IXODES RICINUS, THE SHEEP TICK : ECOLOGY AND DISEASE.

A thesis submitted for the Degree of Doctor of Philosophy in the Faculty of Veterinary Medicine of the University of Glasgow.

By

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August, 1987.

" Modern science has convinced us that nothing that is obvious is true, and that everything that is magical, improbable, extraordinary, gigantic, microscopic, heartless or outrageous is scientific."

George Bernard Shaw

from the preface to Saint Joan.

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ACKNOWLEDGEMENTS.

The author wishes to express her appreciation towards the following people for their help during the course of the present studies.

Firstly to Dr. G.B.B. Mitchell under whose supervision these studies were performed, for his guidance and support throughout the last three years.

Appreciation is also due to Professor G.M. Urquhart and Dr. T.A. Brodie for helpful discussion and advice; Ms. Shahida Mohammed and Dr. S. Holmes for help with electronmicroscopy; Dr. J.S. Gray for advice pertaining to ecological studies; the Geography department at Cumnock Academy for meteorological data and to Dr. W. Gardiner for painstakingly running data through his developmental model.

Special thanks to Dr. G.R. Scott who provided T.B.F. stabilates, antigen and interesting discussion; Mr D.P. Arnot for statistical advice; Mr. J. Ritchie for contacting farmers; the stockmen at Brickrow farm unit and to Messrs C.L. Wright, D.M.L.Barber and the staff of the Veterinary Division at the West of Scotland College of Agriculture.

These studies were financed by the William Thompson Scholarship , awarded by the principal, Professor J.A. Cunningham and the Governors of the West of Scotland College of Agriculture.

Finally I would like to express my sincere thanks to the many farmers without whom these studies would have been impossible, especially Messrs Anderson, Baird, Howat, Lammie and Murray for their cooperation, hospitality and sense of humour.

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ABSTRACT.

Relevant literature was reviewed and the ecology of the sheep tick, *Ixodes ricinus* and associated disease problems studied in south west Scotland.

The development and maintenance of a colony of *I.ricinus* in the laboratory to provide tick-borne fever (T.B.F.) infected and T.B.F. free ticks for electron microscopic and tick pyaemia transmission studies was described. Additionally development times for each instar were measured during routine colony maintenance.

The activity and development of *I.ricinus* was measured over three years at two sites on Ayrshire sheep farms by blanket drags of pasture areas and tick counts on sheep. Engorged stages were placed in nylon mesh tubes under the vegetation mat, in order to monitor development to subsequent stages. In all instances development to the next instar occurred during the late summer or autumn. Activity patterns varied from year to year with a prolonged period of summer activity in 1984, a bimodal distribution in 1985 and a single spring peak in 1986. Meteorological data was recorded in an attempt, only partially successful, to apply the model devised by Gardiner and Gettinby (1983) to data from these studies.

A postal questionnaire was circulated to 300 farmers in Ayrshire and Argyll concerned with seasonal and local distribution of ticks, disease problems and control measures. The replies indicated a high tick incidence in Argyll and marked disease problems in certain regions of Ayrshire where ticks were present. Several of the farms were investigated in more detail by farm visits and

examination of blood samples.

Experimental tick-borne fever (T.B.F.) infections induced in young lambs were monitored by measurement of rectal temperatures, haematology, assessment of parasitaemias and neutrophil function tests. The classical febrile response accompanied by acute parasitaemia, lymphocytopaenia and followed by neutropaenia was recorded. Additionally an impairment of neutrophil function was demonstrated prior to the neutropaenia using an *in vitro* assay of neutrophil function.

A counter immunoelectrophoresis (CIE) test was developed to detect antibodies produced after T.B.F. infection. Sera from experimental infections in lambs and goats were used to determine the interval after primary infection before antibody could be detected, this was shown to be 9-11 days post intravenous inoculation of the organism and the period of persistence 6-10 weeks in lambs. Four hundred and thirteen ovine field sera obtained from sheep of all ages from predominantly tick infested regions of Scotland and the north of England were tested with a positive rate of 18.2%. When CIE serology was coupled with conventional examination of blood smears the detection rate for ovine T.B.F. was doubled. Additionally, antibodies were detected in a number of sera from cattle, goats and deer using CIE.

An electron microscopic technique was developed to demonstrate *C.phagocytophila* in *I.ricinus*. This technique was subsequently applied to ticks collected from one sheep farm in an attempt to estimate the level of infection. *C.phagocytophila* infection was absent from larvae, while 44% of nymphae were infected and 32 % of adults. This

result supports the previous finding that transovarial transmission does not occur.

Recent publications have indicated that an important aspect of T.B.F. infection in lambs is the associated immunosuppression which allows invasion of secondary pathogens, notably *Staphylococcus aureus* the causal agent of tick pyaemia. Several experiments were therefore conducted in lambs and mice to examine this aspect. In mice B-lymphocytes were depressed using cyclophosphamide (CY) [to mimic one aspect of T.B.F. infection] and the mice subsequently challenged with *Staphylococcus aureus* administered by various routes. Death rates , lesion formation and bacteriological isolations were greater in mice pre-treated with CY. The experiment was repeated in young lambs using T.B.F. rather than CY as a potential suppressive agent. Five days after T.B.F. infection, *S.aureus* contaminated ticks were allowed to attach and engorge upon the lambs. At necropsy, abscesses from which *S.aureus* was recovered, were present in the lungs and livers of lambs given T.B.F. and exposed to contaminated ticks, but not in controls which were only exposed to contaminated ticks. This is believed to be the first experimental production of tick pyaemia in lambs using the sheep tick *I.ricinus* as a mechanical vector of *S.aureus*.

Control of *I.ricinus* is traditionally by whole body immersion in acaricidal preparations; however more recently synthetic pyrethroid pour-on formulations have been available. Field trials to assess the efficacy of two synthetic pyrethroid pour-on products are described. Tick counts were performed on treated and control lambs and

hoggs, disease levels assessed and other effects of the treatment monitored. Results indicate a degree of tick control was achieved , but disease problems still occurred.

CHAPTER 1. LITERATURE REVIEW.

SUMMARY.

The literature relating to the sheep tick *Ixodes ricinus* was reviewed. This included : geographical, host and seasonal distributions; activity and development of the tick; length of life cycle under various conditions; tick-borne fever, tick pyaemia, louping ill and babesiosis. The application of mathematical modelling to the biology of *I.ricinus* was also discussed.

1.1 INTRODUCTION.

As far as is known at present, there are 21 species of ticks indigenous to Great Britain (Appendix 1), with a further species *Hyalomma aegyptium* attached to tortoises which until recently were regularly imported and sold as pets. The role of ticks in the human economy merits special consideration. Not only are they annoying pests, but in temperate and tropical countries they surpass all other arthropods in the number and variety of diseases transmitted to man and domestic animals (Arthur,1962 and 1963).

The sheep tick or 'castor bean' tick (plates 1 and 2) *Ixodes ricinus* Linnaeus 1758 is economically the most important tick species occurring in Britain. It exerts its effect primarily as a vector of several diseases of domestic animals, but also occasionally causes debility directly as a result of its blood-sucking habit. It is one of several species of *Ixodes* native to Britain. Only two others, *I.hexagonus* Leach 1815, the hedgehog tick, and *I.canisuga* Johnson 1849, the dog tick, are found on large animals and only the latter on sheep (MacLeod,1939a).

1.2 HISTORICAL REVIEW.

As early as 200 BC. M. Porcius Cato referred to treatments whereby 'there will be no sores and the wool will be plentiful and in better condition and the ticks (ricini) will not be troublesome'. Columella (c.60 BC.) advised that when cattle were bought one should 'pass the hand under the belly that ticks which principally attack cows may be removed'. Certain aspects of the habits and

Plate 1 Adult female *Ixodes ricinus* questing at the vegetation tips.

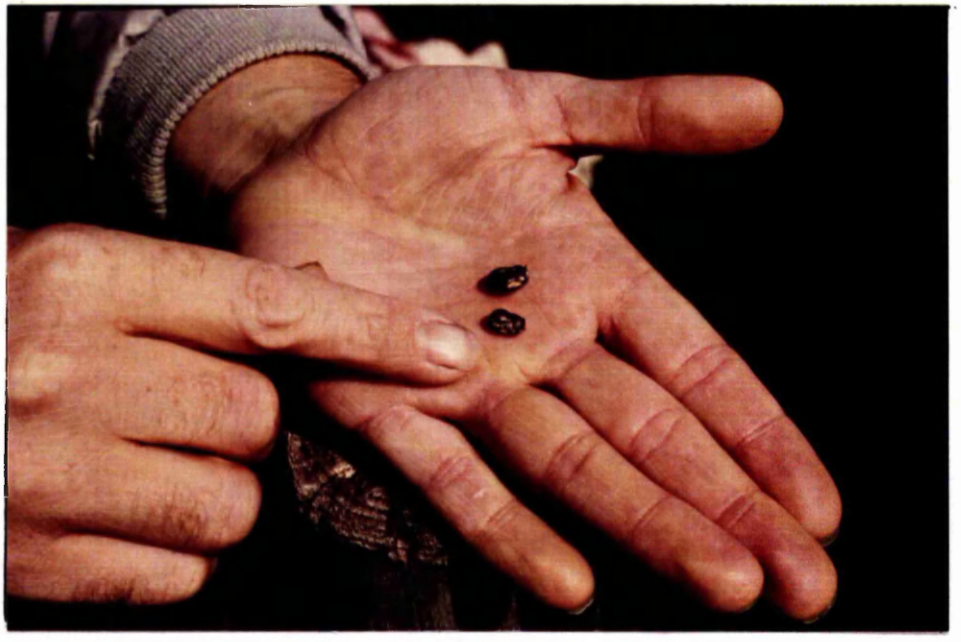


Plate 2 Engorged adult female *Ixodes ricinus*

host relationships of ticks were known to the earliest scholars, thus Aristotle in his famous *Historia animalium* stated that the ass has no lice or ticks, but that oxen have both, and on dogs ticks are plentiful'. (Arthur,1962).

Ixodes ricinus was named and described by Linnaeus in 1758. Despite the early realisation that ticks were ecto-parasites of mammals, no further descriptions were published until the latter half of the nineteenth century. Professor L.G. Neumann of Toulouse (1899) initiated a systemic approach to the study of ticks which was adopted by Nutall (1911) and his colleagues in studying *Babesia divergens* (the protozoan responsible for redwater in cattle), (Nutall 1911 and 1913). This group was hampered by a lack of coordinated knowledge on *I.ricinus*, since the little reliable data which was available was scattered in various journals and not readily accessible. During this period several comprehensive monographs were produced, for example Ixodoidea by Nutall and Warburton (1911) and Argasidae by Nutall *et al* (1908).

One of the most significant discoveries, which probably stimulated further interest in ticks, was the transmission of the Texas fever pathogen by *Boophilus annulatus*. This revelation by Smith and Kilbourne (1893), was the first report in which the transmission of a protozoan parasite by an arthropod was observed. As early as 1806 James Hogg first suggested a link between the sheep tick and louping ill. A paper by Meek and Greig Smith (1897) implicated *I.ricinus* in the transmission of louping ill virus, which was confirmed by MacLeod and his co-workers in the early 1930's (Gordon *et al*, 1932).

Several papers by MacLeod in the 1930's, and Milne and Campbell described the relationship between *I.ricinus* and its physical environment. MacLeod and his co-workers at the Moredun Institute in Edinburgh discovered the existence of tick-borne fever (T.B.F.) and demonstrated that *I.ricinus* was the vector. Subsequently Foggie also contributed greatly to this area of research. The more recent literature on the subject is reviewed in relevent chapters.

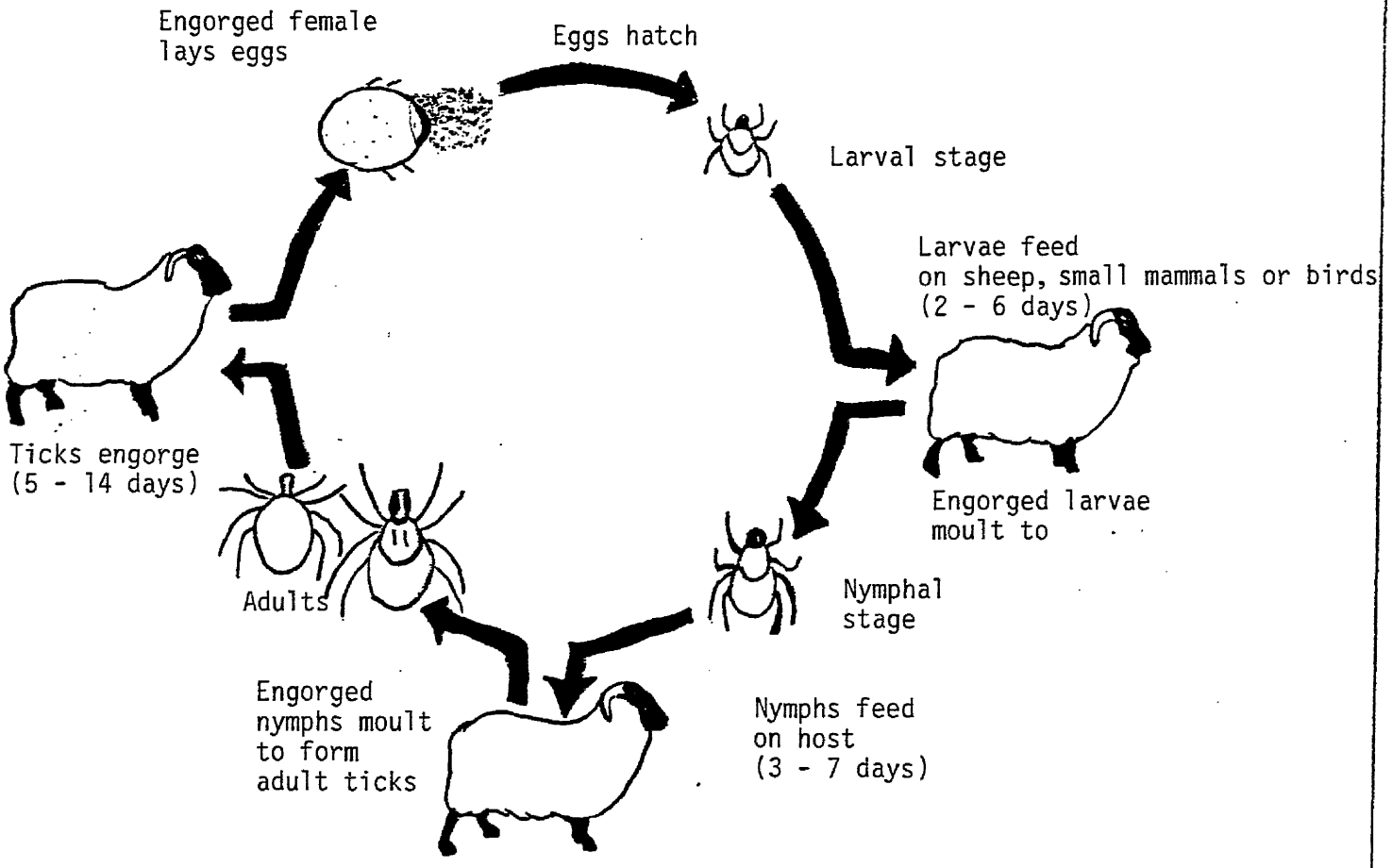
1.3 LIFE CYCLE.

Ticks are obligatory parasites of vertebrates. Hard ticks like *Ixodes ricinus* have three morphologically distinct stages: the larva, nymph and adult. Each of these stages feeds only once by puncturing the skin and consuming the blood of its host. The life cycle is illustrated in figure 1.1.

Five hundred to three thousand eggs are laid by the adult female, in clumps at the roots of grass, rushes and heather (MacLeod,1939a; Perez and Rodhain, 1977). Oviposition lasts between 30 days at an unspecified temperature (Soulsby,1982) and 32-39 days at 19'C (Nutall,1911 and 1913). Females lay very few eggs after the first month (Gray, pers.comm.). Egg production appears to be closely related to the engorged body weight and the size of the blood meal (Gray,1981).

The eggs hatch between 14 and 251 days later (Soulsby,1982; MacLeod,1932). Olenev (1924) reported hatching in 21 days under artificial conditions, while Bauch (1971) reported that August fed females laid eggs after 2-3 weeks, while September fed females entered diapause and laid eggs in May which developed in 79-421

Figure 1.1 LIFE CYCLE OF IXODES RICINUS



days.

Newly hatched larvae are inactive, remaining near the egg cluster for several days depending on temperature. Pomerantzev (1950) found that larvae remained inactive for 10 days at 15'-20'C and 570 days at lower temperatures. After this period, in favourable conditions (15'- 20'C and at a relative humidity of 100%) (Nosek,1969) the tick climbs upwards on the vegetation and reaches the tip. It may remain there for periods ranging from minutes to hours, questing occasionally for a host by waving its first pair of legs in a manner reminiscent of insect antennae. As the ticks begin to desiccate they return to the mat to reabsorb water before beginning questing activity again (Lees,1946). Many larvae fail to find a host, exhaust their energy supplies questing and perish (Gray,1981).

Those larvae which encounter a host, attach and feed for between 2 and 6 days (MacLeod,1939a; Pomerantcev,1950; Soulsby,1982). After feeding, larvae drop to the ground and moult to nymphae. The time before this moult occurs is variable: 28-357 days (Soulsby,1982).

28-56 days at 17'-21'C (Aeschlimann,1972).

80-366 days (Bauch,1971).

The emerging nymphae behave similarly, feeding for between 3 and 7 days (Soulsby,1982), though generally for 5 days (MacLeod,1932). Nymphae then moult to the adult stage 56-196 days later (MacLeod,1932; Soulsby,1982).

Adult males rarely feed, but adult females engorge for 5-14 days, (Pomerantcev,1950; Aeschlimann,1972; Bauch, 1972), the average time required being 8-9 days (MacLeod,1932).

Some authors have suggested that mating occurs on the host (MacLeod,1939a), but more recent work has shown that it occurs mainly off the host, thus Perez and Rodhain (1977) state that 66.84% of unfed females are fertilised before reaching a host; this was also found by Graf (1975). The latter author also presents evidence of a sex pheromone produced by females to attract males and to a lesser extent other females.

After fertilisation and engorgement, females drop from the host and undertake a minor horizontal migration. According to Milne (1950) this is no more than 30 cm, but Cerny (1959) suggested that from his observations in Czechoslavakia several metres displacement may occur in one month. Eggs are then deposited in the vegetation mat or cracks in the ground within two months (Perez and Rodhain 1977) and oviposition is followed by the death of the spent female within 7-21 days (MacLeod,1932).

The length of the life cycle varied from 1.5 to 4.5 years in Britain (MacLeod,1939a). Leow (1964) stated that it was 2 years in Austria and in Switzerland Aeschlimann (1972) thought that it was 3 years. Chmela (1969) reported that under unfavourable conditions in Czeckoslovakia the duration was 6 years.

The above is a record of general development times which occur. Bauch (1971) states that different biotypes are important as they give rise to varying developmental periods, especially for the larva-nymph and egg-larva transitions.

1.4 DISTRIBUTION.

1.41 Host.

1.411 General.

The local and geographical distribution of *I.ricinus* appears to be dependent upon biotic factors (MacLeod,1936b). Host availability also influences the epidemiology of the tick. A study of alternative hosts (MacLeod,1939a) showed that the presence of the chief host, the sheep, is not necessary for the continued presence of the tick. In fact, when the range of alternative hosts from which the tick has been recovered, is considered, (Appendix 2), it is seen to include a wide range of rodents, birds, herbivores, carnivores and sub-tropical lizards of widely differing geographical regions. In view of the life history and habits of *I.ricinus*, almost any hosts above the amphibia, which either feed on the ground or use it as a nesting site, can be parasitised. Bearing in mind the host range, it is unlikely that host availabilty will affect the distribution of *I.ricinus* on a global scale, but the presence or absence of hosts may affect populations on a local basis (Milne,1948a). Thus MacLeod (1934) and Milne (1948a) suggest that in the absence of sheep (and of a large proportion of alternative hosts) over a period of several years the remaining wild fauna will adequately support the tick population.

From the literature it is often impossible to determine which stages of the tick infest a particular host. Arthur (1962) states that nymphae and larvae predominate on birds and mammals smaller than stoats. A comprehensive study on the host relationships of *I.ricinus* was conducted by Milne

(1948a and b), covering hill and moorland sheep farms in northern England and the Scottish borders. He reached the following conclusions:

-it is probable that *I.ricinus* is able to parasitise any bird or mammal, the criterion only being their presence on tick infested ground.

-no invertebrates or reptiles, except *Lacerta vivipara* are parasitised by *I.ricinus*.

-male ticks rarely attach themselves to the host although they are found on them. Female ticks can only parasitise domestic animals and some of the larger wild mammals (red and roe deer, brown and mountain hare, rabbit, fox, badger, otter, stoat, hedgehog) with larger birds (red grouse, pheasant and magpie) occasionally being recorded as hosts.

-all British hosts except the shrews (the smallest British mammals) and possibly the mole (probably because of its underground habit) are recorded as carrying nymphae.

-all British hosts can harbour larvae. The presence of either or both nymphae and larvae depends on sample size, time of year and the care taken in examining the animal, particularly when looking for larvae.

-the smaller the host the fewer adult ticks relative to nymphae and the fewer nymphae relative to larvae.

-generally, the smaller the host the lower the rate of infestation. Smaller organisms present a smaller surface area and cover a smaller area in their movements. That is, the less the area of ground covered in a unit time, the less chances of encountering ticks (Milne,1947b). Self de-ticking may contribute to this lowered infestation,

particularly in birds, where ticks are rarely found within reach of the beak (Milne,1948b).

1.412 SHEEP.

There are often marked variations in the susceptibility to ticks between individual sheep. These variations appear to be dependent both on physical condition and breed. MacLeod (1939b) showed that ewes with lambs had higher infestations than barren ewes, which in turn had higher infestations than hoggs. Previously he had demonstrated that Scottish Blackface sheep were more susceptible to adult female and nymphal tick infestations than Cheviot sheep on the same pasture during the same period (MacLeod,1932). The effects of pregnancy and reduced condition were confirmed by Milne (1947b).

Further work by Milne (1947b) revealed information pertaining to the distribution of ticks on the ovine body. Female ticks were generally found on the relatively bare areas of the axillae and the inguinal regions and the hairy areas of the head (ears, muzzle and chin) and to a lesser extent the short wool adjoining these areas.

Larvae were usually found attached to the lower parts of the legs and around the lips, while nymphae tend to congregate on the face and ears and under the chin.

Milne (1947b) also observed that considerable variations existed between individual infestation levels. These appeared to depend on host size, ewes having more ticks than hoggs which in turn had higher infestations than lambs, confirming the work of MacLeod (1939a). Host activity (ie. the area covered in unit time) was related to the tick burden.

1.413 CATTLE.

The work of Edwards and Arthur (1947) showed that cattle were the preferred host of *I.ricinus* in South Wales. Their observations showed that ticks were more numerous on the hind-quarters, hind legs, folds of flank and the udder at the beginning of the tick season, probably because of the larger area of hairless skin available. As the season progressed, Evans (1951b) found that the percentage of adult females on the fore-quarters increased.

Evans (1951b) also found that on the head, foreleg and hindleg regions, each tick stage had a definite zonal distribution. In the udder region females were evenly distributed, but larvae and nymphae were seldom found.

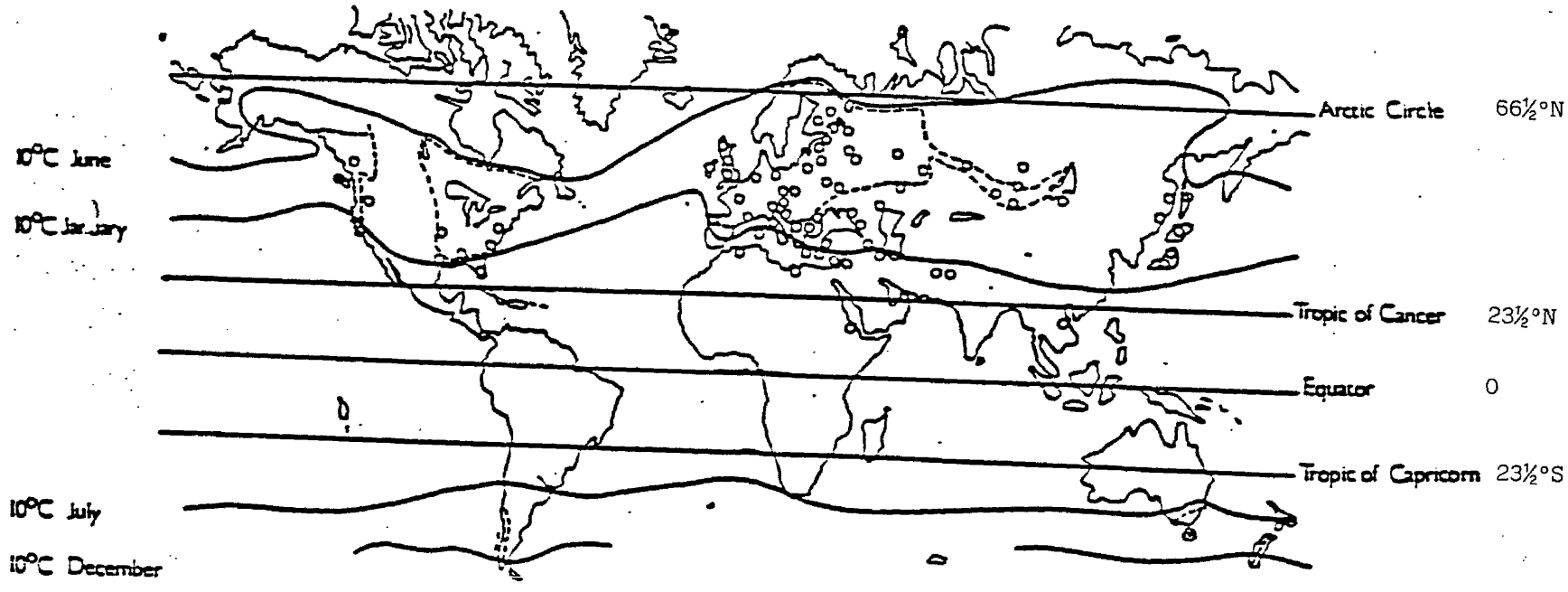
Edwards and Arthur (1947) studied six breeds of cattle (Shorthorn, Hereford, Welsh Black, Friesian, Ayrshire and Red Poll) and found that there was no difference in their susceptibility to ticks. Tick density was not related to physiological condition or the reproductive state of the host.

1.42 GEOGRAPHICAL.

1.421 WORLDWIDE.

Like all ticks, *I.ricinus* is a poikilothermic organism. It might be assumed that climatic factors would have a major influence on its relative abundance and distribution. However, despite extensive work on this subject, the precise nature of 'weather effects' on the ticks' lifecycle remains obscure.

The species is found in much of Europe, parts of North Africa and Asia (figure 1.2), but is absent from Australasia, possibly due to problems in dispersion and



Temperature limits of tick survival

Fig.1.2 Worldwide Distribution of Ixodes ricinus L.

——— Areas of theoretical survival

o Localities of known occurrence

Modified from MacLeod, 1936b

the few reports from North America are probably due to misidentification (Soulsby,1982).

The potential and actual distribution of *I.ricinus* is discussed by MacLeod (1936a). It is limited by a combination of microclimatic and edaphic factors. In any given climatic type, distribution will be determined partly by the separate action of each constituent factor, and partly by the interaction of the different factors. Thus, rainfall is an important factor in determining microclimatic humidity, but this will also depend on wind velocity and direction, soil type and vegetation. Temperature also interacts with other physical effects.

The tick requires a microclimatic relative humidity of at least 80% (MacLeod,1962), and is only present in areas which can provide this throughout the year. These conditions could be satisfied at almost any latitude. In contrast, temperature is largely controlled by latitude and will therefore be the fundamental factor limiting distribution, although a single parallel cannot be expected to represent a limiting factor. Senevet and Rossi (1926) found that, with a few exceptions, the areas from which *I.ricinus* had been recorded were north of 40' latitude. The few recordings south of 40' were attributed to a winter population and inhibition of development by high summer temperatures. Similarly, the northern limit of the distribution of *I.ricinus* lies between 50' and 60' (Olenev, 1934), except between the 30' and 40' meridians where it has been recorded further north.

Tick activity is limited by temperature. Although survival is possible between -14'C and 35'C, development

only occurs during periods of at least 3 months with a mean monthly temperature above 10'C and parasitism is only possible when the temperature of the coldest month exceeds 10'C (MacLeod,1936b).

1.422 GREAT BRITAIN.

The distribution of *I.ricinus* in Great Britain is limited to regions of upland in Scotland, northern and south western England, Ireland and Wales. Thus, it is allied closely to the areas of hill sheep farming (figure 1.3).

In Great Britain, distribution is not limited by host range and density, which are very large, or by temperature as the requirements discussed above are met throughout the year, but by the level of microclimatic humidity available to the free-living stages (MacLeod,1935; 1936b; 1939a). The lower limit of humidity necessary for the survival of larvae and nymphae was shown to lie between 70 and 75% at a temperature of 10'C and between 75 and 80% at 10-30'C (MacLeod,1935). Below these levels mortality of active stages occurs within 15 days, although nymphae are more tolerant of aridity than larvae. Engorged stages require humidities of 85% or over for development and 80% or more for oviposition (MacLeod,1939a).

Survival of unfed stages of *I.ricinus* depends upon the persistence of high humidity in the microhabitat. This is only possible in areas of poor drainage and coarse vegetation. Areas of high humidity in Britain are generally sheep pastures on hills, moors and heaths, where water levels are high and evaporation from the soil is continuous. This evaporation becomes trapped in a layer of

Fig.1.3a Distribution of I. ricinus in Great Britain

Fig.1.3b Sheep Farming in Great Britain

air above the soil, if a dense mat of humus forming vegetation is present. The mat is made up of decaying vegetation and is most dense when the vegetation is rank or coarse and composed of, for example, heather (*Erica* species), rushes (*Juncus*), bracken (*Pteris aquilinium*) or coarse grasses such as *Nardus*, *Agrostis*, or *Festuca* (Macleod,1936b and 1939a; Milne,1944; Arthur,1949; Evans, 1951a).

Generally tick survival is only possible in lowland areas in the spring and autumn. If however, the pasture is allowed to degenerate, allowing ecological succession to alter the vegetation, ticks may become established (MacLeod,1936a; Roberts, cited in Walton,1927). Macleod (1938), stated that 'except during winter low temperatures, neither the unfed nor the gorged tick will survive more than a few days under ordinary air conditions in this country'. However, Mitchell and Renton (1983) reported that ticks apparently survived the winter on in-bye fields, despite cultivation of a pea crop on one field the previous year. No ewes or hoggs which could have carried infestation had been moved to the fields in question during this period.

1.43 SEASONAL.

In most text books it is stated that ticks show a characteristic pattern of seasonal distribution being active in the spring and autumn. Thus as early as 1899 Wheler drew attention to the fact that on some British farms ticks seem to attack the sheep in two waves, a spring wave starting in March which is over by late May or early June and a second wave commencing in August. However, on

some farms only a spring wave has been recorded (MacLeod,1932; Milne,1945; Edwards and Arthur,1947) and on others a summer peak in July (Hendrick *et al*, 1938).

MacLeod (1939a) has published extensive data on the incidence of adult female *I.ricinus* on sheep in Britain. He stated that in Argyll, Perthshire, Selkirk and Northumberland, tick incidence is greatest in the spring (April-May), decreasing markedly in early summer, but showing a slight autumnal increase in activity, particularly in Argyll. Adults appeared first followed by nymphae which outnumbered them until June when again the adults became more numerous. Nymphae were more prevalent in the autumn and larvae, present throughout the season, in May and June.

Studies by Milne (1945) in northern England revealed a bimodal type of activity on a farm in south west Cumberland, with peaks of activity of adult females in May and September. However, only one peak of activity in April-May was observed on a farm in north east Northumberland, while the work of Hendrick *et al* (1938) showed that ticks in Aberdeenshire were active throughout the year with the peak generally being in July.

Several workers have studied seasonal activity in Wales including Edwards and Arthur (1947); Arthur (1949); Evans (1951a) and Herbert *et al* (1981). On experimental plots in south Wales (Margum Moors, Glamorgan), Edwards and Arthur (1947) found that tick activity was bimodal. Numbers of adults and nymphs peaked in early April-May and to a much lesser extent in September. Larvae appeared at the same time as adults, but reached their peak several weeks later.

However, in mid-Wales as in Aberdeenshire there was some evidence that a single peak of activity occurred reaching a maximum in August (Edwards and Arthur,1947). Single peaks of activity have also been recorded in other regions of central Wales, but are unobserved in the south (Arthur,1949). Work in Gwynedd, north Wales (Herbert *et al*, 1981) showed that adult ticks peaked in the spring and larvae in the summer. The peak in nymphal numbers varied between August and October one year and April and June the following year.

Studies in Ireland have shown several differences between the British and Irish tick populations (Gray *et al*, 1978; Gray, 1980 and 1984). These differences include marked bimodal activity for nymphae, almost unimodal activity (June to August) for larvae with a peak in midsummer, and greatest adult activity in the summer months. However, adult spring activity became dominant by the third year of the study, with no alteration in stocking regime.

Wheler (1899) suggested that the absence of ticks during midsummer was due to the available ticks having fed by June at which time the autumn brood had not completed development. Later studies showed that the situation could not be so simply explained.

In his discussion, MacLeod (1939a) states 'that the seasonal incidence would thus seem to be most easily reconcilable with the theory of temperature control of tick activity. In north Africa, the southernmost limit of distribution of *I.ricinus*, the species is active only in the winter, whereas in northern Russia, the northernmost

limit, it is active in the summer'.

In some areas of Britain a bimodal seasonal curve of tick distribution does not occur and only a single peak of tick activity in the late spring or early summer is found (Hendrick *et al*, 1938; Edwards and Arthur, 1947). This suggested a theory of physiologically different populations of ticks in different regions (MacLeod,1932), which was superseded by Campbell's (1948) theory that there were two seasonal populations, one exclusively spring active and one autumn active; some areas had both populations and showed a bimodal distribution of ticks and some had only one population showing a single peak.

Both theories were formulated with the aim of explaining why a cold-blooded organism, whose development in the laboratory could be seen to be dependent on temperature (MacLeod,1939b) showed a pattern of incidence which was at variance with the general trend of seasonal temperature levels.

Throughout its life cycle *I.ricinus* alternates between the engorged state, where developmental processes are of paramount importance and the unfed state in which activity patterns predominate. MacLeod (1934; 1935; 1936b) pointed out that the basic functions of the engorged state were survival and development, whereas those of the unfed state were survival and host-finding activity. He suggested that engorged and unfed ticks should be examined separately as it must not be assumed that environmental conditions best for one would even be good for the other. In the laboratory, studies made mainly by MacLeod (1939b), confirmed this tenet. In particular the range of

temperatures suitable for development was above the maximum suitable for activity and the following values for physical conditions were established:

a) relative humidity must be above 80% for survival.

b) temperature range for survival of at least one day; -15'to 30'C

c) optimum temperature range for activity of unfed stages; 14' to 24'C.

d) optimum temperature range for oviposition; 10' to 27.5'C.

e) optimum temperature range for development of eggs; 15' to 30'C.

f) optimum temperature range for the development of engorged ticks; 15'to 35'C.

It emerged that the tick had a great tolerance for moist conditions, larvae surviving for 80 days and females for 21 days, submerged in water.

In summary, MacLeod concluded that temperature and humidity exercise a controlling effect, the former limiting periods of the ticks' activity and the latter its local distribution. Milne (1947a; 1948a; 1950) also produced evidence that the mat at the base of permanent, rough vegetation produced a microclimate sufficiently humid all year round to support *I.ricinus*. The tick was limited to such a microhabitat and in consequence was a tick of wetter areas with coarse vegetation.

Lees (1946) examined the activity of unfed ticks and showed that an increase in temperature stimulated increased activity which tended to be negatively geotropic resulting in the tick climbing up the vegetation, entering a zone of

drier air and questing at vegetation tips. Lees and Milne (1951) placed newly emerged, unfed ticks of all three stages in tubes at the base of the vegetation and observed their behaviour. They showed that when unfed larvae were placed in position at the time of the autumn rise, some became active that autumn and some not until the following spring; and that all activity had ceased by June of the following year.

Chmela (1969) conducted experimental observations on the development of ticks under field conditions in Czechoslovakia. He placed newly fed ticks of all stages in tubes at the base of the vegetation and observed when the next stages emerged. He found that irrespective of when the ticks were put in place, all newly metamorphosed ticks emerged in the autumn. If they were too late to complete development in the same year, then emergence did not occur until autumn of the followimg year. Thus emergence did not occur during the low temperatures of the winter months, nor in the spring when temperatures rose to suitable levels. Therefore, it appears that the spring population of active ticks is a residue from the previous autumn. Since all of the previous season's ticks had died by June, it was proposed that the autumn population was composed entirely of the current seasons' additions.

Chmela (1969) observed that development ceased in the engorged ticks of all stages due to low autumnal temperatures and was not immediately resumed even in the spring. This phenomenon is generally known as diapause and has been confirmed by Cerny *et al* (1974).

Bauch (1972) working near Magdeberg in the German

Democratic Republiic, also reported that only part of the large number of unfed ticks which appeared in the autumn became active in that season, whereas all the overwintered population became active in the spring.

In re-examining seasonal activity of the tick, it was suggested that the tick year be reckoned from June to June, and not from January to December (Donnelly,1978). Using this approach the apparently anomalous seasonal distribution of ticks is more easily appreciated.

This summary of tick development prevails as the generally accepted view.

1.5 DISEASE TRANSMISSION

1.51 INTRODUCTION.

I.ricinus has been implicated in the transmission of viruses, piroplasms, rickettsiae and associated with bacterial infections. In addition a number of conditions arise directly from infestation with *I.ricinus*, including tick paralysis, tick worry, bite injuries and anaemia.

1.52 TICK PARALYSIS.

Tick paralysis is caused by acute intoxication due to the bites of adult female and sometimes nymphal ticks. The toxin, a neurotoxin, particularly affecting neuro-muscular junctions in the peripheral nervous system, is injected with saliva. It originates either in the ovaries and passes to the salivary glands in the later stages of engorgement, or is produced in the salivary glands. It causes fever and acute ascending flaccid paralysis. The degree of paralysis depends upon the number of ticks attached and the length of time that they have been

feeding. Recovery occurs very quickly if the ticks are removed before respiratory and cardiac failure. Young animals are more susceptible than older animals and acquired immunity in recovered animals may last up to 8 months (Robertson,1976; Smith,1973; Soulsby,1982).

Tick paralysis is associated with a number of tick species. In the near and middle East the most frequently involved species is *I.ricinus gibbosus* (Robertson,1976). The condition is also found in Australasia, North America, South Africa, Europe and Russia. Domestic animals which are commonly affected are cattle, sheep, pigs, horses, goats, dogs, cats and poultry. Man may also be affected. The condition has not been recorded in Great Britain but *I.ricinus* has been implicated in the paralysis of sheep and goats in Crete and Israel.

1.53 TICK WORRY

Tick worry is caused by a heavy infestation of ticks. It causes irritability and affects general behaviour patterns, preventing resting and feeding. This leads to reduced levels of weight gain and productivity. Injuries from bites are due to the characteristics of the mouthparts of the tick. These are debilitating in themselves, but also predispose the affected animal to secondary infections with bacteria, fungi, blowfly, headfly and screwworm larvae. The associated irritation causes rubbing leading to wool loss in sheep and hide damage in cattle (Al-Shadebi,1978).

Blood loss and therefore anaemia may occasionally be severe. A single female *I.ricinus* can remove 0.5-2ml of blood in addition to that lost in clotting when the tick drops off (MacLeod,1939a). Thus, a heavy infestation of

ticks may cause death directly and indeed Al-Shadebi (1978) states that tick infestations in general cause greater economic losses than tick transmitted diseases. Barnett (1968) has also suggested that the simultaneous feeding of 6-10,000 female ticks can kill an adult bovine, although in practice, cattle can withstand much larger numbers as has been shown in Australia where 20,000 adult *Boophilus* failed to kill a steer, but 500 killed a calf (Barnett,1968).

1.54 VIRUSES

1.541 GENERAL.

Ticks have been shown to harbour and in some cases transmit 22 viruses infective to man and animals (Balashov,1968). Tick-borne viruses transmissible to man were reviewed by Hoogstraal (1967) who discussed the role of *I.ricinus* in the transmission of several encephalitides in the northern hemisphere. These are often known by their geographical location and include: Bukovian haemorrhagic fever, Omsk haemorrhagic fever and Central European Tick Borne Encephalitis (T.E.) which is also known as biphasic milk fever. The latter is a member of the Russian Spring Summer (R.S.S.) complex.

The incidence of viral infection is generally low in both animals and man. Host infection is by tick bites and also by ingestion of raw milk from infected animals. *I.ricinus* acts as both vector and reservoir of infection, and the natural cycle of both tick infestation and viral infection also involves wild mammals and birds (Chatelain *et al*,1979). Virus is excreted in tick faeces and tick transmission is generally transtadial (Lvov,1978).

A number of other viruses have been isolated from *I.*

ricinus, but the tick has not yet been implicated in their transmission. These include Tribec virus in Czechoslovakia and Japanese-B-encephalitis (normally transmitted by the culicine mosquitoes) in the Far East. (Moore,1984).

Experimentally *I.ricinus* has been shown to act as an overwintering host for Eastern Equine Encephalomyelitis and as an active vector of Langat Fever which is normally transmitted by *I.granulatus*.(Smith,1973).

1.542 LOUPING ILL (TREMBLING).

The British representative of these encephalitides is louping ill which is caused by a neurotropic virus of the R.S.S. complex. Louping ill is restricted mainly to northern England, Scotland and Ireland and the virus has been isolated from sheep (most commonly affected), cattle, pigs, dogs, horses, deer, hares, various small mammals and red grouse (Smith *et al*, 1964; Varma,1964). Several human cases especially amongst laboratory workers have been reported (Smith,1973). As early as 1806 James Hogg, the Ettrick shepherd noted the association of louping ill with ticks, this was supported by Meek and Greig-Smith (1897) who implicated *I.ricinus* in the transmission of louping ill, which was confirmed by MacLeod and Gordon (1932).

In sheep, louping ill produces a febrile infection (up to 107'F) followed by invasion of the nervous system, where viral replication causes trembling, jerkiness, incoordination with kicking of the limbs in the final stages (Kavanagh and Purcell,1972) hence the term 'louping' which means jumping or jerkiness. In a proportion of animals, virus replication in the brain causes

sufficient damage to result in death. Diagnosis of louping ill is dependent on the recovery of virus, or the presence of neuropathological lesions of a severity sufficient to cause death from acute viral encephalitis.

The incubation period is generally three weeks, although 39 days was recorded by Brotherston *et al*, (1971).

In endemic areas, flock immunity builds up with lifelong protection for recovered animals and annual mortality amongst susceptible sheep of up to 10 per cent. These are usually animals in their second year at pasture. Lambs are protected in their first year by effective long term colostral immunity (Kavanagh and Purcell,1972). Nevertheless if animals of any age are brought in from non-endemic areas, or if louping ill appears in a previously 'clean' area, mortality can reach 50-100% (MacLeod,1939a). However, severe clinical louping ill mainly affects one year old sheep when they are returned to tick infested pastures in the spring, after over-wintering on tick free ground (Brotherston *et al*,1971).

The disease is transmitted by nymphal or female *I.ricinus* which have aquired infection in the preceding stage. Infection does not pass through the egg (MacLeod,1939a), and in endemic areas only 1 in every 2,000 ticks may be infective (Reid, pers.comm.).

Gordon *et al* (1962) suggested that a concurrent infection of tick-borne fever (T.B.F.) may aggravate louping ill infection. Experimental animals harbouring both diseases had louping ill virus in the central nervous system (C.N.S.), but in those without T.B.F., virus was isolated only from the spleen and was absent from the

C.N.S.

Reid *et al* (1986) infected 18 sheep with louping ill virus 5 days after T.B.F.. Sixteen of these died having developed high viraemic titres, although death was asociated with a haemorrhagic syndrome, rather than classical louping ill, while 8 sheep given louping ill virus alone survived showing little or no clinical signs. The probability of this syndrome occurring naturally will depend on the duration of the enhanced susceptibility to louping ill virus infection following exposure to T.B.F. Reid *et al* (1986) administered louping ill virus only five days after injection of T.B.F. and thus, if this interval is critical, the syndrome may occur only infrequently. A further feature militating against the natural occurrence of the syndrome is that colostral antibody is very efficient at protecting lambs from louping ill virus (Reid *et al*, 1976) but does not normally provide any protection from T.B.F. Thus the interval between lambs becoming infected with T.B.F. and louping ill is likely to be considerable. However, sheep introduced to tick infested pasture for the first time are most likely to become infected with both agents together and the consequences of dual infection reported by Reid *et al* (1986) could explain the very high mortality level attributed to louping ill virus in this category of animal.

An apparently similar syndrome has been described in British sheep on two previous occasions. Haëmorrhagic enteritis was reported in sheep following experimental inoculation of T.B.F. (Foster *et al* ,1968) and intestinal mycosis was reported in a lamb (Angus *et al*,1971) also

given T.B.F.. In the light of the findings of Reid *et al* (1986) , it is possible that on these two occasions blood used for T.B.F. infection also, incidentally, contained louping ill virus.

From the early 1930's until 1967 a formalin inactivated vaccine was available to protect sheep, but because of danger to laboratory workers engaged in its production it was withdrawn. Thus the main method of combatting louping ill was no longer available until a suitable alternative was found. It appeared in the form of a new inactivated oil-adjuvant vaccine in 1971. The majority of farmers in areas where louping ill is present protect their flocks with the vaccine, and protection lasts at least one year and probably two or three. In areas where its use has been discontinued, sporadic outbreaks have occurred (Brotherston *et al*, 1971).

1.55 BACTERIA

Tick pyaemia is associated with infection by the bacterium *Staphylococcus aureus*, although not all strains are capable of causing tick pyaemia. In Great Britain the disease is always associated with the presence of *I.ricinus* (section 1.572). The syndrome was first reported by M'Fadyean (1894). Walton, (1927) described a condition on hill farms in north Wales involving 'sores, swellings and blisters on the leg joints.' He found that lambs were most seriously affected, and those which survived remained unthrifty for life. They were generally affected between two and six weeks of age, McEwan,(1947) reported a mean age of 3.8 +/- 1 weeks.

Presumably tick pyaemia may either be due to the

contamination of tick bite lesions, *S.aureus* being normally present on the skin surface, or to the injection of the bacterium by the feeding tick (Kavanagh and Purcell,1972). In either case the organisms pass into the bloodstream causing pyrexia. Subsequent symptoms depend upon the site(s) of abscess formation following localisation of bacteria. Sites include: the brain and spinal cord, leading to nervous signs such as paralysis and incoordination; joints leading to swellings and lameness (joint ill) and parenchymatous organs including the liver, spleen, and lungs with consequent impaired function and unthriftiness (Reid, 1978).

No correlation has been noted between the severity of tick infestations and the number of lambs affected with tick pyaemia (Foggie, 1962).

1.56 PIROPLASMS.

A number of tick-borne protozoan infections of sheep occur in the U.K., including *Babesia*, and *Theileria*. Two species of *Babesia* are known to occur in British sheep. The first, probably *B.capreoli* from deer, was originally isolated from a sheep in Argyll which had been splenectomised at the veterinary faculty in Glasgow and subsequently developed a transient babesial parasitaemia (Reid *et al* ,1976). This *Babesia* species was subsequently passaged in sheep both at Glasgow and at the Animal Diseases Research Institute, Compton (Purnell *et al*, 1981). In addition, *B.motasi* was isolated from *Haemaphysalis punctata* ticks on sheep pasture in Wales (Lewis and Herbert,1980). Neither of these appear to be a cause of economic loss in sheep. The same can be said of another

haemoprotozoan parasite *Theileria ovis* , which has been isolated from British sheep (Lewis *et al*, 1981).

In Britain, piroplasmosis can be debilitating in cattle (Reid and Martin, 1982) and is caused by *Babesia divergens* and *Babesia major*, both transmitted by *I.ricinus*. *B.major* is restricted to the south east of England and produces a benign, subclinical condition (Adam and Blewett, 1978). *B.divergens* causes an important disease which is widespread in Britain, being endemic in Wales (Evans, 1951b), parts of south west England, (Arthur, 1963) and in Northern Ireland (Kavanagh and Purcell, 1972). It occurs only occasionally in Scotland. Blewett and Adam (1978) have demonstrated the presence of benign babesiosis in Scottish red deer. It is thought to be caused by a piroplasm distinct from *B.divergens*, but may be identical to *B.capreoli*, which affects roe deer. The similarities between these piroplasms have suggested a common ancestry.

Babesiosis is also referred to as redwater as one of the symptoms is the presence of blood in the urine. Other symptoms reported by Kavanagh and Purcell (1972) include fever, occasional diarrhoea, weakness, increased respiratory and heart rates with anaemia, jaundice and death in some cases.

Young calves are innately immune for about 6-9 months (Purnell *et al*,1978). Infection of this age group is usually mild resulting in decreased clinical disease in closed herds and life long non-sterile immunity (premunity). Recovered animals remain carriers of the disease. It is only when animals aged more than 9 months encounter disease for the first time, usually through

transport to infected pastures, that the clinical disease of redwater ensues (Reid and Martin,1982).

Premunity does not extend to different strains of *B.divergens* and there is evidence to suggest that the pathogenicity of the different strains varies according to area (Purnell *et al*,1978).

Gray and Lohan (1982) studied the distribution of ticks affected with *B.divergens*. They found that where inoculation rates (and tick density) were high, redwater fever was rarely seen. The highest disease incidence occurred where inoculation rates were not high enough to ensure infection of the cattle as calves and consequent development of immunity, but adequate to result in infection over a longer period of time.

1.57 RICKETTSIAE

1.571 GENERAL.

Two species of rickettsia have been recognized as causing widespread mortality among cattle and sheep, in Africa, Australasia, Asia and parts of Europe, though not in Great Britain. *Anaplasma marginale* is the single most important rickettsia in cattle. It is a parasite of the red blood cells in which it appears as a small dense structure towards the margin. The symptoms of anaplasmosis are somewhat similar to those of babesiosis and there is severe anaemia. Also since *A.marginale* shares tick vectors of the genus *Boophilus* with the tropical *Babesia* species, *B.bovis* and *B.bigemina*, it often occurs with them.

The other important pathogenic rickettsia is *Cowdria ruminatum*, the causative agent of heartwater. It is not

found in Great Britain although it is found in other parts of Europe, Africa, Asia and Australia. The symptoms of infection are fever and a variety of nervous signs, such as incoordination, twitching, and continuous chewing. Unfortunately, by the time these signs are detected the animal is beyond treatment. Diagnosis is complicated by the lack of an identifiable blood stage. The disease is confirmed by the detection of the rickettsia in Giemsa stained smears of brain tissue and by sub-inoculation of blood into a susceptible animal (Moore,1984).

I.ricinus may also be implicated in the transmission of the rickettsial organism *Eperythrozoon ovis* (Overas, 1959) normally transmitted by horse flies, (Tabanidae). Eperythrozoonosis is an occasional disease of sheep in South Africa, Australasia, U.S.A. and Europe (Moore,1984), but has only been recorded in Great Britain on three occasions. The first isolations occurred in Scotland and in Somerset (Foggie,1961; Rouse and Jackson,1966), more recently *E.ovis* has been found as a contaminant in blood from a sheep in Argyll which was also shown to be infected with *Babesia capreoli* (Purnell *et al*, 1981).

Eperthrozoon organisms are pleomorphic and are found in or between the erythrocytes. In experimental infections the incubation period is between 2 and 26 days (usually 5-7) and symptoms include intermittent fever (up to 107'F) haemolytic anaemia, increased respiratory and heart rates, anorexia and poor condition. The varying pathology suggests the existence of several different strains (Soulsby,1982). In nature, the infection is usually benign, although sub-clinical infection in lambs decreases growth rates

(Sutton,1970).

It has been suggested by some workers, that in France and Belgium *I.ricinus* may be the vector of *Rickettsia conorii* the causative agent of Boutonneuse fever (reviewed by Smith, 1973). This rickettsia cycles in nature between ticks, wild rodents and dogs via the tick bite.

