TICKS AND TICK-BORNE DISEASES SURVEILLANCE:

Longitudinal and seasonal changes in the relative abundance of *Amblyomma* tick infestations in relation to the prevalence of heartwater (*Ehrlichia ruminantium* infection) in goats in the area of Mnisi (Mpumalanga), South Africa

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Abstract

The present study was conceived with the main objective of establishing more insight in the epidemiology of heartwater and investigating the longitudinal and seasonal changes in the relative abundance of *Amblyomma* tick infestations on indigenous goats in the area of Mnisi (Mpumalanga), South Africa. One hundred eighty out of 184 goats examined for tick infestation in November 2013 were infested by ticks. Two Ixodid tick species were found, with *Amblyomma hebraeum* being the predominant tick encountered followed by *Rhipicephalus (Boophilus) microplus*. In total 2732 ticks were collected, of which the majority were immature ticks (557 adults, 1449 nymphs and 726 larvae). Compared to a previous study by F. van der Steen (2013), wherein collections were carried out during winter (June and July 2013), a substantial increase in the number of *A. hebraeum* adults was observed. The tick:goat ratio for *A. hebraeum* adults during November was 2.8:1 compared to 0.2:1 during June and July. *A. hebraeum* adults were present on goats in all villages and most numerous in the village of Share. *R. (B.) microplus* ticks were present on goats in small numbers. PCR and RLB assays were applied to study the prevalence of *E. ruminantium* infection in *A. hebraeum* ticks. The rates of infection ranged from 25% (17 out of 68) in *A. hebraeum* adults and 23,5% (4 out of 17) in pooled nymphal samples recovered from goats during November 2013 to 13% (3 out of 23) in adult *A. hebraeum* ticks and 11,8 % (2 out of 17) in pooled nymphal samples collected during June and July 2013 as reported by F. van der Steen (2013). Severe damage to feet, peri-anal region, udders and teats caused by the ticks was observed at 47 out of 53 households. The observed damage, infection rates of *A. hebraeum* and the fact that goats can act as important alternative hosts for cattle ticks underscore the necessity of including goats in tick control programmes designed for cattle at the same locality. The results of this study combined with a previous study by F. van der Steen (2013) and future studies can provide an important insight in the longitudinal and seasonal changes in the relative abundance of *Amblyomma* tick infestations in relation to the prevalence of heartwater in goats in the area of Mnisi. These insights can be used as a basis for the development of well-targeted tick control programmes.

1 Introduction

Heartwater (also known as cowdriosis), is a tick-borne disease affecting domestic livestock and is caused by the obligate intracellular rickettsial organism *Ehrlichia ruminantium* (formerly *Cowdria ruminantium*)(Swai et al., 2009). The three-host tick *Amblyomma hebraeum* (The South African bont tick) is the main vector of *E. ruminantium* in southern Africa (Allsopp, 2009; Walker & Bouattour, 2003). The disease has an economically significant impact on livestock and is regarded as one of the most important vector-borne diseases of domestic livestock and wildlife in Sub-Saharan Africa, according to the World Organization of Animal Health (OIE). The disease can cause a high mortality (up to 90%) in susceptible ruminants such as goats (Martinez, Vachiéry, & Jongejan, 2012).

Different clinical forms of heartwater have been described, but the acute form is most commonly presented. Symptoms of an acute infection are a sudden high fever, listlessness, inappetance, diarrhea, nervous signs and a high mortality (Leask, Pettey, & Bath, 2013). Before the animal dies, it may fall to the ground making pedalling movements and exhibiting opisthotonos, nystagmus and chewing (Martinez et al., 2012). Commonly associated post-mortem signs are hydropericardium, hydrothorax and oedema in the lungs and brain (Ahmadu et al., 2004; Allsopp, 2009). The presence of *Amblyomma* vectors, specific nervous signs and the presence of transudates in pericardium and thorax during the post-mortem examination may lead to a tentative diagnosis of heartwater. However, to exclude other possible diseases and make a definitive diagnosis, specific diagnostic tests are needed (Martinez et al., 2012). Nevertheless the occurrence of heartwater is frequently taken for granted in endemic areas and the prevalence of heartwater is still under-reported since definitive diagnoses are often not performed (Allsopp, 2010).

Recovered animals become carriers of *E. ruminantium*. These animals appear healthy but can carry *E. ruminantium* organisms at very low levels and can be infective to ticks for long periods. For cattle the infective period lasts at least 246 days (Andrew & Norval, 1989) and recovered goats can infect ticks for over 11 months (Barre & Camus, 1987). Sheep were shown to remain carriers for up to 3.5 years post-infection (Bekker et al., 2002). Certain wild animals can also play a role as reservoir. A subclinical carrier state has been experimentally shown to occur in different wild ruminants such as blesbok, black wildebeest, blue wildebeest, African buffalo, eland, giraffe, greater kudu and sable antelope (Peter et al., 1999). This has to be taken in consideration while investigating the epidemiology of heartwater in the area of Mnisi.

The main agricultural activity in the area of Mnisi is livestock farming, of which goats account for a decent part of the total livestock. These indigenous goats play, besides a significant economic role, an important cultural role in rural farming communities (Bryson et al., 2002b). The area of Mnisi has a sub-tropical climate, which, in combination with high host densities, provides very suitable conditions for the survival and maintenance of ticks. Monitoring of tick infestations and collection of field data concerning the prevalence and intensity of infection in both adult and nymph *A. hebraeum* are required for the development of new practical and meaningful (population based) control strategies in the area of Mnisi (Peter et al., 1999). Research on ticks and tick-borne diseases affecting the livestock of small-scale, traditional farmers has been overlooked in South Africa (Bryson et al., 2002a). Therefore, studies have been initiated to get more insight in the species composition and dynamics of ticks infesting livestock of small-scale farmers.

Bryson et al. (2002a) collected ticks from cattle belonging to small-scale farmers at the Geluk communal grazing area, Mpumalanga Province, South Africa. *Boophilus decoloratus* was the most numerous, followed by *A. hebraeum, Rhipicephalus appendiculatus* and *Rhipicephalus evertsi evertsi*. This is in agreement with other studies such as the survey by Rechav et al. (1991), who found *R. evertsi evertsi*, *R. appendiculatus* and *A. hebraeum* to be the most abundant tick species on indigenous goats on a northern Transvaal farm. Other species like *B. decoloratus*, *Hyalomma truncatum* and *Hyalomma marginatum rufipes* were found in small numbers (Rechav & De Jager, 1991).

During June and July 2013, Francine van der Steen from Utrecht University conducted a study in the area of Mnisi, entitled '*The relative abundance of Amblyomma tick infestations in relation to the prevalence of heartwater (Ehrlichia ruminantium infection) in goats in the area of Mnisi (Mpumalanga), South Africa'*. Despite the winter period, fairly high numbers of *A. hebraeum* nymphs were collected from goats from selected households in the area. Moreover, a few adult *A. hebraeum* were also found. Besides *A. hebraeum,* other tick species found by F. van der Steen (2013) were *R. (B.) microplus*, and some *R. appendiculatus, Rhipicephalus simus* and *Rhipicephalus zambeziensis*. Twenty-three adult *A. hebraeum* ticks and 1267 *A. hebraeum* nymphs were identified at the Department of Veterinary Tropical Diseases (DVTD) and a selected number of ticks was examined by polymerase chain reaction (PCR) analysis and reverse line blot (RLB) hybridization at the Utrecht Centre for Tick-borne Diseases (UCTD) to determine the infection rate for *E. ruminantium* and identify possible other pathogens.

The present study is a continuation of the work initiated by F. van der Steen (2013), but added a seasonal dimension to the project. This study will continue to determine the relative abundance of *A. hebraeum* ticks on goats in the described area during summer and will provide insight in the seasonality of *Amblyomma* ticks on goats in the area of Mnisi. The control of tick-borne diseases depends, to a large extent, on the availability of accurate data on the epidemiology of the infection in the vector and host. Therefore, the main goal of this study was to gain more insight in the epidemiology of heartwater and the role of *A. hebraeum* ticks in transmission of disease to livestock in the area of Mnisi.

2 Research questions

2.1 Main research question

What are the longitudinal and seasonal changes in the relative abundance and geographical distribution of *Amblyomma* tick infestations in relation to the prevalence of heartwater (*Ehrlichia ruminantium* infection) in goats in the area of Mnisi (Mpumalanga), South Africa?

2.2 Sub-questions

- Investigate the role of *A. hebraeum* ticks in transmission of heartwater to goats in the area of Mnisi;
- Determine the overall tick infestation rate and the tick:goat ratio
- Comparison between the collection during June-July 2013 and the current collection during November 2013
- Conduct a questionnaire for local farmers/owners (see APPENDIX A).

3 Materials and methods

3.1 Study area

Ticks for this study were collected in 17 villages in the area of Mnisi, province of Mpumalanga, South Africa. This area covers about 29 500 hectare and is situated in the north-eastern corner of the Bushbuckridge Municipal Area. The area falls within the savannah ecosystem and in the Mpumalanga Province the life cycle of *A. hebraeum* continues throughout the year. Over 40,000 people are living in this region, surrounded by the adjacent Andover and Manyeleti provincial game reserves and the Kruger National Park. These residents are divided over an estimated 8555 households of which 917 different goat farmers own a total of approximately 6000 goats (Kriek, 2009). The area is part of the Mnisi Community Programme, an initiative by the University of Pretoria and the Mnisi Traditional Authority (Hartley, 2011). Dip-tanks are operational for cattle in all 17 villages at no cost to the farmers.

