Seasonal dynamics of *Amblyomma hebraeum* ticks on goats in the Mnisi Area (Mpumalanga), South Africa.

Towards a sustainable tick control policy

By Iris Anne Deetman, June/July 2014

Department	Utrecht Centre for Tick-borne Diseases (UCTD), FAO Reference Centre for Ticks and Tick-borne Diseases, Faculty of Veterinary Medicine, Utrecht University, The Netherlands.
Faculty supervisor	Prof. dr. F. Jongejan (Email: F.Jongejan@uu.nl) Utrecht Centre for Tick-borne Diseases (UCTD), FAO Reference Centre for Ticks and Tick-borne Diseases, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 1, 3584 CL Utrecht, The Netherlands, and, Department of Veterinary Tropical Diseases, Faculty of Veterinary Medicine, University of Pretoria, Private Bag X04, Onderstepoort 0110, Republic of South Africa.
External supervisor	Dr. H. Stoltsz (Email: Hein.Stoltsz@up.ac.za) Department of Veterinary Tropical Diseases, Faculty of Veterinary Medicine, University of Pretoria, Private Bag X04, Onderstepoort 0110, Republic of South Africa.

Table of contents

Abstract

The area of Mnisi (Mpumalanga), South Africa is a heartwater endemic area. Heartwater is a disease of all domestic ruminants, caused by the rickettsia *Ehrlichia ruminantium*, transmitted by ticks of the genus *Amblyomma*. Due to a mortality rate up to 90%, heartwater limits import possibilities and development of lifestock. Also direct effects of the ticks, like inflammation of the skin and abscesses resulting in lameness are found to be a problem in the Mnisi area.
This study was performed in March and April 2014, as a continuation of previous studies performed by F. van der Steen and S. Busser in June, July and November 2013.
2884 ticks were collected from 169 goats in 17 different villages in the area of Mnisi, South Africa. The predominant tick was *Amblyomma hebraeum*, followed by *Rhipicephalus (B.) microplus.*
By performing a PCR/RLB , infection rates of *E. ruminantium* could be detected.
47% of the nymphs and 7,8% of the adults turned out to be positive for *E. ruminantium*, indicating the presence of heartwater in the Mnisi area.
The presence of clinical cases of heartwater is likely, since the occurrence of pedaling movements in goats before death were described by the residents.
Since this research was performed in June and July 2013, November 2013 and March and April 2014, some things can be said about the seasonal dynamics. Only 23 adult *Amblyomma* ticks were found in the winter months (June and July 2013), whereas the summer months (November 2013) peaked with 522 adults. Most larvae were present in winter and the number of nymphs did seem to correlate to different seasons. Some seasonal fluctuations were observed, however, for the implementation of a sustainable intervention plan, more research in the remaining months is required.

1. Introduction

Ticks and tick-borne diseases have a negative impact on the development of livestock industries resulting in loss of food and income for humans in developing countries {De Castro 1997}.

Heartwater (also known as Cowdriosis) is a common disease in some wild, and all domestic, ruminants in sub-Saharan Africa, Madagascar and in some Caribbean Islands {Allsopp 2010, Vachiéry 2006}. The disease is caused by the rickettsia *Ehrlichia ruminantium* (formerly known as *Cowdria ruminantium*), an intracellular gram-negative bacterium, transmitted by ticks of the genus *Amblyomma* {Allsopp et al. 2007a}. The presence of heartwater in Sub-Saharan Africa is a major obstacle to upgrade local stock, since the introduction of high producing animals is risky {Allsopp 2010}. Introduction of fully susceptible animals from Heartwater-free to endemic areas causes serious disease, death and therefore great economic loss. Mortality rates of 50 to over 90% are found in nonindigenous goats and sheep {Stuen, Longbottom 2011a}.

Four clinical forms of heartwater exist: a peracute, acute (most common), subacute and subclinical form. Infected animals can show clinical signs such as sudden high fevers, neurological signs, tachypnea and abdominal breathing, due to pulmonary edema and hydro pericardium. In severe cases of infection, goats are lying flat, experience convulsions and can die within 24 hours {Vachiéry et al. 2006}.

When animals recover from an infection with *E. ruminantium*, they become carriers and can serve as a reservoir. Beside domestic ruminants, some wildlife can also play a role in the disease transmission, through subclinical infections {Peter et al., 1999}. The original reservoir of *E. ruminantium* are thought to be wild African ruminants, mainly the blesbuck, African buffalo, black wildebeest and eland (Wesonga et al. 2001, Allsopp et al. 1999, Allsopp 2010). Since the area of Mnisi borders the Kruger National Park, this could be of significance to farmers in this area.

In June, July, and November 2013, the relative abundance of *Amblyomma* tick infestations in relation to the prevalence of heartwater (*E. ruminantium*) in *Amblyomma* ticks in goats in the Mnisi Area (Mpumalanga), South Africa, was examined. In June and July 2013, the winter period, few adults (n=23) of *A. hebraeum* ticks were collected, whereas large numbers of adults were found during the summer in November (n=522). The number of nymphs was comparable in these different seasons, namely 1267 nymphs in June and July and 1430 nymphs in November 2013. After use of PCR and reverse line blot (RLB) hybridization, both studied showed a substantial percentage of *E. ruminantium* positive outcomes (13% of the adults and 11.8% of the nymphs in winter vs 25% of the adults and 23.5% of the nymphs in summer {Steen van der 2013, Busser 2014}. This shows that the causative agent of heartwater is present in ticks infesting goats, which justifies further research on the seasonal impact of *Amblyomma* ticks and heartwater on the wellbeing of goats in the Mnisi area.

Beside the descriptions of clinical signs, similar to those found in goats infected with *E. ruminantium*, were made by owners, additional tick-related problems became evident. Direct harmful effects caused by ticks were clearly present; long mouthparts of ticks can cause considerable tissue damage, resulting in irritation, hypersensitivity, inflammatory reactions, edema, ulceration, abscesses and lameness in livestock {Schwalbach 2003}. Especially in the summer (November 2013), lameness was common in the area of Mnisi, due to heavy tick infestations. Most abscesses were found in the interdigital space, which correlates with findings from *Schwalbach* (2003). Other predilection places of *A. hebraeum* were the perineum, udder and scrotum.