1.572 TICK BORNE FEVER (T.B.F.).

The only important rickettsia affecting sheep in the U.K. is *Cytoecetes phagocytophila*, the causative agent of tick-borne fever and this is reviewed in the subsequent section.

1.5721 HISTORY.

In the course of an experiment originally designed to investigate the transmission of louping ill virus, at the Moredun Institute in Edinburgh, Gordon *et al* (1932) discovered that the tick *I.ricinus* harboured another disease. It was characterised by high fever and was reproduced by sub-inoculating infected blood into susceptible sheep. It was later recognised that these workers had produced the first experimental case of T.B.F., although the disease had probably been encountered by Stockman in 1918, but misdiagnosed as louping ill.

Many aspects of T.B.F. were elucidated in subsequent studies at the Moredun Institute, and its transmission by ticks confirmed. The nature of the agent responsible remained largely questionable until the discovery by Gordon *et al* (1940) of 'bodies' in the cytoplasm of granular leucocytes and monocytes of the diseased animal. Taylor *et al* (1941) studied the haematological disorders that accompany the disease. Foggie (1951) elaborated the nature

of the causal agent and made important contributions to the understanding of immunity against the disease.

1.5722 AETIOLOGY.

The causative agent of T.B.F. was unknown until Gordon *et al*, (1940) demonstrated the organism in the cytoplasm of granular leucocytes and monocytes. Descriptions of the inclusion bodies were subsequently presented by other authors (McEwan, 1947; Hudson, 1950; Foggie,1951). The pleomorphic tendencies of the agent led to the postulation of a complex life cycle. For instance, Gordon *et al* (1962) thought that there was a progression from 'initial bodies' to 'morulae' and then to the infective 'elementary bodies' Toumi and von Bonsdorff (1966) clarified the situation using electron microscopy. They observed the organism in cytoplasmic vacuoles which contained single particles or clumps, suggesting that what had appeared as initial bodies, morulae etc. were simply particles of differing sizes, closely or loosely packed (Woldehiwet, 1983). Studies by Woldehiwet and Scott, (1982c) have confirmed these findings. Furthermore, the latter authors suggested that the developmental life cycle was simple viz: the organisms were phagocytosed by granulocytes and monocytes and enclosed within a membrane which developed into a vacuole. The particles enlarged and divided by binary fission within the vacuole producing what appeared as morulae. Some particles then left the vacuole to initiate new infections.

The classification of the organism has aroused some controversy. Gordon *et al* (1940) tentatively classified the organism as a rickettsia, then a newly formed group of

micro-organisms. Foggie, (1951) proposed the name *Rickettsia phagocytophila*, because of its arthropod vector, intracellular location and morphological similaritiies to what are now known as *Chlamydia*. Some years later he rejected this classification on the grounds that T.B.F. agents had little resemblance to the true rickettsiae, apart from its arthropod host. However, because of its morphological similarities to *Cytoecetes microti*, an organism found in the granulocytes of the vole (Tyzzer,1938), Foggie (1962) proposed to include the agent of T.B.F. in the genus *Cytoecetes* of the tribe Ehrlichieae, (to distinguish it from the genus *Ehrlichia* of the same tribe), as *Cytoecetes phagocytophila*.

However, the classification of the organism has not been finally settled. While some workers accept the latest classification (Kreig, 1984) as *Ehrlichia phagocytophila*, others prefer to use Foggie's (1962) classification of *Cytoecetes phagocytophila*, (Evans, 1972; Pierce *et al*, 1974; Lewis, 1979; Woldehiwet and Scott,1982a).

1.5723 EPIDEMIOLOGY.

T.B.F. was first recognized on tick infested farms in Scotland, but was later recorded in other areas of Britain. (Hudson, 1950; Tutt and Loving, 1955). The disease has also been reported from Norway, (Overas,1959); Holland, (Bool and Reinders, 1964); Finland, (Toumi, 1966); Ireland, (Collins *et al*, 1970); Austria, (Hinaidy, 1973); India, (Raghavachari and Reddy, 1959) and South Africa, (Neitz, 1969).

The disease affects sheep (Gordon *et al*, 1932); cattle

(Hudson, 1950); red, fallow and roe deer (Foggie, 1962; McDiarmid, 1965) and feral goats (Foster and Greig,1969).

Experiments carried out by MacLeod and Gordon (1933) and MacLeod (1936a) established that T.B.F. was transmitted by *I.ricinus*. These workers discovered that the disease organism was transmitted transtadially, ie. it passes from stage to stage, for example from nymph to adult, but not transovarially, ie. from the adult through the egg stage to the larva. They also showed that the agent survived in infected ticks for a considerable period of time (at least one year in laboratory maintained ticks , [MacLeod,1936a]).

The spread of the disease in a susceptible flock is influenced by two main parameters. The immunity (which is dealt with in a later section) and the carrier state of individual animals. The lamb crop provides an input of susceptible individuals in the spring which can become infected with T.B.F. It is not known how long colostral immunity plays a part in protecting the lamb. Toumi (1967a) thought that young animals were less susceptible to T.B.F. while Gordon (1934) suggested that the disease may be more acute in the young.

The infected lambs, once recovered, act as carriers for a considerable period. Foggie (1951) stated that most infected animals became carriers, and that the carrier state could persist for up to two years, thus providing a reservoir of infection for any susceptible animals, for example a newly introduced ram. In cattle the organism could be recovered from the blood of some animals 14 days after inoculation, but blood collected 54 days after inoculation was not infective to naive animals and 11 out

of 14 animals inoculated 6 - 12 months after previous experience of the disease reacted like fully susceptible cattle; thus the carrier state in cattle is apparently of shorter duration and so plays a lesser role in disease transmission (Hudson,1950).

The maintenance of the organism in the tick (MacLeod, 1936a), its persistence in the blood of recovered animals (Foggie, 1951) and the presence of a wildlife reservoir (Foggie,1962) ensure dissemination of the organism.

1.5724 TRANSMISSION.

Tick-borne fever is spread by the hard tick *I.ricinus*, the highest incidence of primary infection occurring in young susceptible lambs in late spring, associated with the 'spring peak' in tick numbers (Scott, 1984). Transtadial (stage to stage), but not transovarial transmission occurs within the tick (MacLeod, 1936a). In a paper published in 1962, MacLeod stated "that there is a transovarial barrier is probable, but not yet certain, since only a limited number of tests have been made with nymphs arising from infected females". Lewis (1980) discovered rickettsiae in the ovaries of *I.ricinus* which had been experimentally infected with T.B.F., but they could not positively be identified as *Cytoecetes phagocytophila*. Accidental transmission can also occur through any agent which is capable of transferring a small amount of blood, such as a contaminated needle.

Lewis (1980) showed that male *I.ricinus* do harbour T.B.F. parasites. Although they do not feed to any great extent they must be considered a factor in the transmission of the disease. Parasitism of male *Ixodes trianguloceps* by

females of the same species has been reported by Nilsson (1975). Should this phenomenon occur with *I.ricinus*, it may be possible for male ticks to transmit T.B.F. rickettsiae to the females, who may subsequently transmit them to the vertebrate host on which they are feeding (Lewis,1980).

Epithelial cells of the mid-gut diverticula represent the first barrier to the development of rickettsiae ingested by the invertebrate host. Lewis (1980) proposed that the subsequent proliferation of these pathogens in the cells of the mid-gut and their migration to the haemolymph could lead to their dissemination to the other tissues including the brain, Malpighian tubules, ovaries and salivary glands. Lewis (1980) and Smith *et al* (1976) reported that the multiplication of the rickettsiae in the mid-gut cells seemed to be triggered by the onset of tick feeding.

The presence of T.B.F. rickettsiae in the gut epithelium indicate that regurgitation of the gut contents, which occurs during tick feeding (Balashov, 1968) could be an important factor in transmission.

1.5725 CLINICAL SIGNS.

The main clinical feature of T.B.F. is fever, other signs being absent or mild. When transmitted by the intravenous inoculation of infected blood, T.B.F. has an incubation period of 3-6 days according to MacLeod and Gordon (1933), about 4 days according to Toumi (1966) and 5-13 days according to Scott (1984). This was followed by a sudden high fever which fluctuated between 40.5'C and 42'C (Scott, 1984) and persisted for 7-10 days. The highest

point of fever is usually reached on the second day of the febrile period.

During the febrile period the causative agent could be detected in peripheral leucocytes. Foggie (1951) reported that 90% of granulocytes may be infected. Woldehiwet (1981) reported that monocytes were also affected to a lesser degree and usually during the latter stages of parasitaemia.

Death was rare (Foggie and Allison, 1960), but during the febrile period animals often appeared listless and lost weight (Gordon *et al*, 1932) due to anorexia (Venn and Woodford, 1956; Toumi,1967a). Hudson (1950) reported that T.B.F. in cattle was milder than the corresponding disease in sheep. If pregnant animals (cattle or sheep) were exposed for the first time late in the gestation period they aborted 2-8 days later (Stamp and Watt, 1950; Wilson *et al* 1964), after the onset of fever while still parasitaemic. In ewes this was commonly followed by the onset of post-parturient sepsis (Scott, 1984). T.B.F. is also thought to be involved in the infertility of rams and bulls (Watson, 1964; Retief *et al*, 1971). The high fever associated with T.B.F. resulting in impaired spermatogenesis. Toumi (1967a) reported a drop in milk yield of lactating cows, which varied from a slight decrease to a 50% reduction. The same author also noticed persistent coughing in affected cattle.

Perhaps the most important aspect of T.B.F. is its implication as a predisposing factor to other infections. It has been reported that louping ill might be aggravated by the simultaneous presence of T.B.F. (MacLeod and Gordon, 1933; Gordon *et al*, 1962 and Foggie, 1962) although Dunn

(1952) disagrees with this hypothesis. In the young indigenous animal grazing on tick infested pasture simultaneous infection is unlikely to occur since a young animal born to and suckled by a mother immune to louping ill has a very strong immunity to that disease for several months (Dunn,1952, suggested about 3 months while Kavanagh and Purcell, 1972, proposed one year). On the other hand, in T.B.F. there is little or no maternal immunity conferred as has been shown by McEwen (1947) in his observations on lambs. In these circumstances, by the time the young animal becomes susceptible to an attack of louping ill, it will probably have undergone T.B.F. infection in early life and be resistant or only partially susceptible to further attack. This is discussed further in section 1.542.

Many other reports have named T.B.F. as a predisposing factor to a variety of viral and bacterial diseases both experimentally and in the field. These include; pasteurellosis (Overas, 1962 and 1972; Gilmour *et al* 1982); chlamydial pneumonia (Munro *et al*, 1982); para influenza-3 infection (Batungbacal and Scott, 1982b); tick pyaemia (Taylor *et al*, 1941; Foggie,1956); septicaemia due to *Listeria monocytogenes* (Gronstol and Ulvand,1977; Gronstol and Overas, 1980); enterotoxaemia (Naerland, 1956). Additionally Greig and his co-workers (1977) considered that T.B.F. played an major part in a complex disease syndrome involving mucosal disease and cobalt deficiency in young calves.

However, the exact relationship between T.B.F. and the above diseases needs clarification and the mechanism by which the supposed predisposition functions, especially

with respect to tick pyaemia requires investigation.

1.5726 HAEMATOLOGY.

The appearance of *C.phagocytophila* in the peripheral blood (and the onset of fever) has been associated with gross haematological changes. Thus there is pronounced leucopaenia coinciding with the onset of illness (Taylor *et al* 1941), initially eosinophils and lymphocytes are reduced, followed 2-3 days later by a profound neutropaenia (Toumi, 1967a; Snodgrass, 1974; Woldehiwet, 1981; Scott, 1984). An apparent reduction in the number of platelets also has been reported (Foster and Cameron,1968).

Batungbacal *et al*, (1982), demonstrated that the lymphocytopaenia was due to a decrease in the number of B-lymphocytes and suggested this was associated with a suppression of antibody response to other antigens (Batungbacal and Scott, 1982a). The mechanism by which T.B.F. causes leucopaenia is not clearly understood. Taylor *et al* (1941) suggested that it may be due to marrow aplasia, however Woldehiwet and Scott (1982c) found that when sheep infected with T.B.F. were treated with the corticosteroid betamethasone there was an increase in the number of neutrophils in the peripheral blood arising from the bone marrow, suggesting that there was no marrow aplasia. They also found that cells were not infected before their arrival in the peripheral blood. Hudson (1950) speculated that the destruction of infected cells was the probable cause. Foster and Cameron (1970a) reported that the presence of the T.B.F. agent may render neutrophils non-functional. It is generally believed that the occurrence of bacterial infections secondary to T.B.F. can

be explained in terms of neutropaenia (Toumi, 1966).

1.5727 PATHOLOGY.

Very little is known about the pathogenesis of T.B.F. The only gross pathological feature reported is enlargement of the spleen (Gordon *et al* 1932; Hudson, 1950), but sometimes this is absent. Other, less common changes, included petechiation of the thymus, serosal and subendocardial haemorrhages and haemorrhage into the colon (MacEwen, 1947). Hudson (1950) observed that lymphoid organs were drained of lymphocytes and that there was a cloudy swelling of the convoluted tubules of the kidney. Recent studies by Munro *et al* (1982) have shown a diffuse cellular reaction in the alveolar walls 24 hours after infection. Foster *et al* (1968) reported a haemorrhagic enteritis in sheep after experimental infection with T.B.F. This is thought to be associated with the thrombocytopaenic state during the early febrile phase of T.B.F.

1.5728 IMMUNOLOGY.

The existence of some degree of immunity to T.B.F. has been suspected since the early part of the century (Gordon *et al*, 1932). Some degree of resistance based on age and breed has been reported. McEwen (1947); Jamieson (1947); Hudson (1950) and Toumi (1967a) all suggested that calves were less susceptible to T.B.F. than older animals, while Gordon (1934) proposed that the disease may be more acute in young lambs. There is little or no maternally conferred immunity, as has been shown by McEwen (1947) in his observations on lambs. Breed differences have been investigated in cattle by Toumi (1967a) and in sheep by

Scott and Koske (1976). The former found that the incidence of T.B.F. in the Java region of Finland was higher in cattle brought into the area from other regions than in indigenous animals. He did not state whether this was due to innate resistance or immunity acquired from continuous exposure to infection. Scott and Koske (1976) reported that Scottish Blackface sheep were less susceptible to T.B.F. than other breeds or their crosses.

It is generally accepted that animals acquire some degree of resistance to reinfection after being exposed to T.B.F. However, precisely when the immunity develops and for how long it lasts is unresolved. Foggie (1951) reported that immunity to reinfection in sheep developed 5 weeks after natural or experimental infection, while Hudson (1950) found that cattle did not resist a challenge several days after infection.

Recent work has suggested that the duration of immunity is variable, some animals remaining immune for a few months while others resisted infection for more than a year (Woldehiwet, 1981; Woldehiwet and Scott, 1982d). This variation could be related to the level of humoral antibodies in the carrier state. Both IgM and IgG antibodies have been recorded in the sera of infected sheep within 2 weeks of the onset of parasitaemia. Initially the IgM titre was significantly higher than that of IgG, but IgM antibodies declined and were replaced by a predominance of IgG within a month (Scott, 1984). However, carriers have been reported to retain high levels of IgM immunoglobulins (Woldehiwet and Scott, 1982c). Humoral antibodies have been detected by the immunofluorescence test (Toumi, 1967a;

Lewis,1980) and the complement fixation test (Snodgrass and Ramachandran, 1971; Woldehiwet and Scott,1982d).

The mechanism by which intracellular organisms are affected by humoral antibodies is unresolved. Some workers believe that humoral antibodies coat the extracellular stages of organisms and so reduce their ability to penetrate and infect host cells (Krier,1976). Humoral antibodies are also thought to act as opsonins, enhancing phagocytosis of free organisms and infected cells (Schroeder and Ristic, 1968).

The role of cell mediated immunity in the pathogenesiss of T.B.F. has not been extensively studied. Woldehiwet and Scott (1982f), demonstrated the inhibition of *in vitro* leucocyte migration when the leucocytes were collected from parasitaemic sheep. This inhibition was due to soluble factors which inhibited the migration of leucocytes from normal sheep. Brodie (1985) demonstrated that the ability of seven month old lambs to mount a cell mediated immune response to tuberculin was impaired when they were undergoing a T.B.F. reaction. Young lambs aged 4 weeks at the time of tuberculin challenge were unable to mount a cell-mediated response regardless of whether or not they were infected with *C.phagocytophila*.

C.phagocytophila and other related organisms not only have the ability to effectively evade the defence system, but they can also enter macrophages by creating a suitable environment inside a vacuole (Woldehiwet, 1983). Hirch (1972) suggested that this may be due to their ability to escape destruction by partial or complete inhibition of the process of fusion with macrophage lysosomes.

The blood of animals infected naturally or experimentally with T.B.F. remains infective for some time (Foggie, 1951; Toumi, 1967a). It is not clear how long the carrier state persists or its role in protective immunity. Gordon *et al* (1932) proposed that continuous exposure was necessary for the development of lasting immunity while Foggie (1951) suggested that when animals were in a state of premunition (ie. immune while infected), immunity was present. This question remains unresolved. There does appear to be a necessary minimum length of exposure to infection before immunity ensues, since Synge (1976) found that if a curative drug was administered early in the course of infection, no protective immunity developed.

The description of bovine T.B.F. by Hudson (1950) provoked a number of studies on the cross-protection between ovine and bovine strains of T.B.F. Hudson (1950), found that there was considerable cross-protection, while Foggie and Allison (1960) reported no evidence of immunity to ovine strains after previous infection with bovine strains and *vice versa*.

It is now accepted that differences between the bovine and ovine strains and also within strains exist (Toumi, 1967b). Strains have been differentiated by experiments based on cross-protection and the level of parasitaemia and febrile response produced (Toumi, 1966). Foster and Cameron (1970b) studied the length of incubation period and the degree and duration of fever. Woldehiwet and Scott (1982e) used a complement fixation test and concluded that the strains of T.B.F. studied had common antigens. They could be differentiated by titrating immune sera against

homologous and heterologous strains.

1.5729 DIAGNOSIS, TREATMENT AND CONTROL.

A tentative diagnosis of T.B.F. may be based upon the detection of high fever in young lambs and calves and in ewes and cows the occurrence of abortion, supported by the history and pathological changes described previously (section 1.5727), in animals which have been exposed to ticks. Confirmation depends upon the demonstration of *C.phagocytophila* in granulocytes and the ability of infected blood to transmit the disease when inoculated into susceptible animals (Scott, 1984).

In uncomplicated T.B.F. very few lambs are treated because of the mild nature of symptoms. If treatment proves necessary a single dose of oxytetracycline or sulphadimidine is usually recommended which reduces fever and parasitaemia, but mild relapses have been reported (Synge, 1974).

The traditional control measure is to purchase the sheep on a farm when the farm is bought (hefted sheep). This avoids moving animals from tick free areas onto infested ground, which is the usual history of disease outbreaks. Pasture improvement, especially drainage often lowers tick numbers (Milne, 1944) and therefore also lowers the rate of infection. This can have adverse effects when the flock is moved onto unimproved tick-infested ground (Scott, 1984). Other methods of good farm management which reduce tick populations include: the burning off of long grass, heather and rushes; ploughing as part of the rotation on a mixed farm and the draining and limeing of wetter areas of pasture (Purnell, 1981). Alternatively small wet areas can

be fenced off. Studies by Brodie (1985) have shown the benefit of a prophylactic treatment of long acting tetracycline, especially when combined with dipping.

Bevan and Sykes (1983) demonstrated the possibility of reducing the risk of contraction of tick-borne diseases by applying chlorpyriphos to the pasture, in the spring before the main period of tick activity.

Treatment of hosts with ixodicides such as cypermethrin can drastically reduce the chances of successful engorgement and can prevent oviposition by the female even if engorgement is successful (Arthur, 1962).

NATURAL PREDATION.

Milne (1950) suggests that the primary predators of the engorged female tick are the common shrew and to a lesser extent birds, predatory beetles (carabids), ants and parasitic wasps. The tick is highly vulnerable when questing and during the pre-oviposition period. Milne (1950) suggested that 2/3 of all engorged females could be killed by predators.

1.6 DISEASE MODELLING.

Descriptive medical statistics, begun in Britain by John Graunt who published 'Bills of Mortality' in the seventeenth century, was developed over the next 200 years and by the mid nineteenth century data was available detailing the incidence and mortality of diseases such as smallpox. Mathematical models were proposed by medical statisticians to explain the course of these diseases (Greenwood, 1943). The course of the 1866 rinderpest epidemic, a viral disease affecting cattle, was predicted

by one of these early investigators, William Farr (Brownlee, 1915).

The early models concentrated on natural epidemics of human infectious diseases. Only in the last 20 years has attention turned to the modelling of parasitic animal diseases (Thrushfield and Gettinby, 1984).

There are two basic types of model: small simple models and sophisticated simulations. The simple models ignore many of the parameters affecting the disease, and are based on temperature and moisture. Meteorological data does not measure the temperature and conditions in the microhabitat, the soil and vegetation where the infective stages are found. However a relationship between the two has been clearly established and widely available meteorological records can be substituted for the microclimatic data which is more difficult to obtain (Thrushfield and Gettinby, 1984).

The work of Ollerenshaw and Rowlands (1954) on liver fluke disease showed the potential of climatic factors as indicators of disease. Subsequently Ross (1978); Thomas *et al* (1978); Paton and Gettinby (1983) and Paton and Thomas (1964) and other authors have made extensive use of climatic data to describe and predict ovine fascioliasis and nematodiriasis in lambs.

The second type of model is a complex, computer simulation, which attempts to include every possible biological and physical factor which may influence the course of the disease. This will not only indicate population patterns of parasites but also population size and therefore potential disease. Gettinby *et al* (1979) have

produced a prediction model for bovine ostertagiasis in calves, which incorporates the parasite populations in the host and on the pasture. The latter model estimates larval development in terms of time and temperature above a developmental minimum. Migration is determined by rainfall and uptake based on herbage consumption. The most difficult influence to estimate is that of immunity, but this is avoided by only considering calves in their first grazing season, the group most susceptible to parasitism. Good correlation was obtained between observed and predicted results for both parasite populations and the occurrence of disease. Thomas (1982), suggested that such a model would be a valuabe aid to the timing of within season grazing management and treatment programmes.

A weather based prediction model for the life cycle of the sheep tick *I.ricinus* has been postulated by Gardiner and Gettinby (1983). The incidence of sheep tick activity depends upon the climatic conditions in the tick habitat, rates of fecundity, development, activity, engorgement and mortality at each stage in the life cycle. Gardiner *et al* (1981) found that much of the variation in tick development in Ireland could be explained by consideration of soil maximum and minimum temperatures measured at a depth of 50mm . Soil temperatures were thought to reflect microclimatic conditions more accurately than air temperatures, the exception being the development of eggs from spring fed females for which screen temperatures gave a better correlation.

CHAPTER 2.

MAINTENANCE OF A CULTURE OF *IXODES RICINUS* IN THE LABORATORY.

SUMMARY.

A colony of *Ixodes ricinus* was developed and maintained in the laboratory to provide a supply of T.B.F. free and T.B.F. infected ticks for other studies. Larvae and nymphae were allowed to engorge upon mice and adults upon lambs and calves. An opportunity was taken to measure the developmental periods of the various tick stages. It was demonstrated that the life cycle could be completed in a minimum of 240 days.

2.1 INTRODUCTION.

A number of tick species including *I.ricinus* have been maintained successfully in the laboratory (Bailey,1960; Branagan,1970; Joyner,1971; Lewis,1980) despite problems due to two characteristics of the Ixodid lifecycle. These are firstly the long feeding periods which require special measures for the containment and collection of ticks and secondly the tendency for *I.ricinus* to assume the dormant state of diapause (Lewis,1980).

To facilitate the studies described in this thesis it was necessary to develop and maintain a culture of *Ixodes ricinus*. Once established, this provided tick-borne fever (T.B.F.) free larvae and nymphs for the staphylococcal pyaemia transmission experiments; T.B.F. free and T.B.F. infected ticks for electron microscope studies and engorged larvae for developmental studies.

2.2 METHODS.

2.21 SOURCE OF ORIGINAL TICKS.

The colony originated from 30 fully engorged adult female *I.ricinus* collected from a sheep farm in Argyll in the spring of 1985.

2.22 STORAGE.

The storage vessels were 6 cm long X 4 cm in diameter, made of clear plastic with screw top lids. They had a 1cm thick layer of a 10% charcoal : 90% plaster of paris mixture at the base (Lewis,1980). This was soaked with distilled water to maintain a high humidity, in excess of 90%, within the vessels and helped to prevent the

formation of condensation on the walls and the subsequent growth of moulds.

The vessels were kept at between 20' and 25' C in a 14 hours light 10 hours dark regimen. These conditions were important to prevent the induction of diapause (Belozerov,1971 ;Lewis,1980).

Three engorged adult females were placed in each storage vessel, the lids were removed at weekly intervals to admit fresh air and the plaster of paris dampened if necessary. The date of the commencement of egg laying and the first appearance of larvae was noted.

2.23 HOSTS USED FOR TICK FEEDING.

Larval and nymphal *I.ricinus* were allowed to engorge on white mice. The Auchincruive strain and the National Institute of Health (N.I.H.) strain (obtained from Interfauna Limited, Cambridge) were both used successfully. In addition, a number of adults and nymphs were fed on either lambs or calves. Each host was used once only.

2.24 METHOD OF HANDLING TICKS.

The cuticle of newly emerged ticks was allowed to harden, denoted by a darkening in colour, for 10 days before feeding. Unfed ticks were removed from the storage vessel with a dampened fine brush and counted onto a petri dish filled with distilled water. The ticks floated on the surface and were confined, but undamaged.

2.25 MICE.

The mouse was introduced into a 60 ml plastic syringe, cut off at the 1 ml mark and plugged at one end with the plunger and after the mouse was inside the other end was closed with a cork which had a hole cut in the base for the

mouse's tail. This constraint prevented the mouse from grooming and so damaging attaching ticks. The ticks (200 larvae or 30 nymphae) were introduced through holes in the top of the syringe. The whole apparatus was placed in a large white plastic tray to catch any ticks which escaped. The mouse remained *in situ* for 1 hour until all the ticks had attached. It was then removed from the syringe to a cage with a mesh floor, placed over a water tray. The engorged ticks fell through the mesh onto the water surface beneath and were collected daily. They were removed with a fine dampened brush, washed in distilled water, blotted dry and placed in batches of 50 engorged larvae or 20 engorged nymphae in the storage vessels.

2.26 CALVES.

Adults , nymphs and occasionally larvae were fed on the tails of separately penned Friesian calves aged 6 weeks. The tail was shaved with electric clippers two days before the application of ticks. The ticks (20 adult females and 20 adult males, 100 nymphs or 500 larvae) were placed in a bag (50 cm long X 5 cm in diameter) made of a double layer of fine nylon. This was secured to the top of the tail with tail tape. (Method adapted from Bouvard, pers.comm.). The bag remained in place for one week. It was emptied by shaking into a plastic bag and replaced for a further 3 or 4 days until all the ticks had detached and fallen into the bag. The engorged ticks were treated as detailed in section 2.25. After development was completed adult males and females were placed with adults from other batches in an equal male to female ratio.

2.27 LAMBS.

Nymphal *I.ricinus* were fed in tail bags (30 cm long X 5 cm in diameter) or ear bags (10 cm long X 5cm in diameter) on lambs (plate 9). The ticks were 'injected' into nylon bags with an adapted wide mouthed 1ml syringe . The bags were secured to the unshaven ears or tail with elastoplast type tape. They remained in place for 9 days, by which time all the nymphs had engorged. The replete nymphs were treated as above.

2.3 RESULTS.

2.31 LARVAE.

Twenty three batches of 200 larvae displayed a successful engorgement rate of 83%. The engorgement periods of *I.ricinus* larvae fed on mice, maintained at 20 -25'C are shown in table 2.1.

TABLE 2.1 The Engorgement Times of *I.ricinus* Larvae Fed on Mice Maintained at 20-25'C.

Time in days.	2	3	4	5	6	7	8
% detached	9	55	26	6	4	<1	<1

2.32 NYMPHS

Nine batches of 30 nymphs were fed on white mice with a successful engorgement rate of 56%.

The engorgement times are shown in table 2.2.

Table 2. The Engorgement times of *I.ricinus* Nymphs Fed on Mice Maintained at 20-25'C.

Time in days.	2	3	4	5	6	7
% detatched	7	27	57	4	1	4

2.33 The Pre-oviposition period.

This is defined as the period elapsing between the detachment of an engorged female and the first appearance of eggs (Branagan,1973). In this colony, maintained at 90-95% R.H. and 20-25'C, the pre-oviposition period ranged between 9 and 23 days with a mean of 15.6 days.

2.34 The Pre-eclosion period.

The pre-eclosion period is the length of time elapsing between the commencement of oviposition and the first appearance of larvae (Branagan, 1973). The range recorded varied from 36 - 70 days with a mean of 50.4 days.

2.35 The Pre- moulting period.

The pre-moulting period (Branagan,1973) is defined as the interval which elapses between engorgement of a larva or nymph and the subsequent emergence of a nymph or an adult.

Engorged stages of *I.ricinus* undergo a short pre-immobilisation period before beginning the true moulting process (Branagan,1973). The latter process is indicated by a colour change, from the dark blue/black of newly engorged ticks to a creamy fawn, which proceeds from anterior to posterior. The end of moulting is indicated by a uniform pale colour and the emergence of the next stage through a

circular split in the dorsal cuticle between the capitulum and the scutum (Sutton and Arthur,1962).

The pre-moulting periods for larvae ranged from 36 to 79 days with a mean of 61.1 days and for nymphae from 37 to 53 days with a mean of 45.8 days (Table 2.3).

Table 2.3 Observations on the Developmental Periods of *I.ricinus* Maintained at 20-25'C and 90-95 R.H.

	Pre-ovip	Pre-eclos	L - N	N - A
Mean time in days.	15.6	50.4	61.1	45.8
Standard deviation.	5.47	18.1	13.7	6.9
No. Batches.	7	7	12	5

L - N The engorged larva to nymph metamorphosis.
N - A The engorged nymphal to adult metamorphosis.

The engorgement times on calves and lambs were not obtained as the ear and tail bags were only removed at the end of the feeding period. All tick instars fed on calves with a similar successful engorgement rate of 60-70%. This was irrespective of the time of year that the ticks were applied. The animals were kept inside , but the conditions were not controlled and temperatures fluctuated considerably.

2.4 DISCUSSION.

The colony was maintained under conditions similar to those described by Lewis (1980), although it was not found necessary to refrigerate ticks prior to feeding. The majority attached successfully provided their cuticle had hardened ; this state was achieved 10 days post-detachment. In this respect the colony resembled that of Aeshlimann

(pers. comm.).

The pre-oviposition, pre-eclosion and pre-moulting periods were slightly longer than those described by Lewis (1980). This disparity may be attributed to a temperature regimen which varied between 20 and 25'C, whereas Lewis's culture was maintained at a constant 22'C. Branagan (1973) reported that the development of *Rhipicephalus appendiculatus* was controlled by temperature and that its rate could be accelerated or retarded by raising or lowering the temperature. However, it is doubtful that *I.ricinus* encounters temperatures in the field in excess of those described . Campbell (1946) mentioned abnormalities in *I.ricinus* which had hatched from eggs exposed to temperatures greater than 25'C. Conversely the differences may be due to physiological variation in ticks originating from discrete gene pools.

The life cycle could be achieved in a minimum of 240 days, substantially less than the 400 days estimated by Lewis (1980). This was attributed either to the differing regimes of temperature and humidity to which the tick cultures were exposed or to the two strains of ticks from different geographical regions which were used to start the colonies.

The problem of fungal contamination during the storage of ticks maintained at high temperatures and humidities has been described by Joyner (1971) and Lewis (1980). The use of a charcoal/ plaster of paris medium to contain water helped to prevent the accumulation of condensed water on the storage vessel walls which precedes mould formation. When moulds did appear the ticks were washed in distilled

water, blotted dry and transferred to a new jar. It was encouraging to note that fungal formation appeared to be restricted to the old instars' cuticle and did not prevent the emergence of the next stage.

The advancement of the moulting process is heralded by colour changes in the tick, from a dark blue/black to cream, which progressed from anterior to posterior. Sutton and Arthur (1962) attributed the external colour of the tick to its caecal contents. So the majority of ticks, feeding mainly on blood are blue/black in colour, whilst the few engorging on tissue fluid are the rarer white ticks. It seems conceivable that gradual digestion of the blood meal during the pre-moulting period is reflected by these colour changes. However, Balashov (1968) described the formation of an anterior fluid filled moulting cavity visible through the integument which enlarges as moulting progresses. The primordia of the capitulum and legs withdraw from the old cuticle and lie within the moulting cavity. Epidermal detachment begins with the capitulum and extends backwards. This process proceeds more rapidly ventrally than dorsally. A new cuticle is secreted after complete epidermal separation.

Female *I.ricinus* are capable of copulating before feeding (Graf,1975; Perez and Rodhain, 1977). The policy of maintaining an equal male : female ratio in the storage vessels and introducing both males and females into the feeding bag maximised the chance of fertilisation. The males did not attach to the host and soon died unless *in copula*.

A new host animal was used to feed each batch of ticks.

This follows the practice of Branagan (1973), but differs from the methods of Morzaria (1973) who fed relatively large numbers of *Haemaphysalis punctata* on individual calves on more than one occasion, without the apparent development of resistance. This is unexpected in view of earlier work (Trager, 1939 a and b; Reik, 1962 ; Roberts, 1968 a, b and c) and later investigations by Schleger *et al* (1976) and Alani and Herbert (1987) who described partial immunity of the host to secondary infestations of ticks. This resistance was denoted by a reduction in the percentage of ticks engorging and a reduced mean engorgement weight. The apparent development of resistance, resulting in a reduction in attachment was also noted in the colony described when the same mouse was inadvertently used to feed two batches of larvae.

Larvae were fed very successfully on mice with 83% completing engorgement, whilst only 56% of nymphs fed successfully. In the field larvae are found in great numbers on small mammals (Milne,1948b) with nymphae present to a lesser extent. Gray (1981) commented on the tendency of a proportion of larvae to quest at a lower level in the vegetational strata, thus increasing the probability of encountering small mammals. This was not observed in questing nymphae. The laboratory evidence may corroborate these field observations of the suitability of the mouse as a host for larvae rather than nymphae.

The main objective of this work was to establish a colony of *I.ricinus* to guarantee a supply of ticks when required. The observations on feeding and development times were made during routine colony maintenance and require

substantiation. However, the aim was accomplished and a small easily maintained colony was successfully developed.

CHAPTER 3. ECOLOGICAL STUDIES.

SUMMARY.

1. The seasonal activity of *Ixodes ricinus* was monitored on two sheep farms in Ayrshire over 3 years, by counting ticks attached to sheep and blanket dragging of pasture areas.

2. Variability of seasonal activity was highlighted by the results. In 1984 there was a summer activity peak of prolonged duration, in 1985 there was bimodal activity with a major spring peak and minor autumnal peak and in 1986 and a unimodal distribution confined to the spring.

3. Data from blanket drags correlated well with the seasonal pattern of ticks attached to sheep.

4. Development times for engorged instars of *I.ricinus*, confined in mesh tubes under the vegetation mat were recorded. Emergence of all new instars occurred in the autumn.

5. The mathematical model for tick development devised by Gardiner and Gettinby (1983) was applied to the data generated in development studies, in an attempt to predict development times in south west Scotland.

3.1 INTRODUCTION.

The ecology of the sheep tick *Ixodes ricinus* L has been studied extensively in the north of England and Scotland by various authors (MacLeod, 1932, 1936b, 1938, 1939b ; Hendrick *et al* ,1938; Milne, 1944 and 1945). This work, conducted in the first half of the twentieth century was based solely on results of tick counts on sheep and blanket drags of pasture areas. Recent work in Czechoslovakia (Chmela,1969) and in Ireland (Gray, 1981 and 1982) using small mesh tubes to hold captive ticks and study their development times has elucidated certain aspects of the life cycle of *I.ricinus* which were unclear. The study described, aimed to use the latter approach in conjunction with tick counts on sheep and blanket drags of pasture to model the population of *I.ricinus* and to relate this to tick-borne disease problems.

3.2 MATERIALS AND METHODS.

Sites were chosen on two farms, both of which had experienced tick associated problems in previous years and offerred differing habitats.

3.21 SITE 1.

Site one, situated at 200m above sea level on an Ayrshire sheep farm which comprised of 40 hectares (ha) of inbye fields and an exposed area of hill (120 ha) which was very wet throughout the year, densely covered with coarse grasses such as *Nardus*, *Agrostis* and *Festuca* and by some areas of bracken (*Pteris aquilinium*) and moss (*Sphagnum* species) (Plate 3). The soil was a non-calcareous gley derived from carboniferous sandstones and shales with a varying admixture of basic igneous material. The hill was

Plate 3 Tick habitat at site 1.

Plate 4 Tick habitat at site 2.

grazed by approximately 400 Scottish Blackface ewes, which were removed to an inbye area prior to lambing in late April, and occasionally in the summer months by a group of 20 cross bred cattle.

3.22 SITE 2.

Site 2 was situated on the banks of Loch Doon in Ayrshire. The vegetation was similar to that at site one with the addition of sedges (*Cyperaceae*), rushes (*Juncus*) and a large proportion of heather (*Erica* species). The underlying soil was peat, which maintained a moist microclimate with little waterlogging (Plate 4). A breeding flock of approximately 300 Scottish Blackface ewes grazed 150 ha of hill and marginal ground present on the farm.

3.23 TICK COUNTS ON SHEEP.

Tick counts were performed on a random sample of at least 12 ewes, whenever flocks were gathered from the hill. Lambs were also sampled in the spring and summer months. Ticks were counted on the face, ears, neck, axillae, inguinal regions and above the coronet using a combination of sight and touch. The latter was useful for detecting small darkly coloured larvae on the muzzle and above the coronary band. The fleece was not examined as very few ticks are thought to feed over the general body surface (Milne, 1947b) and such examination is long, laborious and unreliable.

3.24 BLANKET DRAGS.

Areas of pasture were sampled by the blanket dragging method (Milne,1943; Gray and Lohan,1982) which involved pulling a light coloured blanket (2m x 2.5m) attached at

one end to a stick with reins and weighted at the base to keep it on the ground. Tick challenge was estimated by the mean number of questing ticks obtained from fortnightly blanket drags (2 x 5 minutes) on a 20m strip of pasture. Blanket dragging was carried out at weekly intervals immediately before and during the spring rise of tick activity.

3.25 DEVELOPMENTAL STUDIES.

Engorged *I.ricinus* ticks collected from sheep at the two sites and from other farms in the area, were divided into two batches and half placed at each site in cylindrical tubes (8cm x 2cm in diameter) made from nylon mesh (20 x 15 threads per cm^2, obtained from Fothergill Engineered Fabrics LTD.,P.O.Box 1, Littlebrough, Lancashire, England.) The tubes were assembled with nylon thread and seams proofed with clear varnish. Each end of the tube was stoppered with a piece of cork around which adhesive tape was wound for additional security. One adult female, 10 nymphae or 20 larvae were deposited in each tube which was then placed under the vegetation mat. Tubes were numbered consecutively, their contents recorded and examined at weekly intervals from the beginning of March until the end of October and fortnightly for the remainder of the year.

The tubes were placed in conditions as close as possible to those encountered under the vegetation mat in an area naturally infested with *I.ricinus*. Occasionally it was necessary to transport ticks from one site to another. In these cases the interval between removal from one site and addition to the new site was a maximum of 24 hours and usually transfer occurred on the same day. Observation of

ticks necessitated removal of the vegetation mat and a certain amount of disturbance which was kept to a minimum.

A few unfed nymphs and adults were placed in activity tubes (Gray,1982) which were about 30 cm in length and placed vertically in the vegetation. Initially it was also attempted to record the completion of oviposition and hatching.

3.26 METEOROLOGICAL RECORDING.

Soil temperatures were measured at each site using 4 thermometers placed at depths of 0cm, 10cm, 20cm and 30cm below the soil surface. Wet and dry air temperatures were recorded at each visit. Site one was also equipped with maximum and minimum thermometers, housed in a Stevenson Screen, which recorded the highest and lowest air temperatures attained between visits to the site. Daily temperatures recorded at the nearest meterological station situated 5 miles from site one and 12 miles from site 2 were also available.

3.27 MATHEMATICAL MODEL.

The data obtained from developmental studies was processed using a mathematical model of the developmental periods of *I.ricinus* produced by Gardiner and Gettinby (1983) and modified by Gardiner and Gray (1986).

Duration of tick development was calculated, based upon between day and within day temperature variations. This was achieved using the developmental fraction Fi, for each day, i, obtained from the equation:

$$Fi = \int_{t1}^{t2} \frac{1}{D(T)}\, dt$$

D(T) was the relationship between tick development time and constant habitat temperature.

t = time of day.

The terms t1 and t2 refer to the times of day when the daily temperature curve passed through the developmental temperature threshold, below which development was assumed to cease.

The daily temperature cycle could be related to the time of day, t, by a sinusoidal curve based on the daily maximum and minimum temperatures.

Daily developmental fractions were summed and development predicted whenever the sum first exceeded one.

A more complete explanation of the prediction method can be found in Gardiner *et al*, (1981).

3.3 RESULTS

3.31 ACTIVITY.

Tick counts on sheep and blanket drags of pasture were carried out at site one in 1984, 1985 and 1986 and at site 2 in 1985 and 1986. The results of these studies are shown in figures 3.1 - 3.5 and in tables 1-5 in Appendix 3. Consistently more ticks were captured at site one than site two by both methods although there was considerable annual variation.

The blanket dragging method captured a preponderance of nymphae, some larvae and occasional adults, whereas counts of ticks on sheep accurately reflected the numbers of ticks attached to and feeding on sheep. However, the numbers of ticks captured by the blanket dragging method did resemble the annual distribution of ticks on sheep (figures 3.1-

Figure 3.1

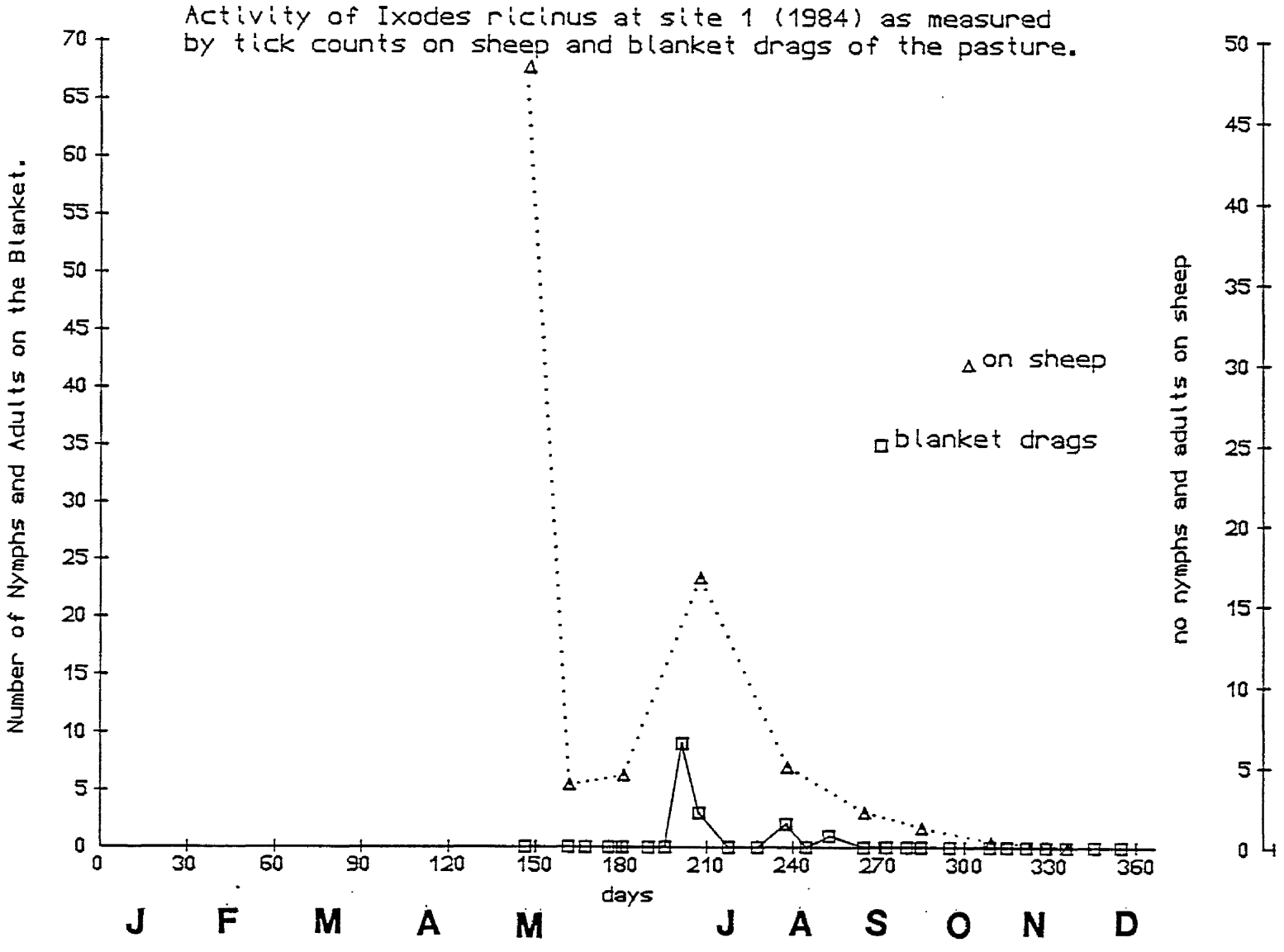

Activity of Ixodes ricinus at site 1 (1984) as measured by tick counts on sheep and blanket drags of the pasture.
Number of Nymphs and Adults on the Blanket.
no nymphs and adults on sheep
on sheep
blanket drags
days
0 30 60 90 120 150 180 210 240 270 300 330 360
J F M A M J A S O N D

Figure 3.2

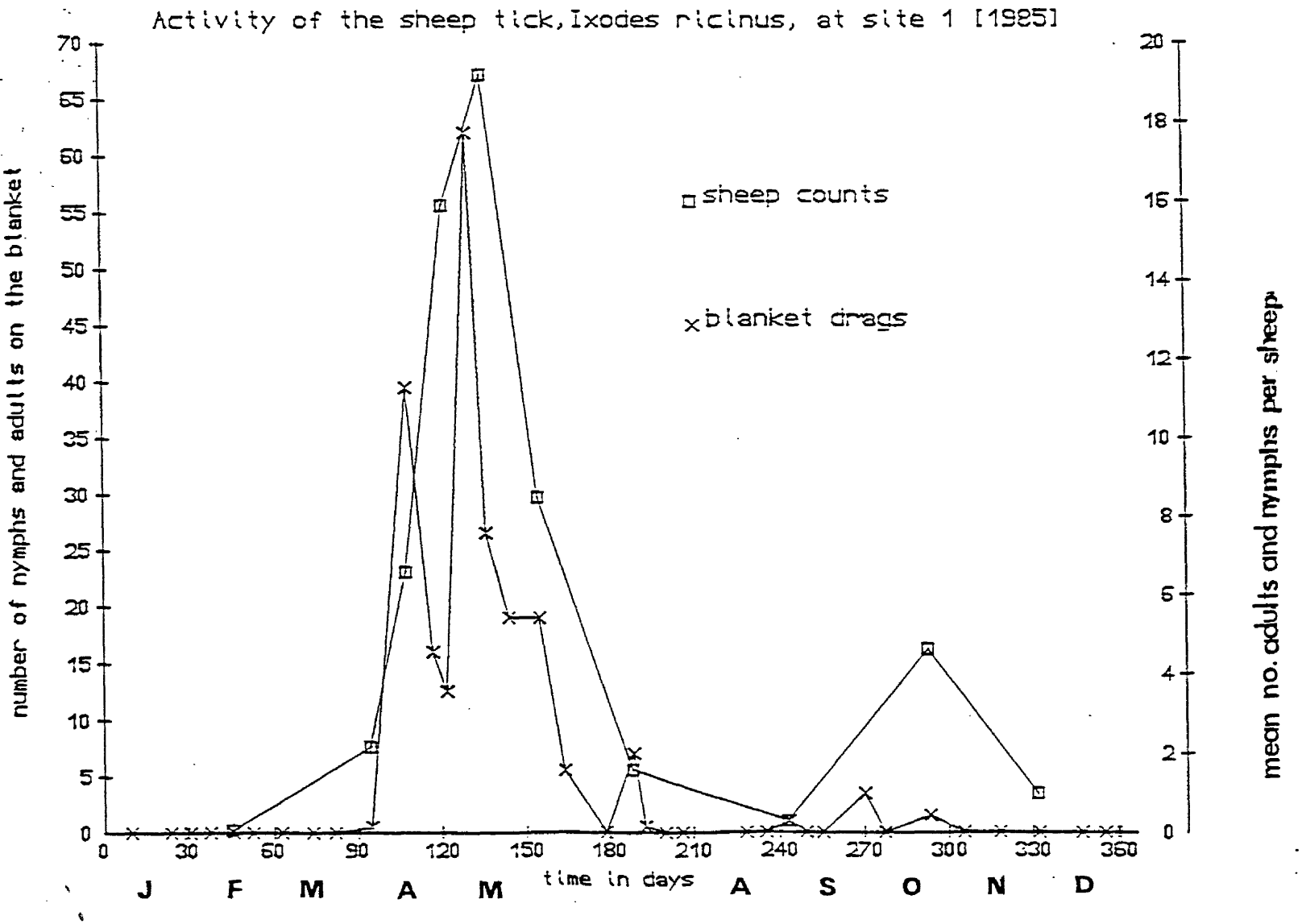

Activity of the sheep tick, Ixodes ricinus, at site 1 [1985]
number of nymphs and adults on the blanket
mean no. adults and nymphs per sheep
sheep counts
blanket drags
time in days
0
30
60
90
120
150
180
210
240
270
300
330
360
J
F
M
A
M
A
S
O
N
D

Figure 3.3

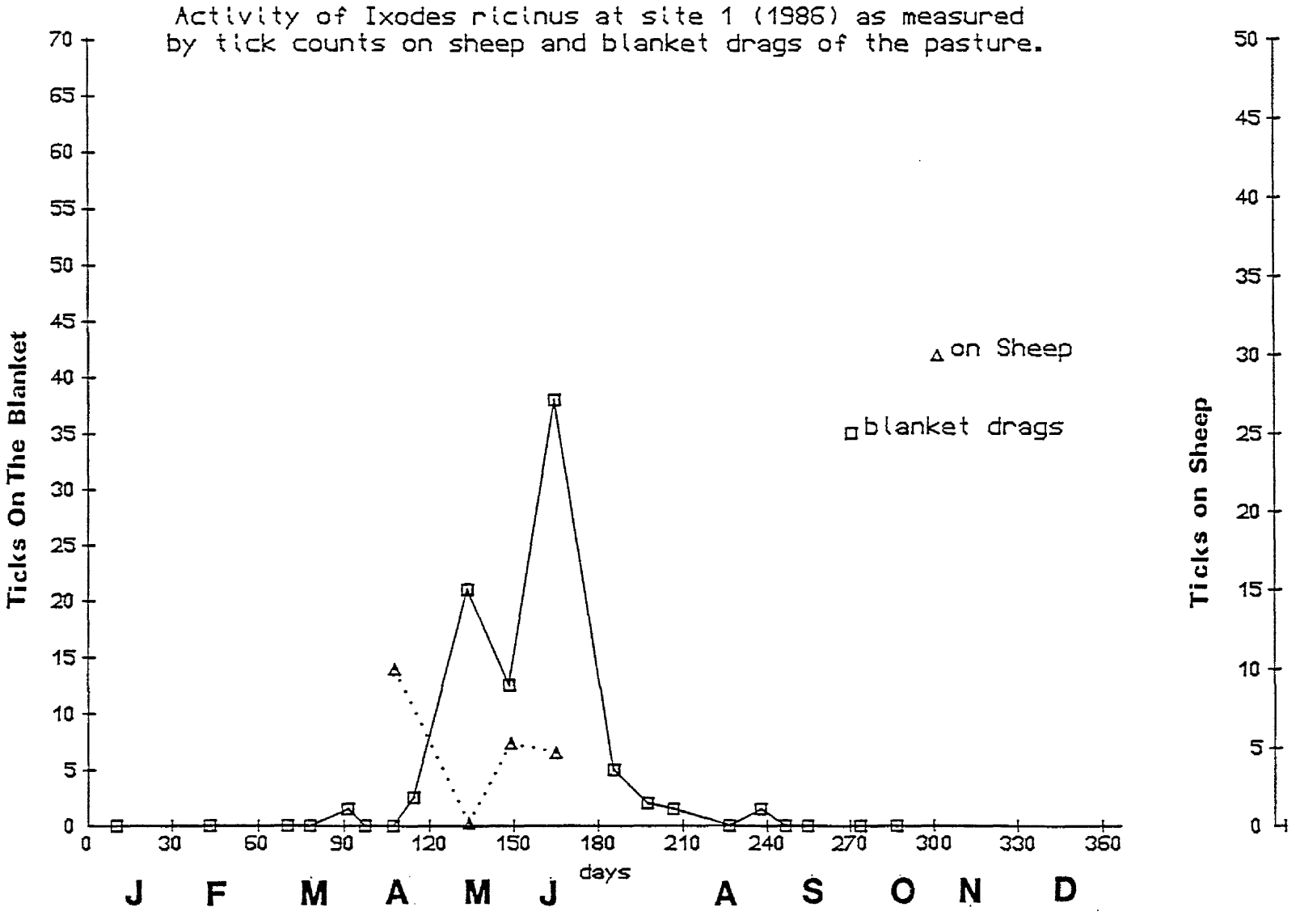

Activity of Ixodes ricinus at site 1 (1986) as measured by tick counts on sheep and blanket drags of the pasture.
Ticks On The Blanket
Ticks on Sheep
on Sheep
blanket drags
days
0
30
60
90
120
150
180
210
240
270
300
330
360
J
F
M
A
M
J
A
S
O
N
D