Since this study is a continuation, the villages used for sampling have been the same as during the study by F. van der Steen (2013). The following villages located in the area of Mnisi have been visited: Ludlow (24°40'54.5"S 31°16'37.7"E), Utha A (24°41'36.0"S 31°26'49.5"E), Utha B (24°42'33.9"S 31°24'13.1"E), Shorty (24°40'22.2"S 31°22'28.2"E), Clare A (24°37'59.0"S 31°18'05.4"E), Clare B(24°38'43.5"S 31°20'11.7"E), Athol (24°42'34.2"S 31°20'45.8"E), Share (24°40'36.0"S 31°18'46.5"E), Gottenburg (24°38'21.6"S 31°24'38.8"E), Hlalakahle (24°37'13.1"S 31°25'50.9"E), Hluvukani (24°38'42.9"S 31°20'47.5"E), Welverdiend A (24°34'46.3"S 31°19'26.7"E), Welverdiend B (24°35'18.4"S 31°20'22.9"E), Thorndale (24°39'01.1"S 31°26'58.4"E), Dixie (24°41'40.3"S 31°28'29.6"E), Tlhavekisa (24°37'30.9"S 31°22'31.6"E) and Seville (24°40'10.1"S 31°25'01.9"E) (see APPENDIX B).

3.2 Study animals / Population

One hundred and eighty-four goats from 53 small-scale farmers divided over 17 villages in the area of Mnisi were sampled. The goats were kept in a traditional manner, often in a kraal (an enclosure for livestock, located within an African settlement or village surrounded by a fence of thorn-bush branches, roughly circular in form). Kraals of goats and cattle are frequently positioned adjacent to each other. During the day goats are taken to communal grazing areas. Animals from different farmers are in close contact at communal grazing areas and dip-tanks.

3.3 Tick collection

Tick collections were conducted between November 4th and November 28th 2013. Larvae, nymphs and adult ticks were collected from goats in 17 different villages (APPENDIX B). At 53 different households goats were randomly selected and sampled in the kraal. The aim was to sample 5 goats at every household. This, however, was not always possible as sometimes the animals were out grazing. Whole body collections were performed. However, if the number of larvae was too high to collect all ticks, half body collections (the left side of the animal) were performed. The animals were restrained by the Environmental Monitor and removal of ticks was always performed by the same researcher. The animals were closely scrutinized for ticks, and those found were manually removed with forceps. Between November 4th and 28th, 2013, 2732 ticks (557 adults, 1449 nymphs and 726 larvae) were collected. The ticks were stored at room temperature and preserved in labelled bottles containing 70% ethanol until further analysis. The bottles were labelled with date and specific ID number. A special sheet was composed with ID numbers for every sampled household. For every ID number the collection site (the name of the village, to keep track of geographical distribution), date of sampling, host species, number of sampled goats, total number of goats in the kraal and the number of ticks found at every household was recorded.

3.4 Tick identification

Tick identification and counting was performed at the Hans Hoheisen Wildlife Research Station, managed by the University of Pretoria. The collected ticks were identified to species level and counted under a stereoscopic microscope. 'Ticks of Domestic Animals in Africa: a Guide to Identification of Species' guide by Walker, A. R. & Bouattour (Walker & Bouattour, 2003) was used as a reference for identification. Genera, species, sex and stage of the life cycle were determined. Larvae were only counted. Collections from every village were pooled after counting and identification. To allow comparison with the previous study by F. van der Steen (2013), the number of ticks collected during November 2013 is displayed per village in Table 1.

3.5 Questionnaire survey

A questionnaire survey (See APPENDIX A) was conducted to establish the status of ticks and tick-borne diseases and to get more insight in the epidemiology of heartwater in the area of Mnisi. The questionnaire involved several open questions, yes/no questions and, in addition, the small-scale farmers were able to express their personal opinion on the subject. They were also asked to name any unusual signs they had observed in their goats during the last year. Furthermore, details of tick-control history of the sampled animals and the mortality rate were obtained at every household. A questionnaire was submitted to every farmer that participated in this study and a total of 53 questionnaires were completed. Preferably the questionnaires were completed by owners of the sampled goats, however, on several occasions the owners were not present. In such cases the questionnaire was completed by family members or, as on two occasions, by an observant neighbour. The questionnaires were translated by and completed with the help of the Environmental Monitors.

3.6 Detection of *E. ruminantium* in ticks

The ticks collected during this study were tested with a specific polymerase chain reaction (PCR). DNA has been extracted from *A. hebraeum* ticks at the UCTD (Utrecht University), whereupon prevalence of *E. ruminantium* in the ticks was determined by the analysis of pooled nymphs and single adult ticks with a specific PCR/RLB protocol as described by Bekker et al. (2002). This is a broad screening test for *E. ruminantium* and other pathogens.

3.7 DNA extraction

Based on a study by B. Faburay (Faburay et al., 2007) *A. hebraeum* nymphs were pooled for DNA extraction and tested in batches of 10 ticks. Due to a lack of collected nymphs in the village of Shorty, only 4 nymphs were pooled from this particular village. Two partly engorged male *A. hebraeum* adults and two partly engorged female *A. hebraeum* adults were randomly selected from every village and the adult ticks were tested individually. Two hundred and thirty-two ticks (164 nymphs and 68 adults) were analyzed.

The DNA extraction was performed according to UCTD protocols from 2012 (UCTD, 2012), see APPENDIX C, 11.1. As described, the ticks were washed in a sonification bath with distilled water for up to 30 seconds. Afterwards, the ticks were put in eppendorf tubes prefilled with 70% ethanol. The ticks were dried on clean tissue paper, placed in sterile 2ml

tubes with 180 µl T1 lysis buffer and the samples were frozen at -80 for 15 minutes. Metal beads of 7mm were added to each frozen sample. The ticks were disrupted by putting the samples in the TissueLyser LT for 3 minutes at 50 oscillations per second. DNA was extracted from each sample according to the NucleoSpin® Tissue kit protocol (Machery-Nagel, 2012). The DNA samples were stored at 4°C until analysis within the next few days.

3.8 Polymerase Chain reaction (PCR)

The PCR assay was performed as previously described by Bekker et al. (2002a), Gubbels et al. (1999), and Schnittger et al. (2004) with modifications found in the UCTD protocols from 2012 (UCTD, 2012), see APPENDIX C, 11.2. Eighty-five samples were analyzed (16 DNA samples which each contained DNA of 10 nymphs, 1 sample which contained DNA from 4 nymphs due to a lack of nymphs in one village and 68 samples which each contained DNA of 1 adult *A. hebraeum*). Each sample was tested for *Ehrlichia*/*Anaplasma* and *Theileria*/*Babesia* species.

During the PCR assay, one set of primers was used for *Anaplasma*/*Ehrlichia* species. The forward primer, Ehr-F (5' – GGA ATT CAG AGT TGG ATC MTG GYT CAG) and the reverse primer Ehr-R (5' – Biotin – CGG GAT CCC GAG TTT GCC GGG ACT TYT TCT)(UCTD, 2012). The primers for *Anaplasma*/*Ehrlichia* are degenerate primers, a set of primers which have a number of options at several positions in the sequence, where M = A+C and Y = C+T (UCTD, 2012). For *Babesia*/*Theileria*, the forward primer, RLB-F2 (5' – GAC ACA GGG AGG TAG TGA CAA G), and the reverse primer, RLB-R2 (5' – Biotin – CTA AGA ATT TCA CTT CTG ACA GT) have been used for amplification (UCTD, 2012).

3.9 Reverse line blot hybridization (RLB)

The development of species-specific oligonucleotide probes, allows simultaneous detection and identification of several *Ehrlichia, Anaplasma, Theileria* and *Babesia* species in an RLB assay (Bekker et al., 2002; Gubbels et al., 1999). This technique provides the possibility to analyze multiple samples against multiple probes at the same time, which is a major advantage.

Three blots (miniblotter) were used, 1 blot was used for 17 different nymphal samples and 2 blots were used for the remaining 68 adult samples. The preparation and subsequent hybridization of the RLB membrane was done as described in UCTD protocols from 2012 (UCTD, 2012), see APPENDIX C, 11.4. A B100 control, PCR negative control, PCR positive control and T100 control were added to each blot. The slots were filled with the diluted and

denaturized PCR samples and all empty slots were filled with 2 x SSPE/0.1% SDS, to avoid cross flow. After hybridization, de blotter was removed from the hybridization oven and the samples were removed by aspiration. The membrane was removed from the blotter and washed twice with preheated 2x SSPE/0,5% SDS. Subsequently, the membrane was incubated with preheated 2x SSPE/0,5% SDS + 5 µl streptavidin and the membrane was washed twice again, with preheated 2x SSPE/0,5% SDS. Next, the membrane was washed twice with 2x SSPE. Afterwards, 10 ml ECL (5 ml ECL1 + 5 ml ECL2), which enhances light emission, was added to the membrane. After the whole membrane was fully covered with ECL, the fluid was collected and the membrane was packed in foil and in the film cassette. The membrane was exposed to the film for 10 minutes in the dark room and the film was developed. Finally, the membrane was rinsed and stored in a sealed plastic bag with 20 mM EDTA at 4°C until stripping.

4 Results

4.1 Tick collection

A total of 184 whole-body tick collections were made from goats in the area of Mnisi during the survey in November 2013 (Ludlow, n = 6; Utha B, n = 7; Shorty, n = 14; Clare B, n = 10; Athol, n = 10; Share, n = 5; Gottenburg, n = 19; Hlalakahle, n = 9; Clare A, n = 7; Hluvukani, n = 10; Welverdiend A, n = 9; Thorndale, n = 13; Thlavakisa, n = 15; Seville A, n = 13; Welverdiend B, n = 13; Utha A, n = 7; Dixie, n = 7; Seville B, n = 10). The number of ticks collected in each of the 17 villages in the area of Mnisi is summarized in Table 1.

