18
6	36	37	18	14	27
9	42	42	16	14	29
13	37	36	0	8	22
16	4	4	0	0	2
20	0	0	0	0	0
Total	143	144	46	48	95.5
Infested with nymphs Day of infection	Rabbit 3	Rabbit 4	Rabbit 9	Rabbit 10	Median
3	16	18	14	23	17
6	25	29	18	32	27
9	28	33	18	32	30
13	28	19	17	23	21
16	4	2	4	5	4
20	0	0	0	0	0
Total	101	101	71	115	86
Not exposed to ticks Day of infection	Rabbit 5	Rabbit 6	Rabbit 11	Rabbit 12	Median
3	12	22	14	15	14.5
6	15	26	19	22	20.5
9	21	28	18	25	23
13	21	6	14	26	17.5
16	6	0	3	17	4.5
20	0	0	0	0	0
Total	75	82	68	105	78.5

Table 7.2 Comparison of primary *Dermatophilus congolensis* infections on rabbits with simultaneous infestations of adult or nymphal *Amblyomma variegatum* or not exposed to ticks.

Day of infection	Kruskal-Wallis H	k	n	P	Interpretation
3	0.66	3	4	P > 0.05	ns
6	0.76	3	4	P > 0.05	ns
9	0.62	3	4	P > 0.05	ns
13	0.50	3	4	P > 0.05	ns
16	1.50	3	4	P > 0.05	ns
20	0	3	4	P > 0.05	ns

k = number of groups compared
n = number of rabbits within each group
ns = not significant

rabbits infested with adult *A.variegatum*, the maximum ranked clinical score for one day during the primary infection ranged from 42 (rabbits 1 and 2) to 14 (rabbit 4).

The ranked clinical scores given to the secondary dermatophilosis lesions on the individual rabbits are shown in Table 7.3. These secondary infections were less severe than the primary infections on all three of the groups (Figure 7.5). The dermatophilosis lesions on the group of rabbits with simultaneous infestations of adult *A.variegatum* were more severe than on the other two groups, with the median ranked clinical scores being 65, 39.5 and 45.5, respectively (Table 7.3). Secondary infections were only produced on five rabbits and because of the small sample size, it was not possible to test for differences between the infections on the three groups of rabbits on each of the individual assessment days. Instead, one Kruskal-Wallis test was carried out using the median ranked clinical scores given to the three groups on each of the six assessment days. There was no significant difference between the secondary infections on the three groups of rabbits ($P > 0.05$, $k = 3$ = number of groups compared, $n = 6$ = number of assessments of the *D.congolensis* infections, for each group).

After 12 primary and five secondary *D.congolensis* infections on the rabbits, comparison of the infections on the three groups of rabbits showed no significant differences in the *D.congolensis* infections ($P > 0.05$, $k = 3$ = number of groups compared, $n = 5,6,6$ = number of *D.congolensis* infections assessed on each group). In fact the median ranked scores for the three groups of rabbits were 65 for the rabbits infested with adult *A.variegatum*, 86 for the rabbits infested with nymphal *A.variegatum* and 71.5 on the rabbits not exposed to tick feeding.

Table 7.3 The systemic effect of adult or nymphal *Amblyomma variegatum* on secondary *Dermatophilus congolensis* infections on rabbits.

	Total ranked clinical scores						
	Infested with adults*	Infested with nymphs			Not exposed to ticks		
Day of infection	Rabbit 2	Rabbit 3	Rabbit 4	Median	Rabbit 5	Rabbit 6	Median
3	13	12	10	11	15	15	15
6	18	10	10	10	14	11	12.5
9	16	10	8	9	11	9	10
13	14	10	6	8	11	5	8
16	4	0	3	1.5	0	0	0
20	0	0	0	0	0	0	0
Total	65	42	37	39.5	51	40	45.5

* Rabbit 1 died.

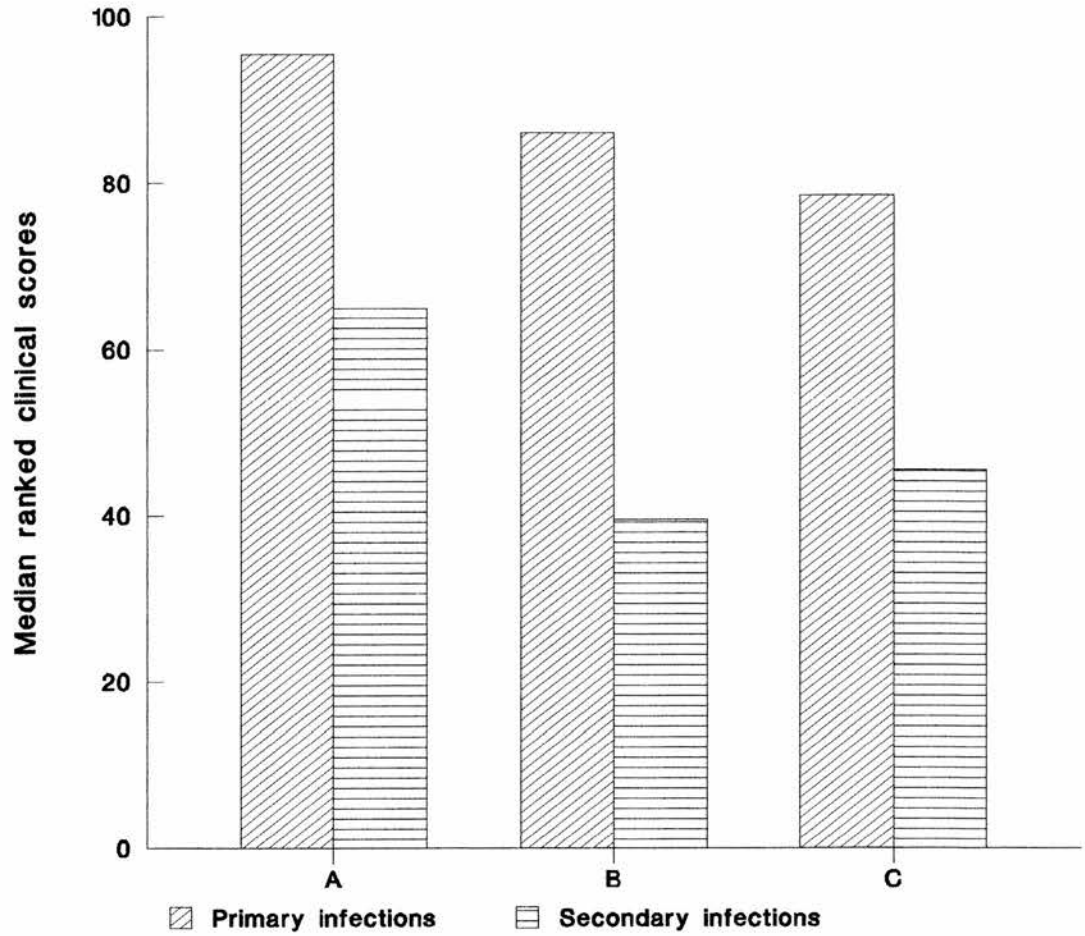


Figure 7.5 Median ranked clinical scores for primary and secondary *Dermatophilus congolensis* infections on rabbits. A, With simultaneous infestations of adult *Amblyomma variegatum*; B, With simultaneous infestations of nymphal *A.variegatum*; C, With no exposure to *A.variegatum*.

7.3.2 THE SYSTEMIC EFFECT OF ADULT AND NYMPHAL *AMBLYOMMA VARIEGATUM* ON SIMULTANEOUS *DERMATOPHILUS CONGOLENSIS* INFECTIONS ON SHEEP

7.3.2.1 *Dermatophilus congolensis* infections

The ranked clinical scores given to the dermatophilosis lesions on the individual sheep are recorded in Appendix 7.3. Differences in the median ranked clinical scores of the three groups were not large, with the highest median score on the sheep infested with adult *A.variegatum* being 24 compared with 28 on the sheep infested with nymphs and 26 on the control sheep.

Differences in the progression of *D.congolensis* infections on the three groups of sheep became apparent approximately four weeks after infection with *D.congolensis* (Figure 7.6 and Plate 7.2). By day 41, three of the four sheep infested with adult ticks still showed signs of infection, whereas all of the lesions on the sheep infested with nymphs had healed and only one control sheep showed slight signs of infection.

Separate Kruskal-Wallis tests were used to compare the ranked dermatophilosis scores on the individual sheep in the three groups at each of the assessment days. During the first infection, a significant difference between the groups was recorded only on day 27 with $P < 0.05$ ($k = 3$, $n = 4$), (Table 7.4). The median ranked scores of the three groups on this day were 13.5 for the group infested with adult ticks and 1.5 for the sheep infested with nymphs or not exposed to ticks (Appendix 7.3).

The differences between the dermatophilosis lesions on the test and control sheep were enhanced on a secondary infection (Figure 7.6). The ranked clinical scores for the secondary dermatophilosis lesions on the individual sheep are

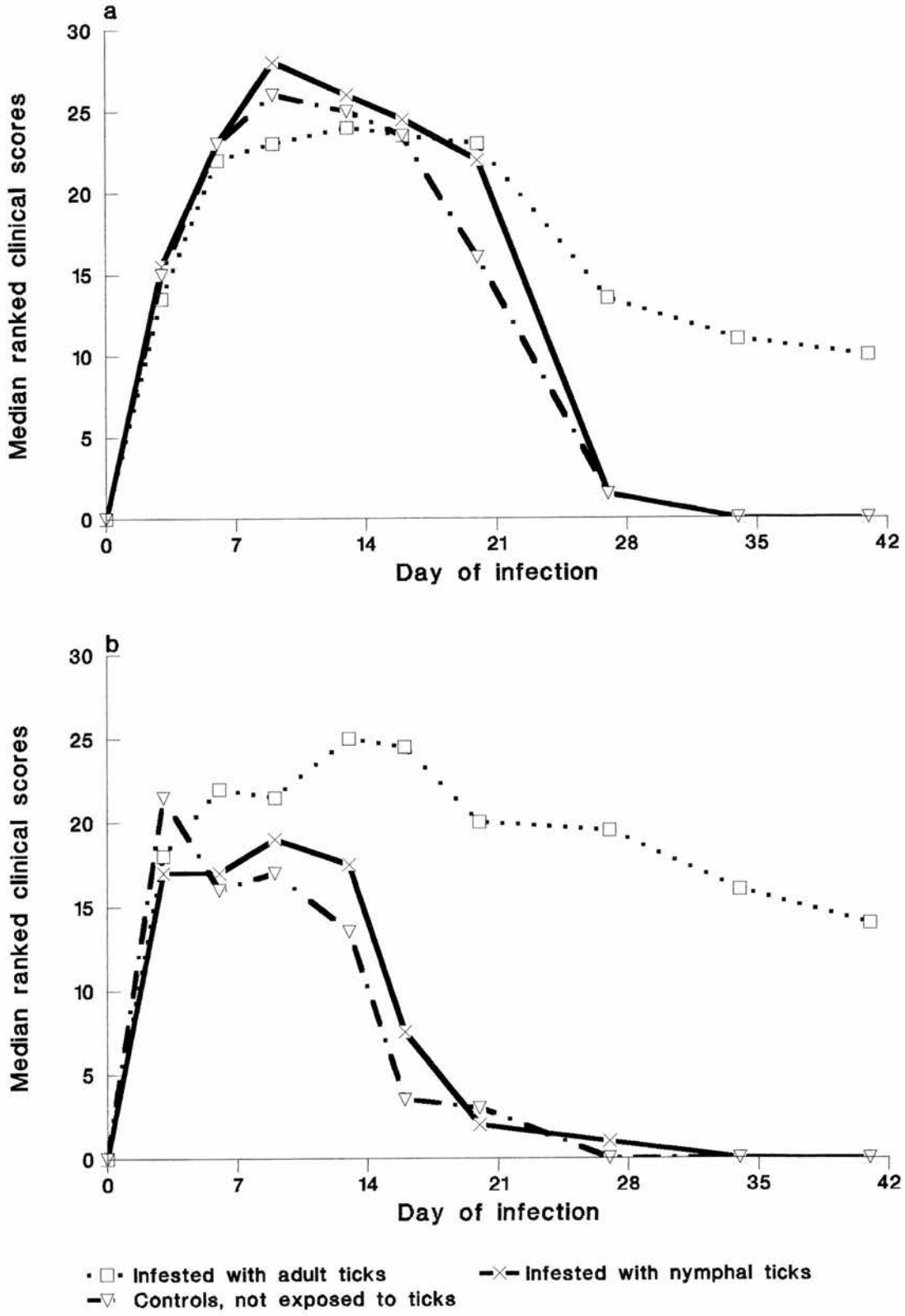
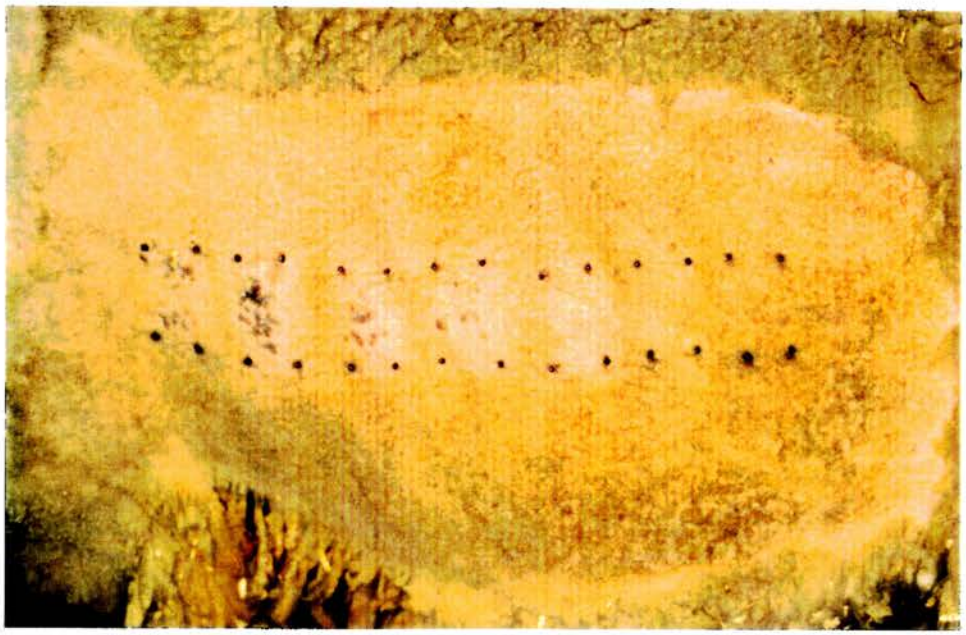


Figure 7.6 The effect of simultaneous infestations of adult or nymphal *Amblyomma variegatum* on the progression of *Dermatophilus congolensis* infections on sheep. a. Primary *D.congolensis* infections, b. Secondary *D.congolensis* infections.

a



b

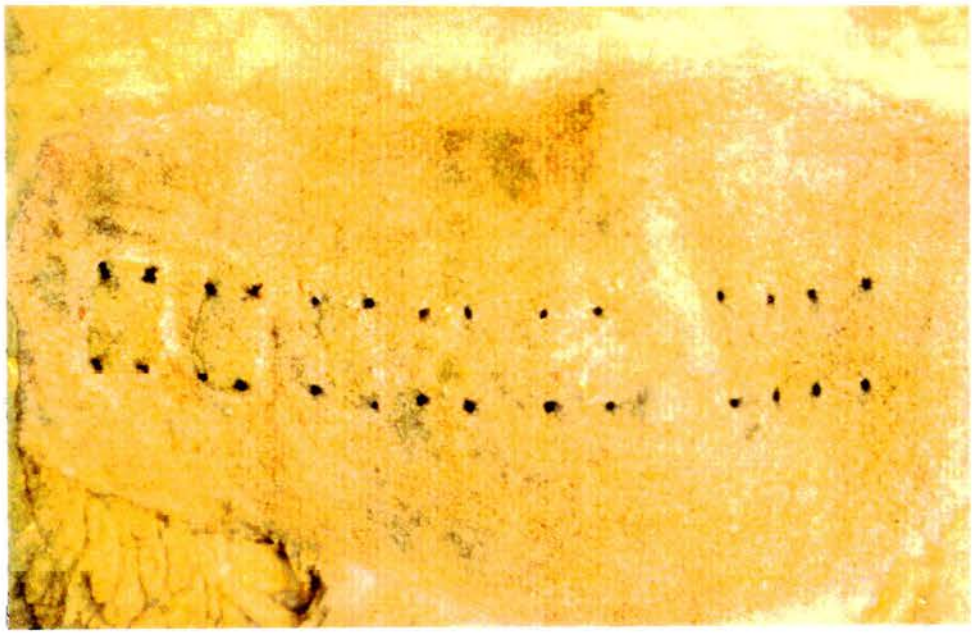


Plate 7.2 Day 27 of primary *Dermatophilus congolensis* infections on sheep. a, On this sheep, infested with nymphal *Amblyomma variegatum*, nearly all the scabs have detached with the only signs of previous infection being the pigmented areas in the skin; b, The dermatophilosis lesions on the sheep infested with adult *A.variegatum* are still quite severe and showing no signs of resolving.

Table 7.4 Comparison of the ranked clinical scores of primary *Dermatophilus congolensis* infections on three groups of sheep; one with a simultaneous infestation of adult *Amblyomma variegatum,* the second with an infestation of nymphs and controls with no exposure to ticks.

Day	Kruskal-Wallis H	k	n	P	Interpretation
3	0.18	3	4	P > 0.05	ns
6	0.36	3	4	P > 0.05	ns
9	1.43	3	4	P > 0.05	ns
13	0.13	3	4	P > 0.05	ns
16	0.04	3	4	P > 0.05	ns
20	0.36	3	4	P > 0.05	ns
27	6.11	3	4	P < 0.05	s
34	5.68	3	4	P > 0.05	ns
41	5.68	3	4	P > 0.05	ns

k = number of groups compared
n = number of sheep in each group
ns = not significant; s = significant

recorded in Appendix 7.4. Using the Kruskal-Wallis test on the scores obtained on individual assessment days during the second infection demonstrated that there was a very significant difference ($P < 0.01$, $k = 3$, $n = 4$) between the severity of the dermatophilosis lesions on the three groups of sheep at day 27, which was maintained up to day 41 when the experiment was terminated (Table 7.5). At day 41, persistent dermatophilosis lesions were recorded on all four of the sheep infested with adult *A.variegatum*, and the lesions on one of these sheep persisted until day 108 after the initial infection.

7.3.2.1.1 Variation in breed susceptibility

Due to restricted availability of stock, six of the sheep used for this study were Blackface x Suffolk, the other six were Blackface. It was not anticipated that a 50% breed difference would lead to such large differences in clinical response as were seen. Two sheep of each breed were included in each of the three experimental groups. It appears from the assessment of the dermatophilosis that the Blackface x Suffolk were more susceptible to infection compared with the Blackface sheep (Figure 7.7).

The Blackface sheep came from a different source than the Blackface x Suffolk sheep and it is possible that they had previously been exposed to *D.congolensis* and developed antibodies to it. Control sera from all twelve sheep was tested for antibodies to *D.congolensis* and it was found that the six Blackface sheep had very high levels of antibodies compared with the six Blackface x Suffolk sheep (Table 7.6). This suggests that the difference in the severity of the dermatophilosis lesions on the two groups of sheep was due to the development of immunity in the six Blackface sheep, rather than difference in breed susceptibility.

Table 7.5 Comparison of the ranked clinical scores of secondary *Dermatophilus congolensis* infections on three groups of sheep; one with a simultaneous infestation of adult *Amblyomma variegatum,* the second with an infestation of nymphs, and controls with no exposure to ticks.

Day	Kruskal-Wallis H	k	n	P	Interpretation
3	0.94	3	4	P > 0.05	ns
6	0.55	3	4	P > 0.05	ns
9	0.13	3	4	P > 0.05	ns
13	0.50	3	4	P > 0.05	ns
16	3.22	3	4	P > 0.05	ns
20	4.23	3	4	P > 0.05	ns
27	7.81	3	4	P < 0.01	vs
34	9.37	3	4	P < 0.01	vs
41	9.41	3	4	P < 0.01	vs

k = number of groups compared
n = number of sheep in each group
ns = not significant, vs = very significant

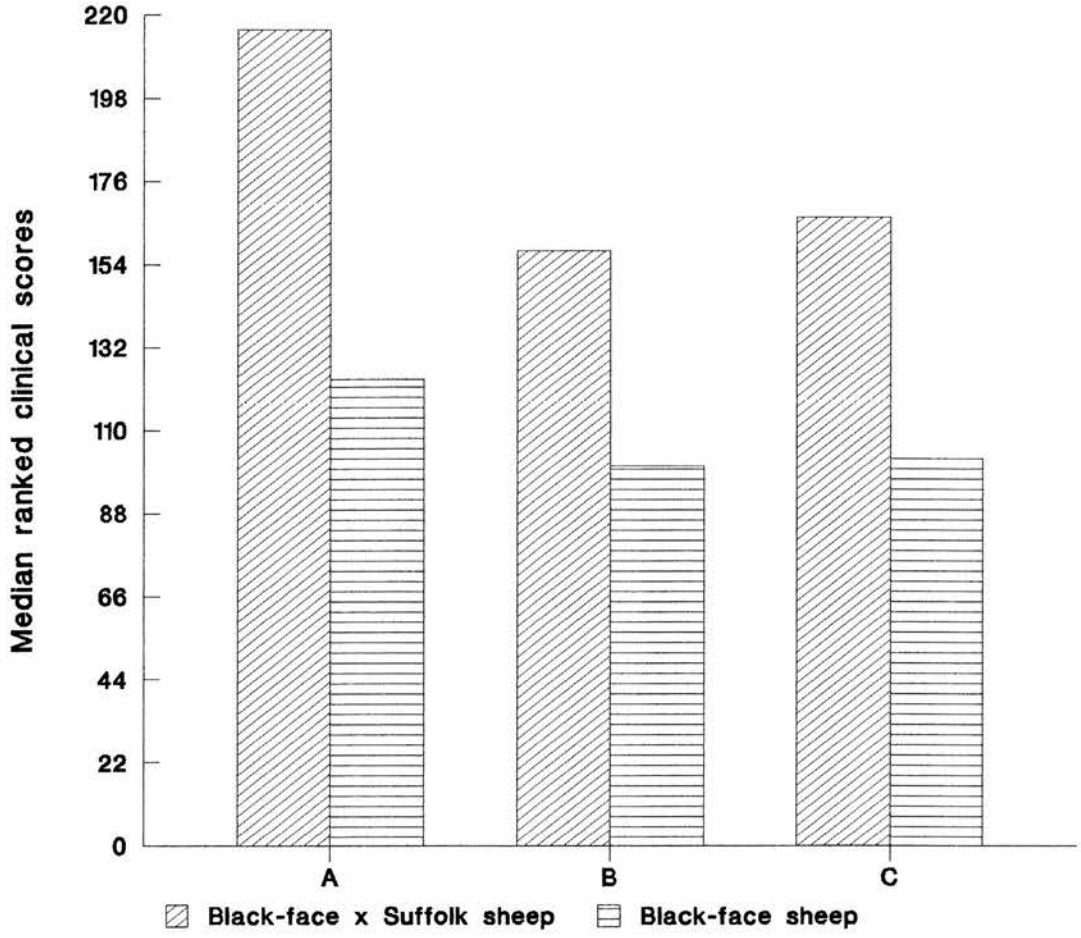


Figure 7.7 Comparison of the severity of dermatophilosis lesions on sheep with a 50% breed difference. A, With simultaneous infestations of adult *Amblyomma variegatum*; B, With simultaneous infestations of nymphal *A.variegatum*; C, With no exposure to *A.variegatum*.

Table 7.6 Highest positive dilutions of sheep sera in response to *Dermatophilus congolensis*, prior to first experimental infection.

Sheep number	Highest positive dilution
Blackface x Suffolk sheep	
1	1:1000
2	1:500
3	1:500
4	1:500
5	Completely negative
6	1:1000
Blackface sheep	
7	1:4000
8	1:8000
9	1:8000
10	1:8000
11	1:4000
12	1:8000

7.3.2.2 Skin test

Using Friedman's test on the median reactions to ovalbumin and *B.abortus* at the five individual skin test sites a very significant difference, $P < 0.01$ and $P = 0.01$ respectively, was observed between the reactions of the three groups of sheep to the antigens. Table 7.7 shows the median reactions of the three groups to ovalbumin and *B.abortus*. Plate 7.3 shows the reduced skin test reaction of a sheep infested with adult *A.variegatum* in response to *B.abortus*, compared with a sheep infested with nymphs of this species.

Distinct immune reactions also occurred at the sites of the booster injections of ovalbumin and *B.abortus*. There were visible differences between the degree of reaction in the three groups of sheep. Skin fold measurements of the individual inoculation sites were taken from the six sheep and compared using Kruskal-Wallis; there was found to be a significant difference between the three groups $P < 0.01$ ($k = 3$ = number of groups compared, $n = 10$ = number of inoculation sites in each group), (Table 7.8). Using the Mann-Whitney test to compare two groups at a time, the skin fold thickness of the sheep infested with adult *A.variegatum* was found to be significantly less ($P < 0.01$) than the skin fold thickness on either of the other two groups of sheep.