Figure 3.4

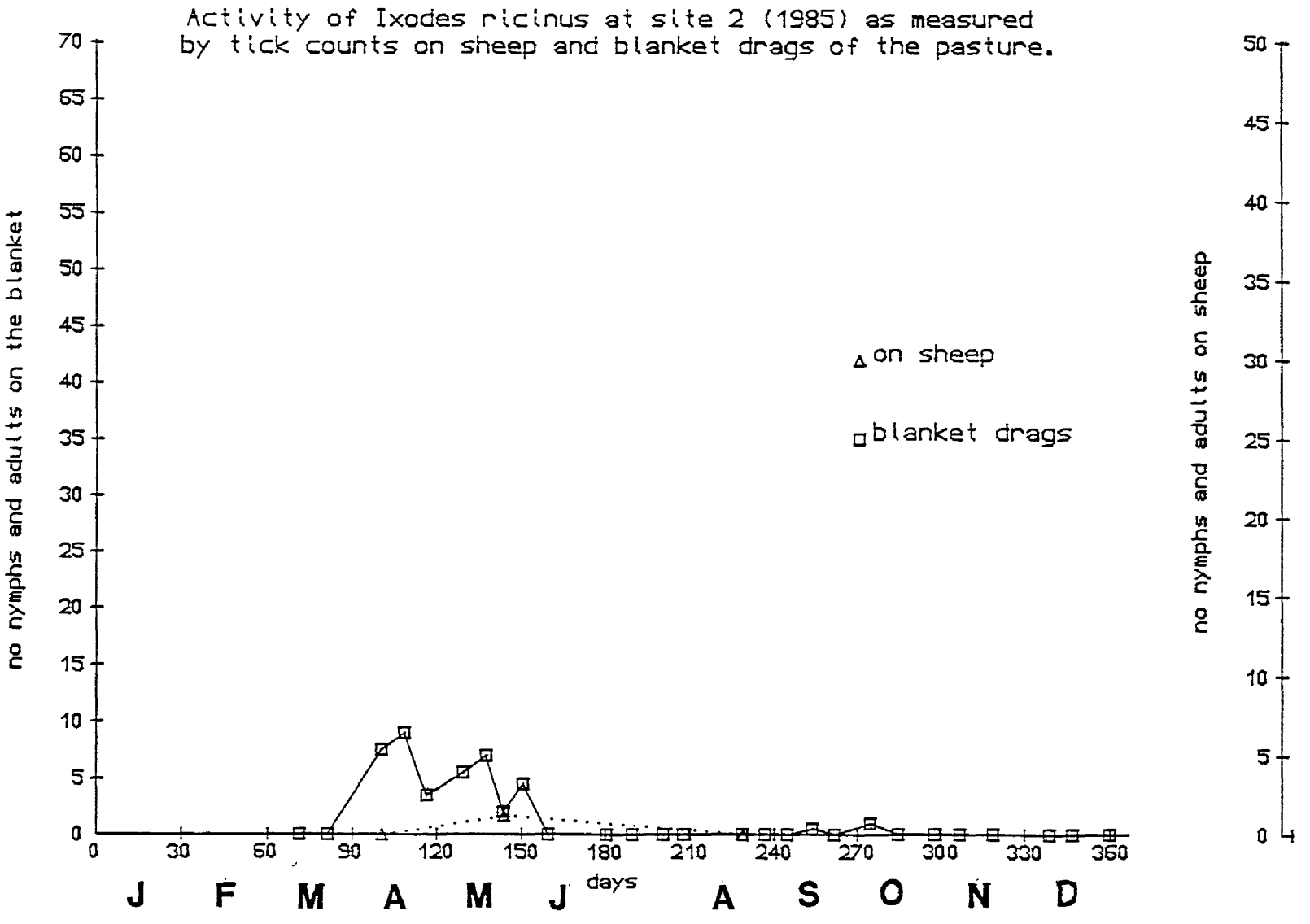
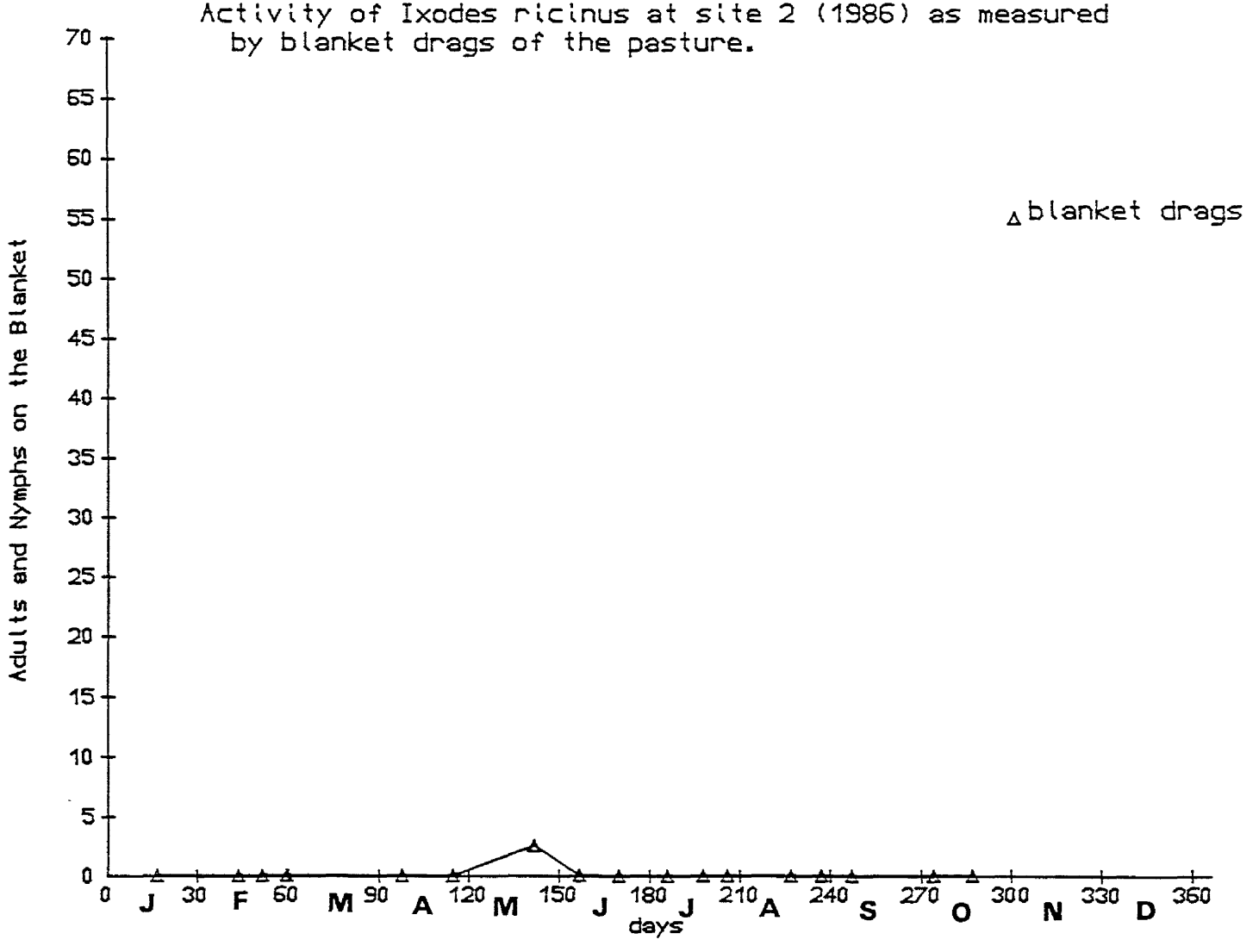

Activity of Ixodes ricinus at site 2 (1985) as measured by tick counts on sheep and blanket drags of the pasture.
no nymphs and adults on the blanket
no nymphs and adults on sheep
on sheep
blanket drags
days
0
30
60
90
120
150
180
210
240
270
300
330
360
J
F
M
A
M
J
A
S
O
N
D

Figure 3.5


Activity of Ixodes ricinus at site 2 (1986) as measured by blanket drags of the pasture.
blanket drags
Adults and Nymphs on the Blanket
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
0
J
30
F
60
M
90
A
120
M
150
J
180
J
210
A
240
S
270
O
300
N
330
D
360
days

3.5). The former sampling method could be used at more frequent intervals as it did not rely on such close cooperation with farmers as the latter, which necessitated collection of sheep from the hill at times suitable to individual farmer's husbandry regimes.

The number of ticks collected at site 2 was uniformly low in the two years sampled (figures 3.4 and 3.5), whereas there were distinct annual variations in the numbers collected and in seasonal distribution at site one; 1984 (figure 3.1) had one wave of tick activity which reached a maximum in early May when a mean number of 48 nymphs and adults were present on each host. At this time this particular group of sheep were dipped in acaricide, but despite treatment ticks were found on sheep on 7 subsequent occasions between the 8th June and the 2nd of November. Blanket dragging of the pasture recovered only small numbers of ticks (a mean of <10 on each occasion in 1984). In 1985 (figure 3.2) there was a spring rise peaking in early May (65 nymphae, 7 adults and 25 larvae on the blanket) with a slight recrudescence in September when a mean blanket drag of 3.5 nymphae was recorded. However 1986 had one period of activity beginning in early April, peaking in mid June (with a mean blanket drag of 34.5 nymphae and 3.5 adults) and persisting until August.

There was a marked zonal distribution of *I.ricinus* ticks on sheep. Adults were usually found on the axillae, the inguinal region and on the neck; nymphae tended to be present on the face and ears; larvae on the lips and sometimes on the legs just above the coronary band.

Sampling over the whole experimental period at the two

sites using both blanket dragging and tick counts on sheep yielded only *I.ricinus* species ticks.

3.32 DEVELOPMENT STUDIES.

The developmental periods of each stage of *I.ricinus* in a given season were characteristic of that stage and similar at both sites, although consistently less by several days at site two. This coincided with recordings of slightly higher temperatures (figure 3.6) when compared with those measured at site one over the same period. The following data was recorded for ticks placed at each site, which had engorged in different seasons of the year and is shown in tables 6-9 of Appendix 3.

Pre-oviposition period - table 6A and 6B.

Egg Development - table 7A and 7B.

Larval Development - table 8A and 8B.

Nymphal Development - table 9A and 9B.

Development times predicted by Gardiner (pers.comm.) using the mathematical model of *I.ricinus* development are also shown in tables 6-9 of Appendix 3.

3.321 THE PRE-OVIPOSITION PERIOD.

This is defined as the period elapsing between the detachment of an engorged female and the first appearance of eggs (Branagan, 1973). The pre-oviposition period varied from 9 days at site one for one spring engorged female to eight and a half months for one female at site two which completed engorgement on 19th October,1985 and did not begin to lay eggs until the 4th of July 1986.

The mean pre-oviposition period for spring engorged

ticks was 28.5 days (Standard Error [SE] =6.9 days) at site one and 34.6 days (SE =14.6 days) at site 2 and for autumn engorged females 119.5 days (SE = 20.4 days) at site one and 244.5 days (SE =11.0 days) at site 2.

3.322 EGG DEVELOPMENT (PRE-ECLOSION PERIOD).

This is the length of time elapsing between the time that eggs are laid by the female and the appearance of larvae (Branagan,1973). Variations from 20 days during the summer of 1986 at site one to 295 days for eggs laid in early November, 1984 which overwintered and did not hatch until the end of August 1985 were recorded.

The mean pre-eclosion period for spring engorged ticks was 106 days (SE =31.2 days) at site one and 79 days (SE = 31.5 days) at site two and for autumn engorged females 229 days (SE =38.2 days) at site one and 61 days at site two (one sample only as eggs from 3 other females did not hatch). In the former case the female overwintered in the engorged state and oviposition commenced in early July.

3.323 LARVAL DEVELOPMENT (LARVAL PRE-MOULTING PERIOD).

This is defined as the interval which elapses between engorgement of larvae and the subsequent emergence of nymphae (Branagan,1973).

In the field situation the emergence of all new instars occurred in the autumn (ie. the latter half of August and September). The larval development periods varied between 82 days for spring engorged ticks at site one in 1984 to 321 days for those larvae at site one which engorged in mid October 1985 and emerged in early September 1986.

The mean pre-moulting period for spring engorged ticks was 136 days (SE = 21.5 days) at site one and 118 days (SE

= 14.3 days) at site two and for autumn engorged ticks, 316 days (SE =3.3 days) at site one and 310 days (SE =5.0 days) at site two.

3.324 NYMPHAL DEVELOPMENT (NYMPHAL PRE-MOULTING PERIOD).

This is defined as the period which elapses between engorgement of nymphae and the subsequent emergence of adults (Branagan,1973).

In these studies metamorphosis always occurred in the autumn (between late August and late October) irrespective of the season of engorgement. Developmental periods varied from 97 days for one batch of June engorged nymphs at site one in 1984 to 405 days for one batch of nymphs at site one which engorged in August 1984 and only underwent nymphal to adult metamorphoses in October 1985.

The mean nymphal pre-moulting period for spring engorged ticks was 142 days (SE = 9.0 days) at site one and 135 days (SE =4.4 days) at site 2 and for autumn engorged nymphae 335 days (SE = 17.4 days) at site one and 337 days (SE = 18 days) at site two. Nymphal development periods were generally slightly longer at site one than at site two (n.s) and nymphal development periods were longer than larval development periods by several days (ns).

Table 1. Observed Mean Developmental Periods of *I.ricinus* at field sites 1 and 2.

	SPRING		AUTUMN	
	Site 1	Site 2	Site 1	Site 2
Pre-oviposition	28.5 (6.9)	34.6 (14.6)	119.5 (8.3)	244.5 (11.0)
Pre-eclosion	106 (31.2)	79 (31.5)	220 (38.2)	61*
L - N metamorphosis	136 (21.5)	118 (14.3)	316 (3.3)	310 (5.0)
N - A metamorphosis	142 (9.0)	135 (4.4)	355 (17.4)	337 (18.0)

L Larval
N Nymphal
A Adult

Standard Error ( ).
* one value only.

3.325 MORTALITY.

Subjective observations of mortality rates were made during developmental studies. The greatest mortality occurred in the larval stage which appeared to be particularly susceptible to desiccation, possibly because of its high surface to volume ratio. More than 80% of developing larvae died during the hot summer of 1984. Mortality of engorged nymphae and adults was low (<25%) and probably linked to cold winter temperatures (Gray,1981). Post mortem examination of several adult female ticks showed the presence of invading dipterous larvae which were thought to be secondary invaders (Stewart, pers comm.) and not associated with the tick's demise.

3.326 ACTIVITY.

Activity was denoted by ticks clambering to the top of

the tube and questing. The onset of activity was somewhat subjective as ticks could be induced to move by slight disturbances to the tube. This criteria for activity was deemed unsatisfactory and the method discontinued.

Initially it was also attempted to record the completion of oviposition and hatching. This involved the transfer of the ovipositing female or egg mass to another tube and the ensuing disruption often halted the process for several days, negating the final result.

3.33 PREDICTED DEVELOPMENTAL PERIODS.

The prediction model (Gardiner and Gettinby, 1983) was applied to the data in tables 6-9 (Appendix 3), using daily temperatures recorded at a meteorological station 5 miles from site one and 12 miles from site two. The predicted completion dates are also shown in tables 6-9 (Appendix 3). A small proportion (12.7%) of the predicted results agreed with those observed (ie were within the error limits of +/- 1 week imposed by weekly sampling).

3.331 PREOVIPOSITION PERIOD.

At site one 17.4% of the predicted values were within one week of the observed values and 34.8% within 10 days. At site two 14.3% of the predicted values were within 7 (and 10) days of the observed values. The errors between observed and predicted preoviposition periods varied between 3 (within the limits of error) and 69 days at site one and between 4 and 76 days at site two. The mean predicted preoviposition period was 7.5 days later than that observed (SE = 4.7 days) at site one and 12.0 days (SE = 16.1 days) at site 2.

3.332 PRE-ECLOSION PERIOD (EGG DEVELOPMENT).

Twelve and a half per cent of the predicted values were within 7 days of those observed at site one. The errors between observed and predicted values varied between 5 and 75 days with a mean error between observed and predicted values of 4.8 days (SE =15.3) days. At site two 25% of the predicted values were accurate to +/- 7 days.

3.333 LARVAL DEVELOPMENT.

At site one none of the predicted values were within 7 days of those observed but, 12.5% were within 10 days. None of the predicted values at site two fell within 10 days of those observed.

3.334 NYMPHAL DEVELOPMENT.

At site one 18.2% of the predicted values fell within 7 days of those observed. None of the observed values were within 10 days of those predicted at site 2.

The errors occurred in both directions. The predicted date was before the observed date for 47.8% of the samples, after the observed date for 34.7% of samples and within the limits of error for 16.7% of batches.

3.4 DISCUSSION.

Tick population studies began at site one in 1984. Sheep were treated with a whole body acaricide preparation on several occasions during the summer of 1984, despite which large numbers of ticks (<40) were counted on sheep in late May and on 7 subsequent dates between 8th June and the 2nd of November 1984. Therefore, clearly *I.ricinus* was active and attached to sheep for a much larger proportion of the

year (Webster *et al*, 1985) than previously reported by other authors (MacLeod,1939b, Milne,1943). Tick activity was also measured by blanket drags of the area grazed by these sheep. Initially, the results were erratic with some negative drags recorded when substantial numbers of ticks were observed attached to sheep. These results were probably due to the blanket dragging technique which was later improved by the use of reins and weights to increase the area of pasture in contact with the blanket. In 1985 and 1986 the capture of ticks by the blanket dragging technique was always accompanied by the presence of ticks on sheep (if they were examined on the same day). Low densities of ticks were occasionally detected by counts of ticks on sheep without a corresponding positive blanket drag, suggesting that the latter method is not as sensitive as examination of sheep in estimation of tick activity. If blanket dragging was carried out whilst it was raining or on very wet herbage, negative results were invariably produced.

Annual differences in the numbers of ticks captured and the seasonal distribution of different stages were shown by blanket dragging and tick counts on sheep. The greatest period of intense tick activity occurred during the spring of 1985 (based upon the numbers of ticks captured at both sites). This can be explained in terms of the climatic conditions experienced in 1984.

MacLeod (1939a) showed that the survival of unfed larvae and nymphae depended on a level of humidity in excess of 75% at temperatures between 10'C and 30'C. Temperatures below this induce a cessation of normal questing activity

(MacLeod,1939a) and continuing low temperatures coupled with short daylength can result in a state of behavioural diapause (Belozerov,1971) which enables unfed ticks to overwinter with minimal depletion of energy reserves. Belozerov (1971) identified two types of diapause in ticks; 'behavioural diapause' due to changes in temperature and photoperiod where unfed ticks undergo quiescence with no developmental changes and 'morphogenetic diapause' where engorged ticks undergo diapause during their developmental phase.

The majority of instars which emerged in the autumn of 1984 probably overwintered as unfed stages and sought a host in the spring of 1985. This hypothesis is supported by the presence of only small numbers of ticks on sheep and on the blanket during the autumn of 1984 (figure 3.1). Gray (1981) reported that the majority of tick mortality occurred during the winter months. In the present study the relatively mild winter of 1984/5 favoured survival of overwintering ticks and large numbers of ticks were seen in the spring of 1985 (figures 3.2 and 3.4). Gray (1982) also suggested that high temperatures during the summer of the previous year (1984) would tend to accentuate the spring rise in 1985. If temperatures were high enough when eggs emerged from diapause, their development would be accelerated and questing larvae could appear in the vegetation from late June (1984) onward, well before the diapause threshold period of early August. In addition, temperatures in excess of 21'C have been shown to break diapause already induced in larvae (Campbell, 1948) so that under these conditions even larvae which fed after the

normal diapause threshold period would join the 1985 spring population as nymphs. Excessive autumn temperatures also tend to accentuate the spring nymphal peak of the following year by killing large numbers of larvae which have already entered diapause and would have become autumn feeding nymphs during 1985 (Gray,1982).

The lower number of active ticks captured at site one in 1986 when compared with 1984 and 1985 can also be attributed to a direct temperature effect. In 1986 ticks were captured on the blanket in early April, but the rise was checked, probably by the cold temperatures experienced at this time which prevented normal questing activity, until the end of April when it recommenced, persisting until the end of August.

The adverse conditions encountered in the spring of 1986 necessitated survival of ticks in an active state for a longer period. Ticks store their food reserves as lipid (Lees and Milne,1951) and have only a finite amount of fuel to survive each non-feeding stage. Questing for a host at the vegetation tips is a strenuous occupation (Steel and Randolph, 1985). The delay in the onset of conditions suitable for tick activity in 1986 probably saw a depletion of the lipid stores of many ticks before they located a host, resulting in death.

Consistently more ticks were captured at site one than at site two despite similarities in the location and vegetation. The reasons for this are unclear especially as the development of ticks held captive in mesh tubes progressed with equal success at both sites. On site temperature and humidity records show that site two was

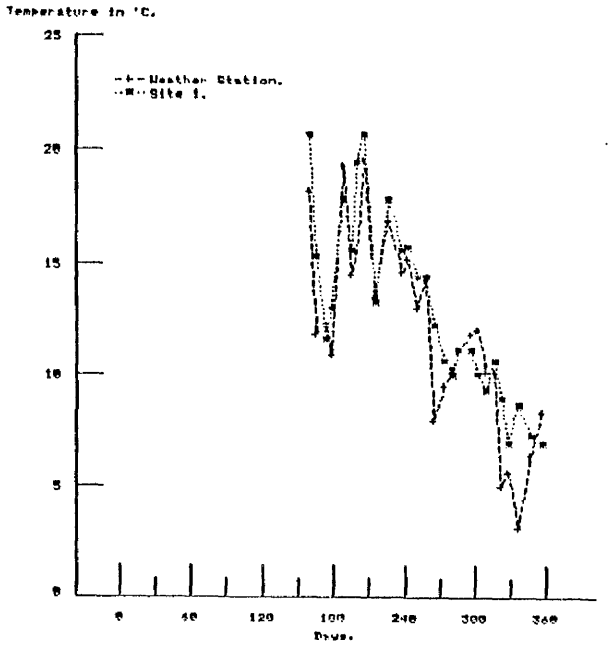
generally warmer and drier than site one (figure 3.6) and its location in a 'fenced field' situated in a small valley was less exposed than the hillside aspect of site one. The stocking rate at site two (2 ewes/ha) was also less than at site one (3.3 ewes/ha). The density of *I.ricinus* at each site was also related to the husbandry methods employed by the farmer to control ticks. This was achieved by acaricide application to sheep in both cases, with differing measures of success which were intrinsically linked to each farmers' individual opinion of acceptable tick burdens. At site 2 sheep were dipped regularly to protect them against ticks and lambs routinely dipped in acaricide prior to turnout onto tick infested hill areas in spring. At site one regular treatment of sheep with acaricide was only incorporated into the husbandry regime in 1984 after losses due to tick-borne diseases. A higher tick burden, however, was 'acceptable' on this farm.

A distinct zonal distribution in the areas of the sheep to which different instars attach has been described (Milne,1947b), which was substantiated by these studies. Each stage comes into contact with areas of the sheep host which are in contact with the vegetation. These areas are the feet, legs and the head. Small larval instars migrate only a short distance before they attach just above the coronary band on the leg or onto the lips; nymphae a little further attaching to the face and ears and adults the greatest distance being found on the axillae, inguinal region and on the neck. The thickness of ovine skin in different areas may also determine which instars can penetrate and attach.

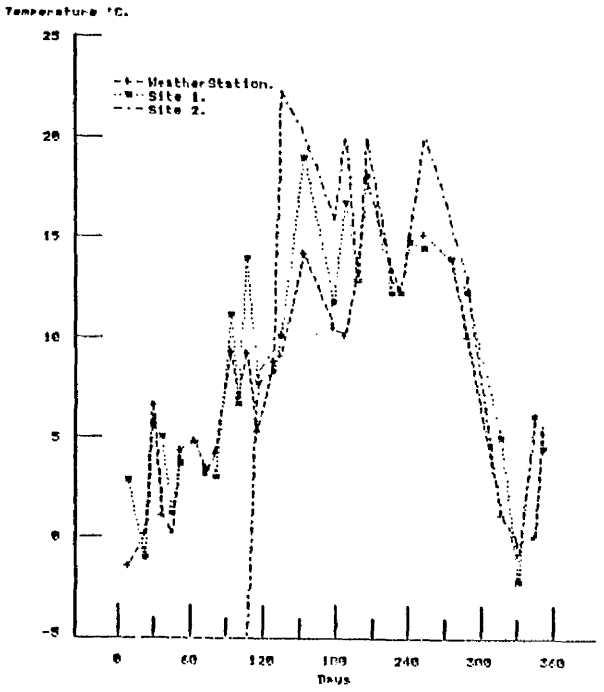
Figure 3.6

AIR TEMPERATURES.

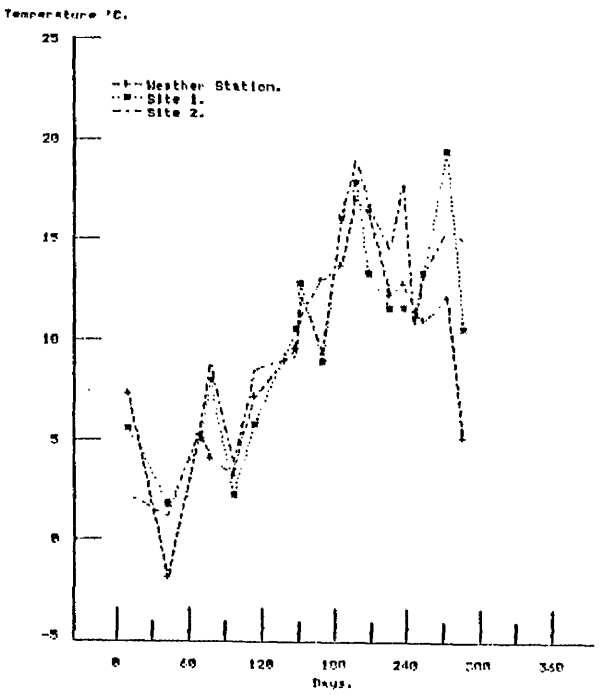
1984.



1985



1986



Examination of sheep has shown that ticks can attach and feed in November and February (Webster *et al* 1985), outwith the traditional activity periods (MacLeod,1939b; Milne,1947a). Increased acaricide application during the spring rise in tick activity may favour the survival of biotypes able to feed in other seasons and tend to result in a divergence in activity periods.

I.ricinus has an extensive host range (Milne,1948 a and b) and is able to feed on most mammals and birds. Rabbits, hares and a diverse range of avian hosts were common at both sites, in addition site one was also frequented by deer. It has been estimated that a single deer can act as host to over 2,000 ticks at one time. However, Milne (1948a) suggested that on hills grazed by sheep, the sheep themselves were host for 94 -99% of engorging adult female ticks.

An adult female tick lays 500 - 3,000 eggs (MacLeod,1939a; Perez and Rodhain,1977). In order to maintain the population at a consistent level only a small proportion of a batch of eggs need survive to maturity. If acaricide treatment of sheep achieved complete control, the tick population could still be maintained by those ticks feeding on alternate hosts.

Studies of the development times of engorged ticks held captive in nylon mesh tubes have yielded some interesting results. Four aspects of tick development were measured:

1. The pre-oviposition period.
2. Egg Development.
3. Larval Development.
4. Nymphal Development.

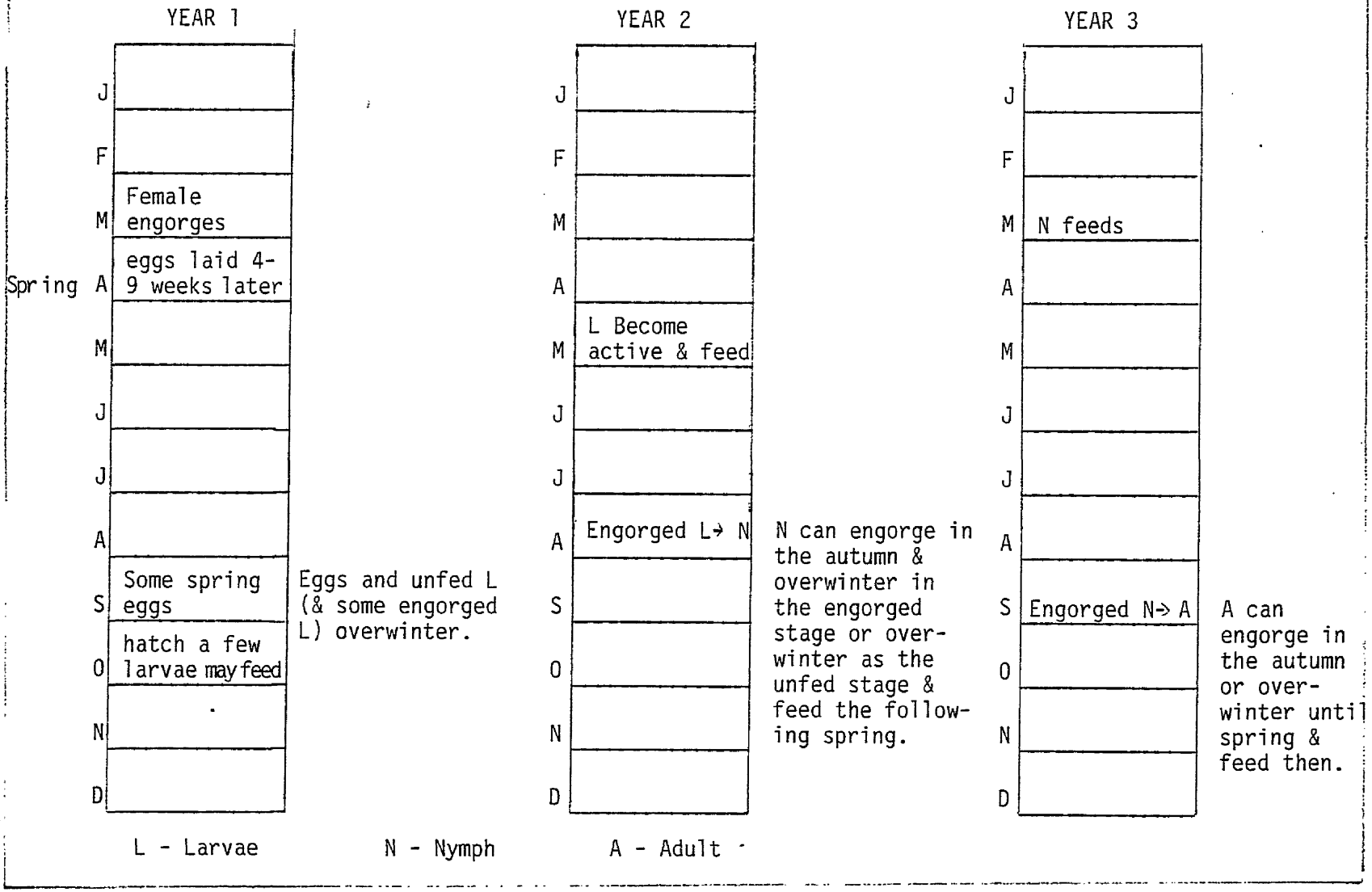
These four aspects of development listed could be examined with apparently little effect on the ticks in the tubes under scrutiny. Developmental studies based on this data (tables 6-9, Appendix 3) have led to a proposal of the following life cycle for *I.ricinus* (figure 3.7).

An adult female which engorged in the spring of year 1 has a pre-oviposition period of 3 or 4 weeks before egg laying commences. The first hatch of such eggs occurs in the late summer or autumn of year 1. If climatic conditions are mild some larvae may harden and feed that season, the remainder overwintering as unfed stages to feed the following spring (year 2). Adverse climatic conditions could result in all larvae feeding in the spring or summer of year 2 and moulting to nymphae in late August or September. These nymphs feed either in the autumn of year 2 or the spring of year 3, giving rise to adults in the autumn of year 3 in both cases. Oviposition can occur either in the autumn of year 3 or if unfavourable conditions are experienced in the spring of year 4. In the former case eggs do not hatch until year 4.

These values result in a 3 year life cycle for *I.ricinus* although in some instances this can be shortened, for example an adult female engorging early in the spring of year 1 can produce eggs which hatch in the early summer of year 1; these larvae feed later in the summer of year 1 and metamorphose to nymphs in the autumn of year 1. The nymphs could feed in the autumn of year 1 if mild conditions prevail or alternately in the spring of year 2. Metamorphosis to the adult instar occurs in the autumn of year 2, the adults feeding in that season if mild

Figure 3.7

DEVELOPMENT OF IXODES RICINUS



conditions prevail or alternatively in the spring of year 3. Thus it is possible for the tick to achieve its life cycle in a minimum of 2 years in the field. Ticks in the laboratory maintained tick culture described in chapter 2 had a minimum life cycle time of 240 days under a 14 hours light 10 hours dark regime at temperatures which fluctuated between 20 and 25'C. Acceleration of the life cycle in this manner relies upon conditions suitable for tick development and activity prevailing for most of the year. The transition from autumn engorgement to spring engorgement agrees with Gray (1982) and disagrees with the physiologically differing spring and autumn populations proposed by Campbell (1948).

There have been reports of the life cycle of *I.ricinus* being extended for up to 6 years in adverse conditions in Czechslovakia (Chmela, 1969) and for up to 4 1/2 years in Britain (MacLeod,1939a). Steel and Randolph (1985) explained a unimodal activity pattern observed in Wales in the following terms. Instars which emerged in the autumn met with very cold conditions and so delayed questing until the following spring. These authors also estimated the lipid contents of unfed stages and deduced a maximum survival period of 122 days for a questing nymph. The metabolic rate of a diapausing tick is extremly low and it is possible that if in adverse conditions ticks are not aroused from the somnolent state, they may survive for longer periods.

It appears therefore that the life cycle of *I.ricinus* is highly dependent upon climatic conditions prevailing at any given time. The tick adopts a flexible, pragmatic approach

using the strategy most likely to ensure survival.

The mathematical model of *I.ricinus* development (Gardiner and Gettinby,1983) was applied to the data generated in this experiment. The model had previously been successfully used with results of development studies obtained in Ireland (Gardiner and Gray, 1986) but had not previously been applied in Scotland or used to provide a forecast of development times for a specific site based upon temperature data from a recording station several miles away. Observations of developing ticks were made at weekly intervals, therefore a possible error of up to 7 days was inherent to the results.

The predicted results pertaining to preoviposition periods were variable. Sixteen point seven per cent differed from the observed interval by less than 7 days and fell within the limits of error imposed by the observation intervals, the remainder differed by varying periods up to 76 days. The errors occurred in both directions ie. the predicted date preceded the observed date in 47.8% of the samples, succeeded the observed date for 34.7% of the samples and 16.7% of samples fell within acceptable limits (ie. +/- 7 days).

Two predicted dates were given for each developmental phase : A, assumed the occurrence of complete diapause from August to February in autumn feeding stages.

B, assumed diapause was broken by maximum daily temperatures exceeding 21'C for 3 consecutive days.

The observed egg development periods were consistently later than those predicted in 1984 (X = 34.3 days SE =10 days), earlier than predicted in 1986 and

varied from -75 days to +29 days of the predicted date in 1985. Similar erratic results were also obtained for the prediction of larval and nymphal development periods (table 1). From the raw data it appeared that development occurred at a similar time each year namely late August - early September for the larval to nymphal transformation at site one, mid to late August at site two. The nymphal to adult transformation occurred in late August to mid October at site one and between mid August and mid October at site two.

The reasons for the aberrations between predicted and observed values are not obvious. Possibly the mesh tubes did not approximate the ticks natural habitat sufficiently well or the frequent disturbance necessary to study development affected tick behaviour. However these factors were also present in the Irish studies (Gray 1981,1982) in which similar anomalies were not recorded. The main difference between this work and that conducted in Ireland was the distance of the meteorological recording station from the observation site. In the Irish studies the meteorological station and development site were very close together, but in the present study, as daily results were required to predict developmental periods a meteorological station several miles from the sites was used.

There has also been a suggestion (Gardiner, pers.comm) that maximum and minimum air temperatures are not wholly applicable to the tick habitat and that soil temperatures may approximate this more closely. Gardiner *et al* (1981) reported that accurate predictions could be calculated from 50 mm soil temperatures except for the duration of

development of eggs laid by spring engorged females where screen temperatures gave a better prediction. Prediction of oviposition was unsuccessful. These authors also suggested that temperatures obtained from nearer the soil surface than the 50 mm depth used in their study could be more useful for accurate prediction.

Maximum and minimum soil temperatures were not recorded at the meteorological station, and it was not possible to test this theory.

I.ricinus has an extremely complex lifecyle and a prediction model must take account of many factors. The present study would suggest that regional temperature based predictions for *I.ricinus* activity and development are unlikely to occur in the forseeable future.

CHAPTER 4. TICK-BORNE DISEASES SURVEY.

SUMMARY.

A questionnaire was circulated to 134 farmers in Ayrshire and 180 farmers in Argyll. The questions related to tick associated problems and tick-borne diseases. There was a 65% and a 47.2% reply in Ayrshire and Argyll respectively. A massive tick problem (amongst those who replied) was highlighted in Argyll and a significant problem in Ayrshire, not usually thought of as a 'ticky' area. Several of the farms with problems were investigated in more detail, the results of which are described in chapter 6.

4.1 INTRODUCTION.

TICK-BORNE DISEASES SURVEY - JANUARY 1985.

The reasons for conducting this survey were twofold. The prime objective was to locate farms with heavy tick infestations for epidemiological studies and secondly, as an adjunct it was also hoped to generate information on the incidence of tick-borne disease in sheep on these farms. It was anticipated that this work would contribute to a greater knowledge of the distribution of *I.ricinus* in south west Scotland, about which relatively little is known.

4.2 METHOD.

After consultation with Mr Cliff Wright, Regional Veterinary Investigation Officer and Mr Jim Ritchie, Secretary of the Blackface Breeders Association, a questionnaire was prepared and this together with an accompanying letter explaining the significance of tick-borne disease, were dispatched with pre-paid postcards to increase the response. The questionnaires were sent to 138 members of the Ayrshire region Blackface Breeders Association and to 180 sheep farmers in Argyll who were in contact with the Oban Advisory Office of the West of Scotland College of Agriculture.

TICK-BORNE DISEASES SURVEY.

1. Have you seen ticks on your flock ? this year/last year/every year/never*.

2. Are ticks present on : ewes yes/no; hoggs yes/no; lambs yes/no *.

3. Is the infestation : heavy(more than 10 ticks)
light(less than 10 ticks per animal)
rare*.

4.In which months are ticks normally seen ? spring /summer /autumn /winter* .

5. Do you routinely dip for ticks ? Yes/no*.

6. Do you experience problems with louping ill (trembling)? Yes/no*.

7. Do you vaccinate against louping ill ? yes/no*.

8. Are ticks restricted to certain areas of the farm ? Yes/no*.

* Delete where inapplicable.

Name.....

Address.....

.....

FURTHER COMMENTS.

Any information given for individual farms will be treated in the strictest confidence.

4.3 RESULTS.

4.31 AYRSHIRE SURVEY.

Four survey forms were returned because the addresses were incorrect. Eighty seven forms were returned completed, giving a 64.9% return (87/134).

It appeared that if ticks were a problem on a particular

farm they tended to appear every year at a similar time. On all of the farms which reported a 'tick problem', ticks were present in the spring and on some farms also in the summer and autumn, but this was variable (figure 4.1b).

Table 1. The Distribution of *I.ricinus*.

Ticks were present:

Last year 4.6%	Every year 27.6%	Never 67.8%
Ewes 71%	Hoggs 46%	Lambs 96%
Heavy 25%	Light 43%	Rare 32%

Pie charts 4.1a and b illustrate the distribution of *I.ricinus* in Ayrshire. Forty per cent of farmers replying dip specifically for ticks, a high percentage when only 32.2% report ticks present on their farms (table 1.) . Twelve per cent of farmers reported that ticks were restricted to certain areas of the farm, while the remaining 20.7% have a widespread tick distribution. Fourteen per cent of farmers reported a louping ill problem and 10.3% vaccinate to reduce losses due to this.

4.32 ARYGYLL SURVEY.

47.2% of farms replied (85/180) with 3 forms returned unanswered.

Table 2. The distribution of *I.ricinus*.

Ticks were present:

Every Year 97.6%	Occasionally 1.2%	Never 1.2%
Ewes 98.5%	Hoggs 95%	Lambs 96.2%
Heavy * 58%	Light 30%	Rare 5%

* 6% of the replies did not specify the level of infestation.

Percentage Distribution.

Figure 4.1a Ticks were present on:

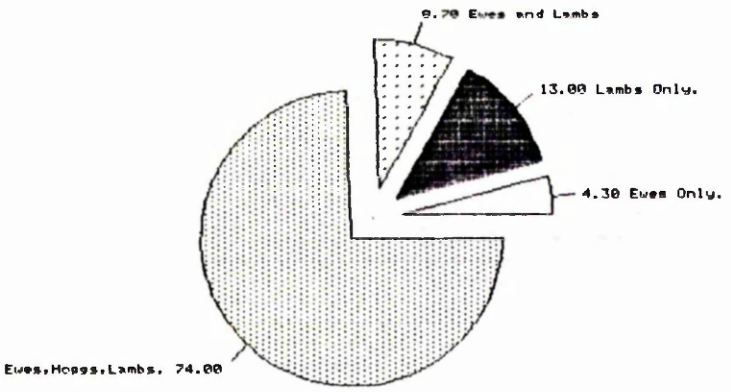
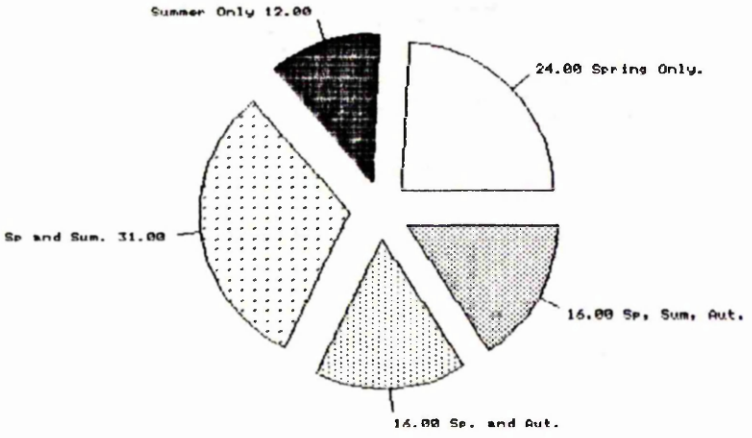


Figure 4.1b Ticks were present in:



AYRSHIRE

Figure 4.1a illustrates the percentages of farmers who reported that ewes, hoggs and lambs were infested with ticks.

Figure 4.1b illustrates the seasonal distribution of tick infestation.

Pie charts 4.2a and b illustrate the distribution of *I.ricinus* in Argyll. Ninety six per cent of farmers dipped specifically against ticks, indicating that they are a widespread problem. Fifty per cent of farms have a louping ill problem and 48% use vaccination to reduce losses due to this.

4.4 DISCUSSION.

The survey indicated that ticks are a problem in both Ayrshire and Argyll, but are more widely distributed in Argyll both spacially and seasonally. Disease aspects appear to be similar, but the problems in Argyll are due to the wider tick distribution.

AYRSHIRE RESULTS.

Lambs, ewes and hoggs in that order were most likely to be infested with ticks. MacLeod,(1939b) reported marked variations in susceptibility to ticks between individual sheep. Several factors may be involved in this including breed, age, body condition and hormonal status.

Lambs are usually born on hill farms in late April. If they are born on lower lying relatively tick free ground they are usually moved onto the hill shortly after birth. There is thus an approximate doubling of the sheep population on a given area of land, the influx of naive lambs coinciding with the spring rise in tick activity. This increase in animals potentially susceptible to louping ill and tick-borne fever is exacerbated on farms where the hoggs are sent away to winter on low, tick-free ground and are returned to the hill at the beginning of April.

Thirty two per cent of the farmers stated that ticks

Figure 4.2a

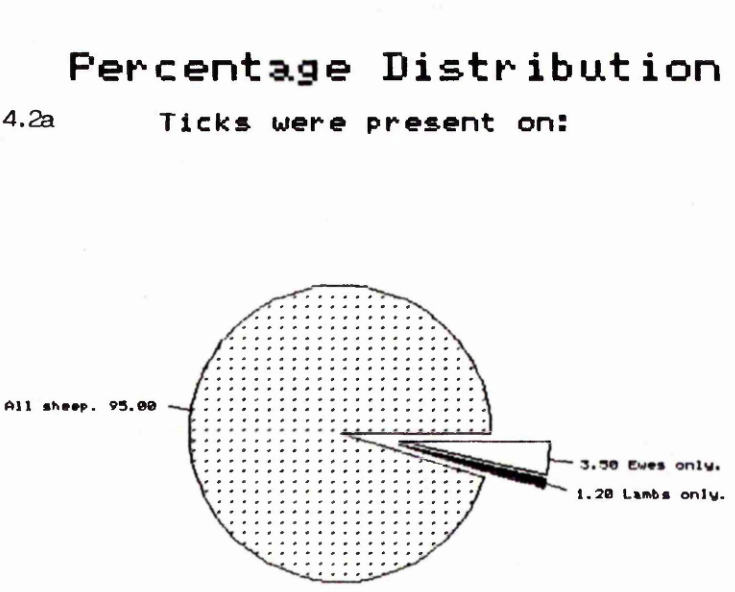
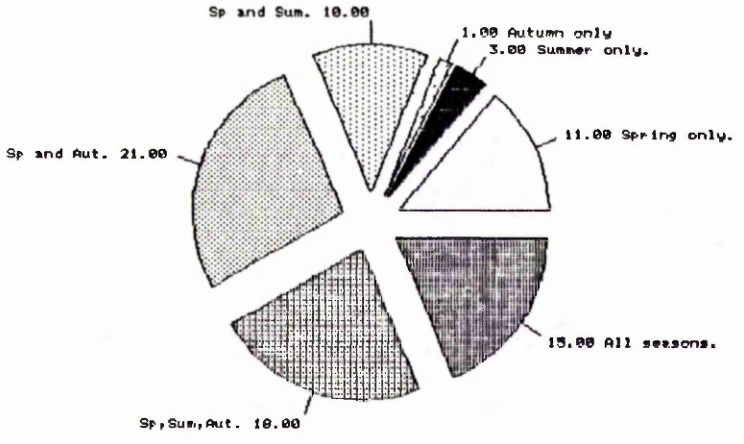


Figure 4.2b



ARGYLL

Figure 4.2a illustrates the percentages of farmers who reported that ewes, hoggs and lambs were infested with ticks.

Figure 4.2b illustrates the seasonal distribution of tick infestation.

were present on their farms. A quarter of these indicated a heavy infestation with an average of 10 or more ticks per sheep, the remainder having light or rare infestations. Ninety six per cent of those who reported ticks present routinely dipped for ticks with a variety of products. Ten per cent of those farmers without ticks also routinely dipped for ticks. Thus, a total of 40.2% of those replying used specific tick control measures. Farmers are aware of the prevalence of ticks on certain types of high ground and vegetation and of the value of dipping.

Fourteen per cent of those replying had problems with louping ill. Three quarters of these (10.3% of the total replying) vaccinated against louping ill. The vaccine is expensive, approximately £1-60 per dose, so the problem would have to be severe to render vaccination economically viable relative to losses.

On one third of farms ticks were confined to certain areas of their farms, the remaining 64.3% (20.7% of the total) had a widespread problem. Louping ill did appear to be linked to the widespread distribution of the tick as seven of the ten louping ill reports came from such farms. However, half of the farms with widespread tick populations did not have louping ill problems suggesting that the virus was not present in these populations. Half of the farms with louping ill reported heavy tick burdens on animals, one third of the farms light infestations and on the remainder ticks were rare. Seven out of the eight farms with a heavy tick infestation had louping ill, the exception occurring on Arran.

It has been estimated that 1/2000 *I.ricinus* in endemic

areas carry the louping ill virus (Reid, pers.comm.) so a heavy tick burden would increase the chances of contact with an infected tick. It has been suggested that the incidence of babesiosis in cattle is increased by a low tick density (Gray,1980) as a sporadic tick challenge may allow an animals' immune status to wane to a very low level, whereas a more regular challenge would enable it to persist, affording protection against reinfection. By analogy this situation may also be involved in immunity to louping ill. One reply complained of losses due to pasturellosis, exacerbated by ticks. This observation would support the work of Overas (1962) and Gilmour *et al* (1982) who demonstrated that the immunodepressive tendencies of tick-borne fever predispose affected animals to secondary infections such as pasturellosis which may be fatal.

The 65% return indicated that there is considerable interest amongst farmers in the Ayrshire area in the sheep tick and tick-borne diseases. The general picture gleaned from the replies is that a few farms in Ayrshire have large tick populations which cause problems. Many more through a stringent dipping and vaccination programme manage to keep the problems at bay in all but the most 'favourable tick years' (chapter 1).

ARGYLL.

The questionnaires were sent out to 180 farms in Argyll during the spring of 1985, slightly later than to the Ayrshire farms. Eighty five replies were received, which represented a 47% response.

The situation was dissimilar to that found in Ayrshire. Ninety seven per cent of farms reported that ticks were

present on their flocks every year, one farmer occasionally found ticks and only one farmer reported the absence of ticks. If the distribution of ticks mirrors that described by the survey, the sheep tick represents a much more widespread problem in Argyll than in Ayrshire. This may be expected, merely by observing the terrain.

Ninety five per cent of replies noted that ticks were present on lambs, ewes and hoggs. One report stated that ticks were only found on lambs and on three farms they were only found on ewes. The latter may have been due to the replies arriving before lambing had begun.

Fifty eight per cent of the tick burdens were classified as heavy (more than an average of 10 ticks per animal), 30% light and 6% rare. Six per cent did not specify a level of infestation.

The pattern of seasonal distribution showed that on some farms ticks were present throughout the year. This was often accompanied by peaks of incidence in spring and/or summer and autumn with fewer ticks usually being found in winter. The spring 'peak' was greatest, and as this usually coincides with hill lambing is potentially the most damaging and economically significant. Forty per cent of farms reported ticks in spring, summer and autumn and almost half of these also found ticks in the winter. This contradicts the conventionally reported spring and autumn activity peaks (MacLeod,1939b; Milne,1945), and is in agreement with the findings in chapter 3.

Ninety six per cent of the farmers with a tick problem said that they dipped specifically for ticks. This underlines the fact that the sheep tick is a widespread but

well recognized problem.

