

TRAPPING OF FREE-LIVING, UNFED ADULT AND NYMPHAL

Amblyomma hebraeum

IN HEARTWATER ENDEMIC AREAS OF SOUTH AFRICA

AND THE PREVALENCE OF *Cowdria ruminantium*

IN A SAMPLE OF ADULT TICKS

by

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DEDICATION

To my parents who believed

in the

importance of education,

and to

Mark and Danielle

for their support.



DECLARATION

Apart from the assistance received,
which has been reported in the Acknowledgements,
and in appropriate places in the text,
this Dissertation represents the original work of
the author.

The investigations in this Dissertation
have not been presented
for any other degree at any other University.

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Trapping of free-living, unfed adult and nymphal *Amblyomma hebraeum* in heartwater endemic regions of South Africa, and the prevalence of *Cowdria ruminantium* in a sample of adult ticks

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ABSTRACT

The main objective of this study was to use the Attraction-aggregation-attachment-pheromone/carbon dioxide (AAAP/CO₂) trap on a sustainable basis at six different field sites in South Africa. This trap was developed in Zimbabwe, but had not been used successfully in the field for the collection of free-living adult and nymphal *A. hebraeum*.

A two-year collection survey was carried out at one of the sites, the Rietgat communal grazing area (CGA) where 1 196 adult and 292 nymphal *A. hebraeum* were trapped with the AAAP/CO₂. Only free-living, unfed adult and nymphal *A. hebraeum* were collected, as these ticks were considered to be epidemiologically more credible than ticks collected off hosts. A distinct seasonal appearance of adult ticks was noted in both 1996 and 1997, and this could explain the difficulty experienced in collecting these ticks in the field in the past.

Peak numbers of adult ticks were collected from late spring (September/October) to midsummer (November - January). This was followed by a sharp decline to very low counts for the remainder of the year (February - August).

Field work was also conducted at five other sites in South Africa. At three of these sites, the AAAP/CO₂ trap was used successfully, these included a farm near East London (n = 187 adults, 17 nymphs) Kruger National Park (KNP) (n = 447 adults) and the Songimvelo Game Reserve (SGR) (n = 48 adults). At the two other sites, namely the MEDUNSA campus (n = 31 adults) and at a farm near Warmbaths (n = 25 adults), the AAAP/CO₂ trap was not really successful. A total of 1 934 adult and 309 nymphal *A. hebraeum* were collected with the AAAP/CO₂ trap.

A sample (n = 570) of the adult ticks collected from the Rietgat CGA (n = 434), the KNP (n = 88) and the SGR (n = 48) was tested for *C. ruminantium* with a specific PCR assay developed at the UF/US AID/SADC Heartwater Research Project in Harare, Zimbabwe. Nearly nine per cent (8.9%) of the ticks from the Rietgat CGA, 5.7% from the KNP and 25% from the SGR were positive for *C. ruminantium*. The overall infection rate of 9.8% for the total sample (n = 570) is similar to others recorded in southern Africa.

This was the first time that a large, statistically-relevant sample of free-living, unfed adult *A. hebraeum* collected with a AAAP/CO₂ trap, from a variety of different ecological areas has been processed with a *C. ruminantium*-specific PCR. The epidemiological data from this project should be more credible than those from many of the previous surveys, where feeding ticks were collected off hosts, and indirect methods used to determine *C. ruminantium* prevalence.

Vang van vrylewende, ongevoede volwassenes en nimfe van *Amblyomma hebraeum* in inheemse hartwatergebiede van Suid Afrika, en die teenwoordigheid van *Cowdria ruminantium* in a monster van volwasse bosluise

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SAMEVATTING

Die hoof doel van hierdie studie was om die "Attraction-aggregation-attachment-pheromone (AAAP/CO₂)" lokval op 'n volgehoue grondslag op ses verskillende terreine in die veld te gebruik. Hierdie lokval wat in Zimbabwe ontwikkel is, is nog nie met sukses vir die versameling van vrylewende, ongevoede *A. hebraeum* in die veld gebruik nie.

Tydens 'n twee-jarige ondersoek by een van hierdie terreine, die Rietgat gemeenskapsweiding, is 1 196 volwassenes en 292 nimfe van *A. hebraeum* met die AAAP/CO₂ lokval versamel. Slegs vrylewende, ongevoede *A. hebraeum* is versamel, aangesien hierdie bosluise epidemiologies meer geloofwaardig beskou word as bosluise wat vanaf gashere versamel is. 'n Duidelike seisoenale patroon in volwasse bosluisgetalle het gedurende 1996 sowel as 1997 voorgekom, en dit kan moontlik verklaar waarom daar in die verlede probleme ondervind is met die versameling van hierdie bosluise. Die grootste getalle bosluise is in die laat lente (September/Oktober) tot middel somer (November - Januarie) versamel, gevolg deur 'n skerp daling in getalle gedurende die res van die jaar (Februarie - Augustus).

Die versameling van bosluise is ook in vyf ander gebiede in Suid Afrika uitgevoer. Die AAAP/CO₂ lokval is in die volgende drie gebiede suksesvol gebruik: SABS plaas, Oos London (n = 187 volwassenes, 17 nimfe), Nasionale Kruger Wildtuin (n = 447 volwassenes) en Songimvelo Wildreservaat (n = 48 volwassenes). By die MEDUNSA kampus (n = 31 volwassenes) en die Warmbad plaas (n = 25 volwassenes) was die AAAP/CO₂ lokval minder suksesvol. 'n Totaal van 1 934 volwassenes en 309 nimfe is met die AAAP/CO₂ lokval versamel.

'n Monster (n = 570) van die volwasse bosluise wat by die Rietgat (CGA) gemeenskapsweiding (n = 434), die Nasionale Kruger Wildtuin (n = 88) en Songimvelo Wildreservaat (n = 48) versamel is, is vir die teenwoordigheid van *C. ruminantium* met behulp van 'n PCR metode getoets. Hierdie toets is ontwikkel deur die UF/USAID/SADC Hartwater Navorsingsprojek in Harare, Zimbabwe. Byna nege persent (8.9%) van die bosluise wat by die Rietgat gemeenskapsweiding, 5.7% in die Nasionale Kruger Wildtuin en 25% in die Songimvelo Wildreservaat versamel is, was positief vir *C. ruminantium*. Die algehele persentasie besmetting van die totale aantal bosluise (n = 570) was 9.8%, en is soortgelyk aan die in ander gebiede in suidelike Afrika.

Dit is die eerste grootskaalse, statisties-aanvaarbare monster van vrylewende, ongevoede volwasse *A. hebraeum* wat met die gebruik van die AAAP/CO₂ lokval versamel is in 'n verskeidenheid ekologiese areas, en met 'n *C. ruminantium*-spesifieke PCR getoets is. Die epidemiologiese data wat verkry is met hierdie projek, behoort meer geloofwaardig te wees as enige vorige opnames waar gevoede bosluise vanaf gashere versamel is, en indirekte metodes gebruik is om die teenwoordigheid van *C. ruminantium* te bepaal.

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ABBREVIATIONS

AAAP/CO ₂	Attraction-Aggregation-Attachment-Pheromone/Carbon dioxide
ASF	African swine fever
ASFV	African swine fever virus
α ³² P (dCTP)	Alpha phosphorus (32) deoxycytidine triphosphate
BAE	Bovine aortic endothelial
bp	Base pair(s)
c-ELISA	competitive-Enzyme-linked Immunosorbent Assay
CGA	Communal grazing area(s)
CO ₂	Carbon dioxide
°C	Degrees Celsius
DNA	Deoxyribonucleic Acid
dATP	2'-deoxyadenosine-5'triphosphate
dCTP	2'-deoxycytidine-5'triphosphate
dGTP	2'-deoxyguanosine-5'triphosphate
dNTP	2'-deoxynucleoside-5'triphosphate
dTTP	2'-deoxythymidine-5'triphosphate
ECF	East Coast fever
ELISA	Enzyme-linked Immunosorbent Assay
FAT	Fluorescent antibody test
fg	Femtogram(s)
Fig.	Figure



g	Gram
i-ELISA	indirect-Enzyme-linked Immunosorbent Assay
IFAT	Indirect fluorescent antibody test
I/P	Intraperitoneally
I/V	Intravenously
Kb	Kilobase
Kbp	Kilobase pair(s)
KCl	Potassium chloride
kDa	Kilodalton
kg	Kilogram(s)
KNP	Kruger National Park
MAP	Major Antigenic Protein
MEDUNSA	Medical University of Southern Africa
mg	Milligram(s)
MIA	Mouse Infectivity Assay
MgCl ₂	Magnesium chloride
ml	Millilitre(s)
mm	millimetres
mM	Millimolar
N	Normal
NaOH	Sodium hydroxide
ng	Nanogram(s)

ORF	Open Reading Frame
³² P	Phosphorus 32
P	Prevalence
PBS	Phosphate buffered saline
PCIA	Phenol:chloroform-isoamyl-alcohol
PCR	Polymerase Chain Reaction(s)
pCR9	Kenyan heartwater probe
pCS20	Zimbabwean heartwater probe
pg	Picogram(s)
RH	Relative humidity
RNA	Ribonucleic Acid
r-RNA	Ribosomal RNA
RVF	Rift Valley fever
SABS	South African Bureau of Standards
s/c	Subcutaneously
SDS	Sodium dodecyl sulphate
SGR	Songimvelo Game Reserve
TAR	Tick attack rate
TBD	Tick-borne diseases
TID	Tick infectivity decay
Tris HCl	Tris (hydroxymethyl) aminoethane

UF/USAID/SADC University of Florida/United States Agency for International
Development/Southern African Development Community

μg	Microgram(s)
μl	Microliter(s)
μM	Micromolar
UV	Ultraviolet
w/v	weight/volume.

1. INTRODUCTION

1.1 JUSTIFICATION OF THE PROJECT

Heartwater is a virulent, infectious, tick-borne and non-contagious disease affecting domestic and wild ruminants. It is characterized in domestic ruminants by a high fever, followed by severe nervous symptoms, hydro-pericardium and acute gastro-enteritis in its acute and peracute forms (Camus, Barré, Martinez & Uilenberg 1996). The causative agent is a *Rickettsia*, namely *Cowdria ruminantium*, which is transmitted by ticks of the genus *Amblyomma* (Camus *et al.* 1996). Heartwater appears to be of African origin, and occurs wherever particular *Amblyomma* vectors exist (Uilenberg 1996). The initial research on heartwater was carried out in South Africa, as there was a large and highly susceptible ruminant population in this country (Uilenberg 1996), and one of the main vectors, *Amblyomma hebraeum*, is indigenous to this region.

Provost & Bezuidenhout (1987) stated that heartwater is a major obstacle to the introduction of highly productive animals into Africa, and is also a significant problem when local animals are moved from heartwater-free to heartwater-infected areas. Heartwater is also a threat to the American mainland, following the introduction of the tick, *Amblyomma variegatum*, to the West Indies and other Caribbean islands (Barré, Uilenberg, Morel & Camus 1987).

Intensive tick control has been the method of choice for the control of heartwater in southern Africa since the end of the last century (Bezuidenhout & Bigalke 1987), but today decision makers are aware that these programmes are difficult to sustain, are costly and have adverse

effects on the immunity to the disease. Heartwater is not normally a problem in indigenous livestock which are born in endemic areas, and they soon acquire immunity through natural exposure when young (Norval 1981). In many instances the intensive dipping programmes have left the local cattle population susceptible to the various tick-borne diseases (TBD), resulting in massive stock losses due to TBD (Lawrence & Norval 1979). This has led to a re-appraisal of various government strategies on dipping programmes in Africa.

There has also been a substantial increase in research efforts to give farmers alternative approaches to tick control. This has mainly been directed at increasing our knowledge of the epidemiology of heartwater, and the production of more effective vaccines. In order to use the vaccines effectively, one needs to characterize the *C. ruminantium* strains in the areas where the vaccine is being tested, and know the prevalence or infection rate of *C. ruminantium* in free-living, unfed ticks.

Peter (1995) stated that in an ideal epidemiological scenario, one would aim to collect free-living, unfed wild *A. hebraeum* ticks in order to get the most credible data. These ticks are truly representative of the field population, whilst attached feeding ticks may have acquired their infection during feeding on their present host. Peter (1995) was, however, not able to collect free-living, unfed *A. hebraeum* in Zimbabwe due to problems with the AAAP/CO₂ trap and eventually collected free-living, unfed *A. hebraeum* attaching to sentinel hosts. One of the main objectives of this project was to collect free-living, unfed *A. hebraeum* with the AAAP/CO₂ trap, and have truly representative samples of the field population of ticks.

1.2 **RESEARCH OBJECTIVES**

1.2.1 **OVERALL OBJECTIVES**

To determine the *C. ruminantium* infection rate of free-living, unfed *A. hebraeum* ticks collected from heartwater endemic regions of South Africa.

1.2.2 **SPECIFIC AIMS**

- To develop a reliable Attraction-Aggregation-Attachment-Pheromone/Carbon Dioxide (AAAP/CO₂) trap to collect free-living, unfed *A. hebraeum* ticks in the field, and to test this trap at various field sites in South Africa.
- To determine the infection rate or prevalence of *C. ruminantium* in a statistically relevant sample of these free-living, unfed *A. hebraeum* by means of a *C. ruminantium*-specific Polymerase Chain Reaction (PCR) assay.

1.3 **LITERATURE REVIEW**

1.3.1 ***C. RUMINANTIUM* IN THE HOST (RUMINANTS/WILDLIFE)**

1.3.1.1 **Characteristics of *C. ruminantium***

Cowdry (1925) first described the pathogenic agent as *Rickettsia ruminantium*. He considered the agent to be a *Rickettsia* because of its Gram-staining properties, its intracellular localization, its morphology and the fact that it was transmitted by an arthropod. The organism, which was later renamed *C. ruminantium*, may be found singly, in colonies or in clumps, and has a predilection for the cytoplasm of reticular cells, neutrophilic leukocytes and vascular endothelium (Mebus & Logan 1988). The organism is also very fragile and must be stored at -70°C or lower, using a cryopreservative.

C. ruminantium was successfully cultivated for the first time in tissue culture when Bezuidenhout, Paterson & Barnard (1985) grew it in a bovine umbilical endothelium cell line. Today it is routinely cultured at various research establishments throughout the world. Many different “stocks” (strains) of *C. ruminantium* have been described (Jongejan, Uilenberg, Franssen, Gueye & Nieuwenhuijs 1988).

There appears to be a close phylogenetic relationship between *C. ruminantium* and *Ehrlichia* species (Dame, Mahan & Yowell 1992; Van Vliet, Jongejan & Van der Zeijst 1992), and this has limited the credibility of the serological tests for *C. ruminantium*. Sequencing of the gene coding for the 16S RNA gene has also revealed a close relationship between *C. ruminantium* and various *Anaplasma*, *Rickettsia* and *Ehrlichia* species (Dame *et al.* 1992; Van Vliet *et al.* 1992).

1.3.1.2 Pathogenesis of the disease in the host

The pathogenesis of heartwater is poorly understood (Bezuidenhout, Prozesky, Du Plessis & Van Amstel 1994). Vertebrate hosts are infected directly via the saliva of the attached ticks or by regurgitation of the gut material. Initial replication takes place in the reticulo-endothelial cells and in the macrophages in the regional lymph nodes (Du Plessis 1970). The organisms are disseminated by the blood stream, and have a predilection for endothelial cells of the brain (Bezuidenhout *et al.* 1994). Increased vascular permeability leads to effusion into the body cavities and consequent oedema, especially in the brain. Hydropericardium and nervous signs are a direct result of the oedema.

1.3.1.3 Main clinical signs of the disease

Under field conditions susceptible animals show signs of the disease 14 to 28 days after being introduced into a heartwater enzootic area. The incubation period in sheep and goats is normally 7 - 14 days, and in cattle 10 - 16 days (Mebus & Logan 1988). The course of the disease depends on the susceptibility of the host, and the virulence of the *C. ruminantium* isolate can be peracute, acute, subacute or subclinical (mild). Cows of the *Bos taurus* breeds are particularly prone to develop peracute heartwater, and these animals die within a few hours after developing a fever, often showing no other clinical signs apart from convulsions and marked respiratory distress (Bezuidenhout *et al.* 1994).

The acute disease is the most common form, and after the rapid onset of fever there is increased respiration, inappetence, hyperaesthesia, high stepping gait, twitching of the eyelids, chewing, abnormal tongue movement and individual muscle tremors. Nervous signs increase in severity, often with the limbs stretched out and showing paddling movements, opisthotonos, nystagmus and convulsions (Mebus & Logan 1988). The course of this form of the disease is usually less than seven days, and most of the animals die. The subacute disease is usually seen in animals which have some resistance, and is characterized by much milder signs, and only a small percentage of the affected animals die. In the subclinical form the signs are so mild, that there is only a transient fever and this is more often seen in animals with natural resistance to the disease.

Exotic breeds of goats, such as Angora and Saanen goats, are commonly affected by the peracute form of the disease. Clinical signs in goats are similar to those of cattle, but the nervous signs are less pronounced (Bezuidenhout *et al.* 1994). Black-headed Persian sheep

are thought to possess some natural resistance to heartwater and tend to develop a mild form of the disease (Alexander 1931). Clinical signs recorded in susceptible wild ungulates are generally similar to those recorded in domestic ruminants (Gradwell, Van Niekerk & Joubert 1976).

Mortality rates vary according to the stock (strain) of *C. ruminantium* involved, and the species, breed and age of the host. In untreated cases, mortality is usually in the region of 50% in exotic breeds of cattle, somewhat higher in exotic breeds of sheep and much higher in exotic goats (Uilenberg & Camus 1993).

1.3.1.4 **Immunity and immunization of the host**

1.3.1.4.1 Age resistance

Calves possess an age-linked resistance to bovine babesiosis and anaplasmosis, which is independent of the immune status of the dam, and may last nine months or longer. A state of endemic stability to these TBD is possible if the infection rate in the field ticks is sufficiently high to ensure that all calves develop active immunity before resistance wanes (Uilenberg & Camus 1993).

An age resistance also occurs in cowdriosis, but it is of shorter duration, lasting only the first four weeks of life in calves, and the first week in lambs and kids (Du Plessis & Malan 1987). The resistance is not absolute, as infection of some calves less than three weeks of age and some lambs and kids of less than one week may result in fatal disease (Neitz & Alexander 1945).

1.3.1.4.2 Breed resistance

Bos taurus cattle, especially those with no exposure to heartwater infection, are less resistant to infection than *Bos indicus* breeds (Uilenberg 1983). The resistance of local Zebu breeds is probably due to inherited resistance acquired through years of natural selection (Bezuidenhout *et al.* 1994). Conglutinin in the serum of cattle appears to be involved in non-specific protection against heartwater (Du Plessis & Bezuidenhout 1979).

Sheep and goats appear to be more susceptible to heartwater than cattle, and there is a great range in different breed susceptibilities which seldom develop good immunity. Merino sheep are particularly susceptible, whilst Persian sheep show a degree of natural resistance. This resistance to heartwater can also be inherited, such as in cross-bred goats (Donkin, Stewart, MacGregor, Els & Boyazoglu 1992).

Wild animals show varying degrees of immunity to *C. ruminantium*, depending on the species and their previous exposure to *C. ruminantium*. Some species such as eland (*Taurotragus oryx*), blesbuck (*Damaliscus pygargus phillipsi*), springbok (*Antidorcas marsupialis*) and black wildebeest (*Connochaetes gnu*) can develop clinical disease, whereas the majority of wild ruminants are refractory to the disease or become subclinical carriers (Peter, Anderson, Burridge & Mahan 1998).

1.3.1.4.3 Mechanisms of immunity to *C. ruminantium*

Although much research has been conducted into the serological responses of the various domestic animals to *C. ruminantium*, it is thought that the humoral response is not the most important immune mechanism. Alexander (1931) injected sheep with hyperimmune serum,

but was not able to protect the sheep from consequent challenge with *C. ruminantium*. Du Plessis (1970) also injected heartwater susceptible sheep with gamma globulins from immune sheep, but these sheep were not protected against heartwater challenge.

The immunity appears to be mainly cell-mediated, and the transfer of spleen cells from immune mice conferred protection against homologous challenge in recipient mice (Du Plessis 1981). Cellular immunity in heartwater is likely to be mediated directly via cell cytotoxic effects, and indirectly through the production of T cell cytokines which induce an anti-rickettsial state (Mahan, Smith & Byrom (1994). Mahan *et al.* (1994) also demonstrated that the addition of mitogen-stimulated bovine T cell supernatants to bovine endothelial cell culture inhibited intracellular development of *C. ruminantium*. The authors suggested that the active agent was gamma-interferon. Circulating levels of alpha-interferon have been shown to be higher in cattle resisting experimental *C. ruminantium* infection (Totté, Blankaert, Zilinwabagabo & Werenne 1993a; Totté, de Gee & Werenne 1993b; Totté, Jongejan, de Gee & Werenne 1994; Totté, McKeever, Jongejan, Barbet, Mahan, Mwangi & Bensaid 1998).

1.3.1.4.4 Different stocks (strains) of *C. ruminantium*

There is a great variation in the pathogenicity of the various stocks (strains) of *C. ruminantium*. Certain stocks of *Cowdria* differ considerably in their pathogenicity to mice, sheep, cattle and goats, and this pathogenicity is often independent of geographical differences (Du Plessis, Van Gas, Olivier & Bezuidenhout 1989). Some stocks are pathogenic to mice (Crystal Springs, Kümm, Kwanyanga, Nonile & Welgevonden), and some non-pathogenic to cattle (Kümm & Mara), (Du Plessis *et al.* 1989; Byrom *et al.* 1993).

Almost all stocks give partial to complete cross-immunity, however, total absence of cross-protection is known to exist between certain stocks (Van Winkelhoff & Uilenberg 1981; Uilenberg 1983; Du Plessis *et al.* 1989; Jongejan, Thielemans, Briere & Uilenberg 1991). The recognised antigenic diversity of different stocks may have important repercussions for vaccine production or development.

1.3.1.4.5 Vaccination/immunization of the host against *C. ruminantium* (Historical)

It has long been recognized that animals which recover from heartwater acquire an immunity to the disease (Alexander 1931). Many of the early attempts to produce a vaccine failed, and it was not until 1931 that Alexander described an intravenous blood vaccine which produced clinical heartwater in the host. The inoculated animal had to be treated with tetracycline once the temperature began to rise (Van Amstel & Oberem 1987). The infection and treatment of immunization/vaccination is still in use in South Africa today (Bezuidenhout *et al.* 1994). It is used especially when animals are brought into endemic areas from heartwater-free regions.

The main problem with this method of immunization is the timing of the treatment. If the treatment is given too early no immunity develops, and if too late, the animal dies (Bezuidenhout *et al.* 1994). Very young animals can be immunized without temperature monitoring because of their age-linked resistance. Cattle develop immunity to heartwater following one vaccination, provided they are then put onto heartwater veld. With sheep and goats this immunity may last for less than one year if not re-enforced by natural infection.

A small proportion of vaccinated animals, usually less than 5%, fail to develop immunity after vaccination (Lawrence, Malika, Whiteland & Kafuwa 1995). It may be that the vaccine is not protective because of field strain differences with the vaccine strain which do not exhibit cross-immunity. Lawrence *et al.* (1995) vaccinated 1 094 cross-bred Zebu cattle in Malawi and found that more than 50% of the vaccinated cattle did not react to the vaccine and six (0.6%) cattle died during vaccination. Van der Merwe (1979) vaccinated 2 743 cattle in South Africa and most of these reacted to the vaccine ($\pm 98\%$), and there were also some mortalities (0.83%).

Because it is necessary to inject animals intravenously (I/V) and to monitor the temperature daily, it is difficult to give mass immunization. The block method of immunization, first described by Fick & Schuss (1952) and Poole (1962), is widely practised in South Africa, especially when vaccinating large numbers of kids and lambs (Bezuidenhout *et al.* 1994). The procedure involves treating vaccinated animals on a specific day, irrespective of whether the animals have reacted or not. In South Africa it is generally advised that *Bos taurus* cattle be treated on day 14, *Bos indicus* cattle on day 16 and sheep and goats on day 11 and 12, respectively (Bezuidenhout *et al.* 1994). The main criticism of this method is that one is uncertain whether the vaccinated animals developed a temperature reaction and whether immunity develops or not.

A further disadvantage of the vaccine is that the organisms are very labile, and need to be stored at -70°C , using special facilities which are not available on farms and in other rural areas. Apart from the difficulties experienced in giving an intravenous vaccine, blood-

borne pathogens can also be transmitted (Uilenberg & Camus 1993). There are also reports of anaphylactic reactions to the vaccine (Lawrence *et al.* 1995), and when using the vaccine in does and kids a high mortality (close to 20%) might be experienced (Gruss 1983).

1.3.1.4.6 New developments with the heartwater vaccine

It is therefore clear that there are a multitude of problems when using the infection and treatment method of immunization for heartwater, but in spite of all the disadvantages, exotic stock cannot be maintained in endemic areas without immunization, unless excellent tick control can be sustained (Uilenberg & Camus 1993). If progress is to be made, then a user-friendly, inactivated vaccine, that can be given subcutaneously (s/c), has to be developed, and the successful growth of *C. ruminantium* (Bezuidenhout, Paterson & Barnard 1985) in cell culture has made this goal more achievable.

Jongejan (1991) showed that a live, attenuated Senegalese stock of *C. ruminantium* vaccine could be used to protect sheep against certain strains of heartwater. Martinez, Maillard, Shiekboudou & Coisne (1993) vaccinated nine goats s/c with an inactivated elementary body preparation of *C. ruminantium* (Gardel stock) mixed with Freund adjuvant. Six of the nine goats were protected on challenge as opposed to none of the controls.

Mahan, Andrew, Tebele, Burridge & Barbet (1995) immunized three groups of five Merino sheep from a heartwater-free area with five inoculations of a s/c inactivated vaccine. Group 1 were vaccinated with the vaccine only, Group 2 with vaccine and Freund's complete and incomplete adjuvants, and Group 3 were the control group. None of the sheep in Group 2

died, but there was a 50% and 60% mortality in Groups 1 and 3, respectively. Clearly the sheep vaccinated with the inactivated vaccine plus the adjuvant were protected.

Mahan, Kumbula, Burridge & Barbet (1998a) did extensive laboratory and field trials using 178 sheep to test the inactivated *C. ruminantium* vaccine against heterologous and field strains. They demonstrated that the vaccine with Freund's adjuvant protected sheep against lethal intravenous challenge and against laboratory-raised infected ticks. The vaccination was also able to protect against heterologous strains from geographically diverse areas and against natural tick challenge. The Freund's adjuvant has, however, been shown to cause numerous clinical problems and would not, therefore, be the adjuvant of choice. Montanide ISA 50 was shown to be the most effective adjuvant for the vaccine (Mahan *et al.* 1998a).

More recently Nyika, Mahan, Burridge, McGuire, Rurangirwa & Barbet (1998) demonstrated that the Major Antigenic Protein-1 (MAP-1) gene of the Crystal Springs stock, cloned into an eukaryotic expression vector VCL 1010 induced a degree of homologous protective immunity in mice.

Nearly all the developments and testing of the inactivated vaccines for heartwater have been tested in sheep. Future developments must be extrapolated to cattle before the vaccine can bring practical benefits to farmers.

1.3.1.5 **Reservoir hosts**

There appear to be three important reservoirs of heartwater (Norval, Andrew, Yunker & Burridge 1992):

- Ruminant carriers,

- Free-living, unfed *Amblyomma* ticks, and
- Attached male *Amblyomma* ticks.

C. ruminantium is widely spread throughout the host and vector populations, and this appears to be the reason why enzootic stability to heartwater is common in areas where *Amblyomma* ticks are prevalent throughout the year (Norval *et al.* 1992).

The early investigators (Alexander 1931; Neitz 1939; Ilemobade 1978) found that blood from infected cattle and sheep, when injected I/V into another ruminant host, was infective for a variable period of 35 to 60 days. Andrew & Norval (1989a) demonstrated that sheep, cattle and African buffalo (*Syncercus caffer*) could remain carriers for as long as 250 days. Peter, Mahan, Barbet, Norval & BurrIDGE (1995) showed that this reservoir state in cattle can last for up to a year.

Many wildlife species are considered to be reservoirs of heartwater, and these have been reviewed by Oberem & Bezuidenhout (1987), and more recently by Peter, Anderson, BurrIDGE and Mahan (1998a). As many as 28 different wildlife species have been implicated as hosts of *C. ruminantium*, but few have been conclusively proved to be susceptible to infection. Eight wildlife species have been experimentally proven to be susceptible to *C. ruminantium* infection, and seven of these have become carriers of the infection (Peter *et al.* 1998a).

Wildlife hosts play a very important role in the epidemiology of the disease. These asymptomatic carriers can infect free-living ticks (Norval *et al.* 1992), and although intensive tick control may lead to the localized eradication of *Amblyomma* ticks, it is

unlikely to do so if alternative hosts for the ticks are available (Norval *et al.* 1992). This also has important consequences for veterinary quarantine decision makers, and makes the translocation of potentially infected animals over long distances into heartwater-free areas such as the United States of America (USA) more important, and they should be carefully screened (Peter *et al.* 1998a).

With the improvement of diagnostic tests for *C. ruminantium*, more and more wild animals will probably be shown to be important carriers of diseases. Recently, Kock, Van Vliet, Charlton & Jongejan (1995) used the PCR assay to demonstrate that the blood and bone marrow of tsessebe (*Damaliscus lunatus*), waterbuck (*Kobus ellipsiprymnus*) and impala (*Aepyceros melampus*) were positive for *C. ruminantium*.

1.3.1.6 Treatment of clinical cases

Historically, a variety of different drugs have been used to treat clinical cases of heartwater, and these have been reviewed by Van Amstel & Oberem (1987). The tetracyclines, especially oxytetracycline (10 to 20 mg/kg live mass), are the most effective drugs used for specific treatment of the disease. Long-acting tetracyclines have the advantage that only a single injection is usually sufficient, whilst with the short-acting preparations at least two injections, 24 to 48 hours apart, are required (Uilenberg & Camus 1993). Treatment of heartwater during the early febrile stages presents very few problems if a diagnosis can be made soon enough. If, however, as is commonly the case, treatment is delayed until nervous symptoms are present, then the prognosis becomes poor (Van Amstel & Oberem 1987). Various drugs, such as diuretics, can also be used to reduce the oedema, and corticosteroids can be used in the supportive treatment of clinical cases (Van Amstel & Oberem 1987).

1.3.1.7 Main post mortem signs

Lesions in cattle, sheep and goats are very similar and differ only in their severity and frequency (Prozesky 1987). Severe hydropericardium and hydrothorax, and in some cases ascites, are striking changes in the most fatal cases of the disease (Bezuidenhout *et al.* 1994). A moderate to severe lung and brain oedema is also present (Prozesky 1987). In spite of marked clinical signs in goats, post mortem lesions are not always present (Uilenberg & Camus 1993).

1.3.2 THE DIAGNOSIS OF HEARTWATER

1.3.2.1 Clinical signs

Unlike many other TBD, *C. ruminantium* is not detectable in a blood smear, making a specific confirmation of heartwater in the living host very difficult. The early investigators relied heavily on the clinical signs seen in domestic stock. These included a variety of nervous manifestations, many of which are not pathognomonic for the disease (Van der Pypekamp & Prozesky 1987). Fever is present throughout the course of the disease, and the nervous signs include inco-ordination, exaggerated high-stepping gait, chewing movements, as well as blinking and twitching of the eyes (Synge 1978). As the disease progresses certain signs, such as hyperaesthesia and scours, become more prominent and eventually affected animals collapse in galloping convulsions (Synge & Scott 1978).

There are, however, a wide range of other diseases of ruminants which have similar clinical signs to heartwater (botulism, babesiosis, theileriosis, anaplasmosis and coccidiosis) (Camus *et al.* 1996; Uilenberg 1971), as well as various neuro-toxicoses of plant origin (Coetzer, Kellerman & Naude 1985).

1.3.2.2 **Detection of the organism (antigen) in the live animal**

1.3.2.2.1 Biopsy

The biopsy method, described by Syngé (1978) for the diagnosis of heartwater in goats, involves collecting brain sections for histopathology. This method has been used successfully by a number of field workers (Camus & Barré 1987a), but requires specialized techniques.

1.3.2.2.2 Xenodiagnosis

■ Inoculation of blood

In the past sub-inoculation of blood into a susceptible ruminant was often necessary to confirm the presence of *C. ruminantium*. If it was present, the recipient animal reacted within 16 days (Syngé & Scott 1978). This method was used as one of the confirmatory tests for the presence of *C. ruminantium* in the Caribbean (Burrige, Barré, Birnie, Camus & Uilenberg 1984).

■ Inoculation of tick homogenates

The inoculation of a supernatant of homogenized *Amblyomma* ticks into a susceptible animal, can also demonstrate the presence of *Cowdria* (Camus *et al.* 1993).

■ Tick feeding transmission

Cowdria-free laboratory-reared *Amblyomma* nymphs can also be fed on suspected infected hosts. After feeding, the ticks are allowed to moult and the resulting adults are fed on a heartwater susceptible host to see whether they have become infected (Camus *et al.* 1996).

Intrastadial transmission of *C. ruminantium* has been demonstrated by infecting heartwater-free *A. hebraeum* ticks after feeding on heartwater infected hosts (Andrew & Norval 1989). Intrastadial transmission has also been achieved with male *A. variegatum* (Kocan, Norval & Donovan 1993).

1.3.2.2.3. Post mortem signs and Histopathology

It is much easier to detect the *C. ruminantium* organism in a dead host, as access to the brain of the animal is then possible. In addition, the pathological changes associated with heartwater are well-documented (Bezuidenhout *et al.* 1994), and include a characteristic hydropericardium, hydrothorax and lung oedema. Examination of crush smears of the endothelial cells of the renal glomeruli or of the cerebral cortex will show colonies of *C. ruminantium* (Purchase 1945). The *Cowdria* organisms can be demonstrated in brain smears even in an advanced state of putrefaction, but detection of the organisms in treated animals can be difficult (Bezuidenhout *et al.* 1994).

C. ruminantium has also been demonstrated in histological sections of various organs in ruminants (Cowdry 1926), and in various endothelial cells (Jackson 1931). Detailed histopathological and electron-microscopic studies have been done (Du Plessis 1970; 1975), but demonstration of the organism remains difficult outside of the endothelial cells of the kidney and the brain. Various stains have been used to demonstrate *C. ruminantium*, but those incorporating toluidine blue or Giemsa are preferred (Prozesky 1987).

1.3.2.3 **Detection of antibodies**

1.3.2.3.1 Development of antibodies to *C. ruminantium*

Early research workers attempting to develop serological tests to determine the kinetics of antibody production to *C. ruminantium*, were hampered by the inability to produce adequate quantities of antigens. The successful cultivation of *C. ruminantium in vitro* in endothelial cell cultures (Bezuidenhout *et al.* 1985) provided a pure source of antigen for the various diagnostic tests, and the tests became more repeatable.

Semu, Mahan, Yunker & Burridge (1992) experimentally infected cattle with a Zimbabwean (Palm River) and a South African (Ball 3) isolate of *C. ruminantium*. A specific antibody response was detected by an indirect fluorescent antibody test (IFAT) using *C. ruminantium*-infected bovine aortic endothelial (BAE) cell cultures as antigen. The first detection of antibodies to *C. ruminantium* generally coincided with the peak of the febrile reaction, and the antibodies remained detectable for a period of 8 - 30 weeks. Peak reciprocal antibody titres in both groups ranged between three and six weeks post-infection. Neitz, Viljoen, Bezuidenhout, Oberem, Van Wyngaardt & Vermeulen (1986) used an Enzyme-linked immunosorbent assay (ELISA) to demonstrate that IgG-specific antibodies to *C. ruminantium* were detectable in cattle and sheep up to 22 weeks post-inoculation.

Mice develop a specific immune response following infection with mice pathogenic strains of heartwater, and the duration of immunity in the case of the Kümm strain can be as long as 18 months (Du Plessis 1985; Stewart 1987). Byrom, Mahan & Barbet (1993) found that mice antibody to *C. ruminantium* develops in the second week post-infection, and the antibody level is dependent upon the inoculation dose.

The exact role of antibody in the protection of domestic animals has been debated over a long period. However, neither passive transfer of immune sera in ruminants (Alexander 1931) or mice (Byrom *et al.* 1993) appears to reduce the severity of the disease. Cell-mediated immunity is presumed to be more important than humoral immunity in protecting against heartwater (Mahan *et al.* 1994).

1.3.2.3.2 Isolation and culture of *C. ruminantium*

For many years all efforts to propagate *C. ruminantium in vitro* failed (Uilenberg 1983). The first successful culture method was that of Bezuidenhout *et al.* (1985). This provided the basic information necessary for the refinement of techniques and to the supply of organisms required for molecular and serological studies (Byrom & Yunker 1990). Problems were, however, often experienced with the special irradiation and chemical retardation deemed necessary to delay cell growth. Byrom & Yunker (1990) reported that the addition of 0.4% glucose to the medium greatly improved the culture conditions, and *C. ruminantium* could be passaged routinely in untreated bovine vascular endothelial cells.

Byrom, Yunker, Donovan & Smith (1991) described a culture technique where no cell growth retardation by irradiation or chemicals was used. They used heparin-derived plasma samples obtained from heartwater-infected animals exhibiting a prolonged high temperature. They demonstrated that by using this technique the infection rate of cultures increased from 1% to 25%. More recently Smith, Anderson, Burrige, Peter & Mahan (1998) infected various wild animal culture cells with *C. ruminantium* by inoculation of plasma from infected animals or infected bovine cell cultures.

1.3.2.3.3 Immunogenic proteins of *C. ruminantium* (MAP-1)

With the advances in the culture of *C. ruminantium*, and the great improvement in *Cowdria*-specific immunological techniques, various antigenic proteins have been identified, namely the 21, 27, 32, 40, 46, 58, 85 and the 160 kilodalton (kDa) proteins. Any of these antigenic proteins may be used in the serological tests or recombinant vaccines for heartwater. Jongejan & Thielemans (1989) first described a 32-kDa immuno-dominant protein referred to as MAP-1 (Barbet, Semu, Chigagure, Kelly, Jongejan & Mahan 1994). This protein was also conserved within nine geographically different stocks of *Cowdria*, and was considered a good candidate for antigen in sero-diagnostic tests (Jongejan & Thielemans 1989).

Due to the various problems with the IFAT such as cross-reactions to various *Ehrlichia* spp., new serological diagnostic techniques have been developed. Most of these new tests use ELISA immuno technology and are based on the use of the immuno-dominant MAP-1 32-kDa surface protein of *C. ruminantium* (Barbet *et al.* 1994). Several other immunogenic proteins have also been identified (Mahan 1995), but the 32-kDa remains the most immuno-dominant. Most of these immunogenic proteins are surface exposed, and this facet would be important in the research and development of new vaccines and diagnostic tests for heartwater (Mahan 1995). Monoclonal antibodies are also available for the 21 and 32-kDa proteins (Jongejan, De Vries, Nieuwenhuijs, Van Vliet & Wassink 1993).

Two immunogenic regions of the MAP-1 protein, have recently been described (Van Vliet *et al.* 1995) and designated MAP-1A and MAP-1B. MAP-1A reacted to *C. ruminantium*

antisera and *E. ovina* antisera, whilst MAP-1B reacted only with *C. ruminantium* antisera. The gene encoding MAP-1 of the Senegal isolate of *C. ruminantium* has also been cloned and expressed in *Escherichia coli* (Van Vliet *et al.* 1994). This allowed identification of the immunogenic regions on MAP-1, and an evaluation of their possible use in the construction of a specific diagnostic test.

1.3.2.3.4 Antibody detecting diagnostic tests

- Indirect fluorescent antibody test

Du Plessis (1981) was the first researcher to use an IFAT to detect antibodies to *C. ruminantium* in ruminants and mice. He used infected mouse peritoneal cells as antigen and the test was known as the mouse IFAT, and the test was used extensively in South Africa and the Caribbean. Logan, Quintero, Whyard & Mebus (1985) used infected neutrophils and macrophages in an IFAT and a direct Fluorescent antibody test (FAT). Semu *et al.* (1992) used bovine endothelial cells infected with *C. ruminantium* in their IFAT to detect antibodies in experimentally infected cattle (refer to section 1.3.2.3.1).

With the advances in cell culture techniques the IFAT's became more sophisticated. Martinez, Swinkles, Camus & Jongejan (1990) used cultured bovine endothelial cells as antigen, and the test could detect antibodies as early as two weeks after infection and for 30 weeks after the initial infection.

- Immunoblots

Mahan, Tebele, Mukwedeya, Semu, Nyathi, Wassink, Kelly, Peter & Barbet (1993) used the immunoblot diagnostic assay, which was first described by Jongejan & Thielemans (1989), to test for heartwater antibodies in cattle, sheep and goats in Zimbabwe. They used this test because of the known sensitivity limitations of the IFAT and the ELISA. Questions, however, arose as to the credibility of the results obtained with the immunoblot diagnostic assay, especially when many “positive” samples were found in heartwater-free areas of Zimbabwe (Mahan *et al.* 1993).

- Enzyme-linked immunosorbent assays

- Competitive ELISA

A competitive ELISA (c-ELISA) was first described by Jongejan, Thielemans, De Groot, Van Kooten & Van der Zeijst (1991), which detected *Cowdria* specific antibodies in goat, sheep and cattle sera. The c-ELISA used a monoclonal antibody directed against the immunodominant MAP-1 antigenic protein. The c-ELISA detected antibodies in 55 out of 70 (79%) goats experimentally infected with eight different *Cowdria* stocks. There was a 89% agreement between the results of the IFAT and the c-ELISA, and there were apparently, no cross-reactions with *Ehrlichia phagocytophila* antibodies in goat sera, and *Anaplasma marginale* in bovine sera.