7.3.2.3 ELISA

The highest positive dilution of the serum samples was taken as the highest dilution to produce an optical density larger than the lowest dilution of the negative control sera (Voller *et al.*, 1979). Figure 7.8 shows the Log_{10} of 1/the median highest positive serum dilution of the serum samples from each of the three groups of sheep, at each assessment day. Serum samples were collected and analysed

Table 7.7 Skin test reactions of three groups of sheep, the first infested with adult *Amblyomma variegatum*, the second infested with nymphal *Amblyomma variegatum* and the third group not exposed to ticks. Twelve sheep were sensitized with ovalbumin and six sheep with *Brucella abortus*.

Skin test reactions to ovalbumin

Amount of antigen (µg/100µl)	Median reactions (Skin fold x average diameter mm) Infested with adults	Infested with nymphs	Not tick infested
2500	102.8	369.92	246.43
500	74.64	224.57	168.74
100	25.95	91.52	64.65
20	6.6	8.2	7.65
4	4.75	6.4	5.5

Skin test reactions to *Brucella abortus*

Amount of antigen (µg/100µl)	Median reactions (Skin fold x average diameter) Infested with adults	Infested with nymphs	Not tick infested
2500	257.02	489.56	420.99
500	142.45	516.72	516.33
100	175.38	420.97	320.16
20	105.09	303.93	229.52
4	12.31	76.48	167.1

a b

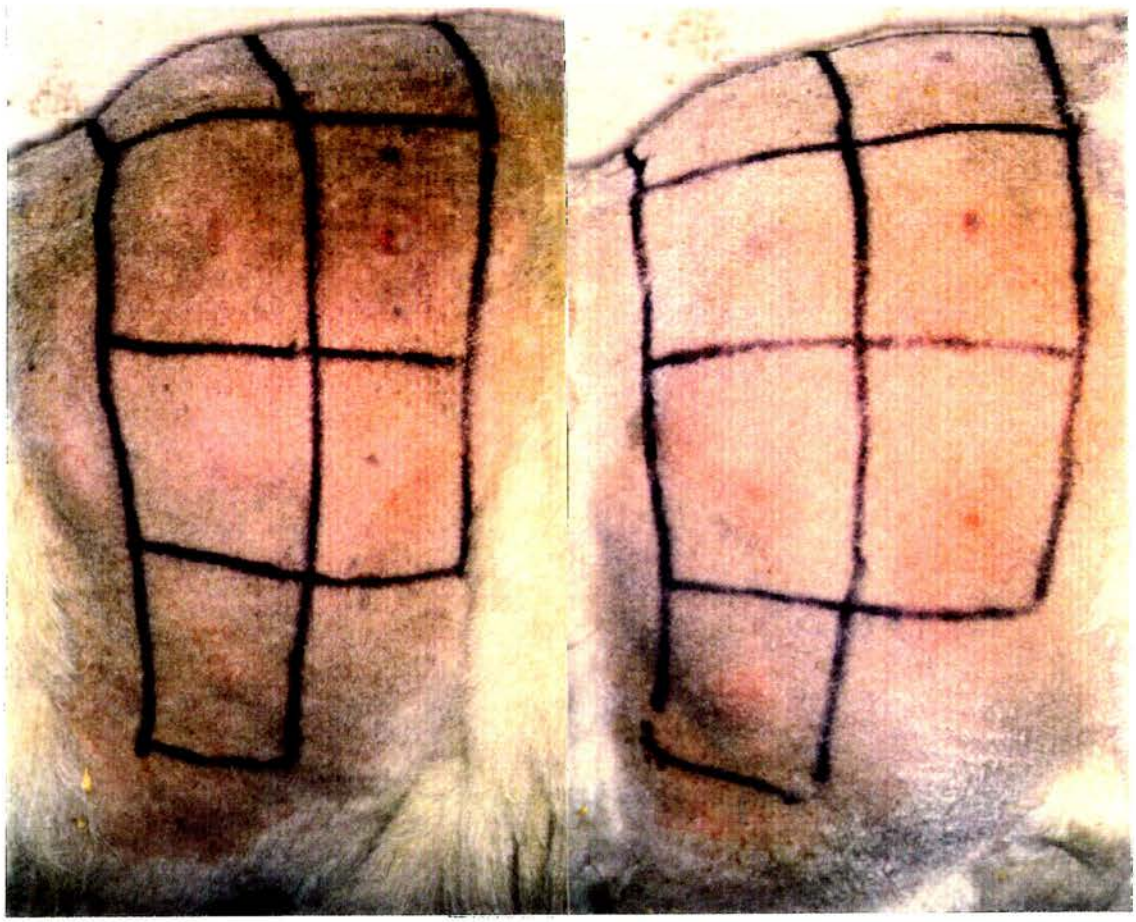


Plate 7.3 Skin test reactions of sheep 24 hours after challenge injections of *Brucella abortus*. a, This sheep has a simultaneous infestation of adult *Amblyomma variegatum*; b, This sheep has a simultaneous infestation of nymphal *A. variegatum*. Note the stronger skin reactions in this sheep compared with the skin reactions of the sheep infested with the adult ticks.

Table 7.8 Skin fold measurements (mm) of the booster inoculation sites of ovalbumin and *Brucella abortus* on sheep.

	Infested with adults	Infested with nymphs	Not tick infested
	9.3	14.6	11.0
	7.1	12.4	12.8
	7.7	10.3	12.1
	7.2	9.1	10.3
	7.0	9.5	10.2
	9.7	13.5	13.6
	8.5	18.4	18.5
	8.4	12.5	17.1
	9.5	10.5	16.3
	10.3	8.7	13.3
Median (Range)	8.45 (7.0-10.3)	11.45 (8.7-18.4)	13.05 (10.2-18.5)

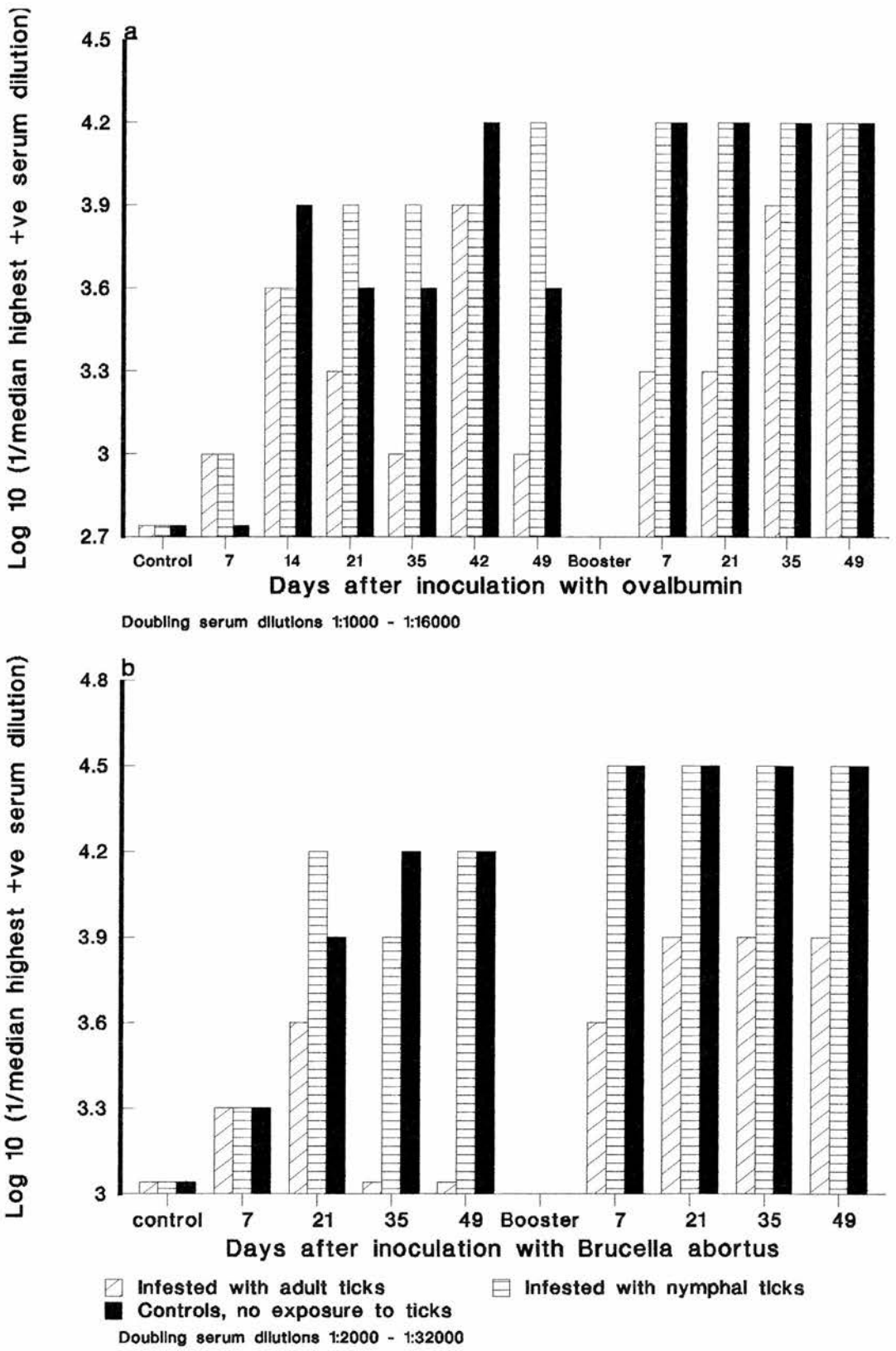


Figure 7.8 The effect of adult or nymphal *Amblyomma variegatum* infestations on the antibody response of sheep to foreign antigens. a. Ovalbumin; b. *Brucella abortus*.

from all 12 sheep from day thirteen after the initial sensitization until 49 days after the booster inoculation.

The antibody response of the three groups of sheep were compared using Log_{10} of 1/the median highest positive dilution for each of the serum samples collected from the individual sheep. Using the Kruskal-Wallis test, a significant difference ($P < 0.01$, $k = 3$ = number of groups compared, $n = 26$ = number of serum samples tested in each group) was recorded between the immune response of the three groups of sheep to ovalbumin. Using Mann-Whitney the responses of the three groups were shown to divide into two very significant classes ($P < 0.01$), with the sheep infested with nymphs and the controls in one class and the sheep infested with adult ticks in another class producing a significantly lower response.

When the results obtained using *B.abortus* were subject to the same analysis as above, the immune responses of the three groups of sheep divided into the same significant classes ($P < 0.01$, $k = 3$, $n = 16$).

7.4 DISCUSSION

The feeding of adult *A.variegatum* ticks has already been shown to aggravate simultaneous *D.congolensis* infections on sheep, resulting in the development of chronic dermatophilosis lesions which persisted for several months (Walker and Lloyd, 1993). The experiments reported in this chapter were carried out to investigate whether the nymphs of this species also have a systemic effect on dermatophilosis lesions. Previous experiments (Chapter Six) have shown that *A.variegatum* nymphs affect the distribution of local dermatophilosis lesions on rabbits, but it is not known whether they also have a systemic effect on remote *D.congolensis* infections.

Simultaneous infestations of adult *A.variegatum* and *D.congolensis* infections on rabbits did not result in the formation of chronic dermatophilosis lesions. The results from the primary infections indicated that the feeding of the adult *A.variegatum* was resulting in more severe dermatophilosis lesions. However, these differences were only manifested in more severe lesions without any delay in the healing. Failure to produce chronic dermatophilosis lesions on rabbits by applying simultaneous infestations of adult *A.variegatum* raises doubts as to whether it is possible to produce chronic dermatophilosis lesions on rabbits.

It may be that it is not possible to produce chronic lesions on rabbits, they are not after all a natural host of *D.congolensis* (Macadam, 1962). There are known to be differences in the cellular immune response to *D.congolensis* in different host species. Roberts (1967) states that rabbit neutrophils phagocytize and destroy *D.congolensis* zoospores, whereas sheep neutrophils do not. The comparison of the systemic effect of nymphal and adult *A.variegatum* on dermatophilosis lesions was therefore repeated on sheep, which are natural hosts of *D.congolensis*.

In this investigation chronic dermatophilosis lesions were reproduced only on sheep simultaneously infested with adult *A.variegatum*, with nymphal tick feeding having no significant effect on the progression of the disease. Evidence from the assessment of the clinical dermatophilosis of reduced immune response in the sheep infested by adult *A.variegatum* has been supported by the results obtained from the skin and serological tests.

The 50% breed difference between the two groups of experimental sheep was not expected to result in a distinct difference in susceptibility to *D.congolensis* infection. Serological analysis to test for antibodies to *D.congolensis* was carried out on control sera from all twelve sheep. This revealed that all six Blackface sheep had relatively high antibody levels to *D.congolensis* compared to the six Blackface x

Suffolk sheep. These two groups of sheep were obtained from different sources and it appears that the Blackface sheep had been previously exposed to *D.congolensis*. However, it is possible that the high levels of *D.congolensis* antibodies were not responsible for the resistance of these sheep to *D.congolensis* infection. There is evidence that circulating antibodies do not provide any protection against further infection (Bida and Kelley, 1976; Roberts, 1965a; Zaria, 1993).

Hart (1976) states that breeds with finer wool, such as Suffolk sheep, are more susceptible to *D.congolensis* infection than hill breeds with coarser wool. The results of the *D.congolensis* infections reported here agree with the observations of Hart (1967) in that the Suffolk x Blackface sheep were more susceptible to infection than the Blackface sheep. It is likely that the 50% breed difference is responsible for the difference in susceptibility to *D.congolensis* infection. The importance of 50% breed difference has been reported in the literature; purebred N'Dama cattle are resistant to dermatophilosis (Coleman, 1967; Oduye, 1975b; Lloyd, 1976) but 50% crossbred N'Dama cattle are killed by dermatophilosis (Coleman, 1967; Lloyd, 1976). These differences in breed susceptibility should have been considered more carefully prior to this investigation into the effect of tick feeding on dermatophilosis. Under the circumstances, only limited stocks of sheep were available and there was no choice but to use the crossbred sheep. However, for future investigations it would be recommended to ensure a reliable supply of sheep, of the same breed.

Serological tests to determine the immune response of the sheep to *D.congolensis* was avoided. Due to the nature of the experiment the immune response of the sheep to *D.congolensis* may have been complicated. The sheep which developed chronic lesions may have had low levels of antibodies which could account for the development of the chronic lesions. On the other hand, the extended exposure to large amounts of *D.congolensis* may have produced relatively high antibody levels,

compared with the sheep with acute dermatophilosis lesions, especially during the late stages of the infections when the acute lesions had healed.

The use of ovalbumin as a T-cell activator proved to be very successful, with strong skin test reactions and distinct ELISA results. Although polyamino acid has been suggested as a suitable foreign antigen (Sigma Catalogue, 1992) it did not work well as a B-cell activator, not producing any skin test reactions in the sheep. It was also not suitable as an antigen for use with the ELISAs. This B-cell activator was replaced by *B.abortus* which had been used as a sensitizing antigen in studies by Ellis *et al.*, (1989).

The humoral and cellular immune responses of the sheep in response to the two foreign antigens were assessed using ELISA and skin testing respectively. Previous attempts in this laboratory to use lymphocyte transformation tests (LTT) to assess cellular immune reactions in sheep have proved unsuccessful and skin testing was included in this study as replacement for LTT.

Due to the simplicity of the skin test procedure this test would be a useful tool for the assessment of cellular immune responses of animals in the field; unlike the more complex procedure of the LTT. Distinct differences in the immune responses of the sheep were apparent at the sites of the booster injections. These reactions were easily measured by taking skin fold measurements, showing significant differences ($P < 0.01$) between the sheep which were infested with adult *A.variegatum* compared with the sheep infested nymphal *A.variegatum*.

The results of the serological and skin tests show that the sheep infested with adult *A.variegatum* developed significantly ($P < 0.01$) lower immune responses than the sheep infested with nymphal *A.variegatum* or the sheep not exposed to tick feeding. These results suggest that adult *A.variegatum* cause a general

immunosuppression of B-cells and T-cells. Nymphs of this species do not appear to have any immunosuppressive effects.

Due to the experimental protocol the sheep infested with adult ticks were subjected to prolonged *D.congolensis* infections with the remaining sheep subjected to acute *D.congolensis* infections. It is possible that the significant difference in the immune reactions of the sheep may have been caused by the different levels of exposure to *D.congolensis*. However, the serological tests show that the immune response to both ovalbumin and *B.abortus* was lower in the sheep infested with adult ticks by day 21 of the primary *D.congolensis* infections. At this time there was no obvious difference between the dermatophilosis lesions on the three groups of sheep. Since the differences in the immune responses of the three groups of sheep were apparent before the divergence in the progression of the lesions it has been concluded that the reduced immunological reactions recorded were a result of the feeding of the adult ticks. This role of tick feeding causing immunosuppression in the host is well documented (Martinez *et al.*, 1992; Wikel and Whelen, 1986).

In conclusion, the assessment of clinical dermatophilosis infections on sheep indicates that the systemic effect of *A.variegatum* is confined to the adults. These findings have important implications for the control of dermatophilosis by control of *A.variegatum*. The populations of adult and nymphal *A.variegatum* vary, with adult and nymphal ticks abundant at different times of the year (Wilson, 1946; Garris and Scotland, 1985). These results suggest that control of dermatophilosis by control of *A.variegatum* would be most cost effective if used during the times of peak numbers of adult *A.variegatum*.

Further experiments are required to compare nymphal and adult *A.variegatum* to determine the factor or factors which enables adult *A.variegatum* to have this immunosuppressive effect on the host. Saliva has been shown to have many

physiological effects on the host including immunosuppression (Ribeiro *et al.*, 1985; Ribeiro, 1987a; Ribeiro *et al.*, 1990; Burger *et al.*, 1991). Therefore the comparison of whole salivary glands and saliva is an obvious starting point for the search for the immunosuppressive factor present only in the adult tick. The following chapter contains the results of a comparison of whole salivary glands and saliva from adult and nymphal *A.variegatum*.

7.5 SUMMARY

1. Simultaneous infestations of adult *A.variegatum* and *D.congolensis* infections on rabbits did not result in the formation of chronic dermatophilosis lesions. It may not be possible to produce chronic dermatophilosis lesions on rabbits; they are not natural hosts of *D.congolensis* (Macadam, 1962).

2. Simultaneous infestations of adult *A.variegatum* and *D.congolensis* infections on sheep resulted in the development of chronic dermatophilosis lesions which lasted for several months. Infestations of nymphal *A.variegatum* had no effect on the progression of simultaneous *D.congolensis* infections.

3. A group of Blackface sheep were more resistant to *D.congolensis* infection compared to a group of Blackface x Suffolk sheep. The Blackface sheep had higher levels of circulating antibodies to *D.congolensis* but these have been shown not to give any protection against reinfection (Bida and Kelley, 1976; Roberts, 1965a). Fifty percent breed differences in cattle have been shown to result in very different susceptibility to *D.congolensis* infection (Coleman, 1967; Lloyd, 1976).

4. General immunosuppression of the host, caused by the feeding of the adult ticks was confirmed by the serological and skin tests. The humoral and cellular immune responses of the T-cells and B-cells were significantly lower ($P < 0.01$) in the sheep infested with adult *A.variegatum*, compared with the sheep infested with nymphs and the sheep not exposed to ticks.

5. Further laboratory studies are required to compare adult and nymphal *A.variegatum* to establish the factor or factors unique to the adult ticks which may be responsible for the immunosuppression.

CHAPTER EIGHT
COMPARISON OF SALIVARY GLAND MATERIAL FROM ADULT AND IMMATURE *AMBLYOMMA VARIEGATUM*

8.1 INTRODUCTION

Simultaneous infestations of adult *Amblyomma variegatum* have been shown to potentiate *Dermatophilus congolensis* infections on sheep, via the systemic route (Walker and Lloyd, 1993). This effect does not appear to extend to the immature stages of this tick. The experiments described in Chapter Seven of this thesis compared the effects of nymphal and adult *A.variegatum* on simultaneous *D.congolensis* infections on sheep. Sheep infested with adult *A.variegatum* showed a significant immunosuppression compared with sheep infested with an equivalent number of nymphal *A.variegatum*. This suggests that adult *A.variegatum* ticks expose their hosts to some factor that is not common with immature stages of the species. This factor could be a distinct molecular entity, introduced into the host during the feeding of the tick. However, it could also result from the physical trauma caused by the adult's mouthparts and prolonged feeding.

Progression of *D.congolensis* infections and the immunosuppressive effects are not noted with simultaneous nymphal infestations (Chapter Seven). This suggests that it could result from injection of material during the feeding of the adult ticks. Various substances, originating from salivary glands and other organs from ticks, have been shown to elicit antigenic responses in the host. Tick toxins produced by *Rhipicephalus evertsi evertsi* are found in the salivary glands (Viljoen *et al.*, 1986). Hosts also demonstrate antigenic responses to haemolymph proteins transported to salivary glands in *Boophilus microplus* (Binnington and Kemp, 1980) and materials found in the gut of *B.microplus* (Gregson, 1960) and *Dermacentor andersoni* (Allen and Humphreys, 1979).

Ixodid tick saliva has been shown to have many physiological effects on the host including anticoagulant (Binnington and Kemp, 1980; Ribeiro, 1987b),

antiinflammatory (Ribeiro *et al.*, 1985) and immunosuppression (Burger *et al.*, 1991; Ribeiro *et al.*, 1985; Ribeiro, 1987a; Ribeiro *et al.*, 1990). Neurotoxins, causing host paralysis during the feeding of *R.e.evertsi* are also found in the salivary glands (Viljoen *et al.*, 1986).

Salivary gland material is obviously very important as a source of physiologically active material to which the host is exposed during tick feeding. This material is an obvious starting point for the comparison of two similar ticks, only one of which causes immunosuppression when feeding on sheep. Comparison of whole salivary gland material and saliva from adult *A.variegatum* with nymphs of the same species eliminates differences due to species.

Visual studies using light microscopy were carried out to study the proportions of individual salivary acini, from each of the three instars, occupied by each of the secretory cell types. Two different granular type acini, containing secretory cells, were found in the salivary glands of *A.variegatum*; these were type-2 and type-3 acini. Four distinct granular cell types were recorded in the type-2 acini and three in type-3 acini; classification of these cell types was based on the methods of Walker *et al.*, (1985) for *Rhipicephalus appendiculatus*.

Whole salivary gland material and saliva from adults and immature instars was compared using gel electrophoresis.

8.2 METHODS

8.2.1 COMPARISON OF SALIVARY GLAND MATERIAL

8.2.1.1 Preparation of salivary gland material for light microscopy

Amblyomma variegatum larvae, nymphs and adults (Section 3.3) were fed on the torso of four rabbits (Section 3.3.4.1). Once the ticks had established themselves on the hosts, samples of ticks in the early and late stages of feeding were removed on a daily basis.

Ticks at early and late stages of engorgement were detached from the host and these, as well as fully engorged ticks, were secured to wax in a dish. Whole salivary glands from the adult and nymphal ticks were dissected out under 0.1% NaCl solution and immediately put into Karnovsky's fixative at 0°C. The material was then washed in 0.1M phosphate buffer prior to embedding in methacrylate resin (for details of embedding method see Section 5.2.1.4). Due to the small size of the larval ticks the entire viscera were dissected out from their integuments and embedded in the same way as the salivary glands. The salivary material was sectioned and 1.5µm sections were stained in 5% Giemsa stain for one hour.

8.2.1.2 Analysis of the material using light microscopy

The analysis of the material involved a study of the granular acini, both type-2 and type-3, in the larvae, nymphs and adults (male and female). The type-4 acini which have been recorded in the males of several ixodid species (Gill and Walker, 1987) were not found in *A.variegatum* salivary glands.

The classification of secretory cell types found in the salivary glands of *R.appendiculatus* by Walker *et al.*, (1985) was used to distinguish the different secretory cell types. Four secretory cell types were recorded in type-2 acini; *a*, *b*, *c1* and *c2* (Plate 8.1). Three secretory cell types were recorded in the type-3 acini; *d*, *e* and *f* (Plate 8.1). For each of the acini studied, the following details were recorded: the number of each cell type within the acini, the area within the acini filled by each of the cell types, and the area within the individual cells filled with secretory granules.

These factors provided an indication of the relative amounts of the different salivary secretions being produced by the different acini within the glands. The following ranking systems were set up to record the area within the acini filled by each cell type and the area within the individual cells filled with secretory granules.

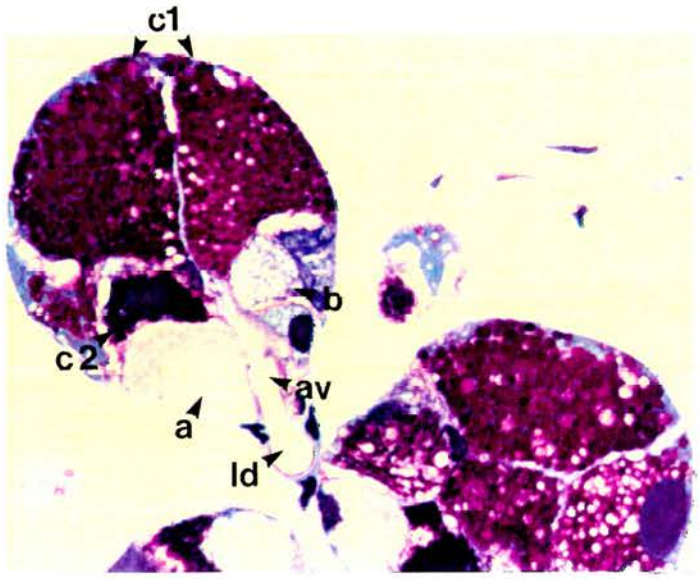
1. The area of the acini filled with a particular cell type, i.e. Cell types *a*, *b*, *c1* and *c2* in the type-2 acini and cell types *d*, *e* and *f* in the type-3 acini.