Fifty per cent of farmers reported louping ill problems, although these were usually qualified as light or 'not severe'. Forty eight per cent of the respondents vaccinated against louping ill, but these were not necessarily the same farmers who reported louping ill as a problem on their farms. Some found that their hefted animals acclimatised, acquiring natural immunity and that vaccination was not an economic proposition.

Tick pyaemia was also a problem in some areas, especially on Iona and parts of Mull. It was recognized that these losses can be reduced by regular dipping, smearing with substances such as Stockholm tar or the use of long acting tetracycline (Brodie *et al*, 1986). A percentage of farmers mentioned treatment of lambs with the new pour-on synthetic pyrethroid acaricides (Mitchell *et al*, 1986) for the first time in the spring of 1985.

Finally one farmer mentioned tick-borne fever (T.B.F.) as a cause of abortion in his flock. The problem in assessing the significance of T.B.F both as a disease in its own right and as a predisposing cause of tick pyaemia, louping ill, pasteurellosis, abortion and other syndromes is the difficulty of confirmation of a current or recent T.B.F. infection even if post mortem material is available.

In conclusion, several interesting points emerged. Farmers in the Argyll area recognise that the sheep tick is a widespread problem, which can to a large extent be alleviated by dipping regularly. Variable numbers of ticks were reported with 1984 having relatively few ticks, while they were abundant in the spring of 1985. The farmers

appeared to recognise areas of their farms most severely affected. South facing slopes were identified on several occasions as having greater tick numbers than other areas of the farm, probably because they are warmer and more equable for tick survival. One farmer mentioned that the top of the hill was not so badly infested as lower slopes, perhaps because it is more exposed and therefore less suitable for tick survival (Chapter 1.)

On the basis of this survey it was possible to select 6 farms for epidemiological studies of tick-borne fever which are described in chapter 6.

CHAPTER 5.

TICK-BORNE FEVER. EXPERIMENTAL INFECTIONS.

SUMMARY.

1. Experimental tick-borne fever (T.B.F.) infections were induced in young lambs and monitored by recording daily rectal temperatures, examination of blood smears for *C.phagocytophila*, haematology and neutrophil function tests.

2. The functional integrity of neutrophils was impaired during the parasitaemic phase of T.B.F. infection. This was assessed using an *in vitro* assay of neutrophil function.

3. A counter immunoelectrophoresis (CIE) serological technique was developed and tested with pre and post T.B.F. infection sera from lambs and goats. Antibody appeared 9-11 days post primary intravenous inoculation and persisted for 6-10 weeks in lambs.

4. Indirect Fluorescent Antibody tests (IFAT) were used to test pre and post T.B.F. infection sera. Antibody appeared 9-10 days after primary infection and persisted for 8-10 weeks.

5. Lambs challenged with the same strain of T.B.F. 16 weeks after initial T.B.F. infection underwent a mild second reaction in terms of fever and parasitaemia.

6. Antibody was detected at low levels for approximately 2 weeks following secondary T.B.F. infection using CIE and for 3-4 weeks using IFAT.

5.1 INTRODUCTION.

Tick-borne fever (T.B.F.) caused by *Cytoecetes phagocytophila* afflicts sheep, cattle, goats and deer (Woldehiwet, 1983). The main clinical feature of T.B.F. is fever, other symptoms usually being absent or mild (Scott,1984).

The appearance of *C.phagocytophila* in the peripheral blood and the onset of fever has been associated with gross haematological changes (Taylor *et al*, 1941). These can be summarised as a short lived lymphocytopaenia followed 2-3 days later by a marked neutropaenia (Toumi,1967a) and the presence of *C.phagocytophila* in neutrophils and to a lesser extent eosinophils and monocytes for between one and two weeks. These haematological changes are thought to be associated with a reduction in the immunocompetence of infected animals, predisposing them to infections such as louping ill (Reid *et al*, 1986); pasteurellosis (Overas,1962 and 1972; Gilmour *et al*,1982); para-influenza-3 virus (Batungbacal and Scott, 1982b) and tick pyaemia (Taylor *et al*, 1941; Brodie *et al*,1986). However, the difficulty of obtaining a definitive diagnosis, even when post mortem material is available makes it impossible to estimate the role of T.B.F. in lamb losses in hill flocks.

In order to help clarify the position, Snodgrass and Ramachandran, (1971) developed a complement fixation test (CFT) for T.B.F. Initially, the quality of antigen obtained from an experimentally infected sheep proved inadequate (Snodgrass,1974), but was later improved by the treatment of donor sheep with cortisone and the culture of whole blood in medium 199 (as described in section 5.2621) before

harvest of leucocytes (Woldehiwet and Scott,1982a). These authors used the C.F.T. with sera from sheep experimentally infected with T.B.F., but the test was not applied extensively to field samples. Lewis (1980) applied the C.F.T. and fluorescent antibody tests (F.A.T.) to sera from sheep previously infected experimentally with T.B.F. and showed the latter technique to be superior. Despite these studies the need remained for a simple rapid test for the detection of antibodies to T.B.F for use with sera from field infections.

5.20 MATERIALS AND METHODS.

5.21 ANIMALS.

Six Scottish Blackface ewes with twin lambs obtained from a tick free farm, were housed indoors and the ewes fed 1 kg concentrate daily, plus hay and water *ad libitum*.

Ten goatlings aged 9 months were kept at the Veterinary Field Station , Edinburgh and supplied with concentrate daily plus hay and water *ad libitum*.

5.22 T.B.F. STABILATES.

T.B.F. stabilates, kindly provided by Dr G.R. Scott, Centre for Tropical Veterinary Medicine, Roslin, Midlothian were stored as 2 ml aliquots of whole blood in 10% dimethyl sulphoxide at -114C. Immediately prior to use a cryopreserved stabilate was rapidly thawed in running water and diluted 10^{-1} with sterile phosphate buffered saline (PBS) at pH 7.2.

5.23 PRIMARY EXPERIMENTAL INFECTIONS.

Nine lambs aged 4 weeks, 4 ewes and 10 goatlings were injected intravenously with 1 ml of a 10^{-1} dilution (in

PBS, pH 7.2) of the Old Sourhope strain of T.B.F. (The ewes were unrelated to the lambs.) This strain was originally isolated from natural cases in sheep by Dr M. Foster on the HFRO farm at Sourhope in the Scottish Borders. There is no record of the number of experimental passages that the strain has undergone in sheep, but the symptoms are well documented (Foster and Cameron, 1970b; Batungbacal and Scott, 1982b).

5.24 MONITORING.

Lambs were weighed prior to infection and at intervals subsequently; rectal temperatures were monitored, plotted against time as in figure 5.1 and the area between the curve and a baseline of 40'C calculated using the trapezium rule. Blood samples collected daily by venopuncture of the jugular vein into two sterile evacuated glass tubes (Monoject, Sherwood Medical,St Louis, U.S.A.) one containing ethylenediaminetetraacetic acid (EDTA) for haematological determinations the other without anticoagulant for serological studies. Only 4-6 ml of blood were removed at each sampling in an attempt to prevent anaemia in the lambs. Three weeks after the initial infections temperature monitoring and daily blood sampling was terminated and thereafter blood samples were obtained at weekly intervals. In addition on days 0,2,5,8,11 and 13, post T.B.F. infection 10 ml of blood were collected into tubes containing 1ml of 1.5% EDTA for neutrophil function tests.

Blood samples were obtained from goatlings prior to infection and at weekly intervals for 3-8 weeks post infection to provide sera .

Table 1. Experimental Design.

	N	T.B.F. infection Primary	T.B.F. infection Second	Temp	Weight	Blood sampling Haem	Blood sampling Serol	Blood sampling N.F	Blood sampling Ag
Lambs 4 weeks	9	+	-	+	+	+	+	+	-
Lambs 20 weeks	6	+	+	+	+	+	+	+	-
Goats	10	+	-	-	-	-	+	-	-
Adult sheep	4	+	-	+	-	+	+	-	+
Adult sheep	2	+	+	+	-	+	+	-	-

N - number of animals

Temp - Rectal temperatures recorded pre-infection and daily post infection for 13 days .

Weight - Lambs weighed prior to infection and at the end of the experimental period (Day 21).

Haem - Full haematology performed on samples from primary infections, limited haematology on second infections (table 2).

Serol - Serology performed on samples (see table 2)

N.F. - Neutrophil function tests performed on samples (see table 2)

Ag - Antigen prepared for CIE determinations.

Table 2 . Monitoring of Lambs.

	Primary Inf.	Secondary Inf.
Temperature	P - D21	P - D21
Parasitaemia	P - D21	P - D21
WBC	P - D21	P - D21
Diff	P - D21	P - D21
Haemoglobin	P - D21	-
RBC	P - D21	-
PCV	P - D21	P - D21
N. Function	P,2,5,8,11,13	P,5,8,
CIE	P-D13 then weekly	P-D13 then weekly
IFAT	P,then weeks 1,2,3,4,6,8, 10,12	P, then weeks 1,2,3,4,6,8

P Pre-infection value.

D Days post-infection

WBC Total leucocyte count

Diff Differential leucocyte count

RBC Total erythrocyte count

PCV Percentage packed cell volume

N.Function Neutrophil Function Test

CIE Counter immunoelectrophoresis serology

IFAT Indirect fluorescent antibody test

Adult sheep were sampled prior to infection and subsequently as described for lambs. Additionally on day 5 post T.B.F. infection, 6 hours after intramuscular injection of dexamethasone at twice the therapeutic dose to promote neutrophilia (Woldehiwet and Scott,1982b) blood was withdrawn for antigen production.

5.25 HAEMATOLOGICAL DETERMINATIONS.

5.251 CELL COUNTS.

Erythrocyte and leucocyte counts were carried out using a Coulter Counter (model TA II) (Coulter Electronics Ltd., Dunstable, England.)

5.252 DIFFERENTIAL LEUCOCYTE COUNT.

Thin blood smears were made on clean glass microscope slides, air dried, fixed in methanol for 2-3 minutes and stained in a solution of 15% Geimsa in Leishmans buffer for 22 minutes. Slides were observed at a magnification of X 400 and 200 cells examined for differentiation.

5.253 ASSESSMENT OF PARASITAEMIA.

The tails of blood smears made for the differential leucocyte counts were observed at a magnification of X 1,000. Two hundred neutrophils were examined for the presence of *C.phagocytophila* and the percentage parasitaemia calculated. The presence of T.B.F. parasites in other cells was noted.

The number of neutrophils per mm^3 blood which contained T.B.F. inclusions was calculated and plotted against days after T.B.F. infection. (figure 5.1). The area under the graph was calculated using the trapezium rule.

5.254 PACKED CELL VOLUME (P.C.V.).

The packed cell volume percentage was determined by the microhaematocrit method using a microhaematocrit centrifuge and reader (Hawksley and Son, London, England).

5.255 HAEMOGLOBIN CONCENTRATION.

The haemoglobin concentration was determined by the cyanmethaemoglobin method as described by the International Committee for Standardisation in Haematology (1967).

5.256 STATISTICAL ANALYSIS.

The Students T test method was used to compare pre and post T.B.F. infection values and results recorded following primary and secondary T.B.F. infections.

5.26 SEROLOGY.

5.261 PREPARATION OF SERA.

Clotted blood samples were placed in a water bath at 37'C for one hour or left on the bench overnight to aid formation of the clot before being refrigerated at 4'C for one hour to assist shrinkage and removal of the clot. The remaining serum was centrifuged at 1,800g (3,200 rpm in a Super Minor centrifuge, Measuring Scientific Equipment, London, England) for 20 minutes during which any remaining erythrocytes formed a sediment at the base of the tube. The supernatant was removed with a pasteur pipette. All serum samples were stored in 200 µl aliquots at -20'C until required.

5.262 PREPARATION OF ANTIGEN.

5.2621 FOR COUNTER IMMUNOELECTROPHORESIS (CIE.).

Antigen was prepared as described by Woldehiwet and Scott (1982d).

Donor sheep were injected with dexamethasone at twice the therapeutic dose rate to increase numbers of neutrophils in the peripheral blood, 6 hours before blood was obtained by jugular venopuncture into evacuated, heparinised glass tubes. The blood was cultured in equal amounts of medium 199 with HEPES buffer (Gibco Ltd., Paisley, Scotland) supplemented with 10% foetal calf serum (Gibco Ltd.) at 37'C stirring continuously for 24 hours. Erythrocytes were lysed with 0.83% ammonium chloride solution and the remaining cells washed 3 times in PBS. The cells were resuspended in veronal buffer to give 2 X 10^8 cells per ml. These were disrupted in a 100 watt ultrasonic disintegrator (Measuring and Scientific Equipment LTD, London, England) at the optimal amplitude. Initially technical problems were encountered with the sonication method when a more powerful machine was used, heat was generated which caused antigenic protein to denature, despite immersion of the sample in an ice bath.

5.2622 INDIRECT FLUORESCENT ANTIBODY TEST (IFAT.).

Antigen smears derived from infected sheep's blood were used for immuno-fluorescent staining procedures. The method adopted was as described by Lewis (1980). Thin smears were made from blood collected from sheep at the height of parasitaemia (5 days post primary T.B.F. infection) and the area of the tail of the smear was marked on the reverse side of the glass slide with a diamond marker. Smears were dried at room temperature in a silica gel desiccator and either used within 24 hours or stored, individually wrapped in paper tissue, at -20'C. Storage was found to be an advantage in that freezing and thawing tended to disrupt

the majority of erythrocytes in the smear thus facilitating recognition of ovine leucocytes. Isolates of ovine leucocytes were obtained using the flash lysis method (Carlson and Kaneko,1973) and thin smears of these concentrates were made in the usual way, which were dried in a desiccator and either used fresh or stored at -20C until required.

5.263 COUNTER IMMUNOELECTROPHORESIS (CIE).

The counter immunoelectrophoresis (CIE.) technique, unlike all other forms of immunoelectrophoresis is performed in a material of high electro-endosmotic flow (Culliford,1964) ; in this case an agarose gel. The conditions (i.e pH) are selected in order that antigen(s) move towards the anode and antibodies were carried towards the cathode by electro-endosmotic flow (Andrews,1981). The interaction of a soluble antigen and its homologous antibody in a supporting medium such as an agarose gel, may give rise to an observable precipitate due to the formation, under conditions of optimal proportions, of an antibody/ antigen complex, which gradually increases in magnitude until an opaque line is visible (Corkill,1977).

Antigen was prepared as described in section 5.2621 and sera from experimental T.B.F. infections stored as described in section 5.261.

Agarose gels, prepared at pH 8.6 were obtained from Corning Medical Instruments U.K. (Halstead, Essex, England). A plastic template with 56 pairs of holes 2mm in diameter, 2mm apart was used to cut the wells. Serial dilutions of sera were made in barbitone buffer (pH 8.6) [Appendix 4]. One microlitre (1µl) of diluted serum was

applied to each of the wells on the anodal side of the gel and 1µl of antigen to the opposite well. All samples were tested in duplicate with a negative control of pre-infection serum and a sample of known infected serum (positive control). One hundred and ninety millilitres of barbitone buffer was placed in the electrophoresis chamber before the gel was inserted into the lid of the chamber and a voltage of 90 V applied for 35 minutes. The gel was then removed from the chamber, pressed dry using 'sta moist' blotting paper (Corning U.K.) and a weight of 1 kg for 15 minutes. Excess unprecipitated protein was removed by soaking in sterile saline for 15 minutes and the pressing process repeated.

The precipitated antibody/antigen complex was stained using Amido Black (Appendix 4). Excess stain was removed by washing in a 9:9:2 solution of methanol, water and glacial acetic acid (destainer) for 1 minute. The gel was then completely dried in an oven at 60'C prior to the final washing in 2 changes of destainer. This drying stage facilitated the removal of all excess stain. Final drying and examination of the gel using background illumination followed.

A total of 580 experimental sera were examined. These comprised of 216 samples from primary T.B.F. infections in lambs, 144 samples from secondary T.B.F. infections in lambs; 96 and 48 samples from primary and secondary infections respectively in adult sheep and 76 samples from primary T.B.F. infections in goatlings. A positive result indicated by the formation of a precipitation arc between antibody and antigen wells on the gel.

5.2632 MERCAPTOETHANOL TREATMENT.

Fifty six samples of sera were treated with mercaptoethanol which breaks disulphide bridges found in IgM molecules (Graham,1974). The sera were then processed using CIE as described in section 5.263. and any positive results attributed to the presence of IgG.

5.264 INDIRECT FLUORESCENT ANTIBODY TEST.

The method described by Lewis (1980) was adopted. Commercially available fluorescein-labelled immunoglobulins (Wellcome Reagents Ltd) were used for Indirect Fluorescent Antibody Tests (IFAT.s).

Antigen smears were removed from storage at -20'C and allowed to 'thaw' for approximately one hour before being air-dried, fixed in cold acetone for 10 minutes and finally placed in a humidified chamber for incubation with specific antiserum.

Serum was diluted 1:4 in Phosphate Buffered Saline (PBS) at pH 7.6 and applied to the area of smear under test using a pasteur pipette.

Slides were incubated for 30 minutes at room temperature, washed with gentle agitation in 2 changes of PBS for a total of 30 minutes and then allowed to drain dry, excess moisture being blotted from the demarcated zone to hasten drying.

Working dilutions of the fluorescein labelled antisera (Wellcome Reagents, Ltd.) were reconstituted as described on the technical data sheet accompanying each vial. The slides were then incubated as outlined above with aliquots of the required dilution at room temperature for 30

minutes. After incubation the slides were washed in two changes of PBS for a total of 60 minutes before draining dry and mounting in Bacto F.A. Mounting Fluid (Difco Laboratories, Detroit, Michigan, U.S.A.).

After the initial characterisation of fluorescence by comparison of smears from healthy and infected animals, the following controls were applied to all tests.

5.2641 POSITIVE CONTROL.

Thin smears were made from the blood of several highly infected sheep (Giemsa stained blood smears showed that peak parasitaemias were in excess of 80% in all cases). These were incubated with lamb serum obtained 3 weeks post primary experimental infection.

5.2642 NEGATIVE CONTROL.

Thin smears were made from the blood of several highly infected sheep (Giemsa stained blood smears showed that peak parasitaemias were in excess of 80% in all cases). These were incubated with sera from lambs with no experience of tick-borne fever.

5.2643 VIEWING IMMUNOFLUORESCENCE.

Immunofluorescence was viewed using a Leitz microscope (model SM LUX) with ploem F.A. system (Ernst Leitz, GMBH, Wetzlar, Germany).

5.27 NEUTROPHIL FUNCTION TEST.

The ability of neutrophils to phagocytose and kill *Staphylococcus aureus* at various times during T.B.F. infection was assessed using the method of Williams, Craven, Field and Bunch (1985).

5.271 PREPARATION OF *STAPHYLOCOCCUS AUREUS.*

One colony of an overnight plate culture of *S.aureus* was placed in 10 ml of Brain Heart Infusion Broth (B.H.I) [Appendix 4] before incubation in rolled bottles at 37'C for 4 hours. The culture was centrifuged at 1,800g for 15 minutes; the supernatant removed by aspiration and the sedimented cells washed in 2 changes of sterile saline *S.aureus* was then resuspended in 10ml of sterile saline. Previously a growth curve (Appendix 4) had established the concentration of this to be 3 X 10^9 colony forming units (CFU) per ml. The culture was adjusted to 6 X 10^6 CFU/ml. 10^{-3} - 10^{-5} dilutions were made and duplicate blood agar plates inoculated with 20µl aliquots.

5.272 PREPARATION OF SERUM.

Sera were prepared as described in section 5.261. Samples originally derived from several lambs (normal and T.B.F. infected) were pooled, decomplemented at 56'C for 30 minutes and diluted in Hanks Balanced Salt Solution (HBSS) to yield 1% serum.

5.273 PREPARATION OF POLYMORPHONUCLEAR LYMPHOCYTES (PMN).

PMN's were isolated using the method described by Carlson and Kaneko (1973).

Whole blood was collected by jugular venopuncture into 10 ml tubes containing 1 ml of 1.5% EDTA in PBS at pH 6.8. The blood was centrifuged at 1,000g for 20 minutes; plasma, the buffy coat and the uppermost portion of the packed red blood cell (RBC) column were removed by aspiration and discarded. The remaining RBC portion, which contained the neutrophils was resuspended in 4 ml of 0.8% buffered saline. This fraction was then transferred to a 50 ml

conical based tube, 20ml of distilled water added to lyse the RBC's and one and a half minutes later isotonicity was restored by the addition of 10 ml of 2.7% buffered saline. The mixture was centrifuged at 200g for 10 minutes, the supernatant removed by aspiration and discarded,the white cell pellet resuspended in 24 ml of 0.86% buffered saline and the white cell button washed twice. The white cells were resuspended in 1 ml HBSS, total leucocytes counted using a Coulter Counter (model TA II) (Coulter Electronics Ltd.,Dunstable, England) and the volume adjusted to give 10^7 /ml.

5.274 TRYPAN BLUE EXCLUSION ASSAY.

The viability of the isolated neutrophils was assessed using the trypan blue exclusion assay (Jain and Jasper, 1967). Viable cells exclude trypan blue. Fifty microlitres (µl) of 0.1% trypan blue in PBS was added to 50 ul of the PMN isolate. A drop of the mixture was transferred to a haemocytometer (Improved Neubauer model, Hawksley and Son, London, England). One hundred neutrophils were examined and the percentage which excluded trypan blue counted.

5.276 PREPARATION OF LYSOSTAPHIN.

Lyophilised lysostaphin (Sigma Chemical Company, Fancy Road, Dorset, England. 240 units/mg) was dissolved in water to a concentration of 500 µg/ml and stored at -20'C in aliquots of 200 µl. On the day of PMN testing a small aliquot of lysostaphin was further diluted 1:60 in HBSS to working strength.

5.277 PREPARATION OF TRYPSIN.

Trypsin (1:250 grade, Difco Laboratories, East Molesey, Surrey, England.) was prepared freshly in PBS (pH 6.8)to a working strength of 25 mg/ml.

5.278 BACTERICIDAL ASSAY.

The following were placed sequentially and incubated at 37'C for 90 minutes in rolled bottles:

0.3 ml 1% pooled decomplemented serum.

0.2 ml PMN suspension (10^7/ml).

0.1 ml suspension of *S.aureus* (6 X 10^6 /ml).

The following negative controls were also employed :

1. 0.2 ml PMN + 0.1 ml *S.aureus* + 0.3 ml HBBS (WITHOUT serum).

2. 0.5 ml of 1% serum + 0.1 ml *S.aureus*

5.2781 TOTAL SURVIVORS.

The total number of *S.aureus* which survived was assessed by the removal of 0.1 ml from the tube, 10^{-3} and 10^{-5} dilutions were made after the incubation and triplicate blood agar plates were inoculated with 50 µl of the dilutions. The plates were incubated at 37'C overnight, examined the following morning and the number of colonies counted.

5.2782 EXTRACELLULAR SURVIVORS.

The percentage of *S.aureus* which were attached to neutrophils, but not phagocytosed was calculated by centrifuging the tubes at 20g for 2 minutes. Neutrophils sedimented to the base of the tube, 0.1 ml of the supernatent was removed, 10^{-3} and 10^{-5} dilutions made in

sterile saline and 50ul of each dilution inoculated onto triplicate blood agar plates. The plates were incubated at 37'C overnight, examined the following morning and the number of colonies counted.

5.2783 INTRACELLULAR SURVIVORS.

The percentage of *S.aureus* which were phagocytosed, but not killed was calculated by the method of Tan, Watanakunakorn and Phaie (1971).

Extracellular *S.aureus* was eliminated by treatment with lysostaphin at a final concentration of 5 µg/ml for 30 minutes at 37'C. Lysostaphin was subsequently inactivated by trypsin (final concentration 1.2 mg/ml) before 10^{-1} and 10^{-3} dilutions were made in sterile saline, 50µl of each dilution was inoculated in triplicate onto blood agar plates to enable colonies of *S.aureus* to grow from viable intracellular survivors. The plates were incubated at 37'C overnight, examined the following morning and the number of colonies counted.

Intracellular survival was calculated as a percentage of the phagocytosed cells.

$$\frac{\text{Mean Survivors} \times 2.1^{*}}{\%\ \text{phagocytosed}} \times 100 = \%\ \text{phagocytosed cells which survived intracellular killing.}$$

* correction factor see table 1.

5.2774 CLASSIFICATION OF SURVIVING BACTERIA.

Since PMN concentrations were all similar (within 10%) and percentage viability was in excess of 85% in all cases, it was not considered necessary to make any adjustment in the analysis to allow for differing PMN numbers.

TO Initial number of bacteria

TS Total number of bacterial survivors

ECS Number of extracellular survivors

ICS Number of intracellular survivors

Phag 1
} Estimates of phagocytosis
Phag 2

For each test therefore, the initial number of bacteria (TO) were recorded together with the total number of surviving bacteria (TS), the number of extracellular survivors (ECS) and the number of intracellular survivors (ICS). The number of bacteria phagocytosed were estimated by two methods. Firstly as TO-ECS, (phag 1) and secondly as TO-(TS-ICS), (phag 2). The second estimate accounts for any bacteria which adhered to the surface of PMN's which were not phagocytosed and any bacterial clumps which sedimented on centrifugation, and is therefore a more precise estimate. The number of PMN associated bacteria (PMS) was estimated by TS-ECS. These six quantities were expressed as percentages, TS, ECS, Phag1, Phag2, and PMS relative to the initial number of bacteria TO, and ICS relative to the number of phagocytosed bacteria TO-(TS-ICS). As phagocytosis is a prerequisite to intracellular killing it seems reasonable to express the number of intracellular survivors as a percentage of phagocytosed bacteria rather than total initial bacteria.

Results obtained from bactericidal assays were analysed using a oneway analysis of variance.

Table 3 .Experimental Method.

TREATMENT	TOTAL VOLUME IN TUBE.
Step 1. Heated pooled ovine serum 1%	0.3 ml
Add 0.2 ml PMN (10^7/ ml)	0.5 ml
Add 0.1 ml *S.aureus* ($6X10^6$/ml Roll at 37'C for 90 minutes Remove 0.1 ml dilute and plate out	0.6 ml 0.5 ml
Step 2. Centrifuge at 20g for 2 minutes	
Remove 0.1 ml supernatent, dilute and plate out	0.4 ml
Step 3. Add 0.6 ml working strength lysostaphin	1.0 ml
Roll at 37'C for 20 minutes	
Add 0.05 ml working strength trypsin	1.05 ml
Roll at 37'C for 10 minutes	
Remove 0.1 ml, dilute and plate out	0.95 ml
Multiply bacterial numbers by 2.1*	

$$* \frac{1.05}{0.5} = 2.1$$

5.28 SECONDARY T.B.F INFECTIONS IN LAMBS.

5.281 EXPERIMENTAL INFECTIONS.

Sixteen weeks after initial infection with T.B.F. lambs were rechallenged as described in section 5.23.

5.282 MONITORING.

The lambs were monitored as described in section 5.24 (Tables 1 and 2).

5.282 HAEMATOLOGY.

Total and differential leucocyte counts were performed and the packed cell volume percentage of blood samples calculated. The measurement of total erythrocyte numbers and haemoglobin concentration was discontinued as no significant changes occurred in these factors during primary T.B.F. infections.

5.283 SEROLOGY.

Sera obtained at daily then weekly intervals post T.B.F. infection were tested using CIE. and indirect fluorescent antibody tests as described in section 5.26.

5.284 NEUTROPHIL FUNCTION TESTS.

Neutrophil function tests were performed on blood samples collected on days 0,5 and 8 post T.B.F. infection as described in section 5.27.

5.30 RESULTS.

5.31 TEMPERATURE MONITORING.

5.311 PRIMARY T.B.F. INFECTIONS.

Rectal temperatures were elevated (>40'C) between days 4 and 8 post T.B.F. infection. The highest temperatures were recorded 5 days post T.B.F. infection and varied between 40.8'C and 42'C. Mean results are illustrated in figure

5.19 The magnitude of fever was calculated from the area under the graph taking 40'C as a baseline , plotted on a 1 mm grid (10mm = 1 day and 10 mm = 0.25'C), using the trapezium rule and varied from 711mm^2 to 2599mm^2 (mean, 1456mm^2, standard error, 63.7mm^2).

5.3112 SECONDARY INFECTIONS.

Rectal temperatures were elevated for a reduced period (between days 5 and 7 inclusive). The mean temperature recorded on day 5 was 40.5'C (range 40.1-41.6'C); on day 6, 40.4'C and on day 7, 40.3'C. Mean results are illustrated in figure 5.1b.

The magnitude of fever was calculated from the area under the graph as described above and varied from 78mm^2 - 613mm^2 (mean 256mm^2, standard error 42mm^2).

5.312 PARASITAEMIA.

5.3121 PRIMARY INFECTION.

C.phagocytophila was first visible in neutrophils 4 days after initial infection with T.B.F.. The peak parasitaemia occurred on day 5 and parasites persisted until 10 days post T.B.F. infection. Figure 5.1 shows $\log_{10}$ number of neutrophils with T.B.F. inclusions per mm^3 of blood. The magnitude of parasitaemia, the area enclosed under the graph plotted on a 1 mm grid (10 mm = 1 day and 10 mm = 0.2 on the $\log_{10}$ number of neutrophils with inclusions) using the trapezium rule varied from 16,520mm^2 -19,240 mm^2 (mean 18060 mm^2, standard error 144.3^2).

5.3122 SECONDARY INFECTION.

C.phagocytophila was visible in neutrophils between days 3 and 10 post challenge. The peak parasitaemia occurred on

Figure 5.1a

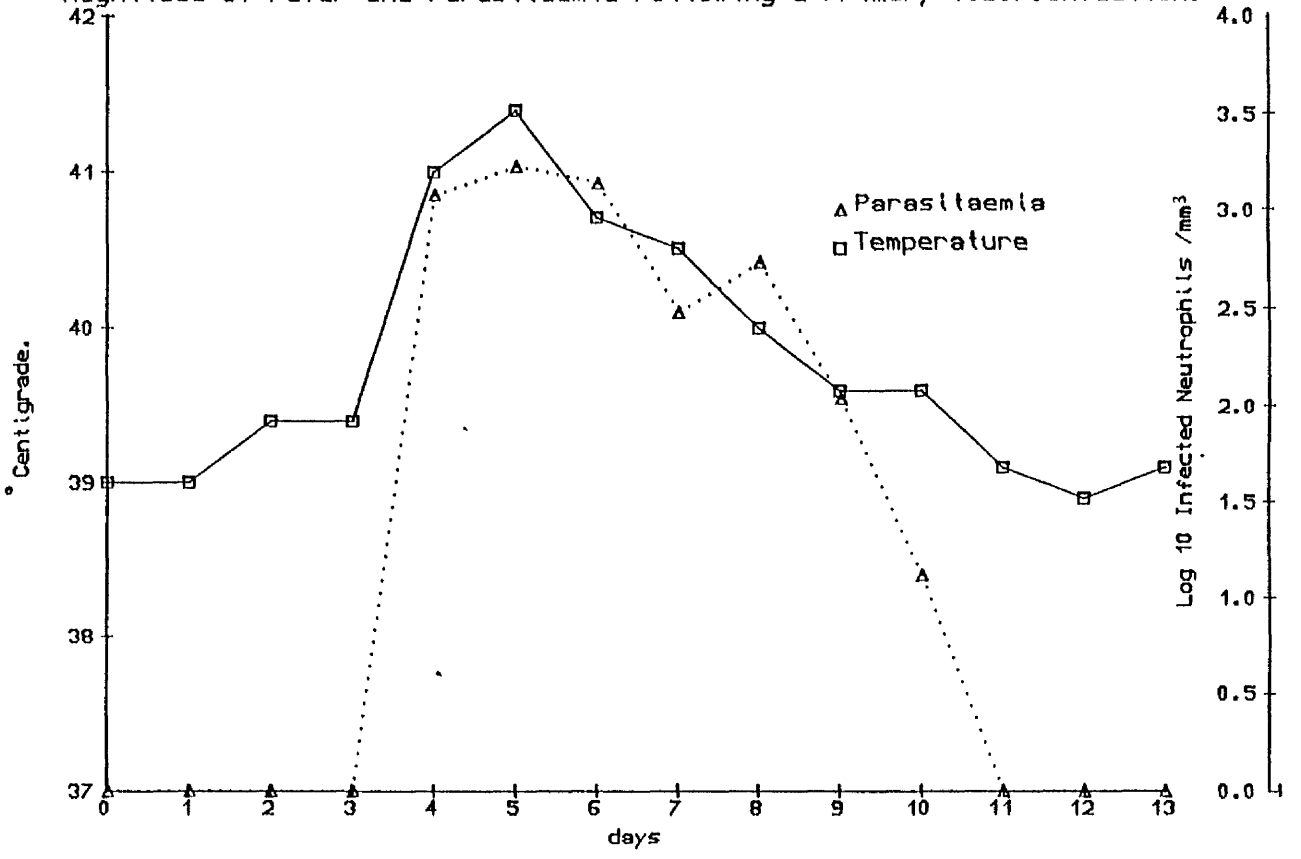
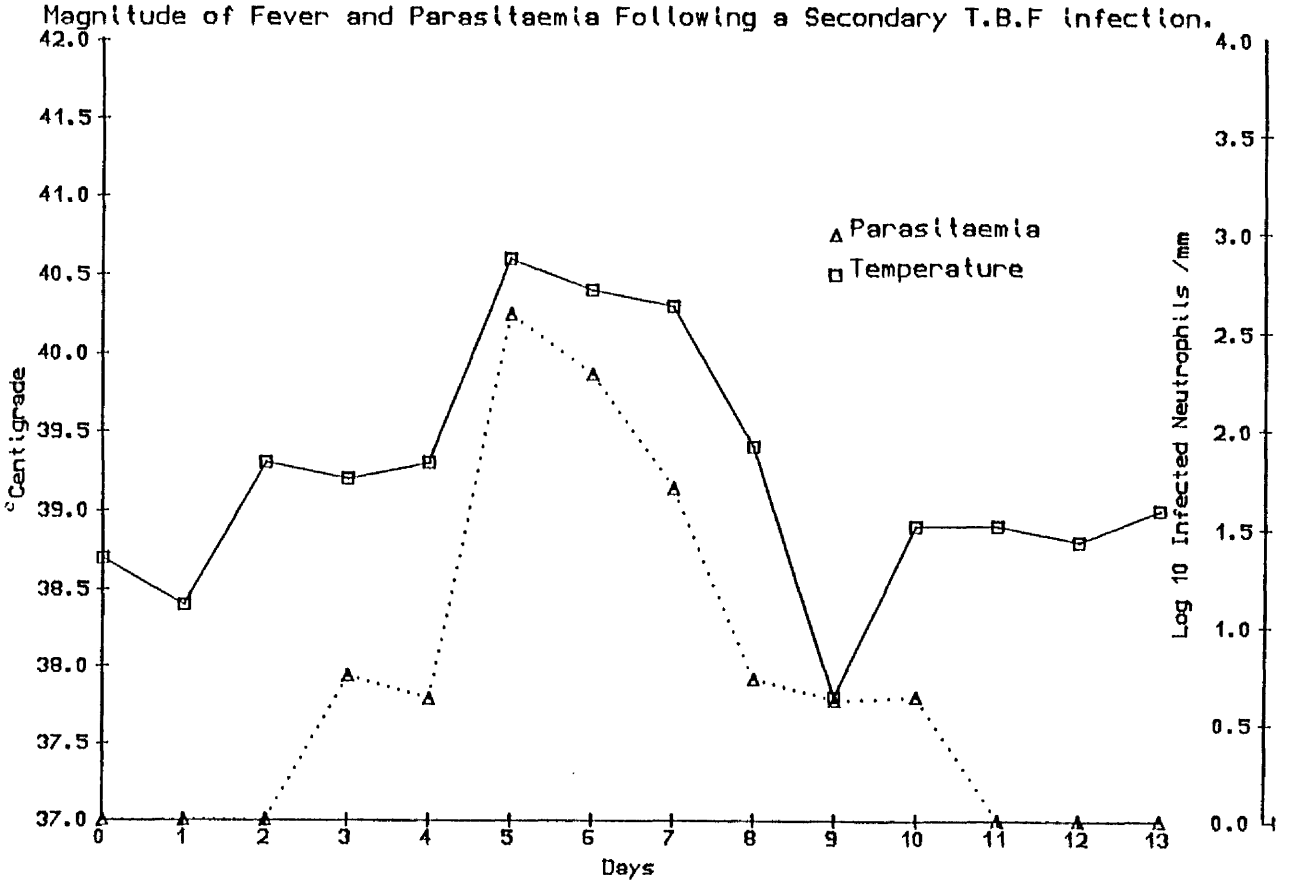


Figure 5.1b



day 5. Figure 5.1b shows $\log_{10}$ number of T.B.F. infected neutrophils per mm^3 blood. The magnitude of parasitaemia, calculated as described before varied from 7810 -12,370 mm^2 (mean 10,258mm^2, standard error 361.2mm^2).

5.3123 STATISTICAL ANALYSIS.

Highly significant decreases were recorded when the magnitude of fever (p=0.0018) and the magnitude of parasitaemia (p=0.0034) following secondary infections were compared using a Mann Whitney U test with those following primary infections (table 4).

Table 4. The Magnitude of Fever and Parasitaemia Following Primary and Secondary T.B.F. Infection.

	Parasitaemia		Fever	
	$\bar{X}$	SE	$\bar{X}$	SE
Primary Infection	18060	144.3	1456	63.7
Secondary Infection.	10258	361.8	256	42
p =	0.0034		0.0018	

5.32 HAEMATOLOGY.

Normal values were calculated from pre-infection bleedings and are shown in table 5.

5.321 PRIMARY T.B.F. INFECTION.

5.3211 TOTAL LEUCOCYTE COUNT.

Mean total leucocyte counts from 9 lambs are shown in table 5 and varied from 8.84 X 10^9 /litre 2 days post infection to 5.5 X 10^9 /litre 13 days post T.B.F. infection. Analysis of the data using Students T test showed that the values recorded after T.B.F. infection

decreased significantly from the normal value of 8 X 10^9/litre (p=0.0049).

Table 5. Normal Haematological Values For Young Lambs.

	$\bar{X}$	Sdi	n
Total leucocytes count	8 X 10^9 /litre	1.80	18
Lymphocyte Count	5.5 X 10^9 / litre	1.51	18
Neutrophil Count	2.32 X 10^9 /litre	1.02	18
Erythrocyte count	7.3 X 10^{12} /litre	0.98	18
Haemoglobin concentration	8g/100ml	1.07	18
Packed Cell Volume	28%	3.94	18

5.3212 LYMPHOCYTE COUNTS.

Mean lymphocyte numbers are shown in table 6 and figure 5.2. The nadir of the lymphocytopaenia occurred on day 5 (3.39 X 10^9 / litre), numbers recovered slightly before falling back to the same level on day 8. Normal levels were recorded on day 10. Analysis of the data using Students T test showed a significant reduction in lymphocyte numbers recorded throughout the infection period (p=0.019) when compared to a normal pre-infection value of 5.5 X 10^9/litre.

5.3213 NEUTROPHILS COUNTS.

Mean neutrophil numbers increased from a normal level of 2.32 X 10^9 per litre to a peak of 3.19 X 10^9 per litre 4 days post T.B.F. infection. This was followed by a steady reduction in neutrophil numbers until the nadir of the neutropaenia was achieved on day 9 (0.69 X 10^9/litre). Neutrophil numbers recovered to normal levels 20 days post T.B.F. infection. These results are shown in table 6 and

figure 5.2. Analysis of data using the Students T test showed that the neutrophil numbers recorded during a T.B.F. infection decreased significantly from normal values ($p=0.0358$).

5.3214 ERYTHROCYTE COUNTS.

Mean erythrocyte counts varied between 5.8 and 8.7 X 10^{12}/litre as shown in table 6. Analysis of this data using Students T test showed no significant differences between erythrocyte numbers throughout the infection period and a normal value of 7.3 X 10^{12} erythrocytes per litre.

5.3215 HAEMOGLOBIN CONCENTRATION.

The mean haemoglobin concentrations are shown in table 6. Values varied between 7.1 and 10.3 g/100ml. Analysis of the data using Students T test revealed no significant differences between the haemoglobin concentrations recorded during the infection period and a normal value of 8g/100 ml.

5.3216 PACKED CELL VOLUME PERCENTAGE.

The mean percentage packed cell volumes are shown in table 6. Variations occurred between 29.3% on day 2 to 20.7% 9 days post T.B.F infection. Analysis of the data using Students T test showed that there were significant decreases between the values recorded throughout a primary T.B.F. infection and the normal value of 28% ($p=0.0022$).

Figure 5.2a

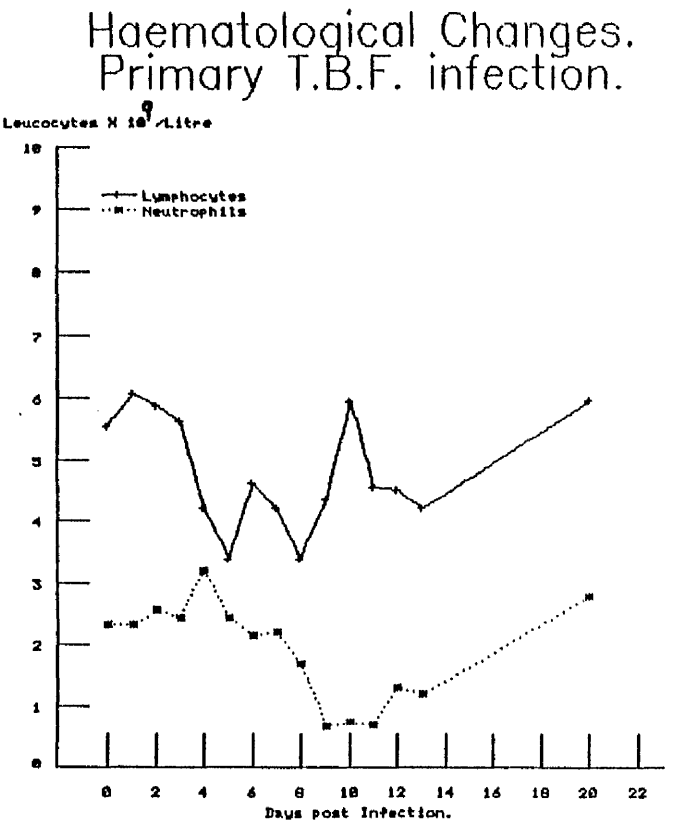


Figure 5.2b

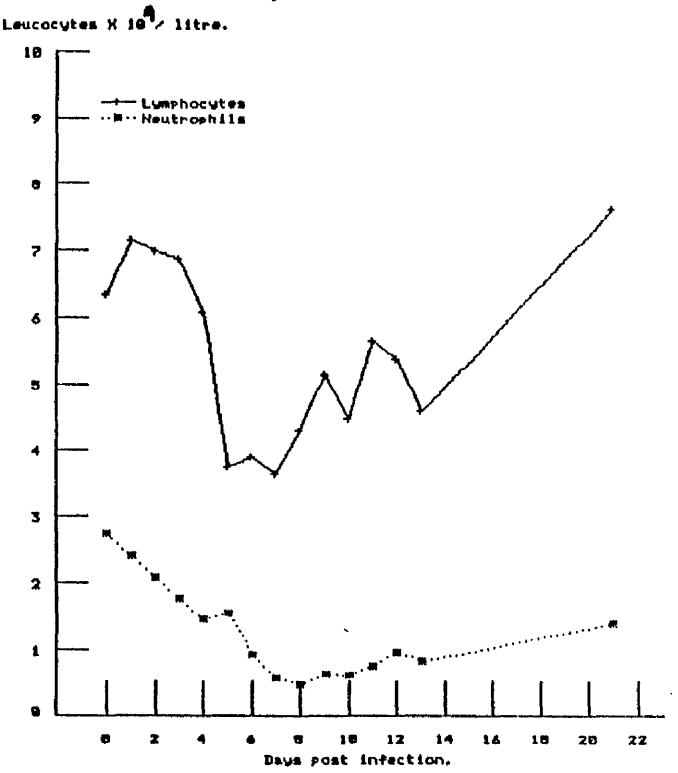


Table 6. Mean Haematological Values Recorded Throughout A Primary T.B.F. Infection (n=9).

Day	WBC	LYM	NEU	RBC	HB	PCV
0	8.05	5.54	2.32	7.2	10.3	27.4
	1.88	1.51	0.98	0.96	1.07	3.90
1	8.64	6.06	2.32	7.8	10.1	29.1
	2.08	1.50	0.82	0.79	0.82	2.20
2	8.84	5.88	2.55	7.4	9.9	29.3
	2.20	1.46	1.27	0.86	0.52	2.12
3	8.31	5.62	2.43	8.6	9.5	28.4
	1.90	1.17	1.41	0.70	0.49	2.30
4	7.64	4.21	3.19	8.7	9.3	27.7
	1.83	1.14	1.38	1.06	0.70	2.74
5	6.10	3.39	2.44	8.1	8.7	25.6
	1.61	0.99	0.86	0.96	0.82	1.88
6	6.91	4.61	2.14	7.8	8.2	24.4
	1.88	1.22	0.79	0.92	0.83	2.60
7	6.64	4.21	2.21	7.5	8.0	24.2
	1.76	1.28	0.95	1.20	0.95	2.83
8	5.70	3.39	1.69	6.7	7.2	22.6
	2.31	1.02	0.89	1.06	0.82	2.27
9	5.95	4.95	0.69	5.8	7.1	20.7
	0.94	0.92	0.27	0.88	1.01	2.78
10	6.94	5.95	0.74	6.7	7.5	21.8
	1.16	1.08	0.21	0.94	1.12	1.86
11	5.66	4.56	0.71	7.2	8.0	23.9
	1.11	0.93	0.21	0.72	0.74	2.52
12	6.14	4.51	1.30	7.0	8.0	23.2
	1.01	0.76	0.41	0.71	0.50	2.44
13	5.50	4.21	1.20	7.1	7.3	22.3
	0.58	0.46	0.32	0.95	0.75	2.06
20	8.79	5.96	2.79	8.7	8.8	27.1
	1.63	0.91	1.19	0.70	0.57	2.47

WBC Total leucocyte count } X 10^9 /litre
LYM Total lymphocyte count }
NEU Total neutrophil count }

RBC Total erythrocyte count X 10^{12} /litre
Hb Haemoglobin concentration (g/100ml)
PCV Percentage packed cell volume.
Upper value is the mean, lower value the standard deviation.

5.322 SECONDARY T.B.F INFECTIONS

Normal values were calculated from pre-infection blood samples and are shown in table 7.

Table 7. Normal Values For Young Lambs Aged 4-5 Months.

	$\bar{X}$	sdi	n
Total leucocytes count	9 X 10^9/litre	1.62	12
Lymphocyte Count	6.5 X 10^9/ litre	1.01	12
Neutrophil Count	2.32 X 10^9/ litre	1.17	12
Packed Cell Volume	34%	4.82	12

$\bar{X}$ Mean pre-infection values.
Sdi Standard deviation
n Number of samples

5.3221 TOTAL LEUCOCYTE COUNTS

The mean total leucocyte counts (table 7) varied between 10.42 X 10^9 /litre 1 day after the challenge infection had been administered and 4.62 X 10^9/litre on day 7 of the infection period. Analysis of the data using Students T test demonstrated a significant reduction ($p=0.0035$) between total leucocyte counts following a secondary T.B.F. infection and the normal pre-infection value of 9 X 10^9 leucocytes/litre.

5.3222 LYMPHOCYTE COUNTS

The mean lymphocyte count varied from 7.14 X 10^9 /litre on day 2 to 3.64 X 10^9/litre on day 7 of the infection period and returned to a normal value of 7.61 X 10^9 /litre by day 20. Mean values are shown in table 8 and figure 5.2. Analysis using Students T test recorded a significant decrease ($p=0.0043$) between the data recorded during a

secondary T.B.F. infection and a normal value of 6.5 X 10^9 lymphocytes/ litre.

5.3223 NEUTROPHIL COUNTS

The mean number of neutrophils per litre varied between 2.76 X 10^9 / litre on day 0 to 0.48 X 10^9 per litre 8 days after challenge with T.B.F.. Mean values are shown in table 8 and figure 5.2. Analysis of the data using Students T test showed that neutrophil counts recorded throughout a T.B.F. infection were significantly reduced ($p<0.001$) when compared to the normal pre-infection value of 2.32 X 10^9 neutrophils /litre.

5.3224 PACKED CELL VOLUME.

The mean packed cell volume percentage varied from 34.8% on day 1 of the secondary T.B.F. infection to 30% on day 10. Mean values are illustrated in table 8. Analysis of the data using Students T test showed that the values recorded throughout a secondary T.B.F. infection decreased significantly ($p=0.0016$) from a normal pre-infection value of 34 %.

5.225 COMPARISON OF HAEMATOLOGICAL VALUES RECORDED DURING PRIMARY AND SECONDARY T.B.F. INFECTIONS.

No significant differences were recorded between the total leucocyte or lymphocyte counts when the results from primary and secondary infections were compared. However, when neutrophil counts were compared there was a significant reduction ($p=0.0152$) in numbers of neutrophils recorded after secondary T.B.F. infection. There was also a significant decrease in the percentage packed cell volume following primary infection when compared to secondary infection ($p<0.001$). However, when values were adjusted to

take account of higher PCV's recorded in older animals, significant differences were not recorded.

5.33 WEIGHT GAINS - PRIMARY AND SECONDARY INFECTIONS.

Lambs were weighed prior to T.B.F. infection (D 0) and at the end of the experimental period (D 21). Mean daily weight gains were calculated and are shown in table 9. These varied between 0.01 Kg per day for the lambs which experienced a secondary infection of T.B.F. and then were challenged with *S.aureus*, to 0.124 Kg per day for the batch of young lambs challenged with *S.aureus* only.

There were no significant differences between the groups, although in the primary T.B.F. infection group lambs challenged with *S.aureus* only (ie.T.B.F. free) had higher daily weight gains than lambs challenged with tick-borne fever alone or tick-borne fever followed by *S.aureus*.

TABLE 8. Mean Haematological Values Recorded Throughout A Secondary T.B.F. Infection.

DAY	WBC	LYM	NEU	PCV
0	10.06	6.33	2.76	34.6
	1.78	1.07	1.42	4.50
1	10.42	7.14	2.41	34.8
	1.61	1.57	1.27	4.44
2	9.68	6.98	2.08	34.6
	2.20	1.85	0.57	4.93
3	9.16	6.85	1.78	34.4
	1.93	1.85	0.35	4.40
4	8.14	6.06	1.47	33.4
	2.00	1.56	0.46	3.58
5	5.90	3.76	1.56	31.8
	1.51	1.34	0.39	3.56
6	5.28	3.90	0.92	32.2
	1.45	1.23	0.45	3.63
7	4.62	3.64	0.58	30.6
	0.64	0.92	0.15	6.11
8	4.84	4.29	0.48	31.4
	1.08	1.42	0.30	4.56
9	6.34	5.13	0.63	32.8
	1.42	1.55	0.19	3.70
10	5.68	4.46	0.62	30.0
	0.89	1.17	0.22	4.30
11	6.96	5.65	0.75	32.6
	0.85	0.93	0.48	3.27
12	6.92	5.38	0.96	31.2
	0.85	0.93	0.48	3.27
13	6.40	4.59	0.83	30.0
	1.24	2.25	0.41	3.24
21	9.22	7.61	1.40	32.6
	2.12	1.41	0.54	3.58

WBC Total leucocyte count } X 10^9 /litre
LYM Total lymphocyte count }
NEU Total neutrophil count }
PCV Percentage packed cell volume.
The upper value for each day post infection is the mean result recorded and the lower value the standard deviation. Both values were calculated from 6 lambs.

Table 9. Mean Daily Weight Gains During Primary and Secondary T.B.F. Infections.

Primary T.B.F. infection only	0.095 kg/ day
Primary T.B.F. infection and then challenged with *S.aureus*	0.089 kg/day
S.aureus infection only	0.124 kg/day
Secondary T.B.F. inf. only	0.097 kg/day
Secondary T.B.F. inf. then challenged with *S.aureus*	0.010 kg/day
S.aureus infection only	0.084 kg/day

5.34 SEROLOGY.

5.341 CIE

A positive result was denoted by the formatiion of a precipitation arc between the wells (Plate 6). Electrophoresis using serum samples originating from animals with no experience of T.B.F. did not produce precipitation lines. Results were expressed as reciprocal antibody titres of the highest dilution producing precipitation lines.