- Indirect ELISA's

Two indirect ELISA's (i-ELISA) have also been described. Martinez, Coisne, Sheikboudou & Jongejan (1993) working in the Caribbean, stated that the overall sensitivity of their

i-ELISA varied between 97 and 98%. The specificity showed only 3% false positives, and was lower for sheep than for cattle and goats. Soldan, Norman, Masak, Paxton, Edelston & Sumption (1993) also used an indirect ELISA to monitor the seroconversion to *C. ruminantium* in Malawian Zebu calves. This ELISA test used a detergent soluble fraction of *C. ruminantium* organisms. This fraction was reported to have a reduced cross-reactivity to *Ehrlichia ondiri* and *E. phagocytophila*. There was no published information on the sensitivity and specificity of the test (Soldan *et al.* 1993).

Recent work (Van Vliet *et al.* 1995; Mahan *et al.* 1998c) with the MAP-1B ELISA test, seems to indicate that the problems with cross-reactivity with *Ehrlichia* have been partially solved by using the MAP-1B ELISA test (refer to section 1.3.2.3.7).

1.3.2.3.5 Advantages of the use of serological tests (Epidemiological surveys)

■ IFAT (Surveys)

One of the main objectives of the various serological tests would be to determine the prevalence of *C. ruminantium* antibodies in domestic animals. Du Plessis (1981) first used the IFAT to determine the relationship between the dipping of cattle and immunity to heartwater. Du Plessis & Malan (1987) then used the IFAT to determine whether endemic stability to heartwater was present or not at certain selected farms in South Africa. Asselbergs, Jongejan, Langa, Nevis & Afonso (1993) also used the IFAT with infected endothelial cell culture antigen to test goat and cattle sera collected from various provinces in Mocambique. Overall 30% of the goat and 43% of the cattle sera were positive to *C. ruminantium*. There was also a noticeable difference between the sera from the north of

Mocambique where *A. variegatum* is prevalent (10% goats, 20% cattle), and the south of Mocambique where *A. hebraeum* predominates (63% goats, 59% cattle).

Camus & Barré (1987b) used the IFAT with the Kümm and Gardel stocks as antigen to determine which islands in the Caribbean were infected with *C. ruminantium*. Most of the areas where *A. variegatum* was present were positive, but of concern were certain islands which were shown to be positive, where no *A. variegatum* were found.

- c-ELISA (Surveys)

De Vries, Mahan, Ushewokunze-Obatolu, Norval & Jongejan (1993) used the c-ELISA and the IFAT to determine if there was any correlation between the antibodies to *C. ruminantium* in cattle and the distribution of *Amblyomma* ticks in Zimbabwe. They reported that in *Amblyomma*-infested areas 52% (c-ELISA) and 26% (IFAT) of the sera were positive for *C. ruminantium* antibodies. In the *Amblyomma*-free areas 11% (c-ELISA) and 10% (IFAT) were positive. The latter result was of some concern because no heartwater was present in these regions, therefore, some other organism, possibly also transmitted by the ticks, must have been responsible for the false positives.

- i-ELISA (Surveys)

The indirect ELISA was also used in two important serological surveys for heartwater in widely differing geographical areas in the Caribbean and Africa (Malawi). Camus *et al.* (1993) published their findings on a survey conducted in Guadeloupe and various islands in the Caribbean. The authors of this major work had to conclude that the serological results

were difficult to interpret due to various factors. At Antigua and Guadeloupe where *A. variegatum* was widespread, the domestic ruminants had a high percentage of positive results. On the other nine islands, however, 4% of the sera were positive and these “positive sera” must have been due to other organisms related to *C. ruminantium*.

Soldan *et al.* (1993) also used the indirect ELISA to test for antibodies to *C. ruminantium* in calves in Malawi, and they compared the antibody levels to heartwater in dipped and undipped cattle. They reported that by 12 months of age almost all the undipped calves had seroconverted to *C. ruminantium*, compared with 41% in the dipped group. They also concluded that a state of enzootic stability exists to *C. ruminantium* in undipped Zebu cattle in the study area in Malawi.

1.3.2.3.6 Disadvantages of the use of serological tests for *C. ruminantium*

As early as 1987, Du Plessis, Camus, Oberem & Malan warned of serious problems with the interpretation of the IFAT due to cross-reactions with related *Ehrlichia* organisms. The specificity was first questioned when positive IFAT results were obtained from sera in the Caribbean where no *A. variegatum* occurred. This was followed with sera from Namibia where again no heartwater was known to exist. Du Plessis *et al.* (1987) believed that *E. bovis*, which is common in Africa and transmitted by various *Hyalomma* and *Rhipicephalus* species, must have been the cause of the false positive reactions.

Holland, Logan, Mebus & Ristic (1987) and other workers demonstrated that there was a close serological relationship between *C. ruminantium* and various members of the genus

Ehrlichia. Du Plessis, Bezuidenhout, Brett, Camus, Jongejan, Mahan & Martinez (1993) proved that five different serological tests for heartwater, at five different laboratories, all showed cross-reactivity with *Ehrlichia* species. De Vries *et al.* (1993) in their serological survey of the antibodies to *C. ruminantium* in Zimbabwe, found high background in the field sera, and they believed that this was due to closely related organisms which recognize similar epitopes in the 32-kDa protein to *C. ruminantium*.

Mahan, Tebele, Mukwedeya, Semu, Nyathi, Wassink, Kelly, Peter & Barbet (1993) did an extensive investigation into the sensitivity and specificity of the 32-kDa-based serological tests. They used immunoblotting techniques on sheep sera from Zimbabwe to investigate whether any of the sheep had in fact been previously exposed to heartwater. Eleven immunoblot-positive and six immunoblot-negative sheep from heartwater-free areas in Zimbabwe were compared regarding their susceptibilities to challenge with *C. ruminantium*. Prior to challenge, *C. ruminantium* could not be detected in any sheep by transmission to *A. hebraeum* ticks or by PCR conducted with plasma samples. All sheep were equally susceptible to the challenge, and infection was confirmed by brain biopsy, necropsy, PCR and transmission to ticks. Mahan *et al.* (1993) suggested that the immunoblot “positive” reactions of the sera were not due to previous *C. ruminantium* infection, but serological cross-reactions with other positive reactors such as *E. canis*, *E. equi*, *E. phagocytophila*, *E. bovis* and *E. ovina*.

They also reported that there was a higher frequency of immunoblot “positive” reactors in heartwater-free areas of Zimbabwe, which appeared to be associated with minimal tick control. Tick species which occur in these areas, such as *Rhipicephalus evertsi evertsi* and various *Hyalomma* species are known to transmit *E. bovis*, *E. canis* and *E. ovina*. Savadye, Kelly & Mahan (1998) later collected *R. evertsi evertsi* adults from these areas of Zimbabwe with “false positives” and fed these ticks on seronegative sheep. The sheep seroconverted to the MAP-1 antigen, but were again totally susceptible to challenge with *C. ruminantium*.

In conclusion the disadvantages of the serological tests for *C. ruminantium* are mainly due to poor sensitivity and specificity, which include serious cross-reactions with various closely related *Ehrlichia* species.

1.3.2.3.7 Recent developments of the serological tests (MAP-1B ELISA technology)
Recent developments (Van Vliet *et al.* 1995; Mahan, Semu, Peter & Jongejan 1998c) with the MAP-1B ELISA test, however, appear to have at least partially solved the cross-reactions with *Ehrlichia*. Van Vliet *et al.* (1995) showed that the MAP-1 contains at least two immunogenic regions MAP-1A and MAP-1B. The MAP-1A contains conserved epitopes which cross-react with *Ehrlichia* species. The MAP-1B region, however, contains one or more epitopes with a high degree of specificity to *C. ruminantium*, and only antibodies to *E. canis* and *E. chaffeensis* reacted with MAP-1B. Mahan *et al.* (1998b) stated that *E. canis* and *E. chaffeensis* are only found in dog and human sera respectively, and should not interfere with routine ruminant diagnostic serological tests.

Mahan, Semu, Peter & Jongejan (1998c) evaluated the MAP-1B ELISA and the immunoblot assay, and compared their specificity and sensitivity on goat, sheep and cattle sera, from heartwater-free and heartwater-endemic areas.

In the heartwater-free areas the MAP-1B ELISA detected only 1% (bovine), 4% (caprine) and 3% (ovine) positives in the various sera. The immunoblot assay detected 40% (bovine) and 83% (ovine) positive sera in the same heartwater-free areas. In heartwater-endemic areas, the MAP-1B ELISA detected 33% (bovine) and >90% (caprine) positive sera.

It was clear that the immunoblot test detected many “positive” samples in heartwater-free areas. The MAP-1B ELISA test did not detect many false positives (1 - 4%) in cattle, goat and sheep sera in the heartwater-free areas, and clearly the specificity was greatly improved. In heartwater-endemic areas, however, the sensitivity of the MAP-1B ELISA was much more difficult to interpret, and cattle and goat sera showed wide differences in sera-prevalence (33% - >90%).

In conclusion great strides have been made with the refinement of serological tests for *C. ruminantium* over the past decade. As a result, the serological tests have increased in sensitivity and specificity, and as a consequence they can now be used with more confidence in serological surveys.

1.3.2.4 **Molecular assays to detect *C. ruminantium***

1.3.2.4.1 Molecular probes (DNA/RNA)

■ Principles of molecular probes

Nucleic acid hybridization can be used to identify specific micro-organisms by using Deoxyribonucleic acid (DNA) or Ribonucleic acid (RNA) probes. The principles involved in the use of the molecular probes are illustrated in Figure (Fig.) 1.

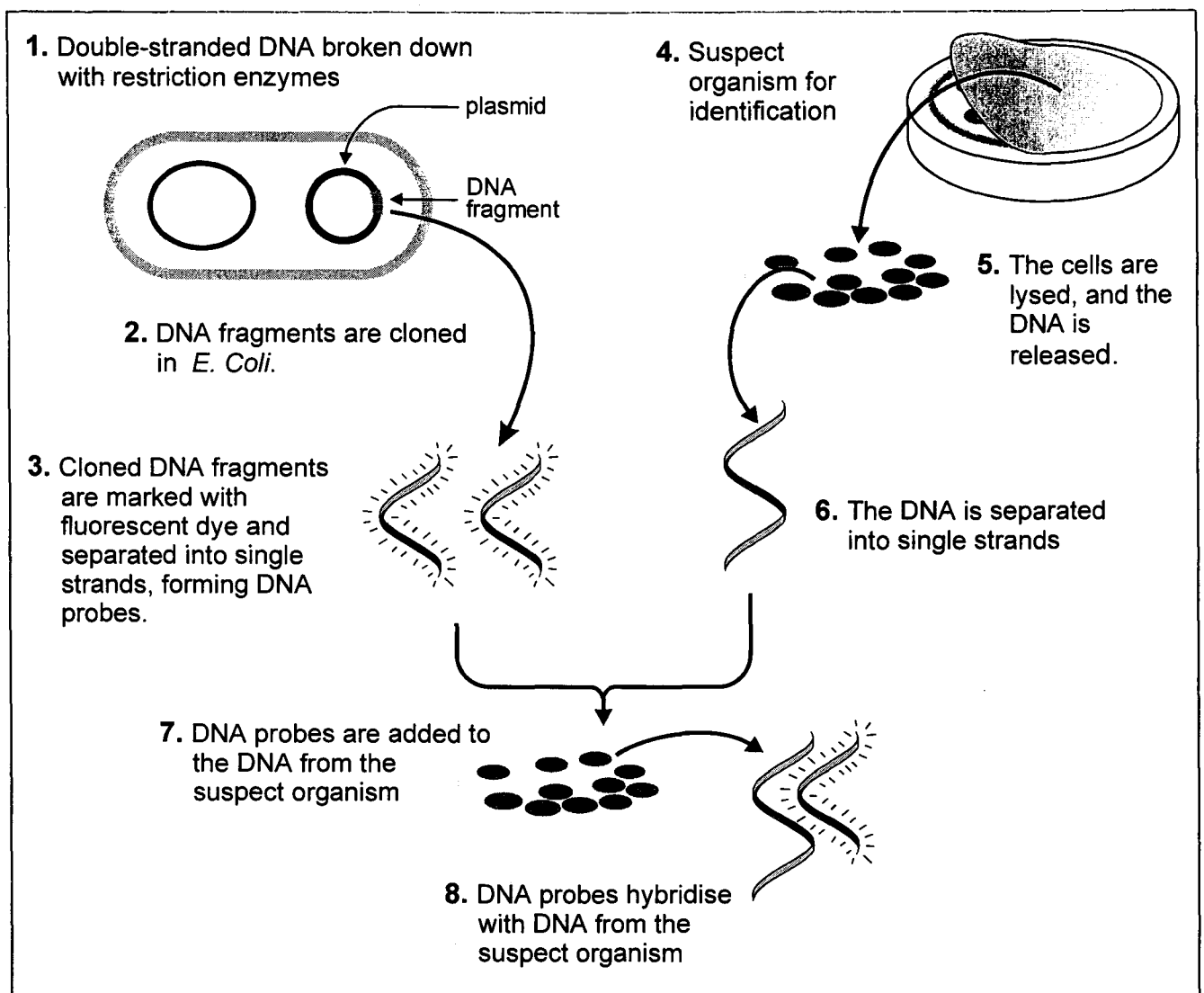


Fig. 1 Principles of a DNA probe used to identify an unknown organism

(Adapted from Tortora *et al.* 1995)

Double-stranded DNA for the probe is initially broken down with restriction enzymes (1), and the small species specific DNA-fragment cloned in *E. coli* (2). The cloned DNA fragments are then marked (3) with fluorescent dye or radioactivity and separated into single strands (DNA probes). These DNA probes can be reacted with target DNA, grown in culture from the suspected organism, which has been blotted onto paper (4 - 6). If the suspected organism is there, the DNA probe will hybridize with its DNA, and this will be detected by radioactivity or fluorescence of the probes.

- Advantages of molecular probes

Molecular probes have certain advantages over conventional diagnostic methods. They are very sensitive, for example, the DNA probe for the diagnosis of *Plasmodium falciparum* malaria can detect 10 picograms (pg) of purified DNA and this is equivalent to only 100 parasites (Barker, Laksami, Rooney, Alecrim, Dourade & Wirth 1986). The *Anaplasma marginale* probe (Eriks, Palmer, McGuire, Allred & Barbet 1989) is 4 000 times more sensitive than existing conventional types of detection. The probes are also very specific and a workable probe should only detect the specific organism and not related organisms. This is because the mechanism of the probe is based on the recognition of the specific DNA structure. The probe for *P. falciparum* for example, only recognizes *P. falciparum* and not two closely related species *P. vivax* and *P. cynomolgi* (Barker *et al.* 1986).

Another advantage of molecular probes is that they give a very quick diagnosis, and are of increased use in the diagnosis of organisms which take a long time to culture or cannot be

cultured. The time taken to process samples is also reduced considerably with molecular probes. Barker *et al.* (1986) stated that with the DNA probe for malaria, a 1 000 samples a day could be done as opposed to only 60 per day with conventional blood smears. Molecular probes can detect antigen directly, and one gets a good indication of present infections as opposed to serological tests which cannot distinguish past and present infections. Finally the sensitivity of the DNA probes can also be exponentially increased through the use of amplification technology such as Polymerase Chain Reaction (PCR).

- Disadvantages of molecular probes

The molecular probe technology is technically demanding, and requires expensive equipment and well-trained laboratory staff. This obviously adds to the cost per sample, so for economic reasons a large throughput of samples is required to bring down the running costs. Although the molecular probes are very sensitive, they cannot detect the very low numbers of organisms circulating in certain reservoir hosts. This was demonstrated by Peter (1995) who found that the Zimbabwean heartwater (pCS20) probe for *C. ruminantium* was not sensitive enough to detect the very low numbers of *C. ruminantium* circulating in reservoir hosts and field ticks.

- Use of molecular probes in medical epidemiological surveys

Much of our present veterinary epidemiological knowledge was gleaned from medical fields. Barker, Banchongaksorn, Courval, Suwongkerd, Rimwungtragoon, Sritong & Wirth (1994) reported on a major epidemiological survey for malaria carried out in Thailand. Their

studies showed that the major advantage of the DNA probe in large-scale epidemiological work was the time saved. Barker *et al.* (1994) were able to increase the time-saving factor by 20 to 40 times, when compared with conventional methods. DNA probes have also been used by research teams to detect *Leishmania* and *Trypanosoma cruzi* in various other epidemiological surveys (Barker 1990).

1.3.2.4.2 Use of molecular probes to detect TBD closely related to *C. ruminantium*

■ Anaplasmosis

Much of the early innovations and development of molecular probes to detect anaplasmosis was done by American research workers, and the technology was then transferred to Africa. Goff, Barbet, Stiller, Palmer, Knowles, Kocan, Gorham & McGuire (1988) developed a 2-kilobase (Kb) DNA fragment from a cloned *A. marginale* gene to be evaluated as a DNA probe. They reported that the sensitivity of the probe exceeded that of blood smears, and believed it would be helpful in identifying chronic carrier animals. The probe was species-specific, and did not hybridize with *A. ovis* DNA. Eriks *et al.* (1989) later reported on the development and use of a RNA probe able to detect the very low parasitaemia present in chronic carriers of anaplasmosis. The probe was an RNA transcript made from a 2-Kb fragment of a gene encoding a portion of an *A. marginale* surface protein. The probe was able to detect *A. marginale* in red blood cells 12 days before microscopic diagnosis, and was 4 000 times more sensitive than the latter. Shompole, Waghela, Rurangirwa & McGuire (1989) described the use of a pA012A DNA probe which identified *A. ovis* in goats in Kenya. *A. marginale* and *A. centrale*, which both occur in Kenya, did not hybridize to the probe.

- Babesiosis

DNA probes for *Babesia bigemina*, *B. bovis* and *B. caballi* have been developed recently, and are sensitive enough to detect clinical infection but not carrier status (Reddy & Dame 1992). A RNA-based probe for the detection of carrier status in *B. bigemina* has been tested and can detect the very low numbers of *Babesia* organisms present in carrier animals (Reddy & Dame 1992).

- Theileriosis

Conrad, Iams, Brown, Sohanpal & Ole-MoiYoi (1987) used three different DNA probes from a genomic library of *Theileria parva* to distinguish the five major stocks of *T. parva* in Kenya. The probe was the most useful in distinguishing between all five stocks, and did not hybridize to *Theileria mutans*, *Theileria taurotragi* or *Theileria annulata* (Conrad *et al.* 1987).

1.3.2.4.3 Molecular probes for *C. ruminantium*

The development of molecular probes for *C. ruminantium* closely followed those for anaplasmosis, and a lot of the highly technological developments were in the USA, Kenya and Zimbabwe. Waghela, Rurangirwa, Mahan, Yunker, Crawford, Barbet, Burrige & McGuire (1991) reported on two cloned DNA probes which identified *C. ruminantium* in *A. variegatum* ticks. These two probes were evaluated, and the pCS20 probe was shown to be more sensitive and specific than the *C. ruminantium* probe (pCR9) from Kenya. More

recently Allsopp, Hattingh, Vogel & Allsopp (1999) evaluated a panel of eight different molecular probes used for the detection of *Cowdria* and *Ehrlichia* species. The development and evaluation of the probes for *C. ruminantium* are discussed in detail in Chapter 3 (section 3.1).

1.3.2.4.4 Polymerase chain reaction

■ Principles of PCR

Very small quantities of DNA can be amplified with a method called PCR, to produce usable quantities of DNA for analysis. The procedure was originally developed by Mullis & Co-workers and he received the Nobel Prize for this work in 1993, and today the technique is standard practice in laboratories throughout the world.

■ Advantages of PCR

The PCR is a very fast, highly sensitive and specific diagnostic procedure which makes it very useful in epidemiological surveys which require extreme levels of sensitivity and specificity (Azad, Webb, Carl & Dasch 1990). It also has other advantages over conventional assays as it does not require frozen samples. DNA from dry dissected organisms, as well as archival specimens and alcohol-preserved samples can be used. The PCR has found wide application in various fields of molecular biology. In human diagnostics it is widely used for the pre-natal diagnosis of inherited diseases, and by evolutionary biologists and forensic pathologists to detect minute quantities of DNA.

■ Disadvantages of PCR

The very sensitivity which makes the PCR a useful technique, is also its most serious

limitation. Because of exponential amplification, targets can be achieved from any air-borne cross-contaminants and can present a serious risk of “false positive” amplification. By far the most common source of “false positive” contamination are products of previous PCR reactions. The sequence of the target material used for the PCR must also be known in order to develop the primers.

- The use of PCR in epidemiological surveys

In the last decade there has been a vast list of reports on PCR techniques in epidemiological surveys world-wide. In the veterinary diagnostic field, the PCR has found most credibility with those organisms which have traditionally been difficult to culture, some of these are described below.

African swine fever virus (ASFV) is enzootic in certain areas of Europe and often difficult to identify. Steiger, Ackermann, Mettraux & Kihm (1992) developed a PCR to detect ASFV which is rapid and safe. Lopez, Osorio & Donis (1991) also developed a PCR for bovine viral diarrhoea which was historically difficult to distinguish from numerous, similar viruses of bovines. Gerritsen, Olyhoek, Smits & Bokhout (1991) have reported on a rapid, specific and sensitive PCR for *Leptospira interrogans* in bovine urine. As culturing of *Leptospira* organisms could take up to six months to confirm a negative sample, this rapid PCR test would have practical benefits to dairy farmers.

1.3.2.4.5 The use of PCR to detect TBD closely related to *C. ruminantium*

- Anaplasmosis

Stich, Bantle, Kocan & Fekete (1993) collected haemolymph from the severed legs of

Dermacentor andersoni ticks, and *A. marginale* DNA was detected by a PCR assay in all the pooled haemolymph samples of ticks which had fed on infected hosts. *A. marginale* was also detected in adult ticks which had fed one day earlier on an infected calf. Stich *et al.* (1993) suggested that this test would be used to detect *A. marginale* in field collected ticks. They also concluded that the PCR/haemolymph test is a simple, rapid and sensitive method to detect *A. marginale* in ticks. The use of haemolymph samples also circumvents any cross-reaction and Taq-polymerase inhibition which were experienced with midgut specimens (Stich *et al.* 1993)

■ Babesiosis

The serological techniques used to diagnose bovine babesiosis do not consistently detect carrier animals, and do not specifically eliminate cross-reactions between *B. bigemina* and *B. bovis*. The use of specific DNA probes and nucleic acid hybridization had several advantages over conventional microscopic, serological and sub-inoculation techniques. According to Figueroa, Chieves, Johnson & Buening (1992) the DNA probe to detect *B. bigemina* in carrier cattle lacked sensitivity. They described the development of a PCR-based assay which detected latently infected cattle over an 11-month period. The PCR-based test was 10 000 times more sensitive than the non-radioactive probe. The authors believed that the high specificity and sensitivity of this PCR assay would provide a valuable tool for epidemiological studies.

■ Theileriosis

There have been numerous reports on the use of the PCR assay to detect various *Theileria*

species in domestic animals. Bishop, Sohanpal, Kariuki, Young, Nene, Baylis, Allsopp, Spooner, Dolan & Morzaria (1992) demonstrated that the PCR using *T. parva*-specific primers was capable of detecting parasites present at low parasitaemias in carrier cattle. D'Olivera, Van der Weide, Habela, Jacquiet & Jongejan (1995) used the PCR technique to detect *T. annulata* in blood samples from carrier cattle, and De Kok, D'Olivera & Jongejan (1993) used the PCR assay on alcohol-preserved specimens to detect *T. annulata* in *Hyalomma* ticks. Both confirmed that the PCR specifically amplified the 372-base pair (-bp) theilerial RNA target.

1.3.2.4.6 Detection of *C. ruminantium* in *Amblyomma* ticks

■ Conventional methods

The morphology and distribution of *C. ruminantium* in *A. hebraeum* was first described by Cowdry in 1925. He used conventional Giemsa staining techniques to demonstrate colonies of *C. ruminantium* in the gut epithelial cells, and sometimes in the lumen of the alimentary tract of the tick. These findings were later confirmed with fluorescent antibody staining techniques, and light and electron microscopy in *A. hebraeum* (Bezuidenhout 1984), and *A. variegatum* (Kocan, Morzaria, Voigt, Kiarie & Irwin 1987). Yunker, Kocan, Norval & Burrige (1987) reported that *C. ruminantium* colonies in the midgut of *A. hebraeum* could be distinctively stained with a Mallory's phloxine-methylene blue stain. They predicted that the stain could be applicable in detecting infected ticks collected in the field.

Kocan, Bezuidenhout & Hart (1987) described the presence of two types of *C. ruminantium*, electron dense and reticulate forms, in the gut epithelial cells of nymphal *A. hebraeum*. They

also observed the first *C. ruminantium* in the salivary glands of known infective *A. hebraeum*. An ELISA for detecting infection in *A. hebraeum* nymphs has been described, but has not been widely used (Neitz, Viljoen, Bezuidenhout, Oberem, Van Wyngaardt & Vermeulen 1986).

- The use of the pCS20 probe/PCR assay to detect *C. ruminantium* in *Amblyomma* ticks

A pCS20 probe for *C. ruminantium* detection in *Amblyomma* ticks has been developed and tested in the field (Waghela *et al.* 1991), but was found to lack the sensitivity to detect the very low numbers of the organisms found in field ticks. A PCR assay was developed by the University of Florida/United States Agency for International Development/Southern African Development Community (UF/USAID/SADC) Heartwater Project in Harare, Zimbabwe, to detect *C. ruminantium* in *Amblyomma* ticks. This will be discussed in detail in Chapter 3 (section 3.1).

Peter, Deem, Simbi, Barbet, Norval, Kelly & Mahan (1995a) evaluated the PCR assay as a method of detecting these very low rickettsaemias in carrier animals and *Amblyomma* ticks. The PCR assay detected *C. ruminantium* in both *A. hebraeum* and *A. variegatum* ticks from different geographical areas. The PCR assay was also specific for *C. ruminantium*, and did not detect other haemoparasitic TBD of domestic animals, or DNA from closely-related serologically cross-reactive organisms such as *E. canis*. Peter *et al.* (1995a) concluded that the PCR supercedes all older detection methods such as microscopy and xenodiagnosis, because of its high sensitivity, specificity and speed.

- The use of the PCR to detect *C. ruminantium* in ruminant and wildlife hosts

Mahan *et al.* (1993) were the first to use the PCR for detection of *C. ruminantium* in infected sheep plasma. They demonstrated that many immunoblot positive sheep from heartwater negative areas of Zimbabwe, were heartwater negative and were in fact, infected with tick transmitted *Ehrlichia* species and not *C. ruminantium* (refer to section 1.3.2.3.6). Mahan *et al.* (1993) demonstrated that the PCR assay can be used to distinguish pre- and post-*Cowdria* infection in the plasma of sheep. They showed that in the pre-infection sheep (uninfected) there was no PCR amplification of the *Cowdria* specific 279-bp DNA products. After challenge with *C. ruminantium*, the 279-bp DNA product was amplified by PCR.

Peter *et al.* (1995a) were able to use the PCR on ticks which had been feeding on recovered carrier cattle, and these ticks were then shown to be infected with *C. ruminantium* by using the DNA probe and the PCR assay. Peter (1995) also demonstrated that *C. ruminantium* can be detected in carrier cattle, by using the DNA probe on the cattle and the PCR assay on the ticks which had fed on the cattle.

Koch *et al.* (1995) developed a PCR assay to detect *C. ruminantium* in whole blood and bone marrow samples in clinically normal, free-ranging Zimbabwean wild ungulates. Prior to this work there was no reference to the use of whole blood or bone marrow for the PCR detection of *C. ruminantium*. PCR inhibition by elements in the haemoglobin was thought to be the reason for negative PCR results. Koch *et al.* (1995) also found positive PCR in tsessebe (*Damaliscus lunatus*), waterbuck (*Kobus ellipsiprymnus*) and impala (*Aepyceros melampus*). They suggested that these wild animals could be important reservoirs for *C. ruminantium*.

1.3.3 THE *AMBLYOMMA* TICK VECTOR: ECOLOGICAL ASPECTS

1.3.3.1 Important tick vectors

Only ticks of the genus *Amblyomma* have been shown to be capable of transmitting *C. ruminantium*. *Hyalomma truncatum* (Du Plessis 1990), and *Rhipicephalus evertsi evertsi* (Savadye *et al.* 1998) have, however, been implicated of transmitting *Cowdria*-like organisms which may interfere with serological surveys.

Heartwater occurs only where its vectors are present, and ten *Amblyomma* tick species are capable of transmitting the organism in Africa. The major species are *A. variegatum* and *A. hebraeum*. Species of lesser importance include *A. pomposum* (Angola), *A. lepidum* (East Africa), *A. astrion* (mainly a buffalo tick), *A. cohaerens* and *A. gemma*, both of which occur in East Africa (Walker & Olwage 1987). *A. sparsum*, *A. tholloni* and *A. marmoreum* are regarded as accidental vectors on domestic hosts and normally feed on wild animals. Only *A. variegatum* has spread to other parts of the world, and is now well-established in the West Indies (BurrIDGE 1985). Three American species of *Amblyomma* (*A. maculatum*, *A. cajennense* and *A. dissimile*) have been shown to be capable of transmitting *C. ruminantium* (Uilenberg 1982; Barré *et al.* 1987; Bezuidenhout 1987; Jongejan 1992).

1.3.3.2 Distribution of the vectors

The distributions of the *Amblyomma* vectors and potential vector species in Africa and the Americas have been well-documented (Walker & Olwage 1987). *A. hebraeum* is restricted to southern Africa (Mocambique, Swaziland, Botswana and South Africa), and in Zimbabwe it co-exists with *A. variegatum*. Walker & Olwage (1987) confirmed that three *A. hebraeum* males were collected in Tanzania, but believed these to have been transported there by birds.

A. variegatum is the most common *Amblyomma* species in Africa, and in southern Africa is widespread in Mocambique, Botswana and Zimbabwe. It has spread to Madagascar, presumably with mainland cattle, many years ago. *A. variegatum* has also spread to various islands in the Caribbean, presumably by the importation of tick-infested cattle from West Africa (Burrige 1985).

Amblyomma pomposum is almost entirely restricted to Angola, and its distribution overlaps that of *A. variegatum*. *Amblyomma lepidum*, *A. cohaerens* and *A. gemma* are common in East Africa and parts of Sudan, Ethiopia and Somalia. *A. lepidum* has been implicated in the transmission of heartwater to sheep and goats in Sudan (Karrar 1960). *A. cohaerens* and *A. astrion* are closely associated with buffalo (Walker & Olwage 1987). *A. gemma* is common in East Africa, and has recently been shown to be a vector of *C. ruminantium* (Wesonga, Mukolwe & Rurangirwa 1993).

A. marmoreum, in the adult stage, is almost exclusively a tick of reptiles (tortoises, snakes and monitor lizards) (Walker & Olwage 1987). The immature stages are found on domestic and a variety of wild animals (Horak, MacIvor, Petney & De Vos 1987). *A. marmoreum* has also been shown to transmit *C. ruminantium* (Bezuidenhout 1987), and has recently been discovered on tortoises in the USA (Allan, Simmons & Burrige 1998). *A. tholloni* is exclusively found on elephants, although MacKenzie & Norval (1980) reported that in Zimbabwe domestic animals were infested with immature ticks of this species.

1.3.3.3 Population dynamics of the *Amblyomma* species

1.3.3.3.1 South Africa

South Africa has a long history of ticks and TBD because of the historical importance to the stock industry. The only *Amblyomma* species of economic importance in South Africa is *A. hebraeum*. In 1900, Lounsbury confirmed the long-standing suspicion that *A. hebraeum* was the vector of *C. ruminantium* in South Africa. Much of the early work was conducted in those regions of South Africa where heartwater was a major problem for the farmers. It was soon realized that more information about the life cycle and seasonal occurrence of the tick was necessary in order to control it. This led to the establishment of Research Centres at Onderstepoort, Grahamstown and Pietermaritzburg.

Alexander (1931) stated that farmers in the Eastern Cape region of South Africa blamed their heavy stock losses (2 000 - 3 000 sheep and goats per annum), to the introduction of *A. hebraeum* from Zululand where the tick was endemic. Heartwater also had devastating effects on the highly susceptible Angora goat flocks, and this resulted in increased research into the life cycle and population dynamics of *A. hebraeum*. Norval (1974; 1977) was responsible for the early research on the ecology, life cycle and population dynamics of *A. hebraeum* in the Eastern Cape Province. He concluded that the life cycle of *A. hebraeum* can last for up to three years, the adults were more prevalent in mid- to late summer, while the immature stages were found throughout the year, and concluded that the absence of nymphal activity was due to a decrease in photoperiod (Norval 1977).

To investigate the dynamics of various tick populations in the Eastern Cape, a detailed five-year survey was then conducted at the Tick Research Unit in Grahamstown. *A. hebraeum* adults were found to be present on cattle throughout the year, but the highest numbers were encountered in summer. This was followed by larval peaks in late summer and early winter, and two nymphal peaks, one in early midsummer and the other in winter (Rechav 1982). Rechav concluded that the life cycle in the Eastern Cape probably only lasted one year and not three years as suggested by Norval (1977).

Horak & Williams (1986), also working at the Tick Research Unit in Grahamstown, reported that helmeted guinea fowls (*Numida meleagris*) were important hosts of *A. hebraeum* immatures, especially the larval stages. Howell, Petney & Horak (1989) also looked at the four-striped grass mouse (*Rhabdomys pumilio*) as a possible host of *A. hebraeum*, but concluded that it was not a significant carrier of these ticks. MacIvor (1982) noted that there was a clear relationship between foot abscesses and the seasonal abundance of *Rhipicephalus glabroscutatum* and *A. hebraeum*, the two most important tick species found on the feet of Angora goats in this region.

An early investigation in KwaZulu-Natal on the tick infestation of livestock by Baker & Du Casse (1967) confirmed that *A. hebraeum* was common on cattle on the farms studied. The adult *A. hebraeum*, in this study, were active from the beginning of September and their numbers only declined towards the end of January. They also noted that although Theiler (1962) had concluded that most immature *A. hebraeum* occur on birds, in their study, a significant proportion also occurred on cattle, and then especially on their limbs.

Much of the early work on tick taxonomy and population dynamics at the Onderstepoort Veterinary Institute was initiated by Gertrud Theiler (1948; 1962), and this culminated in a publication on the ixodid ticks found in this region (Theiler 1962). Londt, Horak & De Villiers (1979) working in the Naboomspruit district, in the Northern Province of South Africa, collected ticks off cattle and only 4% of these were *A. hebraeum*. Although they were unable to conclude exactly when the various peak abundances occurred, they hypothesized that the peak larval period was in late summer/autumn, the nymphal peak in winter/spring and the adult peak periods August or September to January, or February.

Horak has published extensively on the ectoparasites associated with wild animals in South Africa. Some of his more important findings associating *A. hebraeum* with these animals are summarized below:

- The size of the host animal does not appear to have a strong influence on the total tick loads, but with certain exceptions, the larger hosts tended to carry more adult ticks (Horak 1995). Two exceptions to this observations were, warthogs (*Phacochoerus aethiopicus*) which are relatively small animals yet carry fairly large burdens of *A. hebraeum*, and blue wildebeest (*Connochaetes taurinus*) which carried virtually no ticks (Horak 1995). The smaller hosts, such as helmeted guinea fowls (*Numida meleagris*), and scrub hares (*Lepus saxatilis*), tended to carry more immature ticks.
- Certain host species, such as blue wildebeest, seem to have a natural resistance to infestation, and as a consequence carry very low tick burdens (Horak, De Vos & Brown 1983).

- A complete absence of *A. hebraeum* on gerbils (family *Gerbillinae*) and veld rats (*Aethomys* spp.) and very low numbers on striped mice (*Rhabdomys pumilio*). Rodents probably play no role in the epidemiology of heartwater (Howell *et al.* 1989; Horak 1995).
- Burchell's zebra (*Equus burchelli*), shot in the Kruger National Park (KNP), were quite good hosts of all stages of *A. hebraeum*, and they were present on these zebras throughout the year (Horak, De Vos & De Klerk 1984).
- Warthogs must be considered a preferred host of *A. hebraeum*, especially the adult stages (Horak, Boomker, De Vos & Potgieter 1988).
- *A. hebraeum* was the most abundant larval tick collected by drag sampling in the KNP (Spickett, Horak, Braack & Van Ark 1991).

Rechav & De Jager (1991) found that *A. hebraeum* was one of the three most abundant ticks on goats in the Northern Province of South Africa. Adult *A. hebraeum* were present on the goats during summer but absent during winter. The nymphs were present during most of the year, but absent in July (Rechav & De Jager 1991).

1.3.3.3.2 Zimbabwe

The history of tick and TBD in Zimbabwe has been extensively reviewed (Lawrence & Norval 1979). The first recorded heartwater case was in 1927, and it was thought that the disease had been introduced from South Africa (Jack 1942). In 1930, the bont tick (*A. hebraeum*) had spread to large areas of the western lowveld region of Zimbabwe (Norval,

Perry, Meltzer, Kruska & Booth 1994). Between 1942 and 1975 the vector was subjected to a very intensive dipping campaign, and in 1975 only three isolated foci of the tick remained (Norval *et al.* 1994). The disruption of dipping during seven years of conflict in Zimbabwe lead to the breakdown of dipping services, and therefore to the spread of *A. hebraeum* in large areas of Zimbabwe (Norval *et al.* 1994), and this spread has continued up to the present (Bruce & Wilson 1995; Peter, Perry, O'Callaghan, Medley, Shumba, Madzima, Burridge & Mahan 1998).

A. hebraeum has always been absent from the highveld regions of Zimbabwe, and it was thought that the climatic conditions in these areas were not conducive to the survival of *A. hebraeum* (Theiler 1962), as well as the lack of alternative hosts in the region (Norval 1983).

A. hebraeum immatures can feed on a wide range of domestic hosts, as well as wild animals and the translocation of the latter hosts to game reserves may have played an important role in the rapid spread of heartwater in Zimbabwe.

Norval, Andrew & Meltzer (1991) looked at the seasonal occurrence of *A. hebraeum* in Zimbabwe and concluded that adults and nymphs were present on cattle at all times of the year, and they showed less seasonal periodicity than the other three host ticks in the region. Mooring, Mazhowa & Scott (1994) investigated the effects of rainfall on tick challenge at the Kyle Game Reserve near Masvingo, Zimbabwe, and noted that in the case of *A. hebraeum*, only the larval stage could be correlated with the monthly rainfall pattern. The distribution of *A. variegatum* in Zimbabwe has remained static since the early part of the century, and is restricted to the north-western region (Norval *et al.* 1994).

1.3.3.3.3 *A. variegatum* in Zambia

The population dynamics of *A. variegatum* in Zambia are well-documented, with the adults active during the rainy season, and peak abundance on cattle from November to January (MacLeod 1970; Pegram, Perry & Schels 1984). The larvae are present from March to May, and the nymphal population during the cool, dry season May - June (Pegram *et al.* 1984). *A. variegatum* is one of the most important tick species in Zambia, and has been reported from a wide variety of habitat types (Mangani 1997). Very few *A. variegatum* larvae were, however, collected by Zieger, Horak & Cauldwell (1998), and only about 200 of the nearly 40 000 larvae collected by drag-sampling at Mtendere Game Ranch in Central Province, Zambia, were *A. variegatum*.

1.3.3.3.4 *Amblyomma* species in Botswana

Both *A. hebraeum* and *A. variegatum* are present in Botswana, but *A. hebraeum* causes the greatest economic problems (Chimbombi 1997). This is usually when heartwater-susceptible cattle are moved to the market area bordering South Africa, where *A. hebraeum* is found. *A. variegatum* is found in the extreme north of Botswana, which borders Zambia and Zimbabwe.

1.3.3.3.5 *Amblyomma* species in Mocambique and Angola

Heartwater occurs throughout both countries, but mainly during the rainy season. In southern Mocambique *A. hebraeum* is the main heartwater transmitter, and *A. variegatum*

is found in the north of the country (Mavale 1997). In Angola *A. variegatum*, *A. pomposum* and *A. astrion* are the most common *Amblyomma* species (Gomes 1993). *A. variegatum* is only found in the eastern region of Angola, whilst *A. pomposum* is far more widely spread throughout the southern and northern regions of Angola (Ricardo 1997). *A. pomposum* is found in those areas of Angola where most of the domestic animals are kept, and is the most important vector of heartwater. Adults occur throughout the year, but the tick loads are heavier on cattle during the rainy season (Gomes 1993).

1.3.3.3.6 *Amblyomma* ticks in Swaziland

Heartwater occurs in all four agro-ecological zones in Swaziland, and the incidence of the disease follows that of the vector *A. hebraeum* (Dlamini 1997). Heartwater is the second most important disease affecting cattle, and *A. hebraeum* is present at nearly 80% of the diptanks surveyed in the Lowveld and Lubombo region (Dlamini 1997). *A. hebraeum* is also common on wild animals in Swaziland, and Gallivan (1995) found that adult ticks were common on the five species of ungulates studied. Gallivan, Culverwell, Girdwood & Surgeoner (1995) also found that impala (*Aepyceros melampus*), at the Mlawula-Mbuluzi-Simunye Nature Reserve were infested with *A. hebraeum* adult ticks, and these ticks cause a loss of body condition.

1.3.3.3.7 *Amblyomma* species in East Africa (Kenya, Tanzania, Uganda and Burundi)

A. variegatum on Ruzinga Island in Western Kenya appears to have only one generation per annum (Punyua, Latif, Nokoe & Capstick 1991). The high adult infestations of cattle with *A. variegatum* reached a peak between January and February, and were followed by a

sudden drop with the onset of the rainy season (Punyua *et al.* 1991). In Uganda, Kaiser, Sutherst & Bourne (1982) found that *A. variegatum* was the second most common tick on Zebu cattle, although all species of ticks were present throughout the year, with no seasonal variation in abundance of the two- and three-host species (Kaiser *et al.* 1982).