1% - 20%	-	ranked score of	1
21% - 40%	-	" "	2
41% - 60%	-	" "	3
61% - 80%	-	" "	4
81% - 100%	-	" "	5

2. The area within the cytoplasm of the secretory cells filled with secretory granules.

1% - 25%	-	ranked score of	1
26% - 50%	-	" "	2
51% - 75%	-	" "	3
76% - 100%	-	" "	4

a



b

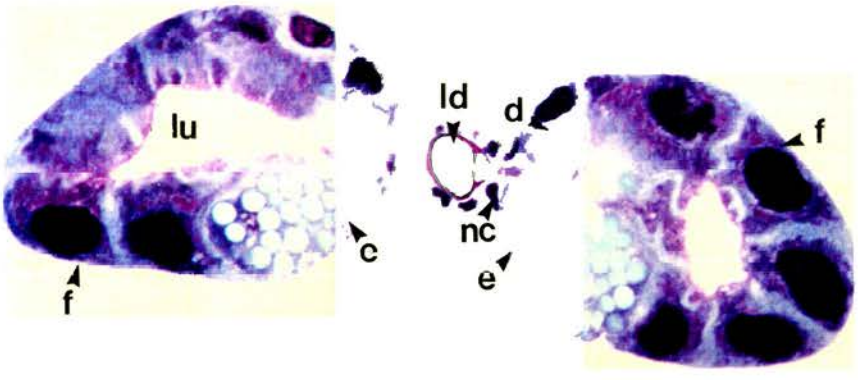


Plate 8.1 *Amblyomma variegatum* salivary glands, stained with 5% Giemsa and alkali buffer (pH 7.2). a, Type-2 acini from a partially engorged adult male; b. Type-3 acini from a partially engorged nymph. *a*, *b*, *c1*, *c2*, *d*, *e*, *f*, = secretory cell types; av = acinar valve; ld = lobular duct; lu = lumen; nc = neck cell.

These factors provided an indication of the relative amounts of the different salivary secretions being produced by the different acini within the salivary glands.

8.2.1.3 Preparation of salivary gland material for gel electrophoresis

All three instars of *A.variegatum* were fed on rabbits; salivary glands from partially fed ticks were dissected out for this study. It was important that no other materials were collected with the glands. To reduce contamination of the secretory gland material the tick gut was removed carefully, with minimum damage. The glands were dissected out under 0.02 TRIS/HCl buffer (pH 8.5) which was replaced regularly to remove contaminants in the buffer solution. As an added precaution, the dissected glands were flushed with fresh buffer before being transferred directly into sodium dodecyl sulphate buffer (SDS) held at 0°C.

The SDS dissolved the glands sufficiently without any prior mechanical trituration. The glands were placed directly into the SDS buffer to reduce loss of the larval salivary glands. Separate stocks of adult male, adult female, nymphal and larval salivary glands were stored at -20°C.

The relative size of salivary glands from all three stages had already been calculated (Section 3.3.3.2) giving a ratio of one adult salivary gland : 30 nymphal salivary glands : 800 larval glands. The following numbers of glands were prepared and equivalent sample sizes for application on to the gel were calculated using the above ratio.

Larvae - 107 pairs of salivary glands were collected into a solution of 100µl SDS + 50µl distilled water.

Nymphs - 28 pairs of salivary glands were put into a solution of 100µl SDS + 50µl distilled water.

Adult males - 6 pairs of salivary glands were put into a solution of 500µl SDS + 500µl distilled water.

Adult females - 6 pairs of salivary glands were put into a solution of 500µl SDS + 500µl distilled water.

8.2.1.4 SDS-Page of whole salivary gland material

The protein content of whole salivary glands dissected from larval, nymphal and adult *A.variegatum* was compared using SDS-polyacrylamide gel electrophoresis. Polyacrylamide gels with gradients of 7 to 20%, with stacking gels of 4.5% were used for the electrophoresis.

The gel was run overnight and stained with silver stain (Morrissey, 1981). The number and position of all the bands were recorded and the similarity of the protein content of salivary glands from the three instars was calculated using the dissimilarity index (Lawson *et al.*, 1980).

8.2.2 THE PRODUCTION OF SALIVA FOR ELECTROPHORESIS

Adult and nymphal *A.variegatum* ticks were fed on rabbits with no previous exposure to ticks (for details of ticks and rabbits see 3.3 and 3.2.1). The adult infestations consisted of five males and five females and the nymphal infestations consisted of 500 ticks. In each case the infestations were applied to the torso and enclosed with a body-bag (Section 3.4.1).

The ticks were left to feed until the final stages of engorgement, then removed from the host for the collection of saliva. Pre-engorged ticks in the final

stages of engorgement, and recently detached fully engorged ticks were easiest to work with. Partially engorged or fully engorged ticks produced more saliva than ticks in the initial stages of engorgement. Moreover, the small size of nymphs in the initial stages of engorgement would have created practical difficulties.

Ticks were used for the production of saliva on the day of engorgement, or for pre-engorged ticks, on the day they were manually detached from the host. Any attachment cement was removed from the mouthparts using forceps. Ticks were secured, dorsal side up, on double sided adhesive tape. In the case of the adult ticks, tape was placed over them to hold them securely. Once a tick was held firmly, the hypostome was inserted into the end of a haematocrit tube, leaving the palps free. The ends of the tubes were constricted by flaming so that they fitted closely around the hypostome, the close fit of the haematocrit tubes was important as a tactile stimulus (Gregson, 1960; Tatchell, 1967) and to ensure that the mouthparts were held securely in place during the saliva production.

Two different drugs were used to stimulate salivation; pilocarpine (Day, 1951; Tatchell, 1967; Howell, 1966; Ribeiro, 1987a); Kerlin and Hughes, 1992) and dopamine (Kerlin and Hughes, 1992). These two drugs were used because of their different modes of action. Pilocarpine is a parasympathetic stimulant and acts on the cholinergic receptors, whilst dopamine stimulates the adrenergic receptors. Artificially stimulated saliva does not necessarily contain all the components of naturally produced saliva; therefore, these two different types of drugs were used in an effort to obtain as many components of natural saliva as possible.

Stimulation of salivation using dopamine (3-hydroxytyramine, Sigma) was done by injection into the ticks. A fresh solution of dopamine at a concentration of 20mg dopamine/ml distilled water was prepared on the day of use and stored at 4°C. For the injection of the dopamine into the ticks the dopamine solution was diluted in

saline, with 100µl of the dopamine solution added to 900µl of 1%w/v NaCl, giving a working dilution of 0.2%w/v dopamine. This solution had to be used within 20 minutes.

Glass needles, made by drawing out haematocrit tubes over a Bunsen flame, were used for the injections. Each adult tick was injected with 20 to 30µl and each nymph with three to five microlitres. Immediately after injection the ticks were placed in 28°C and 100% RH for 45 minutes.

Saliva from the two instars was collected into two separate Eppendorf tubes containing SDS electrophoresis buffer held at 0°C. Histological assessment of adult male and female *A.variegatum* salivary glands (Section 8.3.1.2) and electrophoresis of whole glands (Plate 8.2) showed no differences between the male and female ticks. Saliva from adult males and females was pooled. The haematocrit tubes had previously been calibrated to calculate volume from the length of the saliva column.

Pilocarpine was used for topical stimulation of saliva production. Fresh solutions were prepared each day. The pilocarpine was mixed in an organic solvent at a concentration of 5%w/v of pilocarpine. Two different solvents were used initially, methanol and acetone, but it was found that methanol did not produce very successful results. The final working solution was 25mg pilocarpine in 0.5ml acetone (5%w/v) and 30µl distilled water added to produce a solution of pilocarpine (4.7%w/v).

As soon as the pilocarpine solution was made it was brushed onto the ticks, wetting the entire dorsal surface. Care was taken to ensure none of the solution was drawn into the haematocrit tubes by capillary action. The ticks were left for 45 minutes at 100% RH at a temperature of 37°C (the production of saliva at 28°C was virtually nil when using this method of stimulation).

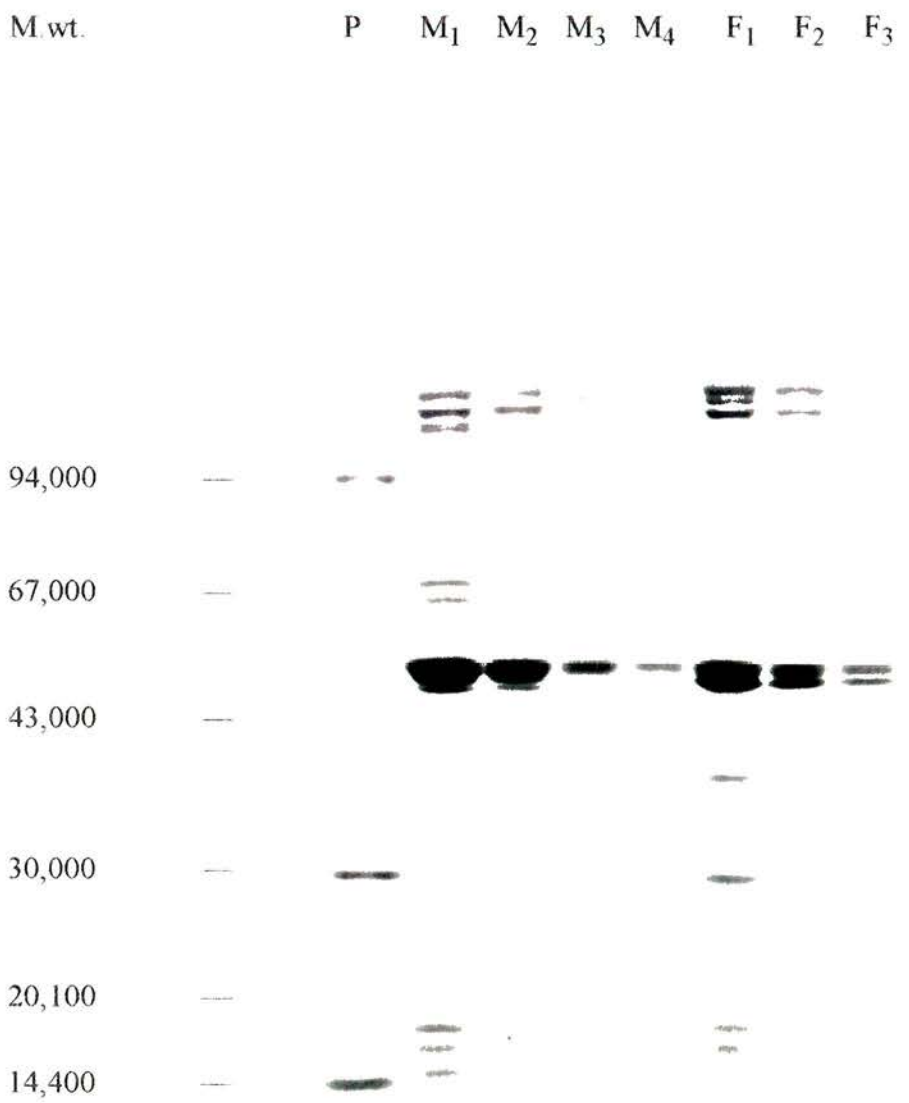


Plate 8.2 SDS-PAGE of whole salivary glands from partially fed adult *Amblyomma variegatum*, stained with Coomassie. P = protein markers; M_1, M_2, M_3, M_4 = doubling dilutions of salivary glands from male ticks; F_1, F_2, F_3, F_4 = doubling dilutions of salivary glands from female ticks. Dilutions start at a concentration of one pair of salivary glands/100µl with 30µl samples applied to gel.

Saliva from adult and nymphal *A.variegatum* was compared using the same method of electrophoresis, staining and analysis as for the whole salivary gland material (Section 8.2.1.4).

8.3 RESULTS

8.3.1 HISTOLOGICAL STUDY OF WHOLE SALIVARY GLANDS

The ranked scores for the proportions of the different secretory granules found in larval, nymphal and adult *A.variegatum* salivary glands at various stages of engorgement, are recorded in Appendices 8.1 to 8.3.

8.3.1.1 Comparison of nymphal acini at first and second stages of engorgement

Separate Mann-Whitney tests were used on the ranked scores for the proportion of each of the seven different acini cell types to compare first and second stage engorged nymphs (Table 8.1). There was found to be a significant difference in the proportion of the *f* cells found in the type-3 acini, with significantly more being observed in the salivary glands of nymphs at the first stage of engorgement compared with the second stage of engorgement ($P < 0.01$, $n_1 = 6$, $n_2 = 6$).

The changes in the median ranked scores of the proportions of the secretory granules in first and second stage nymphal salivary glands are shown in Figure 8.1.

8.3.1.2 Comparison of larval, nymphal and adult acini at the second stage of engorgement

Separate Kruskal-Wallis tests were used to compare the proportions of each of the seven secretory cell types found in the granular acini of the three instars at

Table 8.1 Comparison of the ranked scores of the different cell types found in the salivary acini of *Amblyomma variegatum* nymphs at the first and second stages of engorgement.

Cell type	Mann-Whitney U	n_1	n_2	P	Interpretation
a	12	6	6	P > 0.05	ns
b	7	6	6	P > 0.05	ns
c1	16.5	6	6	P > 0.05	ns
c2	9.5	6	6	P > 0.05	ns
d	18	6	6	P > 0.05	ns
e	11	6	6	P > 0.05	ns
f	0.5	6	6	P < 0.01	vs

n_1 = number of acini in the first group
n_2 = number of acini in the second group
ns = not significant, vs = very significant

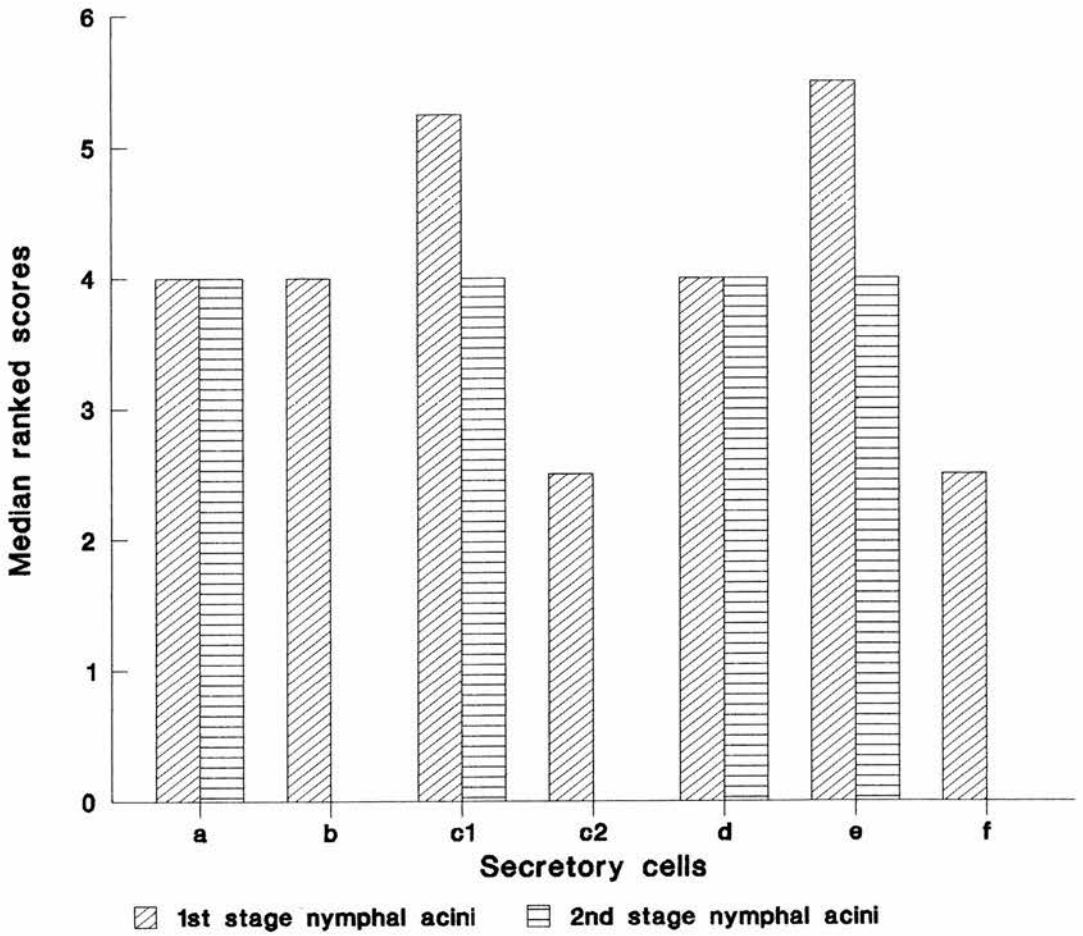


Figure 8.1 Comparison of the median ranked scores of the secretory granules in the salivary glands of nymphal *Amblyomma variegatum*, at first and second stages of engorgement.

the second stage of engorgement. Results from adult males and females were combined as there was no significant difference ($P > 0.05$, Mann-Whitney, $n_1 = 6$, $n_2 = 6$) between the proportions of the secretory cell types found in the salivary glands in the two sexes.

There were significant differences in the space occupied by *c1* ($P < 0.01$, $k = 3$, $n = 14, 6, 12$) and *e* granules ($P < 0.01$, $k = 3$, $n = 14, 6, 12$) in the acini from the three instars (Table 8.2).

The Mann-Whitney test was used to determine where the significant differences occurred. There was significantly more space taken up by *c1* cells in the adult acini ($P < 0.01$), compared with both of the immature stages. There was no significant difference in the amount of *c1* in the larval or nymphal salivary glands. There was significantly greater proportion of *e* type material in the adult salivary glands compared with the larvae ($P < 0.01$) but there was no significant difference between the larvae and the nymphs or the nymphs and the adults.

Figure 8.2 shows the median ranked scores for the proportions of the different secretory granules in the salivary glands of larval, nymphal and adult *A.variegatum* at the second stage of engorgement.

8.3.1.3 Comparison of larval, nymphal and adult acini in engorged ticks

The proportions of the space occupied in the individual salivary acini of salivary glands from engorged ticks was analysed using the same statistical methods as for the glands from ticks at the second stage of engorgement (Table 8.3). There was a significant difference in only one of the cell types *c2* ($P < 0.05$, $k = 3$, $n = 5, 6, 6$). Comparisons of two instars at a time, using the Mann-Whitney test, were all

Table 8.2 Comparison of the ranked scores of the different secretory materials found in larval, nymphal and adult *Amblyomma variegatum* salivary glands at the second stage of engorgement.

Cell type	Kruskal-Wallis H	k	n	P	Interpretation
a	2.3	3	14,6,12	P > 0.05	ns
b	2.47	3	14,6,12	P > 0.05	ns
c1	13.08	3	14,6,12	P < 0.01	vs
c2	4.05	3	14,6,12	P > 0.05	ns
d	4.02	3	11,6,12	P > 0.05	ns
e	11.46	3	11,6,12	P < 0.01	vs
f	4.94	3	11,6,12	P > 0.05	ns

k = number of instars compared
n = number of acini in each of the three groups
ns = not significant, vs = very significant

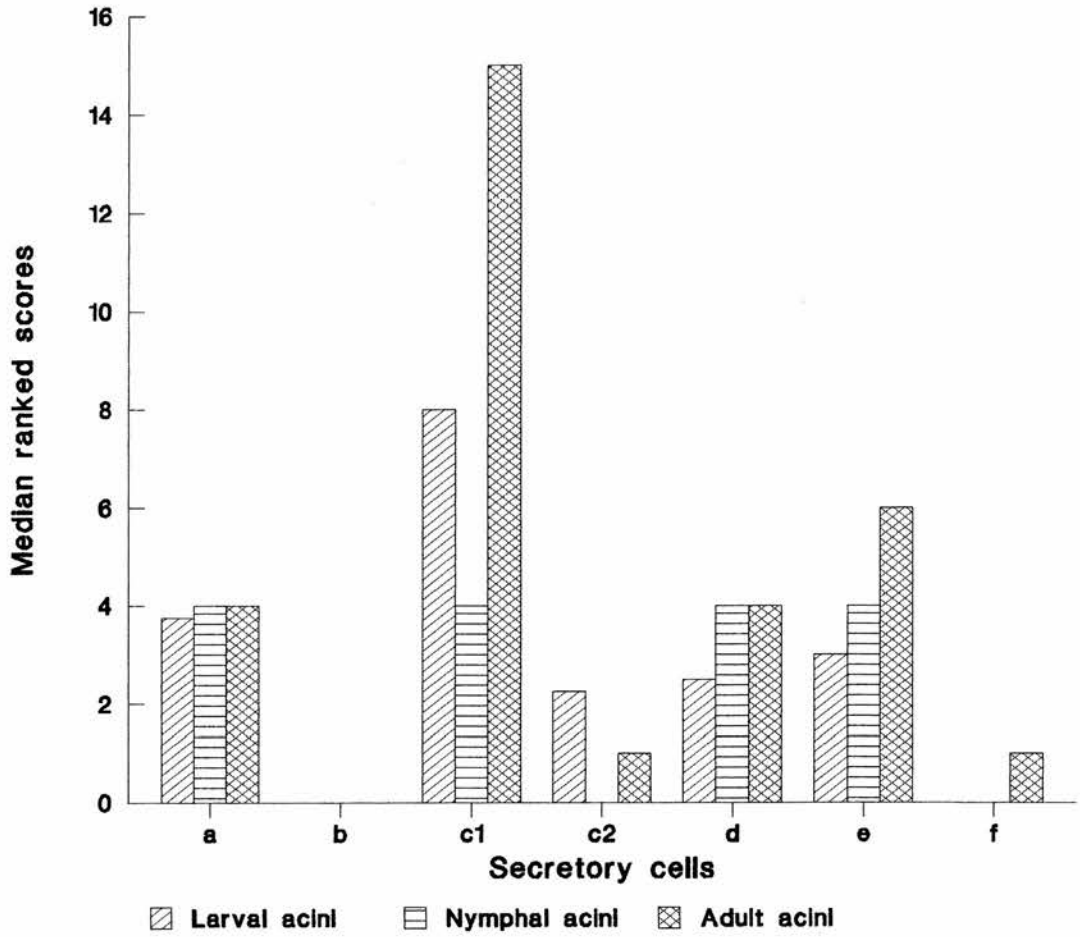


Figure 8.2 Comparison of the median ranked scores of the secretory granules in the salivary glands of larval, nymphal and adult *Amblyomma variegatum* at the second stage of engorgement.

Table 8.3 Comparison of the ranked scores of the different secretory materials found in larval, nymphal and adult *Amblyomma variegatum* salivary glands of engorged ticks.

Cell type	Kruskal-Wallis H	k	n	P	Interpretation
a	3.61	3	5,6,6	P > 0.05	ns
b	2.70	3	5,6,6	P > 0.05	ns
c1	5.10	3	5,6,6	P > 0.05	ns
c2	6.07	3	5,6,6	P < 0.05	s
d	1.46	3	6,6,6	P > 0.05	ns
e	3.70	3	6,6,6	P > 0.05	ns
f	2.42	3	6,6,6	P > 0.05	ns

k = number of instars compared
n = number of acini in each of the three groups
ns = not significant, s = significant

insignificant; therefore, it was not possible to determine where the significant difference occurred.

Figure 8.3 shows the ranked scores for the proportions of the acini occupied by different secretory granules found in salivary glands of engorged *A.variegatum* larvae, nymphs and adults.

8.3.2 SDS-PAGE OF WHOLE SALIVARY GLANDS

There were large numbers of protein bands present in salivary glands from all three instars: 71 bands in the adult female material and 73 bands in salivary glands from both of the immature stages.

However, not enough male salivary gland material had been applied to the gel, and although the major bands were visible, it was not possible to see all of the minor protein bands in the male sample. All of the visible bands in the two adult samples were common to salivary gland material from both male and female *A.variegatum*. Also, histological studies and electrophoresis of whole salivary glands from male and female ticks did not reveal any differences between the two sexes (Section 8.3.1.2 and Plate 8.2). Therefore, comparison of protein bands in the adult material with the salivary gland material from the immature stages was done using the female sample.

Using the index of dissimilarity (Lawson *et al.*, 1980) there was shown to be an 11% dissimilarity between the proteins in adult and immature salivary glands and 0% dissimilarity between nymphal and larval salivary glands.