5.3411 PRIMARY INFECTIONS IN LAMBS

Antibodies to T.B.F. were first detected between 9 and 11 days post T.B.F. infection. Peak titres occurred 2 weeks after initial infection (mean = 35.2 ,S.E. = 7.9) and antibodies persisted for 6 to 10 weeks. These results are illustrated in figure 5.3a.

5.3412 SECONDARY INFECTIONS IN LAMBS

Antibodies to T.B.F. were first detected 9 days after administration of the challenge infection, but titres were

Figure 5.3a

C.I.E. Method.Lambs. Primary T.B.F. Infection.

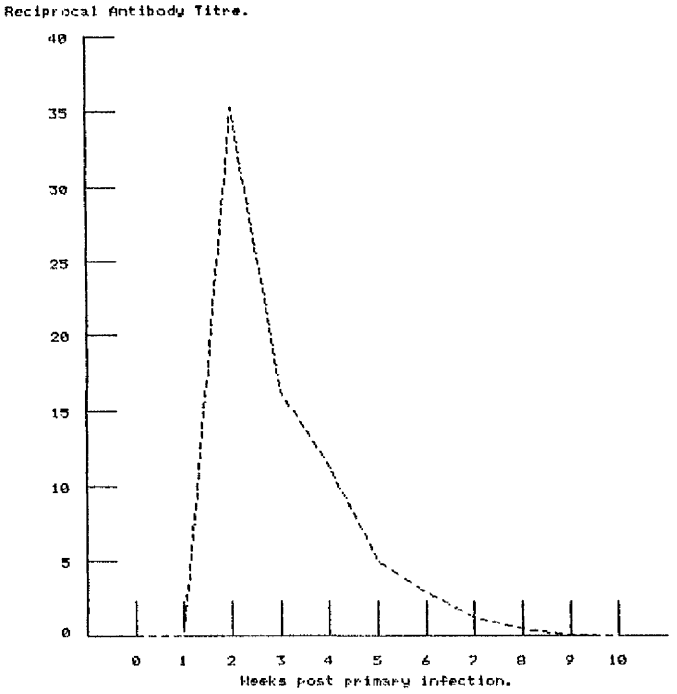


Figure 5.3b

C.I.E. Method. Lambs. Secondary T.B.F. Infection.

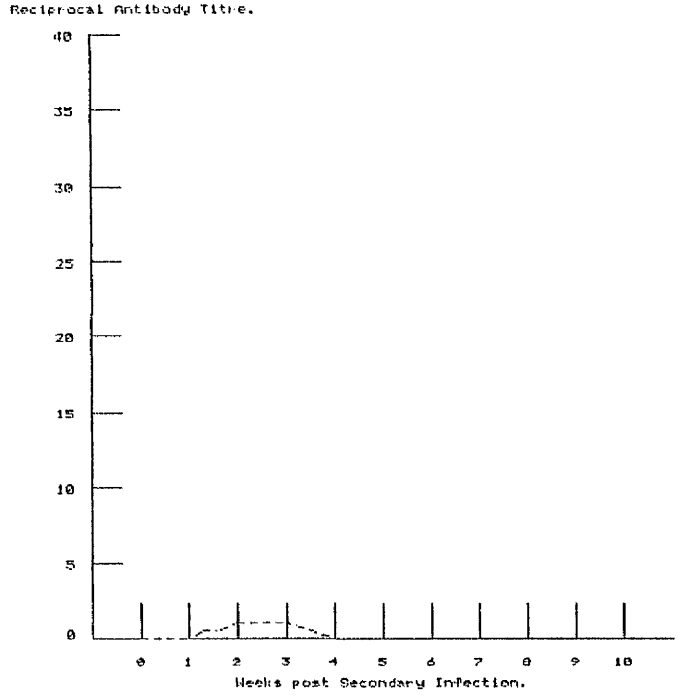
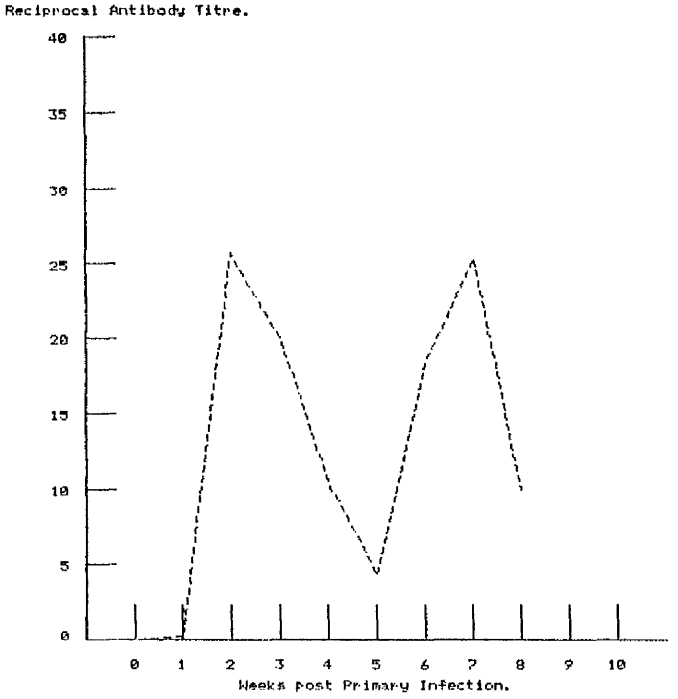


Figure 5.3c

C.I.E. Method.Goats. Primary T.B.F. Infection.



The use of C.I.E. to detect antibodies produced following TBF infection.

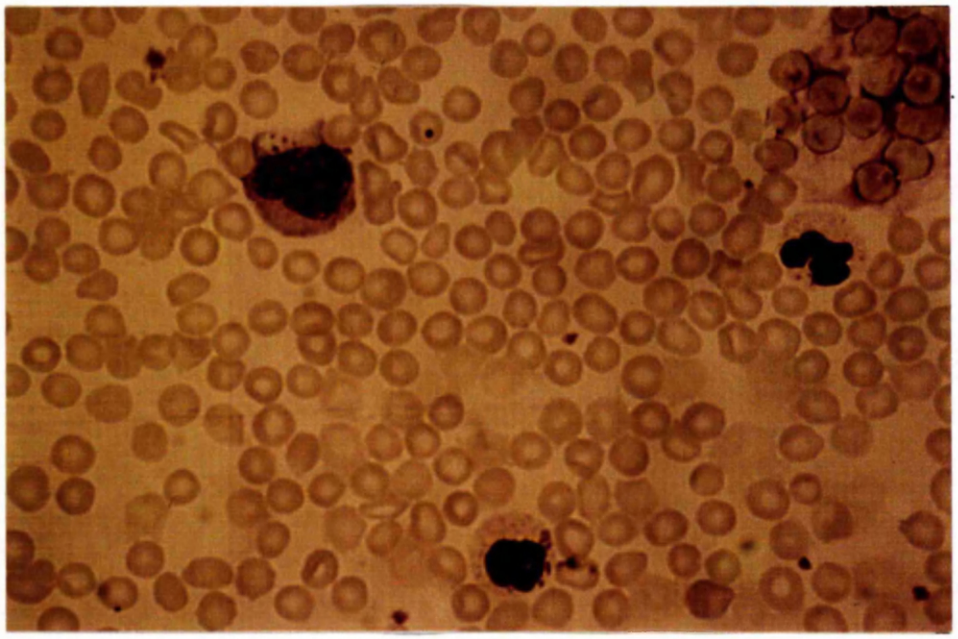


Plate 5 Intracytoplasmic inclusions of *Cytoecetes phagocytophila* within neutrophils. The blood smear was stained with Giemsa and viewed at x 1,000 under oil emersion.

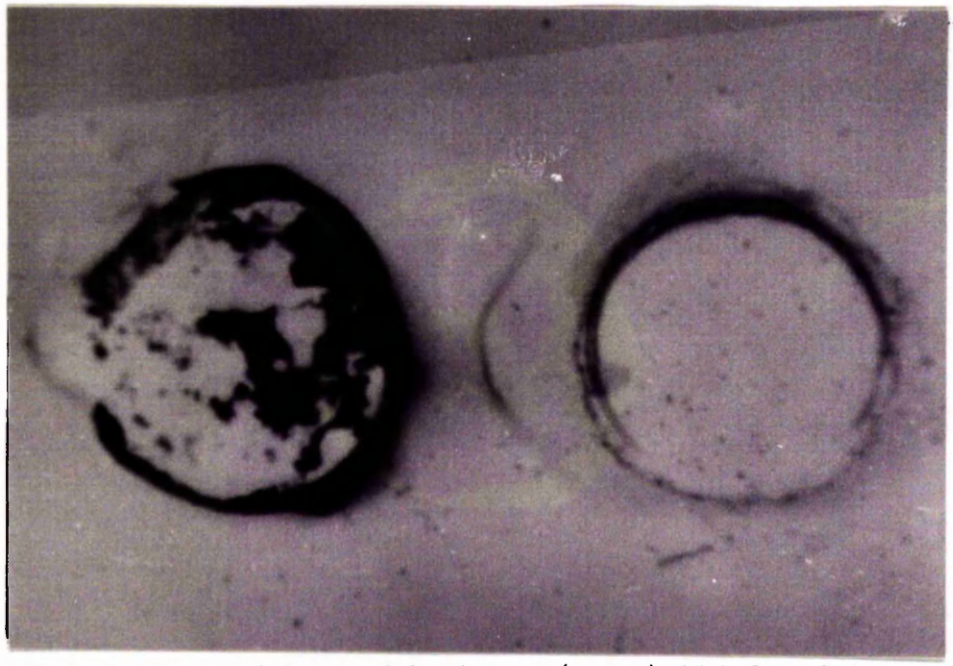


Plate 6 Dark staining precipitation arc (centre) which forms between antigen well (left) and serum well (right) following CIE to indicate a positive result.

at much lower levels than those recorded after primary infections and persisted for only 2-3 weeks. Results are illustrated in figure 5.3b.

5.3413 PRIMARY INFECTIONS IN GOATS

Serum samples were available only at weekly intervals. Three out of ten goats sampled were sero-positive 7 days post infection and the remainder had seroconverted by day 14. The results are illustrated in figure 5.3c. The curve produced had 2 peaks of antibody concentration which occurred at weeks 2 and 7 with a pronounced trough reflecting lower antibody titres in the interim.

5.3415 MERCAPTOETHANOL TREATED SAMPLES.

Serum samples collected throughout the infection period and at weekly intervals until 12 weeks post primary and secondary infection were incubated with mercaptoethanol and lost any precipitating antibody which had been detectable by CIE prior to incubation with mercaptoethanol. However, if the wells were 'triple loaded' with serum (ie three times the volume applied to the gel) antibodies could be detected at low levels in mercaptoethanol treated sera.

5.342 IFAT.

A positive result was indicated by the presence of fluorescing intracytoplasmic inclusions within neutrophils. Neutrophil nuclei appeared as grey, green irregular lobed structures and the inclusions as small bright green dots within the cells. There was also a great deal of extracellular non-specific fluorescence.

Erythrocytes which had not been lysed during storage at -20'C exhibited auto fluorescence.

Figure 5.4a

I.F.A.T.
Primary T.B.F. Infection.

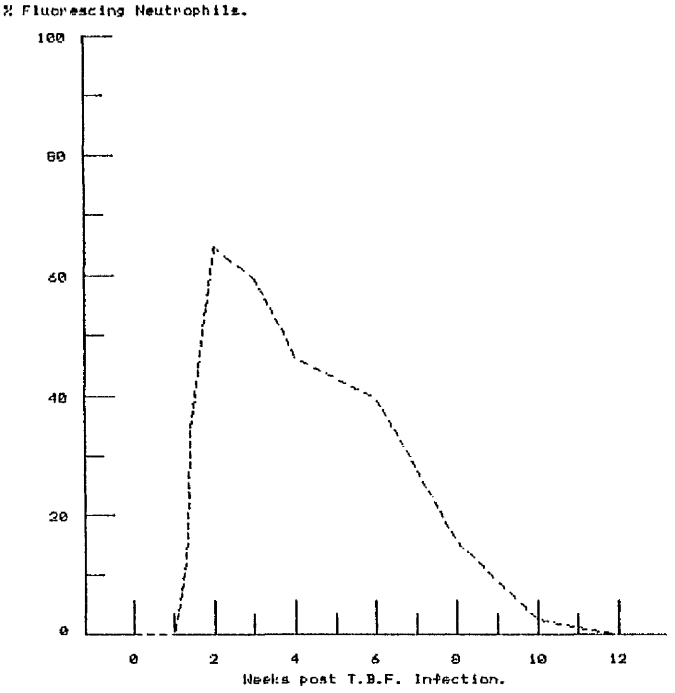
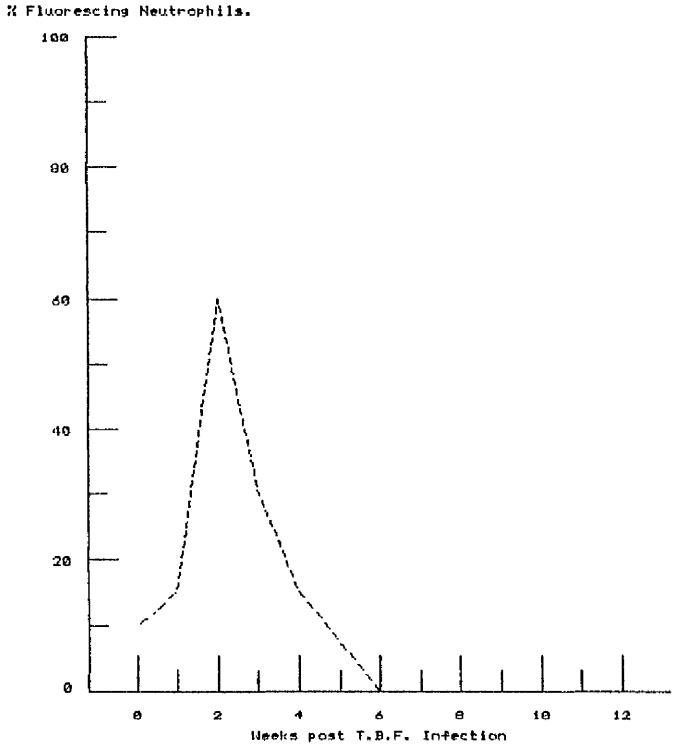


Figure 5.4b

Secondary T.B.F. Infection.



The use of IFAT to detect antibodies produced by lambs following primary and secondary TBF infections.

5.3421 NEGATIVE CONTROLS.

Fluorescing inclusions were not observed in negative control smears.

5.3422 POSITIVE CONTROLS.

Thirty three positive control smears were examined and 20 neutrophils observed on each smear. A mean of 64 percent of the neutrophils contained fluorescing inclusions (S.E.= 2.26). Erythrocytes appeared to be more pronounced than in the negative control smears.

5.3423 PRIMARY T.B.F. INFECTIONS IN LAMBS.

Antibody to *C.phagocytophila* was first detected 9-10 days post initial infection with T.B.F. The greatest number of neutrophils exhibiting fluorescence were found 2 weeks after initial infection and antibody persisted for 8 - 10 weeks. Mean results are shown in figure 5.4a and table 10.

5.3424 SECONDARY T.B.F. INFECTION IN LAMBS.

A low percentage (10%) of neutrophils with fluorescing inclusions were observed in the pre-infection sera. The largest percentage of neutrophils with fluorescing inclusions (60%) was found 2 weeks after administration of the challenge infection and antibodies persisted for 3 - 4 weeks.

Mean results are shown in figure 5.4b and table 10.

5.35 NEUTROPHIL FUNCTION TESTS.

5.352 PRIMARY T.B.F. INFECTION.

The total number of *S.aureus* which survived incubation with the P.M.N./ serum mixture (TS) varied from 6.6% (Sdi=2.23) 2 days after T.B.F. infection to 58.1% (Sdi=6.94) 11 days after T.B.F. infection. Mean numbers of

Table 10. The Percentage of Neutrophils Which Exhibited Specific Fluorescence Using The IFAT Method.

WEEKS POST T.B.F.	PRIMARY INF			SECONDARY INF.		
	n	$\bar{X}$	sdi	n	$\bar{X}$	sdi
0	8	0		2	10	14.1
1	8	0		2	15	7.1
1.3	8	12.5	14.1	-	-	
1.4	8	35.0	21.0	-	-	
2	8	65.0	11.3	2	60	14.1
3	8	60.0	11.6	2	30	21.2
4	8	45.0	15.4	2	15	21.2
6	8	36.2	11.9	2	0	
8	8	15.6	5.0	2	0	
10	8	2.5	3.8	2	0	
12	8	0		2	-	

- sample not tested

$\bar{X}$ mean result sdi standard deviation
n number of samples

extracellular survivors followed a similar pattern with the minimum number of bacteria (1.7%, Sdi=0.85)) surviving on day 2 and the maximum number (53% ,Sdi=9.69) on day 8 post T.B.F. infection. Intracellular survival varied from 0.417% (Sdi=0.27) prior to T.B.F. infection to 0.024%)Sdi=0.007) 2 days after T.B.F. infection. The results of these studies are shown in table 11 and figure 5.5a and b and figure 5.6.

Statistical analysis of the data revealed significant increases in total survivors and extracellular survivors and a decrease in intracellular survivors throughout the

infection period ($p<0.01$) when compared with pre-infection values (table 11).

Table 11. Mean Values for Samples Obtained at Various Intervals after a Primary T.B.F. Infection.

Days post T.B.F.	n	Estimated mean % Survival of *S.aureus* % TS	% ECS	TS-ECS %PMS	% phagocytosis of *S.aureus* % ICS	Phag 1	Phag 2
0	6	16.4 (2.95)	13.7 (6.47)	2.7	0.417 (0.27)	86.3	84.0
2	6	6.6 (2.23)	1.7 (0.85)	4.9	0.024 (0.007)	98.3	93.4
5	6	34.0 (8.84)	18.0 (9.19)	16.0	0.038 (0.036)	82.0	66.0
8	6	57.5 (7.88)	53.0 (9.69)	4.5	0.038 (0.023)	47.0	42.5
11	6	59.4 (6.94)	32.0 (10.2)	26.5	0.053 (0.036)	68.0	42.6
13	6	11.4 (2.98)	9.3 (3.83)	2.1	0.141 (0.025)	90.7	88.7

n number of lambs tested

TS Total Survivors

ECS Extracellular Survivors

PMS PMN associated bacteria

ICS Intracellular Survivors

Phag 1 = %TO-ECS } estimates of phagocytosis,
Phag 2 = % TO-(TS-ICS) } defined in the text.

( ) standard deviation

Table 11b. Statistical Analysis Using a Oneway Analysis of Variance for Samples Obtained at Various Intervals after a Primary T.B.F. Infection.

	Estimated mean % Survival of *S.aureus*			
Days post T.B.F. inf.	n	% TS	% ECS	ICS
p	6	<0.01	<0.01	<0.01

5.353 SECONDARY T.B.F.

The total numbers of surviving bacteria varied from 15.5% prior to T.B.F. challenge to 50.3% 8 days after T.B.F. challenge. The extracellular survivors followed a similar pattern and varied from 12.9% prior to challenge to 48.1% 8 days after T.B.F. infection. The number of intracellular survivors was consistently low and varied from 0.327% to 0.030%. Complete results are shown in table 12.

The results obtained from bactericidal assays were analysed using a oneway analysis of variance. A significant decrease in the number of intracellular survivors occurred following T.B.F. infection when compared to pre-infection levels, otherwise significant differences were absent (table 12).

Table 12. Mean Values for Samples Obtained at Various Intervals After a Secondary T.B.F. Infection.

		Estimated mean % Survival of *S.aureus*			% phagocytosis of *S.aureus*		
Days post T.B.F.	n	% TS	% ECS	TS-ECS %PMS	% ICS	Phag 1	Phag 2
0	4	15.5 (12.9)	12.9 (11.0)	2.6	0.327 (0.174)	87.1	84.8
5	4	50.1 (33.0)	35.7 (24.6)	14.4	0.031 (0.006)	64.3	49.9
8	4	50.3 (15.6)	48.1 (14.6)	2.2	0.030 (0.015)	57.9	49.7

n number of lambs tested

TS Total Survivors

ECS Extracellular Survivors

PMS PMN associated bacteria

ICS Intracellular Survivors

Phag 1 = TO-ECS } estimates of phagocytosis,

Phag 2 = TO - (TS-ICS)} defined in the text.

( ) standard deviations are shown in brackets.

Table 12b. Statistical Analysis Using a Oneway Analysis of Varience for Samples Obtained at Various Intervals After a Primary T.B.F. Infection.

	Estimated mean % Survival of *S.aureus*			
Days post T.B.F. inf.	n	% TS	% ECS	ICS
p	4	ns	ns	<0.01

5.354 COMPARISON OF PRIMARY AND SECONDARY INFECTIONS.

A twoway analysis of variance was used to compare data obtained from primary and secondary infections at various

Figure 5.5a

Percentage Survival. Primary T.B.F. Infection.

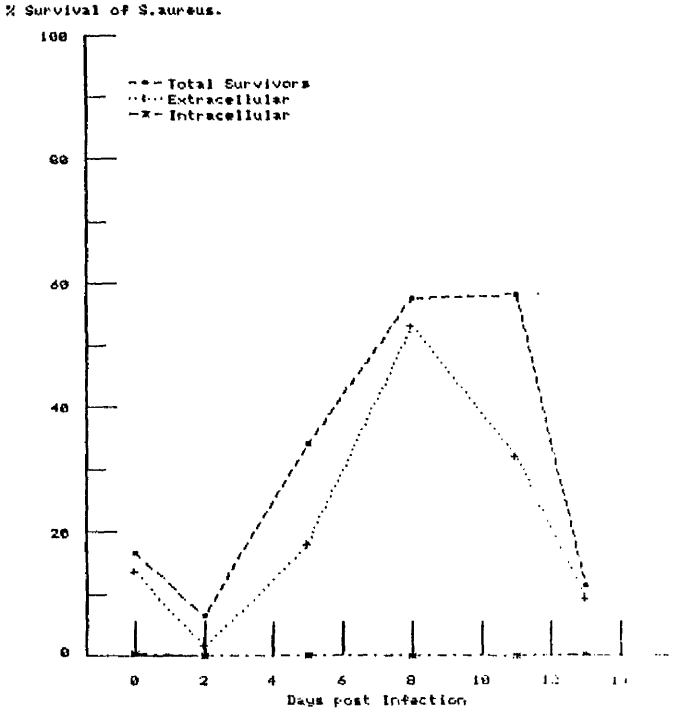
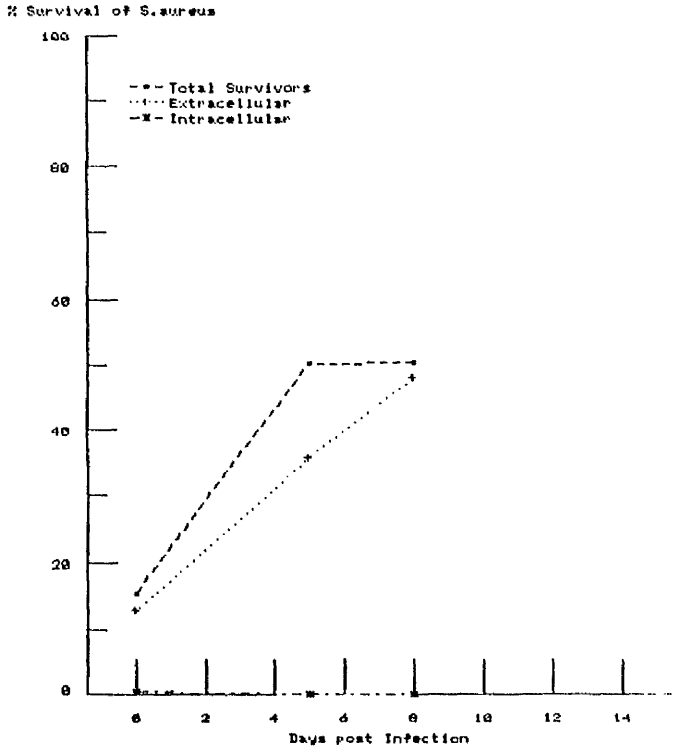


Figure 5.5b

Secondary T.B.F. Infection.

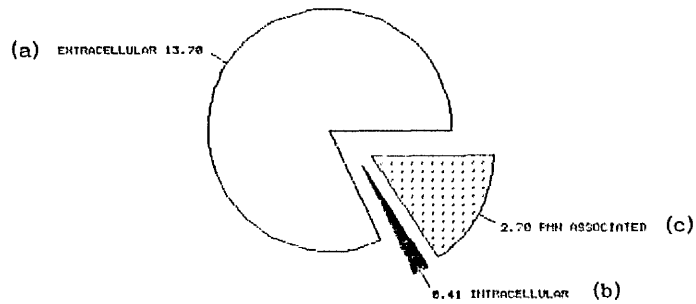


Percentage survival of *Staphylococcus aureus* following incubation with a neutrophils obtained from lambs at various stages of a tick-borne fever infection. The results are expressed as total survivors, extracellular survivors and intracellular survivors.

Figure 5.6

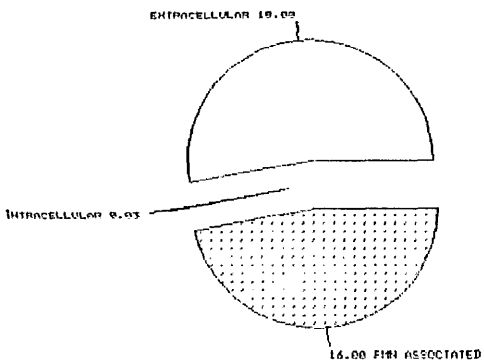
PRE-INFECTION

Total survivors = 16.8%



DAY 5 POST PRIMARY INFECTION.

Total survivors = 34%



DAY 11 POST PRIMARY INFECTION

Total survivors = 58.6%

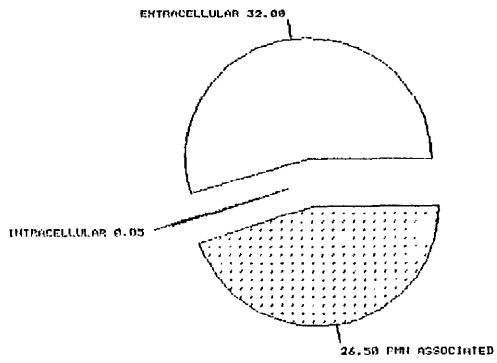


Figure 5.6 illustrates an attempt to study the functional integrity of P.M.N. leucocytes in T.B.F. infected lambs in terms of phagocytosis and intracellular killing. Three criteria were measured in an *invitro* assay of neutrophil function and expressed as percentages: a – extracellular survivors
b – intracellular survivors
c – PMN associated bacteria.

times during the infection period. Highly significant differences ($p<0.01$) were found between values recorded at differing times after infection, but there were no significant differences between results following primary and secondary infection.

5.4 DISCUSSION

Young lambs infected with T.B.F. on day 0 began to exhibit signs of their infection such as a parasitaemia and thermal response 3-4 days after infection. Fever is initiated by pyrogens, which are compounds that can be isolated from bacteria and may also be released by granulocytes such as neutrophils and act directly on the hypothalamus (Svendsen and Carter,1984). The high fever characteristic of T.B.F. infection is probably due to pyrogens released by decaying neutrophils.

Following challenge with the same strain of T.B.F. sixteen weeks after initial infection lambs underwent a milder secondary reaction with a significantly reduced level of fever and parasitaemia when compared to primary infection. This agrees with observations recorded by Woldehiwet and Scott (1982d) who reported that immunity to T.B.F. was often incomplete and secondary infection often resulted in a milder reaction.

The haematological changes recorded during this study were principally a decrease in lymphocyte numbers which occurred 5 days after primary T.B.F. infection and persisted at low levels for only a few days to be followed by a more marked neutropaenia. These observations agree with those of Brodie *et al* (1986) who reported a lymphocytopaenia between days 4 and 9 post initial

infection. In the present study neutrophil numbers declined from day 7 post primary infection (figure 5.2a) and reached a trough during days 9, 10 and 11 after which neutrophil counts increased, reaching normal levels by day 20 post infection. The haematological changes which occurred after secondary infection varied slightly from this pattern, in that the lymphocytopaenia followed a similar distribution with the nadir occurring between days 5 and 7, before subsequent recovery, but highly significant differences were recorded between the numbers of circulating neutrophils following primary and secondary T.B.F. infections (5.354). Numbers of circulating neutrophils decreased gradually from the time of secondary challenge reaching a low point 8 days post T.B.F. infection before recovering to normal levels. This decline was especially marked between day 5 , which corresponded with the high point of the parasitaemia and day 8 when $\log_{10}$ number of infected neutrophils = 0.75. By day 11 *C.phagocytophila* were no longer visible in blood smears and numbers of circulating neutrophils had started to increase.

These results suggest that the significantly lower level of parasitaemia recorded following secondary T.B.F. infection when compared with primary infection is due to a more rapid removal of T.B.F. infected neutrophils from the peripheral blood. Although free antibody was not detected by the CIE method at this time (before day 8 post secondary T.B.F. infection), low levels of antibody were detected by the IFAT method. Thus antibody or cell mediated mechanisms may be involved in the elimination of *C.phagocytophila*, the latter perhaps via primed lymphocytes

which were capable of trapping extracellular *C.phagocytophila* or killing infected cells.

Measurement of the total numbers of circulating erythrocytes and the concentration of haemoglobin revealed no significant differences between pre and post infection levels following primary infection (table 6) and these parameters were not recorded during secondary infections. A significant reduction in percentage packed cell volume (PCV) was noted following both primary and secondary T.B.F. infections. This agrees with studies by Taylor *et al* (1986) who reported a 20% reduction in P.C.V. following primary T.B.F. infection.

Serologically CIE first detected antibodies to *C.phagocytophila* 9 - 11 days after primary T.B.F. infection, titres peaked 2 weeks after initial infection and antibodies persisted for 6 - 10 weeks. The IFAT detected antibodies for 2 weeks longer than CIE. after T.B.F. infection and again the peak occurred 2 weeks after initial infection. However, in our hands IFAT was a less satisfactory method for a number of reasons: problems were encountered in accurately locating fluorescing inclusions in neutrophils and non-specific fluorescence commonly occurred. The method used was that described by Lewis (1980) who successfully detected antibodies following experimental T.B.F. infections.

In the present studies the peak of antibody titres at 2 weeks post infection and the gradual decline over subsequent weeks together with the absence of precipitation arcs (CIE.) following pre-treatment of sera with mercaptoethanol suggest that antibodies produced in

response to infection with *C.phagocytophila* and detected by CIE. and IFAT. are principally composed of IgM. Triple loading of the wells with mercaptoethanol treated sera resulted in the reappearance of precipitation and suggested either that IgG is present in very small quantities or that the smaller IgG molecules form precipitates less well than larger molecules of IgM.

Serological results using CIE and IFAT were very similar following primary T.B.F. infection, but the pattern differed after secondary infection. CIE detected antibodies at a very low level between days 9 and 21 post secondary infection, whereas using the IFAT a low positive value was recorded on day 0 of the secondary challenge (despite negative values at the end of the primary monitoring period), values rose to a peak 2 weeks after challenge and had disappeared 6 weeks post infection. This suggests that IFAT is slightly more sensitive than CIE, which is supported by the results following primary infection of lambs (figures 5.3 and 5.4), where antibody was detected for approximately two weeks longer using IFAT.

The pattern of antibody titres recorded in goats following primary infection was somewhat unexpected (figure 5.3c) and may be an artefact caused by incomplete batches of sera from individual animals.

Woldehiwet and Scott (1982d) used complement fixation tests to detect antibodies to *C.phagocytophila* following experimental T.B.F. infection. Fifty two weeks post infection IgM was detectable at a titre of 1.4 $\log_2$ and IgG at 2.7 $\log_2$. The maximum titres of both IgM and IgG occurred 4 weeks after initial infections and were 7.5

$\log_2$ and 6.0 $\log_2$ respectively. The same strain of T.B.F. (Old Sourhope) was used in the present study with IFAT and CIE and antibody could only be detected for 10-12 weeks which would suggest that differences in peak antibody titres are due to detection techniques.

The precise effect of humoral antibodies on intracellular parasites is unclear. Krier (1976) suggested that humoral antibodies can coat naked organisms and reduce their ability to penetrate host cells. Humoral antibody is also thought to enhance phagocytosis of free organisms or even of infected cells (Schroeder and Ristic,1968). Lewis *et al* (1978) described the suppressive effect of canine immune serum, collected from *Ehrlichia canis* carrier dogs, on ehrlichial growth in normal macrophage cell cultures, supporting this theory.

Woldehiwet and Scott (1982d) calculated that a total antibody titre greater than 4 $\log_2$ was protective ie. sheep producing such levels of antibody were resistant to challenge with the same strain of T.B.F. and did not manifest clinical signs. However, when the antibody titre fell below 4 $\log_2$, a mild secondary reaction followed and sheep reacted with parasitaemia with or without fever. Demonstration of protective humoral immunity to T.B.F. is complicated by the 'carrier state' (Foggie,1951 and Toumi,1967a). Thus, inoculation of blood or serum from a convalescent sheep which is not displaying pyrexia or parasitaemia, into a naive animal results in the production of T.B.F. in the latter. The duration of the carrier state and its significance in terms of protective immunity is unclear. Foggie (1951) stated that infected sheep may

harbour the organism for 15 months and this is similar to observations in other related organisms. Buhles *et al* (1974) demonstrated that dogs which had been inoculated with blood from dogs infected up to five years previously with *Ehrlichia canis* developed canine ehrlichiosis. Consequently serum transfer could not be used to monitor protective antibody production, as it results in infection.

It is not clear whether resistance to re-infection is intrinsically linked to the carrier state, but there is evidence to suggest that a minimum patent period is necessary for the development of protective immunity. Synge (1976) found that if a drug was administered which curtailed the course of a T.B.F. reaction, protective immunity did not develop. Alternatively if a drug was administered which only suppressed the organism a relapse reaction occurred followed by the development of immunity.

The location of *C.phagocytophila* in carrier animals is unknown, but there are several possible sites. T.B.F. organisms have been detected in splenic impression smears (Scott,1984) and gross splenic enlargement is one of the few pathological features associated with T.B.F. infection which in this study persisted for 16 weeks post initial infection (table 7, chapter 8). Hudson (1950) reported swelling in the spleen due to slight enlargement of Malpighian corpuscles and that the lymphoid tissue had the appearance of being "suddenly drained" of the many lymphocytes usually present; thus the cuffs of lymphocytes around the reaction centres were less dense and frequently narrower than normal and the pulp of the spleen appeared to have a little oedematous fluid separating the elements

between sinusoids. The sinuses of the lymphatic glands were dilated with fluid and contained lympohocytes of normal appearance together with an unusually large number of large round cells. Limited histopathology in the present study (Hunter, pers. comm.) revealed no significant changes.

Munro *et al* (1982) isolated *C.phagocytophila* from alveolar macrophage cells and neutrophils in the lungs after experimental T.B.F. infection. These relatively long lived cells are also a possible reservoir of infection.

Foggie (1951) demonstrated that whole blood, plasma and serum from 'carrier' animals was infective when injected into a naive host. In the present study use of IFAT was complicated by non specific fluorescence and auto fluorescence of red blood cells which may have been associated with the presence of T.B.F. inclusions on the surface of erythrocytes. This would also account for the significant depression in percentage packed cell volume recorded in the present study and by Taylor *et al* (1986).

Neutrophils are phagocytic cells which engulf foreign particles and remove them from the bloodstream. An *in vitro* assay of neutrophil function was used to assess the functional integrity of polymorphonuclear leucocytes (PMN) in animals infected with T.B.F. The assay measured three parameters, namely extracellular survivors, intracellular survivors and PMN associated bacteria. The mean total survival of *S.aureus* varied significantly throughout a primary T.B.F. infection. Peak survival occurred between days 5 and 11, when neutrophils were parasitised by *C.phagocytophila* and many immature band neutrophils were present in circulating blood. Paradoxically there was a

highly significant decrease in numbers of intracellular survivors throughout the infection period. Thus during both primary and secondary infections less than 0.05% of phagocytosed *S.aureus* survived, compared to pre infection values of less than 0.5% intracellular survival. Between days 5 and 11, there was an increase in the numbers of P.M.N. associated *S.aureus* which were in contact with the external surface of the P.M.N.s (tables 11 and 12) but had not been phagocytosed and also an increase in the numbers of extracellular survivors. At this time during T.B.F. infection, many mature neutrophils contain *C.phagocytophila* inclusions and may be functionally impaired or removed from the circulation and immature band neutrophils recruited into the peripheral blood to compensate. These results suggest that the increase in PMN associated *S.aureus* and extracellular survivors is due to an exhaustion of phagocytic capacity rather than a reduction in intracellular killing.

Similar levels of impairment of phagocytosis were recorded following primary and secondary T.B.F. infections despite a significantly reduced parasitaemia in the latter. However, during secondary T.B.F. infection numbers of circulating neutrophils declined from day 0 of the infection, suggesting that animals were 'primed' to begin discarding infected neutrophils immediately.

In addition Foster and Cameron (1970a) suggested that the influence of neutrophils on *in vivo S.aureus* infections may be related to:

1. diapedesis (the escape of phagocytic cells from a blood vessel) and migration to the site of infection.

Using the skin window technique , which involved comparing the composition of neutrophils which adhered to a serum bathed coverslip attached to an area of abraded skin to the percentage of T.B.F. infected neutrophils in peripheral blood, the above authors showed that diapedesis was severely inhibited during the period when T.B.F. inclusions were present within neutrophils and concluded that there is a functional impairment of neutrophils which occurs prior to the neutropaenia of tick-borne fever, which depends upon the absolute numbers of infected cells in the blood.

2.Bactericidal properties.

Batungbacal *et al* (1982) demonstrated that the marked lymphocytopaenia accompanying primary T.B.F. infection, recorded between days 4 and 7 in the present studies was principally due to a depletion in B-lymphocytes which are responsible for antibody production. Opsonic antibodies aid neutrophil phagocytosis by coating foreign particles enabling easier recognition and binding by neutrophils (Huber and Fudenberg ,1968). Phagocytic leucocytes have on their plasma membranes , receptors for the Fc portion of immunoglobulin G (IgG) (Griffin *et al* ,1976) and for a cleavage product of the third component of complement (C3). Particles coated with IgG or C3 will therefore attach to the corresponding receptors on the plasma membranes of phagocytic cells.

Chapter 6. TICK-BORNE FEVER - FIELD STUDIES.

SUMMARY.

1. Differential white blood cell counts, assessment of parasitaemia by examination of blood smears and CIE serology have been applied to field samples from sheep of all ages from predominately tick infested areas of Scotland and the north of England.

2. Three hundred and thirty five ovine blood smears were examined for *Cytoecetes phagocytophila* , 14.3% of these proved positive; 413 ovine sera were tested using CIE, 18.2% were positive. On no occasion was an animal positive on smear and CIE on the same date. CIE is a simple, rapid, reliable test which doubled the detection rate of T.B.F. when combined with the conventional examination of blood smears.

3. CIE serology was successfully applied to samples from goats, cattle and deer, however examination of 38 samples from red grouse did not detect antibodies to T.B.F.

6.1 INTRODUCTION.

Samples of blood were collected from farms with tick problems to assess the incidence and timing of the acute symptoms of tick-borne fever and the subsequent development of antibodies. The most successful techniques used to monitor experimental infections were adopted, these include detection of parasitaemias using smears of peripheral blood, total and differential white blood cell counts, measurement of packed cell volume and the detection of antibodies to *C.phagocytophila* using the counter immuno-electrophoresis (CIE) technique . Samples were collected over a two year period during 1985 and 1986 , mainly from sheep, but samples from cattle, deer, goats; red grouse and a horse were also tested.

6.2 MATERIALS AND METHODS.

6.21 FIELD SAMPLES.

Blood samples were collected from sheep of varying ages during the spring and summer of 1985 and 1986 from a number of predominately tick infested farms in Ayrshire and Argyll. Serum samples from suspected cases of T.B.F. were also sent to the laboratory from various regions of Scotland and the north of England. Additionally samples of red grouse blood, obtained during the shooting season in late August and early September were provided by Mr D. Newborn of the Game Conservancy (North of England Grouse Research Project based at Askrigg in North Yorkshire.)

6.22 HAEMATOLOGY.

Total and differential white blood cell counts were

performed on whole blood as described in sections 5.251 and 5.252 and the percentage packed cell volume estimated as described in section 5.254.

6.23 ASSESSMENT OF PARASITAEMIA.

Thin blood smears were prepared, stained with Giemsa and examined as described in section 5.253.

6.24 PREPARATION OF SERA.

Serum was extracted from clotted blood samples as described in section 5.261. All sera were stored at -20'C until processing.

6.25 SEROLOGY.

The CIE technique was applied to samples of sera as described in section 5.263. Each run was controlled with positive and negative samples of sera of known titres from experimental T.B.F. infections. Samples of sera from field infections were applied to the gel without dilution in order to obtain a positive or negative result.

6.3 RESULTS.

6.31 HAEMATOLOGY.

Total and differential white blood cell counts recorded varied from 3 X 10^9 to 13.6 X 10^9/ litre and from 15% to 46% neutrophils respectively (table 2). The wide range of values recorded was probably partially due to individual variation. A number of values however, were recorded which fell outwith the range of normal values used in the Veterinary Investigation Centre , detailed in table 1 (Schalm *et al* 1975).

The results for farm A (1986) are used to illustrate

this and are shown in table 2.

Table 1. Range of Normal Ovine Values.

	Range	Mean
WBC	4-12	8
PCV	27-45	35
%N	10-50	30
%L	40-75	62

WBC Total leucocyte count X 10^9/litre
PCV Percentage packed cell volume
%N Percentage neutrophils
%L Percentage lymphocytes

T.B.F. parasites were detected in blood smears on 5 occasions, 4 of these samples also had a total white blood cell count outside the normal range and three of the samples had neutrophil counts outwith the normal range. However, these values were both lower and higher than normal and there was no obvious relationship between the parameters. Intracytoplasmic inclusions were visible in Giemsa stained smears of peripheral blood at the two sampling dates in May, by June smears had become negative, but 5/8 samples examined using CIE were positive for T.B.F.

PERCENTAGE PACKED CELL VOLUME.

Percentage packed cell volumes varied between 25% and 34% (mean = 24.3% , SE = 6.4%) . There were no significant differences in PCV's between animals which had positive or negative blood smears.

6.32 PARASITAEMIA.

1985.

One hundred and eighty one ovine blood smears were examined for *C.phagocytophila* with a percentage positive rate of 12.7%. The majority of positive samples (15/23)

Table 2. Farm A (1986) Haematological Determinations (Lambs).

Sampling date	%PCV	WBC	% Differential WBC N	L	M	E	B	Smear	CIE
13/5/86	30	8.3	32	64	4	0	0	-	-
	27	5.7	24	74	1	1	0	-	-
	26	4.2	28	70	1	1	0	+	-
	25	3.5	18	80	1	1	0	-	-
	33	13.5	41	55	4	0	0	-	-
	31	3.4	15	82	1	2	0	-	-
	29	5.9	20	77	2	1	0	-	-
29/5/86	33	13.6	45	53	2	0	0	+	-
	31	11.2	40	59	1	0	0	+	-
	27	7.9	30	66	3	1	0	-	-
	27	7.8	31	66	2	1	0	-	-
	26	3.0	24	75	1	0	0	-	-
	26	3.7	24	73	2	1	0	+	-
	29	3.3	17	80	2	1	0	+	-
13/6/86	31	13.2	20	78	2	0	0	-	+
	30	8.3	17	82	1	0	0	-	-
	26	12.6	17	79	3	1	0	-	+
	35	5.6	17	80	3	0	0	-	+
	27	13.5	22	73	4	1	0	-	+
	31	9.1	16	72	1	1	0	-	-
	32	6.2	20	76	1	3	0	-	-
	34	12.4	46	50	4	0	0	-	+

%PCV Percentage packed cell volume

WBC Total leucocyte count X 10^{9}/ litre
N Neutrophils
L Lymphocytes
M Monocytes
E Eosinophils
B Basophils
Smear Assessment of parasitaemia from smear of peripheral blood
CIE Serology using counter immunoelectrophoresis
+ positive result
- negative result

were recorded in May although 7/23 and 1/23 occurred in June and April respectively. Twenty two samples were collected during the other months with no positive results obtained. In addition, 2 blood smears from farmed red deer were examined which were both negative. Full results are shown in tables 3 and 7.

1986.

144 ovine blood smears were examined for T.B.F. parasites with a positive rate of 17.4%. The majority of positive smears occurred in May (24/25), the other positive sample was detected in July. Additionally three blood smears from farmed red deer were examined, one of these proved positive. Full results are shown in tables 4 and 7.

Table 3a . Ovine Samples Examined During 1985.

Farm	Date sampled	n	Age	CIE +	Smear +
A	4/4/85	9	H	0	0
	15/5/85	10	H	0	2
	3/6/85	10	6W	4	2
	7/7/85	21	H	2	ns
	30/8/85	12	H	0	0
	18/10/85	10	H	0	0
B	1/4/85	7	G&E	1	0
	10/5/85	10	3W	0	0
C	12/4/85	10	G&E	2	0
	9/6/85	10	6W	0	2
	9/6/85	2	H	0	0
D	23/4/85	5	2-3W	3	1
E	26/4/85	8	H	0	0
	13/5/85	1	H	0	0
F	29/4/85	3	2-3W	1	0
	13/5/85	8	3W	0	3
	5/6/85	10	6-8W	2	2
G	2/5/85	10	L,E,H	NS	0
	13/6/85	8	6-8W	NS	1
H	20/5/85	1	H	ND	1
I	1/5/85	1	H	1	0
J	22/5/85	2	H	0	1
	24/5/85	6	4W	0	1
K	24/5/85	2	4W	0	1
	23/5/85	8	4W	1	4
L	31/5/85	9	4-6W	NS	2
M	26/6/85	10	8-10W	NS	0
N	22/11/85	20	E	3	NS

E Ewe
H Hogg
G Gimmer
L Lamb
NS no sample
W Weeks

Table 3b Other Species Sampled During 1985.

Animal	Date sampled	n	Age	CIE +	Smear +
Deer	7/10/85	32	6M	1	(0/1)
	20/11/85	55	7M	0	ns
	3/12/85	1	A	0	0
Cattle	6/12/85	7	A	0	0

A Adult
M Months
NS no sample

Table 3c. The Total number of Samples Examined During 1985.

Species	Test	n	% Positive
Sheep	Smear	181	12.7
	CIE.	185	10.8
Cattle	CIE.	7	0
Deer	Smear	2	0
	CIE.	88	1.1

Table 4a. Ovine Samples Examined During 1986.

Farm	Date sampled	n	Age	CIE +	Smear +
A	29/5/86	7	5W	0	2
	13/6/86	8	7W	5	0
B	22/4/86	11	2W	0	0
	9/5/86	10	4W	0	0
	29/5/86	4	7W	1	1
	16/6/86	8	10W	4	0
N	10/4/86	13	E	0	0
	13/5/86	11	3W	0	9
	27/5/86	12	5W	4	5
	2/7/86	20	10W	10	1
O	10/4/86	8	E	0	0
	13/5/86	7	3W	0	2
	27/5/86	10	5W	3	5
	23/6/86	15	9W	4	0
	1/4/86	1	E	0	ns
	16/4/86	2	E	1	ns
	28/4/86	7	E	0	ns
	21/5/86	12	2-3W	2	ns
	7/5/86	2	3W	0	ns
	14/5/86	2	4W	0	ns
	1/6/86	10	6W	6	ns
	5/6/86	1	6W	0	ns
	30/6/86	1	10W	1	ns
	2/7/86	10	E	4	ns
	11/6/86	1	6W	0	ns
	3/9/86	2	3-4M	0	ns
	4/9/86	3	5M	3	ns
	17/9/86	3	6M	1	ns
	18/9/86	6	G	0	ns
	1/10/86	1	6M	0	ns
	9/10/86	1	7M	0	ns
	10/10/86	1	7M	0	ns

Table 4a. Ovine Samples Examined During 1986 (continued).

Farm	Date sampled	n	Age	CIE +	Smear +
	11/12/86	2	6M	0	ns
	10/12/85	1	H	0	ns
	4/12/86	1	8M	0	ns
	4/11/86	1	6M	0	ns
	3/10/85	5	E	0	ns
	29/10/86	1	E	0	ns
	27/10/86	1	6M	1	ns
	4/6/86	6	E	1	ns

E Ewe
G Gimmer
H Hogg
W Weeks
M Months
ns no sample

Table 4b Other Species Sampled During 1985.

Animal	Date sampled	n	Age	CIE +	Smear +
Cattle	4/6/86	1	A	1	ns
		1	-	0	ns
	3/9/86	4	A	0	ns
	29/5/86	1	A	1	ns
Deer	23/4/86	14	A	0	(1/3)
Goats	22/10/86	25	A	13	ns
Red Grouse	16/9/86	8	A	0	ns
	6/9/86	14	A	0	ns
	27/8/86	16	A	0	ns
Horse	14/5/86	1	A	0	ns

A Adult
ns No sample

Table 4c The total number of samples examined during 1986.

Species	Test	n	% Positive
Sheep	Smear	154	16.2
	CIE	228	21.9
Cattle	CIE	7	28.6
Deer	Smear	3	33.3
	CIE	14	0
Goats	CIE	25	52.0
Grouse	CIE	38	0
Horse	CIE	1	0

6.33 CIE SEROLOGY.

1985.

One hundred and eighty five ovine sera were examined, 10.8% proved positive. Positive results were seasonally distributed as shown in table 5.

Table 5. Percentage of Samples Tested Positive on T.B.F. Serology.

Month	April	May	June	July	Aug	Sept	Oct	Nov
%positive	6.7	3.7	18.7	9.5	0	0	0	15%

Seven bovine sera were examined, none proved positive and 1/88 samples taken from farmed red deer was positive . Full results are shown in tables 6.3b and 6.7.

1986.

Two hundred and twenty eight ovine sera were tested with a positive rate of 21.9% distributed as shown in table 6. The majority of positive samples occurred in June and July.

Table 6. Percentage of Samples tested positive on T.B.F. serology (1986).

Month	April	May	June	July	Aug	Sept	Oct	Nov	Dec
%positive	2.4	17.5	42.8	46.6	ns	28.5	10	0	0

Two out of seven bovine samples tested proved positive (28.6%); 0/14 samples from red deer; 0/38 samples from red grouse and 13/25 (52%) samples from goats were positive.

Full results of serological tests are shown in tables 4 and 7.

Table 7. Field Samples Tested Using Serology and Smears During 1985 and 1986.

	n	% Positive CIE	n	% Positive SMEAR
Sheep	413	18.2	335	14.3
Cattle	14	14.3	ns	-
Goats	25	52.0	ns	-
Deer	102	1.0	5	20.0
Grouse	38	0	ns	-
Horse	1	0	ns	-

When samples for serology and assessment of parasitaemia were taken from lambs at the same time, on no occasion was an animal sample positive on smear and serology on the same date.

6.4 DISCUSSION.

The results of these examinations showed that in many cases when T.B.F parasites were visible in smears of peripheral blood, the total leucocyte counts and percentage neutrophils were outwith the normal range, being sometimes greater and sometimes less than normal values. Typical T.B.F reactions result in a transient lymphocytopaenia followed by a more profound neutropaenia (Woldehiwet,1983; chapter 5) and thus from uncomplicated T.B.F. reactions one would expect to record values lower than the norm. However, T.B.F. is often followed by secondary infections such as staphylococcal pyaemia (Brodie *et al*,1986). This can result in an increase in numbers of circulating leucocytes, especially neutrophils, in an attempt to combat bacterial

infection.

During 1985 12.7% of the ovine blood smears examined proved positive. However, this figure included many samples from ewes, gimmers and hoggs. When only young lambs were considered, which from the experimental study would be expected to be susceptible to infection, the figure increased to 18.8%, which was very similar to the figure of 20.3% positives recorded from lambs in 1986. In that year when ewes were included in the 1986 figures the positive rate declined to 16.2%.