Two species of Ixodid ticks were collected from goats in the area of Mnisi. *A. hebraeum* was the most numerous tick species in the area of Mnisi. *A. hebraeum* nymphs accounted for the major portion of the collected ticks (n=1430). *R. (B.) microplus* was found in 9 out of 17 villages (Ludlow, Shorty, Athol, Share, Gottenburg, Hlalakahle, Welverdiend A, Thorndale and Seville A). In Hlalakahle and Clare A many larvae were found and not all of these were removed (indicated with * in Table 1). At all 53 households infested goats were found. Out of 184 sampled goats, 180 goats were infested with adults, nymphs, larvae or several stages at once. On 4 sampled goats no ticks were found, however, well-hidden larvae might have been missed. In total 522 adult *A. hebraeum* ticks were collected, of which 71,5 % were males and 28,5 % were females. Adult numbers peaked in Share with 48 adults recovered during 5 whole-body collections. A Species composition and total number of ticks collected from goats in the area of Mnisi (Mpumalanga), South Africa during November 2013 can be found in Table 1.

During tick collections from goats in June-July and November 2013, no standardized number of animals was sampled. Consequently, the total numbers of adult ticks collected from these animals are not comparable. However, to get a general idea of the seasonality of *A. hebraeum,* the mean number of *A. hebraeum* adults/infested hosts sampled has been compared. A graphic illustration (see Figure 1) of the seasonality of *A. hebraeum* adults has been created by calculating the average number of *A. hebraeum* adult ticks collected per host, comparing the survey during June-July 2013 (summarized in Table 2) and the collection in November 2013. In all sampled villages the mean number of adult *A. hebraeum*/infested hosts is higher during the collection in November 2013 than in June-July 2013. Share peaks with a mean number of 9,6 adult *A. hebraeum* ticks per infested host.

During examination, skin lesions caused by ticks were found, especially around the feet. Adult *A. hebraeum* ticks were often found in the inter-digital space, causing purulent ulcers and abscesses accompanied by lameness, sometimes in combination with an abnormal or necrotic smell. This finding is in accordance with the study by F. van der Steen (2013) who described 16% of farmers observed lameness in tick-infested goats. Predilection sites for attachment of adult *A. hebraeum* appeared to be around the feet, the hairless areas under the tail, the lower perineal region, on the udder, around the genitalia and in the axilla. Many larvae were found on the face and ears. *R. (B.) microplus* ticks were mainly found in or around the ears of goats.

Table 1. Species composition and total number of ticks collected from goats in the area of Mnisi (Mpumalanga), South Africa during November 2013.

Tick species	Adults					Nymphs		Larvae	Total	Mean no. of A. hebraeum adult ticks/ host
	Amblyomma hebraeum	Rhipicephalus (B.) microplus	Rhipicephalus appendiculatis	Rhipicephalus simus	Rhipicephalus zambeziensis	Amblyomma hebraeum	Boophilus microplus			
Ludlow (n=6)	6	11	0	0	0	114	2	24	157	1
Utha B (n=7)	9	0	0	0	0	68	0	28	105	1,3
Shorty (n=14)	17	4	0	0	0	20	3	9	53	1,2
Clare B (n=10)	34	0	0	0	0	51	0	5	90	3,4
Athol (N=10)	13	3	0	0	0	102	2	48	168	1,3
Share (n= 5)	48	4	0	0	0	17	0	3	72	9,6
Gottenburg (n=19)	67	7	0	0	0	88	0	41	203	3,5
Hlalakahle (n=9)	40	0	0	0	0	58	10	96*	204	4,4
Clare A (n=7)	32	0	0	0	0	16	0	17*	65	4,6
Hluvukani (n=10)	15	0	0	0	0	102	0	46	163	1,5
Welverdiend A (n=9)	22	1	0	0	0	67	1	10	101	2,4
Thorndale (n=13)	37	1	0	0	0	156	1	97	292	2,8
Thlavakisa (n=15)	71	0	0	0	0	304	0	85	460	4,7
Seville A (n=13)	16	4	0	0	0	62	0	66	148	1,2
Welverdiend B (n=13)	8	0	0	0	0	28	0	34	70	0,6
Utha A (n=7)	33	0	0	0	0	50	0	46	129	4,7
Dixie (n=7)	9	0	0	0	0	30	0	3	42	1,3
Seville B (n=10)	45	0	0	0	0	97	0	68	210	4,5
Total (n=184)	**522**	**35**	**0**	**0**	**0**	**1430**	**19**	**726**	**2732**	**2,8**

Table 2. Species composition and total number of ticks collected from goats in the area of Mnisi (Mpumalanga), South Africa during June and July 2013 (Collection by F. van der Steen).

Tick species	Adults					Nymphs	Larvae	Total	Mean no. of A. hebraeum adult ticks/ host
	Amblyomma hebraeum	Rhipicephalus (B.) microplus	Rhipicephalus appendiculatis	Rhipicephalus simus	Rhipicephalus zambeziensis	Amblyomma hebraeum			
Ludlow (n=8)	0	11	0	0	0	38	55	104	0
Utha B (n=5)	0	2	0	0	0	46	31	79	0
Shorty (n=14)	5	1	0	0	1	72	13	92	0,4
Clare B (n=3)	0	1	0	0	0	32	77	110	0
Athol (N=11)	0	3	0	0	0	23	236	262	0
Share (n= 11)	0	2	0	0	0	35	77	114	0
Gottenburg (n=2)	0	0	0	0	0	14	2	16	0
Hlalakahle (n=14)	0	0	0	0	0	115	85	200	0
Clare A (n=3)	1	16	1	0	0	21	60	99	0,3
Hluvukani (n=7)	2	4	0	0	0	70	92	168	0,3
Welverdiend A (n=14)	1	71	0	1	1	113	108	295	0,1
Thorndale (n=4)	1	1	0	0	0	36	38	76	0,3
Thlavakisa (n=8)	9	0	0	0	0	135	62	206	1,1
Seville A (n=3)	0	1	0	0	0	59	0	60	0
Welverdiend B (n=6)	0	6	0	0	0	37	106	149	0
Utha A (n=4)	3	30	0	0	0	327	650	1010	0,8
Dixie (n=1)	1	12	0	0	0	94	240	347	1
Total (n=117)	**23**	**161**	**1**	**1**	**2**	**1267**	**1932**	**3387**	**0,2**

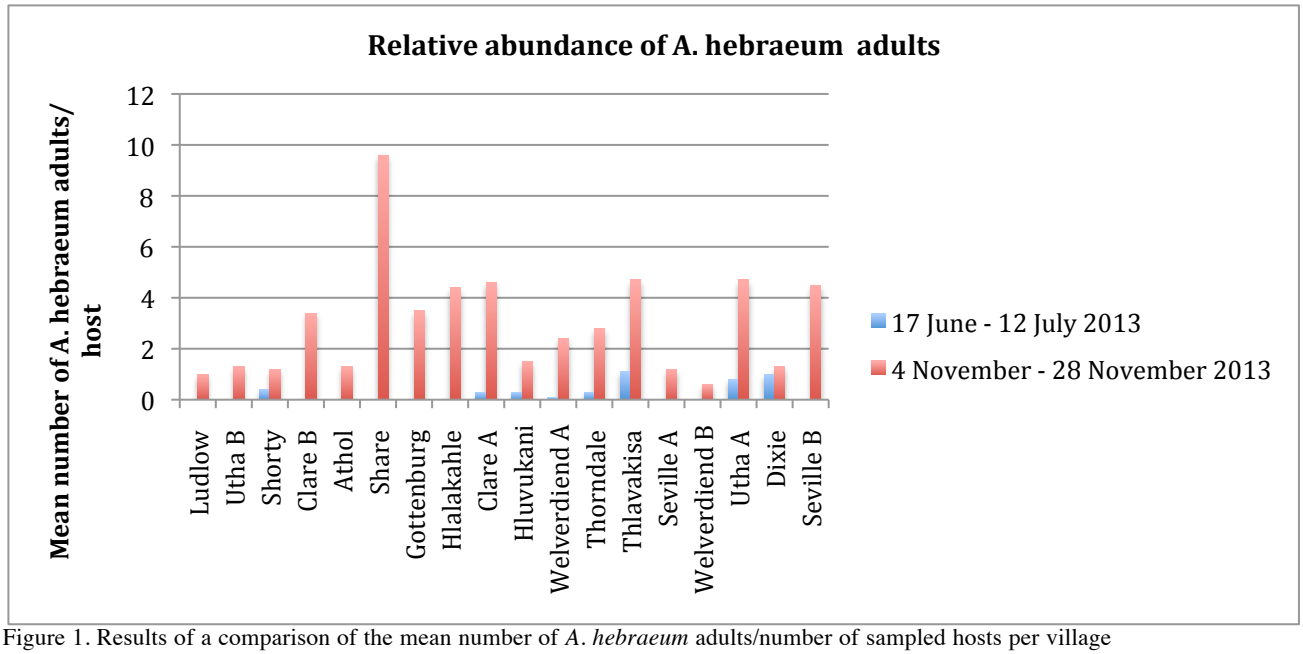

Figure 1. Results of a comparison of the mean number of *A. hebraeum* adults/number of sampled hosts per village collected in June-July and November 2013.