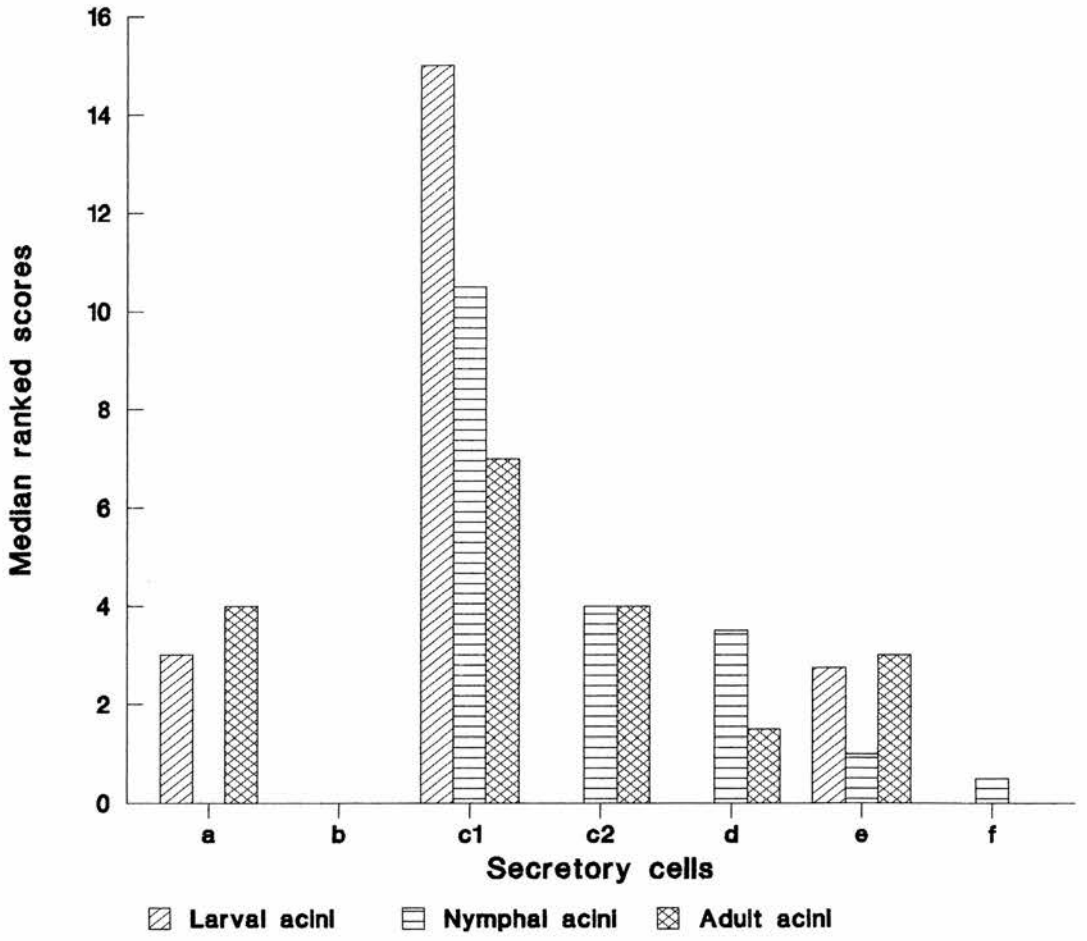


Figure 8.3 Comparison of the median ranked scores of the secretory granules in the salivary glands of engorged larval, nymphal and adult *Amblyomma variegatum*.

There were seven bands unique to the adult salivary gland material, with molecular weights of 79, 77, 51, 37, 35, 31 and 27 kDa (Plate 8.3). Of these seven bands, three were major bands at 37, 35 and 31 kDa.

8.3.3 SDS-PAGE OF SALIVA

Out of a total of 20 bands, 16 were found in the adult saliva and only 11 in nymphal saliva.

Nine bands were unique to adult saliva only, with molecular weights of 82, 76, 67, 42, 40, 38, 37, 36 and 32 kDa (Plate 8.4).

Of these nine bands found only in the adult saliva, one was a major band containing protein with a molecular weight of 67 kDa. However, there was one other major band of protein in the adult saliva, with a molecular weight of 13 kDa, which was only present in very small amounts in the nymphal saliva.

The saliva from the adults and nymphs was compared using the dissimilarity index (Lawson *et al.*, 1980) and there was found to be a 48% dissimilarity (52% similarity) between the saliva produced by the two instars.

8.4 DISCUSSION

Histological comparison of whole salivary glands from *A.variegatum* ticks revealed several differences between the proportions of the salivary acini filled by the different secretory cell types. The most significant differences occurred during the second stage of engorgement with significantly greater proportions of individual adult acini filled with *c1* ($P < 0.01$) and *e* cells ($P < 0.01$) compared with both immature stages and larvae respectively.

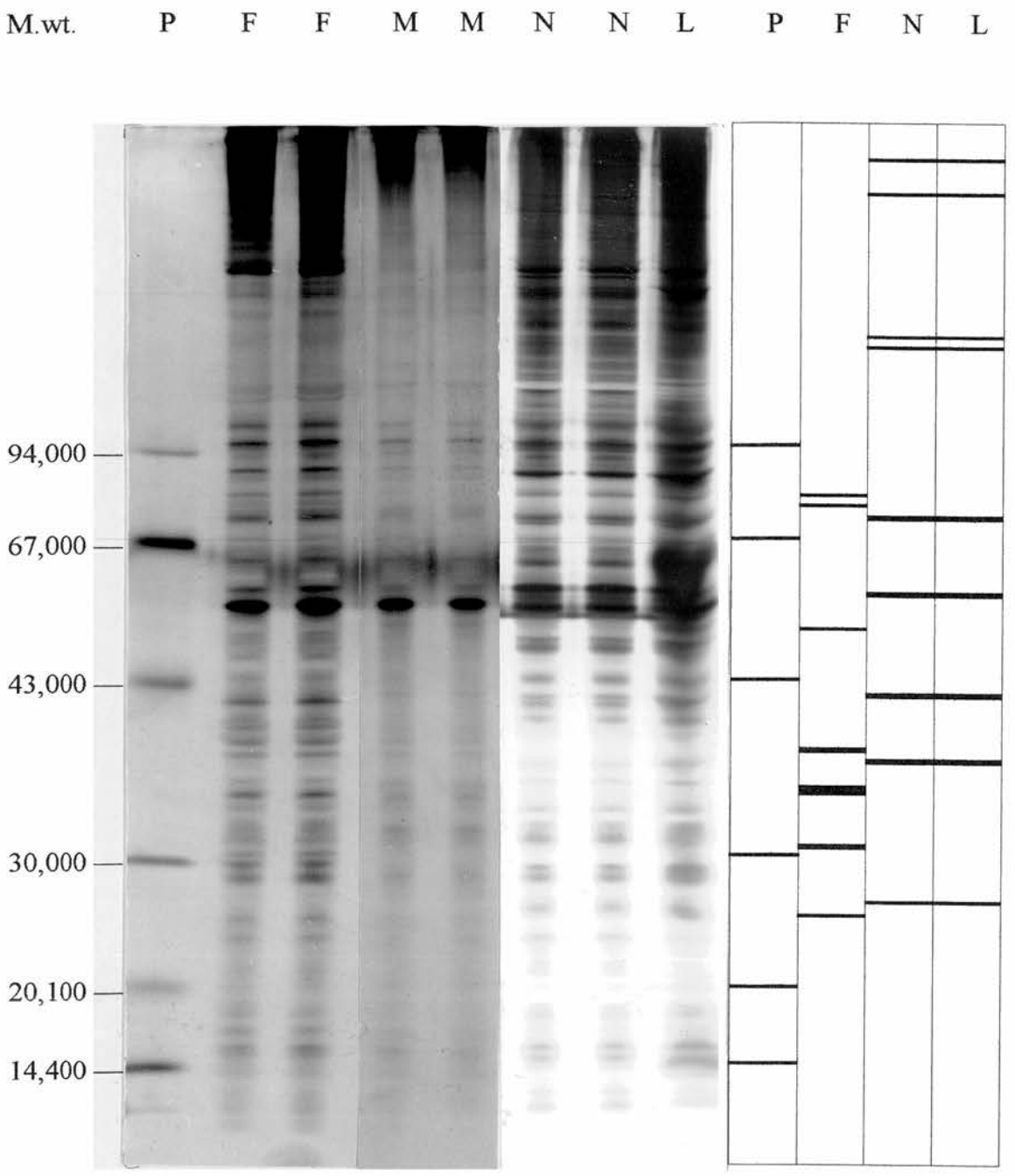


Plate 8.3 SDS-PAGE of whole salivary glands from partially fed female, male, nymphal and larval *Amblyomma variegatum*, stained with silver stain. P = protein marker; F = salivary glands from female ticks; M = salivary glands from male ticks; N = salivary glands from nymphal ticks; L = salivary glands from larval ticks. All samples, except salivary glands from larval ticks, are duplicated. The schematic diagram shows only the bands not common to all instars.

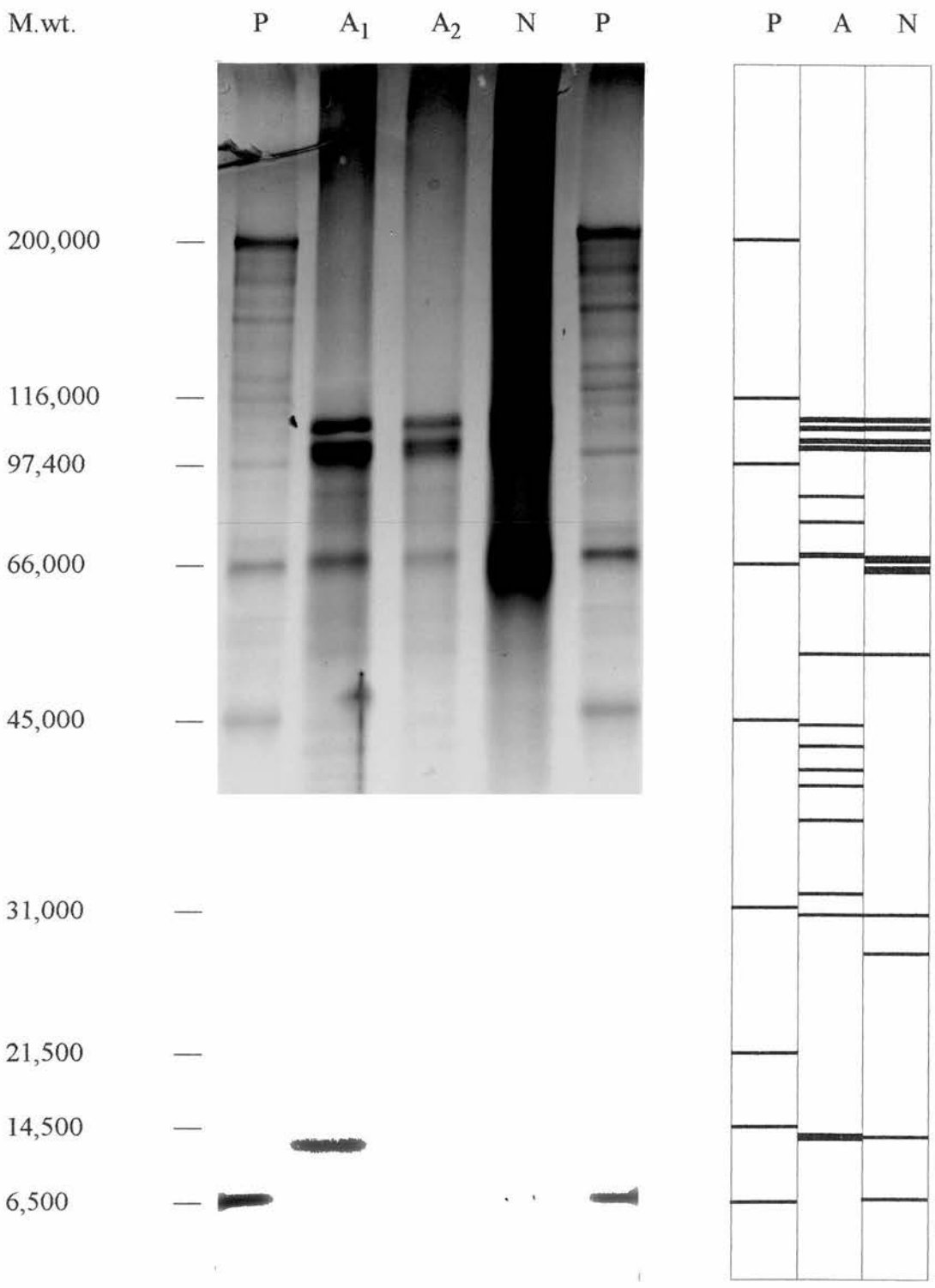


Plate 8.4 SDS-PAGE of artificially induced saliva from adult and nymphal *Amblyomma variegatum*, stained with silver stain. P = protein markers; A_1 = saliva from male and female ticks (30µl); A_2 = saliva from male and female ticks (15µl); N = saliva from nymphal ticks (30µl).

The secretory cell type *c* in *Hyalomma anatolicum anatolicum* and *R.appendiculatus* contain glycoproteins and non-specific esterases (Gill and Walker, 1988; Walker *et al.*, 1985). Geczy and Naughton (1971) suggest that esterases in the saliva of *B.microplus* may increase vascular permeability by hydrolysis of mast cell membranes. The hydrolytic properties of the non-specific esterases may be responsible for causing immense tissue and vascular damage associated with tick feeding lesions (Gill and Walker, 1988). Glycoproteins and other materials from the *c* type cells found in the type-2 acini may be involved in various functions of the saliva, including anticoagulant, vasoactive effects and immunosuppression (Gill and Walker, 1988).

The activity of *c* type cells increases in ticks that are fed on immune hosts (Walker *et al.*, 1985). They suggest that these cells may produce inhibitors that block the action of pharmacologically active substances released from infiltrating leucocytes involved in the host's immune reaction. These *c* cells have been shown to be significantly ($P < 0.01$) more active in adult *A.variegatum* compared with either of the immature instars. This *c* cell may be in response to a greater host immune reaction to the adult tick feeding. Adult *A.variegatum* may elicit a stronger host immune response as a result of the deeper penetration of the mouthparts and the larger volumes of salivary material injected into the host (Latif *et al.*, 1990).

There was also a significant difference ($P < 0.01$) in the proportions of the individual type-3 acini occupied by *e* cells in second stage adult salivary glands compared with larval salivary glands at the same stage of engorgement. However, no significant difference was found between the proportions of type *e* cells found in adult salivary glands compared with nymphal salivary glands at the same stage of engorgement. These cells produce precursors of attachment cement in various species of ticks (e.g. *H.a.anatolicum*, Gill and Walker, 1988).

Electrophoresis of whole salivary glands showed seven polypeptides found only in the adult glands; these were 79, 77, 51, 37, 35, 31 and 27 kDa. Electrophoresis of saliva showed nine polypeptides found only in the adult saliva; these were 82, 76, 67, 42, 40, 38, 37, 36 and 32 kDa.

Using gel electrophoresis and western blotting, Burger *et al.*, (1991) compared whole salivary gland extracts from *Hyalomma truncatum* which did or did not induce sweating sickness. This toxaemic disease is associated with the feeding of *H.truncatum* on cattle and sheep and is thought to be caused by toxins introduced into the host from the ticks (Burger *et al.*, 1991). Only certain strains of *H.truncatum* appear to cause this toxicosis.

Burger *et al.*, (1991) found four polypeptides between 30kDa and 34kDa which occurred in sweating sickness inducing *H.truncatum* and only in very small amounts or not at all in non sweating sickness inducing ticks. Of these four polypeptides only one, with a molecular weight of 32kDa, was demonstrated to be unique to the salivary glands of the sweating sickness inducing *H.truncatum* ticks and to elicit an antigenic response in cattle (Burger *et al.*, 1991). Gel electrophoresis of saliva from adult and nymphal *A.variegatum* has shown that there is a 32kDa polypeptide present, in small amounts in the saliva of the adults only. Electrophoresis of whole salivary glands from the three instars of *A.variegatum* revealed a major band at 31kDa present only in the glands from the adult ticks.

Saliva produced by artificial stimulation may be very different from saliva produced under natural circumstances. Further studies of the protein content of whole salivary gland material could be carried out using two dimensional electrophoresis or high pressure liquid chromatography.

Willadsen and Riding (1979) have characterized a proteolytic-enzyme inhibitor from larval *B.microplus*. This protein was shown to produce an allergic reaction in cattle, indicating that the hosts were exposed to the material during tick feeding. This protein was shown to inhibit trypsin and chymotrypsin and they suggest that it may also affect similar enzymes involved in the hosts immune response. It may be that the important factor associated with the immunosuppression caused by adult *A.variegatum* is also a protein introduced during feeding. However, the material may be regurgitated by the tick rather than being produced as a functional component of saliva (Gregson, 1967).

The study of polypeptides present in salivary material from the different instars of *A.variegatum* is looking only at one of the many possible sources of the factors which may be involved in the immunosuppression associated with adult *A.variegatum* (Chapter Seven). The material produced by adult *A.variegatum* which is responsible for the immunosuppression of sheep, demonstrated in Chapter Seven, may be produced as a by-product of the water regulatory function of the salivary glands when acting as osmoregulatory organs during engorgement (Tatchell, 1969b). In which case the material would only be found passing through the salivary glands during a limited period during the engorgement. The additional polypeptides found in the adults could be associated with reproductive processes and not have a direct role in salivation.

Another possibility is that the material, as produced by the tick, may not be directly responsible for the immunosuppression, but acts indirectly through changing some factor already in the host animal (Gregson, 1973). This may allow small amounts of material produced by tick feeding to have a much larger effect on the host.

This investigation has demonstrated significant differences between the three instars of *A.variegatum*, both in the histological studies of whole salivary glands and the electrophoretic studies of artificially induced saliva. Not only does the feeding of adult *A.variegatum* expose the host to different proportions of secretory materials, but also substances unique to the adults. These differences may be very important in the search for the factor which allows *A.variegatum* adults to have an immunosuppressive effect on sheep (Chapter Seven).

8.5 SUMMARY

1. Whole salivary glands from adult, nymphal and larval *A.variegatum* ticks were compared for structural differences by light microscopy and for protein content by gel electrophoresis.

2. Significantly ($P < 0.01$) greater proportions of individual type-2 salivary gland acini from adult ticks was filled with *c1* cells, compared with either of the immature instars. This same cell type in *H.a.anatolicum* and *R.appendiculatus* contain glycoproteins and non-specific esterases (Gill and Walker, 1988; Walker *et al.*, 1985).

3. Significantly ($P < 0.01$) greater proportions of individual type-3 salivary gland acini from adult ticks was filled with *e* cells, compared with the larval ticks.

4. Gel electrophoresis demonstrated an 11% dissimilarity between salivary glands from adult and immature *A.variegatum*. Seven bands were unique to the adult material, three were major bands at 37, 35 and 31 kDa.

5. Gel electrophoresis demonstrated a 48% dissimilarity between artificially stimulated saliva from adult and nymphal *A.variegatum*. Nine bands were unique to

saliva produced by the adults. One of these bands contained protein with a molecular weight of 32kDa; a band of protein of the same size has been shown to be unique to sweating sickness inducing *H.truncatum*, and to elicit an antigenic response in cattle (Burger *et al.*, 1991).

6. Further studies are required to determine if the proteins, shown to be unique to the salivary glands and saliva of adult *A.variegatum* during this investigation, are introduced into the host during feeding. If so, further analysis and characterization of the proteins need to be carried out, using high performance liquid chromatography.

CHAPTER NINE
GENERAL DISCUSSION

9.1 LOCAL EFFECTS OF THE FEEDING OF *AMBLYOMMA VARIEGATUM* ON DERMATOPHILOSIS LESIONS

There have long been observations in the field associating the feeding of *Amblyomma variegatum* ticks with the development of severe, chronic dermatophilosis lesions. These lesions are often at the attachment sites of *A.variegatum*. Macadam (1964b) observed severe scrotal lesions at the feeding sites of adult *A.variegatum* on White Fulani cattle. There are many reports of initial dermatophilosis lesions developing on the ventral surfaces of cattle (Plowright, 1956; Macadam, 1964c; Oduye, 1975; Bwangamoi, 1976) associated with the predilection sites of adult *A.variegatum* (Butler, 1975; Oduye, 1975; Oduye, 1976; Bwangamoi, 1976). However, there are also many reports of primary dermatophilosis on the dorsal surfaces of cattle, remote from the adult tick feeding sites (Zlotnik, 1955; Macadam, 1964b; Macadam, 1964c; Morrow *et al.*, 1989). These dorsal lesions have been associated with immature ticks and biting flies (Plowright, 1956; Macadam, 1964c; Stewart, 1972).

The association between the tick attachment sites and the dermatophilosis lesions may be due to physical damage to epidermis. Physical damage is thought to be required to allow penetration of *D.congolensis* into the epidermis and the initiation of dermatophilosis lesions (Stewart, 1972; Oduye, 1975; Oppong, 1976). Stewart (1972) found *D.congolensis* in puncture wounds caused by the tabanid biting flies *Haematopota albihirta* and *Tabanus taeniola*. However, other workers have found that physical damage alone, at the site of inoculation with *D.congolensis*, was not enough to facilitate the formation of dermatophilosis lesions (Macadam, 1964c; Martinez *et al.*, 1992).

Work by Davis and Philpott (1980) has shown that hypersensitivity reactions in the skin of goats resulted in persistent dermatophilosis lesions. These lesions were prolonged as long as hypersensitive reactions persisted in the skin. The initial experiments reported here were set up to test the hypothesis that the hypersensitive reactions in the host skin in response to the feeding of *A.variegatum* would result in the formation of chronic dermatophilosis lesions.

Neither hypersensitive nor inflammatory reactions in response to immature *A.variegatum*, in the skin of rabbits and sheep, had any significant effect on the progression of subsequent *D.congolensis* infections (Chapter Five). These lesions were produced by inoculation of *D.congolensis* at tick attachment sites, after the removal of the tick. Granulomas were visible in the skin, and biopsies of the attachment sites demonstrated relatively large numbers of infiltrating granulocytes. However, there was no active stimulation of the cellular immune responses by the presence of the ticks during the course of the infections. Davis and Philpott (1980) repeatedly stimulated hypersensitivity reactions throughout the duration of the *D.congolensis* infections on the goats. This repeated stimulation was representative of the situation in the field where infected animals would be subjected to repeated tick and insect attack prior to, and during, any infection.

Infestations of nymphal *A.variegatum* feeding on rabbits were used to study the local effect of immature *A.variegatum* ticks on simultaneous *D.congolensis* infections (Chapter Six). The feeding of nymphal *A.variegatum* was found to have only a limited effect on the local lesions. There was found to be a strong positive association between the inflammatory tick attachment sites and the dermatophilosis lesions, but not between the hypersensitive tick attachment sites and the lesions. This lack of association between the hypersensitive sites and the lesions may be due to the

repeated detaching and reattaching of the ticks as a result of the strong host immune reactions (Latif *et al.*, 1990).

The association between the tick feeding sites and the dermatophilosis lesions may be due to the physical damage allowing the penetration of the zoospores into the epidermis. Also, substances introduced into the host with the saliva may be causing local immunosuppression. Ribeiro *et al.*, (1990) found that neutrophils were inhibited by saliva from the tick *Ixodes dammini*. They only studied saliva from adult female ticks but it is possible that saliva from nymphal *A.variegatum* may have similar immunosuppressive effects. Saliva from numerous species of ixodid ticks has been shown to have immunosuppressive effects (Ribeiro *et al.*, 1985; Ribeiro, 1987; Ribeiro *et al.*, 1990; Burger *et al.*, 1991). This immunosuppression may have evolved to stop the host immune responses interfering with the feeding of the ticks (Wikel and Whelen, 1986). However, this immunosuppression may also interfere with the host's immune response to invading pathogens, at the site of the tick attachment (Wikel and Whelen, 1986).

Whatever the mechanism which allowed the initiation of dermatophilosis lesions at the nymphal tick attachment sites, there was only a limited effect on the resulting dermatophilosis lesions. There was some increase in the initial severity of the dermatophilosis lesions at the feeding sites of the nymphal *A.variegatum*. However, this increased severity was not persistent and there was no progression into chronic dermatophilosis lesions.

These results suggest that the feeding of nymphal *A.variegatum* is not associated with the development of chronic dermatophilosis lesions. However, there is now some doubt that it is possible to produce chronic lesions on rabbits (Chapter Seven). The systemic effect of the feeding of adult *A.variegatum* has been shown to have a significant effect on the progression of dermatophilosis lesions on sheep

(Chapter Seven; Walker and Lloyd, 1993) but it was not possible to produce this effect on dermatophilosis lesions on rabbits (Chapter Seven). It is therefore recommended that further investigations into the effect of immature *A.variegatum* should be carried out using sheep or cattle as the experimental hosts.

9.2 SYSTEMIC EFFECTS OF THE FEEDING OF *AMBLYOMMA VARIEGATUM* ON DERMATOPHILOSIS LESIONS

Adult *A.variegatum*, in particular, have been associated with the development of chronic dermatophilosis lesions in the field (Macadam, 1964c; Oduye, 1975; Bwangamoi, 1976; Morrow *et al*, 1989). However, it has been observed that although adult ticks are associated with severe dermatophilosis lesions, the ticks are often not feeding at the site of the primary lesions. Severe, generalised lesions are often found on the dorsal surfaces of animals infested with adult *A.variegatum* (Plowright, 1956; Macadam, 1964c; Butler, 1975; Oduye, 1976).