Diagnosis of T.B.F is based on the demonstration of *C.phagocytophila* parasites in smears of peripheral blood or from splenic impression smears (Scott,1984). Parasites were present in the peripheral blood for 7 days only following primary experimental infection with T.B.F. (chapter 5), it is therefore unlikely that blood sampling and parasitaemia would necessarily coincide in the field. The existence of many different strains of T.B.F. which vary in the severity of reactions produced, including magnitude of fever and parasitaemia has been reported (Toumi, 1967b). If this is so detection rates may be also related to the severity of presenting clinical signs and the length of time that parasites are visible in stained smears of peripheral blood.

During 1985 and 1986 the majority of positive smears were detected in May and early June. This is linked to husbandry methods in use on many hill farms where ewes are removed from tick infested areas prior to lambing in late April and placed on relatively tick free inbye ground. The ewes and lambs are returned to tick infested hill areas

when the lambs are two to three weeks old in early May which coincides with the spring rise in tick activity (chapter 3). The addition of naive young lambs constitutes an approximate doubling of the ovine population on a given area of hill.

T.B.F. parasites are visible in blood smears 3-4 days after experimental intravenous inoculation (chapter 5 ; Taylor and Kenny,1980). Infection is delayed when the infection route is via the tick; MacLeod and Gordon (1933) reported an incubation period of 3-13 days while Taylor and Kenny (1980) recorded a period of 11-17 days between the introduction of susceptible cattle to tick infested pasture and the onset of T.B.F. parasitaemia.

During 1985, the CIE technique detected a number of positive samples in April, forty three percent of which were from gimmers and ewes, the remainder from lambs which were three weeks old by the end of April. Positive samples (CIE) were also recorded in May, June , July and November. The November samples were obtained from a batch of ewes in Yorkshire, where patterns of tick activity may vary resulting in infection at different times of the year.

Antigen used in CIE determinations was a crude preparation of ovine neutrophils infected with one strain of T.B.F. (Old Sourhope). Precipitation lines resulted from reaction of this one antigen with ovine sera collected in different regions of England and Scotland and with sera obtained from cattle, goats and deer, which were presumably infected with different strains of T.B.F. These results suggest that precipitation is possibly due to an antigenic binding site or epitope which is common to many strains of

T.B.F.

During 1986 the majority of ovine samples were obtained from lambs and positive serological results were recorded in May, June, July, September and October; 96% of positive smears occurred in May. During 1985 and 1986 both smears and serology were performed on 296 ovine sera and 14.9% proved positive on smear and 15.5% on serology. On no occasion was an animal positive on smear and serology on the same date.

The CIE technique is a rapid, reliable test for the detection of antibodies to *C.phagocytophila*, which when used in conjunction with conventional blood smears approximately doubles the detection rate of T.B.F. It is of particular value in investigation of problems such as abortion induced by the thermal reaction associated with T.B.F.. Since abortion presumably occurs several days after the peak of fever and parasitaemia the period when T.B.F. inclusions can be detected in blood smears is very short ie. 3-4 days. The CIE method may be used for several weeks after abortion and the value of this has been demonstrated in two cases of bovine abortion in Argyll. Blood smears taken the day after the cows had aborted were negative, but serum obtained 1 week later proved positive. CIE has also been used to detect antibodies to T.B.F in sera from deer and goats. Foggie (1962) demonstrated T.B.F. inclusions in blood smears from red deer on the island of Rhum. In this study inclusions have been seen in blood smears from farmed red deer from Argyll, other deer from the same farm were positive serologically . Although deer have been shown to be infected with *C.phagocytophila* and

may act as a reservoir of infection, T.B.F. is not recognised clinically.

Goats have been used as experimental hosts of T.B.F. (Macleod and Gordon, 1933) and the disease has also been described in feral goat populations (Foster and Greig,1969). In the present study 25 sera from domestic goats obtained in mid October were examined . Problems had arisen within the goat populaton on Islay resulting in several deaths. Mild nervous signs were reported and louping ill diagnosed by brain pathology in one case. Only one of twenty five samples proved positive on louping ill serology but thirteen were positive when tested for T.B.F. Antibodies were demonstrated in goats experimentally infected eight weeks previously (chapter 5), therefore no accurate assessment could be made of the duration of infection in the goats of Islay.

Thirty eight sera from red grouse collected during the shooting season in early September were tested using CIE, all of which proved negative. If grouse chicks however, were infected with T.B.F. in the spring , antibody could have waned and they may have become seronegative by September when samples were taken. The ideal time to blood sample would be in May and June, as for the lambs , but this would not be an acceptable time to many estate owners because of the attendant trauma.

In summary , differential white blood cell counts, assessment of acute parasitaemias and CIE have been successfully applied to field samples from Scotland and the north of England. CIE offers a new and relatively simple approach to T.B.F. diagnosis and has doubled the detection

rate over the two year study period when coupled with the conventional examination of blood smears. The test is rapid, reliable, simple to perform and could prove to be of value in detection and diagnosis of T.B.F. both experimentally and in the field.

CHAPTER 7. DETECTION OF *CYTOECETES PHAGOCYTOPHILA* IN *IXODES RICINUS*.

SUMMARY.

1. An electron-microscopic technique was developed to examine *Ixodes ricinus* for *Cytoecetes phagocytophila* infection. T.B.F. infected and T.B.F. free ticks were obtained from the tick culture described in chapter 2.

2. All stages of *I.ricinus* collected from one field site were examined; none of the larvae were infected, while 44% of nymphae and 32% of adults were infected with *C.phagocytophila*. This supports the previously held theory theory of transtadial, but not transovarial transmission.

7.1 INTRODUCTION.

In the past, electron-microscopy has been used to study the morphology of *Cytoecetes phagocytophila* in infected sheep neutrophils (Toumi and Von Bonsdorff, 1966 ; Lewis 1980 and Woldehiwet and Scott,1982c) and to establish the organs of *I.ricinus* infected with *C.phagocytophila* (Lewis 1979 and 1980). In the present studies electron microscopy was used purely to establish the level of infection of *I.ricinus* with *C.phagocytophila*. Therefore a crude tick homogenate was used instead of elaborate fixing and sectioning methods, in order to increase the number of samples which could be handled and so obtain an estimate of T.B.F. infection in *I.ricinus*.

7.2 MATERIALS AND METHODS.

7.21 SOURCE OF MATERIAL.

7.211 NEGATIVE CONTROLS.

I.ricinus maintained in a laboratory colony and fed on T.B.F. free hosts as larvae and nymphae were used as negative controls (Chapter 2). They were stored at -20'C in small plastic vials until processed.

7.212 POSITIVE CONTROLS.

I.ricinus larvae and nymphae were allowed to engorge on T.B.F. infected lambs as described in section 2.27 and subsequently allowed to moult to nymphs and adults respectively. A proportion of these were assumed to be infected with T.B.F. and used as positive controls and stored at -20'C until processed.

Antigen prepared from T.B.F. infected neutrophils as

used for serological tests (5.2621) was also used as a known positive. Immediately prior to use it was diluted 1/10 with Sorensen's buffer (Appendix 4).

7.213 FIELD SAMPLES.

Unfed ticks were obtained from the field sites by the blanket dragging technique (Chapter 3). The majority of ticks captured by this method were nymphal *I.ricinus*. which were stored at -20'C until processed.

7.221 PREPARATION OF TICKS.

Initially ticks were washed in 2 small volumes of distilled water to remove any external adhesions. Single ticks were then immersed in 50 µl of distilled water. The cuticle of the tick was removed, sterile needles were used to dissect out the midgut and attached organs which were then gently disrupted by repeated pipetting in the water.

7.222 PREPARATION OF ELECTRON MICROSCOPE (E.M.) GRIDS.

A pasteur pipette was used to place 1 drop of the tick preparation or diluted antigen onto a carbon coated copper E.M. grid. Excess liquid was blotted, the grid washed with 4 drops of distilled water and excess liquid again gently blotted. The grid was then stained with 2 drops of a 1.5% aqueous solution of uranyl acetate, blotted and allowed to dry before viewing with a transmission electron microscope (adapted from Wright,1984).

7.23 ANTISERUM COATED GRID (A.S.C.G.) METHOD.

Specific T.B.F. antiserum raised in sheep, was obtained 4 weeks after initial infection. The antiserum was diluted 1/100 or 1/500 in 0.6 M Sorensen's buffer (pH 6.5). Twenty five microlitre (µl) droplets were placed on a wax coated

surface. Carbon coated E.M. grids were inverted on these droplets and incubated for 1 hour at 37'C. Excess liquid was blotted from the grids which were then washed for 10 minutes in Sorensen's buffer by gently swirling them on the surface of buffer placed in a small container. This removed excess antisera.

I.ricinus ticks were prepared as detailed previously (section 7.221). Twenty five microlitre (µl) samples were placed on a wax surface, on which the blotted antiserum coated grid was inverted and incubated at 4'C overnight (Wright,1984). The grid was then stained with uranyl acetate as before (section 7.222).

7.3 RESULTS.

Tick-borne fever like inclusions were visible on grids coated with positive control antigen when prepared by both the normal and A.S.C.G. methods. These organisms were either coccoid or elongated in shape and varied in size from 1.3 X 1.0 um to 3.3 X 1.2 um. with a mean coccoid size of 1.4 X 1.0 um and elongated size of 2.5 X 1.1 um. There were no significant differences observed in the sizes of the organisms observed in nymphal and adult ticks. The large particles were bounded by 2 membranes, as described by Toumi and von Bonsdorff (1966). The outer cell wall was clearly defined and tended to be slightly rippled . A lightly staining space separated the 'cell wall' from the plasma membrane, which was often fused to the cell contents and less clearly defined. Occasionally dumb-bell shaped particles were observed which were presumed to be dividing by binary fission (Toumi and von Bonsdorff,1966) (Plates 7

and 8).

Table 1. Infection rates in Ticks from the Laboratory Colony fed on T.B.F. Free Hosts.

Stage	No. tested	% Positive
Larvae	20	0
Nymphae	20	0
Adults	5	0

Table 2. Ticks From the Laboratory Colony Fed on T.B.F. Infected Hosts.

Stage	No tested	% Positive
Nymphae	20	85
Adults	7	86

Table 3. Ticks Collected at the Field Site.

Stage	No. tested	% Positive
Larvae	21	0
Nymphae	54	44
Adults	37	32

The A.S.C.G. method added specificity to the system. T.B.F. bodies clumped together indicating a homologous antigen/antisera reaction.

Morphologically similar organisms were not seen in any of the ticks from the laboratory colony which had been fed on T.B.F. free hosts, or from larvae collected in the field. Forty four per cent of nymphs and 32% of adults collected in the field had T.B.F. like bodies visible on the grids (Table 1) The numbers of particles in infected ticks varied from >5 to several hundred.

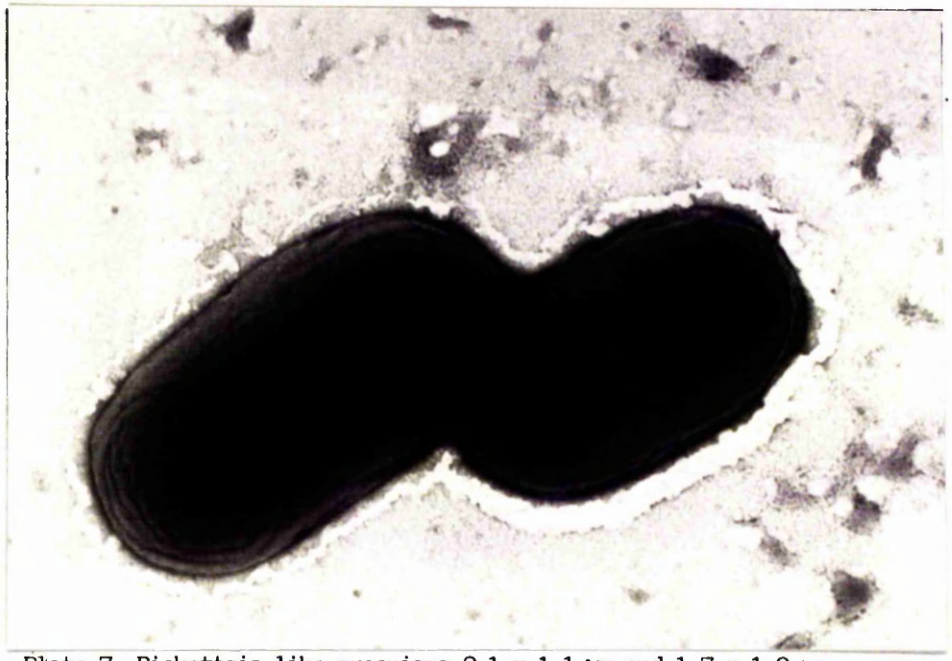


Plate 7 Rickettsia-like organisms 2.1 x 1.1 µm and 1.7 x 1.0 µm respectively stained with urancyl acetate and viewed using transmission electron microscopy. The material originated from ticks collected in the field.

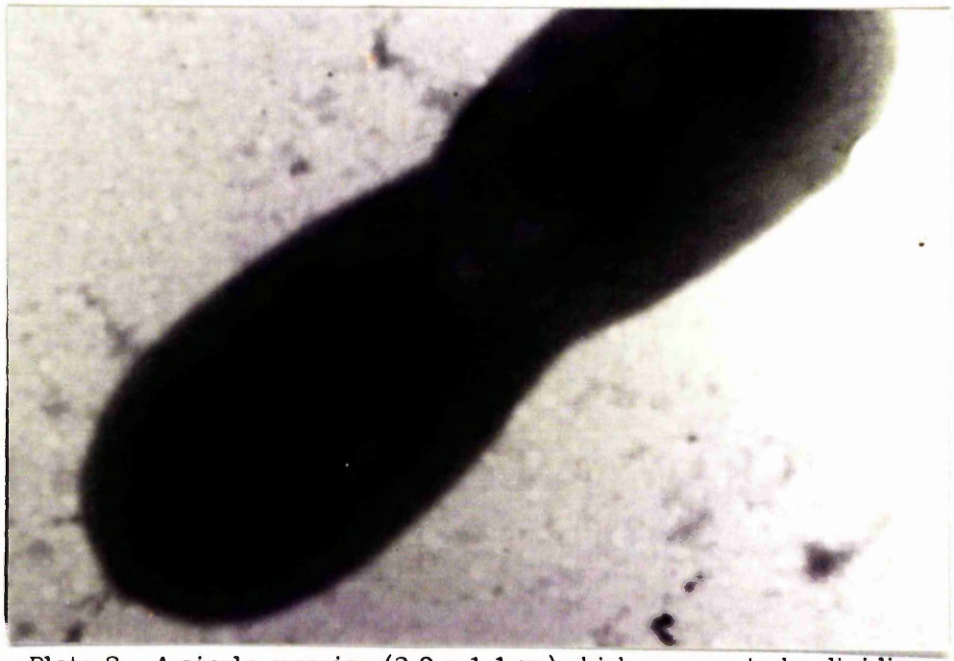


Plate 8 A single organism (3.9 x 1.1 µm) which appears to be dividing.

Table 4. Nymphae.

Date sampled	No. tested	% Positive
Sept.'85	14	71
Oct.'85	2	0
May.'86	4	50
June '86	24	33
July '86	8	50
Aug.'86	2	0

Table 5. Adults.

Date sampled	No. tested	% Positive
Oct.'85	23	52
May '86	9	0
June '86	4	0
July '86	1	0

The ticks were stored at -20'C until processed. The storage time (up to 10 months) did not appear to have an effect upon the level of infection as batches of positive and negative control ticks reared in the laboratory colony were split into two equal aliquots, one of which was tested immediately and the other after freezing and storage at -20'C for 17 weeks. The results shown in table 6, did not differ significantly.

Table 6a. Infection Rates in Ticks From the Laboratory Colony Fed on T.B.F. Free Hosts Tested Before and After Storage.

Stage	no. tested		% positive
	fresh	after storage	
Larvae	10	10	0
Nymphae	10	10	0
Adults	3	2	0

Table 6b. Ticks From the Laboratory Colony Fed on T.B.F. Infected Hosts.

Stage	No tested Fresh	% Positive	No tested after Storage	% positive
Nymphae	10	90	10	80
Adults	3	100	4	75

There were no significant differences between the levels of infection revealed by a Mann Whitney U test.

7.4 DISCUSSION.

This study attempted to estimate the percentage of *I.ricinus*, collected at one field site in Ayrshire, which were infected with *C.phagocytophila*, using transmission electron microscopy of unsectioned material, a method more usually employed by plant pathologists searching for viruses. This is thought to be the first time that the technique has been utilised to search for rickettsia-like organisms in animal tissues.

Previously, other authors (Toumi and von Bonsdorff,1966; Lewis,1980; Woldehiwet and Scott 1982c) had viewed fixed sections of whole blood and found T.B.F. inclusions in the

neutrophils. Lewis (1979 and 1980) also looked at sections of experimentally infected ticks and at sections of separate tick organs. He found rickettsia-like organisms in various parts of the tick including the midgut, salivary glands and the ovaries.

In this study the ticks were collected by blanket dragging pasture sites. This is a simple method for sampling ticks from herbage, but is biased to capture a large proportion of nymphal ticks with fewer adults or larvae (chapter 3). Consequently the field sample was weighted in this manner.

The ticks were stored at -20'C until processed. The storage time (up to 10 months) did not appear to have an effect on the level of infection as batches of positive and negative control ticks reared in the laboratory colony were split into 2 equal aliquots, one of which was tested immediately and the other after freezing and storage for 17 weeks. The two samples did not differ significantly in the percentage infections detected (table 6).

Ticks were collected throughout the activity season (tables 4 and 5), but the number sampled did not permit detection of significant differences in infection levels at different times of the year. Although a variety of stains, dilutions and pH conditions were tried, the uranyl acetate regime was the only one which gave a clear definition to *C.phagocytophila* with minimal background contamination. This stain was used by Sells *et al* (1976) to visualise *Erhlichia equi* organisms and by Lewis (1980) to examine *C.phagocytophila*. These authors also used lead citrate stain as did Toumi and von Bonsdorff (1966) for E.M. work

with *C.phagocytophila*.

The use of antisera coated grids introduced specificity to the system and allowed a greater degree of certainty in the identification of *C.phagocytophila* in tick extracts. Reliance solely on the morphology of such large darkly staining bodies viewed without sectioning would render identification almost impossible. Inoculation of sheep with the tick homogenate, resulting in the production of T.B.F. would have given definitive proof of the organisms identity, however this was not possible because of the small volumes of tick homogenate involved.

Studies by Roshdy (1961 and 1962) ; Hecker *et al* (1968) and Burgdorfer *et al* (1973) reported the presence of symbiotic rickettsiae in the tissues of various species of ticks. However, Balashov (1967) did not find symbiotic rickettsiae in tissues from unfed ticks, but reported their presence in tissue smears made during and after tick feeding.

The absence of T.B.F. bodies in the sample of larvae from the field provides further evidence for the non-occurence of transovarial transmission for T.B.F. in *I.ricinus* (MacLeod and Gordon,1933). Although only a relatively small number of larvae were examined they were collected in several samples at different times of the year and so it is unlikely that they originated from the egg mass of one uninfected female. A larger sample of larvae would have been preferred, but the collection method precluded this.

The 44% infection rate of nymphs tested shows that larvae are becoming infected when they feed. The origin of

infection was probably sheep since cattle and deer were present at this site for only short periods. At this site, a sheep farm, larvae have been observed feeding on sheep (chapter 3) and T.B.F. has been demonstrated in all ages of sheep both by Giemsa stained smears of peripheral blood and serologically (chapter 6).

A higher infection rate was detected in nymphal *I.ricinus* (44%), than in adults (32%). This was surprising as the infected ovine population is parasitised by a greater number of nymphae than larvae and the survival rate for the nymph to adult metamorphosis is greater than that of the larva to nymph transformation (Chapter 3). Ticks which become infected when feeding as larvae maintain that infection even if they feed on a refractory host as nymphs (MacLeod,1962; Balashov,1968). Therefore it would be expected that the level of infection would be greater in adults which have two chances to become infected, feeding as larvae and nymphae, than in unfed nymphs which have only one opportunity. This result may merely reflect the paucity of the sample size. However, it may be attributable to a reduced survival of *C.phagocytophila* in adult ticks as opposed to nymphae; or to the reduced survival of adult ticks infected with the agent. Lewis (1980) reported the presence of *C.phagocytophila* within a degenerate oocyte in the lumen of the ovary of a female tick and the deformation of mitochondria by rickettsiae apparently multiplying within them which would seem to suggest that the presence of microorganisms was responsible for damage to the host cells. Ouheilli *et al* (1982) reported that oviposition, egg hatching and survival of newly hatched larvae was reduced

in *Boophilus annulatus*, kept at 25 or 35'C which were parasitised by *Babesia bigemina* or *Babesia bovis*. It is known that *Babesia* spp. multiply and develop within *Boophilus* and that such development is accompanied by cytopathogenic effects (Hoffmann,1971) and leads to the death of a proportion of infected ticks (Muangyai,1974).

Macleod (1936a) proposed that a moulted tick becomes infective only after a lapse of a definite period of time. He infested a sheep with 2-4 week old nymphs, fed as larvae on a T.B.F. infected host, without the production of a reaction, whereas another sheep infested with 4-6 week old nymphs did react. On the other hand this is possibly more reflective of nymphal ability to attach and feed of which MacLeod makes no mention, than the presence of *C.phagocytophila* in the nymphae. There may however, be a threshold number of organisms below which transmission of T.B.F. does not occur. Lewis (1980) reported the intracellular presence of *C.phagocytophila* within the tissue of ticks. In the present study dividing organisms were noted, so there may be a multiplication stage within the tick, or a trigger to transmission which occurs after the metamorphosis from the engorged to the subsequent unfed stage.

The electron microscopic technique described in this chapter would be of value in several areas of research, estimating the proportion of ticks infected with tick-borne fever in the field, determination of the carrier status of recovered sheep by xenodiagnosis and any studies of T.B.F. where quantification of tick challenge is desirable.

CHAPTER 8. TICK PYAEMIA TRANSMISSION STUDIES.

SUMMARY.

1. B- Lymphocyte numbers were depleted in mice by intravenous injection of cyclophosphamide (CY). Mice were then challenged with *S.aureus* administered by a variety of routes. The death rate was higher, more lesions produced and bacterial recovery greater in mice pre-treated with CY than controls in groups where *S.aureus* was administered intravenously, intradermally, sub-cutaneously and by contaminated ticks.

2. Lambs aged two weeks , were inoculated with T.B.F. stabilate on day 0 and *S.aureus* contaminated ticks applied on day 5. Tick pyaemia was produced experimentally for the first time using *I.ricinus* as a mechanical vector of *S.aureus*.

8.1 INTRODUCTION.

Tick pyaemia is a pyogenic staphylococcal infection of young lambs, common in animals from tick infested areas, but is not found in tick free regions. Foggie (1957) suggested that the tick *Ixodes ricinus* was not a direct vector of staphylococci, but that the immunodepressive symptoms produced by tick-borne fever (T.B.F.), especially the neutropaenia, allowed staphylococci normally present on the skin to invade. However, the mechanism by which staphylococci enter the bloodstream in lambs is still obscure (Anderson,1983).

Recent work (Brodie *et al*,1986) has shown that while only 20% (4/20) of lambs inoculated intravenously with *Staphylococcus aureus* alone developed pyaemia; 57% of those inoculated during a bout of T.B.F. became pyaemic; moreover 9/10 lambs inoculated with *S.aureus* during the febrile phase of a T.B.F. infection (usually 5 days post infection) developed pyaemia. These workers were only able to produce the syndrome consistently if *S.aureus* was injected intravenously. However, Brodie (1985) did produce pyaemic lesions by injecting *S.aureus* 9 days post T.B.F. infection via the intradermal route.

In an attempt to assess the significance of immunodepression in the genesis of *S.aureus* infection it was decided to establish a small animal model for the syndrome. Several years ago Foggie and Hood (1961) adapted strains of T.B.F. to normal guinea pigs and splenectomised mice. However, these strains have been lost and at present cattle and sheep strains are presumably not viable in small mammals in the absence of adaptation.

It was decided to use the cytotoxic drug Cyclophosphamide (CY) which has been used extensively as an immunodepressant since it was first synthesised by Arnold, Bourseaux and Brok (Jun *et al*,1980). The drug has a specific effect upon actively dividing cells, especially B-lymphocytes (Kerckhaert *et al*, 1977) reducing the numbers circulating in the peripheral blood. It has been used in mice (Turk and Poulter,1972 and Stockman *et al*,1973) and in various other species to reduce humoral response and deplete non-thymus dependent areas of lymph node and spleen allowing colonisation of the host by various microorganisms including staphylococci (Rifkind *et al*, 1964), cytomegalovirus (Fulginiti *et al*,1968) and fungi (Hill *et al*,1964).

CY was used in this experiment to mimic the depression of lymphocytes, especially B-cells (Batungbacal *et al*, 1982) occurring on day 5 of a typical T.B.F. reaction.

8.2 MATERIALS AND METHODS.

8.21 ANIMALS.

Female National Institute of Hygiene (N.I.H.) mice aged 5 weeks were obtained from Interfauna U.K. Limited. (Abbots Ripton Road, Wyton, Huntingdon, England). They were allowed to acclimatise to the animal house for 1 week prior to the start of the experiment.

8.22 CYCLOPHOSPHAMIDE (CY).

CY was obtained in a 100ml isopac bottle from the Sigma Chemical Company (Fancy Road, Poole, Dorset, England). It was dissolved in sterile distilled water to a concentration of 30 mg/ml.

8.231 STAPHYLOCOCCI.

The strain of *S.aureus*, which was used in these experiments was isolated from a case of tick pyaemia in Argyll, Scotland and had the following cultural characteristics: α-β- haemolytic; D.N.ase and coagulase positive; resistant *in vitro* to the antibacterial drugs actinomycin and polymixin B and had a characteristic fermentation pattern of serum sugars and phage type (Appendix 4).

8.232 PREPARATION OF STAPHYLOCOCCI.

S.aureus was stored as a lyophilised preparation. When required it was reconstituted in sterile distilled water and plated onto blood agar (Appendix 4). The cultures were subsequently replated onto new plates on three occasions at 24 hour intervals. A growth curve was prepared from the inoculation of 1 colony into 100ml Brain Heart Infusion broth (B.H.I.) (Appendix 4) in a 250 ml volumetric flask incubated at 37'C. Samples were withdrawn at 30 minute intervals for the first three hours and then hourly up to hour 9, which were diluted in sterile B.H.I. and plated onto blood agar. Sterile conditions were observed throughout.

S.aureus was prepared for inoculation by incubation at 37' C for a time indicated by the growth curve, until the required concentration had been achieved, when the organisms were then washed in sterile saline and resuspended in an equivalent volume.

8.30 EXPERIMENT 1.

8.31 AIM.

To assess the minimum intravenous inoculum of *S.aureus*

necessary to produce pyaemic symptoms in mice .

8.32 METHOD.

Pairs of mice were inoculated intravenously in the tail vein with 10^2 - 10^6 *S.aureus* contained in 0.1 ml of sterile distilled water. They were fed and watered *ad libitum* and observed twice daily. Any animals which died were examined immediately after death while the survivors were sacrificed 7 days after initial infection with *S.aureus* for post mortem examination.

At necroscopy, viscera were examined macroscopically and gross lesions were noted. Swabs were taken from lesions and placed in 5ml volumes of B.H.I. ; liver, lungs, heart, spleen and both kidneys were cultured on blood agar and mannitol salt agar (M.S.A.) (Appendix 4). The right stifle joint was skinned, swabbed with sodium hypochlorite, flamed and then cut with a sterile blade and dropped into 5ml B.H.I. The broths were plated onto blood agar and M.S.A. 24 hours later.

All plates were incubated at 37'C overnight and examined the following morning. The densities of α-β haemolytic *S.aureus* were scored on a scale from 0-4, corresponding to gradations from nil to confluent growth respectively. Any other bacteria were identified, although these results are not reported in this thesis.

8.33 RESULTS.

Abscesses up to 0.5 cm in diameter formed on many of the internal organs and muscles when relatively large doses (more than 10^4) *S.aureus* were injected intravenously into mice from which, in many instances an α-β haemolytic strain

of *S.aureus* was isolated. In all cases this organism showed identical characteristics to the original *S.aureus*. ie. sugar fermentation patterns, *in vitro* antibiotic sensitivity pattern and phage type as listed in section 8.231. Full results are available in tables 1 and 2 of Appendix 5.

8.34 DISCUSSION.

Staphylococcal pyaemia was produced in mice inoculated with varying doses of *S.aureus* from 10^4 -10^6 organisms. When 10^2 or 10^3 organisms were injected abscess formation was not apparent, but *S.aureus* could be isolated when samples of liver, heart and kidneys were cultured.

8.40 EXPERIMENT 2.

8.41 AIM.

To follow the haematological changes induced by CY in mice. The dose rate was 300 mg/Kg body weight.

8.42 METHOD.

Eight female N.I.H. strain mice aged 6 weeks were used in this experiment. Five microlitres (µl) of blood was withdrawn from the tail vein with a 'snap off' capillary tube and diluted in 195 ul of 1.5% ethylenediaminetetra-acetic acid (EDTA) in Teuk's solution (1% glacial acetic acid tinged with crystal violet). This gave a 1:40 dilution. Leucocytes were counted in a haemocytometer to assess the total white blood cell count (WBC).

Thin blood smears were made on glass microscope slides, fixed in methanol for 5 minutes and stained in 15% Giemsa for 22 minutes. Slides were examined at X400 magnification

; 200 leucocytes were counted to estimate the differential white blood cell count.

Normal values were obtained prior to the intravenous injection of 6mg of CY contained in 0.2 ml of sterile saline (300 mg per Kg). Blood samples were taken daily from groups of four animals sampled on alternate days to monitor haematological changes.

8.43 RESULTS.

The mean total leucocyte count prior to the administration of CY was 11 X 10^9 per litre. This declined rapidly to 1.3 X 10^9 per litre 24 hours after the administration of CY reaching a low point of 1.2 X 10^9 WBC per litre 3 days after CY administration. The differential W.B.C. also shifted from a pre-inoculation ratio of 74% lymphocytes : 23% neutrophils, to 35% lymphocytes : 61% neutrophils, 3 days after the administration of CY. This resulted in a massive decrease in the number of circulating lymphocytes which is illustrated in fig 8.1; results for individual mice are available in Appendix 5, tables 3 and 4.

8.44 DISCUSSION.

The mean total leucocyte count and the number of circulating lymphocytes fell dramatically after CY was administered with the nadir of the lymphocytopaenia occurring 3 days post CY administration . No attempt was made to distinguish between B-cells and T-cells and it was assumed that the same B-lymphocyte depletion occurred as reported by Stockman *et al* (1973). It has been extensively reported (Turk and Poulter,1972; Sharbaugh,

Lymphocyte Numbers. Following CY Administration.

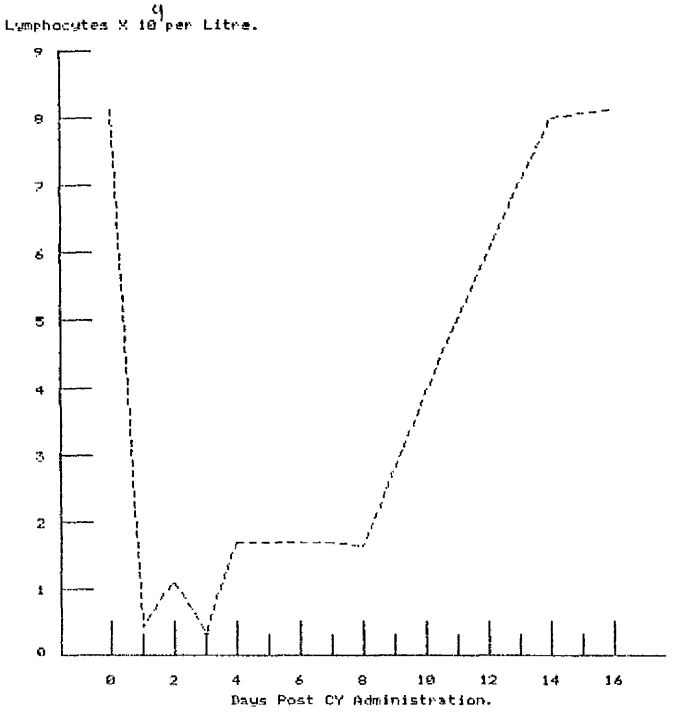


Figure 8.1 illustrates fluctuations of lymphocyte numbers x 10^9/ litre in mice injected with cyclophosphamide (CY) at a rate of 300 mg/Kg body weight on day 0.

1976; Kerckhaert,1977 and Jun *et al* 1980) that CY acts as an immunodepressent by destroying actively dividing cells primarily B-lymphocytes with little direct effect on other cells. Sharbaugh (1976) attributed an increased level of microbial infection in CY treated rats to a decrease in available opsonin(s) necessary for optimal phagocytosis. This effect only occurred *in vitro*, when serum from CY treated rats was used to opsonize neutrophils from either CY treated or normal rats and was probably due to a decrease in opsonic antibody produced by B-lymphocytes.

It was decided that the haematological state of mice on day 3 post CY administration corresponded most closely to that of lambs 5 days after T.B.F. infection (chapter 5).

8.50 EXPERIMENT 3.

8.51 AIM.

A preliminary study of the interaction between CY and *S.aureus* infection.

8.52 METHOD.

CY was administered as described previously in section 8.252 to groups of 4 mice ; 0, 3, 5 and 7 days prior to the intravenous inoculation of 5 x 10^4 *S.aureus* organisms in 0.1 ml of sterile distilled water, prepared as described in section 8.232. Any mice which died were examined immediately after death the remainder were sacrificed on day 7 for post mortem examination as described in 8.32.

8.263 RESULTS.

Three out of four of the mice infected with *S.aureus* 3 days after the administration of CY died 36 hours after *S.aureus* infection. The other mice in the experiment all

survived until the end of the experimental period.

Abscesses were noted in 5 of the mice examined. One mouse treated with CY 5 days prior to the inoculation of staphylococci had a single lesion in its left kidney; one mouse from the day 0 group had an abscess in the muscle of the right thigh. Three out of four mice which received CY three days prior to staphylococcal inoculation had multiple abscesses in the liver, lungs and right kidney; all four mice in this group showed signs of pneumonia; splenomegaly was noted in mice from all groups. Enlarged adrenal glands were visible in all mice given CY 7 seven days prior to staphylococci in three out of four of the day 0 group and in one mouse each from the other two groups. Results for individual mice are shown in table 5 of Appendix 5.

Haemolytic *S.aureus* was isolated from tissues cultured from mice injected with CY three days prior to *S.aureus* administration and from the mouse with the kidney abscess, which had been injected with CY 5 days prior to the administration of *S.aureus*. It was consistently isolated from all organs cultured, with the exception of the stifle joints. These results are displayed in their complete form in table 6, appendix 5.

8.54 DISCUSSION.

This experiment demonstrated that CY predisposed mice to *S.aureus* abscess formation which did not occur in mice injected with similar numbers of *S.aureus* without prior CY challenge, although abscesses were formed when the infective dose of *S.aureus* was increased (experiment 1).

Assuming that the results from this small number of mice is valid the fact that the greatest degree of

susceptibility to *S.aureus* seemed to occur 3 days after the administration of CY is interesting. This coincides with the nadir of lymphocytopaenia induced by CY and measured in experiment 2. The adverse reactions exhibited by this group were severe, with three of the four mice dying 36 hours after inoculation with *S.aureus*. There were no fatalities in the other groups. There thus appeared to be a synergistic combination when *S.aureus* was administered 3 days after CY, producing symptoms of disproportionate severity. The interval between the two injections was critical to the manifestation of staphylococcal abscesses.

Eighty eight per cent of the mice treated with CY and *S.aureus* had either slightly or grossly enlarged spleens. Fifty five per cent had enlarged adrenal glands. The causes of these changes are unknown.

These results suggest that the mouse's ability to eliminate *S.aureus*, administered 3 days after CY was impaired, allowing the establishment of abscesses and the subsequent culture of staphylococci from tissues. The timing of the bacterial challenge appeared to be critical.

8.60 EXPERIMENT 4.

8.61 INTRODUCTION.

The previous experiments established the dose of staphylococci necessary to produce pyaemia in normal mice and that CY administered on day 0 depresses the number of circulating lymphocytes to a minimum level on day 3. At this time 5×10^4 staphylococci inoculated intravenously produce pyaemic lesions in the mouse. The aim of experiment 4 was to use different doses of staphylococci and more natural routes of administration to model pyaemia as it

occurs in lambs.

8.62 METHOD.

EXPERIMENTAL DESIGN.

Each group consisted of 6 mice.

log 10. Dose of *S.aureus*	I.V.	I.D.	S.C.	* Ticks	** Paint
	Group Numbers.				
6	1,8	4,11	5,12	6,13	7,14
4	2,9				
2	3,10				

I.V. intravenous.
I.D. intradermal
S.C. subcutaneous.
* Ticks. Larval *I.ricinus* contaminated with *S.aureus* allowed to feed on the mice.
** Paint. *S.aureus* suspension was painted onto the abdomen which was then pricked.

Groups 8-14 were pre-treated with CY administered as described in section 8.252, 3 days prior to treatment with *S.aureus*.

All intravenous inoculations were made into the tail vein. Subcutaneous and intradermal inoculations were performed on mice lightly anaesthetised with diethyl ether. Intradermal inoculations were made into the ventral abdomen and subcutaneous inoculations into the flap of skin in front of the right hind leg.

Groups 6 and 13.(Ticks *).

Larval *I.ricinus* were placed on a confluent growth plate

of *S.aureus* for 30 minutes before 20 were allowed to feed on each mouse as described in chapter 2.

Groups 7 and 14. (Paint **).

These mice were also lightly anaesthetised. Point one of a millilitre of the *S.aureus* suspension was placed onto the abdomen of each mouse, spread with a sterile swab and the area traumatised by 10 slight punctures of the skin with a sterile needle. The area was marked with an indelible pen.

The mice were kept in pairs and observed twice daily. Any casualties were examined immediately after death; up to 2 mice from each group were sacrificed 9 days after *S.aureus* infection and the remainder 7 days after infection for post mortem examination and bacteriological culture of samples of liver, lungs, heart, both kidneys, spleen, the stifle joint and the inoculation site as described in section 8.32.

A scoring index was used to take into account the effect of both acute toxaemia followed by death and pyaemia resulting in abscess formation. One point was awarded for each organ containing abscesses at post mortem examination and one point for each day of the experimental period during which the mouse was dead. For example if a mouse died one day after the administration of *S.aureus* it was awarded 7 points, if it died 7 days after the administration of *S.aureus* (the final day of the experimental period) 1 point was awarded.

The data was analysed by a two way analysis of variance, to take into account the effects of pre-treatment with or without CY, the various treatment types, and any interaction between the two.

8.63 RESULTS.

Mice which were treated with CY prior to staphylococcal inoculation exhibited a greater degree of susceptibility when staphylococci were administered, than those treated with *S.aureus* alone. Nineteen per cent (8/42) of the staphylococci only treated mice died. These were equally distributed between groups 1 and 2, which received 10^6 and 10^4 staphylococci intravenously respectively. Forty two per cent (17/40) of the CY pretreated mice died. (5/6 from group 8, (10^6 *S.aureus* I.V.); 4/5 from group 9, (10^4 *S.aureus* I.V.); 2/6 from group 11 (I.D.); 4/6 from group 12 (S.C.), and 2/6 from group 13 (tick group). These differences in survival between the use or absence of CY pretreatment were significant at the 1% level.

There were highly significant differences, at the 0.1% level between treatments.

Mean results are shown in table 1.

Table 1. Percentage Death Rate.

GROUP NUMBER	1	2	3	4	5	6	7
% DEATH RATE	50	66	0	0	0	0	0

GROUP NUMBER	8	9	10	11	12	13	14
% DEATH RATE	83	80	0	33	66	33	0

TABLE 1A. Twoway Analysis of Variance.

	P value.
PRETREATMENT	0.001
TREATMENT	0.001
GROUPS 1&8,2&9,3&10, 4&11,5&12,6&13,7&14.	N.S

It is interesting to note the differences in death rates between the intradermal (groups 4 and 11) and subcutaneous (groups 5 and 12) inoculation groups and especially between the two groups which were infected by *S.aureus* contaminated ticks (6 and 13), with or without CY pre-treatment. There were no differences in death rates between the groups pre-treated with CY and controls subsequently challenged with *S.aureus* administered intravenously. High doses of *S.aureus* resulted in fatalities and low doses did not, irrespective of CY pre-treatment.

If the scoring index, described in the method was used to incorporate the effect of toxaemia and rapid death; pyaemia and abscess formation, significant differences were noted between CY treated and untreated mice at the 0.1% level and between treatments at the 0.1% level. There was no interaction between the treatments. This is due in part to the large degree of variation between individual animals and the small group size.

Mean index scores are shown in table 2.

Table 2. Mean Index Score.

GROUP NUMBER	1	2	3	4	5	6	7
MEAN INDEX SCORE	3	5	0	1	1	0	0

GROUP NUMBER	8	9	10	11	12	13	14
MEAN INDEX SCORE	6.2	5.2	0	2.8	4.7	2.2	0.7

Table 2a. Twoway Analysis of Variance.

	P value.
PRETREATMENT	0.001
TREATMENT	0.001
GROUPS 1&8,2&9,3&10, 4&11,5&12,6&13,7&14.	N.S

Scores were generally higher in the CY treated mice, even when high doses of *S.aureus* were administered intravenously. The differences in scores between groups 6 and 13 where larval *I.ricinus* were used to transfer *S.aureus* and between groups 7 and 14, where a suspension of *S.aureus* was painted onto the mouse and the skin then punctured, were interesting although not statistically significant.

Mice which died during the first 3 days after *S.aureus* administration become dull, listless and ceased eating about 24 hours after administration of *S.aureus*. This was especially noticeable in the group 13 mice, who all huddled together in the middle of the cage. Two of these mice subsequently died and the remainder appeared to recover.

BACTERIOLOGY

Bacteriological culture was carried out on liver, lungs, heart, spleen, both kidneys and the inoculation site. For

statistical analysis the culture of joints was ignored as very few staphylococci were isolated from this area. Each organ of each animal had a variable score from 0 representing no growth, to 4 corresponding to confluent growth. Mean scores for each group are shown in table 3.

Table 3. Mean Culture Scores.

GROUP NUMBER	1	2	3	4	5	6	7
MEAN CULTURE SCORE	14.2	11.0	1.8	6.9	7.5	0.7	1.5

GROUP NUMBER	8	9	10	11	12	13	14
MEAN CULTURE SCORE	12.2	16.8	2.4	15.3	16.2	10.7	6.0

Table 3a. Twoway Analysis of Variance.

	P value.
PRETREATMENT	0.001
TREATMENT	0.001
GROUPS 1&8,2&9,3&10, 4&11,5&12,6&13,7&14.	N.S

There were highly significant differences at the 0.1% level between CY treated and untreated mice and between the different treatment regimes. There was no interaction between CY pre-treatment and treatment.

It is interesting to note the differences between groups 4 and 11; 5 and 12; 6 and 13 and 7 and 14. There was also a difference in the scores between groups 14, where a suspension of *S.aureus* was painted onto the mice and the skin then punctured and the mice in group 13 where *S.aureus*

was administered via larval *I.ricinus*. Pre-treatment with CY made little difference to the mean bacterial culture scores when *S.aureus* was administered intravenously.

The results of a two way analysis of variance on the isolation of staphylococci from the various tissues cultured are shown in table 4.

There were significant differences between the CY treated and untreated mice at all sites except the lungs; while significant differences were noted between treatments at all sites sampled and interaction between the two at the inoculation site only.

The individual results of both post mortem examination and bacteriological culture are presented in tables 7 and 8 in Appendix 5.

Table 4. Differences in the Recovery Of *S.aureus* from Various Tissues Analysed by a Twoway Analysis of Variance.

	P	T	PT
LUNG	NS	1%	NS
HEART	5%	0.1%	NS
KIDNEY 1	1%	0.1%	NS
KIDNEY 2	5%	0.1%	NS
LIVER	0.1%	1%	NS
SPLEEN	0.1%	0.1%	NS
INOC	0.1%	0.1%	5%

P - CY Pre-treatment.

T - Treatment with *S.aureus*.

PT - Interaction between CY pre-treatment and treatment with *S.aureus*.

INOC - Inoculation site.

8.64 DISCUSSION.

The administration of CY alone, as in experiment 2, produced no fatalities, suggesting that the fatalities occurring in experiment 4 were a result of a synergistic reaction when treatment with CY and *S.aureus* are combined. There were however no deaths in the two groups of mice which received only 10^2 *S.aureus*, indicating that CY aids the establishment of staphylococci only when relatively large numbers are administered. When however the inoculum of *S.aureus* is increased further (ie.$<10^6$) deaths occur whether CY is used or not. Sharbaugh (1976) reported a decrease in *in vitro* phagocytic capabilities of polymorphonuclear leucocytes when they were opsonized with serum from CY treated donors. This was attributed to decrease in available opsonins necessary for optimal phagocytosis. Supporting evidence for this hypothesis lies in reports that CY causes decreases in both B-lymphocytes (Stockman *et al*,1973 and Jun *et al*, 1980) and gamma-globulin (Sharbaugh and Grogan,1970 and Mackiewicz,1967).

The route of *S.aureus* infection is also important in determining resultant disease. Anderson (1986) reported that while 10^9 staphylococci were necessary to kill a mouse when administered intravenously, the intramammary inoculation of only 9 bacteria of some strains would result in death, seemingly bypassing the animals' defences.

In this experiment intravenous inoculation resulted in a distribution of abscesses throughout parenchymatous organs, both with and without prior CY injection. Fewer visceral abscesses were observed in the CY pre-treatment group. This was probably due to rapid toxaemia and death of many

animals in groups 8 and 9 prior to the development of pyaemic lesions. Intradermal and subcutaneous inoculation produced few abscesses other than local lesions. It was interesting to note that in the groups where *S.aureus* was introduced by tick feeding or by traumatising the skin, local abscess formation occurred only in the CY pre-treatment groups, (13 and 14).

Haemolytic *S.aureus* was isolated from 6/12 mice in groups 6 and 7 (which did not receive CY pre-treatment), and had no other signs of *S.aureus* infection, suggesting that CY aids the development of lesions rather than the initial penetration of *S.aureus* into the mouse.

The mice in group 13 appeared to be very dull about 24 hours after infected ticks were applied. This was unusual in that the whole group was affected. In the other groups only the mice which subsequently died 2 or 3 days later became dull. Two of the group 13 mice died 3 days after the administration of *S.aureus* infected ticks, the remainder recovered. The reason for this is uncertain, but may be associated with direct effects of tick feeding in addition to treatment with CY and *S.aureus*, which had already stressed the host.

Analysis of variance revealed differences in the numbers of *S.aureus* cultured between CY pretreated and CY untreated groups at all sites except the lungs. The relatively large numbers of *S.aureus* isolated from the lungs of mice in both groups suggests that the lungs may be involved in clearing staphylococci from the mouse's circulation, perhaps by alveolar macrophages (Veit and Farrell,1978).

8.70 EXPERIMENT 5.

8.71 INTRODUCTION.

Tick pyaemia is a serious disease of young lambs most frequently noted as suppurative polyarthritis resulting in lameness. However illthrift is also often associated with visceral or soft tissue abscessation in affected lambs. This pyaemic staphylococcal infection is frequently found in young lambs from tick infested areas, but does not occur in lambs from tick free farms.

The aim of this experiment was to attempt to produce tick pyaemia experimentally, using laboratory reared *I.ricinus* contaminated with *S.aureus* as the source of staphylococcal infection.

Experimental work previously described in this thesis has shown that when B -lymphocyte numbers in mice are depressed using CY, prior to challenge with *S.aureus*, there is a significant increase in fatalities and in numbers of *S.aureus* isolated on bacterial culture of tissues. From the work of Sharbaugh (1976) this could reasonably be attributed to the depletion of B-lymphocytes and therefore antibody opsonins resulting in impaired phagocytosis of invading bacteria by polymorphonuclear lymphocytes (P.M.N.). A similar reduction in the numbers of circulating B-lymphocytes has been noted 5 days after a primary T.B.F. infection (Batungbacal *et al*, 1982), associated with a reduced *in vitro* P.M.N. phagocytic activity (chapter 5). On the basis of this finding lambs were challenged with T.B.F. 5 days before *S.aureus* infected ticks were allowed to attach and feed in an attempt to induce tick pyaemia experimentally.

8.72 METHOD.

Four (Scottish Blackface) ewes with twin lambs were obtained from a tick free farm and treated as follows. When two weeks old, one of each pair of lambs (group A) was infected intravenously with 1ml of a 10^{-1} dilution (in phosphate buffered saline, pH 7.2) of the Old Sourhope strain of T.B.F. while siblings remained as uninfected controls, (group B). The experimental design is illustrated in table 5.

Table 5. Design of Experiment 5A.

	T.B.F. infection Day 0.	Application of *S.aureus* infected ticks. Day 5.
Group A	+	+
Group B	_	+

T.B.F. free nymphal and larval *I.ricinus* from a laboratory maintained culture were placed on a blood agar plate with a confluent growth of *S.aureus*, for 30 minutes. The strain of *S.aureus* used was originally isolated from a field case of tick pyaemia in Argyll, Scotland and had the characteristics listed in section 8.231. Five days after T.B.F. infection 10 nymphs and 100 larvae were placed in ear and tail bags (described in section 2.27) respectively on all 8 lambs (Plate 9). The ticks were allowed to attach and feed normally (chapter 2).

Two pairs of twins were sacrificed on days 20 and 27 post T.B.F. infection. Lambs were weighed prior to post

mortem spleens removed entire and weighed. At necroscopy viscera were examined macroscopically and gross lesions noted. Swabs were taken from lesions, tick bites and skin and from stifle and carpal joints (which were cut open with a sterile blade) and placed in 5 ml volumes of B.H.I.. The broths were plated onto blood agar and M.S.A. 24 hours later. Samples of liver, lung, heart, spleen, brain and kidney were cultured on blood agar and M.S.A.. All plates were incubated at 37'C overnight and bacterial growth examined the following morning. The densities of $\alpha\beta$ haemolytic *S.aureus* were scored on a scale from 0 - 4 corresponding to gradations from nil to confluent growth respectively. Any other bacteria were identified, although these results are not reported in this thesis.

Results were analysed using the mann whitney U test.

8.73 RESULTS

Post mortem examination revealed no significant changes in group B lambs, while all group A lambs had a number of small abscesses in the right diaphragmatic lobe of the lung (table 6 and Plate 10). Liver abscesses were present in 2/4 of the lambs , while splenomegaly (table 7), associated with T.B.F. infection (Gordon *et.al*,1932 ; Hudson,1950) was noted in every case.

Plate 9 Nylon ear and tail bags attached to lambs to contain engorged ticks.

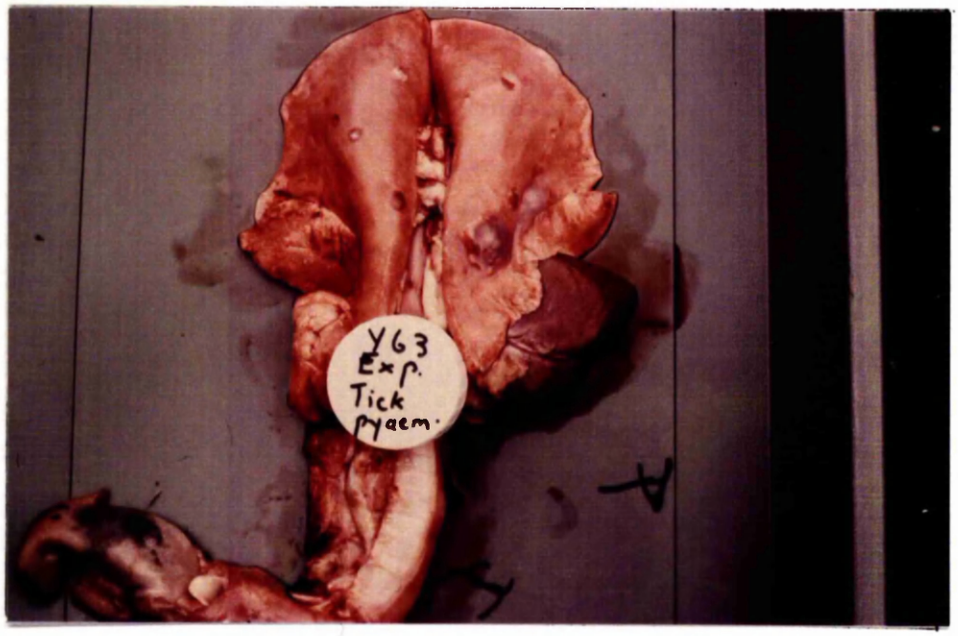


Plate 10 Gross lung lesions in an experimental case of tick pyaemia.