4.2 Questionnaire survey

The questionnaire survey was conducted in 17 villages in the area of Mnisi. The target group consisted of 53 goat owners who participated during this study. The survey showed that a significant proportion of farmers (79,2%) do not use tick control. Only 11 (20,8%)($\chi 2 = 18.1$; $P \leq 0.05$)(Preacher, 2001) farmers do use tick control. Figure 2 shows tick control measures used by farmers in the area of Mnisi. Eleven percent of farmers irregularly used forceps for manual removal of ticks, 5,7% used paraffin for monthly tick control, 1,9% dipped their goats monthly with a commercial acaricide and another 1,9% treated the goats twice a week with a mix of used engine oil and commercial dip (Delete X5®, Intervet, South Africa Contains Contains: 5% m/v Deltamethrin)(Intervet International, 2014)

Different reasons for controlling ticks were mentioned. The main reason was to 'clear' the animals, since a 'cleared' goat is considered easier to sell. Other reasons for controlling ticks included protection against diseases and to kill ticks. Several farmers were convinced there was no need for tick control since the ticks are on the 'outside' of the animal and therefore would not affect the meat. This underscores the fact that there is a need for farmer education providing farmers with information on the benefits of tick control and tick management (Hlatshwayo & Mbati, 2005).

Heartwater is often under-reported in endemic areas. To gain more insight into the current situation in the area of Mnisi, the farmers were asked if they recognized some of the most characteristic heartwater symptoms such as falling on the floor, pedalling movements, tight muscles or chewing movements just before the goat died. Nine farmers (17%) indicated to have seen pedalling movements before one or several goats died. Thirteen of the respondents (25%) were certain not to have seen any characteristic symptoms. The farmers either described the animals to be peaceful and quiet before they died or were certain there was another cause of death, such as a dog attack. Thirty-one farmers (58%) indicated they had found the goats in the morning and therefore were not able to describe any symptoms prior to death. None of the farmers had observed a hydropericardium or hydrothorax since goats in the area of Mnisi are usually not slaughtered but used during traditional ceremonies.

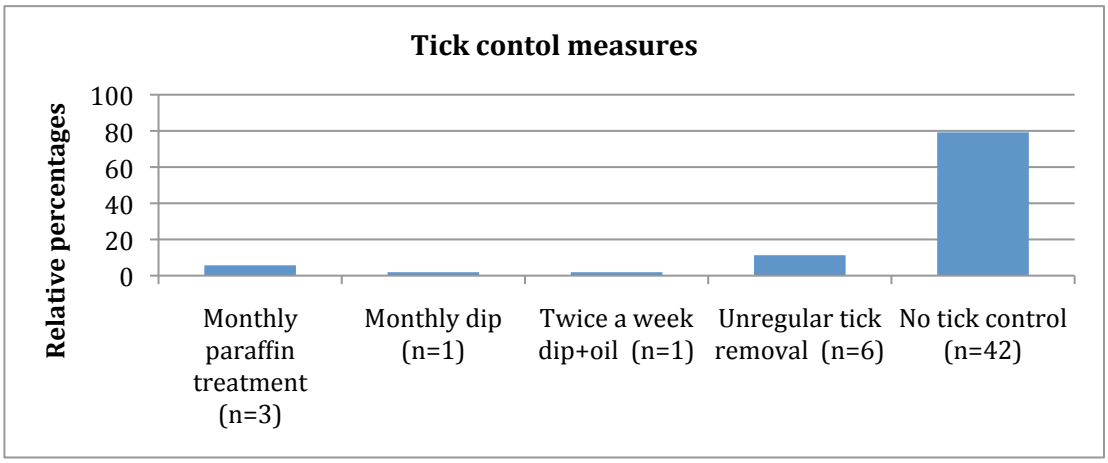

Figure 2. Results of a questionnaire survey on tick control measures on goats used by farmers in the area of Mnisi in November 2013

4.3 Polymerase Chain reaction (PCR)/Reverse line blot hybridization (RLB)

The collected ticks were assessed for *E. ruminantium* infection with polymerase chain reaction (PCR) and reverse line blot hybridization (RLB) assays. Positive and negative controls were included in all tests. Seventeen out of 68 (25%) screened adult *A. hebraeum* ticks, tested positive for *E. ruminantium*. Table 3 shows the prevalence of *E. ruminantium* infection in adult *A. hebraeum* ticks and pooled nymphal samples from the 17 sites in the area of Mnisi from the collection during November 2013. The overall prevalence of infection in male adult *A. hebraeum* ticks was 35,3% (12 out of 34 ticks) and 14,7% (5 out of 34 ticks) of female adult *A. hebraeum* carried *E. ruminantium*. Infected adults were recovered from Athol, Dixie, Gottenburg, Hluvukani, Ludlow, Seville B, Share, Shorty, Utha A, Welverdiend A and Welverdiend B. Highest rates of infection were detected at Athol, where 2 out of 2 male ticks and 2 out of 2 female ticks tested positive for *E. ruminantium* (Table 3). At Clare A, Clare B, Hlalakahle, Thlavekisa, Thorndale and Utha B none of the sampled adult ticks carried *E.*

ruminantium, as detected by PCR. DNA of *E. ruminantium* was identified in 4 out of 17 (23,5%) pooled nymphal samples (10 nymphs per sample). The PCR assay detected infection in pooled samples from Clare A, Dixie, Hluvukani and Ludlow. Both pooled nymph samples and adult samples recovered from Clare B, Hlalakahle, Thlavekisa, Thorndale, and Utha B tested negative for *E. ruminantium* DNA.

Besides *E. ruminantium, Rickettsia massiliae* was also detected in ticks from several villages. *R. massiliae* was detected in samples from 13 out of 17 villages. In Athol, Clare A, Gottenburg, Hluvukani, Shorty, Thorndale, Utha A, Utha B, Welverdiend A and Welverdiend B adult ticks were tested positive for *R. massiliae*. For Clare B, Seville B and Thlavekisa infection was only detected in pooled nymph samples.

Three out of 23 (13%) adult *A. hebraeum* ticks collected during a previous study by F. van der Steen (2013) tested positive for *E. ruminantium* (Table 4). In addition, 2 out of 17 (11,8%) pooled nymphal samples (10 nymphs per sample) tested positive for *E. ruminantium*. Infected adults were collected from goats in Hluvukani, Shorty and Thlavekisa. The PCR assay detected infection in pooled nymph samples from Ludlow and Seville B. Four samples (1 adult and 3 pooled nymph samples) tested positive for *Rickettsia* catch-all and *Rickettsia massilliae*. Five samples (4 adults and 1 pooled nymph sample) were positive for *Babesia* catch-all 1.

Table 3. *E. ruminantium* infection rates determined by RLB in collected *A. hebraeum* ticks from goats in the area of Mnisi (Mpumalanga), South Africa, during November 2013.

Village	Positive Nymphs	Infected/no. analyzed		
		Male	Female	Overall
Athol	No	2/2	2/2	4/4
Clare A	Yes	0/2	0/2	0/4
Clare B	No	0/2	0/2	0/4
Dixie	Yes	½	0/2	1/4
Gottenburg	No	2/2	1/2	3/4
Hlalakahle	No	0/2	0/2	0/4
Hluvukani	Yes	½	0/2	1/4
Ludlow	Yes	0/2	1/2	1/4
Seville B	No	½	0/2	1/4
Share	No	½	0/2	1/4
Shorty	No	½	0/2	1/4
Thlavekisa	No	0/2	0/2	0/4
Thorndale	No	0/2	0/2	0/4
Utha A	No	½	0/2	1/4
Utha B	No	0/2	0/2	0/4
Welverdiend A	No	½	1/2	2/4
Welverdiend B	No	½	0/2	1/4
Total	4/17	12/34	5/34	17/68

Table 4. *E. ruminantium* infection rates determined by RLB in collected *A. hebraeum* ticks from goats in the area of Mnisi (Mpumalanga), South Africa, during June and July 2013 (Collection by F. van der Steen).

Village	Positive Nymphs	Infected/no. analyzed		
		Male	Female	Overall
Athol	No	-	-	-
Clare A	No	0/1	-	0/1
Clare B	No	-	-	-
Dixie	No	0/1	-	0/1
Gottenburg	No	-	-	-
Hlalakahle	No	-	-	-
Hluvukani	No	1/2	-	1/2
Ludlow	Yes	-	-	-
Seville B	Yes	-	-	-
Share	No	-	-	-
Shorty	No	1/4	0/1	1/5
Thlavekisa	No	1/8	0/1	1/9
Thorndale	No	-	0/1	0/1
Utha A	No	0/2	0/1	0/3
Utha B	No	-	-	-
Welverdiend A	No	0/1	-	0/1
Welverdiend B	No	-	-	-
Total	2/17	3/19	0/4	3/23

5 Discussion

The principal ticks that infested indigenous goats in the area of Mnisi were *A. hebraeum* and *R. (B.) microplus*. This is in agreement with the prior study by F. van der Steen (2013), who reported *A. hebraeum* and *R. (B.) microplus* to be the most abundant species, however, she also encountered small numbers of *R. appendiculatus, R. simus* and *R. zambeziensis*.

Since the larvae of *A. hebraeum* and *R. (B.) microplus* cannot easily be recovered during whole-body collections, the method of tick collection in this survey was biased towards the recovery of nymphs and adults (Bryson et al., 2002a). For this reason the relatively larger number of nymphs and adults compared to the number of larvae collected might not be representative of the host seeking tick population.

In the area of Mnisi, almost 3 times as many male as female *A. hebraeum* were collected (71,5% male and 28,5% female ticks). Several authors have observed accumulation of male *A. hebraeum*, mainly on cattle (Bryson et al., 2002a). The significantly higher number of male adults might be explained by the fact that male *A. hebraeum* ticks usually remain attached for long periods, up to about 6 months on cattle, and consequently outnumbered females considerably (Norval et al., 1991). Female *A. hebraeum* tend to feed rapidly and

detach (Bryson et al., 2002a). The long attachment period enables the male ticks to engage in multiple mating and to act as a source of the aggregation-attachment pheromone. This pheromone allows unfed adults to locate hosts and discriminate between suitable hosts with male *A. hebraeum* ticks already attached and potentially unsuitable hosts without males attached (Norval et al., 1989).