Adult *A.variegatum* ticks have been shown to have a significant systemic effect on remote *D.congolensis* infections on sheep, resulting in the development of chronic dermatophilosis lesions which last for several months (Walker and Lloyd, 1993). The results in Chapter Seven of this thesis demonstrate that this systemic effect is restricted to the adult ticks only.

This finding has important implications for the control of dermatophilosis by dipping to control *A.variegatum*. Populations of adult and immature *A.variegatum* peak in abundance at different times of the year (Wilson, 1946; Garris and Scotland, 1985). Effective control of both adult and immature *A.variegatum* would involve extended periods of dipping, during the times of peak abundance of both adult and

immature *A.variegatum*. The comparison of the systemic effect of adult and nymphal *A.variegatum* suggests that the most cost effective control of dermatophilosis, by control of this tick, would be achieved by dipping to control the adult population.

Serological and skin tests have demonstrated significantly ($P < 0.01$) lower T-cell and B-cell immune responses in sheep infested with adult *A.variegatum* compared with the immune responses of sheep infested with nymphal *A.variegatum*. The epizootiology of chronic dermatophilosis is very complex and the disease is often associated with immunosuppression (Munz, 1976) and intercurrent diseases (Plowright, 1956; Stewart, 1972; Munz, 1976; Oppong, 1976). It could be that the general immunosuppression caused by the feeding of adult *A.variegatum* is connected with the frequent occurrence of intercurrent diseases. However, the immunosuppression of these ticks in relation to the intercurrent diseases would probably be only part of a complex interaction of immunosuppression caused by the chronic dermatophilosis (Oduye, 1976) and other factors associated with chronic dermatophilosis.

9.3 COMPARISON OF ADULT AND IMMATURE *AMBLYOMMA VARIEGATUM*

The investigation into the systemic effect of *A.variegatum* on dermatophilosis lesions on sheep has demonstrated that the feeding of only the adults of this tick is associated with the development of chronic dermatophilosis lesions (Chapter Seven; Walker and Lloyd, 1993). Sheep that are infested with adult *A.variegatum* have a significantly ($P < 0.01$) impaired immune response. Thus, it appears that the feeding of adult *A.variegatum* introduces a factor, or factors, into the host that is responsible for the immunosuppression. Experiments were carried out to

compare whole salivary glands and artificially induced saliva from adult and immature *A.variegatum* (Chapter Eight).

Significantly ($P < 0.01$) greater proportions of individual type-2 salivary gland acini from adult *A.variegatum* were filled with *c1* cells, compared with either of the immature instars. This cell type in *Hyalomma anatolicum anatolicum* and *Rhipicephalus appendiculatus* contain glycoproteins and non-specific esterases (Gill and Walker, 1988; Walker *et al.*, 1985). There were also significantly ($P < 0.01$) greater proportions of individual type-3 salivary gland acini from adult *A.variegatum* filled with *e* cells, compared with larval *A.variegatum*.

Electrophoresis of whole salivary glands and saliva revealed several polypeptides unique to adult *A.variegatum*. These comparative studies of material in adult and immature *A.variegatum* are only a small part of the search for a possible candidate for the immunosuppression caused by the adult ticks. Further analysis of the polypeptides unique to the adult saliva could be carried out using high performance liquid chromatography. However, the important factor may not be a protein.

Various tick materials, besides salivary gland material or saliva, have been shown to produce antigenic responses in the host. Binnington and Kemp (1980) demonstrated antigenic responses of the host to haemolymph proteins which were transported via the salivary glands into the host. Other materials which have been shown to produce antigenic responses in the host are the gut (Gregson, 1960; Allen and Humphreys, 1979) and tick attachment cement (Gregson, 1973).

The important factor may be a by-product of sexual maturation of the adult ticks which may be excreted into the host during the later stages of engorgement (Morel, 1989). Tick paralysis, associated with the feeding of adult female

Dermacentor andersoni and other ticks, does not occur until four of five days after attachment and coincides with egg development in the tick (Gregson, 1973).

Obviously, the comparative studies of material from adult and immature *A.variegatum* which have been carried out in this study have covered only a small part of a very extensive subject. However, differences between salivary material from adult and immature *A.variegatum* have been demonstrated and these may provide important clues in the search for the immunosuppressive material unique to the adults.

9.4 CONCLUSIONS

The feeding of nymphal *A.variegatum* appears to have only a limited effect on dermatophilosis lesions by affecting the distribution but not the persistence of the lesions. However, the investigation into the effect of immature *A.variegatum* was carried out using rabbits as the experimental hosts. Rabbits are not natural hosts of *D.congolensis* (Macadam, 1962) and the results of chapter seven indicate that it is not possible to produce chronic dermatophilosis lesions on them. Further investigations of the local effect of *A.variegatum* need to be carried out using sheep or cattle as the experimental hosts.

The feeding of *A.variegatum* ticks significantly affects the progression of *D.congolensis* infections on sheep, resulting in the development of chronic dermatophilosis lesions. However, this systemic effect is confined to the adults.

There have been found to be different relationships between the feeding of *A.variegatum* and *D.congolensis* infections on different host animals. Simultaneous infestations of adult *A.variegatum* and *D.congolensis* infections resulted in the development of chronic dermatophilosis lesions on sheep but not on rabbits.

Therefore, further experiments are required, using cattle as the experimental hosts, to confirm the systemic effect of adult *A.variegatum* on dermatophilosis lesions.

In this study, the effect of individual and breed variation in susceptibility to *D.congolensis* infection was a problem. The greatest difficulty was the acquisition of suitable sheep. Some sheep were obtained from the Moredun Research Institute, but this was not always possible. Other sheep came from various farms and may have had previous exposure to ticks or other parasites. The ubiquitous nature of *D.congolensis* was also a problem, with some of the experimental sheep showing high antibody levels to *D.congolensis*, prior to experimental infection (Chapter Seven).

A reduction in the effect of individual variation on the experimental results could be achieved by using larger sample sizes. This was not possible in this situation due to the very limited space available for isolated experimental animals. Another option would be to establish a reliable source of experimental animals kept under known, constant conditions from birth. The use of identical twins as experimental pairs could also reduce intrinsic host variation.

Some of the important questions concerning the relationship between the feeding of *A.variegatum* and chronic dermatophilosis lesions have been answered, at least in part. The feeding of adult *A.variegatum* does result in the progression of *D.congolensis* infections into chronic dermatophilosis lesions. This systemic effect is confined to the adult ticks. The feeding of nymphal *A.variegatum* affects the distribution of local dermatophilosis lesions but does not result in chronic lesions.

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APPENDICES

Appendix numbers correspond to the relevant chapter number.

APPENDIX 3.1

Staining of whole salivary glands in 1% Methyl green.

1. Dissect out whole glands under phosphate buffered solution.

2. Fix in 100% ethanol for 2 minutes.

3. Stain in 1% methyl green for 10 minutes.

4. Wash in tap water to remove excess stain.

5. Dehydrate the glands by placing in 70% ethanol, followed by two quick changes of 100% ethanol (it is easier to change the ethanol rather than transfer the glands).

6. Transfer dehydrated glands to xylene.

7. The glands are then ready for mounting.

APPENDIX 3.2

Total volumes of type-2 and type-3 salivary gland acini found in larval, nymphal and adult *Amblyomma variegatum*. Measurements recorded from whole salivary glands stained in 1% methyl green.

	Type-2 acini		Type-3 acini		
	Average diameter (μm)	Average number	Average diameter (μm)	Average number	Total volume of type-2 & 3 acini/gland (μm^3)
Adult *Amblyomma variegatum*					
Male	69.80	519	60.56	2038	3.29×10^8
Female	65.67	379	70.62	2256	4.72×10^8
	Average of male and female				4.01×10^8
Nymphal *Amblyomma variegatum*					
	66.83	31	63.20	82	1.57×10^7
Larval *Amblyomma variegatum*					
	40.92	9	55.64	2	5.03×10^5

APPENDIX 5.1

Karnovsky (1965) fixative and phosphate buffer

0.2M Phosphate buffer

1. Calcium chloride anhydrous = 0.08g ($CaCl_2 2H_2O$ = 0.10g).

2. Sodium dihydrogen orthophosphate $NaH_2PO_4.2H_2O$ = 3.94g.

3. Disodium hydrogen orthophosphate $Na_2HPO_4.12H_2O$ = 23.2g (or Na_2HPO_4 = 9.2g).

To obtain 0.2M phosphate buffer the above compounds were dissolved in 450ml of distilled water using the following procedure.

a. Dissolve 1. in 200ml of the distilled water. Compound 2. is added to the solution when 1. has dissolved completely. This is important because 2. will react with any undissolved 1.

b. Dissolve 3. in the remaining 250ml of the distilled water. When it has completely dissolved it is added to the above solution.

0.2ml phosphate buffer is used to make the Karnovsky fixative; for washing biopsies it is diluted to 0.1ml

0.1M Karnovsky Fixative (pH 7.2)

1. 40ml 25% glutaraldehyde.

2. 22.2ml 36% formaldehyde.

3. 200ml 0.2M Phosphate Buffer.

a. Measure out the glutaraldehyde and formaldehyde in a fume cupboard.

b. Add 3. to the above mixture.

c. Add distilled water to bring the volume up to 400ml

The buffer and fixative can be stored at 4°C until required.

APPENDIX 5.2

Changes in average mass, engorgement success and moulting success of repeated infestations of *Amblyomma variegatum* larvae feeding on rabbits.

HOST	Average mass of engorged ticks mg (S.D.) (X)	Number of engorged ticks/Number applied (Y)	Number of moulted ticks/Number put to moult (Z)	X.Y.Z
1st infestation				
Rabbit 1	2.5 (0.7)	320/500 = 0.640	177/188 = 0.941	1.506
Rabbit 2	2.3 (0.5)	358/500 = 0.716	195/199 = 0.980	1.614
Rabbit 3	2.4 (0.7)	374/500 = 0.748	197/200 = 0.985	1.768
Rabbit 4	2.2 (0.4)	294/500 = 0.588	168/174 = 0.966	1.250
2nd infestation				
Rabbit 1	2.5 (0.7)	415/500 = 0.830	198/200 = 0.990	2.054
Rabbit 2	2.9 (0.5)	436/500 = 0.872	217/228 = 0.952	2.407
Rabbit 3	2.2 (0.5)	401/500 = 0.802	148/162 = 0.914	1.613
Rabbit 4	1.9 (0.6)	117/500 = 0.234	73/99 = 0.737	0.328
3rd infestation				
Rabbit 1	2.1 (0.6)	303/500 = 0.606	170/200 = 0.850	1.082
Rabbit 2	2.3 (0.7)	216/500 = 0.432	116/136 = 0.853	.848
Rabbit 3	2.4 (0.6)	450/500 = 0.900	186/189 = 0.984	2.125
Rabbit 4	N/A*	N/A	N/A	N/A
4th infestation				
Rabbit 1	3.7 (0.8)	442/500 = 0.884	191/200 = 0.995	3.254
Rabbit 2	3.2 (0.9)	239/500 = 0.478	130/132 = 0.985	1.507
Rabbit 3	3.6 (0.9)	250/500 = 0.500	126/127 = 0.992	1.786
Rabbit 4	1.5 (0.5)	36/500 = 0.072	12/18 = 0.667	0.072

* = infestation discontinued due to ear infection

APPENDIX 5.3

Changes in average mass, engorgement success and moulting success for repeated infestations of *Amblyomma variegatum* nymphs feeding on sheep.

HOST	Average mass of engorged ticks mg (S.D.) (X)	Number of engorged ticks/Number applied (Y)	Number of moulted ticks/Number put to moult (Z)	X.Y.Z
1st infestation				
Sheep 1	59.4 (11.0)	81/100 = 0.81	50/50 = 1.0	48.11
Sheep 2	47.4 (12.3)	320/400 = 0.80	50/50 = 1.0	37.92
Sheep 3	47.6 (9.3)	85/100 = 0.85	50/50 = 1.0	40.46
Sheep 4	53.7 (9.0)	359/400 = 0.90	50/50 = 1.0	48.33
2nd infestation				
Sheep 1	48.0 (10.9)	84/100 = 0.84	50/50 = 1.0	40.32
Sheep 2	45.0 (9.2)	84/100 = 0.84	50/50 = 1.0	37.80
Sheep 3	46.3 (10.5)	72/100 = 0.72	48/50 = 0.96	32.00
Sheep 4	44.5 (12.3)	75/100 = 0.75	49/50 = 0.98	32.71
3rd infestation				
Sheep 1	40.8 (13.9)	27/100 = 0.27	19/24 = 0.79	8.70
Sheep 2	46.8 (9.4)	67/100 = 0.67	49/50 = 0.98	30.73
Sheep 3	44.0 (8.9)	64/100 = 0.64	45/50 = 0.90	25.34
Sheep 4	37.6 (9.4)	41/100 = 0.41	28/30 = 0.93	14.34
4th infestation				
Sheep 1	N/A*	N/A	N/A	0
Sheep 2	45.8 (8.9)	76/100 = 0.76	48/50 = 0.96	33.42
Sheep 3	42.7 (8.0)	66/100 = 0.66	50/50 = 1.0	28.18
Sheep 4	42.9 (9.2)	39/100 = 0.39	38/38 = 1.0	16.73
5th infestation				
Sheep 1	24.6 (-)	6/100 = 0.06	1/1 = 1.0	1.48
Sheep 2	31.3 (8.6)	90/400 = 0.23	11/13 = 0.85	6.12
Sheep 3	41.8 (5.4)	29/100 = 0.29	2/2 = 1.0	12.12
Sheep 4	25.5 (4.9)	91/400 = 0.23	10/12 = 0.83	4.87

* This sheep lost its bag on the first day of the infestation. A second infestation was applied the following day with an initial attachment success of 80% but none of the ticks fed to engorgement.

APPENDIX 5.4

Cell counts and patholcgical scores for the assessment of individual sections of the dermis at days six to seven of *Dermatophilus congolensis* infections on rabbits.

	Total counts from ten fields (total area 0.13mm^2)[1]						Area[2]	Area[3]	
	Eosinophils	Neutrophils	Mast cells	Basophils	Mononuclear cells	Capillaries	Haemorrhage	Necrosis	Oedema
Lesions at site of hypersensitive reactions to *Amblyomma variegatum* larvae									
Rabbit 1	8, 6	37, 29	10, 6	27, 40	308, 367	9, 9	3, 1	0, 0	602, 341
Rabbit 2	4, 1	103, 107	3, 3	67, 56	269, 252	11, 13	80, 29	54, 96	147, 166
Rabbit 3	10, 1	79, 107	8, 3	46, 62	315, 270	17, 14	8, 11	45, 75	119, 169
Rabbit 4	6, 7	253, 254	5, 5	40, 36	306, 296	15, 17	10, 8	11, 6	524, 451
Lesions at site of inflammatory reactions to *Amblyomma variegatum* larvae									
Rabbit 5	8, 1	216, 233	2, 3	49, 48	338, 364	8, 8	76, 210	67, 45	29, 6
Rabbit 6	1, 0	6, 4	4, 0	12, 14	117, 134	6, 5	6, 4	0, 0	1, 6
Rabbit 7	1, 1	214, 159	8, 4	20, 18	233, 247	15, 12	79, 96	0, 0	410, 248
Rabbit 8	1, 4	25, 11	0, 4	28, 23	200, 194	8, 6	36, 20	0, 0	60, 11
Lesions on hosts not exposed to ticks									
Rabbit 9	0, 0	128, 96	4, 4	60, 51	231, 221	10, 28	109, 86	15, 51	92, 140
Rabbit 10	4, 3	4, 7	0, 2	18, 18	209, 152	14, 6	46, 87	0, 8	38, 92
Rabbit 11	1, 0	204, 231	2, 1	11, 7	228, 223	8, 8	154, 141	10, 6	627, 455
Rabbit 12	1, 4	14, 14	1, 4	38, 48	184, 204	5, 8	26, 30	0, 4	54, 108

[1] Total numbers recorded over 10 fields (12,656μm^2 each), using a x100 light microscope objective

[2] Total number of graticule squares affected (126.6μm^2 each), using a x100 light microscope objective

[3] Total number of graticule squares affected (544.3μm^2 each), using a x50 light microscope objective. Maximum number for [2] and [3] = 1000.

APPENDIX 5.5

Cell counts and pathological scores for the assessment of individual sections of the dermis at day thirteen of *Dermatophilus congolensis* infections on rabbits.

	Total counts from ten fields (total area 0.13mm^2)[1]						Area[2]	Area[3]	
	Eosinophils	Neutrophils	Mast cells	Basophils	Mononuclear cells	Capillaries	Haemorrhage	Necrosis	Oedema
Lesions at site of hypersensitive reactions to *Amblyomma variegatum* larvae									
Rabbit 1	1, 3	5, 4	12, 11	25, 22	180, 246	11, 12	1, 25	16, 7	55, 12
Rabbit 2	2, 2	0, 0	3, 1	22, 32	195, 211	14, 3	2, 3	0, 0	19, 33
Rabbit 3	5, 2	16, 16	1, 1	34, 37	172, 167	1, 4	15, 9	0, 0	16, 26
Rabbit 4	12, 1	1, 8	7, 7	40, 32	518, 420	8, 5	0, 5	10, 38	143, 190
Lesions at site of inflammatory reactions to *Amblyomma variegatum* larvae									
Rabbit 5	3, 6	0, 3	2, 4	12, 22	283, 275	8, 5	3, 1	10, 10	103, 76
Rabbit 6	0, 0	5, 3	10, 11	12, 9	313, 239	8, 9	2, 7	0, 0	17, 20
Rabbit 7	2, 0	6, 4	0, 7	18, 11	291, 289	2, 7	7, 3	0, 0	21, 10
Rabbit 8	2, 2	287, 172	1, 6	13, 14	492, 374	8, 7	43, 7	83, 63	88, 184
Lesions on hosts not exposed to ticks									
Rabbit 9	1, 1	5, 28	10, 8	51, 57	250, 181	5, 8	0, 0	5, 0	29, 41
Rabbit 10	3, 1	9, 6	5, 3	16, 29	221, 207	16, 13	6, 5	36, 40	456, 412
Rabbit 11	6, 3	20, 24	10, 10	16, 20	368, 341	9, 11	21, 66	32, 40	534, 371
Rabbit 12	0, 3	3, 1	6, 6	24, 28	171, 167	7, 8	1, 0	0, 0	108, 69

[1] Total numbers recorded over 10 fields (12,656μm^2 each), using a x100 light microscope objective

[2] Total number of graticule squares affected (126.6μm^2 each), using a x100 light microscope objective

[3] Total number of graticule squares affected (544.3μm^2 each), using a x50 light microscope objective. Maximum number for [2] and [3] = 1000.

APPENDIX 5.6

Cell counts and pathological scores for the assessment of individual sections of the dermis at primary *Dermatophilus congolensis* infections on sheep with inflammatory reactions to the feeding of *Amblyomma variegatum* nymphs.

	Lesions at the site of previous tick feeding								Lesions remote from the previous tick feeding							
	Day 3		Day 6		Day 13		Day 27		Day 3		Day 6		Day 13		Day 27	
Sheep number	1	3	1	3	1	3	1	3	2	4	2	4	2	4	2	4
Eosinophils[1]	36, 66	2, 0	13, 13	12, 11	1, 1	6, 8	2, 1	2, 1	10, 12	2, 1	21, 19	24, 24	N/A	4, 1	43, 44	1, 1
Neutrophils[1]	81, 50	0, 1	89, 51	6, 6	0, 5	0, 2	0, 1	1, 2	10, 9	62, 49	3, 3	56, 48	N/A	0, 0	0, 1	1, 0
Mast cells[1]	2, 2	3, 2	0, 5	6, 2	5, 2	2, 6	2, 4	5, 5	5, 1	2, 1	2, 1	1, 0	N/A	1, 1	6, 5	2, 1
Basophils[1]	7, 7	3, 0	7, 4	1, 4	2, 2	6, 1	1, 1	4, 0	3, 4	2, 2	0, 2	5, 3	N/A	3, 0	2, 6	1, 2
Mononuclear cells[1]	474, 515	290, 472	580, 639	384, 412	309, 272	469, 490	160, 158	306, 317	191, 247	149, 127	230, 250	283, 290	N/A	181, 203	190, 243	94, 161
Capillaries[1]	4,3	2, 3	5, 6	8, 9	8, 11	6, 4	2, 6	7, 4	11, 5	5, 1	6, 10	4, 5	N/A	4, 5	5, 2	5, 6
Haemorrhage[2]	1, 0	1, 2	6, 4	2, 1	1, 2	0, 6	0, 0	5, 2	19, 8	99, 140	3, 13	7, 16	N/A	0, 0	0, 4	0, 0
Necrosis[3]	46, 58	0, 0	0, 5	0, 0	0, 0	0, 0	0, 0	62, 84	0, 0	0, 0	0, 0	0, 0	N/A	0, 0	0, 0	0, 0
Oedema[3]	222, 115	257, 128	256, 190	377, 213	14, 15	24, 47	66, 50	203, 157	206, 97	28, 28	286, 378	26, 18	N/A	76, 67	156, 368	70, 41

[1] = Total numbers recorded over 10 fields (12,656μm^2 each), using a x100 light microscope objective

[2] = Total number of graticule squares affected (126.6μm^2 each), using a x100 light microscope objective

[3] = Total number of graticule squares affected (544.3μm^2 each), using a x50 light microscope objective. Maximum number for [2] and [3] = 1000.

APPENDIX 5.7

Cell counts and pathological scores for the assessment of individual sections of the dermis at secondary *Dermatophilus congolensis* infections on sheep with delayed (type IV) hypersensitive reactions to the feeding of *Amblyomma variegatum* nymphs.

	Lesions at the site of previous tick feeding								Lesions remote from the previous tick feeding							
	Day 3		Day 6		Day 13		Day 27		Day 3		Day 6		Day 13		Day 27	
Sheep number	1	3	1	3	1	3	1	3	2	4	2	4	2	4	2	4
Eosinophils[1]	14, 18	5, 7	N/A	1, 4	8, 6	9, 24	4, 2	2, 4	4, 2	7, 4	22, 13	16, 26	7, 15	28, 21	15, 11	8, 1
Neutrophils[1]	0, 1	47, 51	N/A	50, 48	3, 4	0, 1	2, 2	8, 0	185, 213	128, 151	13, 6	140, 128	0, 1	0, 0	1, 0	5, 0
Mast cells[1]	1, 3	1, 2	N/A	3, 3	6, 9	7, 7	4, 4	4, 3	1, 2	0, 4	1, 2	0, 1	2, 1	4, 4	2, 1	0, 1
Basophils[1]	2, 4	5, 3	N/A	6, 6	7, 9	9, 4	1, 3	3, 0	4, 3	0, 2	6, 3	3, 1	7, 7	6, 3	4, 2	11, 4
Mononuclear cells[1]	259, 258	435, 414	N/A	743, 691	241, 345	533, 444	140, 165	168, 142	392, 361	236, 269	245, 194	389, 369	355, 378	146, 156	278, 242	330, 271
Capillaries[1]	4, 1	2, 8	N/A	5, 4	9, 11	9, 9	14, 8	7, 6	13, 5	2, 3	8, 12	4, 1	6, 11	4, 4	5, 5	17, 18
Haemorrhage[2]	4, 14	122, 137	N/A	24, 9	16, 4	1, 2	46, 23	0, 0	30, 10	4, 25	5, 5	44, 55	0, 8	0, 0	8, 31	0, 0
Necrosis[3]	215, 294	425, 339	N/A	0, 0	0, 0	112, 78	150, 144	0, 0	116, 135	342, 194	0, 0	0, 0	0, 0	0, 0	421, 198	0, 0
Oedema[3]	548, 620	326, 261	N/A	356, 289	28, 48	306, 295	291, 323	122, 128	27, 56	54, 51	6, 6	58, 43	62, 119	62, 28	566, 484	426, 305

[1] = Total numbers recorded over 10 fields (12,656μm^2 each), using a x100 light microscope objective

[2] = Total number of graticule squares affected (126.6μm^2 each), using a x100 light microscope objective

[3] = Total number of graticule squares affected (544.3μm^2 each), using a x50 light microscope objective. Maximum number for [2] and [3] = 1000.

APPENDIX 6.1

Changes in average mass, engorgement success and moulting success for repeated infestations of *Amblyomma variegatum* nymphs feeding on rabbits

HOST	Average mass of engorged ticks mg (S.D.) (X)	Number of engorged ticks/Number applied (Y)	Number of moulted ticks/Number put to moult (Z)	X.Y.Z
1st infestation				
Rabbit 1	46.93(12.3)	20/20=1.0	11/12=0.92	43.18
Rabbit 2	60.80(13.2)	20/20=1.0	14/14=1.0	60.80
Rabbit 3	52.03(11.8)	20/20=1.0	16/16=1.0	52.03
Rabbit 4	N/A*	N/A	N/A	N/A
2nd infestation				
Rabbit 1	32.29(8.7)	18/20=0.9	15/15=1.0	29.06
Rabbit 2	34.63(11.2)	17/20=0.85	16/17=0.94	27.67
Rabbit 3	31.57(14.4)	20/20=1.0	19/20=0.95	29.99
Rabbit 4	33.01(10.7)	13/20=0.65	13/13=1.0	21.46
3rd infestation				
Rabbit 1	27.96(8.8)	19/20=0.95	16/18=0.89	23.64
Rabbit 2	23.88(9.9)	20/20=1.0	3/5=0.6	14.33
Rabbit 3	33.80(11.8)	18/20=0.9	10/18=0.56	17.04
Rabbit 4	29.51(9.5)	17/20=0.85	8/16=0.5	12.54
4th infestation				
Rabbit 1	20.83(10.0)	19/20=0.95	18/19=0.95	18.80
Rabbit 2	25.89(8.8)	19/20=0.95	17/17=1.0	24.60
Rabbit 3	23.11(9.9)	17/20=0.85	16/17=0.94	18.46
Rabbit 4	26.50(11.2)	20/20=1.0	19/20=0.95	25.18

* Infection discontinued due to ear infection.