Towards the end of the rainy season, from the 5th of March until the 8th of April 2014, this study was continued with standardized protocols, in order to obtain fully comparable results with future studies. After identification of the newly collected ticks, DNA extraction,

PCR and RLB was performed at the Utrecht Centre for Tick-borne Diseases (UCTD), Utrecht University.

Seasonal changes and geographical distribution concerning tick infestations and the presence of *E. ruminantium* in these ticks can be analyzed, since this study is being continued in different seasons throughout the year. Hopefully, by combining the information, a better insight in the epidemiology of heartwater in the area of Mnisi arises. Beside this, it is important to focus on the direct damage caused by the ticks, resulting in severe infections and widespread lameness. Before a sustainable intervention strategy concerning tick prevention can be implemented, seasonal dynamics of *A. hebraeum* ticks on goats must be studied.

2. Research questions

2.1 Main research question

"What are the seasonal dynamics of *Amblyomma hebraeum* ticks in goats in the Mnisi Area (Mpumalanga), South Africa?

2.2 Sub-questions

Answers on the following sub-questions were obtained by a questionnaire survey for the residents.

- What can be said about the health of the goats, is it likely that forms of heartwater occur in the area of Mnisi?
- What measurements are used concerning tick prevention and therapy-possibilities?
- Are the residents aware of the direct and indirect problems the ticks are causing?
- Are the owners interested in implementing practical solutions for tick prevention?
- What can be concluded by comparison of the research results of the three studied seasons?

3. Material and methods

3.1 Study area

The study area is a rural area covering about 29500 hectares of ground, situated in the north-eastern corner of the Bushbuckridge Municipal Area. About 40.000 people live in the Mnisi area, divided over 8.555 households. Livestock like cattle, sheep, goats, pigs, donkeys and chickens are owned and estimated 6186 goats are kept in 917 households {Kriek 2009}.

In 2008, the Mnisi Community Programme (MCP) is established. The MCP is a multidisciplinary platform, creating possibilities for research, learning, teaching and community engagement {Website: The Mnisi Community Programme}. The program has a close relationship with the Mnisi Traditional Authority and the University of Pretoria (UP) and it focuses on the "One Health" philosophy, involving animal health, public health, environmental health, wildlife/livestock interactions and socio-economics {Kriek 2009}. Ticks from goats in 17 different villages in the study area were collected and examined in this survey. The villages in which sampling took place were the same as the villages sampled during previous studies from F. van der Steen and S. Busser, namely Athol, Clare A, Clare B, Dixie, Gottenburg, Hlalakahle, Hluvukani, Ludlow, Seville B, Share, Shorty, Thlavekisa, Thorndale, Utha A, Utha B, Welverdiend A and Welverdiend B.

3.2 Study animals

Ticks were collected from 169 different goats in 34 households. After permission of the owner of the household, ticks were collected from a standardized number of 5 goats per households and 2 households per village. Due to a lack of goats in Share, one sampled household only had 4 goats instead of the standardized 5 goats.

3.3 Tick collection

Together with an Environmental Monitor (EM) the villages were visited for tick collection, where the EM served as an assistant and an interpreter. Ticks were collected from the whole body of the goat. The schedule and order of the sampled villages was discussed with the local veterinarian, since possible risk of spreading Foot and Mouth Disease (FMD) had to be limited. Collected ticks were stored in bottles containing 70% ethanol. The bottles were labeled with date, village, host and tick species. GIS (Geographic Info System) data was used for recordings of coordinates in the field. With these coordinates, geographical distribution and owners can be registered more accurately. Also, the number of sampled goats (if other then 5) was recorded. From March and April 2014, ticks were collected from a standardized number of 10 goats per village. In previous studies, no standardized number of examined goats was agreed. In order to compare the three studies, a mean number of *A. hebraeum* adult ticks per host was calculated.

3.4 Tick identification

After collection of the ticks, identification with a stereoscopic microscope took place at the Hans Hoheisen Wildlife Research Station. Only the *A. hebraeum* ticks were taken for further examination, ticks other than the *Amblyomma* genus were only counted after identification.

3.5 Questionnaire survey

A questionnaire for the residents was conducted in order to create insight in the presence and awareness, as well as knowledge of ticks and tick-borne diseases. Questions focusing on tick prevention, heartwater signs and foot problems were added, to get a clearer view of the current situation of the wellbeing of the goats in the Mnisi Area (Appendix A).

3.6 Detection of *E. ruminantium* in ticks

Further examination of the *A. hebraeum* ticks and their pathogens happened at the UCTD, Utrecht University. After DNA extraction, the presence of pathogens in the DNA was simultaneously detected and quantified by PCR, while the reverse line blot (RLB) hybridization detected different genera of tick-borne pathogens {Alessandra, Santo 2012}. The PCR/RLB protocol described by *Bekker et. Al.* (2002) was used for the detection of *E. ruminantium.*

3.7 DNA extraction

From every village, 10 pooled nymphs were randomly used for DNA extraction. Beside the nymphs, two partly engorged males and two partly engorged female adult *A. hebraeum* ticks were analyzed per village. Only in Hluvakani (1 male), Ludlow (1 male, 1 female), Thorndale (2 males, 1 female), Thlavekisa (1 male), Utha B (1 male) and Welverdiend A (3 males), smaller numbers of adult ticks were tested, due to a lack of adult *A. hebraeum* ticks. UCTD protocols from 2013 were used for the DNA extraction, included in APPENDIX B (UCTD, 2013).

3.8 Polymerase Chain reaction

The UCTD protocols from 2014 (UCTD, 2014) were used for the PCR assay. In total, 68 DNA samples were analyzed, of which 17 samples were obtained from 10 pooled nymphs in each sample, and 51 samples contained DNA of adult ticks. As described earlier, a lack of adult ticks in 6 villages limited the amount of adult samples. Each sample was tested for *Ehrlichia/Anaplasma* and *Theileria/Babesia* species. For *Ehrlichia/Anaplasma*, 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) were used. These primers are degenerate primers, with M = A+C and Y = C+T (UCTD, 2013). The primers used for *Theileria/Babesia* species were 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) (UCTD, 2014). Negative and positive control samples have been analyzed with Agarose gel electrophoresis.