Kaiser *et al.* (1982) working in Burundi, gave a detailed record of the population dynamics of the ticks on the local “Ankole” cattle. The life cycle of *A. variegatum* in this region is closely linked to the rainfall, and the adults peak after the heavy rain in September to January (Kaiser *et al.* 1982). The larvae follow after a four-month delay, and the nymphs are present in the dry season (Kaiser *et al.* 1982). Twelve *Amblyomma* species have been identified in Tanzania, but only *A. variegatum* and *A. gemma* are important vectors of heartwater (Otaru 1997).

1.3.3.3.8 *Amblyomma* species in West Africa (Mali, Nigeria and Senegal)

Teel, Bay & Ajidagba (1988) found *A. variegatum* to be a very common tick in southern Mali, and the adult ticks were found to increase after the rains, followed by the larvae and nymphs in the winter/dry season. Bayer & Maina (1984) found that *A. variegatum* was a dominant tick species in their survey in Nigeria. In Senegal, Gueye, Mbengue, Diouf & Sonko (1993) and Gueye, Mbengu & Diouf (1994) found that *A. variegatum* was a common tick on the cattle, sheep and goats which they sampled.

1.3.3.4 **Pathogenesis / Life Cycle in the tick**

In infected ticks the organisms have been seen in the epithelial cells of the midgut (Cowdry

1925). Cowdry (1925) believed that transmission occurred by regurgitation of the gut contents during the feeding of the tick. More recent information suggested that *C. ruminantium* is injected into the host with the tick's saliva (Kocan, Bezuidenhout & Hart 1987). Organ suspension of infected ticks demonstrated that all organs in the tick were infected with *C. ruminantium*, except the brain, haemolymph and ovaries (Bezuidenhout 1988).

1.3.3.5 Behaviour of the vectors in the field

Unfed nymphs and adults of *A. variegatum* and *A. hebraeum* do not ascend the vegetation to await passing hosts, as occurs in so many other ixodid tick species. For many years it was not known where the adult and nymphal stages of *A. hebraeum* survived during the long, unfavourable, non-parasitic period of the life cycle. It is now well-documented that these ticks seek shelter beneath the debris on the soil surface and only become active in response to CO₂ and AAAP (Norval, Yunker & Butler 1987).

The unfed ticks are able to locate suitable hosts from distances of up to 25 metres. The ticks also attach preferentially to suitable hosts, which carry attached fed males secreting AAAP. This largely eliminates their chances of attaching to unsuitable hosts (dipped hosts with no males, or hosts with good grooming behaviour), on which they would be unlikely to survive (Norval, Andrew, Yunker & Burrridge 1992). Game ranches where cattle share grazing with large wild animals, which naturally have a high prevalence of pheromone secreting male ticks, make the control of *A. hebraeum* extremely difficult.

1.3.4 ***COWDRIA RUMINANTIUM* IN THE TICK VECTOR**

1.3.4.1 **Infection rates of *C. ruminantium* in the tick vector**

In order to advise government policy makers on the use of strategic control, more information on the population dynamics of the *Amblyomma* vector species and their infection rates is required (Norval 1988). Many surveys on the *Amblyomma* ticks of Africa have been published, and we can now, with a certain degree of confidence, predict the presence, or absence, of the *Amblyomma* vectors in specific areas, and at specific times of the year. However, this is not the case with the infection rates in free-living or non-parasitic ticks, probably because of the difficulty in detecting with confidence the presence of *C. ruminantium* in *Amblyomma* ticks. In the past it was generally assumed that the heartwater infection rate in tick populations was low, as domestic ruminants could escape infection with heartwater whilst living in endemic regions (Currasson & Delpy 1928; Alexander 1931; Bonsma 1944; Neitz 1939; Uilenberg 1971).

The infection rates in most vector-borne diseases of domestic animals and man have been published and some of these are summarised in Table 1. It is noticeable that most of these infection rates are less than 5%. The infection rates of *C. ruminantium* in *Amblyomma* ticks obtained by the different research teams in Africa and the Caribbean, are summarized in Table 2. It is clear from Table 2 that a wide variety of different methods have been used for determining the infection rates, but only one (Peter *et al.* 1999) uses free-living, unfed *A. hebraeum* collected with a AAAP/CO₂ trap and processed with the PCR assay.

TABLE 1: Infection rates of some important vectors of domestic animals and man which have been reported in the literature

AUTHORS/ YEAR	VECTOR	PARASITE	DISEASE	INFECTION RATE IN VECTORS
Jupp **1994	Mosquito	RVF virus	RVF	0.01%
Plowright <i>et al.</i> **1994	<i>Ornithodoros</i> spp. Tampans	ASF virus	ASF	0.3 - 1.7%
Mahoney & Mirre 1971	<i>Boophilus</i> spp. ticks	<i>Babesia bigemina</i>	Redwater Bovine babesiosis	0.23%
Mahoney & Mirre 1971	<i>B. microplus</i>	<i>Babesia bovis</i>	Redwater	0.04%
Leitch & Young 1981	<i>R. appendiculatus</i>	<i>Theileria parva</i>	ECF	1 - 2%
Dallwitz <i>et al.</i> 1986	<i>Boophilus</i> spp. ticks	<i>Anaplasma marginale</i>	Anaplasmosis	0 - 100%
Burgdorfer <i>et al.</i> 1979	<i>Ixodes ricinus</i>	Swiss <i>Rickettsia</i>	Spotted fever group	8.4%
Burgdorfer <i>et al.</i> 1985	<i>Ixodes pacificus</i>	<i>Borrelia burgdorferi</i>	Lyme Disease	1 - 2%
Magnarelli <i>et al.</i> 1986	- <i>Ixodes damini</i>	"	"	10.5%
	- <i>Dermacentor albipictus</i>	"	"	0.6%
	- <i>Amblyomma americanum</i>	"	"	3.5%
Rijpkema <i>et al.</i> 1994	<i>Ixodes ricinus</i>	"	"	13 - 46%
Piesman <i>et al.</i> 1986	<i>Ixodes damini</i>	<i>Babesia microti</i>	Human babesiosis	11 - 47%

** Coetzer, Thompson & Tustin. 1994. *Infectious Diseases of Livestock*. Oxford University Press

TABLE 2: Infection rates of *Cowdria ruminantium* in *Amblyomma* ticks reported in the literature

AUTHORS & YEAR	STUDY AREA	TICK SPECIES	METHOD OF COLLECTION	METHOD TO DETECT <i>Cowdria</i>	FORMULA TO DETECT INFECTION RATE	TOTAL SAMPLE SIZE	LIFE CYCLE STAGE	INFECTION RATES (% POSITIVES)
Uilenberg 1971	MADAGASCAR	<i>A. variegatum</i>	Field exposure	Sheep blood injected into cattle	-	-	-	1% estimate
Du Plessis 1981	SOUTH AFRICA	<i>A. hebraeum</i> Adults	Off cattle	Tick homogenate injected into mice - confirm IFA	-	n = 240		13/240 5.4%
Du Plessis & Malan 1987	SOUTH AFRICA 23 farms in heartwater endemic regions	<i>A. hebraeum</i> Adults	Off cattle	Tick homogenate injected into mice - confirm IFA	-	n = average 6 ticks per cow	-	1.6% - 30% (mean 7%)
Du Plessis <i>et al.</i> 1992	SOUTH AFRICA Mara	<i>A. hebraeum</i> Adults	Off cattle	Tick homogenate injected into mice - confirm IFA	-	n = 240	-	0% - 5% (mean 2.9%)
Peter <i>et al.</i> 1999	SOUTH AFRICA KNP	<i>A. hebraeum</i> Adults	AAAP trap	PCR assay	-	n = 292	A	1.7%
Norval 1981	ZIMBABWE Various communal grazing areas	<i>A. hebraeum</i> Engorged nymphs	Off cattle	Adults on sheep	Pooled ticks fed on 7 sheep	108		2/7 sheep died 28% positive
Norval <i>et al.</i> 1990	ZIMBABWE - Mbizi - Lemco	<i>A. hebraeum</i> Unfed adults & nymphs Nymphs	AAAP trap Off cattle	Pooled samples fed on sheep Adults on sheep	$P = 1 - \frac{(n-x)}{n} \frac{1}{m}$	32 40 80 49 36	M F N M F	0 → 45% 20 → 36% 0 → 13% 10 → 25% 20 → 34%
Peter 1995	ZIMBABWE Manifest Farm	<i>A. hebraeum</i> Adults	Off sentinel cattle	PCR assay on ticks	-	n = 512		1.76%
Peter 1997	ZIMBABWE - Southern lowveld	<i>A. hebraeum</i> Adults and nymphs	Off cattle	PCR assay on ticks	-	A = 441 N = 95	A M F N	11.48% 10.5% 12.45% 3.16%
Gueye <i>et al.</i> 1993	SENEGAL - Zones: North Guinean. Niayes. South Sudan	<i>A. variegatum</i> Adults and nymphs	Off cattle	Tick supernatant injected intravenously into 30 sheep	3 pools of ticks 1 RO = $\frac{\text{No of ticks Inf.} \times 100}{\text{Total ticks}}$		A N	11% 7.8%
Camus & Barré 1987b	CARIBBEAN (Guadeloupe)	<i>A. variegatum</i> Adults	Partially engorged ticks off cattle	Individual tick homogenate injected into mice - confirm IFA	- Pooled ticks	200 19 pools	-	3/200 Pos (1.5%) 12/19 Pools Overall (1 - 2%)

A = Adult

N = Nymphs M = Male

F = Female

Inf. = Infected

KNP = Kruger National Park

1.3.4.1.1 Infection rates in South African ticks

Du Plessis (1981; 1985) was the first researcher in South Africa to attempt to find a method to detect the percentage of ticks infected with *C. ruminantium* (refer to Table 2). He collected 240 adult *A. hebraeum* (50% males and females) off cattle throughout the heartwater-endemic regions of South Africa. These ticks were then homogenized and injected either intraperitoneally (I/P), or I/V into mice, and the mice either seroconverted to *C. ruminantium* or died of heartwater (Du Plessis 1985). Thirteen out of the 240 ticks tested were positive for *C. ruminantium* (mean of 5.4%), and of these only one mouse died of heartwater and 12 seroconverted. The results of the various experiments ranged from 2.5% to 20% prevalence (Du Plessis 1985).

Du Plessis & Malan (1987) did an extensive epidemiological study of 23 farms, all of which were in heartwater-endemic regions of South Africa. Six adult *A. hebraeum* ticks were collected off each cow in the survey, and the homogenates of these ticks were injected I/V into mice. A mean of 7% (range 1.6% to 30%) of these ticks were positive for *C. ruminantium*. Du Plessis & Malan (1987) also concluded that the *A. hebraeum* burdens on the cattle were the most important determinants of heartwater immunity. They also showed that many of the farms with a high infection rate in the ticks, also had higher seropositivity to *C. ruminantium*. They concluded, however, that the latter relationship was not absolute (Du Plessis & Malan 1987).

In 1992, Du Plessis, Loock & Ludemann carried out an important epidemiological study at the Mara Experimental Farm in the Northern Province of South Africa. Two hundred and

forty adult *A. hebraeum* (125 males and 115 females) were collected and injected into mice. Positive results ranged from 0% to 5%, and never exceeded 5%. They concluded that the seropositivity of calves at six months of age was related to their tick burdens and the infection rates of these ticks. In other words, the higher the *A. hebraeum* burdens and tick infection rates, the more seropositive and immune the calves were. In this particular study 97% of the calves were found to be serologically positive, which indicated exposure to *C. ruminantium* or a closely-related organism. These seropositive calves were not necessarily immune to heartwater.

Du Plessis *et al.* (1992) went a step further, and proposed that if an average of ten adult *A. hebraeum* ticks were found on the calves in a heartwater-endemic area, then 83% of the calves would have seroconverted to *C. ruminantium* at six months of age. If 15 ticks were found on the calves then 97% of them would be serologically positive (Du Plessis *et al.* 1992). They stated that the present tick burdens of ten ticks per animal throughout the year at Mara, coupled with an infection rate in the tick of 3%, was enough to maintain endemic stability to heartwater. They concluded that higher tick infection rates and lower tick burdens, would suffice to induce endemic stability.

More recently Peter, Bryson, Perry, O'Callaghan, Medley, Mlambo, Smith, Horak, Burridge & Mahan (1999) collected 292 adult *A. hebraeum* with the AAAP trap near the Skukuza rest camp in the KNP, and tested them with a PCR assay. Of these ticks 1.7% were positive for *C. ruminantium*.

1.3.4.1.2 *C. ruminantium* infection rates in Zimbabwean ticks

In 1981 Norval collected 108 engorged *A. hebraeum* nymphs from cattle kept on communal grazing in a heartwater-endemic region in the southern lowveld region of Zimbabwe. These nymphs were allowed to moult and the adult ticks were then fed on seven heartwater susceptible sheep, and two (28%) of the seven sheep died of heartwater. Norval (1981) was not able to give an exact infection rate in the ticks after these experiments, but he concluded that the prevalence was high.

Norval, Andrew & Yunker (1990) working on the Lemco Ranch and the Mbizi Quarantine Station in the southern lowveld of Zimbabwe, conducted further experiments in order to calculate the infection rate using a mathematical formula (Norval *et al.* 1990). At Mbizi they collected 152 *A. hebraeum* ticks; 32 were male, 40 female and the remaining 80 were nymphs. Thirty-nine pools of free-living, unfed ticks were then fed on 18 heartwater-susceptible sheep (Norval *et al.* 1990). The infection rates in the various pools of ticks were calculated, using a formula adapted from researchers in mosquito epidemiology (Chiang & Reeves 1962). The infection rate in the male ticks ranged from 0 - 45%, in female ticks from 20 - 36% and in nymphs from 0 - 13%.

At Lemco Ranch engorged nymphal ticks were collected off weaner calves, allowed to moult and 22 pools of adult ticks were fed on 14 sheep. The infection rate at Lemco ranged from 10 - 25 % in the males and 20 - 34% in the females. These results from Zimbabwe were

considerably higher than findings in South Africa and the Caribbean (Du Plessis *et al.* 1992; Camus & Barré 1987).

More recently Peter (1995) working at Manifest Farm in the south-western lowveld region of Zimbabwe, an area considered to be heartwater-endemic, collected *A. hebraeum* adult ticks (n = 512) which had attached onto sentinel cattle. He used the PCR assay to detect *C. ruminantium* and 1.7% of the ticks were positive. Peter (1997) also collected adult and nymphal ticks (adults = 441, nymphs = 95) off cattle, again in the southern lowveld of Zimbabwe and these were also tested with the PCR assay. The overall infection rate in the adult ticks was 11.48% (10.5% in the males and 12.45% in the females), and in the nymphal ticks 3.16%.

1.3.4.1.3 *C. ruminantium* infection rates in Senegalese ticks

Gueye, Mbengue, Dieye, Diouf, Seye & Seye (1993) are part of a research team that has, over a number of years, been studying the population dynamics and various epidemiological aspects of Senegalese ticks. *A. variegatum* has been found to be the most economically important vector of *C. ruminantium* in that country. Gueye *et al.* (1993) collected unfed *A. variegatum* adults and nymphs off cattle in the Niayes, South Sudan and North Guinean regions of Senegal where the ticks were most prevalent.

Some of these ticks were initially fed on rabbits and then divided into three groups, homogenated and injected I/V into 30 susceptible sheep. Using a formula they deduced that 11% of the adults and 7.8% of the nymphs were infected with *C. ruminantium*.

1.3.4.1.4 *C. ruminantium* infection rates in Madagascan ticks

One of the early investigations into the infection rates of *C. ruminantium* in *A. variegatum* was published by Uilenberg in 1971. He noted that most of the cattle in Madagascar were exposed to large populations of the heartwater vector *A. variegatum*, but did not acquire immunity by two years, and sometimes five years of age. He concluded that the infection rate in the free-living, unfed ticks was very low (less than 1%), and as a consequence, few of the cattle were exposed to the organism (Uilenberg 1971).

1.3.4.1.5 *C. ruminantium* infection rates in Caribbean ticks

Camus & Barré (1987b) did extensive epidemiological studies on various aspects of *A. variegatum* in the Caribbean. They collected adult ticks off local cattle and injected individual and pooled homogenated ticks into mice, using the method of Du Plessis (1985). They found that three out of the 200 ticks (1.5%) were positive using IFAT.

1.3.4.2 **Inoculation Rate / Principle of Endemic Stability to TBD**

1.3.4.2.1 *Boophilus*/Babesiosis Model

Much of the early research on the endemic stability to TBD was conducted in Australia, using *Bos taurus* cattle infested with *Boophilus microplus* which were infected with *Babesia bovis* and *Babesia bigemina* (Mahoney 1969; Mahoney & Mirre 1971; Mahoney & Ross 1972). The researchers noted that in places where ticks were abundant there was endemic stability to babesiosis, and clinical cases were rare (Mahoney & Ross 1972). They developed predictive models for the disease, based on the assumption that one infection

confers immunity for life. This relationship between cattle and *Babesia* was maintained chiefly by infection of calves (Mahoney & Ross 1972), and for complete herd immunity, transmission of infection must be adequate to infect all calves in order to avoid clinical illness. Disturbance of this epizootiological balance occurs in places where tick populations have been reduced by dipping or by climatic changes (Mahoney & Ross 1972).

The inoculation rate represents the average probability that an animal in a herd of cattle will be infected with *Babesia* or other TBD (Mahoney & Mirre (1971). The inoculation rate (**h**) is a very important parameter in the maintenance or loss of endemic stability to a TBD. The inoculation rate is dependent on the number of ticks biting per day (**m**), the proportion of these ticks which are infected with the organism (**a**), and the proportion of bites which successfully transmit infection (**b**). The resultant formula to calculate the inoculation rate is illustrated below:

$$h = mab.$$

Mahoney & Ross (1972) concluded that the endemic stability to *Babesia* in cattle was dependent on the inoculation rate, and when the inoculation rate was high enough, then all calves became infected whilst being protected by colostral immunity, and clinical disease would be minimal and endemic stability was achieved (Mahoney 1974). If the inoculation rate was not sufficiently high, then not all the calves were infected, and endemic instability and clinical disease should follow (Mahoney 1974). Mahoney & Ross (1972) were then able to illustrate this process graphically (Fig. 2a).

The relationship between the level of vector challenge, disease incidence, case fatality and antibody prevalence to TBD is illustrated in Fig. 2b (Perry & Young 1995; O'Callaghan, Medley, Peter & Perry 1998). It is clear that with the increase of the inoculation rate, the number of animals which are immune to the disease also increases (endemic stability). The inoculation rate can also be substituted with percentage serologically positive animals, and a similar endemic stability graph can be produced (Fig. 2b).

Norval, Fivaz, Lawrence & Daillecourt (1983) produced a model for bovine babesiosis in Zimbabwe. They divided the country into five different epidemiological zones according to the percentage of serological positive sera (Norval *et al.* 1983). The disease-free regions (0% positive), the minimal disease areas (1 - 20% positive), the enzootically unstable regions (21% - 60% positive), the regions approaching enzootic stability (61 - 80% positive), and finally the enzootically stable regions (81 - 100% positive).

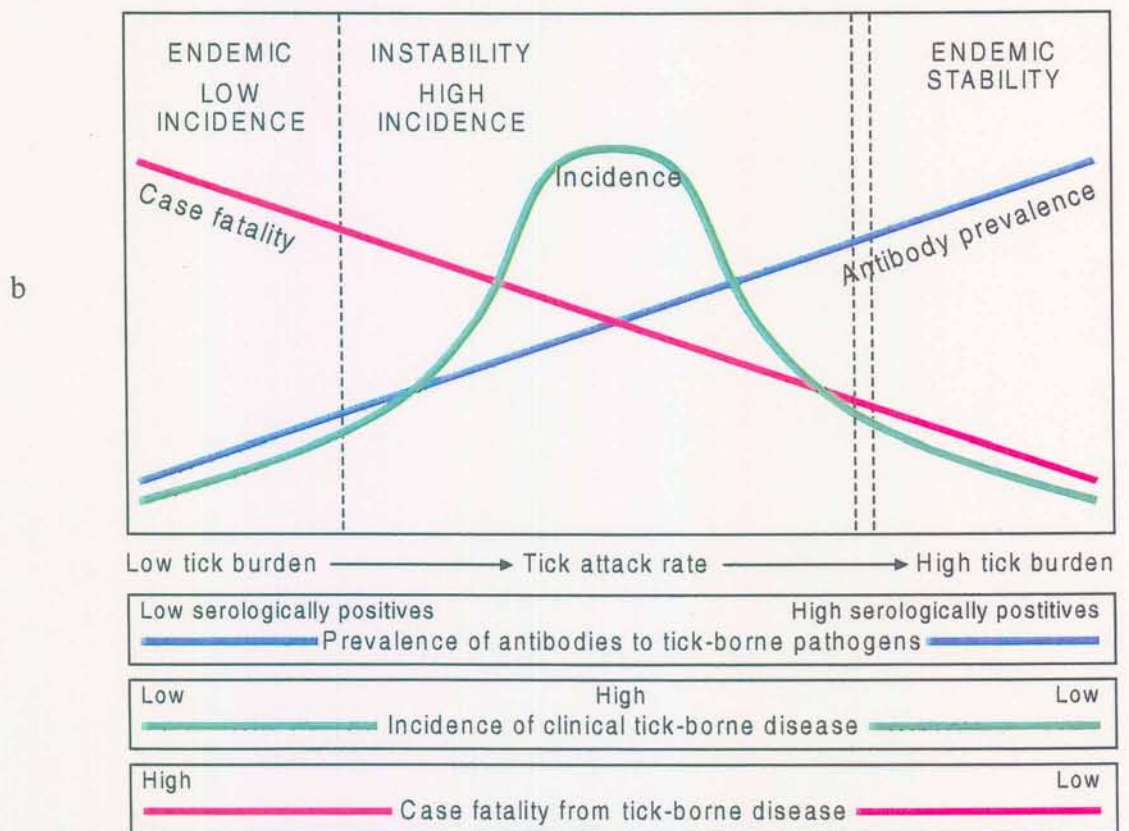
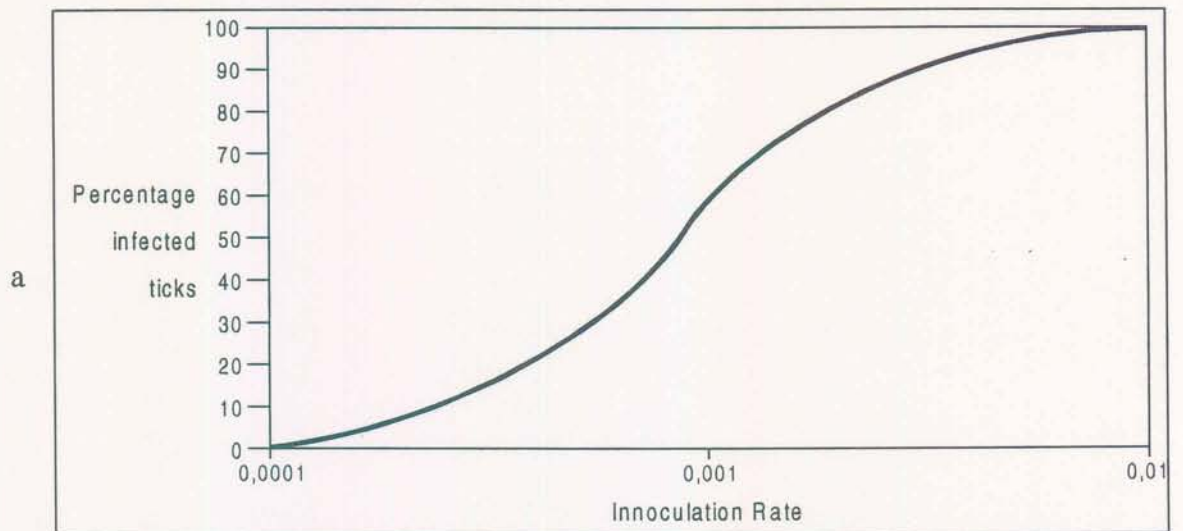


Fig. 2a Percentage infected ticks *versus* inoculation rate, the percentage of calves infected with *Babesia* by nine months of age is illustrated graphically (Adapted from Mahoney & Ross 1972)

Fig. 2b Endemic stability and endemic instability to tick-borne diseases (Adapted from Perry & Young 1995; O'Callaghan, Medley, Peter & Perry 1998)

Mahoney & Ross (1972) found that there were many different factors which could affect the endemic stability to TBD. Changing any of these factors would have serious repercussions for the endemic state of the TBD, some of these factors are listed below:

- Breed of cattle:

Bos taurus cattle were shown to have three times as many *Boophilus* ticks as *Bos indicus*. In a study carried out by Mahoney, Wright, Goodger, Mirre, Sutherst & Utech (1981), increased tick burdens raised the inoculation rate in European cattle, which then had better endemic stability to *B. bigemina*.

- Climatic conditions for the ticks:

Soil and temperature conditions had the potential to affect the tick infection rates and consequent stability to babesiosis (Mahoney *et al.* 1981)

- Tick control:

Tick control reduced the inoculation rates below the minimum level for endemic stability in European cattle, and this resulted in clinical babesiosis.

In South Africa the creation of an endemically unstable situation for bovine babesiosis is mainly due to unfavourable climatic conditions, and the injudicious control of ticks (De Vos & Potgieter 1983). These authors also reported that when tick control was poor there was enzootic-instability to *B. bovis*, while endemic stability to *B. bigemina* was evident. Good tick control reduced *B. bovis* to low levels, but this increased the risk of *B. bigemina* outbreaks (De Vos & Potgieter 1983).

1.3.4.2.2 Endemic stability to heartwater

Historically heartwater was controlled like so many other TBD by dipping, but there was little recognition of the importance of the immunity of the host. In 1945 Neitz & Alexander introduced the first practical field vaccination for heartwater, and for many years this was used in conjunction with dipping. Although these early workers were aware of the importance of breed resistance and the practice of raising domestic stock on heartwater veld, they were not able to convince the commercial farmers to routinely vaccinate and use heartwater-resistant stock.

In Zimbabwe during the civil war (Norval 1981; 1983) cattle could no longer be dipped, and this resulted in high mortalities due to TBD. This led to a re-assessment of the role of dipping in the control of TBD in Zimbabwe, and to more reliance on enzootic stability developed through frequent disease exposure.

Animals which were not dipped, and were heavily tick infested, such as on communal grazing areas (CGA), acquired infection at an early age. They were protected by innate immunity and if repeatedly challenged, become immune. Clinical disease due to TBD on CGA in southern Africa was rare (Tice, Bryson, Stewart, Du Plessis & De Waal 1998), and was only noticed when susceptible or intensively dipped animals were moved into these areas.

1.3.4.3 Tick transmission dynamics

All proven vectors of heartwater are three-host ticks, and transmission has been shown to be transstadial only except for a single report on transovarial transmission (Bezuidenhout & Jacobsz 1986). Thirteen species of *Amblyomma* have been shown to transmit *C. ruminantium* experimentally (Jongejan 1992). Bezuidenhout (1987) reviewed the natural transmission of heartwater, and listed all the proven vectors. *A. variegatum* and *A. hebraeum* were the two most important vectors of *C. ruminantium*, but transmission of the disease appeared to be possible by all *Amblyomma* species normally associated with ungulates in the adult stage (Camus *et al.* 1996). In Mozambique *A. hebraeum* appeared to be a better vector of heartwater than *A. variegatum* (Asselbergs *et al.* 1993).

The hardiness and exceptional longevity of many *Amblyomma* species make them ideal reservoirs of the organism as it can persist in the tick for as long as 15 months (Ilemobade 1978). Male *A. hebraeum* play an important role in the transmission of heartwater as they stay on the host for long periods and they are thus more likely to become infected from an infected host than a female tick. Male *A. hebraeum* can also detach from a dead host and re-attach to another host, and transmit the infection (Norval *et al.* 1992).

Recent work at the UF/USAID/SADC Heartwater Project in Zimbabwe has introduced some new concepts to the accepted view on the transmission of heartwater. The “tick attack rate” (TAR) was used to describe the number of new ticks which attach daily onto animals, and is an important parameter in *C. ruminantium* infection dynamics (Peter 1997). Tick infectivity decay (TID), is the rate at which the infectivity of the clinically infected ruminant

host declines after recovery (Peter 1997). The TID is a new concept, and very little quantitative information was available, but this data will be important for developing epidemiological models of heartwater.

1.3.4.4 Vertical transmission of heartwater

In many heartwater-endemic regions of southern Africa, calves do not become infested with *Amblyomma* ticks until they are three months and older (Du Plessis *et al.* 1992; Norval, Donachie, Meltzer, Deem & Mahan 1995; Deem, Norval, Yonow, Peter, Mahan & Burridge 1996b). If calfhood immunity wanes at four weeks of age, and the initial tick transmission of *C. ruminantium* by *Amblyomma* ticks to calves does not occur, then endemic stability to heartwater would not seem to be possible (Deem *et al.* 1996a). There is, however, strong evidence that heartwater does occur in an endemically stable situation in large areas of Africa, especially where dipping is not carried out (Bezuidenhout *et al.* 1994; Norval *et al.* 1992).

- Deem, Norval, Donachie & Mahan (1996a) demonstrated that vertical transmission of *C. ruminantium* from cows to their calves does occur under natural heartwater-endemic conditions, and that these calves become reservoirs of infection for *Amblyomma* ticks in the area. They believed the epidemiology of heartwater was driven by both vector and vertical transmission. The establishment and maintenance of endemic stability may in fact only be possible because of the high rate of vertical

transmission, which occurred during the perinatal period when there was a high tolerance to clinical disease (Deem *et al.* 1996a). This recent finding of Deem *et al.* (1996a) that vertical transmission of *C. ruminantium* can occur, would obviously have dramatic implications for epidemiological models for heartwater.

1.3.4.5 **Epidemiological models for heartwater**

Accurate models for the various climatic factors are available for many of the African tick species. The CLIMEX model of Norval, Perry, Kruska & Kundert (1991) was applied to both *A. variegatum* and *A. hebraeum*. Perry, Kruska, Mahan & Pfeiffer (1995) were able to predict the changes in the distribution of *A. hebraeum* in Zimbabwe, which had occurred in the last few decades using their Climex model. Randolph & Rogers (1995) were also able to use satellite imagery to produce population and disease transmission models for both *R. appendiculatus* and *Amblyomma* spp., using currently available population dynamics data.

Various heartwater models have been produced at different stages, which explain the accepted epidemiological principles at that time, and these models have had to be updated as new information was discovered. Deem *et al.* (1996b) produced three different epidemiological models (Fig. 3, 4 and 5), which attempt to explain the principles held during these periods (Fig. 3 - 1985, Fig. 4 - 1993 and Fig. 5 - 1996).

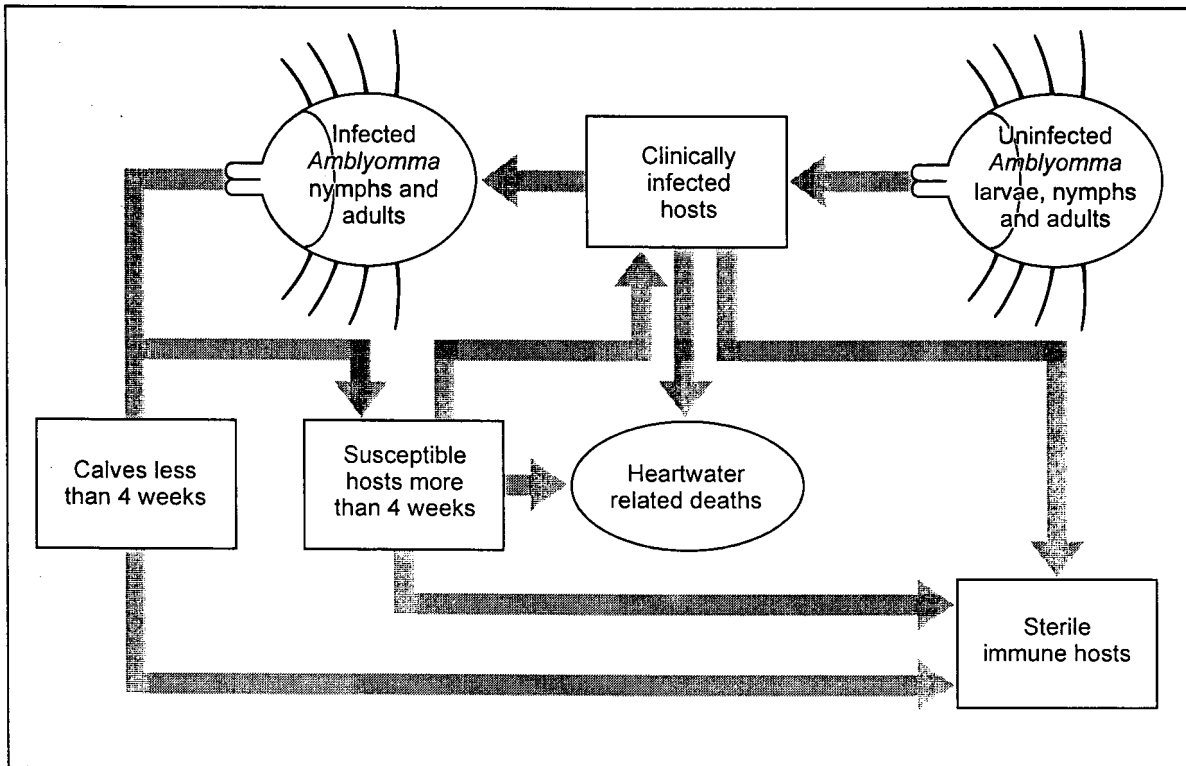


Fig. 3: The epidemiology of heartwater prior to 1985 (Deem *et al.* 1996b).

The first model was the accepted epidemiological model prior to 1985, and was built upon the view held at the time, namely that the dynamics of heartwater did not allow for the development of endemic stability to the disease (Deem *et al.* 1996b). The early model was based on the belief that hosts were only infective for a short period during the clinical reaction, and this led to the very low infection rate in free-living ticks (Alexander 1931; Neitz 1939).

In the second model (Fig. 4), cognisance is taken of the important findings that vertebrate hosts and free-living *Amblyomma* ticks (Norval, Andrew & Yunker 1990), can be long term carriers of *C. ruminantium* and these are included in the model (Andrew & Norval 1989a).

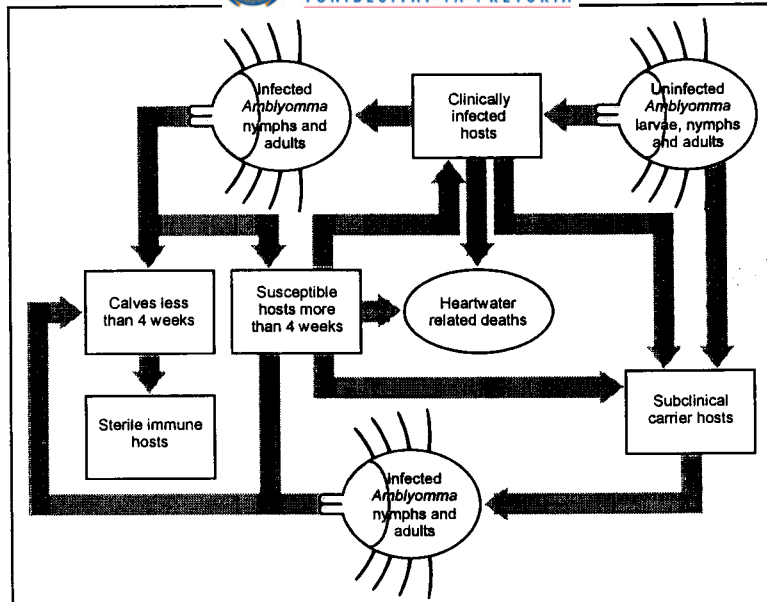


Fig. 4: The epidemiology of heartwater prior to 1993 (Deem *et al.* 1996b)

In Fig. 5 the importance of subclinical carriers and both vertical and tick transmission in establishing and maintaining endemic stability is emphasized (Deem *et al.* 1996b). Carrier calves can also infect clean *Amblyomma* ticks and remain asymptomatic when first infested with *C. ruminantium* infected ticks.

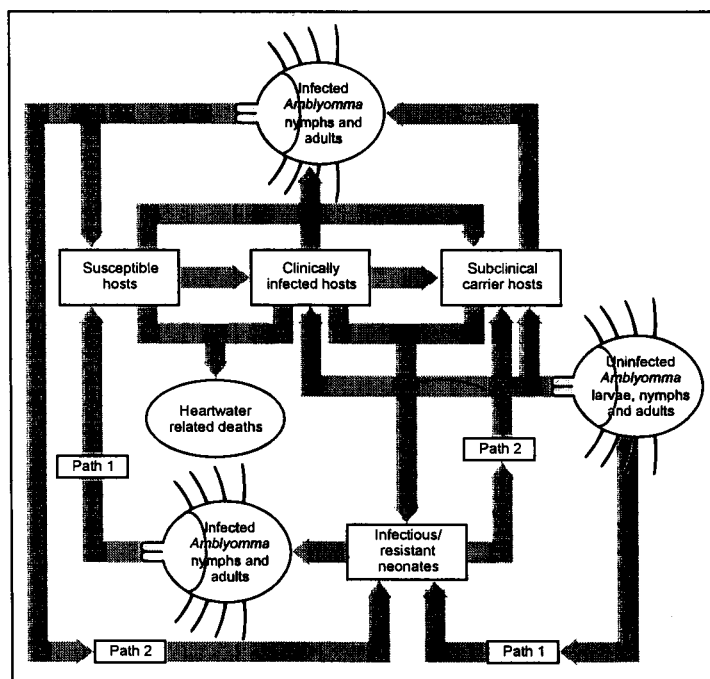


Fig. 5: The epidemiology of heartwater prior to 1996 (Deem *et al.* 1996b)

O'Callaghan *et al.* (1998) have produced a mathematical model which incorporates six mutually exclusive categories of infection which should include all possible host components involved in heartwater. This mathematical model has three components: a deterministic compartmental model of *C. ruminantium* transmission, a mammalian host demographic model and a tick vector population dynamics model. The model will be used to achieve a better understanding of the transmission dynamics of *C. ruminantium*, particularly with reference to the testing of new vaccines in the field (O'Callaghan *et al.* 1998).

Quite recently Yonow, Brewster, Allen & Meltzer (1998) published their 1998 models of heartwater epidemiology. They adapted a malaria epidemiology model and two detailed biological models for heartwater, and predicted various scenarios on their three models. Their results indicated that endemic stability, with a high level of immune carrier infection, in a herd of cattle was likely to be the norm for heartwater. Analyses with the models showed that endemic stability can only be prevented by keeping the cattle *Amblyomma*-free, and even with one tick per host per day, 30% of the cattle could be infected with heartwater.

Yonow *et al.* (1998) concluded that heartwater would readily establish and maintain itself in a cattle population. This would lead to a high percentage of the cattle becoming infected, and the disease should be allowed to spread throughout the cattle population. This would produce immunity in the herd and the risks of the disease minimised. Livestock mortality associated with converting herds from heartwater-susceptible to heartwater-carrier could be kept at a minimum by using heartwater vaccines. Tice *et al.* (1998) have also shown that the process to endemic stability does not always lead to outbreaks of clinical disease, and that direct damage by the ticks themselves is often the biggest problem (Kiwanuka, Stewart, Bryson, De Waal, Tice, Schoeman & Pettey 1995).

1.3.5 THE USE OF TRAPS TO COLLECT FREE-LIVING, UNFED TICKS

There are many different methods of collecting free-living, unfed adult and nymphal ticks and some of these are summarized in Table 3. Most of the traps for adult and nymphal ticks use some form of CO₂ as an attractant. This may be a direct CO₂ source such as dry ice, or indirect such as animals breathing CO₂. Other forms of trapping involve vacuuming the animal's nest for ticks or taking soil samples of the burrow. Some adult and nymphal ticks can also be collected by dragging the vegetation with a flannel apparatus, which the ticks attach to.

1.3.5.1 Different methods of collecting free-living, unfed adult ticks

1.3.5.1.1 CO₂ trapping devices

The early workers in the USA demonstrated that CO₂ emitted by dry ice was attractive to certain species of ticks in the field (Garcia 1962). They were later able to collect ticks and fleas from mammal burrows with these CO₂ traps (Miles 1968). Wilson, Kinzer, Sauer & Hair (1972) described a CO₂ platform trap, which was effective in collecting all stages of *A. americanum*, and demonstrated the advantages of this type of trap over flagging with flannel cloths to collect free-living, unfed ticks.