The regulation of the seasonal occurrence of *A. hebraeum* is still only partially understood. During this study, a substantial increase in number of *A. hebraeum* adults has been observed compared to the study by F. van der Steen (2013) during June and July 2013. Up to date, adults of *A. hebraeum* have not been reported to completely disappear at any time of the year at different locations in southeastern Africa (Norval et al., 1991). This reported absence of a consistent pattern of seasonal occurrence of *A. hebraeum* might be explained by the characteristic behaviour of this tick species. It has been reported that unfed adults and nymphs are able to engage in host-seeking regardless of weather conditions since they do not await hosts on vegetation. Instead they emerge from protected microhabitats in response to specific stimuli from hosts such as carbon dioxide (which initiates searching activity in ticks (Norval et al., 1989)) and the aggregation-attachment pheromone emitted by attached males (Norval et al., 1991). The presence of *A. hebraeum* nymphs on goats throughout the year has important implications for endemic stability to *E. ruminantium* infection as both nymphs and adults can transmit infection (Bryson et al., 2002b).

The occurrence of heartwater in the area of Mnisi might be under-reported since definitive diagnoses are often not performed. The definitive diagnosis of heartwater is based on the observation of rickettsial inclusion bodies within endothelial cells in stained brain crush smears of either the cerebrum or the cerebellum. From the questionnaire survey 9 farmers (17%) indicated to have seen pedalling movements before one or several goats died. Pedalling movements are often shown in the final stages of a clinical heartwater infection however, without a definitive diagnosis other causes cannot be excluded. According to the PCR-RLB assay performed during this study, the prevalence of *E. ruminantium* infection was as high as 23,5% in pooled nymphal samples and 25% in adult *A. hebraeum*. However, the accuracy of these estimates might be uncertain since fairly small sample sizes were used (Peter et al., 1999). Besides, the analyzed ticks have been feeding on goats for unknown periods of time. Therefore, the ticks may have acquired or lost infection during feeding. As a result, the prevalence found in this study might not be representative of the host-seeking tick population.

In addition to *E. ruminantium*, *R. massiliae* has also been detected in *A. hebraeum* ticks collected in the area of Mnisi. *R. massiliae* is a worldwide rickettsia that was first isolated near Marseille in 1992. Thereafter it has been detected in several *Rhipicephalus* species. *R. sanguineus, R. turanicus, R. muhsamae, R. lunulatus, R. sulcatus* have been indicated as recognized or potential tick vetors for *R. massiliae* in Africa (Cazorla & Socolovschi, 2008). *A. hebraeum* has not yet been reported as a recognized or potential vector of *R. massiliae* (Chochlakis et al., 2012; Mediannikov et al., 2010; Vitale et al., 2006). The recognition of the human pathogenicity of *R. mass*iliae occurred in 2005. This species can cause fever, diffuse rash and eschars in humans. Two samples, male adult ticks from Clare A and Shorty, produced *Ehrlichia/Anaplasma* catch-all signals without any species-specific signals. These samples would preferably be sequenced to determine if a novel species or strain of *Ehrlichia/Anaplasma* was present.

The outcome of the questionnaire survey is in agreement with previous observations by R. Coetzee (Coetzee, 1998). Coetzee already reported that very few small-scale farmers send goats to abattoirs. The majority of farmers interviewed in this survey (65%) indicated they do not eat goat's meat and goats are mainly kept as a pet, used during traditional ceremonies, and they are seen as animals of financial security (Coetzee, 1998).

Multiple hosts for *A. hebraeum* were present in all villages in the area of Mnisi, which may have been sources of *E. ruminantium* infection. Due to their greater abundance and high tick burdens, cattle are reported to be a dominant source (Peter et al., 1999). In endemically stable areas, cattle are usually sub-clinically but persistently infected (O'Callaghan et al., 1998). Infection with *E. ruminantium* in ticks collected from goats may have been acquired on persistently infected cattle since cattle and goats are often kept together in the area of Mnisi. Wildlife, including small rodents, may also be a reservoir of infection but the relative contribution is difficult to assess as immature *A. hebraeum* feed on a wide range of wildlife many of which are not know to be susceptible to *E. ruminantium* (Horak et al., 1987; Norval et al., 1994; Peter et al., 1999).

Cattle in the area of Mnisi are subject to an intensive tick control programme and are usually dipped weekly at the communal dip-tanks. Because of the long attachment and feeding periods of adult *A. hebraeum*, eradication of this tick species can be achieved by intensive acaricide treatment of cattle (Norval et al., 1994). However, eradication of ticks such as *A. hebraeum* becomes impossible when alternative hosts for the adult stage are present (Norval et al., 1994). Nyangiwe and Horak reported that *A. hebraeum,* adult *R. (B.)*

microplus, Rhipicephalus evertsi evertsi and *R. appendiculatus*, ticks that usually infest cattle (Bryson et al., 2002a), were found on goats in the eastern region of the Eastern Cape Province (Nyangiwe & Horak, 2007). Cattle usually harbour large numbers of both adult and immature ticks whereas goats are often hosts of large numbers of immature ticks and fewer adults (Macivor & Horak, 2003). However, when cattle are regularly treated with an acaricide, goats can become a major potential host species for *A. hebraeum* and can also become infested with large numbers of adult *A. hebraeum* (Macivor & Horak, 2003). Cattle in the area of Mnisi are regularly dipped and vaccinated against diseases when these procedures are subsidised by the government, whereas goats are seldom dipped or vaccinated (Bryson et al., 2002b). This was also observed by Kunene and Fossey (Kunene & Fossy, 2006) who described that small ruminants like goats were rarely dipped by rural farming communities. Whereupon goats can become reservoirs of ticks, ultimately reinfesting the dipped animals. These findings underscore the necessity of including goats in any tick control programme designed for cattle at the same locality (Nyangiwe & Horak, 2007; De Matos et al., 2009).

A. hebraeum is not only vector of *E. ruminantium,* this tick species can also cause serious damage to feet, hides, udders and scrota because of its long mouthparts and its tendency to form clusters (Bryson et al., 2002a). Several authors have described lesions attributable to the attachment of ticks (Hove et al., 2008). MacIvor et al. (1987) reported a significant relation between the seasonal prevalence of foot abscess affecting Boer goats in Valley Bushveld in the eastern Cape Province of South Africa and the seasonal abundance of adult *A. hebraeum*. Studies have implicated the presence of *A. hebraeum* ticks in the inter-digital space as a contributory cause of purulent ulcers and (foot) abscesses accompanied by lameness in goats (Hove et al., 2008). Lesions and abscesses on other parts of the body have also been reported, such as attachment sites around the peri-anal region characterized by non-purulent to purulent ulcerations, and lesions on the posterior lower abdomen, including the udder (Hove et al., 2008). To limit this considerable injury caused by *A. hebraeum,* control programmes must be aimed at strategically controlling these species while simultaneously attempting to maintain endemic stability to the diseases they transmit (Bryson et al., 2002a). As most ticks recovered in this survey were attached to the feet and ventral surfaces of the goats as well as in and on the ears, acaricide applied in a footbath or tick grease applied on the feet would probably provide adequate control and prevent damage (Bryson et al., 2002a). Due to the socio-economic situation of farmers in the area of Mnisi, many cannot afford to buy conventional acaricides. Therefore, some farmers resort to using old engine oil mixed with acaracides to control ticks (Sekokotla, 2004). One farmer visited during this survey, indicated to treat his goats twice a week with a mix of old engine oil and cattle dip. The result was promising (see household comparison, APPENDIX E). The animals were compared to those

of a neighbouring farmer who applied no form of tick control. At each household 5 goats were sampled, a total of 3 *A. hebraeum* adults was found on the treated animals, whereas a total of 45 *A. hebraeum* adults was found on the untreated animals. Obviously, more factors besides the method of tick control can influence the collected number of adults in the prior example. Even though the use of used engine oil is said to be effective in controlling cattle ticks (Moyo & Masika, 2009), it is not a practice that should be promoted. Components in used engine oil can become concentrated in plant and animals tissues and may be toxic when consumed by humans. Moreover, its components can cause lead poisoning in livestock and contaminate the environment. Furthermore, continuous contact with used engine oil has been reported to cause skin cancer in laboratory animals and swallowing can cause stomach cramps and diarrhoea (Moyo & Masika, 2009).

Tick control can only be effective if it fits well with the day-to-day constraints on farmers. Therefore, instead of expensive intensive tick control programmes, integrated tick control strategies for goats seem more appropriate in the area of Mnisi. Acaricides have been an effective method of tick control in the past. Today, however, acaricide resistance is widespread and environmental pollution is a major focus of concern. Complete prevention of disease transmission would interfere with the endemic stability and result in animal populations that are fully susceptible to heartwater and other occurring tick-borne diseases. This means the population is at risk of disease outbreaks whenever tick control measures break down and this can lead to catastrophic losses (Allsopp, 2009; Faburay et al., 2007b). Therefore, any control programme must involve the farmers, have an integrated approach and be aimed at strategically controlling ticks while attempting to maintain endemic stability to the diseases they transmit (Bryson et al., 2002a; Hlatshwayo & Mbati, 2005).