3.9 Reverse line blot hybridization (RLB)

Simultaneous detection and identification of different tick-borne pathogens is possible with reverse line blot hybridization (RLB). In this RLB assay, multiple samples were analyzed against several *Anaplasma, Babesia, Ehrlichia* and *Theileria* species (Bekker et al, 2002). The 68 samples were added to 2 blots (miniblotter). 1 blot was filled with 17 samples of nymphs and 23 samples of adult ticks. The remaining 28 adults were added to a second blot. In both blots, a *Anaplasma/Ehrlichia* 30 RLB positive, a negative PCR control and a B100 RLB control was added. A positive PCR control was added to the second blot, since there was some extra space. In appendix B, the full RLB protocol as performed in this study is described (UCTD, 2013).

4. Results

4.1 Tick collection

2884 ticks were collected by full body examination from 169 goats in 17 different villages in the area of Mnisi, South Africa. Most present was *Amblyomma hebraeum* (861 larvae, 1272 nymphs and 219 adults) and *Rhipicephalus (B.) microplus* (17 larvae, 295 nymphs and 202 adults). This is in accordance with previous studies from F. van der Steen and S. Busser, who mainly found *Amblyomma hebraeum*, followed by *Rhipicephalus (B.) microplus. Rhipicephalus appendiculatus* was the third spp. found in March and April 2014 with 21 adults. F. van der Steen also found *Rhipicephalus appendiculatus* and some *Rhipicephalus simus* and *Rhipicephalus zambeziensis* in June/July 2013.

During the end of the sampling period in April 2014, fewer adult *Amblyomma hebraeum* ticks were collected, whereas the numbers of *A. hebraeum* larvae drastically increased. In the first two weeks of sampling (5th of March until the 18th of March), an average of 17.8 adults and 54.4 *A. hebraeum* nymphs were collected per village, whereas this was an average of 3.8 adults and 112.3 nymphs in the last two weeks (sampled from 31th of March until the 8th of April). Also the numbers of larvae increased towards the winter period. Numbers of ticks per household varied greatly, but in March and April 2014, an average number of 84.3 ticks per household with an average number of 17 ticks per goats was found.

As mentioned before, a mean number of *Amblyomma hebraeum* adult ticks per host was calculated in order to compare the three studies. November 2013 peaks with a mean number of 2,8 adult *A. hebraeum* ticks per goat. In June and July 2013, a mean number of 0,2 *A. hebreaum* adults/host was found, whereas the mean number of adult *A. hebraeum* ticks/host was 1,3 in March and April 2014. Exact numbers of the collected ticks are shown in the tables below. An overview of the relative abundance of *A. hebraeum* adults is shown in fig. 1.

Most adult *A. hebraeum* ticks were attached in the interdigital space, often with inflammatory reactions, abscesses and necrotic ulcers, frequently resulting in lameness. Other predilection places of adult *A. hebraeum* ticks appeared to be around the feet, on the udder, scrotum, axilla and perineal region. Most larvae were found around the feet and on the head. *Rhipicephalus (B.) microplus* was mainly found in and around the ears of goats.

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*			
Athol (N=11)	0	3	0	0	0	23	236	262	0
Clare A (n=3)	1	16	1	0	0	21	60	99	0,3
Clare B (n=3)	0	1	0	0	0	32	77	110	0
Dixie (n=1)	1	12	0	0	0	94	240	347	1
Gottenburg (n=2)	0	0	0	0	0	14	2	16	0
Hlalakahle (n=14)	0	0	0	0	0	115	85	200	0
Hluvukani (n=7)	2	4	0	0	0	70	92	168	0,3
Ludlow (n=8)	0	11	0	0	0	38	55	104	0
Seville A (n=3)	0	1	0	0	0	59	0	60	0
Share (n=11)	0	2	0	0	0	35	77	114	0
Shorty (n=14)	5	1	0	0	1	72	13	92	0,4
Thlavakisa (n=8)	9	0	0	0	0	135	62	206	1,1
Thorndale (N=4)	1	1	0	0	0	36	38	76	0,3
Utha A (n=4)	3	30	0	0	0	327	650	1010	0,8
Utha B (n=5)	0	2	0	0	0	46	31	79	0
Welverdiend A (n=14)	1	71	0	1	1	113	108	295	0,1
Welverdiend B (n=6)	0	6	0	0	0	37	106	149	0
Total (n=117)	**23**	**161**	**1**	**1**	**2**	**1267**	**1932**	**3387**	**0,2**

Table 1. Species composition and total number of ticks collected from goats in the area of Mnisi (Mpumalanga), South Africa during June/July 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*	*Rhipicephalus (B.) microplus*			
Athol (N=10)	13	3	0	0	0	102	2	48	168	1,3
Clare A (n=7)	32	0	0	0	0	16	0	17*	65	4,6
Clare B (n=10)	34	0	0	0	0	51	0	5	90	3,4
Dixie (n=7)	9	0	0	0	0	30	0	3	42	1,3
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
Hluvukani (n=10)	15	0	0	0	0	102	0	46	163	1,5
Ludlow (n=6)	6	11	0	0	0	114	2	24	157	1
Seville A (n=13)	16	4	0	0	0	62	0	66	148	1,2
Seville B (n=10)	45	0	0	0	0	97	0	68	210	4,5
Share (n=5)	48	4	0	0	0	17	0	3	72	9,6
Shorty (n=14)	17	4	0	0	0	20	3	9	53	1,2
Thlavekisa (n=15)	71	0	0	0	0	304	0	85	460	4,7
Thorndale (n= 13)	37	1	0	0	0	156	1	97	292	2,8
Utha A (n=7)	33	0	0	0	0	50	0	46	129	4,7
Utha B (n=7)	9	0	0	0	0	68	0	28	105	1,3
Welverdiend A (n=9)	22	1	0	0	0	67	1	10	101	2,4
Welverdiend B (n=13)	8	0	0	0	0	28	0	34	70	0,6
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 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*	*Rhipicephalus (B.) microplus*			
Athol (N=10)	16	117	1	0	0	182	152	526	994	1,6
Clare A (n=10)	11	9	3	0	0	51	6	12	92	1,1
Clare B (n=10)	13	9	0	0	0	40	13	1	76	1,3
Dixie (n=10)	19	4	0	0	0	49	0	1	73	1,9
Gottenburg (n=10)	29	1	0	0	0	62	1	10	103	2,9
Hlalakahle (n=10)	5	1	0	0	0	55	0	3	64	0,5
Hluvukani (n=10)	1	0	3	0	0	254	2	58	318	0,1
Ludlow (n=10)	2	5	2	0	0	130	9	97	245	0,2
Seville B (n=10)	14	4	5	0	0	53	9	0	85	1,4
Share (n=9)	25	14	2	0	0	59	26	4	130	2,8
Shorty (n=10)	17	30	0	0	0	39	61	54	201	1,7
Thlavekisa (n=10)	1	0	0	0	0	42	3	21	67	0,1
Thorndale (n= 10)	2	0	0	0	0	77	0	8	87	0,2
Utha A (n=10)	60	1	2	0	0	37	0	0	100	6
Utha B (n=10)	1	5	1	0	0	78	5	71	161	0,1
Welverdiend A (n=10)	2	2	2	0	0	42	5	6	59	0,2
Welverdiend B (n=10)	1	0	0	0	0	22	0	6	29	0,1
Total (n=169)	**219**	**202**	**21**	**0**	**0**	**1272**	**292**	**878**	**2884**	**1,3**