APPENDIX 6.2

Changes in skin fold thickness at dermatophilosis lesions on rabbits with hypersensitive or inflammatory reactions to the feeding of *Amblyomma variegatum* nymphs.

	Skin fold thickness (mm)							
	Dermatophilosis remote from tick attachment sites				Dermatophilosis at the same site as tick attachment sites			
Rabbits with hypersensitive reactions to *Amblyomma variegatum* nymphs								
Day of infection	Rabbit 1	Rabbit 2	Rabbit 3	Rabbit 4	Rabbit 1	Rabbit 2	Rabbit 3	Rabbit 4
0	1.7	1.9	2.1	2.1	1.65	1.7	2.05	1.8
3	1.9	2.15	2.3	2.4	8.3	4.65	4.3	11.2
6	2.65	2.35	3.35	2.9	7.95	3.5	4.0	7.25
9	3.65	2.45	4.45	3.6	7.75	4.2	4.4	5.55
13	3.05	1.95	3.25	2.45	3.35	2.6	2.75	3.8
16	1.95	1.9	3.2	2.7	3.7	2.55	2.5	2.7
20	2.3	2.35	3.0	2.35	2.7	2.6	2.85	2.45
Rabbits with inflammatory reactions to *Amblyomma variegatum* nymphs								
Day of infection	Rabbit 5	Rabbit 6	Rabbit 7	Rabbit 8	Rabbit 5	Rabbit 6	Rabbit 7	Rabbit 8
0	1.8	2.2	2.45	2.3	1.5	1.85	2.5	2.9
3	1.75	2.75	3.6	2.8	3.2	4.7	4.75	5.0
6	2.35	3.9	3.4	3.35	3.6	4.3	6.15	4.55
9	2.65	9.7	3.55	3.35	3.05	4.1	5.5	4.6
13	3.05	2.95	3.65	3.25	4.0	3.75	5.55	4.4
16	2.0	2.9	3.1	4.1	3.6	2.95	5.15	3.4
20	1.55	2.95	2.8	4.7	3.0	2.8	3.95	3.95

APPENDIX 6.3

Mean percentage change in skin fold thickness for dermatophilosis lesions on rabbits with hypersensitive or inflammatory reactions to *Amblyomma variegatum* nymphs.

	Percentage change in skin fold thickness at dermatophilosis lesions									
	Dermatophilosis remote from tick attachment sites					Dermatophilosis at the same site as tick attachment sites				
Rabbits with hypersensitive reactions to *A.variegatum* nymphs										
	Rabbit numbers					Rabbit numbers				
Day of infection	1	2	3	4	Mean (S.D.)	1	2	3	4	Mean (S.D.)
3	12	13	10	14	12.3 (1.7)	403	174	110	522	302.3 (193.1)
6	56	24	60	38	44.5 (16.7)	382	106	95	403	246.5 (168.9)
9	115	29	112	74	82.5 (40.3)	370	147	115	308	235 (123.4)
13	79	3	55	17	38.5 (34.8)	103	53	34	211	100.3 (79.4)
16	15	0	52	29	24 (22.1)	124	50	22	50	61.5 (43.7)
20	35	24	43	12	28.5 (13.5)	58	53	39	36	46.5 (10.7)
Rabbits with inflammatory reactions to *A.variegatum* nymphs										
	Rabbit numbers					Rabbit numbers				
Day of infection	5	6	7	8	Mean (S.D.)	5	6	7	8	Mean (S.D.)
3	-3	25	47	22	22.8 (20.5)	113	154	90	72	107.3 (35.4)
6	31	77	39	46	48.3 (20.1)	140	132	146	57	118.8 (41.6)
9	47	341	45	46	119.8 (147.5)	103	122	120	59	101 (29.3)
13	69	34	49	41	48.3 (15.1)	167	103	122	52	111 (47.6)
16	11	32	27	78	37 (28.8)	140	59	106	17	80.5 (53.8)
20	-14	34	14	104	34.5 (50.3)	100	51	58	36	61.3 (27.4)

APPENDIX 7.1

The following serum samples were collected from the twelve sheep to test for antibody levels to ovalbumin *Brucella abortus* and *Dermatophilus congolensis*.

		Day after sensitisation with antigens								Day after booster dose of antigen						
Sheep	Control sera	1st tick infestation	Day 7	1st *D.congolensis* infection	Day 14	Day 21	Day 35	Day 42	Day 49	2nd tick infestation	Day 7	2nd *D.congolensis* infection	Day 14	Day 21	Day 35	Day 49
1	O,D	+	-	+	O	-	-	O	-	+	-	+	O	O	O	O
2	O,D	+	-	+	O	-	-	O	-	+	-	+	O	O	O	O
3	O,D	+	-	+	O	-	-	O	-	+	-	+	O	O	O	O
4	O,D	+	-	+	O	-	-	O	-	+	-	+	O	O	O	O
5	O,D	-	-	+	O	-	-	O	-	-	-	+	O	O	O	O
6	O,D	-	-	+	O	-	-	O	-	-	-	+	O	O	O	O
7	O,D,B	+	O,B	+	-	O,B	O,B	-	O,B	+	O,B	+	O,B	O,B	O,B	O,B
8	O,D,B	+	O,B	+	-	O,B	O,B	-	O,B	+	O,B	+	O,B	O,B	O,B	O,B
9	O,D,B	+	O,B	+	-	O,B	O,B	-	O,B	+	O,B	+	O,B	O,B	O,B	O,B
10	O,D,B	+	O,B	+	-	O,B	O,B	-	O,B	+	O,B	+	O,B	O,B	O,B	O,B
11	O,D,B	-	O,B	+	-	O,B	O,B	-	O,B	-	O,B	+	O,B	O,B	O,B	O,B
12	O,D,B	-	O,B	+	-	O,B	O,B	-	O,B	-	O,B	+	O,B	O,B	O,B	O,B

O = tested for antibodies to ovalbumin

D = tested for antibodies to *Dermatophilus congolensis*

B = tested for antibodies to *Brucella abortus*

APPENDIX 7.2

Composition of the ELISA washing solution (pH 7.4) of 0.05% PBS/Tween 20.

Sodium chloride	8.0g
Potassium dihydrogen orthophosphate	0.2g
Sodium hydrogen orthophosphate dodecahydrate	2.9g
Potassium chloride	0.2g
Tween 20	0.5ml

Make up to 1000ml with distilled water.

APPENDIX 7.3

The clinical ranked scores of primary *Dermatophilus congolensis* infections on sheep with simultaneous infestations of adult or nymphal *Amblyomma variegatum* ticks.

	Clinical ranked scores														
	Infested with adult *Amblyomma variegatum*					Infested with nymphal *Amblyomma variegatum*					Not exposed to ticks				
Day of infection	Sheep 1	Sheep 3	Sheep 7	Sheep 10	Median	Sheep 2	Sheep 4	Sheep 8	Sheep 9	Median	Sheep 5	Sheep 6	Sheep 11	Sheep 12	Median
3	17	12	15	10	13.5	14	17	9	21	15.5	14	16	23	9	15
6	24	23	17	21	22	22	24	12	27	23	26	23	23	20	23
9	25	26	17	21	23	28	37	11	28	28	29	29	23	20	26
13	27	30	19	21	24	29	31	11	23	26	31	30	20	19	25
16	26	32	19	21	23.5	27	31	11	22	24.5	28	30	19	19	23.5
20	26	26	17	20	23	26	26	5	18	22	25	29	7	3	16
27	18	30	9	6	13.5	3	0	0	3	1.5	3	11	0	0	1.5
34	15	31	7	0	11	0	0	0	0	0	0	6	0	0	0
41	13	31	7	0	10	0	0	0	0	0	0	3	0	0	0
Total	191	241	127	120	159	149	166	59	142	145.5	156	177	115	90	135.5

APPENDIX 7.4

The clinical ranked scores of a secondary *Dermatophilus congolensis* infection on sheep with simultaneous infestations of adult or nymphal *Amblyomma variegatum* ticks.

	Ranked clinical scores														
	Infested with adult *Amblyomma variegatum*					Infested with *Amblyomma variegatum* nymphs					Not exposed to ticks				
Day of infection	Sheep 1	Sheep 3	Sheep 7	Sheep 10	Median	Sheep 2	Sheep 4	Sheep 8	Sheep 9	Median	Sheep 5	Sheep 6	Sheep 11	Sheep 12	Median
3	12	7	24	26	18	13	16	18	28	17	12	13	33	30	21.5
6	22	14	22	28	22	16	18	16	28	17	15	17	34	31	16
9	20	14	23	27	21.5	18	18	20	31	19	16	18	36	30	17
13	25	15	25	27	25	16	4	19	30	17.5	10	17	31	28	13.5
16	25	14	24	27	24.5	3	0	12	30	7.5	0	7	14	16	3.5
20	24	13	18	22	20	0	0	4	30	2	0	6	10	10	3
27	24	15	11	25	19.5	0	0	2	4	1	0	0	2	2	0
34	21	14	10	18	16	0	0	0	0	0	0	0	2	0	0
41	21	14	10	14	14	0	0	0	0	0	0	0	2	0	0
Total	194	120	167	214	180.5	66	56	91	181	78.5	53	78	164	147	112.5

APPENDIX 8.1

The ranked scores for the proportion of *a*, *b*, *c1* and *c2* in type-2 acini and *d*, *e*, *f* in type-3 acini found in nymphal *Amblyomma variegatum* salivary glands at the first stage of engorgement.

	Type-2 acini				Type-3 acini		
	a	*b*	*c1*	*c2*	*d*	*e*	*f*
	4	0	8	3	4	5	2
	4	4	8	2	4	2	2
	4	0	0	8	4	4	3
	4	4	10	4	4	8	3
	4	4	2.5	0	0	6	3
	4	4	2	0	4	9	1
Median	4	4	5.25	2.5	4	5.5	2.5

APPENDIX 8.2

The ranked scores for the proportion of *a*, *b*, *c1* and *c2* in type-2 acini and *d*, *e*, *f* in type-3 acini found in larval, nymphal and adult *Amblyomma variegatum* salivary glands at the second stage of engorgement.

	Type-2 acini				Type-3 acini		
	a	*b*	*c1*	*c2*	*d*	*e*	*f*
Amblyomma variegatum larvae							
	4	4	3	3.5	4	3	0
	4	0	12.5	1	0	6	1
	4	0	4	3	0	0.5	0
	0	0	3	4	4	3.5	2
	4	4	3	2	4	1.5	2
	4	0	14	3	0	0	0
	4	0	8	4	4	2	0
	0	0	10	3	0	4	0
	0	0	6	2	0	0	2
	0	0	10	2.5	2.5	4	0
	3.5	0	8	0	4	4	1
	4	3	1.5	2	-	-	-
	0	0	17.5	0	-	-	-
	0	0	8	2	-	-	-
Median	3.75	0	8	2.25	2.5	3	0
Amblyomma variegatum nymphs							
	4	0	3	4	4	4	0
	4	0	10	0	0	8	0
	3	3	5	0	4	4	0
	4	0	0	0	4	4	1
	2	0	0	0	4	3	0
	4	0	8	0	4	3	0
Median	4	0	4	0	4	4	0
Amblyomma variegatum adults							
	4	4	16	4	4	9	2
	0	4	12	0	4	6	1
	8	4	10.5	0	0	6	1
	4	0	12.5	0	4	8	4
	0	0	20	0	4	4	1
	4	4	1.5	3	4	12	0
	4	0	15	3	4	12	0
	4	0	15	4	4	6	0
	0	0	20	3	4	7	1
	4	0	12	2	4	6	1
	4	4	20	0	4	1	2
	4	0	17.5	0	0	4	1
Median	4	0	15	1	4	6	1

APPENDIX 8.3

The ranked scores for the proportion of *a*, *b*, *c1* and *c2* in type-2 acini and *d*, *e*, *f* in type-3 acini found in larval, nymphal and adult *Amblyomma variegatum* salivary glands from fully engorged ticks.

	Type-2 acini				Type-3 acini		
	a	*b*	*c1*	*c2*	*d*	*e*	*f*
Amblyomma variegatum larvae							
	3	0	15	0	4	4	0
	3	3	12	2.5	0	3.5	0.5
	0	0	17.5	0	3.5	1	0
	3.5	4	6	0	0	2	0
	0	0	20	0	0	4	0
	-	-	-	-	0	0	0
Median	3	0	15	0	0	2.75	0
Amblyomma variegatum nymphs							
	0	0	12	4	0	1	1
	0	3	20	0	4	0	0
	0	0	12	4	3	3	2
	4	0	9	4	0	1	0
	0	0	9	4	4	1	0
	0	4	9	2	4	2	2
Median	0	0	10.5	4	3.5	1	0.5
Amblyomma variegatum adults							
	0	0	15	1	3	4	0
	4	0	4	0	0	1	0
	0	0	8	6	4	3	0
	4	0	9	4	0	2	0
	4	0	0	4	0	3	1
	4	0	6	6	3.5	4	1
Median	4	0	7	4	1.5	3	0

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PUBLICATIONS

The following papers are included with the kind permission of the publishers.

Science and Technology Letters:

LLOYD, C.M. and WALKER, A.R. 1993 The effect of inflammatory and hypersensitive reactions, in response to the feeding of the tick *Amblyomma variegatum*, on the progression of experimental dermatophilosis infections. Experimental and Applied Acarology, 17 (5), 345-356.

LLOYD, C.M. and WALKER, A.R. 1993 The local effect of hypersensitive or inflammatory reactions to nymphal *Amblyomma variegatum* on simultaneous infections with *Dermatophilus congolensis*. Experimental and Applied Acarology, 17(8), 587-596.

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The effect of inflammatory and hypersensitive reactions, in response to the feeding of the tick *Amblyomma variegatum*, on the progression of experimental dermatophilosis infections

by

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EXPERIMENTAL & APPLIED ACAROLOGY HAS NO PAGE CHARGES

The effect of inflammatory and hypersensitive reactions, in response to the feeding of the tick *Amblyomma variegatum*, on the progression of experimental dermatophilosis infections

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ABSTRACT

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Initial infestations of *Amblyomma variegatum* larvae and nymphs, on rabbits and sheep respectively, produced inflammatory reactions in the host's skin; repeated infestations resulted in an increase in development of delayed type hypersensitive reactions. *Dermatophilus congolensis* cocci were applied in titrated doses to hosts at sites of inflammatory or hypersensitive reactions to ticks, and to control hosts with no exposure to ticks. We assessed the resulting infections for three weeks and found no significant difference between the infections on the three groups. We conclude that the local effects of the feeding of immature stages of this tick do not influence the pathogenesis of dermatophilosis.

INTRODUCTION

Dermatophilosis is a bacterial skin disease caused by the actinomycete *Dermatophilus congolensis*. The organism has a world-wide distribution, causing clinical infections on cattle and small ruminants. In the wet tropics, dermatophilosis commonly progresses and develops into severe chronic lesions and is a major cause of low beef and milk production. In some cases it can destroy the viability of cattle farming.

Various arthropods, especially the tick *Amblyomma variegatum*, have been associated with the development of chronic dermatophilosis lesions (Plowright, 1956; Macadam, 1964; Stewart, 1972; Morrow *et al.*, 1989). The spread of *A.variegatum* closely followed by the appearance of severe chronic dermatophilosis is well documented (Thoen *et al.*, 1980; Burridge *et al.*, 1984; Uilenberg *et al.*, 1984; Hadrill *et al.*, 1990; Martinez *et al.*, 1992). Primary dermatophilosis lesions

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have also been associated with the attachment sites of adult *A.variegatum* (Plowright, 1956). Immature ticks and *Haematobia* biting flies will produce inflammatory and hypersensitive lesions scattered widely on the host. These localised reactions may predispose to dermatophilosis (Macadam, 1962; Bida *et al.*, 1976; Davis *et al.*, 1980; Lloyd, 1984). Tick control has been advocated to control dermatophilosis (Plowright, 1956; Macadam, 1962; Macadam, 1970; Stewart, 1972; Samui *et al.*, 1990) but it has not been made clear whether this can be done more cheaply by selective treatment against particular instars of the tick.

The feeding of *A.variegatum* ticks produces delayed (type IV) hypersensitive reactions with the formation of intra-epidermal pustules and increased proportions of infiltrating granulocytes (Latif *et al.*, 1991b). The development of cellular reactions in guinea-pigs in response to repeated tick infestations are characterised by a cutaneous basophil hypersensitivity (Brown *et al.*, 1983). This type of cellular reaction has also been demonstrated by repeated infestations of *Amblyomma americanum* on bovine hosts (Brown *et al.*, 1984). However, with *A.variegatum* feeding on rabbit hosts, the initial cellular reactions are predominantly neutrophil and mononuclear infiltration with eosinophils also infiltrating attachment sites after repeated infestations (Latif *et al.*, 1990).

The production of hypersensitive reactions has been associated with increased resistance of hosts to various tick species, with the pustulation reducing the attachment of the ticks and the increased infiltration reducing the engorgement of the ticks (Walker and Fletcher, 1987). Resistance to immature *A.variegatum* ticks in Zebu cattle has been demonstrated in studies of natural tick burdens (Latif *et al.*, 1991a). Host resistance to experimental infestations of adult and immature stages of *A.variegatum* on cattle is accompanied with increased hypersensitive reactions at the tick attachment sites (Latif *et al.*, 1991b).

Work by Davis and Philpott (1980) has indicated that delayed type hypersensitive reactions in the host's skin at the site of infection with *D.congolensis* can produce dermatophilosis lesions similar to natural, chronic infections. The delayed type hypersensitive reactions in their experiments were produced artificially by the application of chlorodinitrobenzene and were simulations of the reactions which may occur at an arthropod feeding site.

To test the hypothesis that hypersensitive reactions in skin predispose to dermatophilosis, we studied the effect of local reactions in response to the feeding of immature instars of the tick *A.variegatum* on dermatophilosis infections on two species of laboratory hosts.

MATERIALS AND METHODS

Experimental hosts

We used four, two year-old Black-faced × Suffolk sheep (two ewes and two castrated males). We also used three groups of four female New Zealand White rabbits between 2 to 3 kg each. All the hosts were kept in similar environments at

18–20°C with 12 daylight hours/ 12 dark. None of the experimental animals had been exposed to ticks or experimental infections with *D.congolensis* prior to the start of the experiment.

Ticks

(a) Maintenance of ticks: Ticks were from a laboratory colony of uninfected *A.variegatum*. The ticks were fed routinely on laboratory rabbits and sheep, moulted at 28°C and then stored at 16°C. Larvae were used 2 to 5 months after hatching and nymphs 2 to 6 months from the date of the moult. All the ticks were used within 5 months of being transferred to 16°C.

(b) Application of ticks, (i) Rabbits: The immunising infestations were applied to alternate ears and enclosed by ear-bags. The hair was clipped from the top two-thirds of the ears. The challenge infestations were applied to the backs of the rabbits enclosed by cloth patches approximately 10cm square.

Each infestation remained on the host for a maximum of 10 days, by which time the majority of the ticks had engorged and detached leaving attachment sites which displayed visible signs of inflammatory or hypersensitive reactions. To prolong infections by allowing small numbers of ticks to complete their engorgement would have caused unnecessary stress for the experimental animals.

(ii) Sheep: All of the infestations were enclosed by cloth patches or bags glued to the wool on the flanks and shoulders. The area within the bags was clipped of hair before the ticks were applied. The infestations at the shoulder were enclosed with bags that could be opened throughout the infestation for the removal of engorged ticks and assessment of resistance. The infestations on the flanks of the test sheep were enclosed by five sealed patches which were opened at the end of the infestation to remove the engorged ticks and to infect the tick attachment sites with *D.congolensis*.

Any nymphs still attached to the sheep after 10 days were removed, for the same reasons as explained above.

(c) Experimental protocol, (i) Rabbits: Three groups of four rabbits were used to compare the effect of delayed type hypersensitive and inflammatory reactions on the progression of local dermatophilosis infections. Single or multiple infestations of larval *A.variegatum* were applied to produce inflammatory and hypersensitive reactions, respectively.

One of the three groups of rabbits was infested with four consecutive infestations of larval *A.variegatum* to produce delayed type hypersensitive reactions in the host's skin. Three immunising infestations of 500 larvae were applied to alternate ears. After three infestations visible signs of hypersensitive reactions were apparent at the tick attachment sites; a challenge infestation of 500 larvae was

then applied on the backs of the rabbits to produce hypersensitive reactions at the site of the subsequent *D.congolensis* infections. A second group of rabbits was infested once to produce inflammatory reactions at the site of the following *D.congolensis* infection. The third group of rabbits, not exposed to ticks, was used as the control.

(ii) Sheep: Four sheep were used to investigate the local and systemic effect of inflammatory and hypersensitive responses in sheep, in response to tick feeding, on the progression of subsequent *D.congolensis* infections. All four sheep were infested once with *A.variegatum* nymphs to produce inflammatory reactions in the host's skin followed by infection with identical titrated doses of *D.congolensis*. After four more infestations of nymphs had produced hypersensitive lesions in the skin the four sheep were infected a second time, with *D.congolensis*.

The first infestation was a challenge infestation to produce inflammatory reactions in the host's skin. The sheep were infested with 400 nymphs; the controls were infested at one area on the neck, whilst the infestations on the test sheep were divided between one area on the shoulder and five areas along the flank. The infestations on the flanks of the test sheep were at the sites of the subsequent *D.congolensis* infections.

Three immunising infestations of 100 nymphs followed. The ticks were applied to a single bag on the shoulder of all four sheep. Finally a second challenge infestation of 400 nymphs was applied to the four sheep using the same protocol as the first challenge infestation.

Assessment of tissue reactions and host resistance

As an indicator of reactions in skin to the ticks the host resistance was assessed by the method of Walker *et al.* (1990). To demonstrate the development of inflammatory and hypersensitive reactions at attachment sites of ticks, biopsies were taken at day five of the first and last infestations on both host species. Biopsies were taken, processed and examined using the methods of Walker and Fletcher (1986). Two sections from each biopsy were evaluated for signs of acute inflammatory abscesses (Thomson, 1978; Roitt *et al.,* 1985) or delayed (Type IV) cutaneous hypersensitive reactions (Turk, 1967; Roitt *et al.*, 1985).

D.congolensis *infections*

D.congolensis, isolated from local sheep, was used to culture infective cocci foı the production of experimental infections. Suspensions of cocci were counted in a Helber counter and on stained smears. Stabilates, with 7.5% v/v glycerol, were cryopreserved at a concentration of 1.2×10^7 cocci/μL. Suspensions of infective *D.congolensis* cocci were obtained by diluting the previously cryopreserved cocci in Hank's balanced salt solution with pig gelatin at 0.5% w/v.

Before application of *D.congolensis*, any hair or wool was removed from the infection sites and the skin was cleaned using alcohol and ether. The infection areas

were marked using an indelible pen and the skin fold thickness of each area was measured. The *D.congolensis* was then applied without scarification, using a bent pipette tip.

(a) Rabbits: All twelve rabbits were infected with *D.congolensis* at 10 test areas (1×2cm) on the torso, at the site of the previous tick infestations on the test rabbits. A titrated dose of *D.congolensis*, consisting of 50μL doses of five 10-fold dilutions of *D.congolensis* starting at 2.5×10^8 cocci/cm^2 was applied to five of the areas on each rabbit. To allow for any unequal effect on the individual infection sites due to uneven distribution of the ticks, the remaining areas on each rabbit were infected with constant doses of 5×10^7 cocci/cm^2. Also three areas (1×1cm) were infected with *D.congolensis*, at a concentration of 5×10^7 cocci/cm^2, for biopsies throughout the infection.

Both groups of rabbits infested with larvae were infected with identical doses of *D.congolensis* at the tick attachment sites one day after the final infestation of ticks. The third group of rabbits, with no previous exposure to ticks, were also infected with identical titrations.

(b) Sheep: A freshly prepared suspension of *D.congolensis* was used to produce the titration by dilution, as before. All four of the sheep received two titrated doses of *D.congolensis*; one applied after the first infestation of nymphs had engorged, the second after the fifth infestation. The two test sheep were infected at the sites of the previous infestations and the controls were infected at remote sites. The titrated applications were 100μL doses of five 10-fold dilutions of *D.congolensis*, starting at 1.25×10^6 cocci/cm^2.

Two extra areas (2×4cm), on the shoulder's of all four sheep were infected with doses of 1.25×10^4 cocci/cm^2 to provide lesions for biopsy during the infection. These areas were at the same site as the previous tick infestations on the test sheep and at sites remote from tick attachment sites on the control sheep.