6 Conclusion

This survey shows a substantial increase in the number of adult *A. hebraeum* ticks on indigenous goats in the area of Mnisi (Mpumalanga), South Africa, in late spring/summer compared to a prior survey by F. van der Steen (2013) conducted during winter. In addition, an increased prevalence of *E. ruminantium* infection in nymphs and adult *A. hebraeum* ticks has been observed. As detected by PCR-RLB the prevalence rate was 25% (17 out of 68) in adult *A. hebraeum* ticks and 23,5% (4 out of 17) in pooled nymphal samples collected during November and 13% (3 out of 23) in adult *A. hebraeum* ticks and 11,8% (2 out of 17) in pooled nymphal samples collected during June and July 2013. According to the questionnaire survey 9 out of 53 farmers indicated to have seen pedalling movements, a clinical sign which can also be seen in the terminal stage of a clinical heartwater infection. The long mouthparts of *A. hebraeum* can cause considerable injury to feet, teats and scrotum and can lead to ulceration and abscessation accompanied by lameness. This, in combination with the presence of *A. hebraeum* nymphs and adults on goats throughout the year, an increased prevalence of *E. ruminamtium* infection and the observation of clinical signs that could be an indication for heartwater underscore the necessity of including goats in tick control programmes in the area of Mnisi. However, acaricide treatment applied solely to control tick damage could interfere with a possible endemic stability. Therefore, any tick control programme must be aimed at strategically controlling these tick species while attempting to maintain endemic stability to the diseases they transmit. Tick grease already is a preferred tick control method by farmers in the area of Mnisi. However, before this method can be implemented in a sustainable control program, research into seasonal changes in the relative abundance of *Amblyomma* tick infestations in goats, acaricide resistance and possible effects on the endemic stability in the area of Mnisi are vital. Overall, research into sustainable, cost-effective and practical tick control methods for use in traditional livestock husbandry systems should be intensified.

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9 APPENDIX A

Owner questionnaire

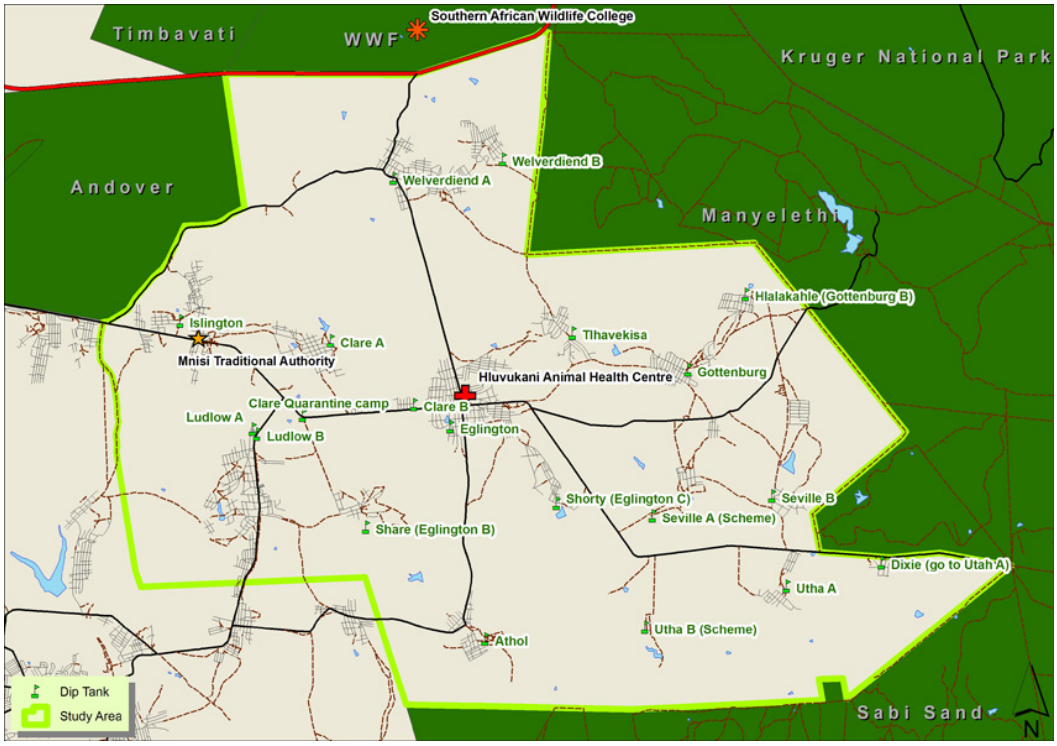
How many goats does farmer own?		
How many goats died during the last year?		

Following signs seen before goat died Yes/No? If yes, how many?		
	Yes	No
Ticks seen on goat		
Inappetance		
Diarrhoea		
Strange behaviour		
Falling on the floor/quick movements with legs/tight muscles/chewing movements just before goat died?		

Has owner slaughtered any goats that suddenly died?		
Noticed 'water' around hart/in thorax (explain!)		

*This questionnaire is meant for the local farmers/owners of goats used for tick collection. The questionnaire has to be explained properly and might have to be translated to local language, depending on the knowledge of the English language of the local goat owners. It is known that other agents can also cause clinical signs similar to those of heartwater. Therefore this questionnaire is only used to get a general idea about the amount of mortalities **possibly** caused by heartwater. Since animals that recovered from a heartwater infection might be even harder to recognize by local famers, these animals are not included in the questionnaire.

10 APPENDIX B



11 APPENDIX C

11.1 DNA extraction of ticks

Utrecht Centre for Tick-borne Diseases (UCTD), laboratory protocols

- To start, turn on a water bath at 56°C and a heating block at 70°C.
- Preheat the BE buffer at 70°C.
- Thaw the proteinase K solution (if needed) and store it at 4°C
- Fill the sonification bath with demineralised water.
- Use filtertip pipettips.

1. Wash the tick in a sonification bath for up to 30 seconds. Check under the microscope whether the surface of the tick is cleaned thoroughly,
2. Put the tick, with a clean forceps, in an eppendorf tube prefilled with 70% ethanol followed by washing in demineralised water,
3. Wash the forceps in 70% ethanol followed by washing in demineralised water,
4. Take the tick from the eppendorf tube and let it dry on clean tissue paper,
5. After the tick has dried, place it in a sterile 2ml tube with 180 µl T1 lysis buffer and freeze the samples at -80 for 15 minutes,
6. Add a 5mm or 7mm, metal bead to the frozen samples,
7. Put the samples in the TissueLyser LT and disrupt the ticks during 3 minutes at 50 oscillations per second,
8. Add 25 µl proteinase K to all samples and vortex,
9. Prelyse the samples during 3 hours in a waterbath at 56°C,
    a. Turn on the heating block at 70°C, i fit is not turned on already
    b. Empty and clean the sonification bath with 70% ethanol.
10. Briefly centrifuge the eppendorf tubes. **1000 x g maximum!**
11. Add 200 µl B3 buffer and vortex,
12. Incubate 15 minutes at 70°C
13. Briefly centrifuge the eppendorf tubes. **1000 x g maximum!**
14. Add 210 µl 96% ethanol and vortex,
15. Briefly centrifuge the eppendorf tubes. **1000 x g maximum!**
16. Transfer the supernatant to a new sterile 1,5 ml eppendorf tube. (Tick parts are allowed to be transferred),
17. Spin the samples at 11.000 x g for 2 minutes,
18. Transfer the liquid onto a spincolom. Avoid pipetting parts of the tick residue as it ccan block the spincolom,

19. Add 500 µl BW buffer and centrifuge the samples at 11.000 x g for 1 minute. Discard the flowthrough,
20. Add 600 µl B5 buffer and centrifuge the samples at 11.000 x g for 1 minute. Discard the flowthrough,
21. Spin the samples at 11.000 x g for 1 minute,
22. Place the spincolum in a sterile eppendorf tube. (Label the eppendorf tube accordingly),
23. Add 100 µl preheated BE buffr directly on the membrane of the spincolum and incubate 1 minute at room temperature,
24. Centrifuge at 11.000 x g for 1 minute,
25. Store the DNA sample at 4°C for use within the next few days or sotre at -20 for long term preservation.

11.2 PCR

- Put frozen DNA samples at 4 °C the day before use (including positive PCR controls).
- Thaw the PCR reagents.
- Turn on the UV in the DNA workstation and flow cabinet for 30 minutes prior to working in them.
- Strictly follow the one way route: clean room!dirty room!PCR room.

PCR reagents for 1 sample are as follows:

5.0 µl	5x Phire reaction buffer
0.5 µl	10mM dNTPs
0.5 µl	F primer (20 pmol/µl)
0.5 µl	R primer (20 pmol/µl)
0.125 µl	2U/µl Phire Hot Start II DNA polymerase
15.875 µl	PCR grade H_2O
y µl (usually 2,5 µl)	cDNA or DNA

End volume of every PCR individual sample is: 25 µl

Leave the polymerase in the freezer until the very last moment.

1. Prepare the PCR mix in an eppendorf tube, multiply the individual reagents by the number of samples. To compensate for pipet accuracy, advisable is that you add 10% additional samples to the total number of PCR samples:

   40 DNA samples + 1 positive PCR control = 41
   41 + 10% = 45 samples.

2. Pipet 22,5 µl of the master mix in every PCR tube, add the leftover mix to an additional tube, which will become the negative PCR control.
3. Close the PCR tubes and clean up everything in the clean room, you are not allowed to come back to the clean room after setting up the PCR.
4. Take the closed PCR tubes to the dirty room and get the DNA samples from the fridge.
5. Pipette 2,5 µl of the DNA sample to the corresponding PCR tube.
6. Pipette 2,5 µl of the positive PCR control, corresponding to the PCR to be performed, to the positive control PCR tube.
7. Vortex and spin down the samples briefly before placing them into the PCR machine.