Table 3. Species composition and total number of ticks collected from goats in the area of Mnisi (Mpumalanga), South Africa during March/April 2014.

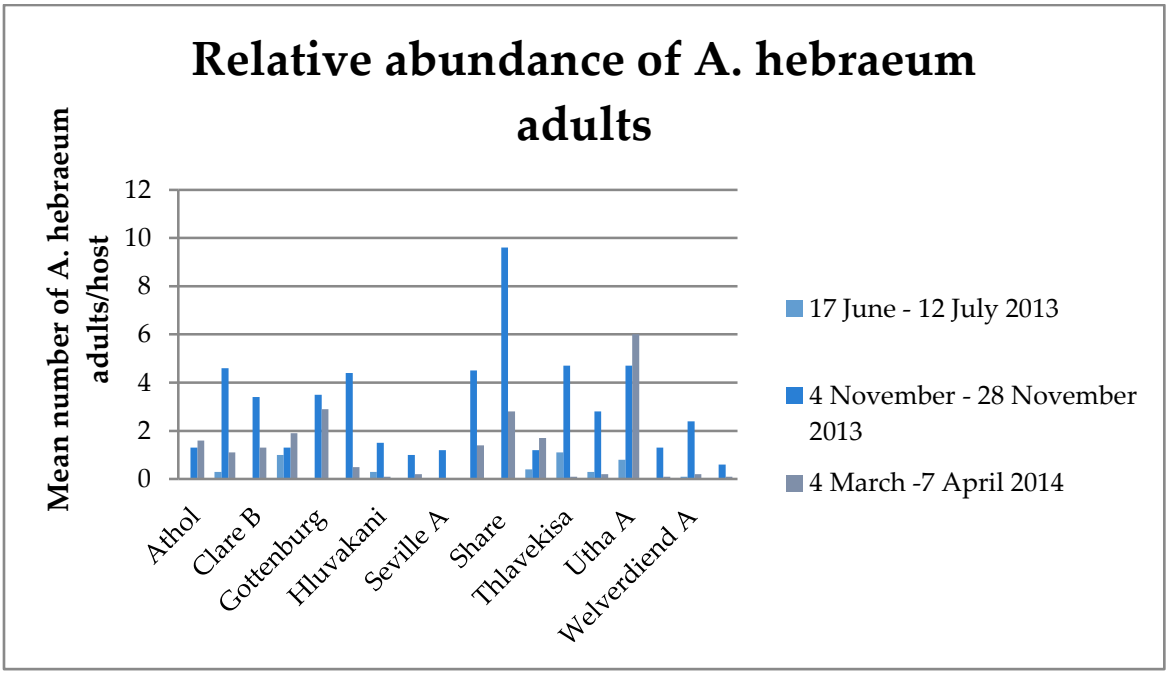


Fig 1. An overview of the relative abundance of *Amblyomma hebraeum* in 3 different seasons.

4.2 Questionnaire survey

In June and July 2013, 55% of the participants of the questionnaire was experiencing problems with ticks, whereas in March and April 2014, this percentage was 85%. When focusing on tick control measures, 37% of the residents was using some form of tick control for their goats in June-July 2013, and 21% in November 2013. In March and April 2014 however, 85% of the residents indicated to use some form of tick control. Also the awareness of ticks as possible vectors for diseases has increased: in June and July 2013, 16% of the owners were familiar with the harmful effects of ticks, which increased to 53% of the questioned residents in March and April 2014 {Steen van der 2013, Busser 2014}. A comparison of the three studies is shown in fig. 3.

As mentioned earlier, 85% of the owners stated to use some form of tick prevention. In fig. 4, the different tick control methods are pointed out. Most popular was a cattle dip (Delete X5), the same dip used at the dip tanks in the Mnisi area. Most residents (71 %) were willing to adopt new tick control methods when available.

35% of the owners experienced death of their goats without visible signs of heartwater. Most common causes of death were diarrhea, dog attacks and worms. 21% of the owners had seen pedaling movements in their goats before death.

67% of the owners observed lameness in their goats and 53% was able to treat an animal when lame. Treatment options varied from cleaning (5%), cleaning and/or the application of wound spray/dip (72%), used engine oil (18%) and the use of terramycine (5%).

In 2 households, Jeyes Fluid was used as a tick preventive method. Jeyes Fluid is an aggressive disinfectant and outdoor cleaning agent, which is harmful for animals and causes severe skin problems. The owners were made aware of the harmful effects and were given advice for other products. Severe skin problems were found in these households as shown below in fig. 2.