Assessment of the dermatophilosis

The resulting infections on both the sheep and rabbits were monitored using a ranking system of 0 to 4 for: skin fold thickness, percentage of each area showing signs of infection, the severity of the scab, ranging from erythema to thick layers of dead, flaking epidermis and extent of exposed dermis at the infection sites. Progression of dermatophilosis on the rabbits was assessed at 3 to 4 day intervals from days 3 to 21. For sheep there was an extra assessment at day 27.

Analysis

Chi-square and Fisher's Exact test, were used for the analysis of the histological results. Friedman's, Kruskal-Wallis and Mann-Whitney tests were used for the analysis of the dermatophilosis.

RESULTS

Reactions to ticks

Resistance in rabbits to larval infestations was extremely varied but all the sheep developed a marked resistance to the nymphs (Table 1). There was a marked increase in resistance to the larvae in only one of the rabbits (No. 4). In the other three rabbits the amount of resistance fluctuated, and at the final infestation two of the rabbits showed no marked change in resistance compared with the first infestation. In the remaining rabbit (No. 1) there was a marked decrease in resistance. In contrast, by the fifth infestation on the sheep, all four had developed a significant resistance to the nymphs.

Intra-epidermal pustules, erythema and exudate were visible on the rabbits and the sheep at the final infestations. A comparison of the reactions at the first and the final infestations revealed that hypersensitive reactions to the ticks had developed by the fourth and fifth infestations on the rabbits and sheep respectively. Table 2 shows the total cell counts and pathological scores for the first and final infestations on both the rabbits and the sheep.

Mann-Whitney test on the counts for individual sections from the rabbits showed significant differences characterising the distinction between initial inflammatory reactions and later hypersensitive reactions ($P < 0.05$ or less).

A Chi-square test on the proportion of eosinophils, neutrophils, mononuclear cells and capillaries at the first and final nymph attachment sites showed significant differences in the proportions of these cells and capillaries ($P < 0.01$). Fisher's Exact test on the number of basophils and mast cells also showed significant differences in the proportions of these cells at the first and final infestations of nymphs ($0.01 < P < 0.02$).

TABLE 1

Changes in resistance to *Amblyomma variegatum* larvae and nymphs feeding on rabbits and sheep respectively, calculated by the method of Walker et al. (1990). Changes in resistance were compared with the initial resistance of individual hosts, in some cases there was a decrease in resistance.

	Resistance (%) at each infestation			
Host	2nd	3rd	4th	5th
Rabbit 1	−36	28	−116	N/A
Rabbit 2	−49	47	7	N/A
Rabbit 3	9	−20	−1	N/A
Rabbit 4	74	N/A	94	N/A
Sheep 1	16	82	100	97
Sheep 2	0	19	12	84
Sheep 3	21	37	30	70
Sheep 4	32	70	65	90

TABLE 2

Histological reactions at the tick attachment sites on rabbits and sheep in response to the feeding of larvae and nymphs of *A.variegatum*, respectively.

	Rabbit hosts (N=4)		Sheep hosts[1]	
	1st	4th	1st	5th
Cell numbers[2]:				
Eosinophils	24	506*	7	16.5
Neutrophils	153	657	711	571.5
Mast cells	13	3	8	0.5
Basophils	62	181*	1	5.5
Mononuclear	1,376	4,523*	545	781.5
Pathological scores:				
Haemorrhage[3]	291	152	369	127*
Necrosis[3]	106	354	768	220*
Oedema[3]	326	1737*	173	78*
Capillaries[4]	54	34	9	7

[1]Total counts for one sheep at the first infestation and the median of the counts for two sheep at the fifth infestation.
[2]Total numbers from 20 fields, 112.5μm square, from each of four biopsies, using a ×100 light microscope objective.
[3]These scores represent the number of areas (126.6μm^2) affected (maximum = 2000).
[4]Total numbers counted over the same fields as [2] but using a ×50 objective, each field being 233.3μm square.
*Statistically significant differences at $P = 0.05$ or less, between 1st and 4th or 5th infestation.

D.congolensis *infections on rabbits*

Using Friedman's non-parametric test on the ranked clinical scores for the dermatophilosis lesions, we tested for any significant difference in the severity of the infections on the individual rabbits within groups (Table 3). With the four rabbits repeatedly infested with larvae prior to infection there was no significant difference in the severity of the infections on them ($P > 0.05$). However, with the rabbits infested with ticks only once or not at all, there were significant differences between the dermatophilosis infections on individual rabbits ($0.01 < P < 0.05$ and $P < 0.01$, respectively).

The infections on the three groups of rabbits were compared at each assessment day using Kruskal-Wallis; there was no significant difference between the three groups at any time during the course of the assessment ($P > 0.05$).

D.congolensis *infections on sheep*

Differences between the severity of the primary dermatophilosis lesions on the four sheep were assessed using Friedman's test. The clinical scores given to each of the four sheep at each assessment were used for the test; there was a significant

TABLE 3

Total ranked clinical scores for the *D.congolensis* infections on rabbits for each assessment day.

Day of infection	Rabbit 1	Rabbit 2	Rabbit 3	Rabbit 4	Median
	Ranked clinical scores Infections at hypersensitive tick attachment sites				
3	52	47	33	36	41.5
6	71	70	58	58	64
9	87	67	64	65	66
13	9	7	30	39	19.5
16	3	3	21	0	3
	Infections at inflammatory tick attachment sites				
Day of infection	Rabbit 5	Rabbit 6	Rabbit 7	Rabbit 8	Median
3	46	31	19	31	31
6	69	54	46	49	51.5
9	66	67	52	58	62
13	17	0	19	52	18
16	13	0	0	31	6.5
	Infections with no previous exposure to ticks				
Day of infection	Rabbit 9	Rabbit 10	Rabbit 11	Rabbit 12	Median
3	35	21	48	29	32
6	59	41	58	36	49.5
9	78	48	72	33	60
13	2	23	54	0	12.5
16	0	10	29	0	5

difference ($P < 0.05$) between the infections on the four sheep (Table 4). However further analysis revealed that one of the control sheep (No. 2) had a significantly lower infection than one of the test sheep (No. 1) and there was no significant difference between the infections on the other control sheep and either of the test sheep.

Assessment of the second dermatophilosis infection, using Friedman's test also showed a significant difference between the infections on the four sheep, ($P < 0.05$). Further analysis revealed that the dermatophilosis infection on the control sheep (No. 2) was significantly lower than the infection on the test sheep (No. 1). However, there was no significant difference between the infections on the other control sheep (No. 4) and the infections on either of the test sheep.

Using the Mann-Whitney test on the median scores of the four sheep for the individual assessment days, we showed that the first infection was significantly more severe than the second infections on the sheep ($P = 0.05$).

TABLE 4

The total ranked clinical scores for primary and secondary *D.congolensis* infections on sheep for each assessment day.

	Ranked clinical scores			
	Infections at same sites as inflammatory tick attachment sites		Infections at sites remote from inflammatory tick attachment sites	
Day of infection	Sheep 1	Sheep 3	Sheep 2	Sheep 4
1st *D.congolensis* infection				
3	21	21	8	15
6	23	22	12	18
9	29	25	15	24
13	31	23	17	29
16	26	18	10	26
20	5	0	5	14
2nd *D.congolensis* infection				
3	13	13	12	13
6	14	21	15	16
9	15	23	14	19
13	14	23	16	16
16	12	21	14	0
20	8	13	0	0
27	6	8	0	0

Duration of the dermatophilosis infections

The lesions on all of the rabbits were beginning to resolve by day 13 of the infections and by day 20 the lesions were completely healed on all the infections assessed at this time.

The infections on the sheep persisted for longer than on the rabbits; however by day 16 all of the infections began to resolve. By day 27, apart from very slight lesions on the two test sheep at the second infection, no lesions were visible on any of the sheep. The lesions on the test sheep at this time may have been the remains of the reaction to tick attachment sites. In any case the lesions at these sites were healing rapidly with the larger part of the dermatophilosis having detached leaving healthy, undamaged skin.

DISCUSSION

Repeated infestations of *A.variegatum* larvae and nymphs on rabbits and sheep produced increasing macroscopic reactions in the host's skin. Macroscopic signs of inflammatory and delayed type hypersensitive reactions, to the first and last infestations respectively, were confirmed by histological assessment of the tick

attachment sites. The development of delayed type hypersensitive reactions in the host's skin was accompanied by an increase in host resistance only with the nymphs feeding on sheep.

D.congolensis cocci were applied to the sites of previous tick attachment where active reactions in the host's skin, particularly after the final infestations, persisted and were visible as distinct granulomas.

Local reactions in the skin of these two host species, in response to the feeding of the immature stages of this tick did not facilitate the progression of the disease to form chronic lesions; neither did differences in the type of host reaction to the ticks have any significant effect on the severity or duration of the dermatophilosis lesions formed. The significant difference between the first and second infections on the sheep is due to the development of immunity to *D.congolensis* infection (Walker and Lloyd, 1993).

Slight increases observed in the severity of the infections at sites of previous tick attachment were not statistically significant and reactions to the ticks at the same sites could be responsible for overestimation of the severity of the lesions. In any case the extended time period for the infections to resolve (Davis and Philpott, 1980) was not observed and differences of a few days are not important when chronic experimental lesions on sheep can persist for months.

The conclusion from these results is that neither inflammatory nor delayed type hypersensitive reactions to the feeding of the immature stages of this tick affect the progression of the disease. These results indicate that the local effects of the feeding of larval and nymphal *A.variegatum* are unlikely to facilitate the development of chronic *D.congolensis* infections once the ticks have been removed. However, the possible influence of this tick on the pathogenesis of dermatophilosis may be due to a systemic effect of ticks continuing to feed at sites separate from dermatophilosis lesions. Further experiments are required to compare the systemic effects of the feeding of adult and immature *A.variegatum* on simultaneous *D.congolensis* infections. However, we suggest that control of dermatophilosis by control of arthropods may not be cost effective if it is directed at immature ticks and other arthropods causing localised skin damage.

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The local effect of hypersensitive or inflammatory reactions to nymphal *Amblyomma variegatum* on simultaneous infections with *Dermatophilus congolensis*

by

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The local effect of hypersensitive or inflammatory reactions to nymphal *Amblyomma variegatum* on simultaneous infections with *Dermatophilus congolensis*

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ABSTRACT

Lloyd, C.M. and Walker, A.R., 1993. The local effect of hypersensitive or inflammatory reactions to nymphal *Amblyomma variegatum* on simultaneous infections with Dermatophilus congolensis. Exp. Appl. Acarol., 17: 587–596.

Amblyomma variegatum nymphs were applied to sites infected with *Dermatophilus congolensis* on eight rabbits. Four rabbits were previously sensitized to the feeding of nymphal *A. variegatum* to produce hypersensitive reactions to the tick feeding; the remaining four rabbits had no previous exposure to nymphal *A. variegatum* and produced inflammatory reactions to the tick feeding.

The resulting dermatophilosis infections were assessed for three weeks and there was a correlation between the position of the inflammatory tick attachment sites and the foci of infection. There was a significant increase in the lesions at sites with inflammatory reactions to the ticks, compared with sites not exposed to tick feeding; these differences appeared to be due to individual variation in the host response and were not sustained throughout the assessment.

INTRODUCTION

Dermatophilosis is a bacterial skin disease caused by a gram-positive actinomycete, *Dermatophilus congolensis*. The causal agent, *D. congolensis* was first described in a mycotic dermatitis on cattle in the Belgian Congo by Van Saceghem (1915). The organism has a world-wide distribution, causing clinical infections on cattle and small ruminants. However, it is usually in the wet tropics that the disease develops into a severe, chronic form. Chronic dermatophilosis can have disastrous effects on the local livestock industry, and in some areas of the Caribbean the disease has been reported to totally destroy the viability of cattle farming (Stewart, 1972; Butler, 1975; Uilenberg *et al.*, 1984).

Dermatophilosis is a superficial infection of the epidermis (Roberts, 1964;

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Amakiri, 1976). The first clinical signs of dermatophilosis lesions manifest in a thickening of the dermis at the site of infection. Initial lesions become visible with the production of exudate, which dries to form a crust. As the infection progresses, the crust thickens as a result of epidermal proliferation and exudation of cells. Initial foci of infection spread and coalesce to form large, confluent scabs.

Previous experiments in this laboratory have demonstrated that the feeding of adult *A. variegatum* has a significant systemic effect on the progression of simultaneous *D. congolensis* infections (Walker and Lloyd, 1993). This systemic effect appears to be confined to the adults only (Lloyd and Walker, 1993a). However, immature *A. variegatum* may have a local effect on dermatophilosis infections (Plowright, 1956).

Work by Davis and Philpott (1980) has shown that delayed (type IV) hypersensitive reactions prolong the progression of local, simultaneous *D. congolensis* infections. Lloyd and Walker (1993b) have shown that hypersensitive or inflammatory reactions remaining in the host's skin in response to infestations of larval or nymphal *A. variegatum* do not significantly affect subsequent *D. congolensis* infections.

There are many observations of dermatophilosis lesions being widespread over the dorsal areas of infected animals, at sites remote from the predilection areas of adult *A. variegatum* (Zlotnik, 1955; Macadam, 1964a and b; Morrow *et al.*, 1989). These dorsal lesions may be aggravated by the systemic effect of adult *A. variegatum*, feeding at sites remote from the dermatophilosis lesions. However, immature ticks and biting insects, such as *Haematobia* species, feed in large numbers on the dorsal surfaces of the animals and may influence the severity or distribution of local dermatophilosis lesions (Macadam, 1964a and b; Stewart, 1972).

This experiment was designed to test the hypothesis that inflammatory or hypersensitive reactions in the skin of rabbits, in response to the feeding of nymphal *A. variegatum* will aggravate simultaneous dermatophilosis infections, resulting in the formation of severe, chronic lesions. The physical damage to the epidermis, at the tick feeding sites was also thought to allow the penetration of *D. congolensis* into the skin, facilitating the initial lesion formation. Therefore, the correlation between the tick feeding sites and position of the initial dermatophilosis lesions was assessed.

METHODS

Experimental hosts

Two groups of four female New Zealand White rabbits 3–5 kg each were used. Four of the rabbits were infested with three immunizing infestations of *A. variegatum* nymphs applied to alternate ears to produce hypersensitive reactions in the host's skin. After the third infestation all eight rabbits were infected, at two areas (25 cm^2) on the back, with identical doses of *D. congolensis* cocci. Infestations of 20 nymphs were applied to one infection area on each of the eight rabbits.

Tick infestations

Amblyomma variegatum ticks from an uninfected laboratory colony were used for the infestations. Ticks were fed routinely on laboratory rabbits and sheep, moulted at 28°C and then stored at 16°C with 14 hours light/10 hours dark at 85% relative humidity.

Three immunizing infestations of 20 nymphs each were applied to alternate ears on four rabbits to produce hypersensitive reactions to the tick feeding. The hair was clipped from the top two-thirds of the ear and the infestations were enclosed using cloth ear-bags. After the third sensitizing infestation, challenge infestations were applied to one of two areas infected with *D. congolensis* on the backs of all eight rabbits. These infestations were contained within cloth bags large enough to allow tick feeding only at the infection sites. The bags were attached to the rabbit's fur using a water based latex rubber glue (Copydex).

Host resistance to experimental infestations of adult and immature stages of *A. variegatum* on cattle is accompanied with increased hypersensitive reactions at the tick attachment sites (Latif *et al.*, 1991). Therefore, the feeding success of the nymphs at repeated infestations was recorded, to provide an indication of the degree of resistance and hypersensitivity developing in the rabbits. The engorgement success, average weight and moulting success were recorded and the percentage change in resistance calculated, using the method of Walker *et al.* (1990).

$$\left[1-\left\{\frac{\text{resistant mass}}{\text{control mass}} \times \frac{\text{resistant numbers}}{\text{control numbers}} \times \frac{\text{resistant survival}}{\text{control survival}}\right\}\right] \times 100$$

Dermatophilus congolensis infections

Dermatophilus congolensis isolated from local sheep, was used to culture infective cocci for the production of experimental infections. Suspensions of cocci were counted in a Helber counter and on stained smears. Stabilates, with 7.5% v/v glycerol, were cryopreserved at a concentration of 3.5×10^7 cocci μl^{-1}. Suspensions of infective *D. congolensis* cocci were obtained by diluting the previously cryopreserved cocci in Hank's balanced salt solution with pig gelatin at 0.5% w/v.

All of the rabbits were infected with identical doses of *D. congolensis* at two sites (5 x 5 cm) on the same day as the challenge infestation of ticks. The suspension of cocci was applied, allowed to dry and then the ticks were applied to one of the two infection sites.

Both infection sites on all eight rabbits were infected with 1×10^7 cocci cm^{-2} at a concentration of 4×10^5 cocci μl^{-1}. In previous experiments, cocci applied at this concentration produced discrete lesions over the application areas. Higher concentrations would have produced stronger reactions which would have disguised any correlation between the position of the ticks and the establishment of infection foci.

Before application of the infective cocci, the hair was removed from the infection sites and the skin was cleaned using ethanol and ether. The infection sites were

marked using an indelible pen and two skin fold measurements were obtained from each of the infection sites. The *D. congolensis* was then applied without scarification, using a bent plastic pipette tip.

The progress of the dermatophilosis was monitored using a non-parametric ranking system to record the percentage area infected, the severity of the developing scabs and the degree of exposure of dermis at the infection sites. Each of the two areas on each rabbit were assessed separately. The scoring for the area, severity and amount of exposed dermis were applied to the whole area. Due to the large size of the areas, two readings of the skin fold thickness were taken for each area. The infections were assessed every three or four days until day 20 when the infections were virtually resolved.

In addition to the assessment of the severity of the dermatophilosis, the number and position of infection foci and tick attachment sites were noted at each assessment. Each 25 cm^2 area was divided into 1 cm^2 plots and the presence or absence of ticks and dermatophilosis was recorded for each plot.

RESULTS

Resistance to ticks

Three out of the four rabbits developed a marked resistance to repeated infestations of *A. variegatum* nymphs (Table 1). The development of resistance to the repeated nymphal feeding manifested itself by fewer ticks engorging, with a reduced mean engorged weight.

The increasing resistance to the feeding of the ticks was accompanied by increased signs of hypersensitivity, including the formation of intra-epidermal pustules, the production of exudate and sloughing of skin. These macroscopic signs of hypersensitivity, in the skin of rabbits have already been confirmed by histological assessment of immature *A. variegatum* attachment sites (Lloyd and Walker, 1993b).

TABLE 1

Changes in resistance to repeated infestations of *Amblyomma variegatum* nymphs on rabbits. The engorgement success, average weight and moulting success of the successive infestations were recorded and the development of resistance was calculated by the method of Walker *et al.* (1990). Changes in resistance were compared with the initial resistance of individual hosts; in some cases there was a decrease in resistance.

	Resistance (%) at each infestation		
Host	2nd infestation	3rd infestation	4th infestation
Rabbit 1	33	45	56
Rabbit 2	54	76	60
Rabbit 3	42	67	65
Rabbit 4	N/A	42*	−17*

*For rabbit No. 4 the results from the 1st infestation were not available, therefore subsequent infestations were compared with the 2nd infestation.

These assessments of tick attachment sites, on naive and resistant rabbits revealed signs of acute inflammatory abscesses (Thomson, 1978; Roitt *et al.*, 1985) and delayed (type IV) cutaneous hypersensitivity (Turk, 1967; Roitt *et al.*, 1985) respectively.

Dermatophilus congolensis infections

All of the control (no ticks) and test (with ticks) areas developed moderate dermatophilosis lesions which persisted for three weeks (Table 2).

Using the Kruskal-Wallis test on the ranked clinical scores, there was no significant difference between the control areas on the individual rabbits in either of the two groups ($P > 0.05$). There was also no significant difference between the severity of the test lesions on the rabbits with hypersensitive reactions to the nymphs ($P > 0.05$). However, there was a significant difference between the test infections on the individual rabbits with inflammatory reactions to the ticks ($P < 0.05$).

TABLE 2

Dermatophilus congolensis infections on rabbits at sites local and remote from hypersensitive or inflammatory reactions to simultaneous infestations of nymphal *Amblyomma variegatum*. The ranked clinical scores for the dermatophilosis lesions were obtained using a ranking system, giving scores of 0 to 4 for the severity of the lesions, the percentage of each infection area showing signs of infection and the amount of exposed dermis at each infection site. The maximum score for one infection area, at a single assessment = 12.

	Ranked clinical scores									
	Dermatophilosis only					Dermatophilosis and nymphal *A. variegatum*				
D. congolensis infections on rabbits with hypersensitive reactions to ticks										
	Rabbit numbers					Rabbit numbers				
Day of infection	1	2	3	4	Median	1	2	3	4	Median
3	4	3	3	3	3	5	5	4	5	5
6	4	3	5	5	4.5	4	4	4	4	4
9	4	4	6	4	4	5	4	4	3	4
13	3	3	4	3	3	6	4	4	5	4.5
16	3	3	3	4	3	3	4	4	4	4
20	3	3	3	3	3	3	4	4	4	4
D. congolensis infections on rabbits with inflammatory reactions to ticks										
	Rabbit numbers					Rabbit numbers				
Day of infection	5	6	7	8	Median	5	6	7	8	Median
3	2	3	2	2	2	4	4	4	4	4
6	2	5	3	2	2.5	4	5	4	3	4
9	3	5	4	4	4	5	6	5	3	5
13	3	4	4	4	4	5	5	6	4	5
16	3	3	4	5	3.5	5	5	5	4	5
20	4	4	0	5	4	5	5	3	4	4.5

A Kruskal-Wallis test on the medium ranked clinical scores given to each of the two areas on the two groups of rabbits (Table 2) showed the infections to fall into two overlapping subsets. The infections on the control areas on both groups of rabbits were significantly milder than the test infections at the site of inflammatory reactions to the ticks, but there was no significant difference between the test areas with hypersensitive or inflammatory reactions to ticks.

Differences in Dermatophilus congolensis infections over time

Separate Kruskal-Wallis tests were used to compare the ranked clinical scores given to the infection areas on each of the eight rabbits for each of the assessment days (Table 2). Significant differences between the four groups of dermatophilosis infections (*i.e.* infections with or without ticks, on rabbits with inflammatory or hypersensitive reactions to nymphs) occurred only at days three and 13, $P < 0.01$ and $P < 0.05$ respectively.

Skin fold thickness at dermatophilosis lesions

At each assessment skin fold measurements were recorded at both infection areas on all of the rabbits. Control skin fold measurements were taken from each infection site at day 0 of the infections, prior to application of the *D. congolensis* cocci. The percentage change in skin fold thickness was calculated for each assessment day, compared with the initial thickness. The mean percentage change in skin fold thickness for the control and test infection areas on both groups of rabbits are shown in Table 3.

The distribution and number of dermatophilosis foci

The position of the tick attachment sites and dermatophilosis infection foci were recorded on hand drawn diagrams for each of the 25 cm^2 infection areas. Gridlines at 1 cm intervals were drawn over the diagrams, dividing each diagram into 25 equal plots. The presence or absence of tick attachment sites and dermatophilosis foci was recorded for each of the individual plots which were divided into four sets; dermatophilosis only, ticks only, ticks and dermatophilosis, and neither ticks nor dermatophilosis present. Table 4 shows the number of plots in each group for the test and control areas on all eight rabbits at day nine of the infection.

A 2×2 contingency table was used to test for significant differences between the number of 1 cm^2 plots occurring in each of the four sets. The association between inflammatory or hypersensitive reactions to the tick feeding and the dermatophilosis infection foci were tested separately, using the total number of plots assessed for each group of rabbits. There was no association between the dermatophilosis and hypersensitive tick attachment sites ($P > 0.05$). However, there was a very significant association between the inflammatory tick attachment sites and the dermatophilosis foci ($P < 0.01$)

DISCUSSION

TABLE 3

Mean percentage change in skin fold thickness for dermatophilosis lesions on rabbits with hypersensitive or inflammatory reactions to *Amblyomma variegatum* nymphs.

	Percentage change in skin fold thickness at dermatophilosis lesions									
	Dermatophilosis only					Dermatophilosis and *A. variegatum* nymphs				
Rabbits with hypersensitive reactions to *A. variegatum* nymphs										
	Rabbit numbers					Rabbit numbers				
Day of infection	1	2	3	4	Mean (S.D.)	1	2	3	4	Mean (S.D.)
3	12	13	10	14	12.3 (1.7)	403	174	110	522	302.3 (193.1)
6	56	24	60	38	44.5 (16.7)	382	106	95	403	246.5 (168.9)
9	115	29	112	74	82.5 (40.3)	370	147	115	308	235 (123.4)
13	79	3	55	17	38.5 (34.8)	103	53	34	211	100.3 (79.4)
16	15	0	52	29	24 (22.1)	124	50	22	50	61.5 (43.7)
20	35	24	43	12	28.5 (13.5)	58	53	39	36	46.5 (10.7)
Rabbits with inflammatory reactions to *A. variegatum* nymphs										
	Rabbit numbers					Rabbit numbers				
Day of Infection	5	6	7	8	Mean (S.D.)	5	6	7	8	Mean (S.D.)
3	–3	25	47	22	22.8 (20.5)	113	154	90	72	107.3 (35.4)
6	31	77	39	46	48.3 (20.1)	140	132	146	57	118.8 (41.6)
9	47	341	45	46	119.8 (147.5	103	122	120	59	101 (29.3)
13	69	34	49	41	48.3 (15.1)	167	103	122	52	111 (47.6)
16	11	32	27	78	37 (28.8)	140	59	106	17	80.5 (53.8)
20	–14	34	14	104	34.5 (50.3)	100	51	58	36	61.3 (27.4)

Rabbits became resistant to tick feeding after repeated infestations of *A. variegatum* nymphs. This resistance was accompanied by macroscopic signs of hypersensitivity, such as pustulation and sloughing of skin. Biopsies of tick attachment sites after repeated infestations have previously confirmed the development of hypersensitive reactions in rabbits and sheep (Lloyd and Walker, 1993b). Other work with *Rhipicephalus appendiculatus* and *A. variegatum* has also demonstrated

the development of hypersensitive reactions with increased host resistance (Walker and Fletcher, 1987; Latif *et al.*, 1991).