Table 6. Lesions Found at Post Mortem Examination.

	L	LIV	H	SP	K	B	TB	S
Group A	+	-	-	-	-	-	-	-
	+	-	-	-	-	-	-	-
	+	+	-	-	-	-	-	-
	+	+	-	-	-	-	-	-
Group B	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-

L. Lung.
Liv. Liver.
H. Heart.
SP. Spleen.
K. Kidney.
B. Brain.
TB. Tick Bite Wound.
S Skin

Table 7. Percentage Spleen to Body Mass Ratio at Post Mortem Examination.

	Weight of Lamb(Kg).	Weight of Spleen (g).	% Spleen Weight / Body Weight.
Group A	7.7	40.3	0.52
	9.1	65.8	0.72
	7.0	58.7	0.84
	7.7	64.0	0.83

$\bar{X}$ = 0.73
sdi = 0.15

	Weight of Lamb(Kg).	Weight of Spleen (g).	% Spleen Weight / Body Weight.
Group B	7.5	17.2	0.13
	8.4	40.3	0.48
	5.7	22.4	0.39
	8.0	33.4	0.37

$\bar{X}$ = 0.34
sdi = 0.15

p = 0.03

S.aureus was recovered from abscesses, the area of skin surrounding bite wounds and from the heart blood of group A lambs (table 8), and all had identical cultural reactions to the original strain of *S.aureus* used for infection (section 8.231).

Table 8. Bacteriological Isolations of *S.aureus*.

	L	LIV	H	SP	K	B	TB	S
Group A	+	-	-	-	-	-	-	-
	4+	-	-	-	-	-	-	-
	3+	+	-	-	-	-	2+	+
	4+	-	+	-	-	+	+	+
Group B	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-
	-	-	+	-	-	-	2+	+
	-	-	+	-	-	-	-	-

L. Lung.
Liv. Liver.
H. Heart.
SP. Spleen.
K. Kidney.
B. Brain.
TB. Tick Bite Wound.
S. Skin.

EXPERIMENT 5B.

8.74 INTRODUCTION.

The results of the previous experiment (5A) showed that staphylococcal pyaemia could be produced experimentally by allowing ticks contaminated with *S.aureus* to feed on lambs five days after a primary T.B.F. infection. The experiment aimed to simulate the conditions encountered by a young lamb on a tick infested hill in the spring.

A second experiment (5b) was designed to mimic the T.B.F. challenge associated with the autumn peak of tick activity reported in some areas (MacLeod,1939b and Milne,1945). Although the experiment grossly simplifies the period of tick challenge in that it supposes no contact

between sheep and ticks in the interim, the approach enabled the immune status of the animals to be measured and interpreted with some accuracy (chapter 5).

8.75 METHOD.

Four Scottish Blackface ewes with twin lambs were obtained from a tick free farm and treated as follows. At the age of two weeks all lambs were infected intravenously with 1 ml of a 10^{-1} dilution (in phosphate buffered saline, pH 7.2) of the Old Sourhope strain of T.B.F. which resulted in normal T.B.F. reactions ie. pyrexia, parasitaemia, lymphocytopaenia, neutropaenia and production of specific antibody as described previously (Chapter 5).

Sixteen weeks after initial infection one of each pair of twins was rechallenged with T.B.F. (Group A), administered as described in section 5.23, while siblings remained as unchallenged controls (Group B).

S.aureus contaminated ticks were allowed to feed on lambs 5 days after reinfection with T.B.F. as described in section 2.27. The experimental design is shown in table 9. The lambs were sacrificed 20 days post T.B.F. challenge for post mortem examination as described in section 8.72.

Table 9. DESIGN OF EXPERIMENT 5B.

	T.B.F at 2 weeks of age.	T.B.F. at 18 weeks of age.(Day 0)	Application of *S.aureus* infected ticks.(Day 5)
Group A	+	+	+
Group B	+	-	+

8.76 RESULTS.

Post mortem examination revealed only non-specific changes in all lambs, except for the presence of splenomegaly in some lambs. Individual spleen weights are shown in table 10. The results of bacteriological culture of various organs, tick bite wounds and skin swabs as described in section are shown in table 11.

Table 10. Percentage Spleen to Body Mass Ratio at Post Mortem Examination.

	Weight of Lamb(Kg).	Weight of Spleen (g).	% Spleen Weight / Body Weight.
Group A	30.4	172.5	0.57
	24.5	65.4	0.26
	27.3	135.4	0.50
	30.4	102.4	0.34

$\bar{X}$ = 0.42
sdi = 0.14

	Weight of Lamb(Kg).	Weight of Spleen (g).	% Spleen Weight / Body Weight.
Group B	27.7	104.5	0.38
	30.0	170.3	0.57
	27.3	71.5	0.26
	30.4	67.5	0.22

$\bar{X}$ = 0.36
sdi = 0.16

p = NS.

Table 11. Bacteriological Isolations of *S.aureus* (Experiment 5B).

	L	LIV	H	SP	K	B	TB	S
Group A	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	4+	+
	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-
Group B	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-

L. Lung.
Liv. Liver.
H. Heart.
SP. Spleen.
K. Kidney.
B. Brain.
TB. Tick Bite Wound.
S. Skin.

8.77 DISCUSSION

This is believed to be the first recorded experimental production of tick pyaemia by *Ixodes ricinus*. Previously Foggie (1962) showed that the subcutaneous or intradermal inoculation of staphylococci either with or without an extract of tick salivary gland, or the feeding of ticks on areas of skin heavily contaminated with *S.aureus* failed to reproduce the disease in both normal and neutropaenic lambs. However, lambs in the neutropaenic phase of T.B.F. were shown to be one hundred fold more susceptible to the intravenous inoculation of staphylococci than healthy lambs (Foggie,1962).

In the present study the staphylococcal challenge was administered 5 days after T.B.F. infection, prior to development of neutropaenia whereas Foggie always administered staphylococci during the neutropaenic phase of T.B.F. infection.

It has also been suggested that staphylococci may be released from a latent state by the immunodepression associated with T.B.F., or that staphylococci invading through minor cuts and abrasions can multiply when the host is stressed by T.B.F. infection (Anderson 1983). However, many lambs have been experimentally infected with T.B.F and there have been no reports of the development of spontaneous pyaemia, which might be expected if the above explanations were valid.

Monitoring of experimental tick infestations has shown that the tick produces a battery of substances to enable it to breach the intact skin barrier and attach to the host. These include anticoagulants (Foggie,1959) and a substance similar to hyaluronidase which allows particles to percolate very slowly through the tissues (Foggie,1959). The penetrating staphylococci are also armed with aggressins including coagulase, DNase, leucocidins and haemolysin (Cruickshank *et al*, 1973).

Staphylococci which have penetrated the skin's defensive barrier are thought to be repulsed by a combination of humoral and cellular mechanisms. *S.aureus* is readily phagocytosed in the presence of adequate amounts of specific antibody, but elimination is not complete since a small proportion of the ingested bacteria survive within the phagocyte (Roitt,1984), also, virulent *S.aureus* resist

phagocytosis. This may be due partly to capsule formation *in vivo* and partly to the elaboration of factors such as protein A which combines with the Fc portion of IgG and inhibits its binding to the polymorph Fc receptor.

The depression of B-lymphocytes, antibody production, neutrophil function and the neutropaenia induced by T.B.F. in the present experiments are probably the reasons for increased staphylococcal infection and pyaemia in T.B.F. infected lambs when compared to healthy lambs.

There were two main differences between experiments 5A and 5B. In experiment 5A the lambs were 3 weeks old when contaminated ticks were placed on the skin; these lambs had received a primary infection of T.B.F. five days previously. In contrast the lambs in experiment 5B were 19 weeks old at the time *S.aureus* contaminated ticks were introduced; these lambs had received a second infection of T.B.F. five days previously.

The group of young lambs (5A) all had pyaemic abscesses in the lungs and some also in the liver, whereas in older lambs undergoing a secondary T.B.F infection (5B), which produced less severe haematological changes than a primary infection and a reduced serological response (chapter 5), abscessation was absent. All the lambs receiving *S.aureus* alone were without abscesses at necroscopy.

These results reflect the field situation where tick pyaemia primarily occurs in the late spring, when lambs born on the hill are about 4 weeks old, but is not normally present in other seasons of the year, despite tick activity.

The differences between the two groups were basically

age, size of the animal related to the doses of *S.aureus* and T.B.F. and the immune status of the lambs in relation to *S.aureus* infection. The immune competence of young lambs, at this critical time when maternally derived antibody to staphylococci is declining is questionable. Foggie (1948) showed that staphylococcal antitoxin derived from the colostrum disappeared from lambs by about the 20th day of life and that it was difficult to stimulate an active immunity in lambs under about 4-6 weeks old. This period coincides with the age at which tick pyaemia occurs in the field. The mean age at the first appearance of clinical signs of 54 naturally occurring cases was 3.8 +/- 1 week (Foggie,1962).

Apart from antibody it has been suggested (Cruickshank *et al*,1973) that the presence of established commensal staphylococci can prevent the colonisation of sites by new invading strains for many months or even years. The age at which a lamb becomes infected with staphylococci varies with environment, but it is likely to occur in the first few days of life. It is possible that this may explain why only some lambs develop tick pyaemia.

Additionally a further reason why older lambs may not be susceptible to tick pyaemia is suggested by observations of experimentally induced (tables 8 and 11) and naturally occurring tick bite wounds (chapter 9). These have indicated that an older lamb may mount a more vigorous immune attack on piercing tick mouthparts, producing increased redness, swelling and oedema at the site of penetration, than a young lamb and so reduce the chances of successful attachment, feeding and disease transmission.

Comparison of our experimentally produced cases of tick pyaemia with those reported from the fields show that clinical signs and pathological findings in the former were relatively mild, there are several possible explanations for this. The experimental lambs in the present studies were kept in 'ideal' conditions; housed inside protected from adverse weather, fed *ad libitum* etc., so the stresses normally experienced by a young lamb on the hill were reduced considerably. Brodie *et al* (1986) reported that young lambs became very dull and were reluctant to suckle when infected with T.B.F. alone. These authors hypothesised that a lamb so afflicted could become lost from its mother, particularly if living in exposed terrain and die of starvation. Perhaps these physiological stresses also exacerbate the severity of opportunist pathogens.

A second reason is that one strain of staphylococci, isolated from a field case of tick pyaemia was chosen and used throughout these experiments. It was subcultured a minimum number of times to obtain a pure culture and stored as a freeze dried culture. However, pathogenicity may still have declined resulting in less severe symptoms than those observed in the field.

In experiment 5B *S.aureus* infected ticks were allowed to attach and feed on lambs 5 days after the initiation of a secondary T.B.F. infection. Reactions to secondary T.B.F. infections are less severe than to primary infections (Woldehiwet and Scott, 1982d; chapter 5) pyrexia, parasitaemia, neutropaenia and lymphocytopaenia being less marked when compared to a primary infection. It is therefore likely that *S.aureus* inoculated by ticks in

experiment 5B encountered a more effective immune attack in terms of B-lymphocytes producing antibodies, opsonins and therefore phagocytotic capacity. *In vitro* assays of neutrophil function showed similar levels of impairment of phagocytic capacity following both primary and secondary infections (chapter 5).

Multiple small abscesses were present in the lungs of all pyaemic lambs in experiment 5A. The reasons for this location are uncertain, especially as in field cases the most common site for abscess formation appeared to be the liver (McDiarmid,1946), although they may be found in almost any part of the body. The T.B.F. stabilate was injected intravenously into the jugular vein but had disseminated throughout the body by the time staphylococcal infected ticks were applied to the ears and tail. In the mouse experiments, where injections were made into the tail vein, the lungs were severely affected in both CY pre-treated and untreated mice. This suggests that the lung is either particularly susceptible to staphylococcal infection or that the lungs are involved in the defence against staphylococci , perhaps via alveolar macrophages clearing stapylococci from the body (Veit and Farrell,1978).

In field outbreaks the mode of staphylococcal infection remains obscure. Foggie (1947) showed that haemolytic staphylococci commonly occurring on the skins of lambs have the same characteristics as the staphylococci isolated from cases of tick pyaemia. McDiarmid (1948) noted that the sites of tick bites are usually inflamed and abscesses may be present from which *S.aureus* can be isolated. This was substantiated by post mortem examination of experimental

(tables 8 & 11.) and field cases of tick pyaemia (chapter 9) at this laboratory. Of 11 field cases examined post mortem at the Auchincruive Veterinary Investigation Centre in 1986, 9 had either attached ticks or bite wounds, although in 2 cases the source of *S.aureus* infection could not be identified. Moreover, staphylococcal abscesses rarely appear at wounds caused by surgical interference such as docking or castration or even at the umbilicus which is a favourite entrance for other pathogenic organisms (McDiarmid,1948).

The experimental model of tick pyaemia described above would be of considerable value in assessment of prophylactic measures currently used in lambs to control tick-borne fever and tick pyaemia on hill farms, particularly in defining more accurately prophylactic regimens.

CHAPTER 9. TICK CONTROL.

SUMMARY.

1. Five millilitres of a deltamethrin pour-on formulation was applied to each of 40 hoggs to assess efficacy against the sheep tick, *Ixodes ricinus*. Significantly fewer ticks were found on treated hoggs than on untreated control animals 13 days (a mean of 2.6 adult ticks on treated hoggs compared to 6.5 on controls); 27 days (a mean of 3.1 adult ticks on treated hoggs compared with 14.2 on untreated controls) and 41 days post treatment, however by day 41 unacceptably high numbers (7.5 adults per treated hogg compared to 17.5 on controls) were attached to the former.

2. Five millilitres of a pour on cyhalothrin formulation was applied to each of 155 lambs (48 untreated controls remained) on four farms in Ayrshire and Arran. Tick challenge was generally low in 1986, but treated lambs had significantly fewer ticks than untreated controls for 15 days (farm 1). Problems arose due to fleece staining and possible liver and kidney damage in treated lambs.

Tick-borne fever and tick pyaemia occurred in normal proportions in treated and control lambs on all farms. Eleven cases of tick pyaemia were confirmed by post mortem examination, all of which had enlarged spleens suggestive of prior tick-borne fever infection.

3. Of the two preparations deltamethrin appeared to be superior and would be of equivalent value to dipping. The ease of use of pour-on preparations is an added advantage.

9.1 INTRODUCTION.

The sheep tick *Ixodes ricinus* is traditionally controlled by spring dipping of ewes, and/or lambs, to coincide with the peak of tick activity. However whole body immersion of heavily 'in lamb' ewes is stressful at a critical period of pregnancy and mismothering problems have also been reported when young lambs are dipped in acaricide.

In an attempt to avoid the disadvantages of dipping, pour-on preparations of synthetic pyrethroids have been shown to be effective in controlling a number of sheep ectoparasites including keds (Kettle *et al* ,1983), headfly (Titchener,1984) and sheep lice (Rundle and Forsyth,1984). MAFF (1981) investigated the efficacy of cypermethrin impregnated collars against *I.ricinus*. Additionally a pour-on cyhalothrin formulation has also been used to control *Dermacentor marginatus* which is, apart from *I.ricinus*, the most important tick species affecting sheep in Germany. A protective period of four weeks was recorded (Leibisch, pers.comm.).

The purpose of the trials described below was to assess the efficacy of two synthetic pyrethroid pour-ons, deltamethrin (Spot On, Coopers Animal Health) and cyhalothrin (Coopers Animal Health) in controlling the sheep tick *I.ricinus*. Details of chemical structures, properties and uses are given in the pesticide manual (Worthing, 1987). Results of the former study have been published elsewhere (Mitchell *et al*, 1986).

9.2 MATERIALS AND METHODS.

9.21 ANIMALS.

1985 Trial.

Fifty two Scottish Blackface hoggs were used of which 40 were treated with a formulation containing deltamethrin 1% w/v in an oily solvent and 12 remained as untreated controls.

1986 Trial.

Two hundred and three Scottish blackface lambs were studied of which 155 were treated with a formulation containing cyhalothrin 2% w/v in an oily solvent and 48 remained untreated as controls.

9.22 METHODS.

1985 FARM 1.

On April 3, 1985 Fifty two Scottish Blackface hoggs from tick free pasture were held overnight on an inbye field before examination for ticks and treatment. Twelve hoggs were left untreated as controls while forty were treated with 5ml deltamethrin applied to the skin between the shoulder blades (table 1). Both groups were moved onto tick infested pasture and examined 13, 27 and 41 days after treatment. Adult ticks were counted on the ears, face, axillae and inguinal regions, classified as fed or unfed and a subjective assessment of numbers of larvae and nymphae made.

Table 1. Design of 1985 Trial.

Group	n	Examined for Ticks on Days
Treated (3.4.85)	40	0,13,27,41 post-Treatment
Controls	12	

1986 FARM 1.

On May 13,1986 forty Scottish Blackface lambs aged three to four weeks old were individually tagged, weighed and examined for ticks as described above. Twelve lambs were selected as untreated controls (group C) and 28 lambs treated with 5ml cyhalothrin applied to the skin between the shoulder blades (group T). Both groups were then moved onto tick infested pasture and examined 15 days after treatment. At the 'marking gather' on June 2, 1986 (day 20 post initial treatment) many of the treated lambs were found to be infested with ticks and the farmer re-treated the pour-on group, which were re-examined 11 days after retreatment (31 days after initial treatment).

1986 FARM 2.

Twenty single Scottish Blackface lambs were individually tagged, treated by the farmer with cyhalothrin on the day of birth, between 16th and 20th of April, 1986 and immediately turned onto tick infested pasture (group P - treated soon after birth).

On April 22 1986, 33 further single lambs were individually tagged, weighed and examined for ticks as described above. Twelve lambs were selected as untreated controls (group C - controls) and 21 treated with cyhalothrin as described previously (group T - treated). Both groups were turned onto tick infested pasture and examined 17, 37, and 46 days after treatment of group T lambs.

1986 FARM 3.

Twenty single lambs were individually tagged, treated with cyhalothrin immediately after birth by the farmer from

April 18 - 25 1986 (group P) whilst on tick infested hill grazing. On April 28, 1986, a further 34 Scottish Blackface lambs were individually tagged, weighed and examined for ticks. Twelve lambs were selected as untreated controls (group C) and 24 lambs treated with cyhalothrin (group T). The lambs from group P which had been treated by the farmer soon after birth were gathered, weighed, examined for ticks and blood samples obtained by jugular venopuncture into heparinised and untreated vacutainer tubes for biochemical analyses. All three groups of lambs were turned onto tick infested hill grazing and examined 14, 28 and 66 days after treatment of group T lambs.

1986 FARM 4.

Nineteen single Scottish Blackface lambs were individually tagged, treated with cyhalothrin on the day of birth by the farmer (group P), between April 18 and 25, 1986, whilst on tick infested pasture. On April 28, 1986 a further 37 lambs were individually tagged, weighed and examined for ticks. Twelve lambs were selected as untreated controls (group C) and 25 treated with cyhalothrin (group T) (table 2). Lambs from group P were weighed and examined for ticks. All lambs were returned to tick infested pasture and examined for ticks 14, 28 and 54 days after treatment of group T lambs.

9.23 TICK-BORNE FEVER INFECTIONS.

Tick-borne fever (T.B.F.) was demonstrated by examination of Giemsa stained smears of peripheral blood for T.B.F. parasites or serologically as described in sections 5.253 and 5.263 respectively.

9.24 POST MORTEM EXAMINATION.

Any lambs which showed severe clinical signs suggestive of tick pyaemia when examined at gathers were sacrificed and examined post mortem for macroscopic lesions. Samples of affected tissues were cultured on blood agar and mannitol salt agar as described in section 8.72.

9.25 STATISTICAL TECHNIQUES.

Statistical analysis was carried out using Students T test when variance ratios of the samples to be compared were approximately equal to one, otherwise the Mann Whitney U test was applied. Percentage infestation levels were compared using the Chi square test.

Table 2. Design of 1986 Trial.

Group	n	Treatment Date	Examined Days post Treatment	Comments
Farm 1				* Retreated by farmer 2.6.86.
C	12	-		
T*	28	13/5/86	0,15,11(31)	
Farm 2				
P	20	16-20/4/86	4,21,41,51	
C	12	-		
T	21	22/4/86	0,17,37,47	
Farm 3				
P	20	18-25/4/86	7,21,35,73	
C	12	-		
T	24	28/4/86	0,14,28,66	
Farm 4				
P	19	18-25/4/86	7,21,35,61	
C	12			
T	25	28/4/86	0,14,28,54	

n number of samples

9.3 RESULTS.

9.31 1985 FARM 1.

As shown in figure 9.1 and table 3 the mean number of adult ticks attached to untreated hoggs varied between 2.3

and 17.5 during the trial period. Significantly fewer adult ticks were noted in the treatment group compared with controls ($p < 0.01$, $p < 0.01$, $p = 0.01$) on days 13, 27 and 41 respectively. The distribution of ticks on both groups of hoggs is illustrated in table 4. Significantly fewer ticks were found on the axillae of treated hoggs until day 41 post treatment, the majority of ticks on this group were on the face and ears.

Table 3. Deltamethrin Pour-on for the Control of Sheep Ticks.

	Mean number of ticks per hogg.			
Days after treatment	0	13	27	41
Treated	2.2	2.6	3.1	7.5
+/- SE	2.9	2.3	2.6	4.0
Untreated	2.3	6.5	14.2	17.5
+/- SE	3.1	3.1	8.4	9.6
Significance	NS	p<0.01	p<0.01	p=0.01

SE Standard error
NS Not Significant

Table 4. Distribution of Ticks on Hoggs.

	% Ticks per site.											
Days after Treatment	0			13			27			41		
	H	A	G	H	A	G	H	A	G	H	A	G
Treated	0	100	0	94	3	3	97	0	3	59	16	25
Untreated	0	100	0	37	58	5	37	56	7	53	34	13
Significance	NS	NS	NS	*	***	NS	NS	***	NS	*	NS	NS

H Head; A Axillae; G Inguinal region.
* P<0.05
*** P<0.001
NS Not Significant

Deltamethrin Trial 1985
Farm 1

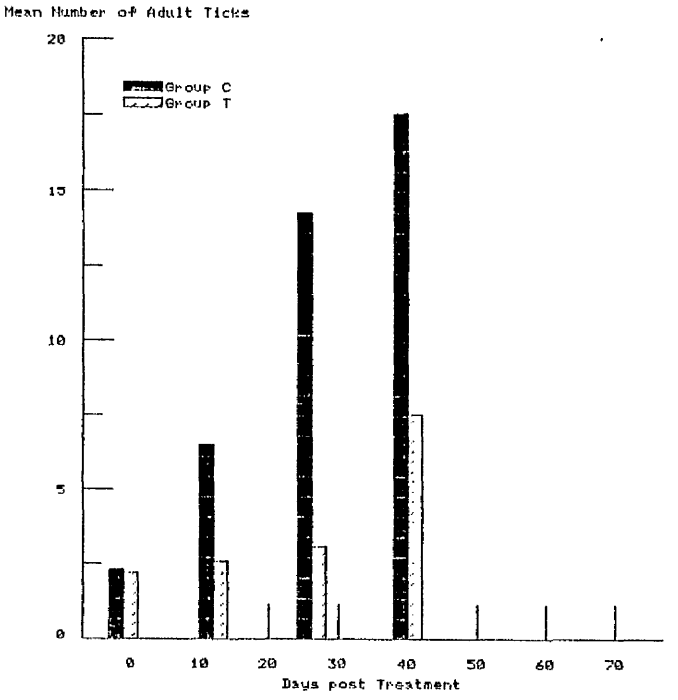


Figure 9.1 - The mean number of adult ticks attached to untreated hoggs (group c) and hoggs treated with deltamethrin (group T) at various times after application of the product.

9.32 1986 Farm 1.

Results of tick counts are shown in figure 9.2a and table 5. The mean number of adult ticks attached to untreated hosts varied between 0.17 and 5.3 during the trial period. Significantly fewer adult ticks were noted in the treated group compared with controls ($p < 0.01$, $p < 0.01$) on days 15 post treatment and day 11 post retreatment respectively. The distribution of ticks on lambs is shown table 6, no significant differences were noted in the percentages attached at each site in treated or untreated lambs.

Table 5. Cyhalothrin Pour-on for the Control of Sheep Ticks - Farm 1.

	Mean no. of ticks per lamb.		
Days after treatment	0	15	11(31)
Treated (Gp T)	0.28	0.7	0.9
+/- SE	0.13	0.17	0.31
% infested	18	59	44
Untreated (Gp C)	0.17	5.3	4.9
+/- SE	0.11	1.32	1.20
% infested	17	100	100
Significance			
Mean no. ticks.	NS	p<0.01	p<0.01
Infestation.	NS	p<0.05	p<0.02

SE Standard error.
NS Not Significant

Table 6. Distribution of Ticks on Lambs at Farm 1

	% Ticks per site.								
Days after Treatment	0			15			11(31)		
	H	A	G	H	A	G	H	A	G
Group T	43	57	0	8	69	23	7	86	7
Group C	0	50	50	8	70	22	9	85	6
Significance	NS	NS	NS	NS	NS	NS	NS	NS	NS

H Head; A Axillae: G Inguinal region.
NS Not Significant

9.321 FATE OF LAMBS.

The fate of lambs, ie. the number of lambs present at the final gather (marking time) as a ratio of the original number of lambs in all groups is shown in table 7. Two lambs, one each of groups T and C were sacrificed on 13.6.86 and 28.5.86 respectively, when post mortem examination confirmed tick pyaemia.

Table 7. Fate of Lambs - Number Gathered/Number in Group.

TREATED	7/12 (1 tick pyaemia)	4 fate unknown
UNTREATED	15/28 (1 tick pyaemia)	12 fate unknown

9.322 LIVEWEIGHTS.

The mean daily liveweight gains (table 8) were 0.16 and 0.17 Kg per day for group T and C lambs respectively. No significant differences were recorded between groups.

Table 8. Mean Daily Liveweight Gains During the Trial Period.

	Mean Weight Gain in Kg (SE)			Mean daily weight gain
Days after Treatment	0	15	11(31)	
Group T	8.5 (0.31)	9.8 (0.83)	13.4 (0.66)	0.16
Group C	7.5 (0.50)	10.0 (0.84)	12.7 (0.90)	0.17
Significance				NS

SE Standard Error
NS Not Significant

9.33 FARM 2.

Results of tick counts are shown in table 9 and figure 9.2b. The tick challenge early in the season was

insufficient to show any differences between groups. On 16.6.86 (day 46 post treatment) ticks were found attached to lambs for the first time; all ticks were attached to the axillae. One of the treated group T lambs was examined post mortem on 9.5.86 and tick pyaemia confirmed.

Table 9. Cyhalothrin Pour-on for the Control of Sheep Ticks - Farm 2.

	Mean no. of ticks per lamb.			
Days after treatment of group T.	0	17	37	46
Group P	*	0	0	0.4
+/- SE		0	0	0.17
% infested		0	0	24
Group C	0	0	0	0.3
+/- SE	0	0	0	0.14
% infested	0	0	0	27
Group T	0	0	0	0.4
+/- SE	0	0	0	0.13
% infested	0	0	0	35
Significance				
Mean no. ticks.	NS	NS	NS	NS
Infestation.	@	@	@	NS

SE Standard error.
NS Not Significant
* treated a median of four days before group T.
@ chi square test not possible as expected frequencies are equal to zero.

9.331 FATE OF LAMBS.

The fate of lambs is shown in table 10.

Table 10. Fate of Lambs - Number Gathered/Number in Group.

GROUP P	17/20	3 fate unknown
GROUP C	11/12	1 lamb removed from trial
GROUP 3	20/21	1 Tick pyaemia (PM 9.5.86)

9.332 LIVEWEIGHTS.

Mean weight gains for each group and the mean daily

weight gain over the trial period are shown in table 11. Mean daily weight gains varied between 0.21 and 0.22 Kg per day and no significant diffences were recorded between groups.

Table 11. Liveweight Gains Over the Trial Period.

	Mean Weight Gain in Kg (SE)				Mean daily weight gain
Days after Treatment	0	17	37	47	
Group P		8.3 (0.25)	12.4 (0.49)	16.5 (0.70)	0.21
Group C	5.3 (0.21)	9.2 (0.15)	12.6 (0.46)	16.7 (0.57)	0.21
Group T	5.8 (0.23)	9.2 (0.39)	12.6 (0.65)	17.8 (0.53)	0.22
Significance					NS

NS Not Significant
SE Standard error

9.34 FARM 3.

Results of tick counts are shown in table 12 and figure 5.2c. Tick challenge was low particularly early in the season and insufficient to show any significant differences between treated and untreated animals. The sites of attachment are shown in table 13. All ticks found on day 0 and day 14 were on the axillae and inguinal region; on days 28 and 66 the majority of ticks were attached to the axillae.

Table 12. Cyhalothrin Pour-on for the Control of Sheep Ticks - Farm 3.

	Mean no. of ticks per lamb			
Days after treatment of group T.	0	14	28	66
*Group P	0	0.5	1.5	3.6
+/- SE	0	0.14	0.3	0.34
% infested	0	38	47	92
Group C	0	0.46	1.85	4.8
+/- SE	0	0.19	1.22	1.66
% infested	0	36	63	100
Group T	0.23	0	0.83	2.2
+/- SE	0.09	0	0.30	0.34
% infested	23	0	50	92
Significance				
Mean no. ticks.	NS	NS	NS	NS
Infestation.	NS	p<0.01	NS	NS

SE Standard error.
NS Not Significant
* treated a median of four days before group 3.

Table 13. Distribution of Ticks on Lambs at Farm 3.

	% Ticks per site											
Days after Treatment	0			14			26			66		
	H	A	G	H	A	G	H	A	G	H	A	G
Group P	0	0	0	0	25	75	6	83	11	15	62	23
Group C	0	0	0	0	25	75	8	84	8	34	53	13
Group T	0	100	0	0	0	0	20	10	70	12	40	48
Significance	NS	***	NS	NS	*	***	NS	***	**	NS	NS	NS

* p<0.05; ** p<0.01; *** p<0.001.
H Head; A Axillae: G Inguinal region.
NS Not Significant

9.341 FATE OF LAMBS.

Two lambs from group P and two from group T had become pyaemic by 3.7.86. The fate of all lambs is shown in table 14.

Table 14. Fate of Lambs - Number Gathered/Number in Group.

GROUP P	12/20 (2/12 pyaemic)	4 known not gathered, 1 dead, 3 fate unknown
GROUP C	6/12	6 known not gathered
GROUP T	12/22 (2/12 pyaemic)	1 killed on road, 9 fate unknown

9.342 LIVEWEIGHTS.

Mean weight gains for each group and the mean daily weight gain over the trial period are shown in table 15. The mean daily weight gain varied between 0.16 and 0.17Kg per day and no significant diffences were recorded between groups.

Table 15. Weight Gains Over the Trial Period

	Mean Weight Gain in Kg (SE)				Mean daily weight gain
Days after Treatment	0	17	37	47	
Group P	5.47 (0.53)	8.0 (0.35)	10.7 (0.64)	16.5 (0.81)	0.16
Group C	6.8 (0.34)	9.6 (0.40)	11.7 (0.54)	18.3 (0.91)	0.17
Group T	6.6 (0.23)	9.7 (0.49)	11.2 (0.49)	17.5 (0.95)	0.16
Significance					NS

NS Not Significant
SE Standard Error

9.35 FARM 4.

Results of tick counts are shown in figure 5.2d and table 16. Tick numbers were low throughout the trial period, mean numbers of adult ticks attached to untreated lambs varied between 0 and 2.8 per lamb. The percentage of ticks attached to different sites on the lambs is shown in

ticks attached to different sites on the lambs is shown in table 17. All ticks found on days 0, 14 and 28 were attached to the axillae and inguinal region as were the majority of ticks recovered 54 days post treatment of group T.

Table 16. Cyhalothrin Pour-on for the Control of Sheep Ticks - Farm 4.

	Mean no. of ticks per lamb			
Days after treatment of group T.	0	14	28	54
*Group P	0.05	0.08	0.44	2.0
+/- SE	0.05	0.08	0.44	0.69
% infested	5	8	11	71
Group C	0	1.00	0.40	2.8
+/- SE	0	0.33	0.4	0.55
% infested	0	67	20	100
Group T	0.04	0	0	0.9
+/- SE	0.04	0	0	0.29
% infested	8	0	0	43
Significance				
Mean no. ticks.	NS	NS	NS	NS
Infestation.	NS	p<0.001	NS	p<0.01

SE Standard error.
NS Not Significant
* treated a median of four days before group 3.

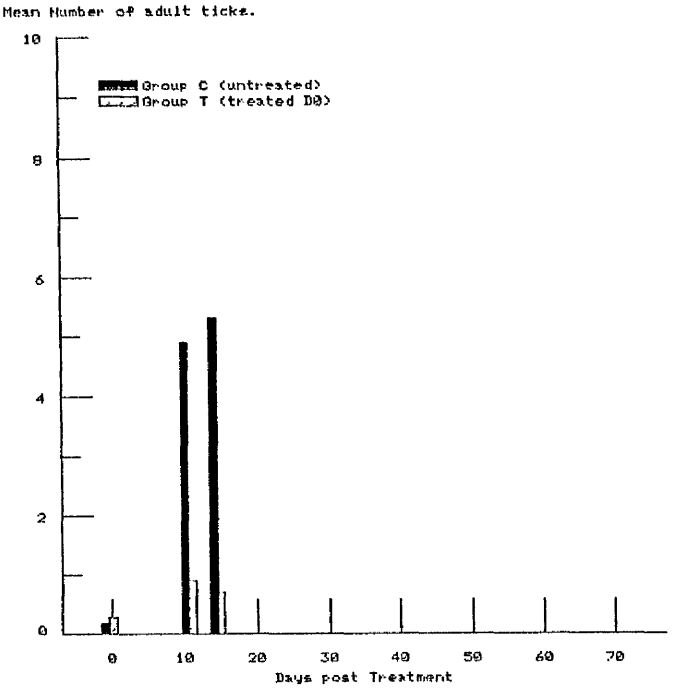
Table 17. Distribution of Ticks on Lambs at Farm 4.

	% Ticks per site											
Days after Treatment	0			14			28			54		
	H	A	G	H	A	G	H	A	G	H	A	G
Group p	0	100	0	0	0	100	0	0	100	7	64	29
Group C	0	0	0	0	62	38	0	0	100	16	60	24
Group T	0	100	0	0	0	0	0	0	0	12	62	28
Significance	NS	***	NS	NS	***	***	NS	NS	NS	NS	NS	NS

* p<0.05; ** p<0.01; *** p<0.001; NS Not Significant.
H Head; A Axillae: G Inguinal region.

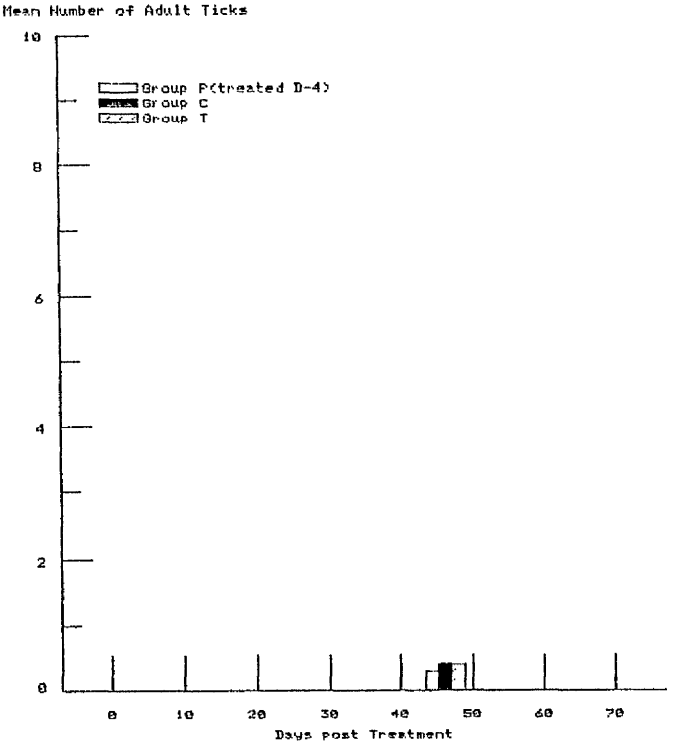
Cyhalothrin Trial 1986
Farm 1

Figure 9.2a



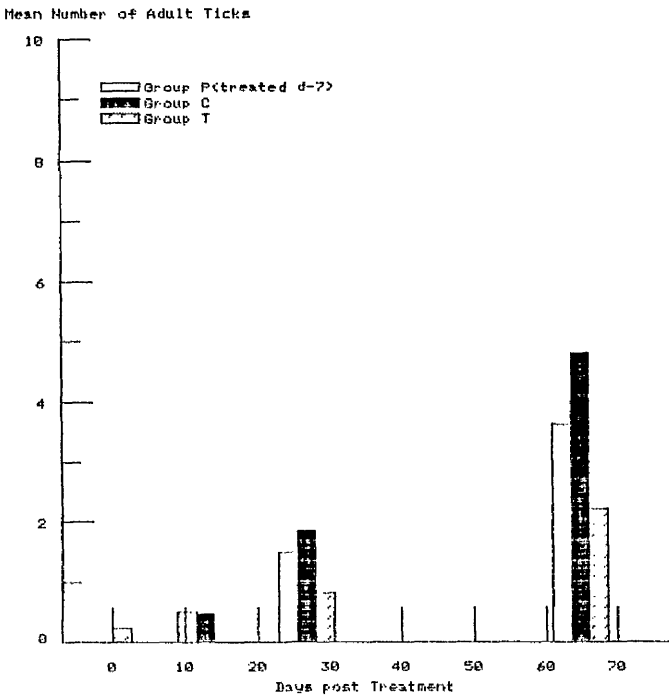
Farm 2

Figure 9.2b



Cyhalothrin Trial 1986
Farm 3

Figure 9.2c



Farm 4

Figure 9.2d

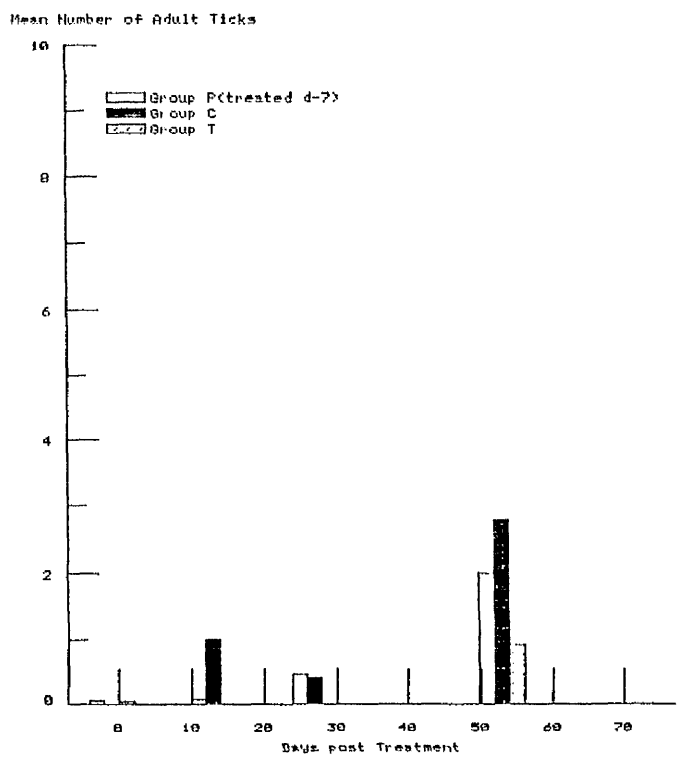


Figure 9.2 illustrates the mean number of adult ticks attached to:

untreated lambs (group C)
lambs treated with cyhalothrin on day 0 (group T)
lambs treated with cyhalothrin on day - 4 (group P)

on four farms and at various times after application of the product.

9.351 FATE OF LAMBS.

The fate of lambs in the trial is shown in table 18, 4 cases of tick pyaemia were confirmed at post mortem examination and the fate of 10 lambs was unknown.

Table 18. Fate of Lambs - Number Gathered/Number in Group.

GROUP P	14/19 (3/14pyaemic)	1 dead, 4 fate unknown
GROUP C	9/12	3 fate unknown
GROUP T	23/25 (1/23 pyaemic)	1 known not gathered, 1 fate unknown

9.352 LIVEWEIGHTS.

Mean weight gains for each group and the mean daily weight gain over the trial period are shown in table 19. The mean daily weight gain varied between 0.16 and 0.19 Kg per day and no significant diffences were recorded between groups.

Table 19. Live Weight Gains Over the Trial Period on Farm 4.

Mean Weight Gain in Kg (SE)				Mean daily weight gain
Days after Treatment 0	14	28	54	
Group P 4.9 (0.24)	6.9 (0.31)	9.4 (0.33)	13.2 (0.47)	0.16
Group C 5.2 (0.36)	7.3 (0.58)	8.9 (1.16)	15.4 (1.09)	0.19
Group T 5.1 (0.22)	7.2 (0.29)		14.1 (0.65)	0.17
Significance				NS

NS Not Significant
SE Standard Error

9.36 BIOCHEMICAL AND HISTOPATHOLOGICAL RESULTS.

On April 28 1986 (approximately four days after treatment) a random four of twenty group P lambs were gathered on farm 3. All four appeared clinically ill and biochemistry showed elevated levels of the enzyme aspartate transferase (AST) and blood urea, indicative of liver and kidney damage (Mitchell pers. comm.). One of the lambs died in early June, but was not available for post mortem examination. A further lamb from this group developed tick pyaemia and was examined post mortem on 3.7.86. Histopathological examination of the liver and kidney revealed fatty degeneration and hepatotoxic changes in the liver and extensive proximal tubular necrosis in the renal cortex (Brodie,pers.comm.).

9.37 FLEECE STAINING.

Staining of the fleece over the treated area was noted in the majority of lambs from all trial farms, although skin lesions were not present. The stain was of an 'oily' nature and allowed dust and other debris to adhere to the fleece.

9.38 TICK PYAEMIA.

During 1986, 203 lambs were involved in the cyhalothrin trials; 48 untreated controls and 155 treated with the product. Tick pyaemia was confirmed by post mortem examination in 10 treated (6.4%) and 1 control (2.1%) lamb. Table 20 shows the percentage of lambs in each group which were affected.

In all cases staphylococcal abscesses were present in joints, perhaps not surprising since lameness was the main criterion for clinical diagnosis. The following tissues were

Table 20. Tick Pyaemia Cases - Cyhalothrin Trials.

Group	Percentage of lambs confirmed to have tick pyaemia
P	10.2% (6/59)
C	2.1% (1/48)
T	4.2% (4/96)
Significance	NS

NS Not Significant,

also affected in one or more lambs: liver, spleen, spinal cord, diaphragm, tendon sheaths and muscle surrounding affected joints (Plate 11). All pyaemic lambs had gross splenomegaly indicative of recent tick-borne fever infection and either variable numbers of ticks attached (between 1 and 20) or evidence of recent tick bites. In one case *Pasteurella haemolytica* was isolated from the lungs although macroscopic changes were absent.

9.39 TICK-BORNE FEVER.

Samples were tested for tick-borne fever by examination of Giemsa stained smears of peripheral blood to demonstrate acute infections and serologically by CIE (5.263) to detect less recent infections. Results of these tests are shown in table 21. On no occasion was a sample positive both on smear and serologically.

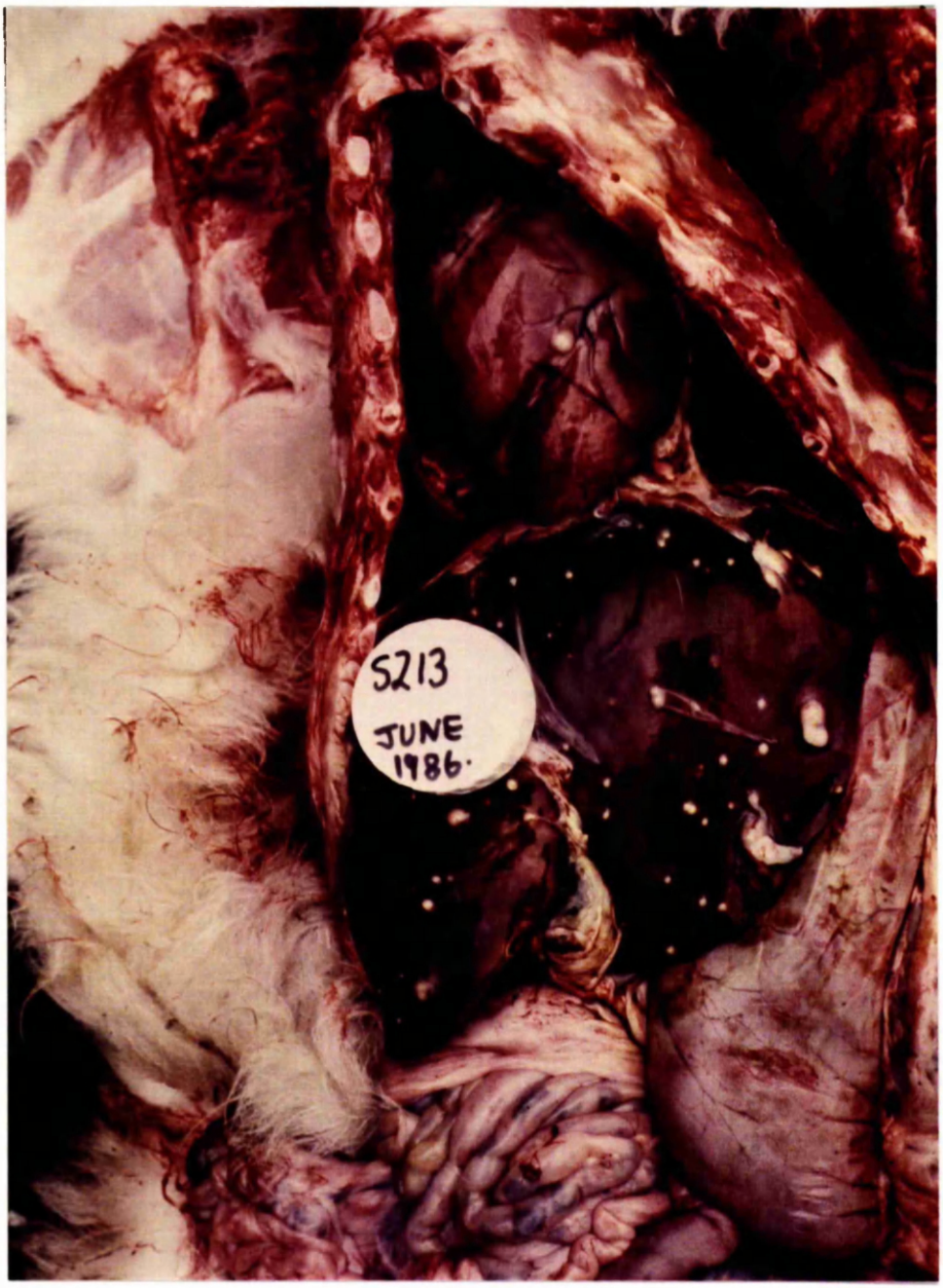


Plate 11 Field case of tick pyaemia showing distribution of lesions.

Table 21.Tick-borne Fever Diagnosis.

	Date	n	% positive Smear	Serology
Farm 1	13.5.86	7	28	0
	29.5.86	7	28	0
	13.6.86	8	0	62
Farm 2	22.4.86	11	0	0
	9.5.86	10	0	0
	29.5.86	4	25	0
	16/6/86	8	0	50
Farm 3	10.4.86	17	0	0
	13.5.86	11	82	0
	27.5.86	14	28	21
	2.7.86	20	5	75
Farm 4	10.4.86	2	0	0
	13.5.86	7	28	0
	27.5.86	10	50	20
	25.6.86	15	0	27

Tick-borne fever positives were equally likely to come from any of the three groups although the timing of infection varied between groups. Results from farm 3 are given in more detail in table 22.

Table 22. Tick-borne Fever Diagnosis.

	Positive samples. Smear			Serology		
Date	P	C	T	P	C	T
10.4.86	0	0	0	0	0	0
13.5.86	5/7	2/2	2/2	0/7	0/2	0/2
27/5/86	2/7	1/4	1/3	2/7	1/4	0/3
2/7/86	0/7	0/6	1/7	6/7	5/6	4/7

9.4 DISCUSSION.

Synthetic pyrethroid pour-ons have several advantages providing their efficacy proves equal to that of organophosphate (OP) dips traditionally used for tick control. Mammalian toxicity levels are much lower than for OP substances , which are the active ingredients of traditional dips, and therefore synthetic pyrethroids are inherently safer for the treated animal and the handler (MAFF,1984). Application of acaricide via a pour-on is less stressful (for all concerned) than plunging heavily pregnant ewes through a dipper or whole body immersion of lambs with the associated problems.

Deltamethrin, Cyhalothrin and other synthetic pyrethroids act by inhibiting the sodium potassium pump in nerve fibres causing a build-up of sodium within the axon, continual firing of the nerve and consequent paralysis of the affected organism (Ware,1978).

1985 DELTAMETHRIN TRIAL

The tick habitat on farm 1 was extensive (chapter 3, Webster *et al* ,1985) and tick attachment had occurred apparently overnight when hoggs were held in inbye fields prior to treatment with deltamethrin (figure 9.1). When hoggs were examined on days 13, 27 and 41 after treatment, there were at least twice as many adult ticks attached to controls as to treated hoggs (table 3), however by day 41 there was an average of 7.5 adult *I.ricinus* on treated hoggs, an unacceptably high figure.

The majority of ticks were attached to the heads of treated hoggs and before day 41 ticks were absent from the axillae (table 4). This may be related to the way the

deltamethrin formulation spreads over the skin surface from the point of application rather than acting systemically. Jenkinson *et al* (1986) showed that cypermethrin applied topically to the dorsal surface of sheep moved radially across the skin within the stratum corneum of the epidermis at a rate which exceeded 11 cm h^{-1}. This spread was accompanied by a degree of dermal infiltration which was most marked at the point of application. It appears likely that the deltamethrin formulation spreads in a similar manner, although not necessarily at the same rate. The face and heads of deltamethrin treated hoggs received a lower level of protection than the axillae and inguinal regions, presumably related to spread of the product over the body surface. It will also take several hours for a product applied at one point to spread to all peripheral regions of the treated animal.

Pour-on treatment with this deltamethrin formulation would appear to be a useful alternative to dipping for tick control as satisfactory results were obtained up to day 41 post treatment.

1986 CYHALOTHRIN TRIAL

During the spring of 1986 tick challenge was lower than in the previous year and on farms 2, 3, 4 insufficient, especially early in the season , to show any significant differences between the number of ticks attached to treated and control lambs. However on farms 1,3 and 4 significant differences were noted between the percentage of lambs in treated and control groups which were infested with adult female *I.ricinus* approximately two weeks after treatment. On farm 1 lambs were retreated at the 'marking gather' on

June 2 1986 when many treated lambs were found to be infested with *I.ricinus*. On this farm the lambs were treated with cyhalothrin approximately 2 to 3 weeks later than on the other three farms and significant differences between numbers of adult ticks attached to treated and control lambs ($p < 0.01$, $p < 0.01$) on days 15 post treatment and 11 post retreatment respectively were noted.

No significant differences were found between the sites of attachment in treated or control lambs probably due to the low tick challenge during the trial period.

No differences were noted between the mean daily weight gains or the incidence of tick-borne diseases in groups of lambs on the same farm. There were however, several adverse reactions. On farm 3 a number of the group P lambs (at least 4/20) treated at birth by the farmer became clinically ill and showed biochemical and histopathological evidence of liver and kidney damage. This phenomenon only occurred on farm 3 where lambs were treated immediately after birth, whilst their coats were still wet. The other farmers treated lambs when they were a few hours old. The reasons for this reaction are inapparent and merit further investigation.