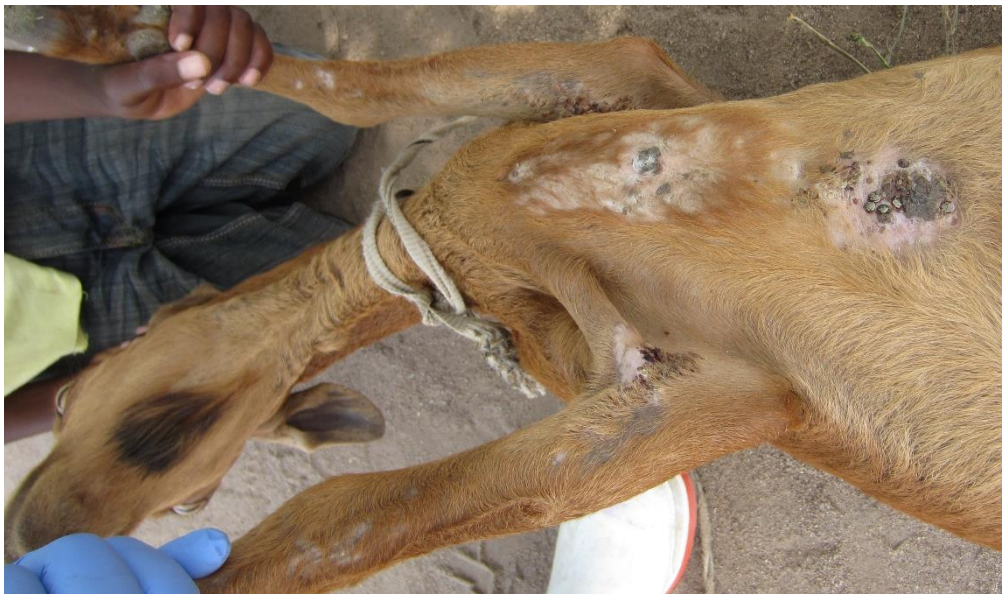


Fig. 2. Skin problems due to the use of Jeyes Fluid as a tick control method. Ticks were abundantly present in the affected areas.

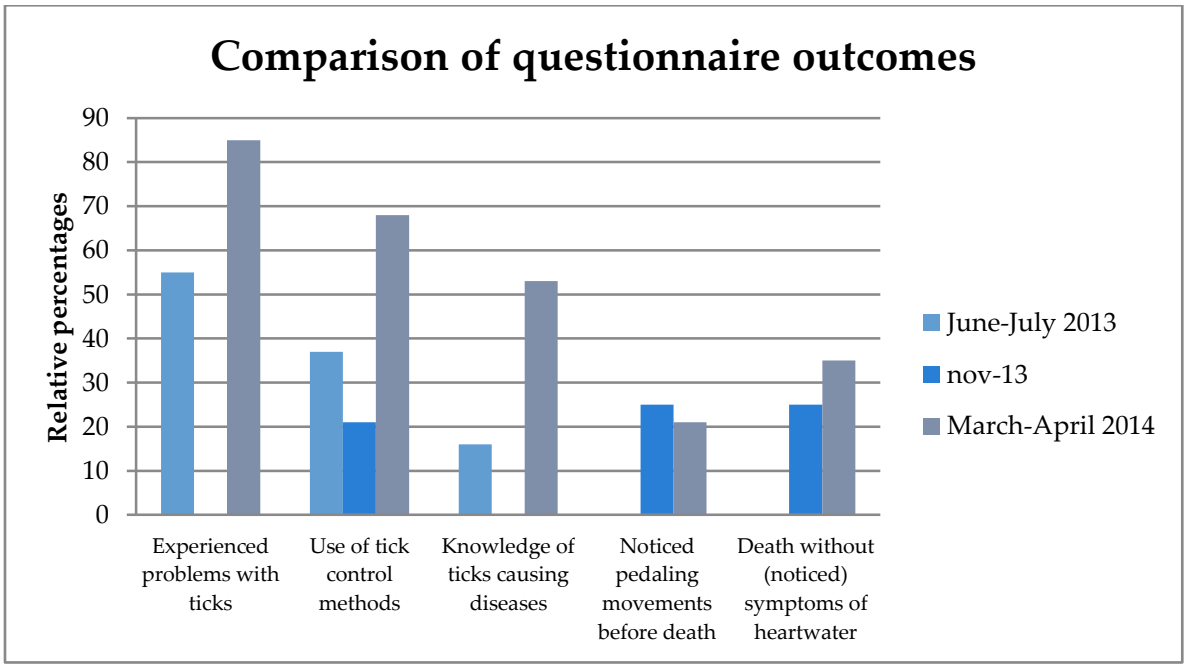


Fig. 3. A comparison of the questionnaire outcomes in three different seasons.

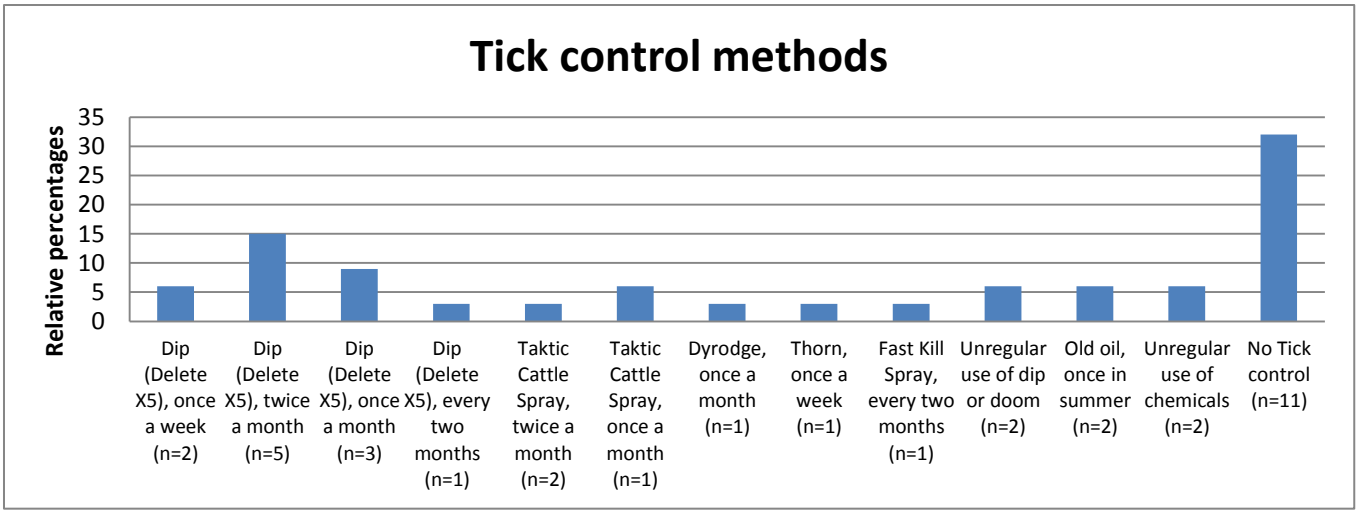


Fig. 4. The different tick control methods, used in March and April 2014.