The results of this experiment indicate that the local effect of the feeding of nymphal *A. variegatum* manifests itself in increased severity of the initial dermatophilosis lesions. However, when the severity of the four groups of infections was compared on individual assessment days, significant differences were found only at days three and thirteen. This shows that even though inflammatory reactions to the ticks had a significant effect on the overall ranked severity, the feeding of these ticks did not result in the progression of the dermatophilosis into severe, chronic lesions. Skin thickness at the sites of dermatophilosis lesions was greatly increased with local tick feeding. This increase in skin fold thickness was probably a result of the strong host reaction to the tick feeding. There was no significant difference between the dermatophilosis lesions on the two groups of infections infested with nymphs. However, there was a large difference in the skin thickness between the two groups, with the greatest increase in skin fold thickness occurring at the hypersensitive tick attachment sites. The hypersensitive host reactions to the tick feeding had resulted in oedematous swelling and the formation of intra-epidermal pustules which would produce large increases in skin fold thickness.

A strong association has been shown between inflammatory tick feeding sites and dermatophilosis foci. Further studies are needed to determine if there is a similar association between inflammatory reactions to biting insects and dermatophilosis lesions. No association has been shown between hypersensitive tick feeding sites and dermatophilosis lesions. However, it may be that any association with hypersensitive sites has been disguised due to the host's resistance causing the ticks to repeatedly detach and reattach (Latif *et al.*, 1990).

These results indicate that the feeding of nymphal *A. variegatum* has only a limited effect on local, simultaneous *D. congolensis* infections, on rabbits. The correlation between the tick feeding sites and the initial foci of dermatophilosis lesions indicates that the physical damage to the epidermis facilitates initial lesion formation, but there is no evidence that *A. variegatum* nymphs facilitate the formation of severe, chronic lesions. However, care must be taken in interpreting these results when considering the effects of these ticks on severe, chronic lesions on cattle and small ruminants. Recent experiments in this laboratory (unpublished data) have failed to produce chronic dermatophilosis lesions on rabbits with simultaneous infestations of adult *A. variegatum*. In similar experiments, using sheep as the experimental hosts, simultaneous infestations of adult *A. variegatum* and *D. congolensis* infections have resulted in the development of chronic lesions, persisting for several months (Lloyd and Walker, 1993a; Walker and Lloyd, 1993). It may not be possible to produce chronic dermatophilosis lesions on rabbits since they are not natural hosts for *D. congolensis* (Macadam, 1962). Further work is required to investigate the local effect of immature *A. variegatum* on simultaneous *D. congolensis* infections on sheep or cattle.

TABLE 4

The association of dermatophilosis lesions and nymphal *Amblyomma variegatum* attachment sites on rabbits at day nine of a primary *Dermatophilus congolensis* infection. Each 25 cm^2 area was divided into 1 cm^2 plots and the presence or absence of ticks and dermatophilosis was recorded for each plot.

Host	Dermatophilosis only (c)	Ticks only (b)	Ticks and dermatophilosis (a)	Neither (d)
Dermatophilosis and hypersensitive tick attachment sites				
Rabbit 1	12	4	5	4
Rabbit 2	14	2	4	5
Rabbit 3	6	3	8	8
Rabbit 4	13	2	8	2
Total	45	11	25	19
Dermatophilosis and inflammatory tick attachment sites				
Rabbit 5	11	1	5	8
Rabbit 6	11	2	6	6
Rabbit 7	13	0	9	3
Rabbit 8	9	0	8	8
Total	44	3	28	25

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THE SYSTEMIC EFFECT OF ADULT AND IMMATURE AMBLYOMMA VARIEGATUM TICKS ON THE PATHOGENESIS OF DERMATOPHILOSIS.

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Abstract

The systemic effect of adult and nymphal Amblyomma variegatum on the pathogenesis of experimental infections of Dermatophilus congolensis was investigated. Three groups of four sheep were used with all 12 sheep being infected with identical titrated doses of D.congolensis. One group of sheep was simultaneously infested with adult A.variegatum the second with nymphal A.variegatum and the third group were the controls, with no exposure to ticks. Assessment of the resulting infections indicate that the systemic effect of this tick is confined to the adults. Skin and serological tests using foreign antigens showed significantly reduced cell mediated and humoral immune response only in the sheep infested with adult A.variegatum.

Introduction

Amblyomma variegatum ticks have long been associated with chronic dermatophilosis lesions on cattle in the wet tropics (4). Until recently the association of these ticks with chronic dermatophilosis lesions has been based on field observations alone. The present report compares the effect of adult and nymphal ticks on the severity and duration of dermatophilosis on sheep. Adult ticks have been implicated as an important factor in the formation of chronic dermatophilosis lesions

(5, 6). However other evidence suggests that the inflammatory and hypersensitive reactions to immature ticks and other haematophagous insects may predispose to dermatophilosis (1,3)

Methods and materials

Experimental animals

Three groups of four sheep were used with equal numbers of Blackface x Suffolk and Blackface in each group. All the sheep were kept in constant conditions throughout the experiment, 18 - 20°C with 12 hours light/12 dark.

Tick infestations

The ticks used in the experiment were adult or nymphal A.variegatum from an uninfected laboratory colony, held at 16°C with 14 hours light/10 dark at 85% relative humidity. One group of sheep (Group A) were infested with 20 adult ticks each, the second group (Group B) were infested with 600 nymphal ticks and the third group (Group C) were not exposed to any ticks. Equivalent numbers of adult and nymphal ticks were calculated by counting the number and size of salivary acini found in salivary glands of both life-stages.

All of the infestations were applied to cloth bags glued to the wool on the shoulders of the sheep. The wool was shaved from inside the bags and the whole area cleaned with alcohol and ether. The adult infestations consisted of 10 males and 10 females with the males being applied 7 to 10 days before the females. The nymphal infestations were applied in three batches of 200, at weekly intervals with the final batch being applied 7 days after the adult females.

Dermatophilus congolensis infections

One day after the final batch of nymphs were applied all of the sheep were experimentally infected with D.congolensis. The D.congolensis was taken from a large batch of stabilate previously cultured and frozen at -20°C at a concentration of 1.2 x 10^7 cocci/µl. This stabilate was diluted to the required concentration of 1 x 10^7 cocci/µl in Hank's balanced salt solution with pig gelatin at 0.5% w/v.

All 12 of the sheep were infected with identical titrated doses of D.congolensis consisting of 100µl doses of seven ten-fold dilutions starting at a concentration of 1 x 10^7 cocci/µl applied to seven areas, 2 x 4 cm on the left flank of each sheep.

Before the application of the D.congolensis the wool was removed from the application sites and the whole area was cleaned using alcohol and ether. Each of the infection sites were marked using an indelible pen and the skin fold of each area was recorded. The D.congolensis was then applied, without scarification, using a bent pipette tip.

The resulting infections were assessed using a ranking system of 0 to 4 for: skin fold thickness; percentage of each area showing signs of infection; the severity of the scab, ranging from erythema to thick layers of dead, flaking epidermis; extent of exposed dermis at the infection sites. The progression of the dermatophilosis was monitored every 3 to 4 days for 4 weeks and then weekly for another fortnight.

After an interval of several weeks the entire procedure of tick infestations and D.congolensis infections were repeated using the same sheep. In previous experiments differences in the infections produced on the test and control sheep were more pronounced at the second infection.

Skin testing

All 12 of the sheep were inoculated with chicken egg ovalbumin, a T lymphocyte activator. Two B lymphocyte activators were used with six of the sheep being inoculated with a polyamino acid, poly-d-glutamate-d-lysine and the remaining sheep being inoculated with freeze-dried Brucella abortus (Central Veterinary Laboratory, Weybridge).

Initial sensitizing doses of 2.5mg of each antigen were injected intramuscularly in an equal mixture of one T and one B lymphocyte activator suspended anhydrously in Freund's incomplete adjuvant approximately one week prior to the first D.congolensis infection. A booster dose of 1.25mg of each antigen was injected in the same way one week before the start of the second infection.

The challenge injections of the individual antigens in phosphate buffered saline (PBS) were applied intradermally in five titrated doses to sites previously shaved and cleaned on the rump of each sheep. The ovalbumin and B.abortus were applied in five 100µl doses of five-fold dilutions starting at a concentration of 2.5mg/100µl. The polyamino acid was also applied in 100µl doses of five-fold dilutions, but the starting concentration was 1mg/100µl due to excessive viscosity of higher concentrations.

The skin test reactions were assessed 24 and 48 hours after challenge by measuring the skin fold thickness and average diameter of the reactions at each of the five sites. The results were analysed using the median values obtained for the reactions at each of the challenge sites.

Serological tests

Enzyme linked immunosorbent assay (ELISA) was used to measure the humoral antibody response of the sheep to the T and B lymphocyte activating antigens. Aseptic sera was collected from all 12 sheep prior to the start of the experiment and at weekly or fortnightly intervals throughout the entire procedure. For each serum sample collected, duplicate series of five doubling dilutions, starting at 1:1000 and 1:2000 were tested for antibodies to ovalbumin and B.abortus, respectively.

The tests were carried out using 96 well ELISA plates (Immulon 1, Dynatech Laboratories). Each well was coated with 0.25μg and 0.051μg of ovalbumin or B.abortus respectively. RASh/IgG(H+L)/PO anti-ovine conjugate (Nordic Immunology-Immunoconjugate) and 3,3', 5,5'-tetramethyl-benzidine dihydrochloride in phosphate citrate buffer were used to complete the ELISA. To stop the substrate reaction 50μl of 2M H_2SO_4 was added to each well, the optical density of each well at 450nm was then recorded using an optical density scanner (Titertek Multiskan, Labsystems).

Results

D.congolensis infections

Kruskal-Wallis test was used to compare the ranked scores of the dermatophilosis on the individual sheep in the three groups at each of the assessment days. During the first infection a significant difference between the groups was recorded only on day 27 with $P < 0.05$. The median ranked scores of the three groups on this day were 13.5 for the group infested with adult ticks and 1.5 for the sheep infested with nymphs or not exposed to ticks.

Using the Kruskal-Wallis test on the scores obtained on individual assessment days during the second infection demonstrated a very significant difference ($P < 0.01$) developing between the severity of the dermatophilosis lesions at day 27 which was maintained up to day 41 when the experiment was terminated.(Figure 1.)

Skin test

Using Friedman's test on the median reactions to ovalbumin and B.abortus at the five individual skin test sites a very significant difference, $P < 0.01$ and $P = 0.01$ respectively, was observed between the reactions of the three groups of sheep to the antigens. Table I shows the median reactions of the three groups to both antigens.

ELISA

Figures 2 and 3 show Log_{10} of 1/highest positive serum dilution of the serum samples when tested against ovalbumin and B.abortus antigens, respectively. Using samples collected from all 12 sheep from day thirteen after initial sensitization until 49 days after the booster inoculation.

Using Kruskal-Wallis a significant difference, $P < 0.01$, was recorded between the immune response of the three groups of sheep to ovalbumin. Using Mann-Whitney the responses of the three groups were shown to divide into 2 very significant classes ($P < 0.01$), with the sheep infested with nymphs and the controls in one class and the sheep infested with adult ticks in another class producing a significantly lower response.

Using the same analyses on the results obtained using B.abortus the immune responses again divided into the same significant classes as above.

Discussion

This comparison of the effect of adult and nymphal A.variegatum follows previous work demonstrating the significant systemic effect of adult A.variegatum on the progression of experimental dermatophilosis on sheep (6).

In this investigation moderate chronic dermatophilosis lesions were reproduced only on sheep simultaneously infested with adult A.variegatum, with nymphal tick feeding having no significant effect on the progression of the disease.

Evidence from the assessment of the clinical dermatophilosis of reduced immune response in the sheep infested by adult A.variegatum has been supported by the results obtained from the skin and serological tests.

B.abortus has been used as a sensitizing antigen in previous studies to demonstrate antibody responses in sheep infected with D.congolensis (2). The method of application of antigen in PBS was used previously by ELLIS and SUTHERLAND (2).

Due to the experimental protocol the sheep infested with adult ticks were subjected to prolonged D.congolensis infections with the remaining sheep subjected to acute infections. It is possible that the significant difference in the immune reactions of the sheep may have been caused by the different levels of exposure to D.congolensis. However it has been concluded that the reduced immunological reactions recorded were caused by the tick feeding, this role of tick feeding causing immunosuppression in the host is well documented (4,7).

In conclusion, the assessment of clinical dermatophilosis infections on sheep indicate that the systemic effect of A.variegatum is confined to the adults. A

significant reduction in both the cell mediated and the humoral immune response of sheep infested with adult A.variegatum has been demonstrated by skin and serological testing.

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Table 1 Skin test reactions of sheep in response to ovalbumin and B.abortus

OVALBUMIN

	Median reactions (Skin fold x average diameter mm)		
Amount of antigen (µg/100µl)	Infested with adults	Infested with nymphs	Not tick infested
2500	102.8	369.92	246.43
500	74.64	224.57	168.74
100	25.95	91.52	64.65
20	6.6	8.2	7.65
4	4.75	6.4	5.5

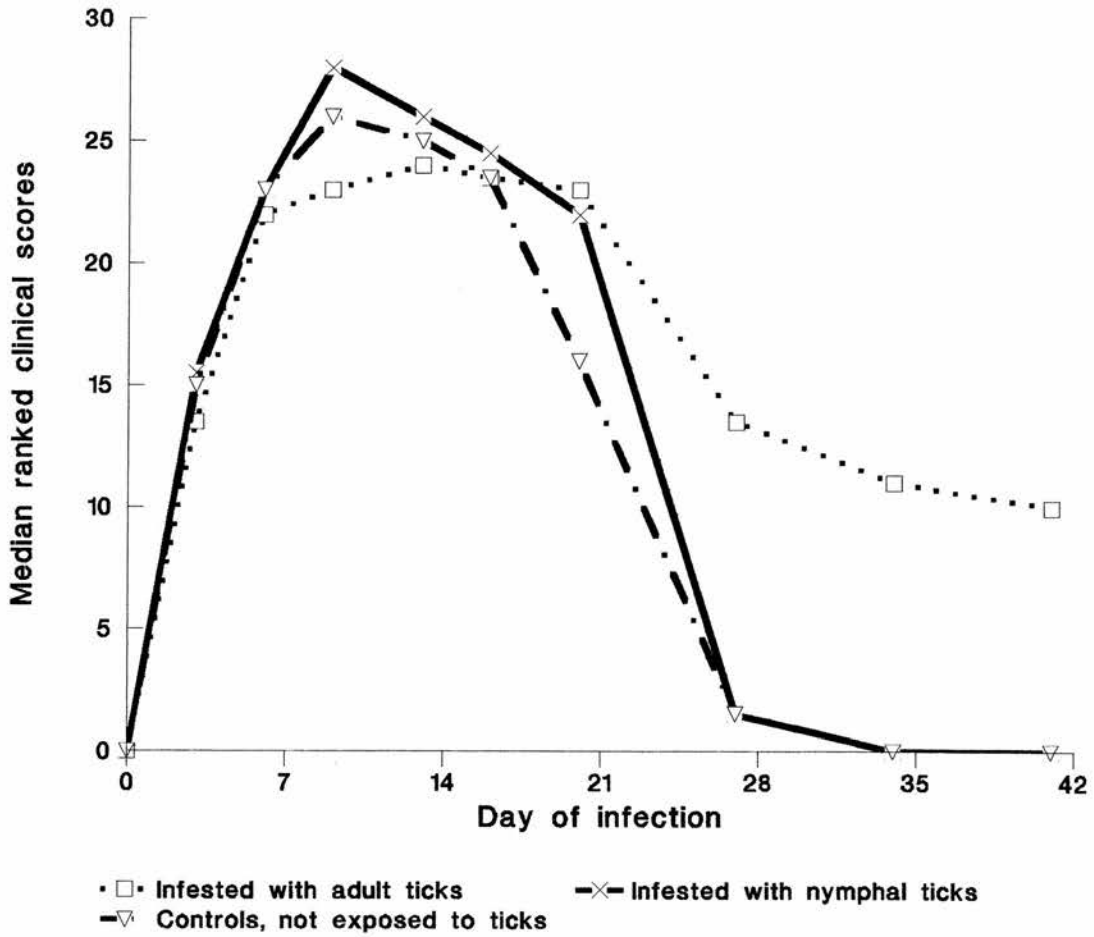
B.ABORTUS

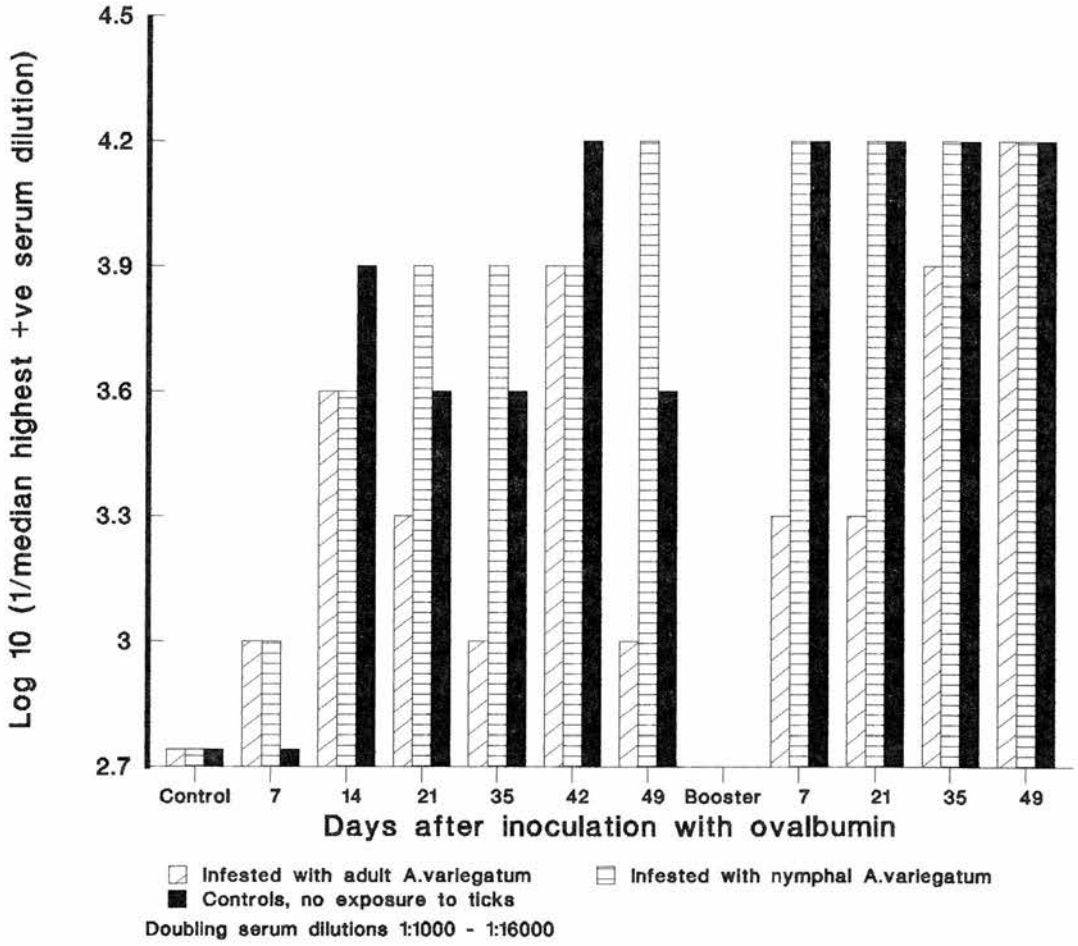
	Median reactions (Skin fold x average diameter)		
Amount of antigen (µg/100µl)	Infested with adults	Infested with nymphs	Not tick infested
2500	257.02	489.56	420.99
500	142.45	516.72	516.33
100	175.38	420.97	320.16
20	105.09	303.93	229.52
4	12.31	76.48	167.1

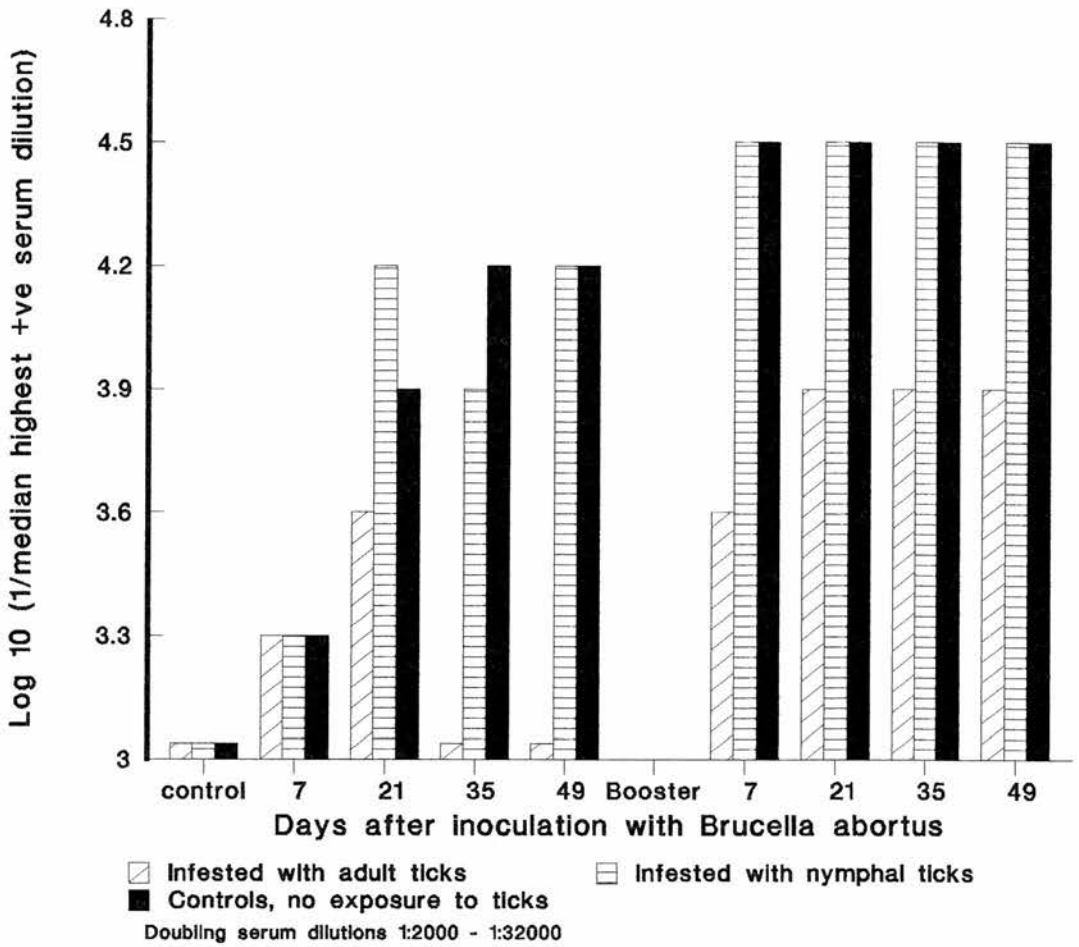
Figure 1. Changes in the median ranked scores of secondary dermatophilosis lesions on sheep. Comparison of the effect of simultaneous infestations of adult or nymphal A.variegatum.

Figure 2. Comparison of the effect of adult and nymphal A.variegatum infestations on the antibody levels of sheep in response to ovalbumin, using ELISA.

Figure 3. Comparison of the effect of adult and nymphal A.variegatum infestations on the antibody levels of sheep in response to B.abortus, using ELISA.


Median ranked clinical scores
30
25
20
15
10
5
0
0
7
14
21
28
35
42
Day of infection
Infested with adult ticks
Infested with nymphal ticks
Controls, not exposed to ticks


Log 10 (1/median highest +ve serum dilution)
4.5
4.2
3.9
3.6
3.3
3
2.7
Control
7
14
21
35
42
49
Booster
7
21
35
49
Days after inoculation with ovalbumin
Infested with adult A.variegatum
Infested with nymphal A.variegatum
Controls, no exposure to ticks
Doubling serum dilutions 1:1000 - 1:16000


Log 10 (1/median highest +ve serum dilution)
4.8
4.5
4.2
3.9
3.6
3.3
3
control
7
21
35
49
Booster
7
21
35
49
Days after inoculation with Brucella abortus
Infested with adult ticks
Infested with nymphal ticks
Controls, no exposure to ticks
Doubling serum dilutions 1:2000 - 1:32000