TABLE 3: Methods used by various authors to collect adult and nymphal ticks in the field

AUTHORS	YEAR	AREA	TICKS	METHOD OF COLLECTION	SAMPLE SIZE	OBJECTIVE
Garcia	1962	USA	-	CO ₂ trap		To collect ticks with a CO ₂ trap
Miles	1968	USA	-	CO ₂ trap		To collect ticks from mammal burrows
Wilson <i>et al.</i>	1972	USA	<i>A. americanum</i>	CO ₂ platform trap		To collect <i>A. americanum</i> in the field
Eads <i>et al.</i>	1982	USA	<i>D. andersoni</i>	CO ₂ platform trap		To collect released laboratory-reared <i>D. andersoni</i> in the field
Bulter <i>et al.</i>	1984	USA, Caribbean	<i>Ornithodoros</i> spp.	Various vacuum-type traps		To collect burrow dwelling ticks to test for ASF
Gray	1985	Ireland	<i>Ixodes ricinus</i>	CO ₂ trap		To collect released <i>I. ricinus</i> (A/N) ticks in the field
Hess & De Castro	1986	Kenya	<i>A. variegatum</i>	AAAP trap (No CO ₂)		To collect laboratory-reared <i>A. variegatum</i> with a AAAP trap
Norval <i>et al.</i>	1987	Zimbabwe	<i>A. hebraeum</i> (A)	Various	n = 2 000	To test the attractiveness of various stimuli to unfed laboratory-reared ticks (A)
Norval <i>et al.</i>	1988	Zimbabwe	<i>A. hebraeum</i> (N)	Various	n = 2 000	To test the attractiveness of various stimuli to unfed laboratory-reared ticks (N)
Ginsberg & Ewing	1989	USA	<i>Ixodes damini</i> <i>A. americanum</i>	Various		To compare the flagging, walking, trapping and host collection methods to collect ticks
Norval <i>et al.</i>	1989	Zimbabwe	<i>A. hebraeum</i>	Calves with AAAP trap		To see whether AAAP calves attract <i>A. hebraeum</i>
Norval <i>et al.</i>	1989	Zimbabwe	<i>A. hebraeum</i>	AAAP/CO ₂ trap		To test whether laboratory-reared <i>A. hebraeum</i> (A) ticks are attracted to the AAAP/CO ₂ trap
Kinzer	1990	USA	<i>A. americanum</i>	Flagging + CO ₂ sticky trap		To compare flagging + CO ₂ traps to collect <i>A. americanum</i> in the field
Norval <i>et al.</i>	1990	Zimbabwe	<i>A. hebraeum</i> (A/N)	AAAP/CO ₂ trap	n = 152	To collect unfed field <i>A. hebraeum</i> (A/N) ticks
Norval <i>et al.</i>	1991	Zimbabwe	<i>A. hebraeum</i> <i>A. variegatum</i>	AAAP/CO ₂ trap		To test if <i>A. hebraeum</i> and <i>A. variegatum</i> are attracted to similar components of the AAAP/CO ₂ trap
Norval <i>et al.</i>	1992	Zimbabwe	<i>A. hebraeum</i> <i>A. variegatum</i>	AAAP/CO ₂ trap		To test if laboratory-reared <i>A. hebraeum</i> and <i>A. variegatum</i> (A/N) are attracted to CO ₂ and AAAP/CO ₂ traps
Falco & Fish	1992	USA	<i>Ixodes damini</i>	Various		To compare mice, drag sampling and CO ₂ traps to detect immature <i>I. damini</i>
Barré	1997	Caribbean (Quadeloupe)	<i>A. variegatum</i> (A/N)	AAAP/CO ₂ trap	1 440 (A) 2 700 (N)	To test the AAAP trap in the Caribbean in order to collect released ticks (A/N), under variable conditions at different times of the year.

A = Adult

N = Nymphs

Eads, Smith & Maupin (1982) working with released populations of *Dermacentor andersoni* in Colorado, USA, devised a platform-type CO₂ trap, which could be filled and re-charged with 650 g of chipped dry ice, and checked on a daily basis for ticks. They found that up to 76% of the marked released ticks could be recovered from a distance of six metres, but when the distance was increased to 12 metres, catches were poor.

Wilson *et al.* (1972) used one kilogram of dry ice over a seven-day period, but recovered only 5% of marked *A. americanum* released at six metres from the trap, and only 3.5% released at 12 metres. Clearly, there were substantial differences in the attractiveness of CO₂, for these two species of ticks with *A. americanum* showing much less response.

Butler, Holscher, Adeyeye & Gibbs (1984) integrated the technology used by previous workers, and designed a complex chemical CO₂ system, and various vacuum extraction systems, to suck out debris from warthog burrows, and this was then filtered and the live ticks separated. They showed that multiple sampling of the burrows at 3 - 4 day intervals, produced more than 2 000 ticks per burrow (Butler *et al.* 1984).

Gray (1985), working in Ireland with free-living, unfed and released adult, nymphal and larval *I. ricinus*, re-designed the trap of Wilson *et al.* (1972), to take much more CO₂ (12 kg), so that it could be dispersed for seven days. The CO₂ disperses in the area surrounding the trap and any field ticks that were attracted were caught on sticky tape as they go up the slopes. A total of 4 777 larvae, 1 092 nymphs and 772 adults were caught with this trap,

and this was substantially more than a blanket-dragging method used as a control (Gray 1985). The trap would attract ticks over a maximum distance of 3.5 metres. This was less than that recorded by Wilson *et al.* (1972) and Eads *et al.* (1982), for ticks of other species in the USA.

1.3.5.1.2 Pheromone trapping devices

Pheromones are information-bearing chemicals used by animals to modify behaviour. These chemical signals are used by a wide variety of animals to influence many aspects of their behaviour, e.g. mating, food gathering, assembly and other vital activities (Sonenshine, 1991). Four different categories of tick pheromones have been recognized since the pioneering discovery of the first tick sex pheromone, 2,6-dichlorophenol [cited in Sonenshine (1991)]. These include assembly, AAAP, sex and primer pheromones (Sonenshine, 1991).

Feeding male *A. hebraeum* emit an AAAP and other host odours which are extremely attractive to unfed males and females of the same species (Norval *et al.* 1989). Ether extracts of fed males and O-nitrophenol were also attractive to these ticks. Male *A. hebraeum* stay on the host for considerable periods, and heavily infested animals would be more attractive to unfed adult ticks than dipped hosts with no pheromone producing males. Male ticks do not transfer to new hosts under normal circumstances, but would leave a dead host and attach to a living one (Andrew & Norval 1989b).

The AAAP induces attachment of ticks to the host's body at sites where feeding males are attached. This pheromone is volatile, soluble in organic solvents, produced solely by males.

and is only produced by members of the genus *Amblyomma* (Sonenshine 1991). Gladney, Grabbe, Ernst & Oehler (1974) and Rechav *et al.* (1977; 1978), were the first groups to carry out experiments with natural extracts of AAAP. Rechav *et al.* (1978), working in the Eastern Cape Province of South Africa, applied highly concentrated diethyl-ether extracts from feeding *A. hebraeum* males to specific sites on cattle. The cattle were then released into a camp where a large population of free-living, unfed *A. hebraeum* was known to occur. They were able to demonstrate that unfed adult ticks were attracted to the treated areas on the cattle, and this was the first recorded evidence of wild, unfed ticks being attracted to a pheromone extract. Rechav *et al.* (1978) thought, however, that the effect of the pheromone was only short-lived (four days), and that ticks responded only over a short distance.

Norval *et al.* (1987) released thousands of adult *A. hebraeum* in a heartwater-endemic region of Zimbabwe, in order to determine which factors stimulated them to emerge. Large volumes of CO₂ from blocks of dry ice, placed upwind from the release site, were the most attractive of the stimuli (Yunker & Norval 1991). The CO₂ did not, however, lure the adult or nymphal ticks to the source, but rather caused them to emerge from their shelters and to run erratically on the surface (Yunker & Norval 1991).

In subsequent field trials in Zimbabwe, various chemicals were tested for their attractiveness to *A. hebraeum*. It was found that adult and nymphal ticks responded positively to the volatile extract of the AAAP produced by feeding males, and this response was enhanced by the addition of CO₂ (Yunker & Norval 1991).

Norval *et al.* (1987) stated that wild *A. hebraeum* adult ticks were attracted over a distance of three metres, to cattle carrying pheromone secreting males. Herds or flocks infested with *Amblyomma* males will emit large quantities of CO₂ and AAAP, and are likely to attract wild, unfed nymphs, as well as free-living, unfed adult *A. hebraeum* (Norval *et al.* 1992). The practice of herding domestic stock during the day and holding them in enclosures overnight is thus likely to increase the exposure of these animals to unfed ticks (Norval *et al.* 1992).

Males of *A. hebraeum* remain on the host for several months (Norval 1974), and are capable of mating with several females. Only after the males have been feeding for a week or longer, do they secrete sufficient AAAP to attract females, other males and nymphal *A. hebraeum* which then attach. As a consequence, these ticks tend to accumulate on suitable hosts in clusters, formed at pre-dilection sites of male attachment (Norval, Andrew & Yunker 1989a).

The responses to both CO₂ and AAAP appear to be part of a selection process that ensures that ticks attach preferentially to hosts on which they are more likely to feed successfully (Norval *et al.* 1989a). Obviously, cattle which are treated with acaricides will have less pheromone secreting males and, presumably, less immunity to heartwater. In 1989 Norval, Butler & Yunker confirmed that host location in adult *A. hebraeum* involved two main responses. Firstly, a response to CO₂ which initiates searching actively, but is non-directional, and secondly, a directional response to AAAP.

They also designed two field traps to collect laboratory-reared *A. hebraeum*, both of which used CO₂ from a cylinder passing over fed AAAP producing males (Norval *et al.* 1989b). Norval, Peter, Yunker, Sonenshine & Burrige (1991) also tested a variety of potential chemicals as long-range attractants for *Amblyomma* ticks, and they concluded that AAAP contained compounds that served as long-range attractants, short-range attractants that induce aggregation, and also attachment stimulants. The major component of the AAAP of *A. hebraeum* appeared to be O-nitrophenol, and the AAAP attracted both *A. hebraeum* and *A. variegatum* (Norval *et al.* 1991).

Norval *et al.* (1992) found that with the AAAP/CO₂ trap large numbers of released adults (64%) were recovered at four metres, but this was not the case with CO₂ alone (7%). As the distance was increased to over ten metres, 30% of adult ticks were still collected with AAAP/CO₂, as opposed to 0% with CO₂ alone.

Barré, Garris & Lorvelec (1997) working with *A. variegatum* in the Caribbean, used an AAAP/CO₂ trap to collect ticks. The trap, consisted of a piece of white cloth and a plastic cup of dry ice (250 g), and various attractants, including tick pheromone and cattle hair. As only a small quantity of CO₂ was used, it had to be replaced every two hours. This study involved laboratory-reared ticks, and was not directed at wild tick capture, and the maximum attraction distance reported for this trap was eight metres.

In conclusion, it would appear from the literature that there are few reports of sustained collections of wild *Amblyomma* adult ticks in the field, and most of the collections to date have been based on the release and capture of laboratory-reared ticks.

1.3.5.2 Different methods of collecting free-living, unfed nymphal ticks

Nearly all of the known, proven experimental vectors of *C. ruminantium* can become infected in the larval stage and the infection passed on to the nymphal stage (Bezuidenhout 1987). *A. hebraeum* and *A. variegatum* in the nymphal stage would then transmit *C. ruminantium* in the adult stage, i.e. the nymphs are infective. In other species of *Amblyommas* (*A. sparsum* and *A. cajenense*) the nymphs lose the infection and the adults cannot transmit heartwater (Bezuidenhout 1987).

Unfed *A. hebraeum* nymphs could be expected to be most abundant in areas where cattle rest at night (Norval 1988), and one would hence look for *A. hebraeum* nymphs in shaded areas near kraals where cattle are kept overnight. Unfed nymphs and adult *A. hebraeum* have not been successfully collected by conventional sampling techniques such as blanket-dragging, flagging or standard CO₂ traps (Norval *et al.* 1992). Norval *et al.* (1987) found that the presence of high concentrations of CO₂, mimics the breath of large hosts, and causes nymphs and adult *A. hebraeum* to emerge from and engage in host seeking activity, but required the addition of pheromone to be more effective. Adult *A. hebraeum* are large and conspicuous, and can easily be collected as they run over the soil surface. Nymphs, however, cannot be readily collected in this manner as they are much smaller, are darkly coloured, and consequently not easily visible on the ground (Norval, Yunker, Gibson & Deem 1988).

Norval *et al.* (1988) released 2 000 nymphs into camps at the Mbizi Quarantine area in

Zimbabwe. They then tried various methods to recover the ticks. Flagging with a drag apparatus was not effective, but it was improved by prior stimulation with CO₂. Vacuuming, following CO₂ exposure, was the most successful way of collecting the nymphs. However, a strong source of CO₂, e.g. dry ice, was needed to attract the nymphs (Norval *et al.* 1988). With released nymphs, 83% were recovered at ten metres with CO₂, and 88% with AAAP/CO₂. The response of the nymphs to CO₂ alone, obviously differed to that of the adults, which showed poor response. The AAAP trap was able to attract nymphs from up to 25 metres from the source (Norval *et al.* 1992).

Rechav & Whitehead (1978) in their field trial with AAAP mixtures on cattle, found that wild, nymphal *A. hebraeum* were also attracted to demarcated treated areas on cattle. Many other reports (Gray 1985; Falco & Fish 1992; Kinzer 1990) have documented the use of traps for collecting nymphs of other species, most have little relevance to this project, as these nymphal ticks quest, and are easily caught with flagging and other means. Other methods besides trapping, used to collect nymphal ticks, are the use of tracer animals, trapping rodents, and the use of sentinel cattle (Peter 1995).

1.3.5.3 **Different methods of collecting free-living, unfed larval ticks**

Ticks acquire *C. ruminantium* whilst feeding on a reacting or carrier animal. Most of the larval stages of the proven vectors of heartwater can transmit *C. ruminantium* transstadially to the nymphal stage. The transmission from the nymph to adult is, however, variable and depends on the ability of the species involved (Bezuidenhout 1987).

Most larval ticks, including *A. hebraeum*, quest and are easily collected off the vegetation with the various forms of drag-sampling. *C. ruminantium* is not normally transmitted transovarially (Bezuidenhout & Jacobsz 1986), so very few larvae of *A. hebraeum* would be infected with *Cowdria*. This stage of development is less important in the epidemiology of the disease. The larvae feed on numerous hosts which could also carry heavy burdens of *A. hebraeum* larvae, and could be sub-clinically infected with *C. ruminantium* (Horak & Williams 1986; Horak *et al.* 1987).

Spickett *et al.* (1991) using drag-sampling in the KNP, collected 18 199 ticks of which 99.53% were larvae. This would imply that this form of sampling is not efficient for adult and nymphal ticks in southern Africa, and especially for those species which do not quest. This is certainly not the case in other parts of the world where other tick species occur, and L'Hostis, Dumon, Dorchies, Boisdron & Gorenflot (1995) reported on a survey in France where blanket-dragging collected 34 adult, 900 nymphal and 2 628 larval *I. ricinus*. In Ireland Gray (1985) collected a large sample of all stages of *I. ricinus* with both a CO₂ trap and blanket-dragging. There have been few reports on the use of a CO₂ trap to collect larval ticks in South Africa. This is probably because of the ease with which these ticks are collected by dragging, as opposed to the difficulty of seeing these very small ticks when they are attracted to CO₂ traps. The larvae also quest on the grass stems and blades, and are unlikely to be found on the ground during the day.

Various workers (Ginsberg & Ewing 1989; Kinzer *et al.* 1990; Falco & Fish 1992; Shulze, Jordan & Hung 1997) have critically analysed live tick sampling methods. Ginsberg & Ewing (1989) concluded that those ticks which seek hosts in leaf litter, are poorly

sampled by attachment onto the investigators' clothing (walking samples), and these ticks can better be sampled by dragging at ground level. Dry ice-baited traps caught far more *A. americanum* than *I. damini* in areas where the latter ticks predominated on flagging samples (Ginsberg & Ewing 1989).

Falco & Fish (1992) compared mice trapping, drag-sampling and CO₂ traps to collect the various stages of *I. damini* in an endemic lyme disease area in the USA, and they concluded that drag-sampling is best for nymphs, and CO₂ traps best for the larvae. Kinzer (1990) on the other hand, compared the efficacy of flagging and CO₂ traps to collect *A. americanum*, and found that CO₂ traps captured more adults and nymphs than did flagging. The traps were more precise and produced less variability between samples. Schulze *et al.* (1997), however, have recently reported that various biases have to be taken into account when looking at the populations of *I. scapularis* and *A. americanum* in the USA. Walking surveys were more efficient for collecting *I. scapularis* adults, but drag-sampling more effective for collecting *A. americanum* adults. Clearly, this contradicted the work of Kinzer *et al.* (1990) and Ginsberg & Ewing (1989).

To conclude one can accept that there are many different methods of trapping larval, nymphal and adult free-living, unfed ticks. It is important, however, that one should carefully analyse the strengths and weaknesses of each method, before using it on a large scale to collect free-living, unfed adult and nymphal ticks. With *A. hebraeum* there have been no sustained collection surveys of adult and nymphal ticks, but it is clear that pheromone/CO₂ traps would be far more successful in collecting adult and nymphal ticks than dragging methods.

CHAPTER TWO

2. THE USE OF THE ATTRACTION-AGGREGATION-ATTACHMENT-PHEROMONE/CARBON DIOXIDE TRAP UNDER DIFFERENT FIELD CONDITIONS IN SOUTH AFRICA

The AAP/CO₂ trap was used at six different areas in South Africa (Fig.6, 1 - 6). A two-year survey was carried out at the Rietgat CGA (1). Further collections were made at the campus of the Medical University of Southern Africa (MEDUNSA) (2), and a well-managed bushveld cattle farm, Dr L van der Merwe's farm "Ludlow" near Warmbaths (3). The SABS Farm in East London (4) was an experimental farm where tick seeding had occurred for a number of years, and was typical of the Eastern Cape coastal vegetation. The AAAP/CO₂ trap was also used at two game reserves. At the Kruger National Park (5) the trap was used in the vicinity of the Skukuza main camp. The trap was also tested at the Songimvelo Game Reserve (6).

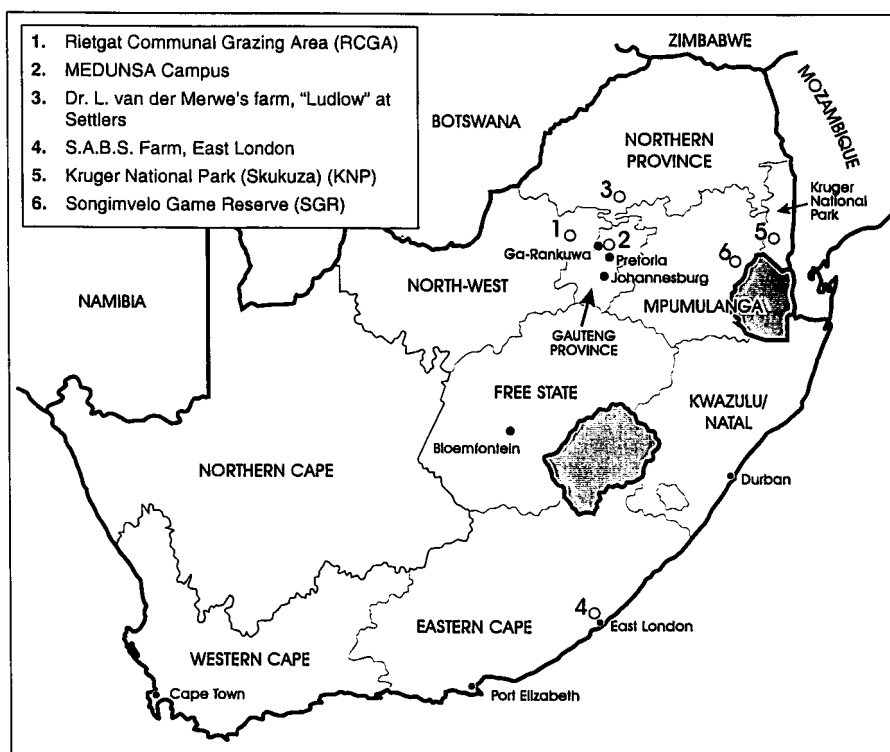


Fig. 6 Map of South Africa, illustrating the six different areas (1 - 6) where the AAP/CO₂ trap was used to collect ticks

2.1 THE COMMUNAL GRAZING AREA AT RIETGAT, NORTH WEST PROVINCE (POOR TICK MANAGEMENT)

2.1.1 INTRODUCTION

The Rietgat CGA belongs to a rural community 50 km north-west of the Medical University of South Africa (MEDUNSA). Rietgat is one of a number of similar communities in the region which include Maboloka, Wintersveldt, Bethany, Madinyane, Lethlambile and Jericho.

The Veterinary Faculty at MEDUNSA has been involved in servicing these regions for over a decade, by outreach clinics which were run at Maboloka, Lethlambile and Winterveldt. Short-term objectives of the clinics were to give veterinary students clinical exposure, as well as providing a clinical service to the local communities. Education in veterinary primary health, extension services, community development and the accumulation of research data were the long-term objectives.

In the early 1990's, Tice, Stewart, Bryson, Du Plessis & De Waal initiated a project to investigate the population dynamics of ticks, and the serological prevalence of TBD in cattle in four communally grazed areas in South Africa. The two-year survey entailed monthly visits to the CGA. Three of these CGA namely, Rietgat, Madinyane and Bethany, all in the North West Province of South Africa, were selected because dipping management was poor and erratic. These CGA were compared with another at Geluk, which borders the KNP (Fig. 6), and where tick control was for many decades historically excellent. At all of

these CGA there had been some subsidization of dipping, which was at the time being phased out, and the cattle owners now had to pay for the cost of tick control.

A survey by Tice (1997) showed that eight species, namely *A. hebraeum*, *Rhipicephalus appendiculatus*, *R. evertsi evertsi*, *R. simus*, *Boophilus decoloratus*, *B. microplus*, *Hyalomma marginatum rufipes*, and *H. truncatum* were present on cattle and goats virtually throughout the year. At Rietgat *A. hebraeum* was present on the cattle throughout the year, and the relative abundance of this tick was greater than that recorded in other bushveld areas of South Africa (Londt *et al.* 1979). There was a distinct seasonal variation in its abundance with a summer peak from October to April, and a spring/winter low from May to September. Indications were that *A. hebraeum* completed only one generation per year at Rietgat (Tice 1997). Up to 70 male ticks were collected per animal during summer and 15 to 20 in the spring/winter. *A. hebraeum* nymphs were abundant on the feet of goats throughout the year and comprised 58% of the total tick burden. As many as 30 nymphs were recorded per goat during summer, and throughout winter at least five nymphs were present.

Young cattle in the CGA were also tested for antibodies to the common TBD and antibodies to *Babesia bigemina*, *B. bovis*, *Anaplasma marginale* and *C. ruminantium* were detected. Seroprevalence of antibodies to *C. ruminantium* was high throughout the study which, when coupled with the high tick burdens, would seem to indicate endemic stability to *C. ruminantium* and other TBD at the Rietgat CGA.

The work of Tice (1997) was extended by Kiwanuka *et al.* (1995) who used participatory research and extension methods to determine which control methods were being employed to control ticks on cattle. It became apparent that tick damage to teats of cows and not TBD was the farmer's primary concern (Kiwanuka *et al.* 1995), and that *A. hebraeum* was the main contributor to this damage. More recently Stewart (1997) conducted a "resource needs' appraisal" in the Jericho region just west of Rietgat. Ticks, and more specifically, tick damage was again shown to be the most important factor affecting cattle in this region.

As a result of these surveys, reliable data on the population dynamics of the ticks and the serology of the common TBD were now available for the CGA. Reasonable progress in defining the needs of the developing farmers in these regions had also been made. Little, however, was known about the free-living stages of any of the ticks found in the region.

Drag-sampling of the vegetation for free-living larvae by Tice (1997) on the CGA in this region had indicated that the common ticks, with the exception of the *Hyalomma* spp., were present. They were, however, collected in much lower numbers than in other bushveld areas of South Africa such as the Eastern Cape Province (Rechav 1982) and the KNP (Spickett *et al.* 1991). At Rietgat Tice (1997) collected a total of only 40 *A. hebraeum* larvae after 16 flannel drags, one could deduce that either the sampling method did not reflect the true prevalence of *A. hebraeum* in the area, or the population was very small. *A. hebraeum* adults and nymphs are seldom collected by flannel dragging techniques and with this in mind, it was decided to test the efficacy of the AAAP/CO₂ trap described by Norval *et al.* (1989b) in attracting adult and nymphal *A. hebraeum* at the Rietgat CGA.

On 20 October 1992 preliminary trials were conducted with Dr Yunker of the Veterinary Research Institute, Onderstepoort, using a diethyl-ether pheromone extract obtained from feeding male *A. hebraeum* with dry ice as a CO₂ source. Thirty AAAP/CO₂ traps were placed in a variety of ecological habitats in which *A. hebraeum* adults might occur at the Rietgat CGA. Initially few of the traps appeared to be attractive although some *A. hebraeum* adults were collected, but were unfortunately frozen by the dry ice.

At only one site (Rietgat area C), which was close to a cattle kraal and had good leaf litter from surrounding *Euclea crispa* shrubs, was high tick activity seen. Consequently the traps were concentrated in this region only, and by the end of that day 78 adult ticks (35 males and 43 females) had been collected. This was the first report of the successful application of the AAAP/CO₂ trap for the sustained collection of *A. hebraeum*.

In 1996 it was decided to use the AAAP/CO₂ trap to collect adult, and possibly nymphal, *A. hebraeum* at the Rietgat CGA, and to determine the *C. ruminantium* prevalence in these ticks. The following two visits to the same site in February and March 1996 yielded negative results. As the identical pheromone and CO₂ sources were used, it was thought that other factors, possibly climatological, were involved. It was then decided to visit the Rietgat CGA on a regular basis over a two-year period, to investigate the factors affecting the attraction of free-living, unfed adult and nymphal *A. hebraeum* in this region.

2.1.2 MATERIALS AND METHODS

2.1.2.1 Geographical location in South Africa

Rietgat (25° 24S - 27° 49E), Madinyane (25° 22S - 27° 52E), Maboloa (25° 26S - 27° 51E) and Jericho (25° 20S - 27° 49E) are all rural villages (Fig. 6) between 30 and 35 km north-west of the commercial centre of Brits (25° 36S, 27° 49E). All these villages and their adjacent CGA are in the North West Province of South Africa.

2.1.2.2 Study area: Different ecological areas sampled

Rietgat is 65 km north-west of MEDUNSA, which is adjacent to the Ga-Rankuwa Hospital, and situated 31 km north-west of Pretoria, the administrative capital of South Africa. A detailed map showing the Rietgat study area (Fig. 7) was compiled from the 1987 1:50 000 map and an aerial photographic survey map (1991) obtained from the Chief Directorate of Surveys and Land Information in Pretoria.

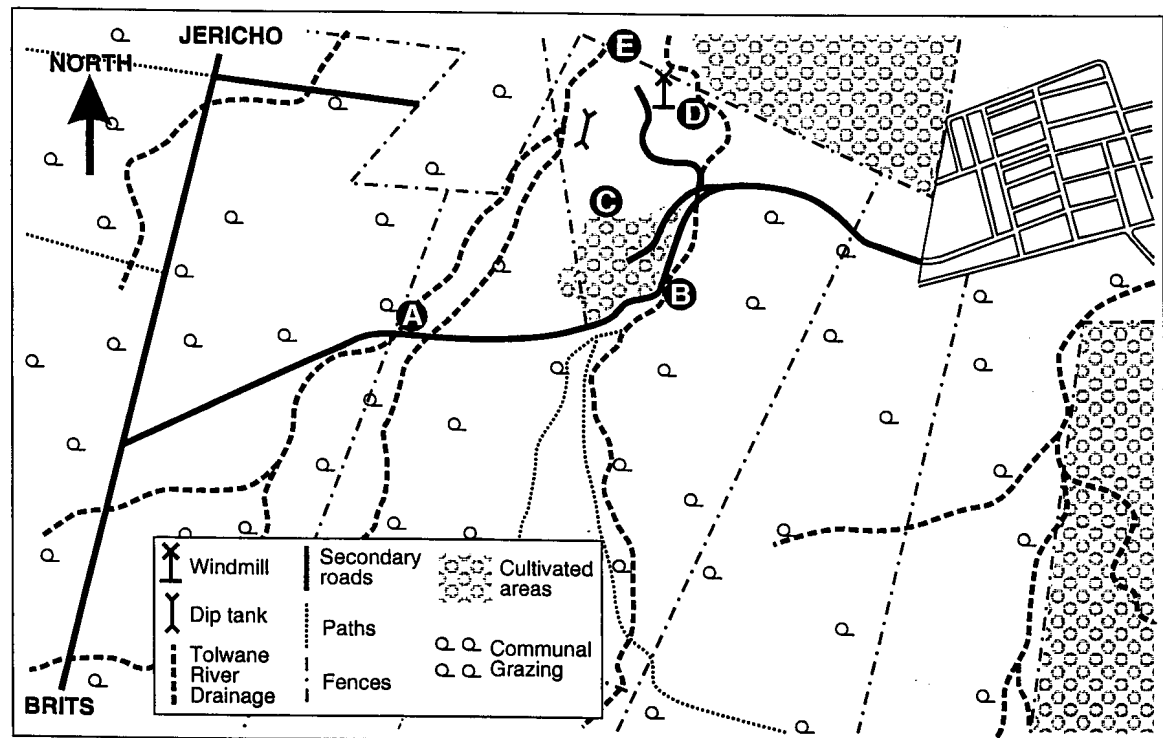


Fig. 7 Map of the Rietgat CGA, North West Province, South Africa, showing the five different ecological areas (A - E), where ticks were collected with the AAAP/CO₂ trap during the two-year field survey

The Rietgat CGA is adjacent to the small village of Lethakaneng (25° 23S - 27° 49E), and access to this area was normally obtained by using the Brits/Jericho road, although it can also be reached from the Maboloka/Jericho road. The altitude in the CGA varies from 1 074 to 1 111 metres above sea level, with the 1 100 metre contour transecting the area. Tributaries of the Tolwane river drain the GCA, and although the river was dry for long periods during the year, there were times when it flowed continually.

Historically this area had been farmed by “white” commercial farmers, but these farms were taken over by the “Apartheid Government” and given to “black” homeland farmers in the former Bophuthatswana. Evidence of previous residences, camps, fences, diptanks, windmills, reservoirs and water points was still visible and many of these structures were still in use. The whole Rietgat area was still fenced and the two access points had gates, which were permanently closed.

Most of the area was set aside for traditional communal grazing for the numerous cattle, goats and donkeys owned by the farmers, whilst other areas were used for cultivated lands, which were left fallow in winter and only used when the summer rains came. A variety of crops were grown in the region and included cotton (*Gossypium* spp.), maize (*Zea mays*), and various vegetables.

Five sampling sites, A to E, were chosen to represent the various ecological areas found at the Rietgat CGA (Fig. 7 and 8). These sampling sites were visited on a regular basis during the two-year survey, and pheromone/CO₂ traps were used to locate ticks.

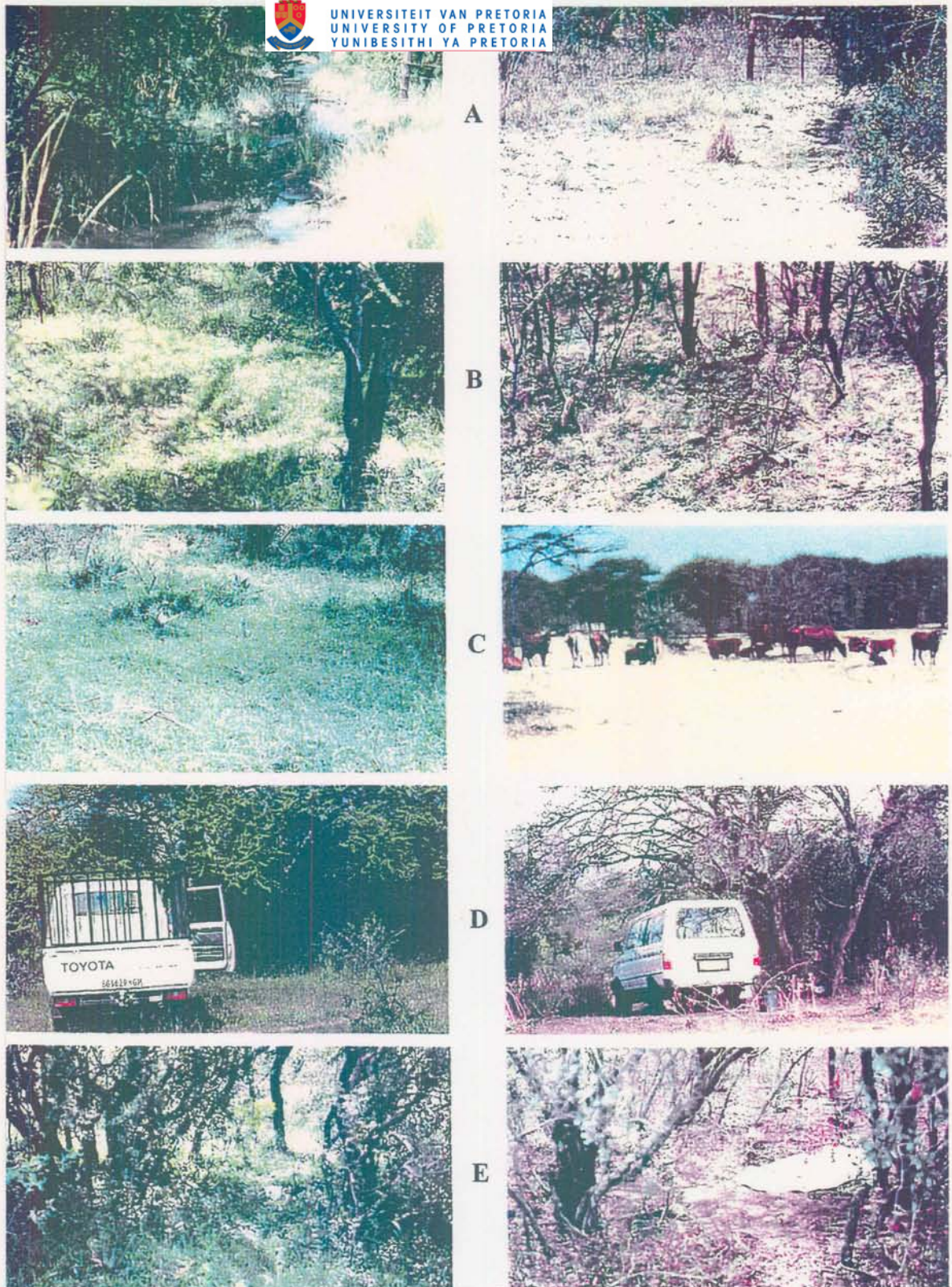


Fig. 8 Typical views of the vegetation types in midsummer (left) and mid-winter (right) at the 5 ecological sites (A - E) at the Rietgat CGA, where the AAAP/CO₂ trap was used: A) Site A, B) Site B, C) Site C, D) Site D, E) Site E

- Site A (Fig. 7 and 8): This was the first site sampled, and was located in the vicinity of the westerly entrance gate in the area where the Tolwane River crosses the road. Four micro-habitats (A/1, A/2, A/3 and A/4) were sampled on a regular basis. A/1 was mainly *Acacia karoo* and *Combretum apiculatum* veld, and was close to a watering point where cattle accumulated. It was very dry in winter, but in summer a number of annual plants were present. A/2, A/3 and A/4 were three sampling sites at the entrance gate A. The Tolwane River crosses the road at this point where there are clumps of tamboti trees (*Spirostachys africana*), with a semi-shaded, deep leaf litter. A windmill was present amongst the trees and there was evidence of cattle and game tracks throughout the area.

- Site B (Fig. 7 and 8): This site also lies close to a branch of the Tolwane river, and six micro-habitats - B/1, B/2, B/3, B/4, B/5 and B/6 - were sampled on a regular basis.
 - B/1: On the banks of the Tolwane river under a clump of *Acacia tortillis* and *Acacia nilotica* trees, semi-shaded and with deep leaf litter.
 - B/2: In the riverbed in the shade of *Combretum apiculatum* and *Rhus lancea* trees and with deep leaf litter.
 - B/3: Open savanna-type grass in the shade of a *Rhus lancea* (karee) tree.
 - B/4 and B/5: A thick overgrowth of *A. karoo* and *A. tortillis*; the traps were placed on or near a path used by grazing cattle.
 - B/6: A clump of *E. crispa* trees with a thick leaf litter which was moist almost throughout the year.

- Site C (Fig. 7 and 8): This was the area where the original 1992 collection of ticks was made. At least 10 collection points were tried and eventually three sites (C/1, C/2 and C/3) were found to be productive for the collection of ticks.
 - C/1: Open site next to *E. crispera* shrubs; very dry in winter, but with a meadow-like appearance in summer.
 - C/2: The original site at which Dr Tice and I collected ticks in 1992. It was close to a kraal and was in mixed *E. crispera* and *Acacia* veld, and in winter there was a deep leaf litter.
 - C/3: Site surrounding C/2, shaded and with a good leaf cover.

- Site D (Fig. 7 and 8): A clump of *E. crispera* in the shade of large *A. tortillis* trees. Deep leaf litter throughout the year.
 - D/1: Deep inside the clump of *E. crispera* with almost permanent shade and deep leaf litter.
 - D/2, D/3 and D/4: Sites surrounding D/1, much more open with numerous cacti and no leaf litter, but a sandy surface.

- Site E (Fig. 7 and 8): Situated near the diptank and characterised by a large variety of trees and shrubs.
 - E/1: Dense clump of *E. crispera* with a very deep leaf litter, which stayed moist almost throughout the year.
 - E/2: In the shade of a large tamboti tree with deep leaf litter, especially just after the tree had lost its leaves.

E/3: Small and large clumps of tamboti trees, mixed with weeping wattles (*Peltophorum africanum*) but more open than E/2.

E/4: *E. crispa* shrubs with good leaf litter.

E/5: Mixed *A. karoo* and *A. robusta* with little ground cover present.

E/6: One large tamboti tree with some leaf litter.

E/7: In the shade of a *Rhus lancea* tree.

2.1.2.3 Climate at the Rietgat CGA

2.1.2.3.1 Recording of the climatic data

Climatic data was obtained in two ways. Firstly, locally where the temperature and the relative humidity were recorded at soil level in the shade. This was done with a thermohygrometer (Solvay Animal Health), which is illustrated in Fig. 9a. The temperature and relative humidity were monitored throughout the day. Secondly, climatological data were obtained from the Brits Agro Meteorological Station which is 20 km south-east of Rietgat, and was considered to be representative for the region. The various devices used to measure climate at Brits are illustrated in Fig. 9b. These included rainfall, temperature (minimum and maximum) and relative humidity (minimum and maximum). Wind speed and evaporation data were also recorded, but were not used in this study.



a



b1



b2

- Fig. 9a) Thermohygrometer used to record the ground temperature and relative humidity at the Rietgat CGA
- b) Climatological data were collected at the Brits Agro Meteorological Station: Temperature (maximum and minimum) and relative humidity were measured on the thermohygrograph illustrated (b/1). The rainfall was recorded with a rain gauge (b/2).

At each visit to the Rietgat GCA all the micro-habitats were photographed (Nikon FE2) to illustrate ecological changes which may have occurred since the last visit. It was thus possible to monitor the climatic, vegetational and animal parameters which may have altered. At each visit the strength of the wind was recorded using a “wind index” scale, zero represented no wind and five heavy gale force winds. The occurrence of recent rains was also noted, particularly when this resulted in standing surface water. A “day length index” was also calculated with the minimum day length being the midwinter solstice (21 June), and equivalent to zero; intermediate day length (autumn and spring) as 2.5 and the midsummer solstice (21 December) as a maximum of five.

2.1.2.3.2 Seasonal variation in rainfall

The seasonal variation in rainfall measured at the meteorological station at Brits during the survey is illustrated in Fig. 10a. There were two distinct seasons, a rainy and a dry season. The rainy season starts in September, has a peak in November/December and another in February/March, and then declines in April/May. The dry season spans June/July/August.

Seasonal rainfall was closely followed by a substantial growth of vegetation at the CGA. The wettest month was March, which was followed with some more rain in April and May. In June/July surface water was still present and the grazing was in good condition. In August, September and October there was very little rain and the surface water had dried up completely. The vegetation and animals on the CGA suffered enormously as the temperature increased and grazing and surface water decreased.