4.3 Polymerase Chain Reaction (PCR)/ Reverse Line Blot hybridization (RLB)

The negative and positive control groups were as expected. Due to a mistake in the procedure of DNA-extraction, the samples from Utha B and Welverdiend A were accidentally combined and devided over 2 tubes. Therefore, these villages are not included in the results from the pooled nymphs. 46.7% (7/15) of the samples from the pooled nymphs turned out to be positive for *E. ruminantium*. From the tested adults, 3.9% (2/51) of the females was tested positive for *E. ruminantium* and 5.9 % (3/51) of the males. In total, 9.8% of the tested adults was positive for *E. ruminantium* (5/51). The total average infection rate of *E. ruminantium* was 17,6%. Some test results in the blotter were indecisive and are therefore not included in the results as positive.

In June and July 2013, 11.8% (2/17) of the nymphs had a positive outcome and 13% (3/23) of the adults were test positive. The average of the *E. ruminantium* infection rate in June and July 2013 was 12.5% {Steen van der 2013}. In November 2013, 23.5% (4/17) of the nymphs and 35% (17/68) of the adults was found positive for *E. ruminantium*, with an average of 24.7% *E. ruminantium* positive ticks {Busser 2014}.

Other pathogens found by performing the RLB were *Ehrlichia canis*, *Babesia* catch-alls, *Theileria/Babesia* catch-alls, *Rickettsia* catch-alls and *Rickettsia massiliae* positive outcomes. Apart from the *E. canis*, these outcomes correlate with previous studies from F. van der Steen and S. Busser in 2013 {Steen van der 2013, Busser 2014}. The exact RLB results are found in Appendix C. In table 4, the RLB results from the three different seasons are pointed out.

	Pos. Nymphs June/July '13	Pos. Nymphs November '13	Pos. Nymphs March/April '14	Adults June/July '13 (%)	Adults November '13 (%)	Adults March/April '14 (%)
Athol	No	No	Yes	/	100%	25%
Clare A	No	Yes	Yes	0%	0%	0%
Clare B	No	No	Yes	/	0%	0%
Dixie	No	Yes	No	0%	25%	0%
Gottenburg	No	No	No	/	75%	50%
Hlalakahle	No	No	No	/	0%	0%
Hluvakani	No	Yes	No	50%	25%	0%
Ludlow A	Yes	Yes	Yes	/	25%	0%
Seville A	Yes	/	/	/	/	/
Seville B	/	No	No	/	25%	0%
Share	No	No	No	/	25%	50%
Shorty	No	No	No	20%	25%	0%
Thlavekisa	No	No	Yes	11%	0%	0%
Thorndale	No	No	Yes	0%	0%	0%
Utha A	No	No	No	0%	25%	0%
Utha B	No	No	Yes	/	0%	0%
Welverdiend A	No	No	Yes	0%	50%	0%
Welverdiend B	No	No	Yes	/	25%	0%
Total	11.8% (2/17)	23.5% (4/17)	47.1% (8/17)	13.0% (3/23)	25.0% (17/68)	9.8% (5/51)

Table 4. The RLB results from June/July 2013, November 2013 and March/April 2014.

5. Discussion

From the 5th of March until the 8th of April 2014, ticks were collected in 17 different villages. This was a relatively long period of collecting, which started in the rainy season and finished at the start of winter. This could be of influence on the results, particularly on the stage in the lifecycle of the ticks. At the end of the sampling period, fewer adult *Amblyomma hebraeum* ticks were collected, whereas the numbers of *Amblyomma* larvae drastically increased. In the first two weeks of sampling (5th of March until the 18th of March), an average of 17.8 adults and 54.4 *Amblyomma* nymphs were collected, whereas this was an average of 3.8 adults and 112.3 nymphs in the last two weeks (sampled from 31th of March until the 8th of April). Also the numbers of larvae heavily increased towards the end of the sampling period. However, these details could provide more insight in the seasonal dynamics.

Despite the fact that large numbers of ticks were collected, only a relatively small amount of ticks was used for the determination of pathogens by PCR/RLB. From every village, 10 pooled nymphs were tested, which increased the chance of positive outcomes. When possible, 2 female adults and 2 male adults were tested from every village, which gives a more detailed outcome than the pooled nymphs. However, samples from some villages did not include 4 adult *A. hebraeum* ticks. Although all the present adult ticks were tested in these cases, the analysis was limited.

During the performance of the DNA-extractions, 2 samples of pooled nymphs of 2 different villages (Utha B and Welverdiend A) were accidentally combined and devided over

2 tubes. Since these samples ended up being positive, no reliable conclusion can be made from this outcome. Either both or one of the two villages was positive. Therefore, there samples were not included in the results.

Of the 219 collected *A. hebraeum* adults, 72.2% were males. This can be explained by the feeding behaviour of *A. hebraeum:* Female ticks detach after a short period of feeding, while male ticks attach for longer periods. This way, male adults can mate multiple times on one host. Moreover, it extends the period for the secretion of an attraction/aggregation- and attachment pheromone, in order to assist unfed nymphs and adults in their search for a host. {Bryson et al. 2002a, Andrew, Norval 1989, Deem et al. 1996a}.

Especially in November 2013 and March and April 2014, interdigital abscesses were frequently observed, resulting in widespread lameness. *Maclvor et. Al*. (1987a) found that the presence of abscessed feet in Boer and Angora goats in Valley Bushveld, South Africa is seasonal. Higher numbers of ticks were correlated with greater numbers of foot abscesses, especially on the hind limbs {MacIvor, Horak 1987a}. In the Mnisi area, greater numbers of adult ticks were present during these months, supporting the findings from *Maclvor et. Al*. (1987a).