11.3 Agarose gel electrophoresis

Be careful! Ethidiumbromide is carcinogenic! Wear gloves during all procedures involving the electrophoresis.

Preparation of the gel:

Prepare 1x TAE solution by diluting the 10x stock. (100ml stock solution 900ml demineralized water):

1. Add 2,25 grams of agarose to an erlenmeyer and add 150ml 1x TAE buffer for a large gel. Add 0.563 grams of agarose to an erlenmeyer and add 37.5ml 1x TAE buffer for a small gel.
2. Heat the solution in a microwave until the agarose is fully dissolved.
3. Let the agarose solution cool down until about 60 °C and add 2,5µl ethidiumbromide solution (10mg/ml).

4. Prepare the gel tray by putting the rubber sides on the edges of the tray and place the comb(s).
5. Pour the gel onto the tray (air bubbles can be removed using a pipet tip).
6. When the gel has solidified, the comb(s) can be gently removed and the gel can be placed in the electrophoresis unit.
7. If needed, fill up the 1x TAE level in the electrophoresis unit until it fully covers the gel.

Preparation of the PCR samples

1. Pipette 1µl of the 6x loading buffer a 0,2ml PCR tube or in one of the wells of a 96 well plate.
2. Add 5µl PCR sample to the loading buffer, mix by pipetting and load onto the gel.
3. Pipette 5µl of the DNA marker and load onto the gel.
4. Run the gel for 30-45 minutes and check the gel using the gel-dock system (LabWorks program)

11.4 Reverse Line Blot (RLB) hybridization

Wear gloves and use non-filter pipet tips.

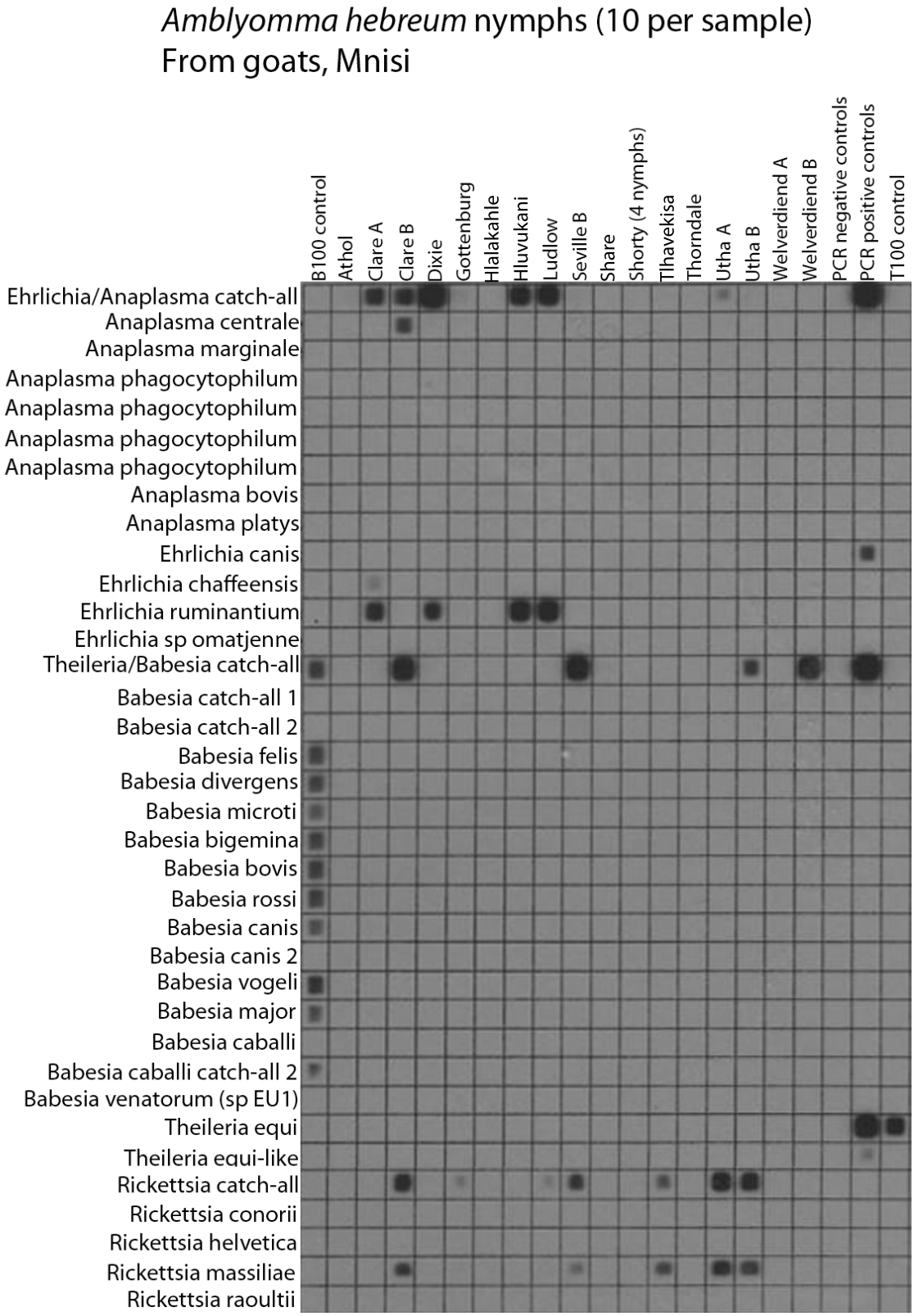
1. Turn on the heating block at 100 °C.
2. Turn on the hybridization oven at 42°C. Preheat 50ml 2x SSPE/0.5% SDS solution in the hybridization oven at the same time.
3. Turn on the waterbath at 50°C. Preheat the 2x SSPE/0.5% SDS solution in the water bath at the same time.
4. Clean working space with 70% ethanol.
5. Combine and dilute the PCR products, per DNA sample, in a 1,5 ml eppendorf tube. Use of every PCR sample 10 µl and add 2x SSPE/0,1% SDS until 160 µl. (For example: 10 µl *Anaplasma/Ehrlichia* PCR + 10 µl *Babesia/Theileria* PCR + 140 µl 2x SSPE/0,1% SDS.)
6. Add 10 µl of the RLB positive controls to 150 µl 2x SSPE/0,1% SDS to give a final amount of 160 µl.
7. Denature the diluted PCR samples at 100 °C during 10 minutes using the heating block and cool down the samples rapidly on ice after. After the samples have cooled down, briefly spin down the samples before opening.
8. Keep the samples on ice.

9. Wash the membrane, during the denaturation step, at room temperature with 2x SSPE/0,1% SDS for 5 minutes under gentle shaking.
10. Place membrane on a support cushion in miniblotter, with slots perpendicular to line pattern of applied probes.
11. Remove residual fluid by aspiration.
12. Fill the slots with the diluted and denaturized PCR samples (150 µl), avoid air bubbles. Fill empty slots with 2 x SSPE/0.1% SDS, to avoid cross flow.
13. Hybridize the blotter at 42°C for 60 minutes in the hybridization oven, without shaking.
14. Turn screws hand tight and remove the samples by aspiration.
15. Remove the membrane from the blotter.
16. Wash the membrane twice with preheated 2x SSPE/0,5% SDS during 10 minutes at 50°C under gentle shaking.
17. Clean the blotter and the support cushion during the washing step. Use the appropriate cleaning product. Do not use ethanol, this will damage the blotter.
18. Incubate the membrane with 50 ml preheated 2x SSPE/0,5% SDS + 5 µl streptavidin during 30 minutes at 42°C in the hybridization oven under gentle shaking. Discard the streptavidin solution in a tube and into the yellow bin. **Do not pour it in the sink.**
19. Turn down the water bath to 42 °C during the streptavidin hybridization with the 2x SSPE/0,5% SDS solution inside. Keep the lid of the water bath open.
20. Wash the membrane twice with preheated 2x SSPE/0,5% SDS during 10 minutes at 42 °C under gentle shaking.
21. Change the water bath temperature to 80 °C and place the 1% SDS solution inside the water bath.
22. Wash the membrane twice with 2x SSPE at room temperature for 5 minutes under gentle shaking.
23. Prepare the film cassette and check if the developing machine is on.
24. Dispose the 2x SSPE solution.
25. Add 10 ml ECL (5 ml ECL1 + 5 ml ECL2) to the membrane and gently shake the membrane by hand until the whole membrane is covered with ECL. Collect the ECL in a tube and dispose in the yellow bin, **do not pour it in the sink.**
26. Place the membrane in foil and place it in the film cassette.
27. Go to the dark room and expose the membrane to the film for 10 minutes.
28. Develop the film using the developing machine.
29. Strip membrane or store membrane in a seal bag wit 20 mM EDTA at 4 °C until stripping.
30. Turn off all equipment and clean working space.