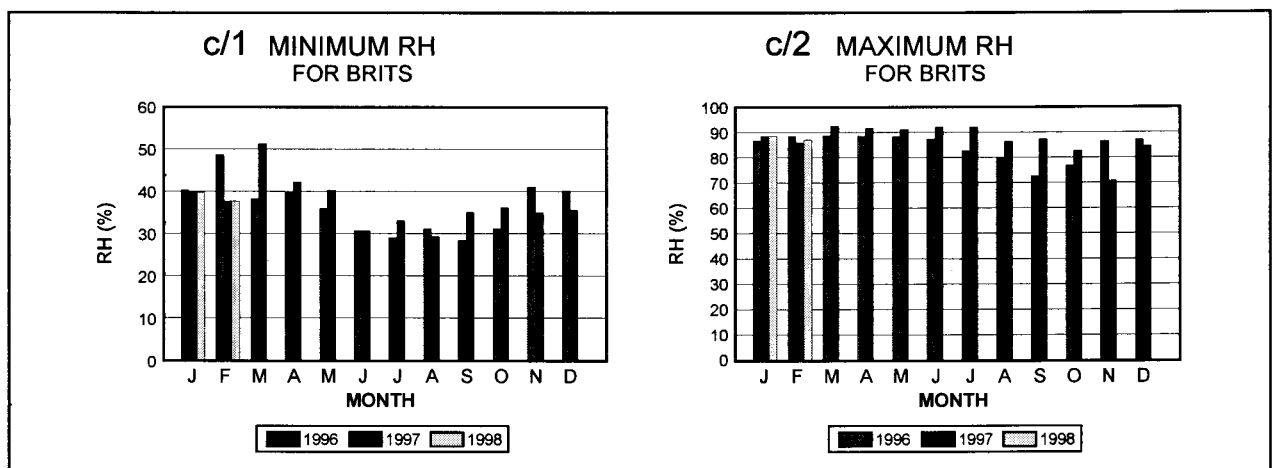
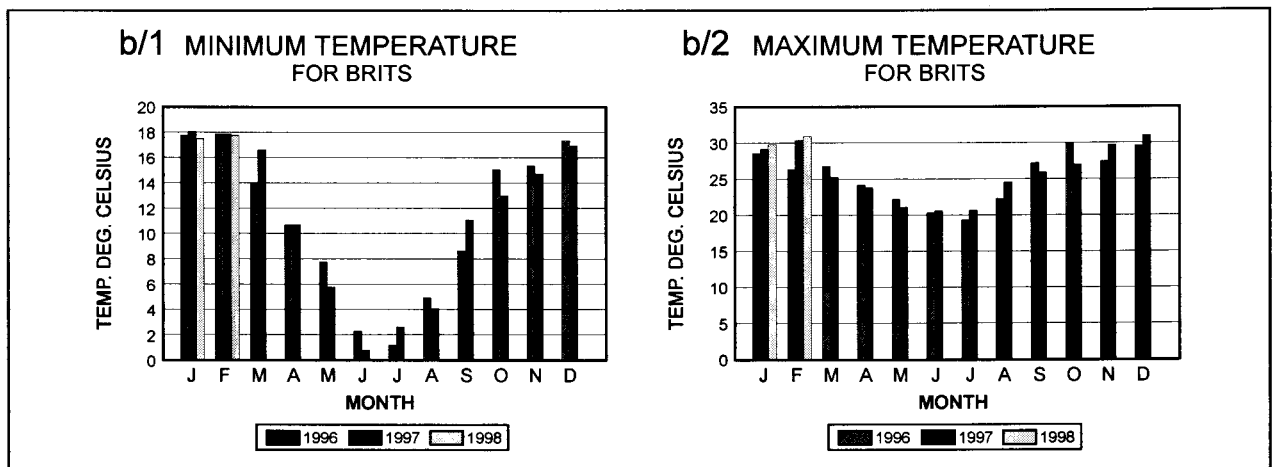
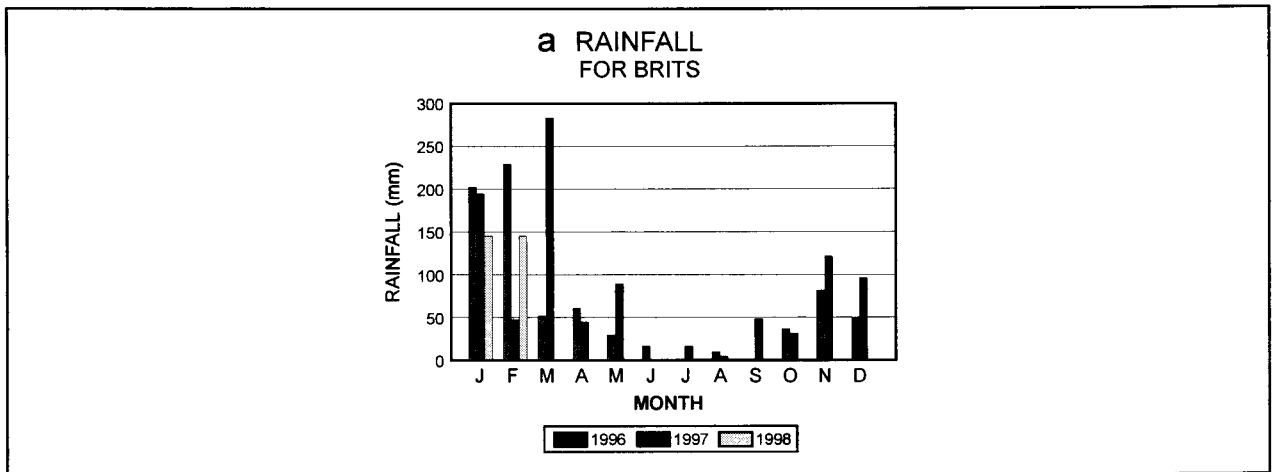


Fig. 10 Climatological data recorded at the Brits Agro Meteorological Station, 20 km south-east of the Rietgat CGA: a) Rainfall, b) Temperature (minimum b/1 and maximum b/2), c) Relative humidity (RH) (minimum c/1 and maximum c/2)

2.1.2.3.3 Seasonal variation in temperature

The minimum (b/1) and maximum (b/2) temperatures recorded at the Brits meteorological station are illustrated in Fig. 10b. The maximum temperatures at Brits (Fig. 10b/2) during the years 1996 to 1998 did not vary greatly and were normally in the range of 20°C to 30°C. Peak maximum temperatures were measured from October to February, and started to decline in March, April and May. The lowest maximum temperatures were recorded in June and July and started to rise again in the spring of August/September. The minimum temperatures at Brits showed greater variation with the lowest minima in June and July and the highest in January/February.

2.1.2.3.4 Seasonal variation in relative humidity

The seasonal variation in the RH measured at Brits is illustrated in Fig. 10c. The maximum RH (Fig. 10c/2) varied little throughout the year (74% to 90%), and was mostly in the range of 80 to 90%. The minimum RH (Fig. 10c/1) ranged from 52% to 28% and for most of the year varied between 30% and 40%.

2.1.2.3.5 Wind and day length

These were initially monitored on a regular basis, but because of difficulties in recording these parameters, a wind and day length index was eventually calculated on the day of the field visit. On the highveld of South Africa certain months of the year are more windy and this was taken into consideration. The photoperiod, i.e. the number of hours of sunshine per day, can affect the behaviour of ticks, and consequently it was decided to calculate a day length index on each visit.

2.1.2.4. Vegetation

2.1.2.4.1 Main vegetation types

The main vegetation types found in the five ecological sites at Rietgat are illustrated in Fig. 8 and 11. Tice (1997) described the vegetation in this site as sourish, mixed bushveld (Acocks 1988), but this site does not fall into any definite bushveld type, but is rather a combination of different types from mixed bushveld (Acocks type 18) through to sour bushveld (Acocks type 20). It is evident that there has been severe overgrazing and bush encroachment by both *A. karoo* and *Dichrostachys cinera*.

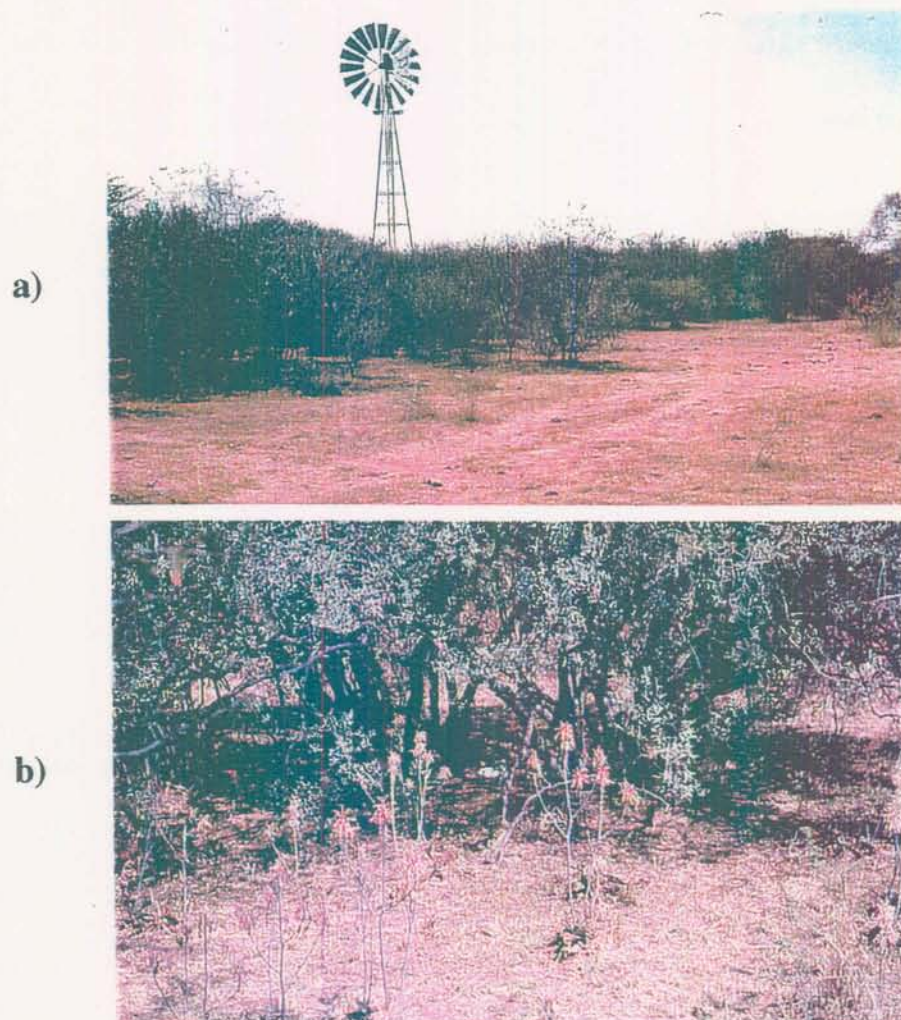


Fig. 11 The CGA at Rietgat comprised mainly *Acacia* sour veld (a) and *Euclea crispa* shrub veld (b).



Rietgat CGA:

1. *Acacia karoo* (sweet thorn)
2. *Acacia robusta* (splendid thorn)
3. *Acacia caffra* (common hook thorn)
4. *Acacia erubescens* (blue thorn)
5. *Acacia mellifera* (black thorn)
6. *Acacia tortillis* (umbrella thorn)
7. *Acacia nilotica* (scented thorn)
8. *Acacia burkei* (black monkey thorn)
9. *Boscia albitrunca* (shepherd's tree)
10. *Dichrostachys cinera* (sickle bush)
11. *Peltophorum africanum* (weeping wattle)
12. *Spirostachys africana* (tamboti)
13. *Sclerocarya caffra* (marula)
14. *Ozoroa paniculosacum* (common resin)
15. *Rhus lancea* (karee)
16. *Rhus leptodictya* (mountain karee)
17. *Rhus pyroides* (common wild currant)
18. *Maytemus heterophylla* (common spike thorn)
19. *Pappea capensis* (jacket plum)
20. *Dombeya rotundifolia* (wild pear)
21. *Combretum apiculatum* (red bush willow)
22. *Combretum zeyheri* (large fruited bush willow)
23. *Combretum hereroense* (russet bush willow)
24. *Euclea crispa* (blue guarri)
25. *Euclea undulata* (small-leaved guarri)
26. *Diospyros lycioides* (Karoo blue bush)
27. *Olea europaea* (wild olive)
28. *Carissa bispinosa* (num num)
29. *Ehretia rigida* (puzzle bush)
30. *Tarchonanthus camphoratus* (camphor bush).

The following grass species were also common at the Rietgat CGA: *Themeda triandra*, *Heteropogon contortus*, *Eragrostis rigidior*, *Aristida congesta* and *Panicum maximum*.

The grassland grazing sites appeared to be of three main types. Firstly the open savanna type grasses which had a predominance of sourveld types. These soon became unpalatable and were frequently burned, so that the cattle could graze the young leaves in spring.

Secondly the open sites where cultivated land had previously existed and which were now taken over by various invader grasses. Thirdly the grasses associated with the trees, which were more sweet veld types, and which were preferred by the cattle.

Within the CGA there were regions where one or more of the tree types predominated and these included:

- Dense *Acacia* veld, usually *A. karoo*, but also mixtures of other *Acacia* species.
- Tamboti (*S. africana*) was found close to the river, often clumped and in dense thickets.
- *Euclea* spp. - the two *Euclea* spp. occurred as shrubs or small clumps of trees often with a dense leaf litter. These areas remained shaded even when most of the other trees had lost their leaves in the very hot, dry season in early summer. The leaf litter remained reasonably moist here throughout the year.
- Mixed *Combretum* veld - the various *Combretum* species were found in forests and were associated with the river.
- *D. cinera* - dense, impenetrable thickets of sickle bush were particularly common where the land had been disturbed for agricultural purposes.
- *Peltophorum africanum* - the weeping wattle was common and also occurred in fairly dense clumps of trees.

2.1.2.4.2 Seasonal variation in vegetation cover

There were enormous changes in the vegetation on the CGA and these are illustrated in Fig. 12. These changes would have direct effects on the collector's ability to locate free-living, unfed ticks. Each ecological site was also photographed and a data bank of slides

representing the ecological changes occurring began to emerge. During the two-year cycle each year was divided into six seasonal cycles which were deemed to be representative of the seasonal changes in vegetation on the CGA.



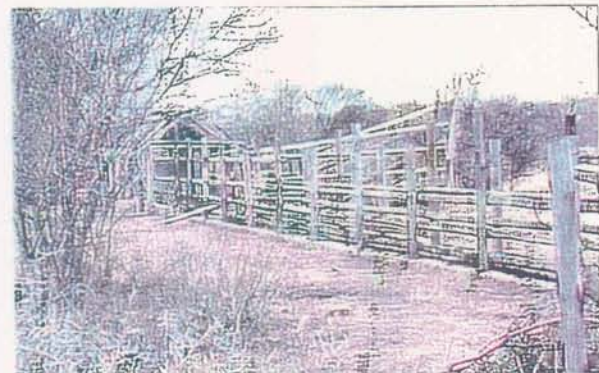
a/1



a/2



b/1



b/2



c/1



c/2

Fig. 12 Seasonal changes in the vegetational cover on the CGA at Rietgat were dramatic:

- ▶ The entrance gate in summer (a/1) and winter (a/2)
- ▶ The diptank site in summer (b/1) and winter (b/2)
- ▶ The grasslands site in summer (c/1) and winter (c/2).

- Midsummer vegetation cover (January/February) (Fig. 12 a/1). The effects of the early rains in October and November were evident on the CGA. Most of the trees had flowered and were in full leaf. The grasses were at their peak and many were seeding. This was a hot, wet period but little of the rain remained as surface water and the river ran intermittently. The increase in vegetation was starting to make tick collection difficult.
- End of summer vegetation cover (March/April) (Fig. 12 b/1). The real effects of the summer rain was evident on the CGA during this period with maximum growth in shrubs, trees and grasses. The dense undergrowth, particularly under the larger clumps of trees, made the detection of ticks almost impossible. The main communal grasslands were at their peak and were seeding. There was a lot of surface water and the Tolwane river was in full flow across the road. The cattle were in peak condition during this period.
- Early winter vegetation cover (May/June) (Fig. 12 c/2). Much of the surface water from the late rains was still present on the roads and the Tolwane river was flowing slowly. Although the CGA was beginning to dry out, as the rains had stopped, much of the grasslands and the cattle were still in good condition. Many of the grasses under the cover of the trees were still green but the open grasslands were now dry and yellow-brown. The asparagus and cotyledonous shrubs were in flower.
- Midwinter vegetation cover (July/August) (Fig. 12 a/2). Four or five months without substantial rains were now beginning to show their effects on the CGA, which were very dry and dusty. The Tolwane river flowed only intermittently, but pools of water were still present. The grassland was a yellow-brown colour, and the veld burning process had started in certain sites. Many of the trees had lost their leaves and this led to a substantial decrease in shade cover. The aloes were in full flower as were the wild pears (*Dombeya rotundifolia*), and the cattle were beginning to lose condition.

- Spring vegetation cover (September/October) (Fig. 12 b/2). The weather during this period was hot and dry although the first rains had come. The surface water was absorbed into the dry dusty CGA. The tamboti trees had also lost their leaves during this period. There was very little surface water and the animals collected at the water points. The Tolwane river was dry and there was little natural shade during this period, and only those evergreen trees and shrubs, such as *E. crispera* and *Rhus lancea*, were able to maintain the moist climate necessary for survival of free-living ticks. The open grasslands were “semi-desert” and the cattle were in very poor condition. The aloes and the cotyledons were in seed. Some of the *Acacias* were beginning to regain their leaf cover and the early *A. caffra* and *A. robusta* were in flower.
- Early summer vegetation cover (November/December) (Fig. 12 c/1). During early summer the first substantial rains fell and the Tolwane river started to flow again. The very hot conditions during this period quickly counteracted the effects of the rain. The early annual plants and grasses started emerging, and the CGA started to recover from the long, dry season. Although there was vegetation growth it was still possible to see adult and nymphal free-living ticks when they were attracted to the trap.

In conclusion the CGA at Rietgat experienced large seasonal variations in vegetation cover which were monitored photographically. This showed that at certain times of the year the vegetation on the CGA was so dense that it was not possible to see the free-living ticks. At other times of the year only a few clumps of shaded vegetation remained and these times were optimal for the collection of free-living ticks which had accumulated there.

2.1.2.4.3 Leaf litter

A. hebraeum is a tick which requires high environmental temperatures and humidity to survive (Norval 1977), and is not found in those regions of South Africa which are too dry

and cold. It is also known to prefer shaded, moist habitats (Rechav, 1982), and with the exception of the larvae, is thought to spend the majority of its non-parasitic life beneath leaf litter (Norval *et al.* 1989b).

During the warm summer rainy season the relative humidity and temperature of most of the ecological micro-habitats at the Rietgat CGA would probably be suitable for the survival of free-living *A. hebraeum*. In the dry, cold season, however, only those sites which remain shaded and moist would be suitable micro-habitats for the free-living ticks. As the majority of the trees on the CGA lose their leaves in winter the surface habitat around their stems would not be an ideal place for the ticks to survive. At Rietgat the *E. crispa*, *Rhus lancea* and *S. africana* clumps have a deep leaf litter which remains moist even in the depth of the dry season. These sites would be ideal for the survival of free-living *A. hebraeum* adults and nymphs.



Fig. 13 Leaf litter, which was especially abundant under the shade of *Euclea crispa* shrubs, remained moist throughout the year. An adult *A. hebraeum* can be seen (arrow) emerging from the leaf litter.

2.1.2.5 Livestock/wildlife on the Rietgat CGA

Cattle on the CGA were in good condition at the beginning of the dry season (Fig. 14), but their condition declined rapidly towards the end of winter.

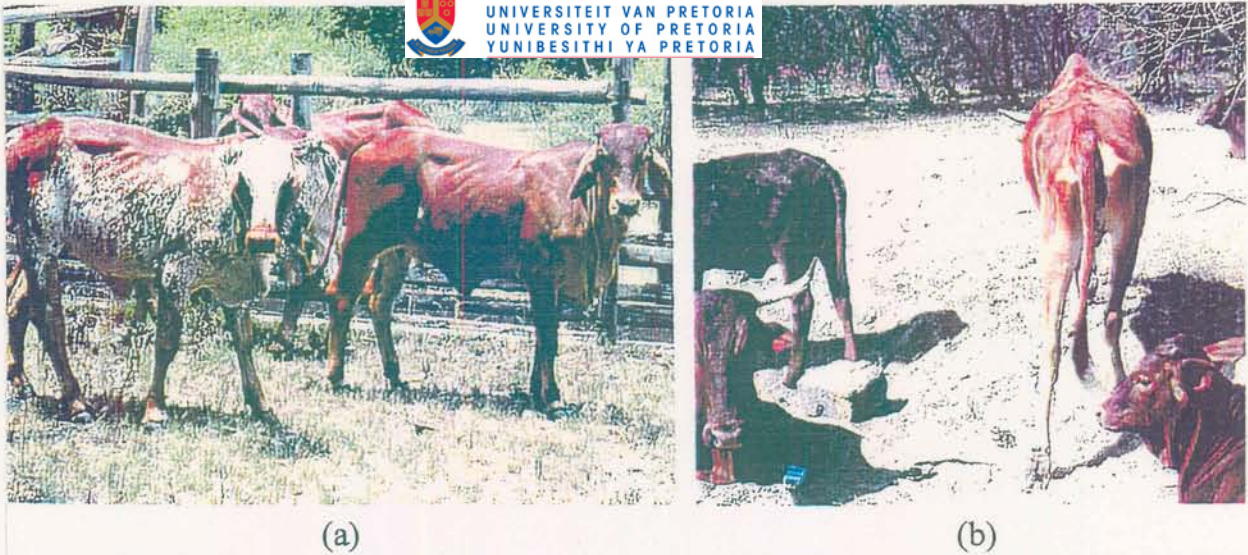


Fig. 14 Mixed type cattle breeds on the Rietgat CGA were in good condition at the beginning of winter (a), but their condition declined rapidly towards the end of winter (b).

In 1991/2/3 there were 153 cattle, 30 sheep and 60 goats belonging to 17 owners (Tice 1995). Most of the cattle were Brahmans and Afrikaners, as well as Brahman-Afrikaner crosses. Some dairy breeds (Simmentaler, Drakensberger, Friesland and Jersey) were also evident in the cross-bred cattle. The goats were all indigenous types and the sheep, Dorper cross-breeds. Pigs (large white and indigenous types) were also present at a small piggery. Donkeys, chickens, geese, turkeys, pigeons and dogs were also common at the communal village. A diptank which was not functioning during the 1991/2/3 project (Tice 1997) was still not functional during this project and the owners appeared to use either no form of tick control, or hand spray or pour-ons. It was difficult to assess the numbers of wild animals present on the CGA. Following discussions with local farmers it was clear that some wild animals were still present, as they were hunted with packs of dogs.

2.1.2.6 Sample size determination

2.1.2.6.1 Factors affecting sample size

Elbers, Stegman, de Jong, Lambers, Koning & Hunneman (1995) stated that during a survey

five essential parameters need to be considered. These are population size, expected prevalence of the disease, permissible error, desired level of confidence and the variance in the disease prevalence.

- Population size

The study population in this survey was the total free-living, unfed adult and nymphal *A. hebraeum* population at the Rietgat CGA. It was presumed that the populations of *A. hebraeum* in the surrounding CGA had similar characteristics to the study population. It was not possible to extrapolate the findings to other populations in South Africa, and the size of the tick population at the Rietgat CGA was assumed to be infinite. The sampling method was not random, as the AAAP/CO₂ trap attracted localised populations (clusters) of ticks (Gummow pers. comm. 1996). To overcome this it was decided to double the size of the required sample. Another sampling bias with this type of survey was that ticks tend to be collected more frequently from one or more high density sites, in order to overcome this problem, up to 25 ecological sites were sampled to get a more representative sample.

- Expected prevalence of disease

There have been no *C. ruminantium* prevalence surveys done on the specific tick population of the Rietgat CGA. Research in other parts of South Africa indicated a prevalence of 5% to 10% (Du Plessis 1985; Du Plessis *et al.* 1992), and in Zimbabwe a much higher prevalence (P) of 0% to 45% in free-living *A. hebraeum* (Norval *et al.* 1990). Because of this it was decided to do a pilot study in which 150 adult ticks were collected and an

estimate of disease prevalence was done on this sub-sample. Once the results of this estimate were available a more accurate sample size could be calculated.

- Permissible error (Desired absolute precision)

The desired absolute precision of a sample is normally the choice of the investigators. In this project, because of financial constraints, it was decided that a 5% level of precision would give a cost-effective sample.

- Desired level of confidence

In general a 95% confidence level gives accurate results.

- Variance

Because our survey population was sampled in clusters, it was decided to double the required sample and to avoid sampling only specific clusters.

2.1.2.6.2 Calculation of the required sample size

It was decided that 2%, 5% and 10% prevalence levels would be used in the initial sample size estimation. These would be adjusted according to the pilot survey results ($n = 150$), and the final estimate would be made when that information became available. The P used for all calculations (Canon & Roe 1982) involved a 95% desired level of confidence and a 5% desired absolute precision.

It was calculated that the prevalence levels would require the following sample sizes, i.e. number of ticks collected:

PREVALENCE	REQUIRED SAMPLE SIZE
● 2% (P = 0.02)	31
● 5% (P = 0.05)	73
● 10% (P = 0.10)	139
● 20% (P = 0.20)	246
● 50% (P = 0.50)	385

As the prevalence increases, the larger the sample size required. The pilot survey using the PCR assay indicated that 4.7% ($\pm 5\%$) of the ticks ($n = 150$) were infected with *C. ruminantium*, and that a field sample of 73 ticks ($P = 0.05$) would probably be sufficient. However, in order to counteract the effects of cluster sampling and other inherent problems with the sampling method, it was decided to substantially increase the required sample size, and eventually 434 adult ticks were collected from the Rietgat CGA for PCR analysis to detect *C. ruminantium*.

2.1.2.7 Field collection of ticks using the AAAP/CO₂ trap

2.1.2.7.1 The apparatus used to collect the ticks in the field

The apparatus used to collect the free-living, unfed *A. hebraeum* is illustrated in Fig. 15.

This included the following:

- Dry ice blocks (5½ kg)
- Pheromone in a small brown glass container
- Metal pegs and foam rubber pieces for pheromone
- 1 pair of large canvas garden gloves to handle the dry ice
- 1 large plastic bucket (black) to carry the dry ice
- 1 smaller plastic bucket (white) to mix pheromone onto the foam rubber pieces
- “Cooler box” to store the pheromone in the field
- Thermohygrometer
- Plastic storage containers for collection of ticks

- Record book, camera, pencil and forceps
- Small mat to lie on
- garden rake to clean site.



Fig. 15 The apparatus used to collect the ticks in the field.

2.1.2.7.2 Pheromone source

The pheromone used in the field collection of free-living, unfed *A. hebraeum* adult and nymphs was obtained from a mixture of feeding male *A. hebraeum* ticks stored in 99% diethyl-ether (Unilab-Saarchem (Pty) Ltd). The adult feeding males were collected from a number of undipped cattle. These cattle were owned by farmers in the area, and were either

restrained on the ground with ropes, or placed in a crush whilst the ticks were collected. Approximately 1 ml of diethyl-ether per tick was used. The main storage bottle was an empty, used 2,5 l brown diethyl-ether bottle with a good screw-on cap. The bottle was kept in the dark in a cool place. Batches of feeding male *A. hebraeum* ticks were added when they became available. The bottle was shaken periodically to mix the volatile pheromone with the diethyl-ether.

For field trips small quantities (5 - 10 ml) of diethyl-ether-pheromone were transferred to 50 ml brown glass bottles which were stored in a cooler box. When field work involved travelling by plane, such as to the East London site, it was necessary to send the pheromone ahead via courier to avoid any dangers involved in the transport of such a volatile and flammable substance.

2.1.2.7.3 CO₂ source

The CO₂ source used throughout the survey was dry ice. Initially small pieces of broken dry ice were used, but it was soon realised that large solid blocks (5½ kg) (Fig. 16) were more efficient. They disseminated CO₂ over a much wider area, were easier to move and lasted longer in the field. Two blocks of dry ice, which were supplied in a box, were collected early in the morning from the Onderstepoort Research Institute and taken to the field. A block of dry ice gave a good, strong source of CO₂ all day.



Fig. 16 A large 5½ kg block of dry ice was used as a source of CO₂. Pheromone-impregnated foam rubber pieces were attached to metal pegs (n = 1 to 4), close to the dry ice.

2.1.2.7.4 Using the AAAP/CO₂ trap in the field

Initial visits were at 07:00 in the morning, but after field experiments at the KNP (Bryson *et al.* 1999) indicated that these ticks only become active after 09:00, then field work commenced at 09:00. On each visit to the Rietgat CGA a certain procedure which was found to give the best results, was followed with the AAAP/CO₂ trap. On arriving at a site the climatological parameters were first recorded. Soil temperature and relative humidity were measured in the shade and recorded throughout the day. Recent rains were noted and the wind index calculated. Any ecological changes were noted and the site was photographed with a Nikon FE camera to record these changes.

(a)



(b)



Fig. 17a) + b) The AAAP/CO₂ traps were easy to use in the field, and were often moved around. Six or more traps were used at a time (a), and the traps could be concentrated in sites where tick activity was highest (b).

A number of dry ice blocks were positioned in close proximity (6 - 8 at a time) around a clump of trees or down a path in order to maximise the effect of the CO₂/pheromone (Fig. 17a). Small (4 cm diameter) pieces of circular foam rubber which were collected from used medicine containers, were fitted onto metal pegs (Fig. 16) and these were placed in a plastic bucket. Ten ml of diethyl-ether/pheromone mixture was then poured onto the foam

rubbers/pegs in the small white plastic bucket. Care was taken to ensure that all the foam rubbers were impregnated with the pheromone ether. The pegs (4 at a time) were then placed around the CO₂ source. It was particularly important that at least one of these pheromone sources was downwind of the CO₂. As the CO₂ gave off a white visible trail it was easy to monitor the direction of the wind. By using six, or even eight blocks of dry ice/pheromone, it was easy to surround a large site with CO₂/pheromone and to maximise the effects of the trap (Fig. 17a).

2.1.2.7.5 Collecting adult ticks

When surveying a new area it was important to locate a site or sites with high tick density. This was not always easy and required patience, time and a good understanding of the ecology of the tick. Certain areas such as the farms at Warmbaths and MEDUNSA were not ideal places to use the trap for reasons which will be discussed later. Rietgat and the KNP were, however, ideal areas because of the high density of ticks in these sites.

When using the traps in an exploratory way it is important to exclude sites where ticks cannot survive, and to rather concentrate on those sites in which ticks are most likely to accumulate. The latter include shade, moisture, leaf litter, cattle paths and kraals, bushes, game tracks and water points. At these sites the traps can be moved quickly, i.e. every 5 - 10 minutes until tick activity is observed (Fig. 17a), then all the traps can be moved into this site and to concentrate the collecting effort here.

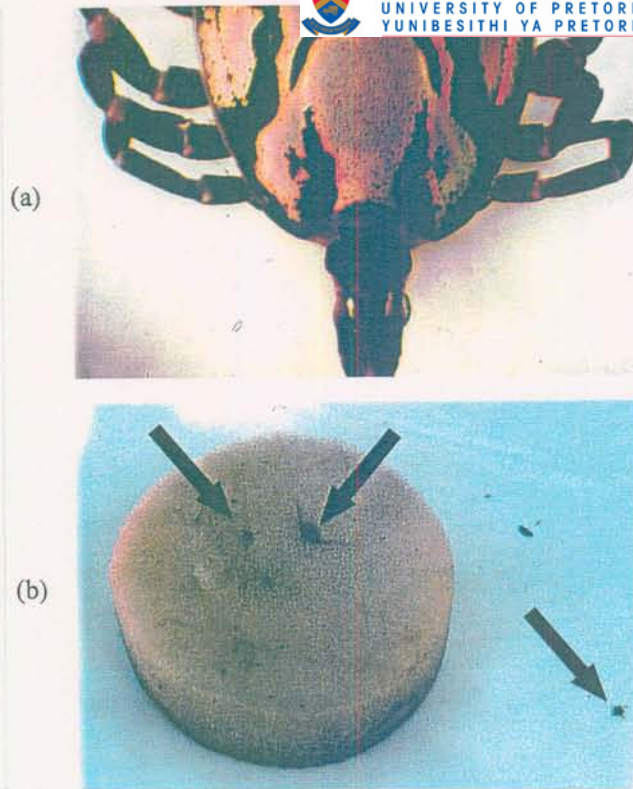


Fig. 18a Adult *A. hebraeum* (male).

Fig. 18b Nymphal *A. hebraeum* (arrows) were also attracted to the AAAP/CO₂ trap, and often attached to the pheromone-impregnated foam rubber pieces.

The collection of adult *A. hebraeum* (Fig. 18a), was comparatively easy as the ticks rapidly move towards the trap and are clearly visible because of their movement. In ecological sites with deep leaf litter, it was imperative to clear the litter with a small rake to make the ticks more visible. Once the ticks had been located they could be collected either by hand or with forceps. The traps could also be placed in an open, sandy space (Fig. 17b), and the ticks could then be enticed out of the dense vegetation and into the open area where they were more easily visible. The same principle was employed when using a cattle path (Fig. 17a), and ticks from the surrounding bushes would be attracted onto the path.

The traps had to be visited on a regular basis in order to stop the ticks from getting too close

to the dry ice, which froze and killed them. Once the adult ticks detected the CO₂/pheromone, they scuttled around in an erratic manner with their front legs waving excitedly. In an area of high tick density, three or more ticks could be caught at a time, all of them making their way to the trap. It was also important to walk around the area where the traps were located, as adult ticks were attracted from several metres and were often collected some distance from the trap. Once the ticks had been caught they were transferred to small plastic containers with an air hole and stored. Care was taken not to leave these containers in the direct sunlight and to store them in the shade. Adult ticks collected during the field surveys were transferred to the containers and stored in a humidifier.

2.1.2.7.6 Collecting nymphal ticks

At Rietgat it was noticed that numbers of small ticks were also attracted to the traps. A sample of these ticks was collected and later identified as *A. hebraeum* nymphs. Certain ecological sites were far better for the collection of *A. hebraeum* nymphs than others. These sites, A/3, B/1, B/6 and E/6 (Fig. 22), all had good shade and a deep leaf litter. The most successful method of collecting these ticks was to put four or more traps in one site. After 30 minutes one would lie down between the traps and carefully view the leaf litter from a distance of 30 cm. The nymphal ticks could be seen moving around in an excited manner, but the collection of the nymphs was difficult as they are small. One could either use fine forceps to collect the nymphs or alternatively one's fingers and take a pinch of soil and leaf litter where the nymph was and place it on white paper and grasp the nymph with fine forceps when it moves.

One or more nymphs were frequently attracted to the pheromone impregnated foam rubbers (Fig. 18b) placed in the vicinity of the CO₂ block. The nymphs collected were also placed in plastic vials and later stored in a humidifier. Both adult and nymphal *A. hebraeum* were transported to Zimbabwe in makeshift humidifiers. An important aspect with free-living, unfed ticks is to keep them moist (cotton wool soaked in water) and to clean the humidifier regularly. These free-living, unfed ticks can survive for several months using this method. This is better than using ticks collected off cattle as these die within a few days or at best weeks. Ticks collected off cattle are also susceptible to fungal and other infections, probably as a result of infected and damaged mouthparts. Field collected ticks are usually clean, flat and easier to keep alive.

2.1.3 RESULTS

2.1.3.1 Total number of ticks collected during the survey at the Rietgat CGA

The total numbers of adult and nymphal *A. hebraeum* collected at the Rietgat CGA are summarized in Tables 4, 5, 6, 7 and 8, and Fig. 19 and 20. There were a total of 23 visits to the Rietgat CGA survey area and 22 of them took place during the 1996/7/8 period. A total of 1 196 adult ticks were collected with the AAAP trap (Fig. 19), with an average of 52 ticks per day (Fig. 20). Fifty-one percent of these ticks were females (n = 604) and 49% were males (n = 592). Sixty-two percent of the total number of ticks (n = 1 934) caught with the AAAP trap were collected at the Rietgat CGA (Table 8, Fig. 19). Ninety-five percent of the total nymphs collected during the survey (n = 309) were from the Rietgat region (n = 292) (Table 8, Fig. 19).

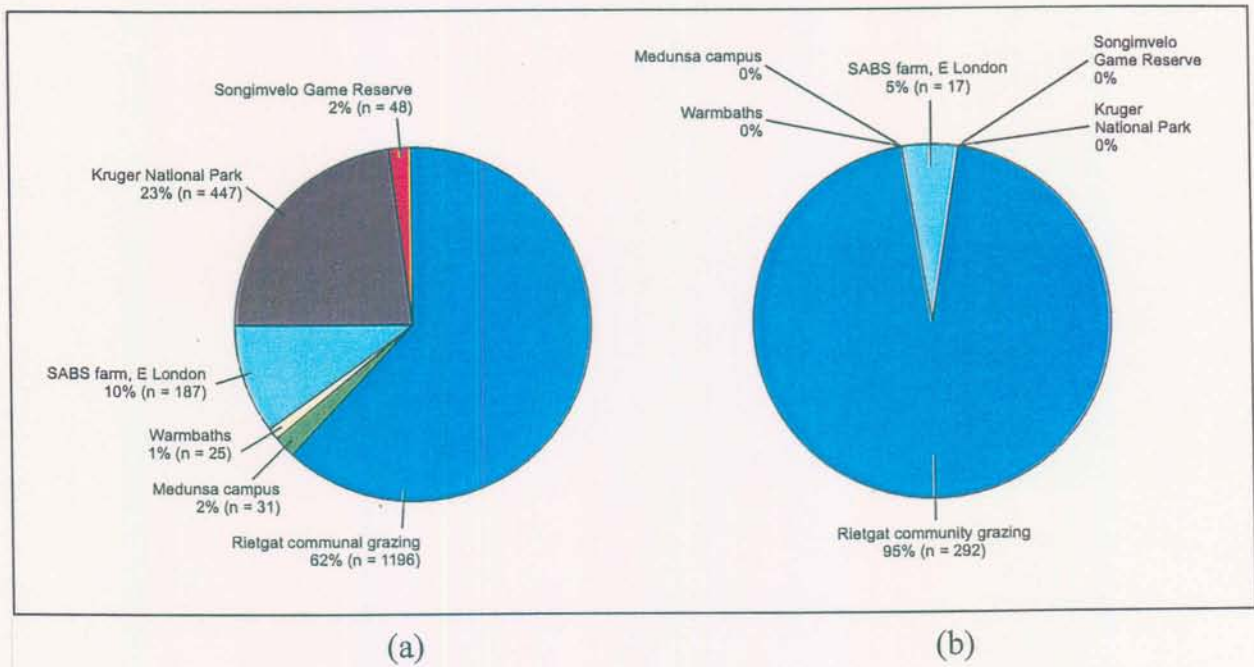


Fig. 19 Total number of adult (n = 1 934) (a) and nymphal (n = 309) (b) *A. hebraeum* collected with the AAAP/CO₂ trap at 6 areas of South Africa.

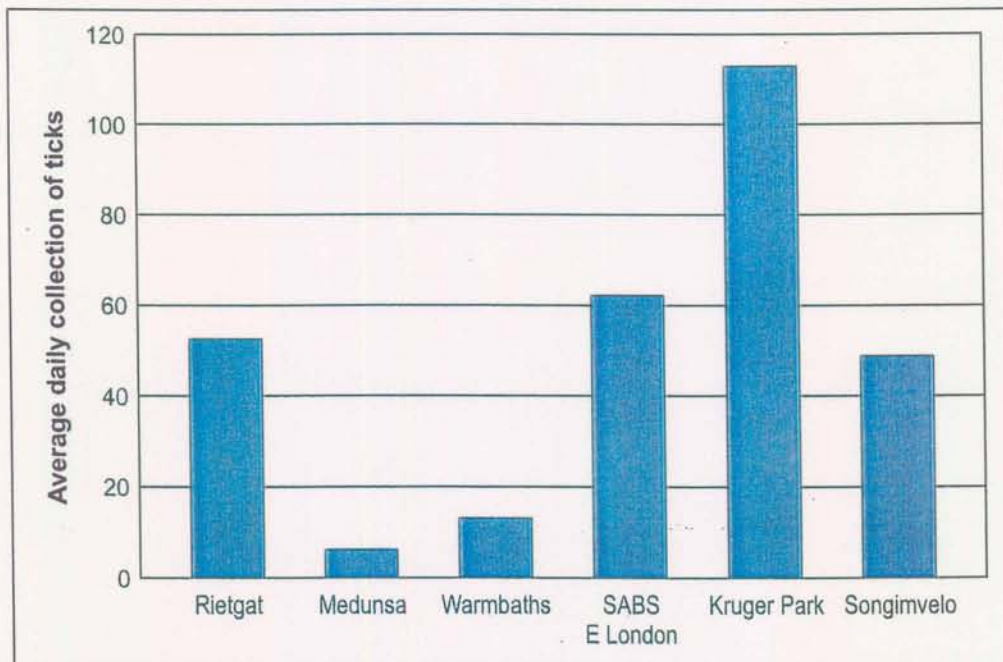


Fig. 20 Average daily collection of adult *A. hebraeum* using the AAAP/CO₂ trap at the different field sites.