Although sampling did not occur in the exact same households visited in earlier studies, expected is that this is of minimal influence when looking at the comparability of results, since the majority of the goats is free roaming and they often graze in the same areas.
Since definite diagnoses are rarely performed, the effects of heartwater are often underestimated and taken for granted. Therefore, the economic impact of heartwater is difficult to quantify, but it is thought to be comparable to the enormous losses of trypanosomosis, East Coast fever, dermatophilosis and rinderpest {provost, Bezuidenhout 1987, Allsopp 2010}**.** Despite the fact that heartwater is one of the most serious diseases concerning livestock in sub-Saharan Africa, no suitable diagnostic test for *Ehrlichia ruminantium* exists {Deem et al. 1996a}. By combining the questionnaires and the RLB results, the presence and effects of heartwater in the Mnisi area can only be estimated. By the exploration of typical post-mortem lesions and detection of *E. ruminantium* colonies in brain smears, concrete numbers can be reported {Peter et al. 2002}. Currently, residents in the Mnisi area tend not to inform a veterinarian about diseased livestock, but stimulating farmers to do so could be a beneficial method to study the prevalence and effects of heartwater in the future.

When looking at the questionnaire outcomes in different seasons, the awareness and knowledge of ticks and tick-borne diseases has clearly increased over the year. A possible explanation for this is that the residents become more informed and aware after the visits. Every visited resident was told about the risk of ticks and possible tick prevention methods. The visible tick removal during the visits showed the residents the presence of and regular damage caused by ticks. The fact that households did not necessarily overlap throughout the different seasons enforces the idea that tick awareness and information is shared within a village.

At this point, 55% of the residents were familiar with the possible harmful effects of ticks and 85% of the owners indicated to use some form of tick prevention. Although these numbers are high, the effectiveness of the used control measures is debatable. The interval of application and the used products varied greatly, even within the same households. Some products were not reliable or even harmful, like old oil or Jeyes Fluid.

In the area of Mnisi, dogs are in close contact with free roaming goats. Previous studies did not show the presence of *E. canis* in the RLB results. In the results from March and April 2014, some indistinct positive outcomes for *E. canis* were found. Although *A. hebraeum* ticks occasionally attach on dogs {Bryson et al. 2002}, these results are probably false positive outcomes of *E. canis*.

Furthermore, endemic stability is a topic worth to discuss. Endemic stability can be obtained in endemic areas where animals of all ages are exposed to a high infection challenge. Several factors are believed to help encourage the creation of an endemically stable area. *Perry et. Al.* (1995) speculated that endemic stability would be obtained by the infection of young hosts within a short period of less susceptibility for clinical disease. This age-specific resistance was believed to be unrelated to the immune status of the mother (Du Plessis & Malan, 1987, 1988).

A new perspective on the development and maintenance of endemic stability includes the role of maternally derived factors through vertical transmission and colostrum {O'Callaghan et al. 1998a}. Vertical transmission of *E. ruminantium* occurs within a period of high tolerance to clinical disease. Later in life, tick infestation ensures a continual exposure to heartwater. Both factors contribute to the spread of heartwater, a high level of herd immunity and thus the maintenance of endemic stability {Deem et al. 1996a}. Other tick-borne diseases like Babesiosis, Theileriosis and Anaplasmosis are primarily transmitted by vectors without the role of vertical transmission.

Beside this, *A. hebraeum* ticks have other qualities that facilitate the achievement of endemic stability. At first, trans- and intra-stadial transmission occur within *A. hebraeum* ticks. By excreting an attachment/aggregation pheromone, other *A. hebraeum* ticks will be attracted to the host, contributing to a successive intra-stadial transmission. Due to the three-host lifecycle, more than one host can be infected by one tick. Moreover, male ticks attach for feeding for a long period and the ticks maintain their infectiveness after infecting a host {Andrew, Norval, 1989, Deem et al. 1996a}.

The relationship between agent, host, vector and environment needs to be stable in order to succeed in terms of endemic stability. Apart from a large reservoir of vector ticks and a high prevalence of infection in vector ticks, a high infection rate in resistant hosts is acquired for endemic stability {Deem et al. 1996a}.

The transmission of *E. ruminantium* depends entirely on infestation by *E. ruminantium* infected *Amblyomma* ticks on susceptible hosts. {Norval, Andrew, Yunker, 1990}.
Carrier animals appear to be the most significant reservoirs of *E. ruminantium* {Deem et al. 1996a}. The presence of these long-term carrier animals in the field play a key role in the maintenance of heartwater endemic stability, since these carrier animals result in a high level of *E. ruminantium* infected ticks in the field {Norval, Andrew, Yunker, 1990}.

As shown in the figure below, tick attack rate must be relatively high in order to maintain an endemic stable situation. Widespread use of tick control methods could negatively interfere with this stability, possibly resulting in a sensitive population. Awareness of this possible complication of tick prevention is essential. Moreover, topics like acaricide resistance and financial possibilities for the residents are important to be taken into account. Uncontrolled use of acaricides might lead to acaricide resistance in tick populations, possibly with an exacerbation of tick-borne diseases as a consequence {Eisler et al. 2003}. Also, synthetic chemicals developed for parasite control can be potentially toxic to humans and the ecosystem {Schwallback et al. 2003}. Vaccination seems to be the best long-term and cost-effective control method {Stuen, Longbottom 2011a}, but will not be a realistic preventive measurement due to the relatively low income of the residents.

In order to determine a cost-effective and realizable intervention plan, a better understanding of transmission models, the establishment of endemic stability and the current situation in the Mnisi area, South Africa, is essential.