12 APPENDIX D

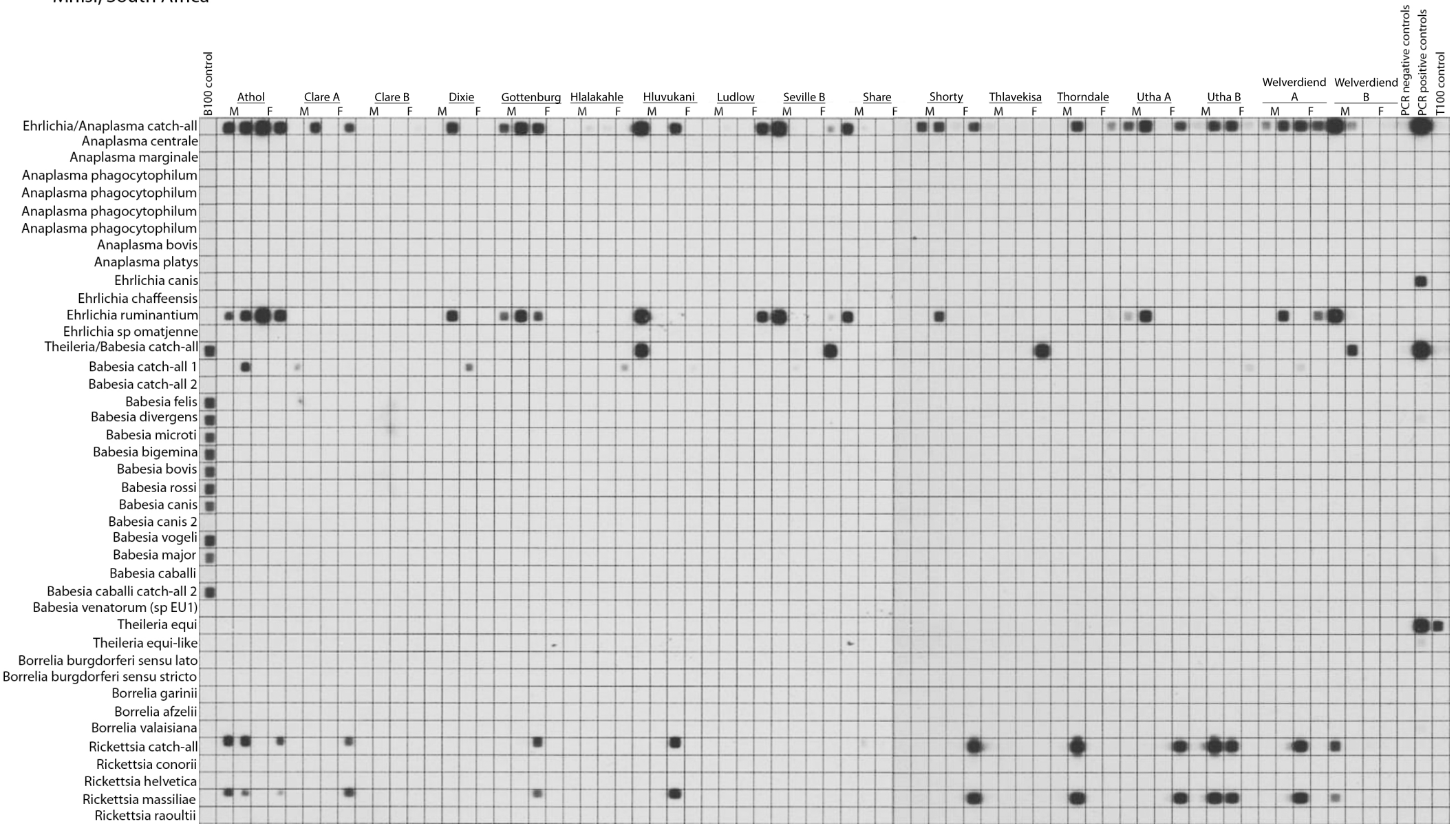
12.1 Results RLB *Amblyomma hebraeum* nymphs and adults

Ticks collected from goats in the area of Mnisi (Mpumalanga), South Africa, during November 2013



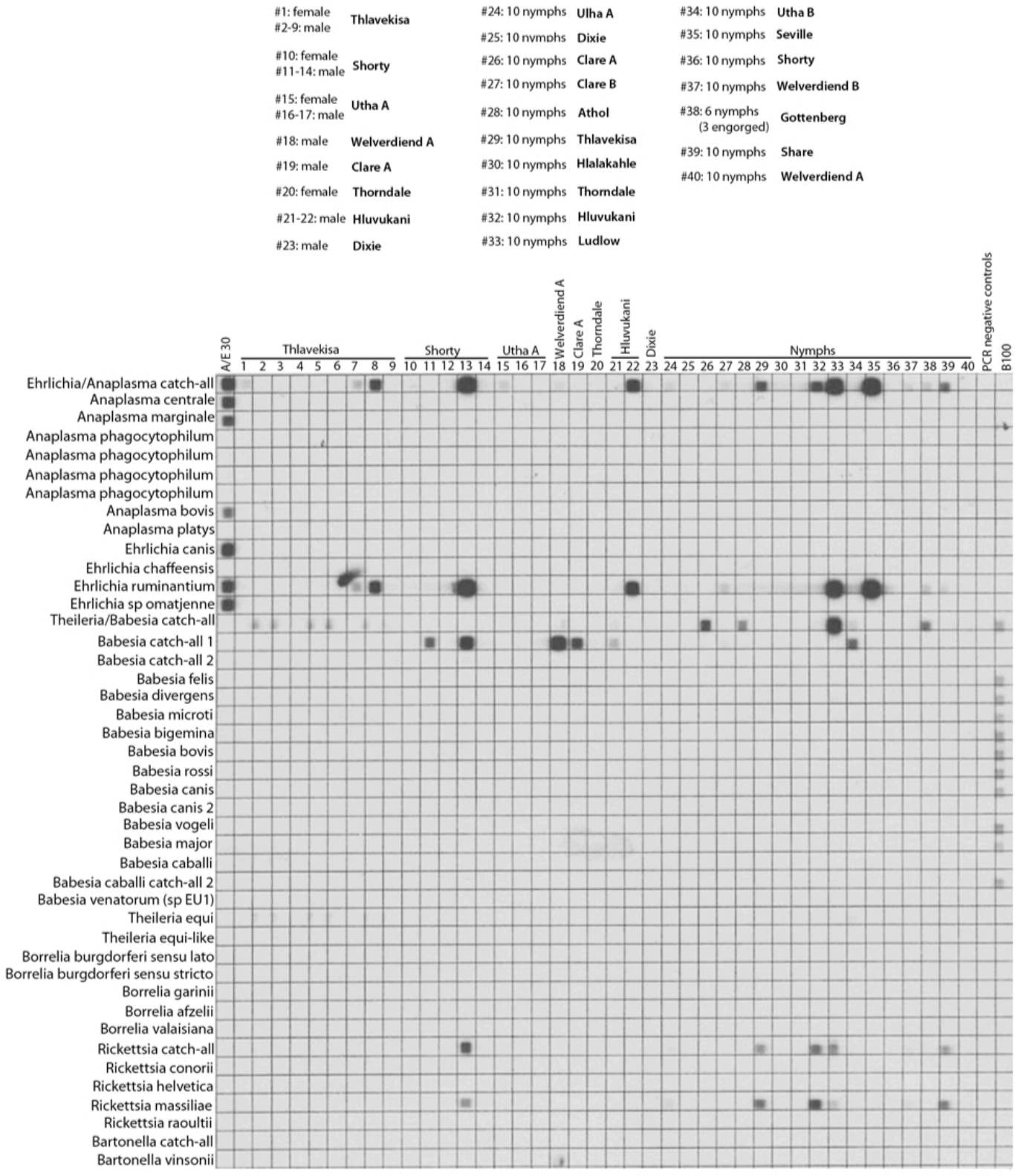
A. hebraeum adults

Mnisi, South Africa



12.2 Results RLB Amblyomma hebraeum nymphs and adults

Ticks collected from goats in the area of Mnisi (Mpumalanga), South Africa, during June and July 2013 (Collection by F. van der Steen)



13 APPENDIX E

Household comparison

Sample date	28-11-2013
Household ID	51
Village	Hlalakahle
Name owner	-
Total number of goats	7
Number of goats sampled (aim for 5)	5
Additional picture ID's	P1100556 - P1100567

Sample date	28-11-2013
Household ID	52
Village	Hlalakahle
Name owner	-
Total number of goats	21
Number of goats sampled (aim for 5)	5
Additional picture ID's	P1100567

Questionnaire:

Does owner do any tick control?	No
What kind of tick control is performed?	-
How often does owner perform tick control?	-
How many goats have suddenly died during the last year?	1
Has owner seen pedalling movements just before goats die?	No
Does owner describe any other signs that might be characteristic for Heartwater?	-
Have any of the animals been treated during the last year?	-
How have these animals been treated and how often?	-
Would owner be interested in new methods of tick control?	-

Does owner do any tick control?	Yes
What kind of tick control is performed?	Dip (Delete) + used engine oil on feet
How often does owner perform tick control?	2 times a week
How many goats have suddenly died during the last year?	-
Has owner seen pedalling movements just before goats die?	21
Does owner describe any other signs that might be characteristic for Heartwater?	5
Have any of the animals been treated during the last year?	P1100567
How have these animals been treated and how often?	-
Would owner be interested in new methods of tick control?	Yes, very interested in footbath if more dip available

Additional comments (e.g comments on housing of the animals, animal management, extraordinary number of ticks on certain body parts other than feet):

Household 51

- Small housing
- Many goats on a small area
- Unhygienic housing
- 5/5 goats had one or more infected feet
- Goats are not checked and no control is performed
- Owner does not express interest in tick control
- Many lame goats, mostly because of infected feet

Household 52

- Spacious housing
- Owner seems knowledgeable, already dips twice a week and is interested in other tick control methods if sufficient dip is available
- Only 1 of the sampled goats has infected feet
- Goats are dipped and checked twice a week
- Sampled goats are very clean, not many ticks are found
- Some ticks are found on parts of the body other than the feet e.g. inguinal area and sides of the animal
- No lame animals seen during sampling

Tick identification household 51:	
Amblyomma Adults	45 (30 Male, 15 Female)
Amblyomma nymphs	31
Amblyomma larvea	11

Tick identification household 52:	
Amblyomma Adults	3 (1 Male, 2 Female)
Amblyomma nymphs	40
Amblyomma larvea	2

*Left household **51**, right household **52***

*Infected foot at household **51***