TABLE 4 Total numbers of adult and nymphal *Amblyomma hebraeum* collected using the AAAP/CO₂ trap, at the Rietgat CGA, North West Province, South Africa

VISIT NO.	DATE	ADULT TICKS			NYMPHAL TICKS	NUMBER OF VISITS THAT MONTH	AVERAGE DAILY COLLECTION OF ADULT TICKS DURING THAT MONTH	AVERAGE DAILY COLLECTION OF NYMPHAL TICKS DURING THAT MONTH
		T	M	F				
1	1992-10-20	78	35	43	0	1	78	0
2	1996-02-06	0	0	0	1	1	0	1
3	1996-03-07	0	0	0	0	1	0	0
4	1996-07-02	2	1	1	0	1	2	0
5	1996-09-18	96	48	48	0	4	47.25	0
6	1996-09-19	28	13	15	0			0
7	1996-09-26	55	27	28	0			0
8	1996-09-27	10	5	5	0	↓	↓	0
9	1996-10-29	258	130	128	0	2	202	0
10	1996-10-30	146	60	86	0	↓↓	↓	0
11	1996-11-19	139	66	73	0	2	111	0
12	1996-11-30	83	49	34	0	↓	↓	0
13	1997-01-21	18	12	6	0	2	9.5	.0
14	1997-01-30	1	0	1	0	↓	↓	.0
15	1997-02-13	2	1	1	0	1	2	.0
16	1997-04-11	7	4	3	0	1	7	.0
17	1997-06-25	3	1	2	0	1	3	.0
18	1997-08-26	3	2	1	10	1	3	10
19	1997-09-04	6	3	3	57	2	9.5	50
20	1997-09-27	13	6	7	43	↓	↓	↓
21	1997-11-20	81	41	40	80	2	111	77.5
22	1997-11-25	141	72	69	75	↓	↓	↓
23	1998-01-30	26	16	10	26	1	26	26
TOTAL		1 196	592	604	292	23		

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RESULTS

- Total adults ticks collected = **1 196**
- Total nymphal ticks collected = **292**
- Total collection days = **23**
- Average adult ticks per day = **52**
- Average nymphal ticks per day = **13**

TABLE 5 Nymphal *A. hebraeum* collected from various ecological micro-habitats during the survey at Rietgat, North West Province, South Africa (1996 - 1998)

AREA	DATE OF COLLECTION							TOTAL
	1996-02-06	1997-08-26	1997-09-04	1997-09-27	1997-11-20	1997-11-25	1998-01-30	
A/1								
A/2								
A/3						30	21	51
A/4								
B/1		10	29		20			59
B/2								
B/3								
B/4								
B/5								
B/6			28	22	10			60
C/1	1							1
C/2								
C/3								
D/1								
D/2								
D/3						15		15
D/4								
D/5								
E/1				10	20	10	0	40
E/2				11	30	20	5	66
E/3								
E/4								
E/5								
E/6								
E/7								
TOTAL	1	10	57	43	80	75	26	292

1. Total nymphs collected = 292

2. BLANK SQUARES = Not sampled

TABLE 6 Adult *A. hebraeum* collected from 25 ecological micro-habitats sampled on a regular basis at Rietgat, North West Province, South Africa

A R E A	1992	1996 DATES OF COLLECTION											1997 DATES OF COLLECTION										1998	TOTAL	
	10-20	02-06	03-07	07-02	09-18	09-19	09-26	09-27	10-29	10-30	11-19	11-30	01-21	01-30	02-13	04-11	06-25	08-26	09-04	09-27	11-20	11-25	01-30	TICKS	
A/1						0		0		0					0	0	0	0		0	0	0	0	0	0
A/2						0		0		0						0	0		0	0	95		0	95	
A/3						13		0		0					0	0	2		2	70			8	95	
A/4						0		3							0	0	0		1	5	0		0	9	
B/1											64		0			0	0	0	4	1	2	0	1	72	
B/2-5											0		0		0	0	0	0	0	0	0	0	0	0	
B/6											0		0		0	0	0	2	1	4	0		0	7	
C/1		0	0	2							0		0		0	0	0	0	0	0	0	0	0	2	
C/2	78	0	0	0	50		55			58			0		2	0	0	0	0	0	0	0	0	243	
C/3		0	0	0						24		45	0	1	0	0	1	0	0	0	0	0	0	0	71
D/1									5	0	0	23	0	0	0	0	0	0	0	0	0	0	8	36	
D/2										35	0	18	15	1	0	0	0	2	0	0	1	0	38	110	
D/3										68		26		0			0	0	0	4	0	0	0	98	
D/4										19		10		0		0	0	0				0	0	29	
D/5										0		0		0		0	0	0	0	0	0	0	0	0	
E.1					46	15		7	96		23		0		0	5	0	0	0	0	0	8	9	209	
E.2					0	0		0			62		11		0	0	0	0	0	3	0	0	0	76	
E.3					0	0		0			0		6		0	2	0	1	0	0		0		9	
E.4					0	0		0	15		0		0		0	0	0	0	0	0	0	0	0	15	
E.5					0	0		0	10		0		0		0	0	0	0	0	0	0	0	0	10	
E/6					0	0		0	10		0		0											10	
E/7					0	0		0			0		0			0	0	0	0	0			0	0	
TOTAL	78	0	0	2	96	28	55	10	258	146	139	83	18	1	2	7	3	3	6	13	81	141	26	1196	

Total number of ticks collected = 1 196 Adults (592 Males and 604 Females)

0 = No ticks collected

BLANK SQUARES = Not sampled

TABLE 7 Ticks other than *Amblyomma hebraeum*, which were attracted to the AAAP/CO₂ trap

DATE OF COLLECTION	SPECIES	SEX		AREA
		MALE	FEMALE	
1996-11-08	1 x <i>Rhipicephalus appendiculatus</i>	1	0	Rietgat CGA
1996-11-11	8 x <i>R. simus</i>	5	3	MEDUNSA
1996-11-15	2 x <i>R. evertsi</i>	2	0	MEDUNSA
1997-09-09	3 x <i>R. evertsi</i>	2	1	Warmbaths
1998-01-31	8 x <i>R. simus</i>	5	3	Rietgat CGA
	4 x <i>R. appendiculatus</i>	3	1	
	4 x <i>R. evertsi</i>	3	1	
	1 x <i>Hyalomma marginatum rufipes</i>	1	0	

TABLE 8 Total *A. hebraeum* adults and nymphs collected with the AAAP/CO₂ trap at the various areas in South Africa

AREA	ADULT TICKS	% OF THE TOTAL COLLECTION OF ADULTS	NYMPHAL TICKS	% OF THE TOTAL COLLECTION OF NYMPHS	DAYS SPENT COLLECTING TICKS	AVERAGE ADULT TICKS COLLECTED PER DAY ON EACH VISIT
Rietgat CGA	1 196	62	292	95	23	52
MEDUNSA	31	1.6	0	0	5	6.2
Warmbaths	25	1.3	0	0	2	12.5
SABS (East London)	187	9.6	17	5	3	62.3
Kruger National Park	447	23	0	0	4	111.75
Songimvelo Game Reserve	48	2.5	0	0	1	48
TOTAL COLLECTION	1 934	100	309	100%	38	

2.1.3.2 Seasonal abundance of the ticks collected

■ Adults

Adult *A. hebraeum* showed a definite seasonal pattern of abundance in both 1997 and 1998 (Fig. 21). The first adult ticks collected with the trap after winter were in August and September. This was followed with peak numbers in October/November. There was a steady decline in January/February and from March through to August very few adult *A. hebraeum* were collected. Seasonal peaks thus occurred during the dry, hot period of September/ October/November. During this period the first rains fell but had not had time to affect the vegetational cover on the CGA.

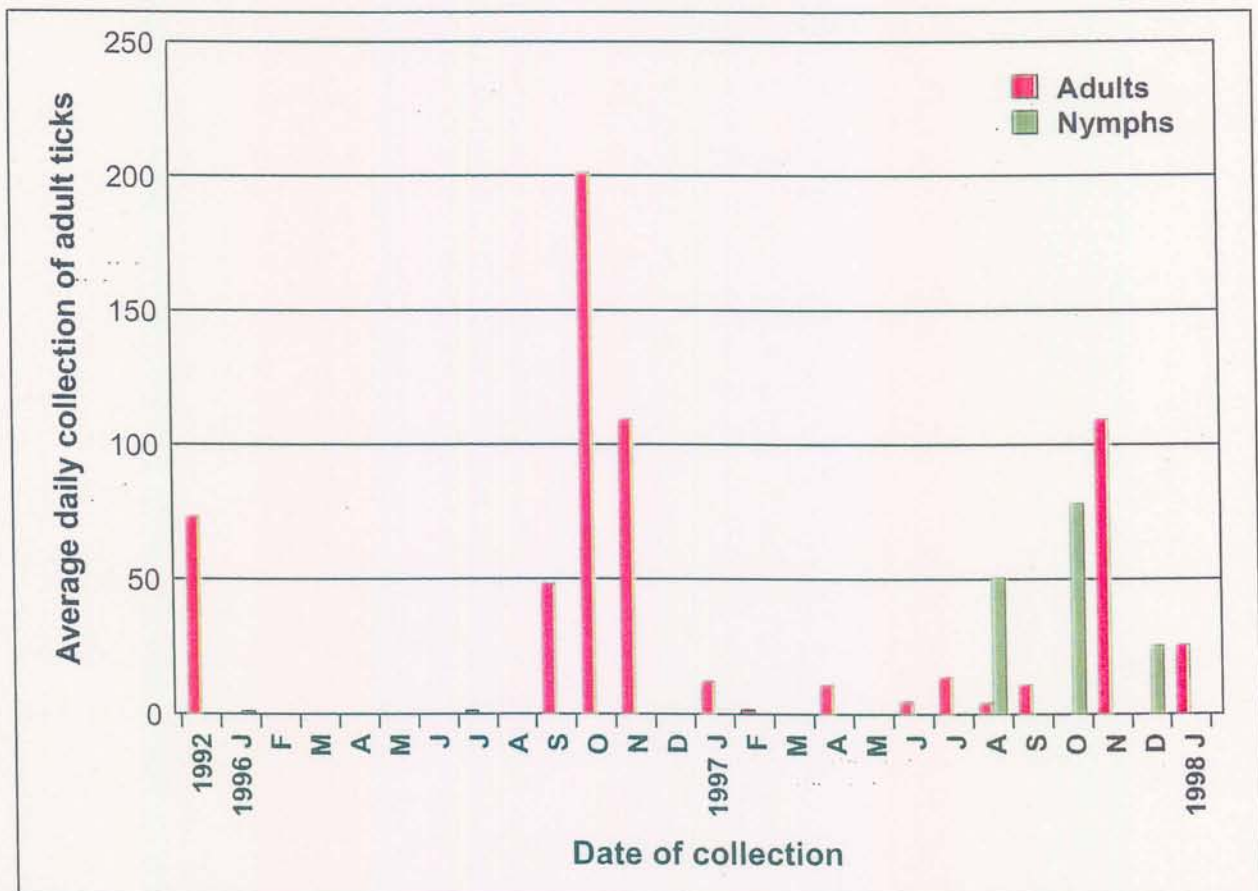


Fig. 21 Seasonal variation in the numbers of adult and nymphal *A. hebraeum* collected with the AAAP/CO₂ trap at the Rietgat CGA.

In October 1992 when the CGA at Rietgat was first visited, ticks were collected successfully with the AAAP/CO₂ trap. Follow-up visits during the late summer (February/March) resulted in no ticks being collected at exactly the same site. This change in tick activity can now be explained by changes in climate and vegetation, and will be discussed later in more detail.

■ Nymphs

Nymphs were only collected during a short period towards the end of the survey. This was mainly because at the commencement of the project, it was not realised that the AAAP/CO₂ trap could attract nymphs. As a consequence no data were collected for the first one and a half years. During the six-month period when nymphs were sampled, successful collections in certain ecological areas were made on a regular basis (Fig. 22). Although they did not appear to have the same seasonal periodicity as the adults, a more in-depth investigation would be necessary to confirm this.

2.1.3.3 Variation in tick numbers collected at the various ecological areas

■ Overall results

Twenty-five ecological micro-habitats were sampled on a regular basis during the survey. It was clear for both the adults and the nymphs that certain ecological areas were preferred, whilst others had no ticks at all (Tables 5 and 6, and Fig. 22).

■ Individual sites sampled

- Areas A/2 and A/3 (Fig. 8A), which were associated with a clump of tamboti trees and were close to the Tolwane river, were good areas for collecting both adult and nymphal ticks.

- Area B (Fig. 7 and 8B) was not a highly productive area with the exceptions of areas B/1 and B/6, both of which were heavily shaded with a good, deep leaf litter and were associated with a high nymphal population.
- Area C (Fig. 7 and 8C) was the original site at which the AAAP/CO₂ trap was tested in 1992. C/2 and C/3 were both good areas for collecting adult ticks but no nymphs were found.

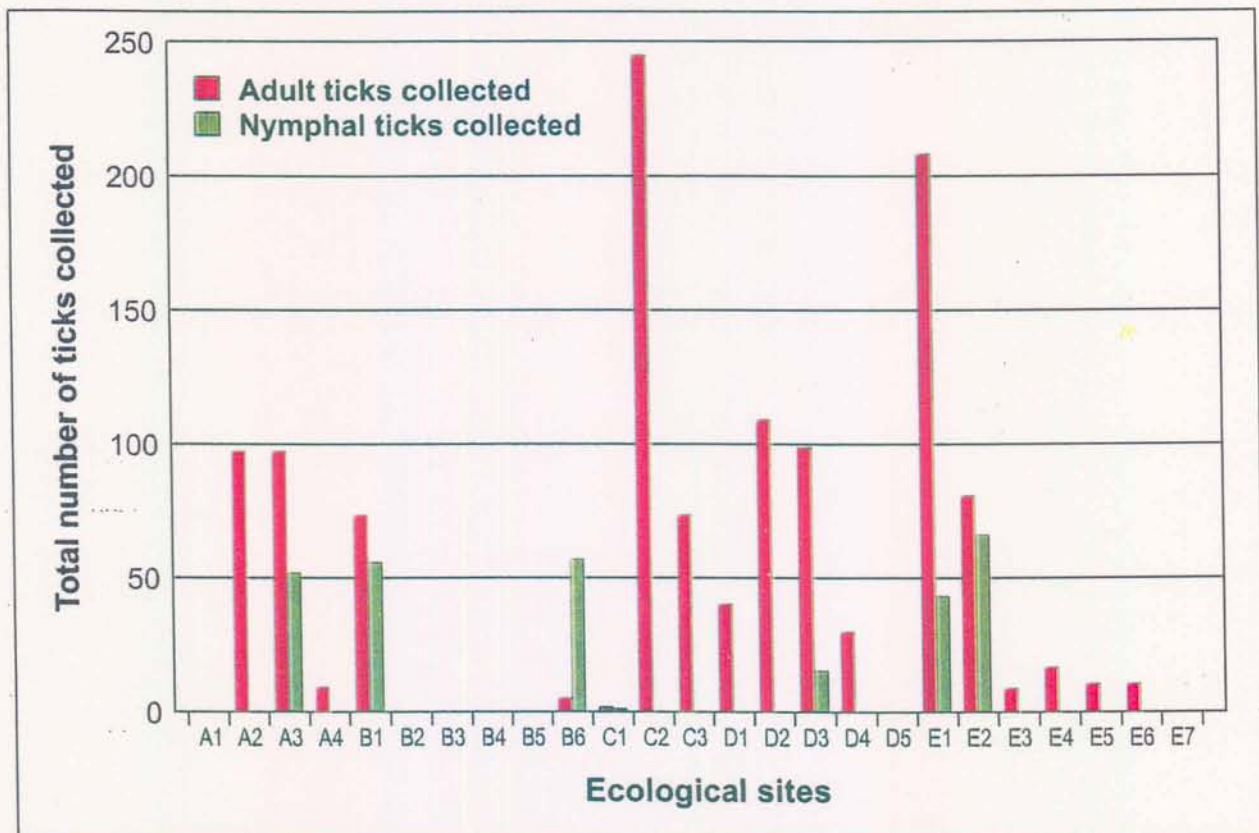


Fig. 22 Numbers of adult and nymphal *A. hebraeum* collected at the various ecological sites at the Rietgat CGA.

- Area D (Fig. 7 and 8D) and the surrounding area was a reasonably good habitat at which to collect adult ticks. D/2 and D/3, which were more open areas, were found to be better than the deeply shaded D/1.

- Area E (Fig. 7 and 8E), which was close to the diptank, encompassed very good sites E/1, E/2, as well as mediocre ones E/3, E/4, E/5, E/6 and E/7, where very few ticks were collected. E/1 was mixed *E. crispera* and *Rhus lancea* trees with a dense leaf litter which was moist throughout the year even during the severe, hot, dry period of the year, when most of the trees and shrubs had lost their leaves. The evergreen *E. crispera* and *Rhus lancea* appeared to lose their leaves throughout the year and this resulted in year round, moist leaf litter and shade for the ticks. The other area E/2, was a typical tamboti clump with a dense leaf litter in late winter/spring and this resulted in a good, moist, shaded environment for the ticks.

2.1.3.4 Sex ratio of the population of *A. hebraeum*

The sex ration of female to male ticks collected with the AAAP/CO₂ trap at the Rietgat CGA was 51% females to 49% males.

2.1.3.5 Other tick species attracted to the AAAP/CO₂ trap

Various other tick species were also attracted to the traps. The ticks collected and identified are listed in Table 7. *R. appendiculatus*, *R. simus*, *R. evertsi evertsi*, and *H. marginatum rufipes* were collected and identified at Rietgat.

2.1.4 DISCUSSION

2.1.4.1 Factors affecting the efficacy of the AAAP/CO₂ trap

2.1.4.1.1 CO₂ Source

Initially small chopped pieces of dry ice were used after breaking up a large 5½ kg block. This method was successful, but the dry ice was difficult to move around, dissipated quite quickly and did not last the entire day. The “lunch box” tick trap of Norval, Butler & Yunker (1989b), using fed males of *A. hebraeum* over which a stream of CO₂ from a CO₂ cylinder was passed, was also tried in the field. A very poor response to this trap resulted probably because of the very low levels of CO₂ emitted from the cylinder. Various other combinations of CO₂ cylinders and fed male ticks were tested at the Rietgat CGA, all with poor results. The CO₂ cylinders were expensive to purchase and maintain, and certainly could not be left unattended because of the possibility of theft. The cylinders were heavy and the CO₂ did not last long under field conditions, and had to be frequently refilled. In certain areas facilities to refill these cylinders were unavailable. The most important disadvantage was the inability of the CO₂ cylinder to disperse the CO₂ over a large area and it was also impractical to use six or eight cylinders at a time.

For the above-mentioned reasons it was deemed necessary to use a more flexible, movable and inexpensive source which dispersed CO₂ over a wide area and mimicked the breath of

a grazing bovine. Large blocks of dry ice were found to be ideal for this purpose and they were easy to move around, either with one's shoes or with gloves. It was possible to concentrate the CO₂ by surrounding the area with 6 - 8 blocks of dry ice. Ticks in the area would then be affected by one or other of the CO₂ blocks in the region. These blocks were easy to move along the paths onto other sites, so that one had a better chance of discovering new habitats. The blocks lasted for at least a day and could be left unattended without fear of theft, and when the day's sampling was finished they could be left in the veld and did not cause any environmental damage. Periodically cattle would investigate the blocks of dry ice, but were careful not to get too close due to the very cold CO₂. The cattle probably made the traps more efficient because of the extra CO₂ they exhaled, but caused concern when they tried to eat the pheromone impregnated foam rubber/metal pegs. The most important advantage of these large CO₂ blocks was their ability to disperse high concentrations of CO₂ over a wide area and deep into the leaf litter.

There are few reports on the sustained capture of *A. hebraeum* adults in the wild. The most important reason for the failure to collect these ticks was probably a poor CO₂ source. It is imperative to have a strong source of CO₂, and to select sites carefully. If an area is sampled during the peak season of tick abundance (September to November), and no ticks are collected, then one can be reasonably sure there are no ticks in the area.

2.1.4.1.2 Pheromone source

When problems were experienced with the AAAP/CO₂ trap and no ticks were collected, it was necessary to examine various factors to determine where the problem was. The same CO₂ source was always used so this was ruled out. However, the pheromone used did vary from batch to batch, and this change brought about variability which needed to be tested under field conditions.

This new diethyl-ether pheromone extract was tested under field conditions at Rietgat and found to be effective, and subsequently was the only pheromone used for the remainder of the project at all six areas in South Africa. Initially in 1992 a few drops of pheromone extract were applied to small pieces of filter paper which were then placed on a twig or a small piece of a branch close to the CO₂. Later it was found that the pheromone on the paper did not last as the paper was unable to absorb sufficient pheromone, and could also easily be blown away by wind. For this reason the small foam rubber pieces collected from pill containers were preferred. These were attached to metal pegs and were more durable. They could also be dipped into diethyl-ether pheromone extract which had been poured into a plastic bucket. This dipping process made it easier to make up multiple pheromone sources at the same time, and also ensured that all the volatile diethyl-ether was absorbed by the foam rubber pieces.

2.1.4.1.3 Day length/Time of collection

The effect of day length and time of collection on tick emergence was difficult to monitor, and should be done under controlled laboratory conditions. The seasonal effect of increased or decreased day length must, however, play an important role in the emergence of adult *A. hebraeum*. This would be in association with temperature, rainfall, RH and other factors. When day length was at a minimum in South Africa (midwinter solstice in June) then no or very few ticks were collected. When day length was at its maximum (midsummer solstice in December) the ticks were far more active. When day length starts to increase in September/October there was a substantial increase in tick activity.

2.1.4.1.4 Temperature

The minimum and maximum temperatures recorded at Brits have already been discussed (section 2.1.2.3.3). It is difficult to assess the direct effect of temperature in isolation from other factors, especially day length and RH. However, when soil temperature and RH were monitored over a 72 hour collection period in the KNP, a relationship between the daytime temperature and the number of ticks collected per hour was evident (Fig. 34). Although trapping ticks in the KNP started at 06:00, few were collected till 08:00. Low temperatures and day length would also contribute to this effect. The rapid decline of temperature, followed by the almost total absence of *A. hebraeum* adults collected at Rietgat during winter could also be attributed to the decline in temperature and the frost associated with this season.

2.1.4.1.5 Rainfall

The seasonal variation in rainfall at the Brits meteorological station has already been discussed (section 2.1.2.3.2). It is apparent that there was an indirect effect on adult tick activity at Rietgat. During the cool, dry winter when no rain fell, there was virtually no adult tick activity. In September/October the first rains occurred and these coincided with an increase in adult tick activity making it the optimal time to collect ticks. These early rains resulted in a flush of vegetation which started to show its effect in January/February/March, resulting in poor visibility of the ticks. This was compounded by further good rains in January, February and March resulting in maximum vegetation cover in April.

2.1.4.1.6 Relative humidity

The seasonal variation in RH at Brits has already been discussed (section 2.1.2.3.4). Data in the KNP (Fig. 34) showed that the RH was initially high at 06:00 (70%). It then declined steadily throughout the day to a minimum of 10% at 14:00, and then began to rise again. No clear relationship between RH and the total number of ticks collected with the trap could be established. The higher RH's measured in summer (Fig. 10c) are probably more conducive to the survival of *A. hebraeum* than the much lower RH's in winter. The very low RH measured at the end of the dry season (August/September) made tick survival difficult, and as a consequence, they congregated in shaded, moist areas with deep leaf litter. During this period it was much easier to collect adult ticks as the traps could be concentrated in these areas.

2.1.4.1.7 Wind

Although wind speed was recorded at Brits, this data was not extrapolated to Rietgat. The wind index at the Rietgat CGA was recorded on each visit, but this area is not very windy and the wind had little effect on the use of the AAAP/CO₂ trap. When strong winds were present such as in East London, they would affect the functioning of the trap. The breezes which were common at the Rietgat CGA assisted the use of the trap, and by placing the trap upwind, the breeze would distribute the CO₂/pheromones over a much wider area and would reach downwind ticks.

2.1.4.1.8 Vegetation cover/leaf litter/shade

The seasonal changes in vegetation on the Rietgat CGA were dramatic, and a major effect was to substantially decrease the visibility of adult ticks. During the summer when the RH was high in most ecological areas on the CGA, adult ticks were able to migrate into the thick bush, and as a consequence, they were no longer visible or easy to trap. As the dry winter progressed, more and more of the CGA dried up.

2.1.4.1.9 Animal presence/kraals

Within its distribution range *A. hebraeum* will always be found in and around areas associated with domestic and wild animals. Kraals are particularly favoured and engorged female ticks detach and accumulate here. Other areas of high tick activity are water points and cattle/game tracks used on a regular basis. The presence of cattle or goat faeces can be

a good indicator of ticks, especially in the case of goats which rest under trees and deposit ticks and faeces in the area (Fig. 27).

2.1.5 CONCLUSIONS

The survey at the Rietgat CGA was the first to employ the AAAP/CO₂ trap on a sustained basis to collect adult *A. hebraeum*. A total of 1 196 adult ticks were collected with the trap at an average of 50 ticks per day on each visit. These results were most satisfactory as workers in Zimbabwe (Peter 1995), and in other parts of Africa (Hassan, pers. comm. 1997), have experienced difficulties in using the trap on a sustained basis, and collected only laboratory-reared ticks. The present survey has demonstrated that if specific parameters are met, the AAAP/CO₂ trap can be used successfully over a sustained period to monitor and collect adult and nymphal *A. hebraeum*.

The ecological factors which affect the efficacy of the trap make it clear that one cannot just go to an area and start collecting *A. hebraeum* adults. Visits should be undertaken on a regular basis throughout the year, and on each visit probable sites selected according to clearly defined parameters. It is imperative that a strong source of CO₂ be used and preferably in the form of large blocks of dry ice.

The pheromone must be extracted from male ticks from undipped cattle, and at least 200 feeding males should be used for the original stock. One of the most difficult and frustrating

tasks was to locate possible new free-living clusters of adult *A. hebraeum*. This requires a good field knowledge of the African bushveld, and a reasonable knowledge of the ecological requirements of the tick. Above all, it requires persistence, as frequently no adult ticks will be collected. It was important then to return on a monthly basis and to spend the entire day testing possible sites. At Rietgat, it is obviously preferable to go in September/October as there would be a much better chance of success. It is also advisable to periodically test the efficacy of the AAAP/CO₂ trap with laboratory-reared ticks.

It is difficult to conclude that there are no *A. hebraeum* ticks in an area, as was the case at Rietgat during the winter, where the ticks were present but were not attracted to the traps. Population dynamics data had, however, indicated that *A. hebraeum* were present on the goats and cattle throughout the year (Tice 1997), but that they were only attracted to the traps for a short period in late spring.

An important new use for the trap at Rietgat was the collection of nymphal *A. hebraeum*. Previous workers (Norval, Peter & Meltzer 1992) had suggested that *A. hebraeum* nymphs were attracted to the AAAP/CO₂ trap. This is the first time that large numbers of *A. hebraeum* nymphs have been collected in the field. These free-living, unfed ticks are going to be assayed by PCR to determine the prevalence of *C. ruminantium* organism in the nymphs.

2.2 THE PRODUCTION ANIMAL UNIT, MEDICAL UNIVERSITY OF SOUTHERN AFRICA (MEDUNSA) (SEMI-INTENSIVE GRAZING; MEDIUM TICK CONTROL)

2.2.1 INTRODUCTION

The campus of MEDUNSA was selected as one of the sites to test the AAAP/CO₂ trap because domestic animals grazing the camps had low numbers of *A. hebraeum*. In addition heartwater had been diagnosed in the indigenous goats in this region. Camps in which animals had a high heartwater mortality (Donkin pers. comm. 1998) were selected. Heartwater mortality in the Saanen goats kept on zero grazing at the Animal Production Unit also occurred periodically (Donkin pers. comm. 1998).

2.2.2 MATERIALS AND METHODS

2.2.2.1 Geographic location

MEDUNSA is 31 km north-west of Pretoria in the Gauteng Province of South Africa (Fig. 6).

2.2.2.2 Study area

The location of the study area is illustrated in Fig. 23. The three main sampling areas, camps A, B and C, were adjacent to the student residences (H), the sewage works (K) and the main entrance (A). The sampling sites are labelled 1 to 10.

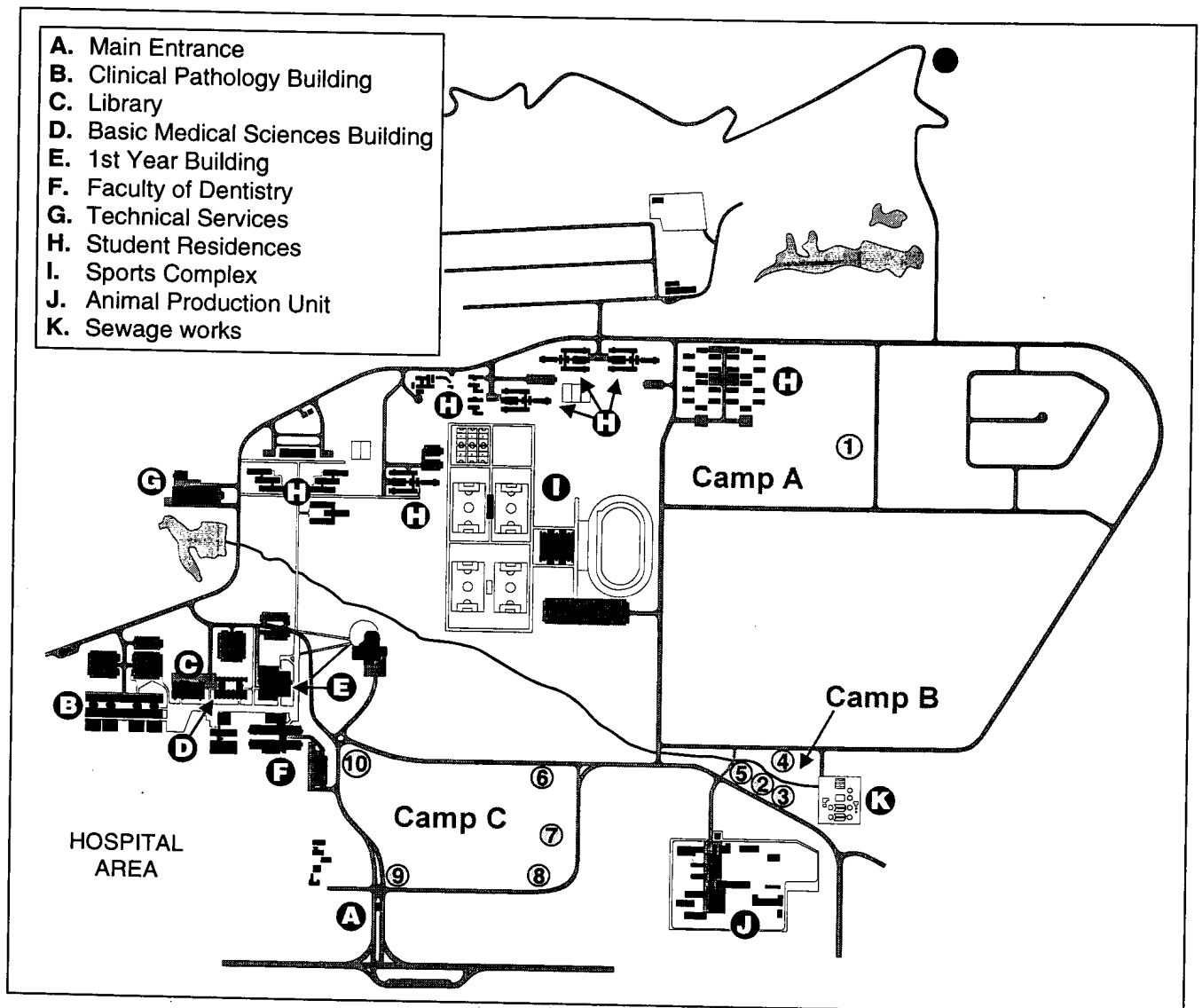


Fig. 23 The different camps at MEDUNSA (A - C), and sites (1 - 10) where the AAAP/CO₂ trap was used to collect ticks.

- Site 1 (Camp A): This site was adjacent to the student residences (H), and was chosen because Nguni cattle were grazing the camp. There was a dense invasion of *A. karoo*, making access to the camp difficult. The soil was the characteristic black loam (turf) (Fig. 24).



Fig. 24 Ticks were not common on the black loam (turf) found in certain camps at MEDUNSA.

- Sites 2 - 5: (Camp B): These sites were situated close to the sewage works (K) and the Animal Production Unit (J). A stream originating from the sewage works (K) crossed the camps to the dam. Invasion by *A. karoo* was evident, and indigenous goats were kept in this camp for most of the year.

- Sites 6 - 10: (Camp C): A large camp next to the main gate (A) which was inhabited by indigenous goats throughout the year and periodically grazed by cattle. Invasion by *A. karoo* was evident and *A. nilotica* and *A. robusta* were also present.

2.2.2.3 Climate

The climate at MEDUNSA was similar to that described for Brits. Soil temperature, the RH, vegetation index, wind index and day length index were all recorded. Recent rain fall was also noted (see Table 9).

2.2.2.4 Vegetation and fauna

All the camps were invaded by *A. karoo* and to a lesser extent *D. cinera*, *Acacia nilotica*, *A. caffra* and *A. robusta*. *Peltophorum africanum* and *Rhus lancea* were also present.



Fig. 25 Typical mixed *Acacia* spp. camps at the campus of MEDUNSA.

2.2.2.5 Livestock

The Nguni beef cattle of the Animal Production Unit grazed the various camps at different times of the year (Fig. 26 a/1). The indigenous goats also grazed camp C, and occasionally camp B (Fig. 26 a/2).

TABLE 9 *Amblyomma hebraeum* collected using the AAAP/CO₂ trap at the Production Unit, MEDUNSA, South Africa

VISIT NO.	COLLECTION DATE	AREA SAMPLED (MAP)	ADULT TICKS			NYMPHAL TICKS	VISITS PER MONTH	NO. OF TRAPS USED	AVERAGE TICKS PER DAY		**1 RAINFALL	**2 RH	**3 TICK ACTIVITY INDEX (0 - 5)	*4 VEGETATION COVER INDEX (0 - 5)	**5 TEMPERATURE	**6 WIND INDEX (0 - 5)	**7 DAY LENGTH INDEX (0 - 5)	TIME OF DAY	
			T	M	F				A	N									
1	1996-07-23	6, 7, 8, 9, 10	0	0	0	0	1	20	0	0	None	N/R	0	1	N/R	1	0	11:00 - 14:00	
2	1996-11-06	2	3	2	1	0	3	5	8	0	Yes	43	1	3	31°C	3	3		
		3, 4, 5	0	0	0	0		5	0	45		31°C							
TOTAL			3	2	1														
3	1996-11-14	6 & 7	0	0	0	0		60			Yes	65	1	3	26°C	2	3	09:00 - 13:35	
		8	10	6	4														0
		9	0	0	0														0
		10	1	0	1														0
		TOTAL			11														6
4	1996-11-15 NOVEMBER 1996	8	9	4	5	0		20		Ground wet - overcast	66	1	3	26°C	2	3	09:00 - 15:00		
		TOTAL			23													67	23°C
5	1997-11-21 NOVEMBER 1997	1	0	0	0	0	1	48	8	0	Yes	43	1	3	25°C	2	3	09:00 - 13:24	
		2	0	0	0							30			2				12:00
		3	0	0	0							15			2				13:24
		4	0	0	0														
		5	0	0	0														
		6	0	0	0														
		7	0	0	0														
		8	6	2	4														
		9	1	1	0														
		TOTAL			8							3			5				

RESULTS: 1. Total adult ticks collected = 31 2. Total days collecting = 5 3. Average adult ticks collected per day = 6

**1 RAINFALL	**2 RH (Relative Humidity)	**3 TICK ACTIVITY INDEX (0 - 5)	**4 VEGETATION COVER INDEX (0 - 5)	**5 TEMPERATURE	**6 WIND INDEX	**7 DAY LENGTH INDEX (0 - 5)
Recent rains Average monthly rainfall recorded at the nearest meteorological station	Measured on the ground in full shade	0 = Zero tick activity 1 = A few ticks present 2 = Some activity - low numbers 3 = Medium tick activity 4 = Ticks active 5 = Ticks very active	0 = None 1 = Little 2 = Some 3 = Medium 4 = Good 5 = Dense	Measured on the ground in full shade. Minimum (min.) and maximum (max.) also recorded if possible	0 = Zero winds 1 = Slight breezes 2 = Medium breezes 3 = Windy day 4 = Strong winds 5 = Heavy, gale force	0 = Minimum 2.5 = Intermediate 5 = Maximum

T = Total M = Male F = Female A = Adult N = Nymphal 0 = No ticks collected NR = Not recorded

BLANK SQUARES = Negative



a/1



a/2

Fig. 26 Nguni cattle (a/1) and indigenous goats (a/2) were grazing on the campus at MEDUNSA.



Fig. 27 *A. hebraeum* was more common in areas where goat droppings accumulated.

2.2.2.6 Sample size determination

The same statistical approach as that used at Rietgat was followed at MEDUNSA to calculate the sample size.

2.2.2.7 Collection of ticks with the AAAP/CO₂ trap

In order to collect *A. hebraeum* adults, five visits were made to the camps at MEDUNSA. During this period 158 traps were used at ten sample sites. The same sampling procedure as that at Rietgat was followed, using multiple blocks of dry ice surrounded by pheromone impregnated foam rubber pegs, and moved around until tick activity was observed. Sampling started at 09:00 and usually ended by mid-afternoon.

2.2.3 RESULTS

Only 31 ticks in total were collected during five sampling days giving an average of six adult ticks per day. This was the lowest figure for all of the sites sampled (Table 9, Fig. 20) and represented only 2% of all the *A. hebraeum* collected with the traps (Fig. 19a). No nymphal ticks were collected (Fig. 19b), but eight *R. simus* adults and two *R. evertsi evertsi* males were attracted to the traps (Table 7).

2.2.4 DISCUSSION

At the MEDUNSA collection site, *A. hebraeum* adults were not common either on domestic animals, (Donkin pers. comm. 1998) or in the veld, as indicated by the poor response to the AAAP/CO₂ trap. No nymphal *A. hebraeum* were found in any of the camps, although this tick occurred on goats in these camps (Donkin pers. comm. 1998). There were considerable differences between tick activity here and at Rietgat or the KNP. Tick activity is difficult to compare, especially if the comparisons are between different areas, different times of the year and different times of the day. In an attempt to assess tick activity subjectively, a tick activity index was produced (see Table 9). Tick activity ranged from no activity (0) to very

active (5). At Rietgat and the KNP the index measured five on several occasions, but at MEDUNSA activity ranged between 0 and 1. It was a difficult place to work, as the veld had been invaded by numerous *Acacias*, and after six or seven hours of collection in the midsummer sun, no or very few ticks had been collected. It was hoped to calculate the prevalence of *C. ruminantium* in the ticks but this was not possible.

An average of only six adult ticks were collected per visit (Fig. 20). Most of these were from a single sampling site in the shade of a large *A. nilotica* tree, and where goats were common, as indicated by piles of their faeces (Fig. 27). Most of the other areas sampled had a characteristic black loam (turf) (Fig. 24), which is a clay-like soil. In summer the turf got wet and it was difficult to walk on. The black colouration of the soil made the visibility of the ticks much more difficult. These areas were also closely associated with *A. karoo* and *A. nilotica*.

2.2.5 CONCLUSIONS

When there was a high concentration of free-living, unfed adult *A. hebraeum* in the field such as at Rietgat, then the response was good. In a low activity area at MEDUNSA, however, the tick response was always poor and despite many hours of trapping very few ticks were collected. As the trap was concurrently being used successfully at Rietgat, there were obviously no inherent problems with the trap design, but rather a very low population of adult ticks at MEDUNSA. The tick control management at MEDUNSA, which included regular acaricide application to cattle and goats, must also have reduced the population. The Nguni cattle were also seldom infested with large numbers of ticks.

2.3 DR L VAN DER MERWE'S FARM (LUDLOW) NEAR WARMBATHS,
NORTHERN PROVINCE, SOUTH AFRICA - COMMERCIAL
BUSHVELD FARM (GOOD TICK MANAGEMENT)

2.3.1 INTRODUCTION

This farm was chosen as a representative of a typical, well-managed bushveld cattle farm with a heartwater problem. A preliminary visit indicated that *A. hebraeum* was present on the farm and that pour-on acaricides were regularly used to control ticks. The farm was owned and managed by Dr Lente van der Merwe, a veterinarian with a particular interest in heartwater. Dr van der Merwe has published on the clinical symptoms of heartwater and done extensive vaccination trial work. Her farm was also selected by the U.F./USAID/SADC Heartwater Project in Zimbabwe to test a new, inactivated heartwater vaccine (Mahan 1997). It was therefore important that live, adult ticks be caught, so that the strain of *C. ruminantium* causing heartwater in this area could be characterised.

2.3.2 MATERIALS AND METHODS

2.3.2.1 Geographical location

The location of this farm is illustrated in Fig. 6. The farm comprises 675 hectares of land and is essentially a cattle/grain farm. It lies 18 km south-east of the town of Warmbaths (25°10' South; 28°20' East) and close to the village of Settlers.

2.3.2.2 Study area

A detailed map, supplied by Dr van der Merwe, is reproduced in Fig. 28.

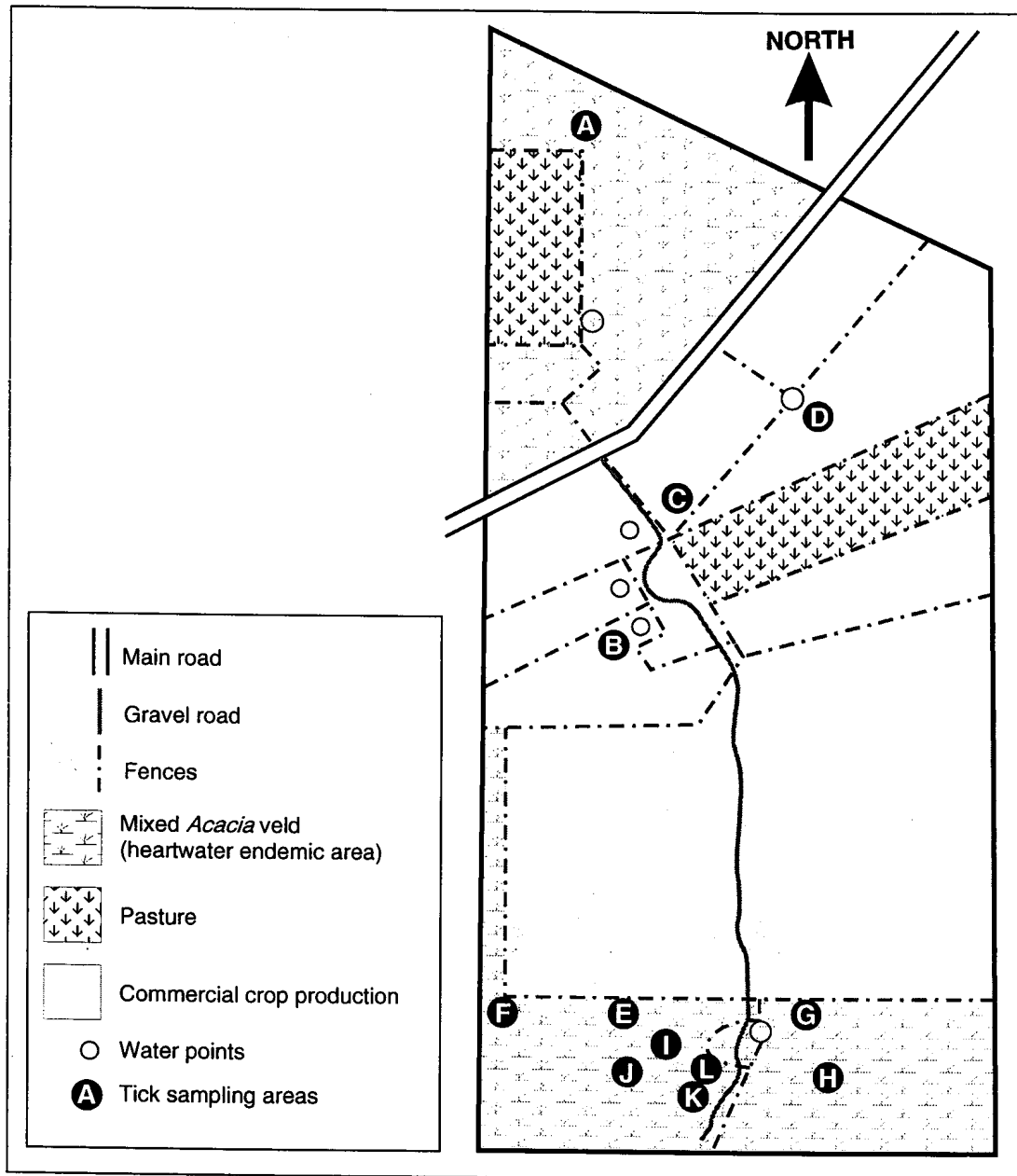


Fig. 28 Diagrammatic layout of the farm "Ludlow", near Warmbaths, Northern Province, South Africa.