However Piercy (toxicologist, Coopers Animal Health) (pers.comm.) stated that " there is little likelihood that the signs seen in the four observed lambs were related to cyhalothrin intoxication. The pyrethroids are basically neurotoxins, and then only in high overdose in mammals. Hepatoxicity and nephrotoxicity, as reported in the present trial are most likely associated with poisoning due to other chemicals/ingesta, or to staphylococcal pyaemia.

Young lambs have been dosed with 5x the therapeutic dose without any ill effects. I doubt the wetness in newborn lambs would alter the safety of the pour-on since the fluid would probably trap the active ingredient extradermally and the stratum corneum would be an effective barrier."

To be of value in the field situation a product is required which can be used to treat lambs born on the hill soon after birth and also older lambs born on tick-free inbye ground as they are turned onto tick infested hill at 2 - 3 weeks of age. Farmers may be reluctant to treat very young lambs of 'first time' mothers in case they desert their lamb on the hill due to the trauma of separation soon after birth. The other problem with the cyhalothrin formulation was fleece staining which occurred in most treated lambs on all farms. The treatment site had an oily deposit and while no skin lesions of the type reported by Britt *et al* (1984) caused by a deltamethrin product (Clout, Wellcome, Australia) formulated with xylene, cyclohexanone and corn oil, were produced , the oily deposit allowed dust and other debris to adhere to the affected area of the fleece and produced an unsightly stain. Discolouration of a similar nature was noted by Leibisch (pers. comm.) who conducted trials in Germany using the same cyhalothrin product as described in the present study. The stain was seen 48 hours after treatment when sheep were exposed to sunlight and was not encountered when sheep were housed indoors.

A total of eleven cases of tick pyaemia from all farms were confirmed at post mortem examination ; 10.2% of group P lambs (treated soon after birth by the farmer), 2.1% of

group C lambs (controls) and 4.2% of group T (treated) lambs became pyaemic. Lameness was the principal presenting clinical sign and in all cases joints were arthritic. Additionally liver, spleen, spinal cord, diaphragm and tendon sheaths or muscles surrounding affected joints were also affected in several cases. This differs from experimental infections (chapter 8) when the lungs were affected in all cases and the liver in 50% of lambs. The experimentally induced cases of tick pyaemia did not exhibit severe clinical symptoms and in the present trial such lambs would probably not have been removed from the hill for post mortem examination. The number of similar lambs in the field , with internal staphylococcal abscesses is open to speculation.

The incidence of tick pyaemia does not appear to be related directly to tick challenge as the farm with the greatest number of tick pyaemia cases (farm 3) did not have the greatest tick challenge as measured by tick counts on sheep. This was reported by Foggie (1959).

Tick-borne fever infection was diagnosed using 2 methods; acute cases were detected by the examination of Giemsa stained smears of peripheral blood whilst less recent infections were monitored serologically using CIE. The number of positive smears on each farm ranged from 0 to 82% the latter taken on 13.5.86 at farm 3. On the other farms the percentage of smears which were positive when lambs were between 3 and 4 weeks old was much lower. Foggie (1948) reported that maternally derived anti staphylococcal antibody would have waned by this time leaving lambs highly susceptible to staphylococcal infection. On no occasion was

an animal positive on smear and serology on the same date.

Experimentally, the causative organism of tick-borne fever was demonstrated in blood smears 4-5 days after intravenous infection (chapter 5), but humoral antibody was not detected until day 9-11. In field outbreaks of tick-borne fever serum antibody was recorded 2-4 weeks after the appearance of *C.phagocytophila* in peripheral blood smears. Allowing for errors associated with the infrequency of sampling , these findings are consistent with experimental infections. The timing of the tick rise on farm three, a high incidence of tick-borne fever infection when lambs were at a critical age of 3-4 weeks and displaying a breach in their defence against staphylococcal infection (Foggie,1948) may account for the high losses on farm three of about 10% of lambs, due to tick pyaemia.

On farm 1 where the tick challenge was present early in the trial, cyhalothrin treatment significantly reduced numbers of adult ticks which attached for at least 15 days. The number of lambs infested with *I.ricinus* on farms 1, 3, and 4 was reduced by cyhalothrin treatment for a similar period. There was however, no effect on the incidence of T.B.F. and cases of tick pyaemia still occurred.

In conclusion therefore, treatment with the deltamethrin pour-on provided a pronounced residual effect and efficacy appeared to be equivalent to traditional dipping controls. Cyhalothrin did not appear to be as effective, although these trials were conducted using lambs and direct comparisons cannot be made. The pour-on method has several advantages over dipping, principally its ease

of use, the reduction of stress factors for treated animals and handlers and a saving of labour which is especially valuable on small farms without convenient dipping facilities.

CHAPTER 10. GENERAL DISCUSSION

GENERAL DISCUSSION.

A recent survey conducted by the North of Scotland Agricultural College in conjunction with the Department of Agriculture Fisheries and Food for Scotland. (Vipond and Gunn, 1985) asked hill sheep farmers to list the most important problems limiting production. The outright 'winner' was the sheep tick *Ixodes ricinus* and tick-borne diseases. Whether or not this is indeed the case, sufficient interest was created for a second survey at the beginning of this project. The latter was conducted during the spring of 1985 and is described in chapter 4. It highlighted a very large tick problem in Argyll together with a significant problem in Ayrshire, which is not traditionally regarded as a 'ticky' area. Farmers were clearly aware of tick associated diseases and were trying to combat them using dips (at that time pour-ons were just becoming available) and if necessary louping ill vaccine.

The aim of the present project was to examine the ecology of *I.ricinus* and associated disease problems in south west Scotland, the findings of which will now be discussed together with contemporary studies.

ECOLOGY OF I.RICINUS.

A colony of *I.ricinus* was established and maintained in the laboratory (chapter 2); larvae and nymphae were allowed to engorge upon mice, adult females on lambs or calves. This provided known T.B.F. free or, if desired, probable T.B.F. infected ticks for electron microscopic observations and transmission experiments. An opportunity was taken to measure development times for different stages and these were compared with those reported by Lewis (1980) and found

to be slightly shorter. This was attributed either to differing regimes of temperature and humidity or to different strains of ticks from different geographical regions which were used to start the respective colonies.

Chapter 3 describes ecological studies which were carried out at two field sites in Ayrshire during 1984-1986. Activity was monitored by counts of ticks attached to sheep and by blanket drags of pasture areas. Seasonal activity varied greatly from site to site and from year to year, but to a large extent could be explained in terms of climatic data. During both 1984 and 1986 a unimodal pattern of activity was recorded . In 1984 tick numbers peaked in mid-June and continued at a lower level until early November. Prolongation of the activity season in 1984 is difficult to explain, but may have been associated with the relatively hot, dry summer of that year. During 1986 ticks were recovered from the blanket between April and September with a peak in mid-June. The lack of activity in the autumn was associated with cold temperatures which prevented activity in that season. In 1985 there was a bimodal pattern of activity with a marked spring peak and a lesser autumnal rise when warm, mild conditions favoured tick activity.

Development times were monitored in the field using the methods applied by Gray (1981) in Ireland. All metamorphoses and therefore new additions to the questing population occurred in the autumn, supporting the work of Gray (1981,1982,1984). In general from the results obtained and described in chapter 3, it is apparent that in favourable years there may be a cross-over between spring

and autumn feeders. Thus a tick could feed in spring, moult to the next instar in late summer (August) and feed again a few weeks later in autumn. This concurs with a report by MacLeod (1939a) that the length of *I.ricinus* lifecycle varies from 2 to 4 1/2 years in Britain.

On site and local meteorological data was monitored and an attempt was made to predict development times using the model developed by Gardiner and Gettinby (1983). This did not prove possible due to the lack, among other things, of daily on site recordings of soil temperatures. Based upon these results the accurate forecasting of development based upon widely available meteorological data does not seem feasible at present.

TICK-BORNE DISEASES.

Until the present study it had been generally assumed that most ticks were infected with tick-borne fever. For example, Macleod and Gordon (1933) reported that two female ticks are sufficient to produce infection in a sheep. Field studies (described in Chapter 6) demonstrated T.B.F. serologically or by examination of blood smears from a proportion of sheep on all farms studied in any detail, lending some support to this tenet. In order to quantify this hypothesis electron microscopic techniques were used to examine unfed *I.ricinus* instars for *Cytoecetes phagocytophila* and to measure the level of infection in a population of *I.ricinus* on one farm (described in chapter 7). Approximately one third of nymphae and adults were found to be infected whilst all larvae examined were uninfected. This finding incidentally, corroborates the

non-occurence of transovarial transmission proposed by MacLeod and Gordon (1933).

Ouheilli *et al* (1982) reported that oviposition, egg hatching and survival of newly hatched larvae was reduced in *Boophilus annulata* which were parasitised by *Babesia bigemina* or *Babesia bovis*. It is known that *Babesia* spp. multiply and develop within *Boophilus* and that such development is accompanied by cytopathogenic effects (Hoffmann,1971) leading to the death of a percentage of infected ticks (Muangyai,1974). A similar effect may account for the decreasing infection levels of T.B.F.in nymphal (44%) and adult (32%) *Ixodes ricinus* in the present study. However, the disparity in infection levels may simply be due to the small sample size. Assuming the levels of infection on farm 1 (1/3 of nymphae and adults infected with *C.phagocytophila*) are typical of tick infested areas then to be effective, control measures must be extremely efficient, literally preventing the attachment of all ticks in order to control disease transmission.

In Chapter 5 monitoring of experimental T.B.F. infections in young lambs, detected the frequently described lymphocytopaenia and neutropaenia following T.B.F. infection. Not only are absolute numbers of neutrophils reduced but additionally a depression in neutrophil function was recorded, demonstrated by an *in vitro* assay, during the the parasitaemic phase of T.B.F. infection ie. before the neutropaenia was manifest. This confirms the work of Foster and Cameron (1970a) who used a skin window technique to demonstrate a reduction in diapedesis.

A counter immunoelectrophoresis (CIE) technique was developed and tested with sera from primary experimental infections. The lag period (9-11 days) before which antibody was detectable was characteristic as was the period of antibody persistence (6-10 weeks in sheep). On no occasion was a false positive detected using pre-infection serum.

Following a homologous challenge infection of T.B.F., administered 16 weeks after the initial primary infection (ie. some weeks after the disappearance of the primary antibody response), antibody was soon detected by CIE at low levels for two to three weeks only after secondary challenge. Perhaps this low level of detectable humoral response was sufficient to eliminate the challenge infection or, alternatively there may have been some cell mediated component which was not measured.

The response of the immune mechanism to *C.phagocytophila* is obscure and requires further investigation particularly because of the existence of the carrier state which lasts for several months. Thus inoculation of blood from a convalescent sheep which is not displaying pyrexia or parasitaemia into a naive animal results in the production of T.B.F. It is probable that the carrier state, whereby T.B.F. organisms are present at low levels, presumably affording protection to the host via non-sterile immunity, and the unusual pattern of antibody production following secondary homologous challenge are in some way linked.

Field studies on tick-borne fever are described in chapter 6. Several sheep farms in Ayrshire with tick

problems were chosen from those replying to the survey. From these samples of sera were obtained and tested using CIE. CIE serology was also requested by several veterinary practitioners on suspect samples from other tick infested farms. The majority of positive samples were collected in June and July which suggests that infection probably occurred approximately 2 weeks before the first positive sample was detected. Examination of blood smears for *C.phagocytophila* detected the majority of positive samples in May and early June also suggesting that lambs become infected at this time.

Toumi (1966) stated that different strains of T.B.F. which are not cross-protective, but may differ in the severity of symptoms and the degree of immunity produced, may co-exist on the same farm. Different strains of T.B.F. cannot of course be separated by examination of blood smears. Positive blood smears could therefore represent infection with a second strain. In our results all of the samples tested originated from tick infested areas and were taken throughout the year. A total of 18.2 % positives were recorded using CIE serology and 14.3% by examination of Giemsa stained smears of peripheral blood. The CIE method detected positive samples from Scotland and the north of England. All of these results suggest that the antigen (Old Sourhope strain) used in the CIE test either has several epitopes or one common antigenic binding site. On no occasion was a sample positive on smear and serology at the same time.

Since the use of CIE the detection rate of T.B.F. infections in sheep has doubled over the two year study

period. It has potential for use as a routine diagnostic test as it is simple. rapid and accurate. A small number of samples from goats (13/25), deer (1/102) and cattle (2/14) were positive on CIE serology, while additionally 1/5 blood smears from red deer was also positive on smear. This agrees with Foggie (1962) who demonstrated T.B.F. inclusions in blood smears from red deer on Rhum.

Gross splenomegaly was a constant feature of T.B.F. infection (chapter 5), although the reasons for this are unknown. Hudson (1950) reported a slight enlargement of the Malpighian corpuscles in the spleen and that the lymphoid tissue had the appearance of being "suddenly drained" of the many lymphocytes usually present; thus the cuffs of lymphocytes around the reaction centres were less dense and frequently narrower than normal and the pulp of the spleen appeared to have a little oedematous fluid separating the elements between sinusoids. The sinuses of the lymphatic glands were dilated with fluid and contained lymphocytes of normal appearance, together with an unusually large number of large round cells. Limited histopathology in the present study (Hunter, pers.comm) did not substantiate these findings.

The most important effect of T.B.F. is immunodepression which allows invasion by pathogens such as *Staphylococcus aureus* and the subsequent development of tick pyaemia. A laboratory model was used to artificially simulate the B-lymphocyte depletion accompanying T.B.F. infection. Initial experiments described in chapter 8 using mice demonstrated increased colonisation by *S.aureus*. This was especially marked when *S.aureus* infected ticks rather than needle

inoculation were used to introduce the staphylococci to a group of mice treated with cyclophosphamide to depress B-lymphocytes compared to untreated controls. One third of the CY pre-treated group died from which greater numbers of staphylococci were recovered on culture compared with the control group. By contrast when staphylococci were introduced by painting a suspension of *S.aureus* onto the abdomen of mice and pricking the area with a sterile needle, there were no deaths in the CY pretreated or control groups and few staphylococci were recovered on culture. Assuming that the doses of *S. aureus* administered by the two methods are broadly comparable, the importance of the tick in disease transmission is underlined. Perhaps secretions produced by the tick mouthparts facilitate the establishment of staphylococci.

Tick pyaemia was produced experimentally for the first time in lambs using *I.ricinus* as a mechanical vector of *S.aureus*. The experiment involved two groups of lambs, one of which was infected with T.B.F. five days before *S.aureus* contaminated ticks were allowed to attach and feed. Abscesses from which staphylococci were recovered were present in all the lambs which were pre-infected with T.B.F., but absent from lambs which had experienced *S.aureus* contaminated ticks only. Foggie (1962) stated that lambs were one hundred times more susceptible to the intravenous inoculation of staphylococci during the neutropaenic phase of a T.B.F. infection, but did not produce the syndrome using intradermal, or subcutaneous inoculation routes. It is believed that a prior T.B.F. infection creates an environment in young lambs, not fully

immunologically competent, which allows dissemination and establishment of *S.aureus*. These gain access either directly through inoculation on the tick's hypostome or indirectly through the wound caused by the tick bite. Anticoagulants and enzymes secreted by the tick during feeding may aid this process. The timing of T.B.F. infection in relation to challenge with *S.aureus* appears to be critical; if staphylococci encounter the "open window" offered by an impaired immune response due to T.B.F. infection at a time when maternally derived immunity to staphylococci has waned and prior to the development of immunocompetence, establishment occurs and tick pyaemia ensues. If however, the immune response is competent staphylococci are quickly eliminated.

CONTROL.

If the onset of tick activity could be predicted, application of control measures could be timed to maximise their efficacy. Most acaricidal preparations, currently available protect lambs for 3-4 weeks (Veterinary Data Sheet Compendium,1987-88). On hill farms lambs are born in late April when ticks are often active, and lambs should therefore be treated as soon as possible. The ideal acaricide should have a rapid 'knock-down' effect, to prevent attachment of ticks and transmission of disease pathogens and this should be combined with long-term persistence to control ticks during the whole activity season (usually 8-12 weeks). Acaricidal treatment however, which requires frequent application is not practical when lambs are on the hill and often not gathered for weeks at a time.

In the present studies the deltamethrin pour-on formulation was shown to have an efficacy against ticks similar to that of traditional dipping methods, significantly reducing the tick burden of treated hoggs for a period of 41 days. The cyhalothrin pour-on formulation was less satisfactory, but the latter trials were conducted on lambs and are not directly comparable. The pour-on method appears to offer an alternative to dipping which is especially valuable where the farmer wishes to reduce the stress involved in dipping proceedures or where there is a shortage of labour. As mentioned in chapter 9, a combination of strategically timed tick control related to activity and antibiotic prophylaxis is the best weapon currently available to combat T.B.F/tick pyaemia.

The experimental tick pyaemia model in lambs described in chapter 8 may be of value in elucidating the precise pathogenesis of tick pyaemia and may also provide accurate information for strategic prophylaxis.

The possibility of developing a vaccine against ticks or T.B.F. in the near future poses a number of problems these include the necessity of immunising either very young lambs not fully immunologically competent or of vaccinating ewes and relying on transfer of immunity from ewe to lamb. Colostrum has been shown to prevent clinical disease, but only when ewes have been hyper-immunised (Brodie,1985). This does not seem to occur naturally in the field. However, with the future development of tissue culture techniques for *C.phagocytophila* and the use of a suitable adjuvant this situation may change.

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APPENDICES.

APPENDIX 1.

BRITISH TICKS.

ARGASIDAE.

Argas (carios) vespertilionis (Latreille 1802)

Argas reflexus reflexus (Fabricius 1794)

Ornithodoros capensis Neumann 1901

IXODIDAE.

Ixodes ricinus (Linnaeus 1758)

Ixodes festai Rondelli 1926

Ixodes caledonicus Nuttall 1910

Ixodes hexagonus Leach 1815

Ixodes canisuga Johnston 1849

Ixodes lividus (C.L. Koch 1844)

Ixodes arboricola Schulze and Schlottke 1929

Ixodes dorriensmithi Turk 1948

Ixodes arvicolae Warburton 1926

Ixodes guernseyensis Arthur 1955

Ixodes pari Leach 1815

Ixodes unicavatus Neumann 1908

Ixodes rothschildi (Nutall and Warburton 1911)

Ixodes vespertilonis C.L. Koch 1844

Ixodes (exopalpiger) trianguliceps Birula 1895

Ixodes (ceratixodes) uriae White 1852

Dermacentor reticulatus Fabricius 1794

Haemaphysalis punctata Canestrini and Fanzago 1877

Hyalomma (hyalommasta) aegyptium (Linnaeus 1758)

From Arthur, 1962.

APPENDIX 2.

BRITISH MAMMALIAN HOSTS OF ONE OR MORE STAGES OF *IXODES RICINUS*.

	man	*Homo sapiens*
Bovidae	cow	*bos*
	sheep	*ovis*
	goat	*capra*
Suidae	pig	*sus*
Cervidae	red deer	*Cervus elaphus*
	sika deer	*Cervus nippon*
	fallow deer	*Dama dama*
	roe deer	*Capreolus capreolus*
Equidae	horse	*Equus*
Canidae	dog	*Canis*
	fox	*Vulpes vulpes*
Felidae	cat (domestic)	*Felis*
	wild cat	*Felis silverstris*
Mustelidae	stoat	*Mustela erminae*
	weasel	*Mustela nivalis*
	ferret	*Mustela furo*
	badger	*Meles meles*
	otter	*Lutra lutra*
Leporidae	rabbit	*Oryctolagus cuniculus*
	brown hare	*Lepus capensis*
	mountain hare	*Lepus timidus*
Sciuridae	red squirrel	*Sciurus vulgaris*
	grey squirrel	*Sciurus carolinensis*
Cricetidae	bank vole	*Clethrionomys glareolus*
	field vole	*Microtus agrestis*
	water vole	*Arvicola terrestris*
Muridae	wood mouse	*Apodemus sylvaticus*
	common rat	*Rattus norvegicus*
Capromyidae	coypu	*Myocastor coypus*
Erinaceidae	hedgehog	*Erinaceus europaeus*
Talpidae	mole	*Talpa europaea*
Soricidae	common shrew	*Sorex araneus*
	pygmy shrew	*Sorex minutus*
	water shrew	*Neomys fodiens*

Rhiolophidae
} bats species ?
Vespertilionidae

minimum total : 35 species
Based on MacLeod (1932); Milne (1948a and b): Arthur (1963) and Corbet and Southern (1977).

In addition, the viviparous lizard *Lacerta vivipara* Jacquin has been recorded as a host of *Ixodes ricinus* (Milne 1948a).

BRITISH AVIAN HOSTS OF ONE OR MORE STAGES OF *IXODES RICINUS*.

	domestic poultry	
Falconidae	kestrel	*Falco tinnunculus*
	sparrowhawk	*Accipter nisus*
	merlin	*Falco columbarius*
Tetraonidae	red grouse	*Lagopus lagopus scoticus*
	black grouse	*Tetrao tetrix*
	capercaillie	*Tetrao urogallus*
Phasianidae	pheasant	*Phasianus colchicus*
	common partridge	*Perdix perdix*
	quail	*Coturnix coturnix*
Charadriidae	lapwing	*Vanellus vanellus*
	golden plover	*Pluvailis apricaria*
Scolopacinae	curlew	*Numenius arquata*
	woodcock	*Scolopax rusticola*
	common snipe	*Gallinago gallinago*
Laridae	black headed gull*	*Larus ridibundus*
Columbidae	wood pigeon	*Columba palumbus*
Strigidae	long eared owl	*Asio otus*
	barn owl	*Tyto alba*
Alaudidae	skylark	*Alauda arvensis*
Corvidae	magpie	*Pica pica*
	carrion (hoodie) crow	*Corvus corone*
	rook	*Corvus frugilegus*
	jackdaw	*Corvus monedula*
Paridae	great tit	*Parus major*
Turdinae	song thrush	*Turdus philomelos*
	redwing	*Turdus iliacus*
	blackbird	*Turdus merula*
	whinchat	*Saxicola ruberta*
	wheatear	*Oenanthe oenanthe*
	fieldfare	*Turdus pilaris*
	redstart	*Phoenicurus phoenicurus*
Prunellidae	robin	*Erithacus rubecula*
	dunnock (hedge sparrow)	*Prunella modularis*
Sylviidae	willow warbler	*Phylloscopus trochilus*
	common white throat	*Sylvia communis*
	chiffchaff	*Phylloscopus collybita*
	blackcap	*Sylcia atricapilla*

Motacillidae	meadow pipit	*Anthus pratensis*
	tree pipit	*Anthus trivialis*
Fringillidae	chaffinch	*Fringilla coelebs*
	linnet	*Carduelis cannabina*
	siskin	*Carduelis spinus*

* hatched on a grouse moor (Milne 1948a).

minimum total : 43 species drawn from MacLeod (1932); Milne (1948a and b); Arthur (1963) and Campbell and Watson (1964).

APPENDIX 3.

Table 1. Tick Counts on Sheep and Blanket Drags of Pasture - Site 1, 1984.

Date	Day	Mean Blanket Drag	Mean Count on Sheep	Comments
25.5	146	0	>50 A&N	Dipped 21/4
8.6	161	0	3.9 A&N (2A)	re-dipped
14.6	167	0		25/5
22.6	175	0		
27.6	180	0	4.5 A&N (1.3A)	
6.7	189	0		
13.7	195	0		
18.7	201	3A, 6N		
24.7	207	1A, 2N	16.7 A&N (12.8A)	
3.8	217	0		
13.8	227	0		
23.8	237	2A	5A&N (2.5A)	
30.8	244	0		
7.9	252	1N		
19.9	264	0	2.2A&N (1.1A)	
27.9	272	0		
9.10	284	0	1.2A&N (0.8A)	
19.10	294	0		
2.11	308	0	0.3A&N (0.1A)	
22.11	328	0	0	
29.11	335	0		
10.12	345	0		
19.12	354	0		

A adult

N nymphae

ND Not done.

Table 2. Tick Counts on Sheep and Blanket Drags of Pasture - Site 1, 1985.

Date	Day	Mean Blanket Drag	Mean Count on Sheep	Comments
9.1	9	0		
23.1	23	0		
30.1	30	0		
6.2	37	0		
14.2	45	0	0.08A	
21.2	52	0		
5.3	62	0		
14.3	73	0		
22.3	81	0		
4.4	94	0.5N	2.2A	
17.4	107	1.5A,38N,1L	6.6A ,7.8N	
26.4	116	1A, 15N		
1.5	121	12.5N	14.5A,1.4N	
9.5	129	7A,55N,25L		
15.5	135	0.5A,26N	17.5A 9.6N	
23.5	143	1.5A,17.5N		
3.6	154	19N	8.1A,0.4N	
12.6	163	0.5A,5N,1.5L		
7.7	188	7N	1.4A,0.17N	
11.7	192	0.5N		
18.7	199	0		
24.7	205	0		
15.8	227	0		
22.8	234	0		
30.8	242	1N	0.24A,0.05N	
11.9	254	0		
26.9	269	3.5N		
18.10	292	1.5N	2.4A,2.2N	
30.10	304	0		
13.11	318	0		
27.11	332	0	0	
11.12	346	0		

A Adult

N Nymphae

L Larvae

Table 3. Tick Counts on Sheep and Blanket Drags of Pasture - Site 1, 1986.

Date	Day	Mean Blanket Drag	Mean Count on Sheep	Comments
10.1	10	0		
12.2	43	0		
11.3	70	0		
19.3	78	0		
1.4	91	0.5A,1N		
7.4	97	0		
17.4	107	0	9.75A,0.25N	
24.4	114	0.5A,2N,0.5L		
13.5	133	3.5A,19N	0.16A	
28.5	148	1A,12N	5.3A	
13.6	164	3.5A,34.5N	4.7A	
4.7	185	0.5A,4.5N		
16.7	197	2N		
25.7	206	1.5N		
14.8	226	0		
25.8	237	1.5N,1L		
3.9	246	0		
11.9	254	0		
30.9	273	0		
13.10	286	0		
31.10	304	0		
20.11	324	0		

A Adult

N Nymphae

L Larvae

Table 4. Tick Counts on Sheep and Blanket Drags of Pasture - Site 2, 1985.

Date	Day	Mean Blanket Drag	Mean Count on Sheep	Comments
12.3	71	0		
22.3	81	0		
10.4	100	2A, 5.5N	2A on 1,000	sheep
18.4	108	9N		
26.4	116	0.5A,3N		
8.5	129	1.5A,4N		
16.5	137	2A,5N		
22.5	143	0.5A,1.5N	1.2A	
29.5	150	0.5A,4N		
7.6	159	0		
28.6	180	0		
18.7	200	0		
25.7	207	0		
15.8	228	0	0	
23.8	236	0		
9.9	253	0.5N		
17.9	261	0.5N		
30.9	274	1N		
10.10	284	0		
23.10	297	0		
1.11	306	0		
13.11	318	0		
3.12	338	0		
11.12	346	0		
24.12	359	0		

A Adult

N Nymphae

L Larvae

Table 5. Tick Counts on Sheep and Blanket Drags of Pasture - Site 2, 1986.

Date	Day	Mean Blanket Drag
16.1	16	0
12.2	43	0
11.3	51	0
19.3	59	0
7.4	97	0
24.4	114	0
21.5	141	0.5A,2N
5.6	156	0
18.6	169	0
4.7	185	0
16.7	197	0
24.7	205	0
14.8	226	0
24.8	236	0
3.9	246	0
30.9	273	0
13.10	286	0
31.10	304	0
20.11	324	0

Table 6a. Preoviposition Site 1.

Engorgement Period	Drop off host	Pre-ovip start	Completion Dates Obs	Pred	Error
Mar-June	21.3.84	22.3.84	17.4.84	17.5.84	-30
	18.5.84	19.5.84	4.6.84	16.6.84	-12
	26.5.84	27.5.84	4.6.84	21.6.84	-17
	26.5.84	27.5.84	13.6.84	21.6.84	-8
Sept	19.9.84	20.9.84	7.11.84	29.10.84	+9
	19.9.84	20.9.84	20.11.84	29.10.84	+22
Oct	10.10.84	11.10.84	dead	27.11.84	
Mar-June	1.4.85	2.4.85	22.5.85	25.5.85	-3
	1.4.85	2.4.85	2.6.85	25.5.85	+8
	12.4.85	13.4.85	14.5.85	30.5.85	-16
	12.4.85	13.4.85	22.5.85	30.5.85	-8
	12.4.85	13.4.85	2.6.85	30.5.85	+3
Oct	17.10.85	18.10.85	18.3.86	11.3.86	+7
	10.10.85	11.10.85	18.3.86	8.1.86	+69
	30.10.85	31.10.85	31.3.86	5.4.86	-5
	4.11.85	5.11.85	31.3.86	11.4.86	-11
Mar-July	9.4.86	10.4.86	3.7.86	5.6.86	+28
	9.4.86	10.4.86	24.7.86	5.6.86	+49
	17.4.86	18.4.86	24.7.86	7.6.86	+47
	12.5.86	13.5.85	13.8.86	17.6.86	+57
	26.5.86	27.5.86	3.7.86	25.6.86	+9
	26.5.86	27.5.86	24.7.86	25.6.86	+29
	3.7.86	4.7.86	16.7.86	28.7.86	-12
	3.7.86	4.7.86	14.8.86	28.7.86	+17

Table 6b. Preoviposition Site 2.

Engorgement Period	Drop off host	Pre-ovip start	Completion Dates Obs	Pred	Error
Mar-June	4.4.85	5.4.85	15.5.85	27.5.85	-12
	12.4.85	13.4.85	19.4.85	30.5.84	-41
Oct	18.10.85	19.10.85	4.4.86	21.3.86	+14
	18.10.85	19.10.85	5.6.86	21.3.86	+76
Mar-Jul	21.5.86	22.5.86	18.6.86	22.6.86	-4
	3.7.86	4.7.86	16.7.86	28.7.86	-12
	3.7.86	4.7.86	30.9.86	28.7.86	+63

Obs - observed date
Pred - predicted date

Table 7a. Egg Development Site 1.

Engorgement Period	Ovip period Begins	Completion dates Obs 1st hatch	Pred A	Pred B	Error
Mar-June	18.4.84	4.10.84	11.8.84	12.8.84	+53
	5.6.84	4.10.84	27.8.84	6.9.84	+28
	5.6.84	27.9.84	27.8.84	6.9.84	+21
Sept	8.11.84	30.8.85	28.9.85	20.7.85	+29
	21.11.84	30.8.85	28.9.85	10.7.85	+29
Mar-June	15.5.85 *	NH by 26/9	15.9.85	27.9.85	
	23.5.85 *	NH by 26/9	23.9.85	27.9.85	
	3.6.85 *	NH by 26/9	30.9.85		
Oct	11.12.85	30.9.86	5.10.86		-5
	19.3.86	25.8.86	7.10.86		-43
	1.4.86	25.7.86	8.10.86		-75

* Site disrupted early October 1985.

Table 7b. Egg Development Site 2.

Engorgement Period	Ovip period Begins	Completion dates Obs 1st hatch	Pred A	Pred B	Error
Mar-June	20.4.85	9.9.85	8.9.85	7.9.85	+1
Oct	4.7.86	8.9.86	*		
Mar-June	18.6.86	3.9.86	*		
	16.7.86	3.9.86			

* prediction not available.

Table 8a. Larval Development Site 1.

Engorgement Period	Drop off host	Dev. starts	Completion Dates Observed	Predicted
Mar-July	8.6.84	9.6.84	30.8.84	6.10.84
	6.3.85	7.3.85	26.9.85	4.10.85
October	16.10.85	17.10.85	3.9.86	
	21.10.85	22.10.85	28.8.86	*
	27.10.85	28.10.85	11.9 86	
Mar-July	7.4.86	8.4.86	28.8.86	27.10.86
	7.4.86	8.4.86	11.9.86	27.10.86
	27.5.86	28.5.86	3.9.86	*

* predictions not available.

Table 8b. Larval Development Site 2.

Engorgement Period	Drop off host	Dev. starts	Completion Dates Observed	Predicted
Mar-July	16.5.85	17.5.85	15.8.85	30.10.85
October	22.10.85	23.10.85	24.8.85	27.10.86
Mar-July	7.4.86	8.4.86	24.8.86	*
	27.5.86	28.5.86	30.9.86	

* predictions not available.

Table 9a. Nymphal Development Site 1.

Engorgement Period	Drop off host	Dev. starts	Completion Dates Observed	Predicted	
Mar-July	1.5.84	2.5.84	19.9.84	30.8.84	
	8.6.84	9.6.84	14.9.84	11.10.84	
				A	B
August	23.8.84	24.8.84	3.10.85	30.10.85	2.10.85
October	9.10.84	10.10.84	26.9.85	30.10.85	30.10.85
	10.10.84	11.10.84	11.9.85	30.10.85	30.10.85
Mar-July	3.4.85	4.4.85	22.8.85	2.10.85	
	4.4.85	5.4.85	26.9.85	2.10.85	
	12.4.85	13.4 85	26.9.85	4.10.85	
	17.4.85	18.4.85	10.9.85	7.10.85	
	17.4.85	18.4.85	26.9.85	7.10.85	
October	17.10.85	18.10.85	11.9.86	*	
Mar-July	27.5.86	28.5.86	13.10.86	*	

* predictions not available.

Table 9b. Nymphal Development Site 2.

Engorgement Period	Drop off host	Dev. starts	Completion Dates Observed	Predicted
Mar-July	12.4.85	13.4.85	15.8.85	4.10.85
	16.4.85	17.4.85	23.8.85	5.10.85
	22.4.85	23.4.85	17.9.85	9.01.85
October	18.10.85	19.10.85	3.9.86	*
	22.10.85	23.10.85	13.10.86	
Mar-June	22.5.86	23.5.86	13.10.86	*
	21.6.86	22.6.86	31.10.86	

* predictions not available.

APPENDIX 4.

1. BARBITONE BUFFER.

Twenty point six grams of sodium barbital and 0.7g disodium EDTA were dissolved in 1 litre of distilled water and the pH adjusted to 8.6.

2. DESTAINER (FOR CIE)

The destainer was a 9:9:2 mixture of methanol (or ethanol), distilled water and glacial acetic acid.

3. AMIDO BLACK.

2g Amido Black (B.D.H. Chemicals Ltd., Poole, England) was dissolved in 1 litre of destaining solution.

4. SORENSENS BUFFER.

Basic Salt - 0.06M solution of dibasic sodium phosphate (Na_2 HPO_4 $2H_2O$).

Acid Salt - 0.06M solution of monobasic potassium phosphate (KH_2PO_4)

To obtain a solution with pH 6.5, 40 ml of the basic salt were added to 65 ml of the acid salt.

5. BLOOD AGAR.

Thirty nine grams of Columbia agar (Oxoid) were suspended in 1 litre of distilled water and brought to the boil to dissolve completely. Sterilisation was achieved by autoclaving at 121'C for 15 minutes, when cooled to hand hot 50ml of ovine blood were added and plates poured. The plates were incubated at 37'C overnight to check for contamination and stored at 4'C until required.

6. MANNITOL SALT AGAR.

One hundred and eleven grams of Manitol Salt Agar (Oxoid) were suspended in 1 litre of distilled water and brought to the boil to dissolve completely. Sterilisation was achieved

by autoclaving at 121'C for 15 minutes, poured into petri dishes and allowed to cool. The plates were incubated at 37'C overnight to check for contamination and stored at 4'C until required.

7. BRAIN HEART INFUSION BROTH.

Thirty seven grams of brain heart infusion were added to 1 litre of distilled water mixed thoroughly and distributed into final containers before autoclaving at 121'C for 15 minutes.

8. DNASE AGAR.

Forty two grams of DNase test agar (Difco) were suspended in 1 litre of distilled water and brought to the boil to dissolve completely. 50 ml of 0.1% toluene blue was added. Sterilisation was achieved by autoclaving at 121'C for 15 minutes. The agar was allowed to cool slightly and poured into pertri dishes. The plates were incubated at 37'C overnight to check for contamination and stored at 4'C until required. Plates were innoculated by making a half to one inch streak across the agar with the test culture. A positive result was indicated by a colour change from blue to pink.

9. COAGULASE TUBE TEST.

The formation of fibrin clots is accelerated by a bacterial product known as coagulase, a pro-thrombin like enzyme present in more than 96% of staphylococci isolated from human infections (Cruikshank *et al*,1973).

Point five per cent (0.5%) citrated rabbit plasma was diluted 1:10 in sterile saline. 5 drops of an overnight broth culture of the test organism was added and the mixture incubated at 37'C for 6-12 hours. The mixture was

inspected hourly for coagulation. All tests were conducted with a known positive strain of staphylococci and a negative control of saline.

10. *IN VITRO* ANTIBIOTIC SENSITIVITY.

Thirty two grams of antibiotic sensitivity agar were suspended in 1 litre of distilled water and brought to the boil to dissolve completely. Sterilisation was achieved by autoclaving at 121'C for 15 minutes. The agar was allowed to cool slightly and poured into petri dishes. The plates were incubated at 37'C overnight to check for contamination and stored at 4'C until required. Sulphonamide and trimethoprin inhibitors are absent from sensitivity agar (Oxoid) which is designed to give large clear zones with all antibiotics without the addition of lysed or whole blood.

Four sensitivity agar plates were sown with the bacteria under test and streaked to give an even growth. One mastring-S for staphylococci was placed on two duplicate plates and one mastring-S special on two further plates. The plates were incubated at 37'C overnight and zones of inhibition noted.

Antibiotics used for the *in vitro* antibiotic sensitivity assay of *S.aureus*.

Mastring-S staphylococci.	Mastring-S special.
Tetracycline	Spectinomycin
Neomycin	Apramycin
Erythromycin	Synulox
Novobiocin	Leotrox
Streptomycin	Poltmixin B
Penicillin G	Framycetin Sulphate
Nafpenzal	Leored
Cefuroxime	Tetradelta

11. A.P.I.

'API STAPH' (Basingstoke, Hampshire) is a biochemical system for the identification of staphylococci and micrococci based on 19 characters. It consists of a strip containing dehydrated test substrates in individual microtubes. The substrates were reconstituted by the addition of an aliquot of API staph media that had been inoculated with the strain of staphylococci to be studied. The strip was then incubated for 18 hours at 37'C afterwhich the results wwere read and interpreted with reference to the information manual. Purity of the inoculation medium was assessed by streaking 2 blood agar plates with the inoculant and incubating them with the strip.

SUBSTRATES.

D-Glucose, D-Fructose, D-Mannose, Maltose, Lactose, D-trehalose, D_Mannitol, Xylitol, D-Melibiose, Potassium Nitrate, -Methyl Phosphate, Sodium Pyruvate, Raffinose, Xylose, Saccharose, -Methyl Glucoside, N-Acetyl Glucosamine, Arginine, Urea.

12.PHAGE TYPES.

Freeze dried samples of the original *S.aureus* used for initial infections and staphylococci recovered on culture of organs were typed by the staff of the bacteriology department, Central Veterinary Laboratory, Weybridge, Surrey. The bovine phage set (16 phages) was used at routine test dilution. The strain of *S.aureus* used was 'strongly' lysed by miscellaneous phage 78.

13.PREPARATION OF GROWTH CURVE.

A freeze dried ampoule which contained an α-β haemolytic strain of *Staphylococcus aureus* was opened, reconstituted with 0.5 ml sterile distilled water, inoculated onto blood agar plates, which were incubated overnight at 37'C. New plates were subsequently inoculated from the first batch of plates and incubated overnight as before. One colony of an α- β haemolytic *S.aureus* was placed in 100ml of Brain Heart Infusion Broth in a 250 ml conical flask at 37'C. At half hourly intervals for the first two and a half hours and then hourly until 8 hours after the broth was inoculated, 1 ml of the broth was withdrawn,diluted in sterile B.H.I. and aliquots inoculated onto triplicate blood agar plates. The plates were incubated at 37'C overnight, examined the following morning, numbers of colonies counted and the number of colony forming units (CFU) in the original broth calculated and plotted against time.

APPENDIX 5.

Table 1. Abscess Formation in Mice Inoculated with Varying Doses of *S.aureus*.

log_{10} *S.aureus*	Died/ killed	ABSCESSES Liv	lung	H	k1	K2	SP
6	D4 pm	+	+		+	+	enl
	D4 pm	+	+		+		enl
5	D1 pm		+				enl
	K D7	+					enl
4	D1 pm						enl
	K D7	+					enl
3	K D7						enl
	K D7						enl
2	K D7						enl
	K D7						

Liv. Liver.
H Heart.
K1 Left Kidney.
K2 Right Kidney.
SP Spleen.
M Muscle.
K sacrificed
D Day
enl enlarged.

Table 2. *S.aureus* Isolated from Tissues of Mice Infected with Varying Doses of the Organism.

$\log_{10}$

S.aureus dose.	Died/ killed	*S.aureus* J	Liv	Lung	H	K1	K2	SP
6	D4 pm		3+	3+	4+	4+	3+	3+
	D4 pm	4+	4+	4+	4+	4+	4+	2+
5	D1 pm		4+	+		+	+	
	D7		+					
3	D7							
	D7							
2	D7					2+	3+	
	D7		2+		+	+		

J. Joint.
Liv Liver.
H. Heart.
K1. Left Kidney.
K2 Right Kidney.
SP Spleen.
AB Abscess.

Table 3. Total and Differential Leucocyte Values of Normal Mice.

Total W.B.C	Differential				
	L	N	M	E	B
7,800	78	19	2	1	0
10,800	77	18	2	3	1
14,000	75	22	2	1	0
6,700	69	28	1	2	0
6,700	69	28	1	2	0
10.300	73	25	1	1	0
14,800	80	17	3	0	0
11,000	73	24	2	1	0
10,300	72	25	2	1	0
10,800	69	30	1	0	0
11,500	75	21	3	1	0

Table 4. Changes in White Blood Cell Numbers After the Administration of Cyclophosphamide.

Days post CY.	WBC $10^9/l$	mean WBC	sdi	% diff. WBC L	N	M	E	B	mean L X$10^9/l$
0		11,000		74	23	3	1	0	8140
	800			47	49	2	2	0	
1	1,000	1,255	350	21	76	0	2	1	447
	1,600			37	58	2	3	0	
	1,400			41	54	3	2	0	
	2,300			52	46	1	1	0	
2	3,400	2,375	713	47	51	1	1	0	1,122
	1,800			44	53	1	2	0	
	2,000			46	50	3	1	0	
	1,200			37	61	1	1	0	
3	1,300	1,150	129	34	60	2	2	1	322
	1,100			31	65	2	2	0	
	1,000			40	57	2	1	0	
	4,500			58	40	2	0	0	
4	2,700	3,525	750	42	55	2	1	0	1,709
	3,300			44	51	2	2	1	
	3,600			50	47	2	1	0	
	5,300			35	61	2	2	0	
7	4,800	5,025	222	32	64	3	1	0	1,696
	4,900			37	60	2	1	0	
	5,100			31	65	2	2	0	
	5,400			23	75	0	1	1	
8	5,300	5,600	316	38	62	0	1	0	1,638
	5,700			30	67	2	1	0	
	6,000			26	70	2	2	0	
	10,700			54	43	2	0	1	
11	9,200	9,050	1,287	63	25	3	1	0	5,000
	7,600			57	45	2	1	0	
	8,700			47	49	2	2	0	
	12,800			71	25	2	1	0	
14	12,000	11,125	1,552	80	17	3	0	0	8,010
	10,300			67	30	2	1	0	
	9,400			70	26	2	2	0	
	9,800			80	17	2	1	0	
16	10,100	11,075	1,315	71	26	2	1	1	8,112
	12,000			73	24	1	1	1	
	12,400			69	27	2	2	0	

WBC Total Leucocyte Count.
% L,N,M,E,B. Percentage Lymphocytes, Neutrophils, Monocytes, Eosinophils, Basophils.
Sdi= Standard Deviation.

Table 5. Post Mortem Findings from Mice Injected with an Immunosuppressive Dose of Cyclophosphamide Prior to Staphylococcal Infection.

CY	STAPH	DIED	LIV	LUNG	H	K1	K2	SP	M	AD
D -7	1.V	S D7						ENL		ENL
	I.V	S D7		P				ENL		ENL
	I.V	S D7						ENL		ENL
	I.V	S D7								ENL
D -5	I.V	S D7						ENL		
	I.V	S D7						ENL		
	I.V	S D7		P				ENL		
	I.V	S D7				+		ENL		ENL
D -3	I.V	D 2	+	+ P			+	ENL		
	I.V	D.2	+	+ P			+	ENL		
	I.V	D.2	+	+ P				+ENL		
	I.V	S D7		P				ENL		ENL
D 0	I.V	S D7								ENL
	I.V	S D7						ENL	+	ENL
	I.P	S D7						ENL		ENL
	I.P	S D7						ENL		

S Sacrificed.
P Pneumonia.
+ Abscess present.
ENL Enlarged.
STAPH *S.aureus*.
I.V. Intravenous.
I.P. Intraperitoneal.
Liv Liver.
H Heart.
K1 Left Kidney.
K2 Right Kidney.
SP Spleen.
M Muscle.
AD Adrenal Glands.

Table 6. Bacterial Isolations From Tissues of Mice Injected with Cyclophosphamide Prior to Staphylococcal Infection.

CY ADMIN	STAPH	DIED	LIV	LUNG	H	K1	K2	SP	J
D -7	I.V.	S D7							
	I.V	S D7							
	I.V	S D7							
	I.V	S D7							
D -5	I.V	S D7							
	I.V	S D7							
	I.V	S D7							
	I.V	S D7	+			3+	2+	+	
D -3	I.V	D2	4+		4+	2+	3+	3+	
	I.V	D2	3+		2+	4+	2+		
	I.V	D2	2+	2+	+	+	4+	2+	
	I.V	S D7	+		+	+	+		
D 0	I.V	S D7	+						
	I.V	S D7							
	I.P	S D7							
	I.P	S D7							

CY	Cyclophosphamide.
Staph	*S.aureus*.
I.V.	Intravenous.
I.P.	Intraperitoneal.
S	Sacrificed.
+	Abscess(es) present.
Liv	Liver.
H	Heart.
K1	Left kidney.
K2	Right Kidney.
SP	Spleen
J	Joint.

TABLE 7. Post Mortem Findings from Mice Injected With Various Doses of Staphylococci Administered by Various Routes With and Without Prior Injection With Cyclophosphamide.

CY	DOSE STAPH	DIED	ABSCESSES LIV	LUNG	H	K1	K2	SP	LOCAL
		D2							
		D3							
	6	S D7							
		D7			+	+	+	+	
	I.V.	D8			+	+			
		S D9							
		D2	+		+	+			
		S D9							
	4	D4			+	+	+		
		S D9							
	I.V.	D2							
		D3	+	+				+	
		S D9							
		S D9							
	2	S D7							
		S D7							
	I.V.	S D7							
		S D7							
		S D9							+
		S D9							+
	6	S D7							+
		S D7							+
	I.D	S D7							+
		S D7							+
		S D7		+					
		S D7			+				
	6	S D9		+					
		S D9							+
	S.C	S D7							+
		S D7							+
		S D7							
		S D7							
	TICK	S D7							
	L.	S D7							
		S D9							
		S D9							

Table 7 continued.

CY	DOSE STAPH	DIED	ABSCESSES LIV	LUNG	H	K1	K2	SP	LOCAL
		S D7							
		S D7							
	PAINT	S D7							
		S D7							
		S D9							
		S D9							
+		D1							
+		D2				+			
+	6	D1							
+	I.V.	S D9							
+		D1							
+		D2		+			+	+	
+		S D9							
+		D1		+					
+	4	D2							
+	I.V	D2							
+		D2		+			+		
+	DIED	D -1							
+		S D7							
+		S D7							
+	2	S D9							
+	I.V	S D9							
+		S D7							
+		D ON NEEDLE							
+		D2				+			
+		S D7							+
+	6	D2							
+	I.D	S D7							+
+		S D9							+
+		S D9							+
+		D1							
+		D2							
+	6	S D9							+
+	S.C	S D9							+
+		D1							
+		D2							
+		D3							
+		D3							
+	TICKS	S D7							+
+		S D7							
+		S D9							+
+		S D9							+

Table 7 continued.

+		S D7							
+		S D7							+
+	PAINT	S D7							
+		S D7							+
+		S D9							+
+		S D9							+

CY Cyclophosphamide.
Staph *S.aureus*.
I.V. Intravenous.
I.P. Intraperitoneal.
Ticks Larval *I.ricinus* contaminated with *S.aureus* allowed to feed on mice.
Paint *S.aureus* suspension was painted onto the abdomen which was then pricked.
S Sacrificed.
+ Abscess(es) present.
Liv Liver.
H Heart.
K1 Left kidney.
K2 Right Kidney.
SP Spleen

Table 8. Bacteriological Cultures From Mice Injected With Various Doses of Staphylococci Administered by Various Routes With and Without Prior Injection With Cyclophosphamide.

$\log_{10}$

CY	DOSE STAPH	DIED	ABSCESSES LIV	LUNG	H	K1	K2	SP	LOCAL
		D2	4+	3+	3+	4+	4+	2+	
		D3	4+	2+	4+	4+	4+	2+	
	6	S D7							
		D7	3+	2+	4+	4+	4+	3+	
	I.V.	D8	4+	4+	3+	+	4+	+	
		S D9	2+	2+	+	+	+	3+	
		D2	+	4+	+			+	
		S D9	2+	+				2+	
	4	D4	3+	3+	3+	3+	3+	+	
		S D9	2+						
	I.V.	D2	3+	4+	4+	3+	3+	2+	
		D3	4+	+	4+	3+	3+	3+	
		S D9							
		S D9		3+					
	2	S D7							
		S D7	3+		+	2+	+	+	
	I.V.	S D7							
		S D7							
		S D9							
		S D9				+	+		
	6	S D7	+					+	4+
		S D7	2+	2+					4+
	I.D	S D7	3+					+	4+
		S D7							
		S D7	3+	4+	3+	2+	2+	2+	4+
		S D7	+	3+	+	+	+		4+
	6	S D9			+			+	4+
		S D9							
	S.C	S D7							4+
		S D7							4+
		S D7							
		S D7							
	TICK	S D7							
	L.	S D7							
		S D9		2+	+				
		S D9	+						

Table 8. continued.
$\log_{10}$

CY	DOSE STAPH	DIED	ABSCESSES LIV	LUNG	H	K1	K2	SP	LOCAL
		S D7							
		S D7							
	PAINT	S D7	+						
		S D7	+				+		
		S D9	2+	2+				+	
		S D9		+					
+		D1	2+	+		2+	4+		
+		D2	2+		4+	4+	4+	2+	
+	6	D1	2+	3+	4+	3+	2+	3+	
+	I.V.	S D9	+	+				+	
+		D1	4+	2+	+	2+	3+	3+	
+		D2	3+	2+	+	4+	+	2+	
+		S D9		+		+			
+		D1	4+	4+	3+	3+	4+	3+	4+
+	4	D2	4+	3+	3+	4+	3+	3+	
+	I.V	D2	+	3+	3+	4+	4+	2+	
+		D2	4+	4+	2+	2+	2+	2+	4+
+	DIED	D -1							
+		S D7							
+		S D7							
+	2	S D9	3+	+		+		2+	
+	I.V	S D9	+			+	+		
+		S D7	+			+			
+		D ON NEEDLE							
+		D2	4+	4+	4+	2+	2+	+	4+
+		S D7	2+		+	+		2+	4+
+	6	D2	4+	4+	4+	3+	N/C	3+	4+
+	I.D	S D7	+	+	+	+			4+
+		S D9	+	+	+	+			4+
+		S D9	4+	4+	2+	3+	2+	3+	4+
+		D1	2+				+		4+
+		D2	3+	3+	3+	2+	2+	+	4+
+	6	S D9	4+		2+				4+
+	S.C	S D9	3+	3+	3+	2+	2+	2+	4+
+		D1	4+	4+	4+	2+	2+	3+	4+
+		D2	3+	3+	2+	2+	3+	2+	4+
+		D3	3+	+	2+	+	+	3+	4+
+		D3	2+	4+	4+	4+	4+	4+	
+	TICKS	S D7	3+	+	2+	4+		+	
+		S D7	+						
+		S D9						+	4+
+		S D9	+	+	2+		+		4+

Table 8 continued.

+		S D7							
+		S D7	2+	+	+		+		4+
+	PAINT	S D7							
+		S D7	3+	+			+	+	4+
+		S D9	3+	2+	2+		2+		4+
+		S D9	+	+		+		+	

CY Cyclophosphamide.
Staph *S.aureus*.
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