■ Tick sampling sites

The farm is transected by the Warmbaths-Settlers road and the first sampling site (A) was on the northern border of the farm, in the mixed *Acacia* veld and close to a well-used cattle kraal. Site B was located in a camp adjacent to the main homestead. Sites C and D were in camps just north-east of the homestead and in dense, mixed *Acacia* veld. Bonsmara bulls were also kept in these paddocks. Sites E to K were in the mixed *Acacia* veld (Fig. 28) in the southern most camp on the farm. Most of the Bonsmara stud cattle grazed in this vicinity, and a well-used cattle kraal, crush and water point were operating at the time of sampling. Sites E to K were all similar type sites, usually in the shade of *A. karoo*, *A. nilotica* and *A. tortillis* which were abundant in these camps. Many cattle tracks were visible in the grass veld and the black turf soil was similar to that found at MEDUNSA.

2.3.2.3 Climate

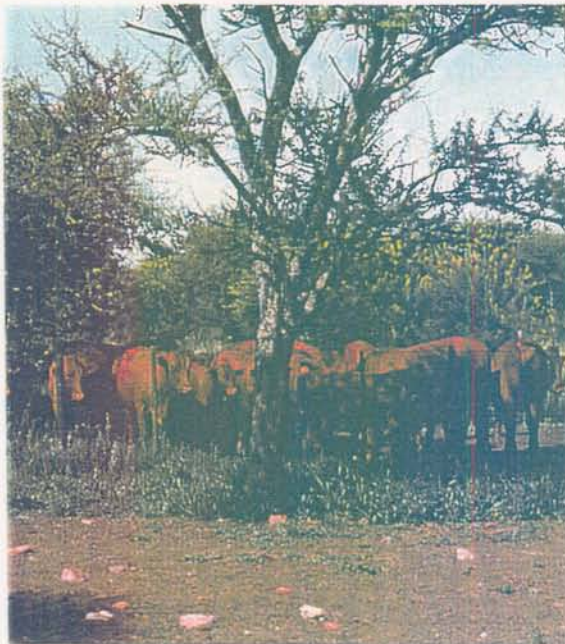
The climate at Warmbaths was similar to that at Brits, and detailed climatic data were not recorded as only five visits were made. The average annual rainfall recorded at Ludlow was 562 mm and this was mainly in summer. On each visit to the farm the RH and soil temperature were measured. The occurrence of recent rain was also noted and wind and day length indices estimated.

2.3.2.4 Vegetation

The vegetation consisted of mixed bushveld and cultivated lands. The mixed bushveld comprised *Acacia* species typical for this area, and was in many ways similar to that at MEDUNSA with *A. karoo* invasion on black turf (Fig. 29a, b and c).



(a)



(b)



(c)

Fig. 29a) Typical black turfveld at "Ludlow", with *A. nilotica*, *A. tortillis* and *A. karoo* the most dominant.

b) Bonsmara stud cattle bred on the farm were fairly resistant to the common TBD.

c) Collection of *A. hebraeum* at the kraal region of "Ludlow" farm, the black loam is clearly visible

2.3.2.5 **Livestock**

The farm is a registered Bonsmara stud farm and the bulls were kept close to the homestead, and the rest of the herd grazed on the mixed *Acacia* bushveld and old cultivated lands. The cattle were treated with a pour-on acaricide when tick levels were considered excessive.

2.3.2.6 **Sample size determination**

The same statistical approach to the sample size determination as that at Rietgat was used at Warmbaths.

2.3.2.7 **Collection of ticks**

2.3.2.7.1 Use of the AAAP/CO₂ trap

The AAAP/CO₂ trap was used on two separate visits to Warmbaths in September and November 1997. On the first visit sites (E - L), all in the southern section of the farm, were sampled. All these sites were close to the main cattle kraal and watering point in this region (Fig. 29b), and where the black turf veld had been invaded by *A. nilotica*, *A. tortillis* and *A. karoo* the dominant tree species (Fig. 29a). Leaf litter on the turf and *Acacia* veld was limited.

On the second visit to this farm, the same sites (E - L) as previously were sampled, but other sites were also sampled including some near the homestead (B, C and D) and in the northern areas (A) of the farm.

TABLE 10 Ticks collected on Dr L van der Merwe's farm "Ludlow", near Settlers, South Africa

VISIT NO.	COLLECTION DATE	AREA SAMPLED (MAP)	ADULT TICKS			NYMPHAL TICKS	VISITS PER MONTH	NO. OF TRAPS USED	AVERAGE TICKS PER DAY		**1	**2	**3	**4	**5	**6	**7	TIME OF DAY
			T	M	F				A	N	RAIN-FALL	RH	TICK ACTIVITY INDEX (0 - 5)	VEGETATION COVER INDEX (0 - 5)	TEMPERATURE	WIND INDEX (0 - 5)	DAY LENGTH INDEX (0 - 5)	
1	1997-06-17										NO	N/R						
2	1997-09-09	E F G H I J K L TOTAL	0 0 3 0 0 0 19 0 22	0 0 1 0 0 0 13 0 14	0 0 2 0 0 0 6 0 8	0 0 0 0 0 0 0 0 0	1	8 8 8 8 8	2 2	0	No	80 80	2	1	20°C 31°C	2	2	09:40 - 14:00
3	1997-11-12	G H I J K L B C D A TOTAL	0 0 0 0 2 0 0 0 0 1 3	0 0 0 0 0 0 0 0 0 1 1	0 0 0 0 2 0 0 0 0 0 0	0 0 0 0 0 0 0 0 0 0 0	1	4 4 4 4 4 4 4 4 4 4	3	0	Over-cast Over-cast	63 62 62	0 0 0 0 1 0 0 0 1	4	26°C 20°C 31°C	1 1	3	09:00 11:00 12:30
4	1997-12-11	*242 adult ticks (mostly male) collected from 30 cattle *Sent to Dr Mahan, Zimbabwe																
5	1998-01-21	Visited Dr Van der Merwe's farm with Dr Mahan, to discuss details of the Heartwater trial																
RESULTS:																		
1. Total adult ticks collected = 25 2. Total days collecting = 2 3. Average adult ticks collected per day = 12,5																		

**1 RAINFALL	**2 RH (Relative Humidity)	**3 TICK ACTIVITY INDEX (0-5)	**4 VEGETATION COVER INDEX (0-5)	**5 TEMPERATURE	**6 WIND INDEX	**7 DAY LENGTH INDEX (0-5)
Presence of recent rains noted. Average monthly rainfall recorded at the nearest meteorological station	Measured on the ground in full shade	0 = Zero tick activity 1 = A few ticks present 2 = Some activity - low numbers 3 = Medium tick activity 4 = Ticks active 5 = Ticks very active	0 = None 1 = Little 2 = Some 3 = Medium 4 = Good 5 = Dense	Measured on the ground in full shade. Minimum (min.) and maximum (max.) also recorded if possible	0 = Zero winds 1 = Slight breezes 2 = Medium breezes 3 = Windy day 4 = Strong winds 5 = Heavy, gale force	0 = Minimum 2,5 = Intermediate 5 = Maximum

T = Total M = Male F = Female A = Adult N = Nymphal 0 = No ticks collected N/R = Not recorded BLANK SQUARES = Negative

2.3.2.7.2 Collection of ticks off cattle

Because of the lack of success in collecting adult ticks at Ludlow farm using the trap, adult *A. hebraeum* were eventually collected from cattle on the fourth visit (Table 10). These ticks were sent to Zimbabwe for characterisation of the local *C. ruminantium* strain.

2.3.3 RESULTS

The results of the visits to Ludlow are summarized in Table 10. A total of 25 adult ticks were collected during two separate visits. An average of 12.5 ticks per visit and no nymphal ticks were collected.

2.3.4 DISCUSSION

It was important for the characterisation of the heartwater strains that a statistically relevant field sample of free-living, unfed ticks be caught with the AAAP/CO₂ trap. However, when two days of trapping involving 12 trapping sites, resulted in only 25 ticks, of which 21 (84%) were from one site (K), it was decided that the trap was not successful in attracting *A. hebraeum* adults and nymphs on this farm. In order to obtain a sample of adult ticks, the cattle were put into a crush (Fig. 29c), and ticks were removed manually. From discussions with Dr van der Merwe and observation of the cattle, it was obvious that medium to heavy burdens of *A. hebraeum* were in fact, present on the farm.

If the different areas where the AAAP/CO₂ trap was used are compared (Fig. 19a & 20), then only 1% of the total ticks were collected at Warmbaths and no nymphs (Fig. 19b). The average daily collection of adult *A. hebraeum* (Fig. 20) at Warmbaths was the second lowest of the six areas sampled.

A number of reasons probably resulted in the poor tick catch at Ludlow farm. Firstly the good management here, coupled with the ongoing pour-on tick control, undoubtedly resulted in decreased ticks in the veld. The landscape type, *A. karoo*/black turf, was similar to that at MEDUNSA and in both areas tick collecting was disappointing.

2.3.5 CONCLUSIONS

The trap can only work optimally if there are medium to high densities of ticks in the area. If one is working on a well-managed farm with good tick control, then there are going to be fewer free-living, unfed adult and nymphal ticks in the veld. Thus it will be more difficult to collect ticks in any significant numbers.

2.4 THE SOUTH AFRICAN BUREAU OF STANDARD'S (SABS) EXPERIMENTAL FARM AT EAST LONDON, EASTERN CAPE PROVINCE, SOUTH AFRICA (MEDIUM TO GOOD TICK CONTROL; TICKS SEEDED)

2.4.1 INTRODUCTION

The Eastern Cape Province of South Africa's coastal zone has a very high population of *A. hebraeum*. The SABS farm at East London was at first chosen to test the AAAP/CO₂ trap primarily to trap ticks to test the new inactivated UF/USAID/SADC heartwater vaccine, and was also ideally suited due to the ongoing tick experiments on the property. Heartwater is an enormous problem for Angora goat farmers in the region.

2.4.2 MATERIALS AND METHODS

2.4.2.1 Geographical location

East London is situated on the south-eastern coastline of South Africa and is 1 100 km from Pretoria (Fig. 6). The exact geographical position is 25°50' - 33°10'. The farm is set amongst coastal vegetation and within view of the Indian Ocean.

2.4.2.2 Study area

The layout of “Little Go” farm is illustrated in Fig. 30. Most of the farm was typical Eastern Cape coastal vegetation but large areas were under pastures. The main gate was at the north-western end of the farm, and the main buildings of the farm where the crush and all the cattle handling facilities were located, were at the southern end of the farm (homestead).

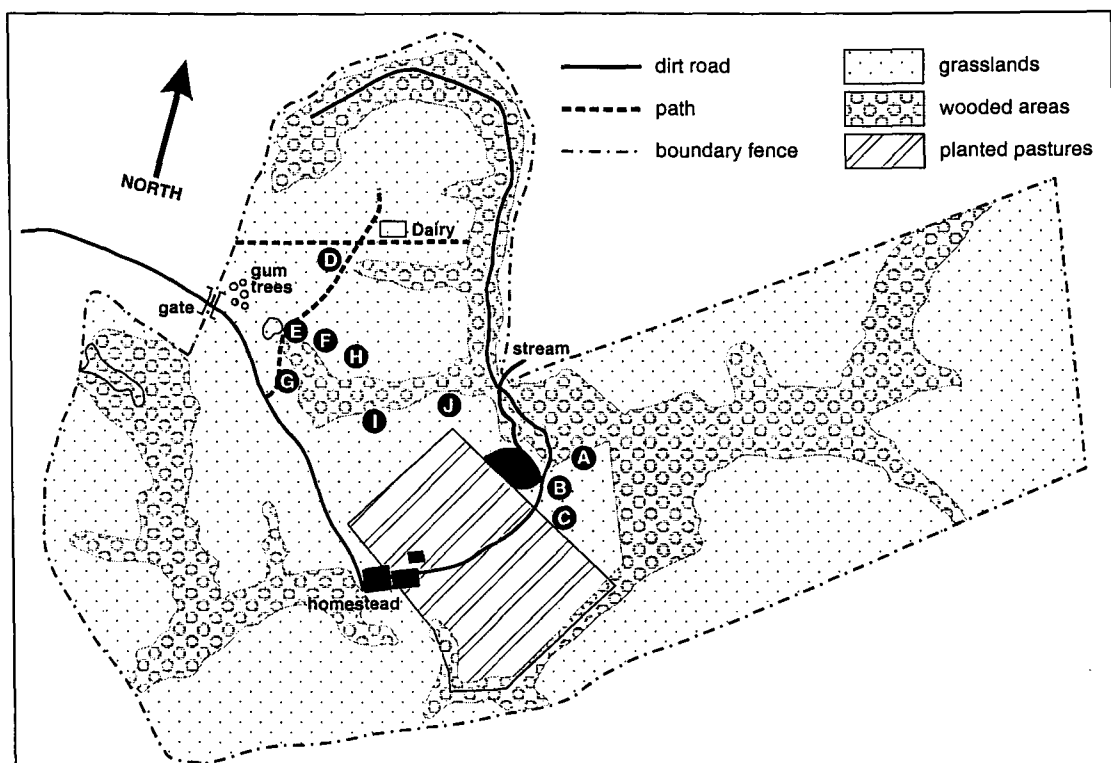


Fig. 30 Diagrammatic representation of the SABS farm, “Little Go”, near East London, Eastern Cape Province, South Africa. The AAAP/CO₂ trap was used at sites A to J.

2.4.2.3 **Climate**

As the East London farm was only visited once for a period of four days during December 1997, no detailed climatic data were collected. However, East London is midway between the humid, tropical East Coast and the temperate Mediterranean type climate of the West Coast of South Africa. Rainfall is mainly during summer and the climate in summer is hot and humid. Strong coastal winds may occur. A heavy, gale force wind (5, wind index, Table 11) was blowing during two of the three days' field collection trips. These winds reduce the summer temperatures and a maximum of 29°C was measured in December 1997.

2.4.2.4 **Vegetation and fauna**

The SABS farm lies within a few kilometres of the Indian Ocean and obviously this has dramatic effects on the vegetation. The vegetation is described by Acocks (1988) as coastal forest and thorn veld. Although most of the coastal forest (Fig. 31a) had been removed, gullies and valleys containing original coastal forest still remained. *A. karoo* and other *Acacias* are invading this region and parts of the farm now correspond to Acocks type 7 vegetation (Fig. 31b) (Eastern Cape Province thornveld). The grasses were dense and sourish mixed species (Fig. 31) (Acocks 1988).

(a)



(b)



Fig. 31a) Typical coastal vegetation where ticks were collected at the SABS farm near East London.

b) The AAAP/CO₂ traps were placed on a path next to a clump of coastal vegetation along which ticks were successfully collected at the SABS farm, East London.

2.4.2.5 Livestock

The species of domestic animals on the experimental farm varied according to the needs of the trials in operation. Beef and dairy cattle, Angora goats and dogs (Beagles) were present during this trial period.

2.4.2.6 Sample size determination

The same statistical approach to sample size determination was carried out as at Rietgat CGA.

2.4.2.7 Collection of ticks

2.4.2.7.1 With the AAAP/CO₂ trap

The collection method used at East London had to be adapted to the facilities available. No dry ice blocks were available, so small quantities of chipped dry ice were used (Afrox, East London) and placed in muslin bags (± 1 kg in total), otherwise the same method and pheromone extract was used as at Rietgat.

Day 1: Tick collecting took place only during the afternoon as it was really only an exploratory exercise. Twenty-four traps were placed in various areas along the road adjacent to the dam (Fig. 30, sites A, B and C). The vegetation was dense coastal forest invaded by *A. karoo* and associated with a stream. The tick activity index in this area was only one, and only two *A. hebraeum* adults were collected. As this field work was designed to assist the UF/USAID/SADC Heartwater project to determine the field strain of heartwater present, it was imperative that more ticks be collected during the four-day visit.

Day 2: After discussions with the SABS personnel and a neighbouring farmer, it was decided to try another area where the cattle apparently pick up many ticks. This area was near the main gate (Fig. 30), and lay between the road and the dairy. This site contained two gullies of deep, coastal forests (Fig. 31b), surrounded by grasslands and two paths ran through it, one to the dairy and another to the dam.

Initial collection (Fig. 31b) started deep in the shade of the coastal forest and soon ticks were being collected by all three collectors. As the tick numbers started to decline it was necessary to move to the periphery of the coastal forest, where once again good tick activity was found (Fig. 31b, Fig. 30 sites F and H). After this the traps were moved onto the paths and into the open (Fig. 30 sites E and G), and eventually when tick activity started to decline here the traps were moved into the open grasslands and again good tick activity was found (Fig. 30 sites I and J).

Day 3: Similar sites to that on Day 2 were sampled. The wind on both days 2 and 3 was described as gale force but appeared not to affect the efficiency of the trap. Trapping occurred throughout the day from 08:00 until 18:00 on both days (Table 11).

2.4.2.7.2 Ticks collected off cattle

A. hebraeum adults (n = 268) were collected off cattle on day 4 (1997-12-18) and these ticks were sent by courier to Zimbabwe for characterisation of the strain of *C. ruminantium*. A sample of these ticks was later fed on heartwater-susceptible sheep at the laboratory in Harare.

2.4.3 RESULTS

A total of 187 adult ticks were collected using the AAAP/CO₂ trap during the three days collecting at the SABS farm at East London (average of 62 adult ticks per day). Seventeen nymphs were also collected during the visit. Certain ecological sites (Fig. 30 D, E, F, G and H) yielded far more ticks than others (A, B, C, I and J).

2.4.4 DISCUSSION

This project was a good example of how the AAAP/CO₂ trap had to be adapted to local conditions. Initially it was thought that there were very few adult *A. hebraeum* on the farm. However, because of the flexibility of the AAAP/CO₂, which could be moved over large areas of the farm, good tick collection areas were eventually located. The traps were then concentrated in these areas. The pockets of coastal forest in the grasslands, were found to be the best tick collecting sites, and by moving the traps quickly, and by having three operators, a wide variety of habitats could be covered.

The 187 adult ticks collected by the trap at the SABS farm at East London represented 9.6% of the total number of ticks collected during the whole study (Table 8, Fig. 19a). The 17 nymphs represented 5% of the total nymphs caught (Fig. 19b). The average, daily collection of adult ticks at the SABS farm, East London (Fig. 20) (n = 62) was considerably more than at MEDUNSA (n = 6) or Warmbaths (n = 12).

2.4.5 CONCLUSIONS

The sample of nearly 200 ticks collected on a well-managed, mixed farm, demonstrated that the AAAP/CO₂ trap can be used to locate and collect an adequate sample of free-living, unfed *A. hebraeum* if the ticks are present.

2.5 SKUKUZA REST CAMP, KRUGER NATIONAL PARK MPUMALANGA PROVINCE, SOUTH AFRICAN GAME RESERVE (NO TICK CONTROL)

2.5.1 INTRODUCTION

The KNP was chosen as one of the six sites to test the AAAP/CO₂ trap because no form of tick control had been applied in the area for many decades. Wild and domestic animals had also been kept apart for almost a century, especially since the foot-and-mouth fencing had been established. The KNP was also one of those areas where the influence of man and domestic animals was minimal. This area was possibly the closest to the original Africa, before modern man, cattle and goats arrived. It was presumed that if *C. ruminantium* was present, it circulated only in the wild animals and the ticks. The main objective of this study was to collect free-living, unfed *A. hebraeum* which had not fed on domestic animals and determine the prevalence of *C. ruminantium* in these ticks.

2.5.2 MATERIALS AND METHODS

2.5.2.1 Geographical location

The KNP lies along the easterly border of South Africa with Mocambique, and in the north with Zimbabwe (Fig. 6). The Skukuza Rest Camp (25°00' - 31° 35'), is 482 km north-east of Pretoria and all the tick collecting was done around this camp.

2.5.2.2 Study area

The trapping area around the Skukuza Rest Camp is illustrated in Fig. 32. This camp is located between the Sabie and the Nwashitsaka rivers, and much of the field work was conducted around the Nwashitsaka Research Guest Camp.

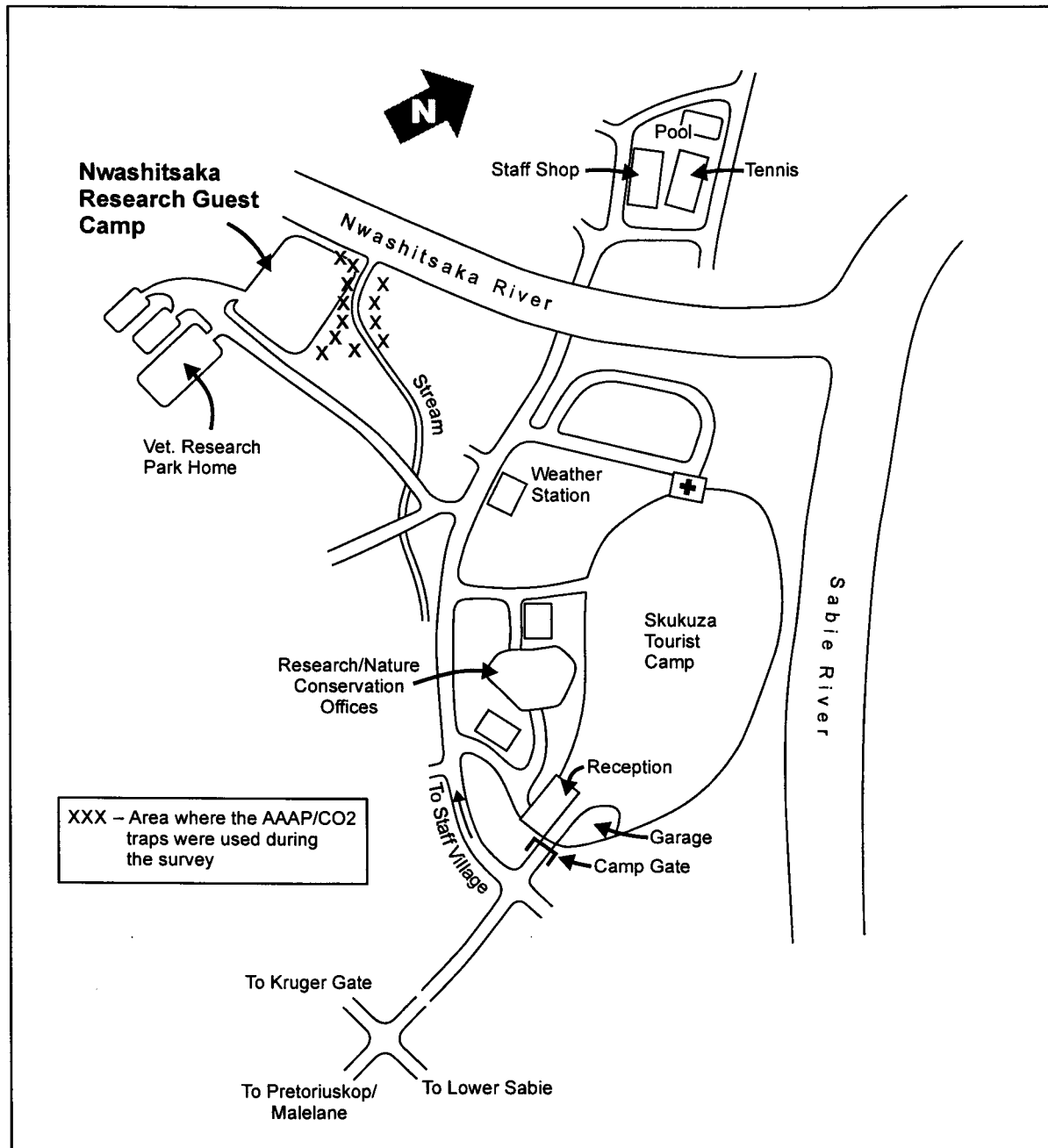


Fig. 32 Map of the Skukuza region in the Kruger National Park illustrating the Nwashitsaka Guest Camp where most of the AAAP/CO₂ traps were used.

2.5.2.3 Climate

The climate was dry and tropical rains normally occur from November to March (average total 561 mm per annum) with the cool period from April to September. The maximum temperatures in midsummer were in the lower thirties and the minimum temperatures in winter between 5°C and 8°C with no frost. The maximum RH in summer was in the upper 90's and the minimum in winter in the lower 20's. The main climatic problem in the KNP is the unpredictability of the rains and this often results in serious droughts, such as the 1998 drought in the northern part of the KNP. During these drought periods many of the rivers dry up and the wildlife is forced to use borehole water.

2.5.2.4 Vegetation and fauna

Gertenbach (1983) described the specific landscape type in the Skukuza area of the KNP as mixed Combretum and Terminalia woodland (type 4). He also indicated that detailed studies on the climate, soils, vegetation and animal life in the KNP have recently been published. This data base has made it possible to zonate the KNP into landscapes.

2.5.2.5 Livestock

There are no domestic livestock in the KNP, and this has been the situation for many decades. This is the result of establishing game-proof fencing to stop the movement of wild animals into surrounding cattle ranches and thus the spread of foot-and-mouth disease. The buffalo (*Syncerus caffer*) herds in the KNP are infected with the foot-and-mouth disease virus.

2.5.2.6 The use of the AAAP/CO₂ trap

2.5.2.6.1 Collection of ticks

The AAAP/CO₂ trap was used on two separate occasions at the Skukuza Rest Camp in the KNP. The first visit in December 1992 was a preliminary trip to test the trap, using Dr Tice's original pheromone solution and small pieces of dry ice, and the trap was used in a gully adjacent to the Nwashitsaka Research Guest Camp (Fig. 32 and 33).



Fig. 33 The gully adjacent to the Nwashitsaka Research Guest Camp at Skukuza, KNP, South Africa, where the ticks were collected.

(Acknowledgement Prof. I G Horak).

The test site was surrounded by various bushveld trees including *Dichrostachys cinera*, *Grewia* spp. and many tamboti (*Spirostachys africana*). There was a good leaf litter under the shade of these trees and various wild animals were seen in the vicinity and game tracks and faeces were common.

On the second visit to the KNP in September 1996, a number of 5½ kg blocks of dry ice were taken along and stored in the -70°C freezer of the State Veterinary office, and were used when required. The same gully, adjacent to the Research Camp (Fig. 32 and 33), near the Nwashitsaka river was again sampled, but this time with a new pheromone solution and with 5½ kg blocks of dry ice. Other possible areas in and around the Skukuza Rest Camp were also sampled with the assistance of a Pretoria Technikon student, Mr Duncan MacFadyen. All the ecological vegetation types tested were within easy reach of the Skukuza Rest Camp.

2.5.2.6.2 Soil temperature and RH measurement

On 16 September 1997 the RH and soil temperature were monitored in the gully adjacent to the Nwashitsaka Research Guest Camp. The objective of this experiment was to determine whether a relationship existed between the soil temperature/RH and the collection of free-living, unfed adult *A. hebraeum*. These parameters were measured with the thermohygrometer used at Rietgat (Fig. 9a), and recorded in full shade and sometimes semi-shade.

2.5.3 RESULTS

2.5.3.1 Collection of ticks

Four hundred and forty-seven ticks were collected in the KNP over a four-day period, with an average daily collection of 112 ticks per day. *A. hebraeum* nymphs were not collected at the KNP during the survey.

TABLE 12 *Amblyomma hebraeum* collected at Skukuza, Kruger National Park, South Africa, using the AAAP/CO₂ trap

VISIT NO.	COLLECTION DATE	AREA SAMPLED (MAP)	ADULT TICKS			NYMPHAL TICKS	NO. OF VISITS PER MONTH	NO. OF TRAPS USED	AVERAGE TICKS PER DAY		**1 RAIN-FALL	**2 RH	**3 TICK ACTIVITY INDEX (0 - 5)	**4 VEGETATION COVER INDEX (0 - 5)	**5 TEMPERATURE (FULL SHADE)	*6 WIND INDEX (0 - 5)	**7 DAY LENGTH INDEX	TIME OF DAY
			T	M	F				A	N								
1	1992-12-06	Skukuza Scientific Camp Site (SSCS)	88	45	43	0	1	10	88	0	Yes	N/R	5	4	N/R	1	5	09:00 - 16:00
2	1997-09-15	SSCS	110	42	68	0	3	30	110	0	No	N/R	5	3	N/R	2	2	15:00 - 17:00
3	1997-09-16	SSCS	112	51	61	0	3	30	112	0	No	Max. 75% Min. + 10%	5	3	Min. 13°C Max. 51°C	1	2	
4	1997-09-17	Tamboi Riverbed	42	22	20	0	3	30	137	0	No	N/R	5	3	N/R	1	2	09:00 - 15:00
5	1997-09-17	Fishing hole	95	38	57	0	3	30	0	0	No	N/R	5	3	N/R	1	2	N/R
	SEPTEMBER	TOTAL	447	198	249	0												

RESULTS: 1. Total adult ticks collected = **447** 2. Total days of collecting = **4** 3. Average ticks collected per day = **112**

**1 RAINFALL	**2 RH (Relative Humidity)	**3 TICK ACTIVITY INDEX (0 - 5)	**4 VEGETATION COVER INDEX (0 - 5)	**5 TEMPERATURE	**6 WIND INDEX	**7 DAY LENGTH INDEX (0 - 5)
Presence of recent rains noted. Average monthly rainfall recorded at the nearest meteorological station	Measured on the ground in full shade	0 = Zero tick activity 1 = A few ticks present 2 = Some activity - low numbers 3 = Medium tick activity 4 = Ticks active 5 = Ticks very active	0 = None 1 = Little 2 = Some 3 = Medium 4 = Good 5 = Dense	Measured on the ground in full shade. Minimum (min.) and maximum (max.) also recorded if possible	0 = Zero winds 1 = Slight breezes 2 = Medium breezes 3 = Windy day 4 = Strong winds 5 = Heavy, gale force	0 = Minimum 2.5 = Intermediate 5 = Maximum

T = Total M = Male F = Female A = Adult N = Nymphal 0 = No ticks collected N/R = Not recorded

2.5.3.2 Temperature and relative humidity, and its possible effect on tick collection with the AAAP/CO₂ trap

Trapping of ticks on the second day (1997-09-17) started at 06:00 and the soil temperature (T°C) and the RH were monitored throughout the day until 17:00 (Fig. 34). At 06:00 the T°C was relatively low (15°C) and no ticks were collected. As the day progressed and the T°C increased to over 20°C (07:00), the ticks then began to respond to the trap. From 09:00 the ticks really started to become active and this continued throughout the day with a peak in the early to late afternoon (14:00 - 16:00). The RH started at a high of 75% (06:00), but declined steadily throughout the day to reach a minimum of 10% at 14:00 and then slowly increased to 40% at 17:00.

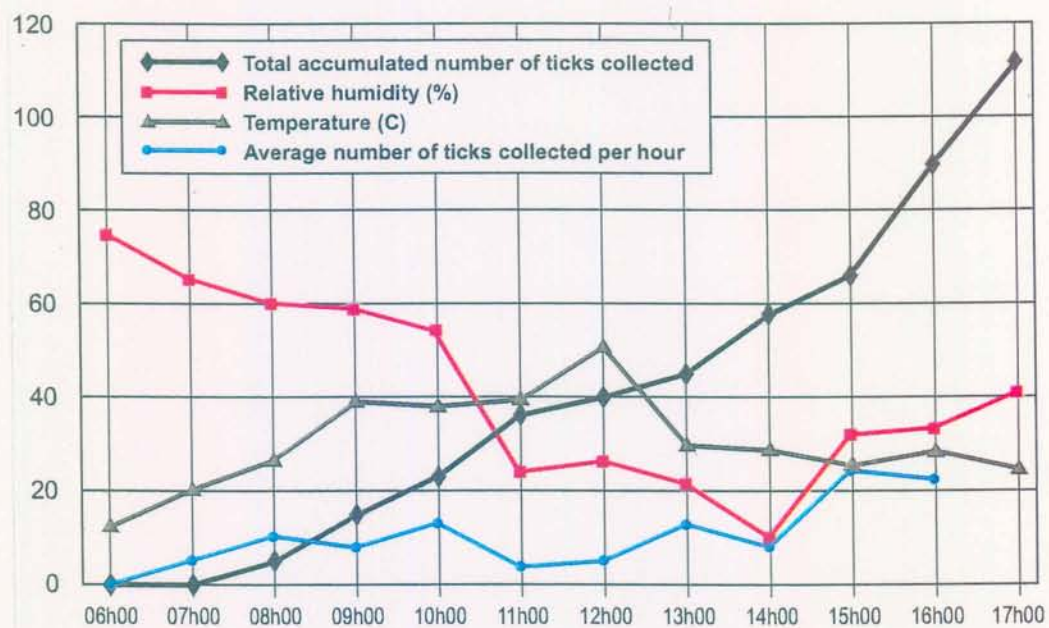


Fig. 34 Total accumulated adult *A. hebraeum* (—◆—) collected with the AAAP/CO₂ trap during one day of collection (1997-09-17) at the Nwashitsaka Research Guest Camp, Skukuza, KNP, South Africa. The average ticks collected per hour (—●—), relative humidity (—■—) and temperature (—▲—) were also recorded.

2.5.4 DISCUSSION

2.5.4.1 Ticks collected

The 447 ticks collected in the KNP comprised 23% of the total number of ticks collected in the study (Table 8, Fig. 19a). No nymphal ticks were collected (Fig. 19b), and the average, daily collection of 112 adult ticks was almost twice that of any other area sampled (Fig. 20). The KNP had the highest density of *A. hebraeum* adults of the six localities sampled (Fig. 20). The AAAP/CO₂ was successfully used in a number of different ecological areas in the KNP, having high tick activity. The tick activity index (Table 12) in all these areas and on each of the collecting days was a maximum of five. This indicated a high population of free-living, unfed *A. hebraeum* in the KNP. Certainly this would confirm the findings of Horak (1995), who found the *A. hebraeum* to be a common tick on many of the KNP wild animals. Spickett *et al.* (1991) also found that free-living *A. hebraeum* larvae were the most prevalent larval species in the KNP. However, a more intensive study needs to be done to locate *A. hebraeum* nymphs in the KNP, which were not detected by drag-sampling or AAAP/CO₂ trapping.

2.5.4.2 Temperature and relative humidity measurements

There appeared to be a direct relationship between the soil T°C and the activity of the ticks. Certainly early in the morning (06:00) there was no tick activity, and this only improved after 09:00 when the tick activity increased substantially. Certainly one can conclude that field work with these ticks should not start until after 09:00, as little tick activity was present before this. The effect of RH on tick collection was more difficult to ascertain, but there appears to be no direct relationship.

2.5.5 CONCLUSIONS

The KNP was an easy place to collect *A. hebraeum* adults with the AAAP/CO₂ trap probably as a result of a high population of ticks due to ideal climatic conditions, no acaricidal control and an abundance of wild animal hosts. All these factors would have led to a sustained high population of free-living, unfed adult *A. hebraeum*. Future research should investigate seasonal changes in the population of adult *A. hebraeum*, and whether it is possible to collect adult ticks in midwinter, as well as where the nymphal ticks can be found.

2.6 THE SONGIMVELO GAME RESERVE, MPUMALANGA PROVINCE, SOUTH AFRICAN GAME RESERVE (NO TICK CONTROL)

2.6.1 INTRODUCTION

The Songimvelo Game Reserve (SGR) was originally chosen as an area to test the AAAP/CO₂ trap because of its proximity to "Geluk". "Geluk" was one of Tice's (1995) CGA used in his project as an example of an intensively dipped CGA (Tice 1995).

2.6.2 MATERIALS AND METHODS

2.6.2.1 Geographical location

The SGR (30° 52' - 31° 00' East; 28° 57' - 26° 05' South) is close to Badplaas, Mpumalanga Province, and also close to Swaziland. It was established in 1986 by the amalgamation of sparsely populated lands which had been communally grazed.

2.6.2.2 Study area

No map of the area was available.

2.6.2.3 Climate

As the SGR is close to the KNP it can be assumed that their overall climatic conditions are reasonably similar, i.e. lowveld, hot summer and no frost (rainfall 700 mm per annum).

2.6.2.4 Vegetation and fauna

Lowveld and sour bushveld. A large variety of wild animals have been introduced into the SGR and these include elephants.

2.6.2.5 Livestock

None.

2.6.2.6 Collection of ticks

Ticks were collected by three operators using the original pheromone and chips of dry ice (Tice 1997).

2.6.3 RESULTS

The results of the one visit to the SGR are summarized in Table 8 and 13, and Fig. 19a & 20. Forty-eight adult (24 male and 24 female) *A. hebraeum* were collected during this visit (Fig. 20) (average of 48 ticks per day).

TABLE 13 *Amblyomma hebraeum* adults collected using the AAAP/CO₂ trap at Songimvelo Game Reserve

VISIT NO.	DATE	ADULT TICKS			NYMPHAL TICKS	NUMBER OF VISITS THAT MONTH	AVERAGE ADULT TICKS COLLECTED PER VISIT
		T	M	F			
1	1992-12-02	48	24	24	0	1	48
TOTAL		48	24	24		1	48

T = Total M = Male F = Female

2.6.4 DISCUSSION

The AAAP/CO₂ trap worked well in both game reserves where there was a high density of free-living adult *A. hebraeum* as indicated by the high tick activity index. The 48 ticks collected in the SGR comprised only 2% of the total number of adult ticks collected (Fig. 19a), and no nymphal ticks were collected (Fig. 19b). The number of ticks collected during a single day (n = 48) was good (Fig. 20), and on the same level as at Rietgat.

2.6.5 CONCLUSION

The game reserves proved to be the best localities for the optimum use of the AAAP/CO₂ trap, probably as a result of the high density of the population of *A. hebraeum* in these controlled environments. The high population of undipped hosts in both reserves would have contributed to the artificially high populations of *A. hebraeum*. Ideal climatic conditions, coupled with the lack of frost would also have had a positive effect on the free-living population of *A. hebraeum* in these game reserves.

2.7 OVERALL DISCUSSION AND CONCLUSIONS ON THE USE OF THE AAAP/CO₂ TRAP

A total of just under 2 000 (1 934) adult *A. hebraeum* and 309 nymphs were collected with the AAAP/CO₂ trap at the geographically different sites. There were certain areas where the trap worked well (KNP, SGR, Rietgat and East London), and others (Warmbaths and MEDUNSA), where the sustained use of the trap could not be justified.

The various factors affecting the use of the trap have been discussed in detail, and the time of year was far the most important. There was a definite seasonal variation in response to the trap, and in winter very few ticks were collected at any of the sites, and it would be enlightening to test the trap in the KNP in midwinter. The effect of temperature on tick collection has important implications because much of the field work, especially in summer, is done early in the morning (04:00 to 06:00). Winter and early morning collections must be avoided and rather concentrate on the early to late afternoon in spring and early summer.

An important observation made during this study was the effect of vegetational cover. In South Africa, as in other parts of the world, many of the trees lose their leaves, especially towards the end of summer and during autumn. Some trees only lose their leaves in spring (tamboti) and some continuously (*E. crispa*). The trees which lose their leaves continuously give rise to deep litter, creating excellent habitats for adult and nymphal ticks during the dry, cold winter. They also constitute excellent sites to collect ticks which concentrate there.

Dense, living vegetation cover had negative effects on the visibility of the ticks to the collector, and especially in late summer when the vegetation was so dense it was almost impossible to see adult ticks.

The effects of RH and rainfall are more difficult to assess. However, it would appear that as the RH declines in winter, especially after all the surface water has evaporated (late spring), conditions become unfavourable for free-living, unfed adult ticks. Some of these ticks probably survive by moving to habitats where RH is maintained. At Rietgat at the end of winter the only areas where these moist conditions are maintained are shaded, moist leaf litter. As the summer progresses and the rains fall, then larger portions of the CGA develop suitable RH conditions. Consequently collections made during the late winter/early summer period (September, October and November) would have the greatest chance of success.

The biggest advantage of the AAAP/CO₂ trap is its simplicity and flexibility. It did not require very specific conditions to work, the CO₂ source is usually easy to obtain and once the pheromone solution has been made it lasts for years if stored correctly. All items used in the trap are inexpensive consumables which are easily replaceable.

CHAPTER THREE

3. THE USE OF THE PCR ASSAY TO DETERMINE THE PREVALENCE OF *COWDRIA RUMINANTIUM* IN FREE-LIVING, UNFED ADULT *AMBLYOMMA HEBRAEUM*

3.1 INTRODUCTION

The advancement of our understanding of the epidemiology of heartwater has undoubtedly been hampered by the poor diagnostic tests available to determine *C. ruminantium* in free-living, unfed *Amblyomma* ticks. Estimations of the prevalence of infection rates in these free-living, unfed *A. hebraeum* are important because they reflect the true prevalence of *C. ruminantium* in the field population (Section 1.1). Previously, indirect methods (Section 1.3.2) were used to detect *C. ruminantium*, but many of these methods were cumbersome, expensive and with unknown sensitivities and specificities. In this project a molecular assay, which was specific for *C. ruminantium*, was used on an epidemiological sample which was known to be truly representative of the free-living, unfed *A. hebraeum* population.

3.1.1 MOLECULAR-BASED DIAGNOSTIC TECHNIQUES FOR THE DETECTION OF *C. RUMINANTIUM*

New molecular-based techniques have been evaluated to detect *C. ruminantium* in both animals and ticks. In 1994/5, a collaborative research project was initiated between the

University of Florida and Washington State University, with the aim of identifying and characterising a diagnostic DNA probe that could be used to detect *C. ruminantium* in animals and ticks (Mahan 1995).

A DNA library was made from DNA isolated from *C. ruminantium*-infected endothelial cell cultures. Seventy-four recombinant plasmids were screened, and two were identified as possible probes. DNA from the one recombinant originated from the Kiswani strain from Kenya, and was referred to as the pCR9. The other DNA was from the Crystal Springs strain from Zimbabwe, and referred to as the pCS20 probe.

The pCS20 probe cross-reacted with strains of *C. ruminantium* from Zimbabwe, South Africa, Nigeria, the Caribbean and Kenya (Waghela *et al.* 1991). It could also detect *C. ruminantium* in infected *A. hebraeum* (Yunker *et al.* 1993), and *A. variegatum* which had been fed on febrile animals (Waghela *et al.* 1991). The use of the pCS20 probe also showed that the *C. ruminantium* infection was mainly in the midguts of the infected ticks (Yunker *et al.* 1993), as well as in infected sheep (Mahan *et al.* 1992), and carrier animals (Peter *et al.* 1995a).

Allsopp *et al.* (1999) listed ten different molecular probes which have been used to detect heartwater associated organisms. Eight of these probes have been used to detect *Cowdria* and two to detect *Ehrlichia*. They classified the probes into three main groups containing the 16S probes, the pCS20 probe and the MAP-1 probe respectively. These three groups of

probes were then evaluated to compare their ability to detect *Cowdria* in field ticks collected in the North and North West Provinces of South Africa. The pCS20 probe was found to be the most specific indicator for *Cowdria* genotypes, other than the Omatjenne stock, and showed no cross-hybridization with the *Ehrlichia* species tested. Allsopp *et al.* (1999) stated that the pCS20 probe should be the probe of choice when initially screening field samples for *Cowdria*. The MAP-1 probe currently in use at the Onderstepoort Veterinary Institute, was less sensitive and specific compared to the pCS20 probe, and did not provide genotypic information.

The DNA sequences of both the pCS20 and pCR9 probes were determined and published (Waghela *et al.* 1991). The pCS20 probe consists of 1 306-bp, and contains two Open reading frames (ORF) of 512 and 459 nucleotides. The pCR9 has a 754-bp insert and had one ORF of 243 nucleotides. Waghela *et al.* (1991) suggested that the sequence of the pCS20 probe should be used to develop a diagnostic PCR.

3.1.2 THE USE OF THE PCR TO DETECT *C. RUMINANTIUM*

Mahan *et al.* (1992), described the use of two oligonucleotide primers (AB 128 and AB 129), developed from the pCS20 probe and which amplified a 279-bp fragment. These primers were specific for *C. ruminantium*, and did not amplify DNA from bovine DNA or any other organisms tested. Mahan *et al.* (1992) concluded that the sensitivity and specificity of the PCR, and the availability of the pCS20 probe, could play a crucial role in the detection of

C. ruminantium in field ticks and carrier animals, as the rickettsaemia was so low it could not be detected by the probe alone. Peter *et al.* (1995) confirmed these results by showing that the PCR could amplify between one and ten *C. ruminantium* organisms, and did not amplify DNA from *E. canis*.

Peter *et al.* (1995) demonstrated that the PCR, using the primers of Mahan *et al.* (1992), can be used to detect *C. ruminantium* infection in geographically different strains, as well as with ticks preserved in ethanol, formalin and glutaraldehyde. Mahan *et al.* (1998b) demonstrated that the PCR can detect 92.5% of infected ticks, which was far better than the DNA probe (76.7%), or mouse inoculation assay (MIA) (8.3%).

It was clear that the combination of the PCR and the pCS20 probe was the most credible combination for the detection of *Cowdria* in free-living, unfed *A. hebraeum*. It was therefore decided that the PCR should be used to detect *C. ruminantium* in free-living, unfed *A. hebraeum* adult ticks.

3.2 MATERIALS AND METHODS

3.2.1 STORAGE AND TRANSPORT OF THE FREE-LIVING, UNFED TICKS

All captured free-living, unfed ticks were kept in special, small plastic containers inside a glass humidifier (750 g glass bottle with 200 ml tap water at the bottom). The majority of the ticks were collected in summer, and it was not necessary to control the ambient temperature. They were kept alive at room temperature until they were transported to the laboratory at the UF/USAID/SADC Heartwater Research Project at the Central Veterinary

Diagnostic and Research Laboratory in Harare, Zimbabwe. At the laboratory the ticks were incubated at 28°C and 75% RH until they were dissected for the PCR work.

3.2.2 SAMPLE PROCESSING

Two batches of free-living, unfed ticks were processed (Table 14). The first was the survey group (A) from Rietgat (n = 150), which was processed during November and December 1996 at the UF/USAID/SADC Heartwater Research Project in Harare. The second group (B) consisted of ticks collected at the Rietgat CGA (n = 284), Kruger National Park (n = 88) and Songimvelo Game Reserve (n = 48), and were processed in the laboratory in Harare during April/May 1997.

All the tick samples (groups A and B, n = 570) were initially digested with proteinase K and extracted with phenol, chloroform, isoamyl-alcohol 25:24:1 (PCIA). However, problems were experienced with PCR inhibition within group B (n = 420), and these samples were then re-extracted using QIA amp tissue DNA extraction columns (QIAGEN method, Qiagen, Ca., USA). The extracted tick DNA samples were then frozen at -20°C until used. An aliquot of the frozen material was PCR amplified and confirmed by gel-electrophoresis. PCR amplified products were dot-blotted and hybridized to the pCS20 probe.

3.2.3 TICK DISSECTION

Ticks were dissected on sterile plastic petri dishes with four to five ticks dissected on each dish and after each batch of dissections, a new dish was used. After every 2 - 3 ticks, gloves were changed. The ticks were pinned down dorsally on the dish and the back legs cut off.

The posterior tip of the ticks idiosoma was dissected with a new scalpel blade. The tick guts were then forcibly extruded with the scalpel blade and transferred to an eppendorf tube with a sterile disposable needle. The flat, free-living, unfed ticks were different to laboratory-reared ticks, as many of them had been starved for months and were very thin and at times it was difficult to get a decent sample of the midgut. Ticks which had dried out were not used, as they were difficult to dissect. Tick midguts were collected in eppendorf tubes and frozen at -70°C . Previous workers (Waghela *et al.* 1991, Yunker *et al.* 1993) have shown that *C. ruminantium* occurs primarily in the midgut tissue of both *A. variegatum* and *A. hebraeum*.

3.2.4 DIGESTION OF TICK GUT MATERIAL

The tick midguts were thawed at 37°C and $200\ \mu\text{l}$ of a PCR digestion buffer (10 mM Tris HCl, pH 8.3, 50 mM KCl, 2.5 M MgCl₂, 0,5% Tween, 0,5% NP40) was added. Proteinase K (10 mg/ml) was added to a final concentration of $250\ \mu\text{g/ml}$. The tubes were then incubated at 56°C for 16 hours.

3.2.5 EXTRACTION OF THE DNA FROM THE DIGESTED MATERIAL

3.2.5.1 Phenol, chloroform, isoamyl-alcohol method (25:24:1)

The DNA in the first batch of ticks (samples 1 - 150) from survey group A was extracted using the PCIA method. An equal volume ($200\ \mu\text{l}$) of phosphate buffered saline (PBS) equilibrated phenol was added to each tube, mixed and centrifuged at 12 000 g for five minutes. The aqueous top layer was transferred to a clean tube, and $200\ \mu\text{l}$ PCIA was added

to the samples, mixed and centrifuged for five minutes at 14 000 g. The aqueous top layer was then transferred to a fresh tube, and equal volumes of chloroform isoamyl alcohol (24:1) was added. After being mixed and centrifuged for five minutes at 12 000 g, the aqueous top layer was transferred to a clean tube. An aliquot from each tube (5 μ l) was then transferred to a 0.6 ml PCR tube.

3.2.5.2 Q1A amp tissue DNA extraction column

The DNA of the tick midguts in the second batch of samples, group B (refer to 3.2.2), were initially extracted with the PCIA method. Problems with PCR inhibitors were experienced and these samples were re-extracted to purify the DNA, using the Q1A amp tissue DNA extraction columns (Qiagen, CA, USA). Briefly, the tick guts were digested using the buffers and proteinase K provided in the kit. The digested material was coated onto spin columns where DNA was bound. After a number of washes, using buffers with different concentrations of salt, the DNA was eluted pure and free of inhibitors.

3.2.6 POLYMERASE CHAIN REACTION

The two primers (AB 128 and AB 129) originally developed from the pCS20 probe by Mahan *et al.* (1992), were obtained from the University of Florida, Interdisciplinary Centre for Biotechnology and Research. The primers were used to amplify a 279-bp fragment of *C. ruminantium* DNA, and had the following sequences:

AB 128 5' ACTAGTAGAATTGCACAATCTAT 3'

AB 129 5' TGATAACTTGGTGC GGAAATCCTT 3'.

PCR was performed with 5 μl of sample DNA in a total reaction volume of 50 μl . The following concentrations of reagents were used for each 50 μl PCR mixture: 10 mM Tris, HCl pH 8,3, 50 mM KCl, 3.0 mM MgCl_2 , 0.001% w/v gelatin, 2.00 μM of each dNTP, 0.5 μM of each of the primers and 1.25 units of Taq polymerase.

Reagents in the master mix

The reagents in the master mix were always made up as follows:

● 10 x PCR reaction buffer*	5 μl
● 25 mM MgCl_2 *	6 μl
● dNTP's (10 mM) (4 x 1 μl each)	4 μl
● Primer AB 128 and AB 129 (2 x 5 μl each)	10 μl
● Sterile (distilled) water	19.75 μl
● *Taq DNA polymerase	<u>0.25 μl</u>
	Total mixture = <u>45.00 μl</u>

* Reagents bought from Perker Elmer

After adding the Taq DNA polymerase, the reagents were well mixed, and put on ice. In the sample preparation room 45 μl of the master mix was added to each 5 μl aliquot of tick DNA sample and 45 μl sterile mineral oil was layered on top of the tube. Positive controls consisted of 1 ng genomic *C. ruminantium* DNA in 5 μl distilled water, as well as 5 μl tick DNA spiked with 1 ng of the same DNA. Distilled water (5 μl), as well as DNA extracted from uninfected female and male ticks served as negative controls. One cycle of

amplification consisted of denaturation at 95°C for one minute, annealing at 55°C for one minute, and extension at 72°C for two minutes. Amplification took place for 45 cycles with the final extension step at 72°C for ten minutes. Amplified DNA was then kept at 4°C until analysed.

PCR products (20 μl) were analysed using a 1.5% agarose gel (Gibco BRL Life Technologies, USA). The gels contained 0.4 $\mu\text{g}/\text{ml}$ of ethidium bromide (Sigma Company, USA) (10 mg/ml), and were photographed on a UV transilluminator. To determine the size of the amplicon a 1Kb molecular weight marker (Gibco, BRL, Bethesda, MD, USA) was electrophoresed together with the samples. The specificity of the PCR products were then confirmed by dot-blotting.

3.2.7 HYBRIDIZATION WITH THE pCS20 PROBE

To denature the DNA the PCR product (40 μl) was added to a tube containing 10 μl of 0.4 M NaOH and 50 μl distilled water for 15 minutes at 37°C. The samples were then dot-blotted onto nylon membranes (Genescreen Plus, Du Pont), using a dot-blot vacuum manifold apparatus (Gibco, BRL, Bethesda, MD, USA). The membranes were rinsed in 2 x SSC buffer (0.3 M NaCl and 0.03 M Na citrate), exposed to UV light (312 nm) for two minutes, to cross-link the DNA to the membrane and allowed to air dry.

The method for the hybridization of the membranes was described by Mahan *et al.* (1992) and Yunker *et al.* (1993). Briefly, the probe was labelled with ^{32}P -dCTP, using the multi

prime labelling kit (Boehringer Mannheim). The blotted nylon membranes containing DNA samples were pre-hybridized for six hours at 42°C in a hybridization solution (2 M Na phosphate [pH 7.2], 5 M NaCl, 25 M EDTA, deionized formamide with dextran sulphate: 25% SDS and 10 mg/ml denatured herring sperm DNA) containing 10% dextran sulphate in formamide. The pCS20 probe was denatured by boiling for ten minutes and immediately put on ice for ten minutes. It was then added to the solution and hybridized at 42°C for at least 16 hours. After hybridization, the blots were washed under stringent conditions of four washes of 15 minutes each at room temperature: one wash in 2 x SSC; one wash in 2 x SSC, 0.1% SDS; one wash in 0.5 x SSC, 0.1% SDS and the fourth wash in 0.1 SSC, 0.1% SDS. Two washes of 30 minutes each at 60°C in 0.1 x SSC, 0.1% SDS. The membranes were then exposed to X-ray films (Kodak), for 24 hours at -80°C (Mahan *et al.* 1992), and the autoradiographs were developed according to the recommended procedure of the manufacturers.

3.3 RESULTS

3.3.1 PCR AMPLIFICATION

PCR amplification of the group A samples occurred without any problems, as indicated by both negative and positive controls. The group B samples were initially processed using the PCIA method for extraction of DNA. However, in some of the positive control samples containing 1 ng *C. ruminantium* DNA, positive amplification occurred (Fig. 35, lanes 2, 4, 9, 12 and 15), but no amplification occurred in the spiked tick samples containing the same DNA (lanes 7, 10, 13 and 16). This indicated that PCR inhibition by tick DNA occurred in

all these samples. The PCR inhibition was removed by purifying the samples using spin columns (QIA amp tissue DNA purification columns), after which the positive PCR amplification was restored (Fig. 36, lanes 7, 10, 13 and 16), except for a few PCIA extracted samples (Fig. 36, lanes 5 and 6). After analysing the results in Fig. 35 and 36, it was decided to re-extract the group B samples with the QIA amp tissue DNA purification columns.

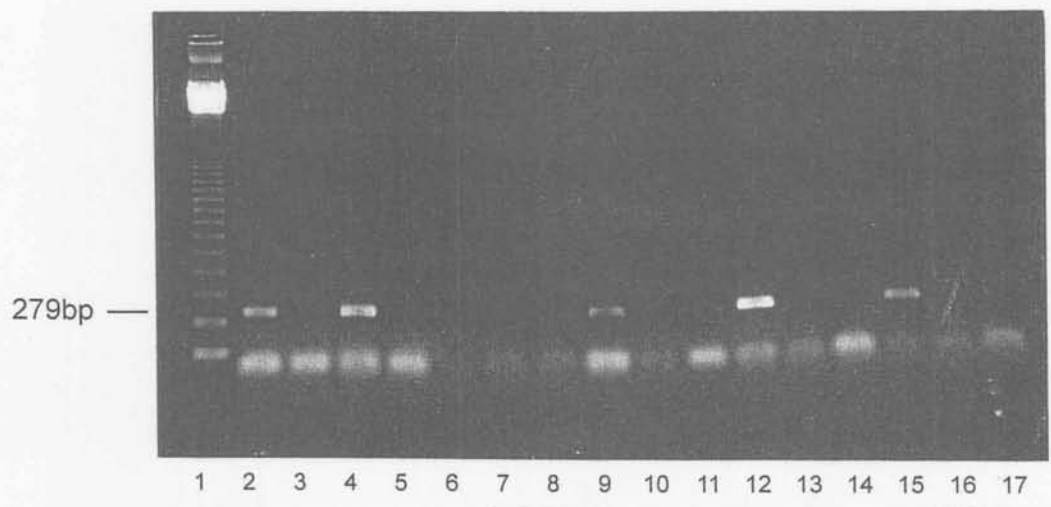


Fig. 35 Agarose gel electrophoresis indicating amplification of genomic *C. ruminantium* DNA after extraction with PCIA. Lane 1: 123-bp molecular marker (Gibco BRL Life Technologies, USA). Lanes 2, 4, 9, 12 and 15: PCR products from amplification of 1 ng *C. ruminantium* DNA. Lanes 3, 5, 8, 11, 14 and 17: negative controls. Lanes 7, 10, 13 and 16: PCR products from amplification of tick DNA spiked with 1 ng *C. ruminantium* genomic DNA.

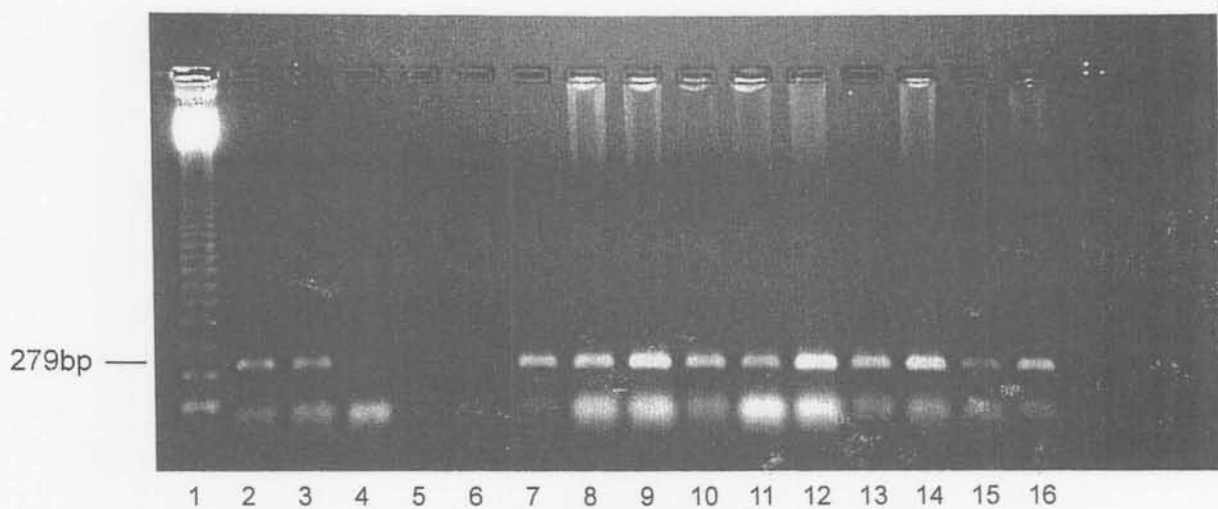


Fig. 36 Agarose gel electrophoresis indicating amplification of *C. ruminantium* DNA after purification with Q1A amp spin columns to remove inhibitors from crude tick digests. Lane 1: 123-bp molecular marker (Gibco BRL Life Technologies, USA). Lanes 2, 5, 8, 11 and 14: PCR products from amplification of 1 ng *C. ruminantium* genomic DNA (PCIA extraction only). Lanes 3, 6, 9, 12 and 15: PCR products from the amplification of 100 ng *C. ruminantium* genomic DNA (PCIA extraction only). Lane 4: PCR negative control. Lanes 7, 10, 13 and 16: PCR products purified with Q1A amp spin columns.

Figure 37 also illustrated PCR amplified DNA from *C. ruminantium* collected from ticks in the field, and extracted using the PCIA method, followed by purification with QIA amp tissue DNA columns.

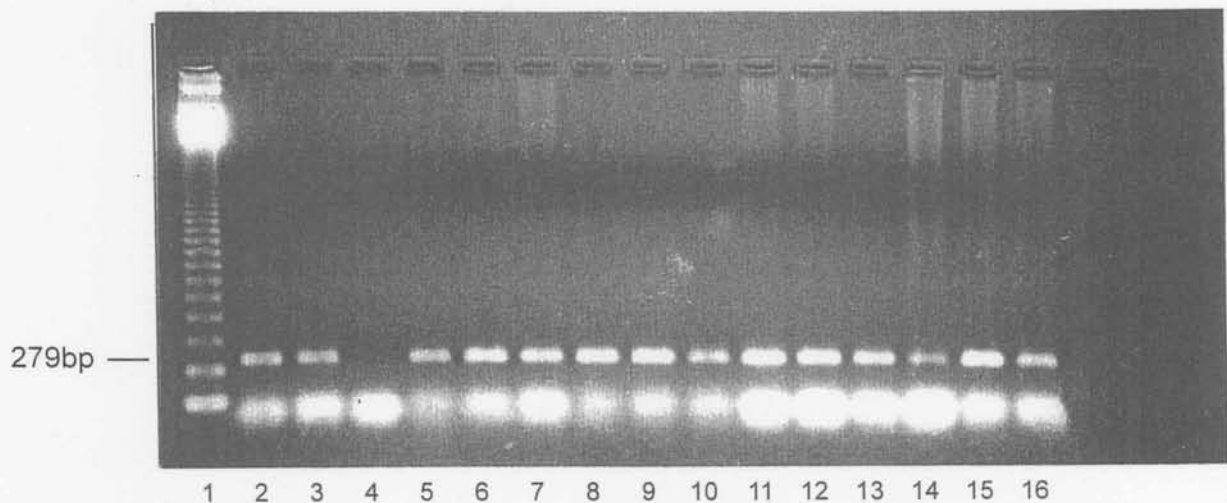


Fig. 37 Agarose gel electrophoresis showing positive and negative amplicons of *C. ruminantium* DNA extracted from field collected ticks. Lane 1: 123-bp molecular marker (Gibco BRL Life Technologies, USA). Lanes 2, 5, 8, 11 and 14: PCR products from amplification of 1 ng genomic DNA. Lanes 3, 6, 9, 12 and 15: PCR products from the amplification of 100 ng *C. ruminantium* genomic DNA. Lane 4: PCR negative control. Lanes 7, 10, 13 and 16: PCR products which had been purified with QIA amp spin columns.

3.3.2 PCR RESULTS FROM THE RIETGAT CGA

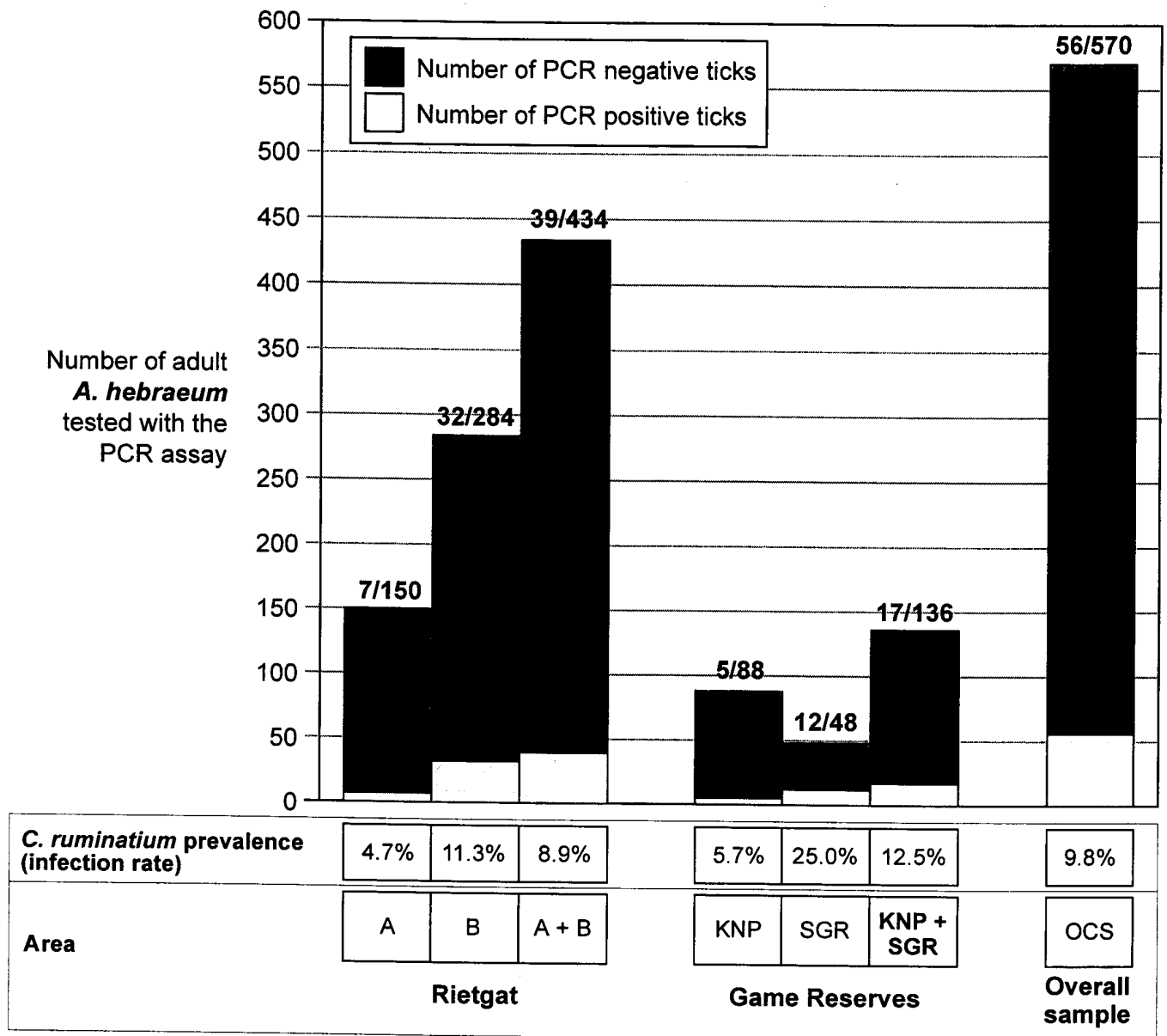
In order to get a preliminary indication of the heartwater prevalence at the Rietgat CGA (n = 150), a sample of free-living, unfed adult *A. hebraeum* were initially tested with PCR. Seven out of one hundred and fifty (4.7%) of these ticks was positive for *C. ruminantium* by PCR (Table 14 and Fig. 38), and confirmed by dot-blotting. In the second batch of adult ticks (group B) collected at the Rietgat CGA (n = 284), 32 (11.3%) were positive for *C. ruminantium*. In the total overall combined sample (OCS) of ticks collected at Rietgat (n = 434) 39 were positive, giving an overall infection rate of 8.9% (Table 14 and Fig. 38).

3.3.3 PCR RESULTS FROM THE SOUTH AFRICAN GAME RESERVES (KRUGER NATIONAL PARK AND SONGIMVELO GAME RESERVE)

Eighty-eight of the total of 447 *A. hebraeum* adult ticks collected at the KNP, were tested with the PCR and five of these were positive (5.7% prevalence) for *C. ruminantium* (Table 14, Fig. 38). The 88 ticks were all from the first KNP collection trip in 1992 (Table 12). Five adult *A. marmoreum* ticks were also collected off a tortoise, but none of these ticks were positive for *C. ruminantium* (0/5 = 0%). A total of 48 adult ticks were collected at SGR, and of these 12 were positive for *C. ruminantium*, giving an infection rate of 25%. A combined total of 136 *A. hebraeum* adults ticks were collected in the two game reserves (KNP and SGR), 17 were positive, and which gave an infection rate of 12.5% for the game reserve ticks (Table 14, Fig. 38).

TABLE 14 Results of the PCR for *Cowdria ruminantium* on the free-living, unfed adult *A. hebraeum* ticks (n = 570), collected from the different regions in South Africa

AREA	NUMBER OF TICKS COLLECTED	PERCENTAGE POSITIVE FOR <i>C. ruminantium</i> (INFECTION RATE)
<u>RIETGAT CGA</u>		
* Survey Group A	n = 150	4.7% (7/150)
* Survey Group B	<u>n = 284</u>	<u>11.3%</u> (32/284)
TOTAL	n = 434	8.9% (39/434)
<u>GAME RESERVES</u>		
* Kruger National Park (KNP)	n = 88	5.7% (5/88)
* Songimvelo Game Reserve (SGR)	n = 48	25.0% (12/48)
TOTAL:	n = 570	9.8% (56/570)
● Five adult <i>A. marmoreum</i> were also tested with the PCR and all proved negative.		



Rietgat CGA

A = Survey group A
 B = Group B
 A + B = Combined group A + B

Games Reserves

KNP = Kruger National Park
 SGR = SGR Songimvelo Game Reserve
 KNP + SGR = Combined Game Reserve Sample

OCS (Overall Combined Sample)

OCS = Rietgat CGA + KNP + SGR

Fig. 38 PCR positive and negative adult *A. hebraeum* assayed at the different collection sites. The *C. ruminantium* prevalence (infection rate) is also illustrated.

3.3.4 HYBRIDIZATION RESULTS

Seven dot-blot plans, representing all 570 PCR reactions done at the UF/USAID/SADC Laboratory in Harare, were recorded (dot-plot plans 1 to 7). An example of one of these dot-blot plans (Fig. 39a) and the corresponding auto-radiograph (Fig. 39b), is illustrated below.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	2	3	4	5	6	7	8	9	10	11	12 ●
B	13	14	15	16	17	18	19	20	21	22	23 ●w	24
C	25	26	27 ●	28	29	30	31	32	33	34	35	36
D	37	38	38	40	41	42	43	44	45	46	47	48
E	49	50	51	52	53	54	55	56	57	58	59	60
F	61	62	63	64	65 ●	66	67	68	69	70	71	72
G												
H	FUN	FUN	FUN	MUN	MUN	MUN	NEG				T/S ●	POS ●

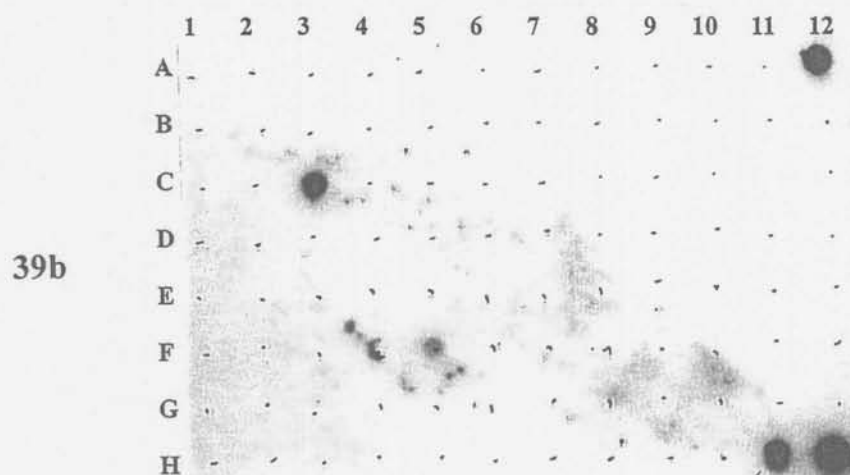


Fig. 39a and b Dot-blot plan 1 of the DNA of tick midgut samples 1 - 72 from the Survey Group A (Rietgat CGA). Corresponding auto-radiograph of the samples 1 - 72, after the hybridization with the pCS20 DNA probe. **Negative control** samples were FUN (female uninfected ticks) at H/1, H/2 and H/3; MUN (male uninfected ticks) at H/4, H/5 and H/6, and negative blank sample with no DNA at H/7. **Positive control** tick DNA samples, spiked with 1 ng genomic *C. ruminantium* DNA tick samples (T/S), and 1 ng *C. ruminantium* DNA (Positive) were at H/11 and H/12, respectively. The three strongly positive hybridization signals of tick midgut samples are indicated as ● at A12, C3 and F5 (Fig. 39b), and represent tick numbers 12, 27 and 65. A fourth weak positive signal, ●w, is at B/11 from tick 23.

3.4 DISCUSSION

The overall infection rate of the ticks collected at the Rietgat CGA was 8.9% (Fig. 38). This indicated that nearly one in ten adult ticks, collected at this CGA, was infected with *C. ruminantium*. This infection rate was higher than many of the previous surveys for *C. ruminantium* prevalence in South Africa (Table 2), (Du Plessis 1981; Du Plessis & Malan 1987; Du Plessis *et al.* 1992 and Peter *et al.* 1999); Madagascar (Uilenberg 1971); Senegal (Gueye *et al.* 1993) and the Caribbean (Camus & Barré 1987b). The results from the Rietgat CGA were more in line with some of the higher values recorded in Zimbabwe (Table 2), (Norval 1981; Norval *et al.* 1990; Peter 1997). The infection rates recorded during this project were considerably higher than many of the vector-borne diseases illustrated in Table 1. Surveys done for Rift Valley fever (Jupp 1994), African swine fever (Plowright *et al.* 1994), *Babesia bigemina* (Mahoney & Mirre 1971), *Babesia bovis* (Mahoney & Mirre 1971), East Coast fever (Leitch & Young 1981), and Lyme disease (Burgdorfer *et al.* 1979) all report prevalence levels below two per cent.

The exact reasons for the higher infection rates in this project are difficult to explain, but it is clear that there are a wide range of results from even within one country. In South Africa, Du Plessis & Malan (1987) recorded prevalence values up to 30%, whilst Peter *et al.* (1999) found only 1.7% of the ticks infected. The consequences of the high infection rates in the free-living, unfed *A. hebraeum* adults at the Rietgat CGA have important repercussions for the endemic stability of heartwater on the communal grazing. Clearly, the free-living, unfed *A. hebraeum* will be important reservoirs of infection for *C. ruminantium*, and the disease will be maintained by a smaller population of infected ticks. Another consequence of the

higher infection rate in free-living ticks would be the direct influence this parameter would have on the epidemiology model of Yonow *et al.* (1998). The high infection rate in the ticks, coupled with the high tick burdens on the cattle (Tice 1997), would produce endemic stability to heartwater in the cattle. A very positive aspect of the higher infection rate in the ticks at the Rietgat CGA would be the increased endemic stability to TBD on the communal grazing. This would be a consequence of the higher exposure to infected ticks, and the farmers would not have to dip their cattle on such a regular basis as a result of the endemic stability to the TBD.

The overall prevalence of 5.7% *C. ruminantium* in the unfed, free-living ticks found at the KNP, was the first documented evidence of the existence of *C. ruminantium* in free-living, unfed *A. hebraeum* ticks collected in a game reserve where no cattle hosts were present. Five *A. marmoreum* adult ticks were also collected from a tortoise in the KNP, but none of these ticks were positive for *C. ruminantium* by PCR. At SGR the 25% prevalence rate was very high, but this area was only visited on one occasion, and it was not possible to revisit to collect more ticks. The prevalence of 25% was by far the highest in our survey, and a larger sample size would have given a more accurate result. It is important not to put too much emphasis on these results which came from a very small sample size (n = 48). Nevertheless, one in four ticks in this game reserve was infected with *C. ruminantium*.

The findings that *C. ruminantium* was present in the ticks collected in the KNP and SGR, was an important epidemiological result, because at neither game reserve was there any contact between the wild and the domestic animals. In Africa wildlife and domestic animals

frequently share grazing areas, but in these game reserves this was not possible. A sylvatic life cycle for *C. ruminantium* has been suspected on the basis of demonstrated infections in wild animals (Norval *et al.* 1992; Peter *et al.* 1998a). The presence of *C. ruminantium* in the free-living, unfed *A. hebraeum* in these two game reserves in this study, adds credibility to the idea of a sylvatic cycle for *C. ruminantium*.

The high infection rate in the *A. hebraeum* adults collected in the two game reserves, was to be expected as *C. ruminantium*, *Amblyomma* ticks and wild animals have a long association, and were probably the original hosts of *Cowdria*. A number of different wild animals have been implicated as reservoirs of heartwater (section 1.3.1.5), and some of these hosts were present in the KNP and SGR, and carried heavy burdens of *A. hebraeum*. These wild animal species, such as buffalo, giraffe and kudu, must play an important role in transporting the ticks, and circulating the *Cowdria* organisms to infect the free-living, unfed *A. hebraeum*. This could lead to the high infection rates seen in this population of ticks. Buffalo have been shown to be asymptomatic carriers of *C. ruminantium* for as long as 161 days. It is clear therefore that these buffalo carriers would be able to infect sylvatic free-living, unfed *A. hebraeum* in the game reserves, and increase the prevalence of *Cowdria* in the ticks.

The PCR used during the project worked well during the investigation, and the results of the combined sample of free-living, unfed *A. hebraeum* collected with the AAAP/CO₂ trap, and *C. ruminantium* detected with the PCR/pCS20 probe, produced the most creditable field data

to date. PCR inhibition was, however, one problem experienced during the investigation, and Peter (1995) stated that his initial PCR's, performed on unextracted DNA of tick tissues, were unsuccessful, and this inhibition was removed by extracting with PCIA. Blood is often an inhibitory substance which PCR laboratories have to deal with on a regular basis. McCusker, Dawson, Noone, Gannon, & Smith (1992) heated the blood to 95°C prior to the addition of the PCR components and this eliminated the inhibitory effects.

Diaz, Nussenzweig & Gonzales (1992) noted that some PCR inhibitors were present in whole human blood, and these inhibitors include heparin, which was used as an anti-coagulant to collect the sample. Gage, Gilmore, Karstens, & Schwan (1992) found that haemoglobin, and especially, the breakdown products such as haematin, are very inhibitory to the PCR reaction. In this project, free-living, unfed ticks would obviously have less haemoglobin products in the midgut than feeding ticks collected off hosts. Stich *et al.* (1993) were aware of the potential problems of PCR inhibitions by haemoglobin products, and chose rather to collect haemolymph from the ticks than use midgut samples.

Kelly, Dasch, Chan & Ho (1994) described the PCIA method of DNA extraction as tedious and potentially hazardous. They also experienced problems with PCR inhibition whilst extracting *Rickettsia tsutsugamushi* from chiggers fleas, and they used a resin to increase the yield of DNA with better results.

Peter (1995) states that organic solvent DNA extractions are hazardous and tedious, and are prone to cross-contamination and damage to or loss of target DNA through frequent sample transfers. He suggested that rapid DNA extraction techniques that remove inhibitors without multiple tube changes should be investigated. These included solid DNA binding media such as filters, resins or selective protein precipitation (Peter, 1995).

To overcome the PCR inhibition, the QIA amp spin column was first used in the mid 1990's. This purification requires no solvents such as phenol, chloroform or alcohol, and involves very little handling of the DNA. In this procedure, DNA bound to the column is washed and therefore there is removal of residual contaminants, including PCR inhibitors (Qiagen Handbook, 1996).

In conclusion, the PCR of the UF/USAID/SADC Heartwater project was able to detect the very low numbers of *C. ruminantium* positive ticks found in the field at three different areas in South Africa. The results of this survey might have been more accurate if this purification method had been used from the start of the project. The PCR was time-consuming and expensive, but the results were credible as the PCR detects only *C. ruminantium* in the free-living, unfed ticks. The recent work of Mahan *et al.* (1998) also confirmed that the PCR was far more specific than the DNA probe or the MIA.

CHAPTER FOUR

4. GENERAL DISCUSSION AND CONCLUSIONS

In Africa the frequent association of *Amblyomma* ticks with wild hosts, means that the eradication of heartwater in ruminants from this continent is simply not feasible. Therefore the first step towards the improvement of the management of heartwater, is to collect more information on the epidemiology and the economic impact of this disease. With more data on *Cowdria* infection rates in the vector populations, it should be possible to determine more accurate levels of endemic stability. Ultimately, this data should be fed into epidemiological and economic models for *Cowdria* to assist farmers to determine their levels of endemic stability to heartwater.

Our early work at Rietgat and the surrounding areas (Tice 1997; Tice *et al.* 1998), involved extensive data collection on the population dynamics of all the ticks found in this region, including *A. hebraeum*, and a serological survey was run concurrently with the tick survey (Tice *et al.* 1998). The overall objective of our study was to advise farmers on the levels of endemic stability to TBD in their domestic animals.

The main aims of this specific project were, firstly to develop a reliable AAAP/CO₂ trap in order to collect free-living, unfed *A. hebraeum* ticks at various field sites in South Africa, and secondly to determine the infection rates of *C. ruminantium* in a statistically relevant sample of these ticks with a *C. ruminantium*-specific PCR assay.

This project has shown that the AAAP/CO₂ trap can be used on a sustained basis to collect adult and nymphal *A. hebraeum*. The survey at the Rietgat CGA demonstrated that there was a definite seasonal response to the trap, with peak collection of adult *A. hebraeum* in late spring to early summer. Many different ecological factors can affect the efficacy of the AAAP/CO₂ trap, and these must be taken into consideration when planning the collection of these ticks. The AAAP/CO₂ trap can also be used to locate new population clusters of *A. hebraeum*, and this was demonstrated at the East London site. There were few disadvantages of this type of AAAP/CO₂ trap, but difficulties in obtaining dry ice in certain areas of Africa could be problematic. If the traps were left unattended then ticks would be frozen by the dry ice, and some means of stopping the ticks getting to the dry ice needs to be constructed.

The PCR was chosen as the diagnostic method because it is fast, extremely sensitive and very specific for *C. ruminantium*. The PCR does not cross-react with any *Ehrlichia* species, and could also be used to detect the very low rickettsaemia present in the free-living, unfed *A. hebraeum* ticks. Peter *et al.* (1999) were the first group to use the combination of a *Cowdria*-specific PCR on AAAP/CO₂ collected ticks, and this same technology has now been used successfully on ticks collected at a communal grazing area, as well as two game reserves.

At the Rietgat CGA, the 8.9% prevalence of *C. ruminantium* in the ticks was higher than many other surveys in southern Africa, with the exception of the study of Norval *et al.* (1990) in Zimbabwe. Previous surveys at Rietgat (Tice 1997) have shown that at least ten *A. hebraeum* ticks were always present on the cattle throughout the year, but clinical heartwater was not observed. The tick burdens and the *C. ruminantium* prevalence in the free-living ticks at Rietgat far exceeded the numbers required to establish endemic stability to heartwater (Du Plessis *et al.* 1992; Yonow *et al.* 1998), and it was clear therefore, that at the Rietgat CGA endemic stability to *C. ruminantium* was present.

Wild animals play a pivotal role in the epidemiology of heartwater in Africa, and their role is only just beginning to be unravelled. The results of this project indicated that the ticks found in the game reserves had a higher infection rate than those in the CGA. This was particularly noticeable in the SGR, where nearly 1:4 of the ticks were infected with *C. ruminantium*, and these results support the viewpoint that *C. ruminantium* is widely circulating in wildlife hosts.

To conclude, this was the first time that a large, statistically-relevant field sample of free-living, unfed adult *A. hebraeum* ticks, collected with the AAAP/CO₂ trap from a variety of different ecological areas, have been processed with a PCR to detect the prevalence of *C. ruminantium*. The data from this project should be epidemiologically more relevant than

previous surveys, and it is hoped that this will contribute to our understanding of heartwater epidemiology, especially the dynamics of the endemic stability of this important disease.

Future research in this field should be aimed at investigating ways of improving the collection methods for nymphal *A. hebraeum*. This was shown to be possible at the Rietgat CGA and at East London, but there was no success at the other sample areas. A survey to determine the possible, seasonal incidence of free-living, nymphal *A. hebraeum* needs to be conducted at one or more of these areas. The PCR could also be used on the free-living, unfed nymphal *A. hebraeum* collected with the AAAP/CO₂ trap, to determine the prevalence of *C. ruminantium* in the nymphal stage of the life cycle of *A. hebraeum*. Data on the prevalence levels in free-living nymphs would contribute important information to the models on heartwater, and this would, ultimately, lead to a better understanding of heartwater epidemiology.

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