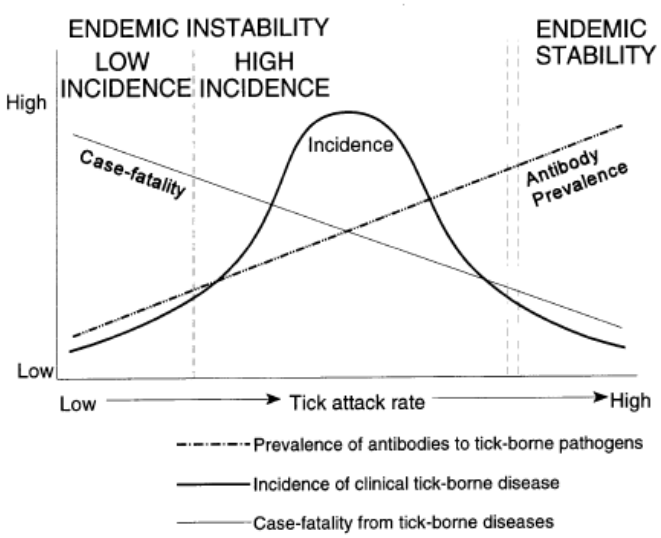


Fig. A demonstration of hypothetical relationship between the level of disease incident, vector challenge, antibody prevalence and case-fatality for tick-borne diseases {Perry, Young 1995}.

6. Conclusion

Amblyomma hebreaum ticks seem to have some form of seasonal abundance. Especially in the summer months (November 2013), high numbers of adult *A. hebraeum* ticks were collected. Larvae were mostly found in the winter period (June and July 2013) and in the period going towards winter (April 2014). The number of nymphs in different seasons seems to be comparable.

When combining the questionnaire outcomes with the detection of *E. ruminantium* in ticks on goats in the area of Mnisi, the presence of heartwater seems very likely. Clinical signs like pedaling movements, possibly related to heartwater, were described by the residents several times, not only in March and April 2014 but also in the studies performed in June and July and November 2013. The infection rate of *E. ruminantium* in *Amblyomma hebraeum* ticks was 12.5% in June and July 2013, 24.7% in November 2013 and 17.6% in March and April 2014. This contributes to the fact that heartwater might be present in the Mnisi area. The presence of heartwater is not the only tick-related problem goats in the Mnisi area are dealing with, abscesses resulting in lameness due to heavy tick infestations in the interdigital space were often found. Skin damage on other places of the body was also present.

Currently, 55% of the residents were familiar with the possible harmful effects of ticks and 85% of the owners indicated to use some form of tick prevention. Although these numbers are high, the effectiveness of the used control measures is doubtful, since the interval of application and the used products vary greatly. 70.6% of the residents indicated to be interested in new tick control methods, emphasizing the willing attitude of the residents.

At this point, the seasonal abundance of *A. hebraeum* ticks and the prevalence *of E. ruminantium* in these ticks has been studied in June, July, November, March and April. Before a sustainable intervention strategy concerning tick prevention can be implemented, seasonal dynamics of *Amblyomma hebraeum* ticks on goats must be determined. Also, the possible interference of tick prevention with endemic stability, the possible creation of acaricide resistance and the economical possibilities for the residents in Mnisi must be taken into account. Therefore, more research in the remaining months of the year is acquired.

7. Acknowledgements
I would like to thank Prof. dr. F. Jongejan (Utrecht Centre for Tick-borne Diseases, Faculty of Veterinary Medicine, Utrecht University, and, Department of Veterinary Tropical Diseases, University of Pretoria, South Africa) and Dr. H. Stoltz (Department of Veterinary Tropical Diseases, University of Pretoria, South Africa) for their supervision. Greatly appreciated are the helpful discussions and instructions given during this study.
I am extremely grateful for the participation of the residents, the possibility to work with their animals and the assistance of all the Environmental Monitors. Dr. Bjorn Reininghaus and Anne Conan are thanked for the help with scheduling and Jeanette Wentzel for all the help in and around Hans Hoheisen.

8. References

Alessandra, T. & Santo, C. 2012, "Tick-borne diseases in sheep and goats: Clinical and diagnostic aspects", *Small Ruminant Research,* vol. 106, no. SUPPL., pp. S6-S11.

Allsopp, B.A. 2010, "Natural history of Ehrlichia ruminantium", *Veterinary Parasitology,* vol. 167, no. 2–4, pp. 123-135.

Allsopp, M.T.E.P., Hattingh, C.M., Vogel, S.W. & Allsopp, B.A. 1999, "Evaluation of 16S, map1 and pCS20 probes for detection of Cowdria and Ehrlichia species", *Epidemiology and Infection,* vol. 122, no. 2, pp. 323-328.

Allsopp, M.T.E.P., Van Strijp, M.F., Faber, E., Josemans, A.I. & Allsopp, B.A. 2007, "Ehrlichia ruminantium variants which do not cause heartwater found in South Africa", *Veterinary Microbiology,* vol. 120, no. 1–2, pp. 158-166.

Andrew, H.R. & Norval, R.A.I. 1989, "The carrier status of sheep, cattle and African buffalo recovered from Heartwater", *Veterinary Parasitology,* vol. 34, no. 3, pp. 261-266.

Bekker, C.P.J., De Vos, S., Taoufik, A., Sparagano, O.A.E. & Jongejan, F. 2002, "Simultaneous detection of Anaplasma and Ehrlichia species in ruminants and detection of Ehrlichia ruminantium in Amblyomma variegatum ticks by reverse line blot hybridization", *Veterinary Microbiology,* vol. 89, no. 2-3, pp. 223-238.

Bryson, N.R., Tice, G.A., Horak, I.G., Stewart, C.G. & Du Plessis, B.J.A. 2002a, "Ixodid ticks on cattle belonging to small-scale farmers at 4 communal grazing areas in South Africa", *Journal of the South African Veterinary Association,* [Online], vol. 73, no. 3, pp. p.98-103.

Busser, S. 2014 "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." *Faculty of Veterinary Medicine, Utrecht University*

Camus, E. & Barre, N. 1992, "The role of Amblyomma variegatum in the transmission of heartwater with special reference to Guadeloupe", *Annals of the New York Academy of Sciences,* vol. 653, pp. 33-41.

Chamboko, T., Mukhebi, A.W., Callaghan, C.J.O., Peter, T.F., Kruska, R.L., Medley, G.F., Mahan, S.M. & Perry, B.D. 1999, "The control of heartwater on large-scale commercial and smallholder farms in Zimbabwe", *Preventive Veterinary Medicine,* vol. 39, no. 3, pp. 191-210.

De Castro, J.J. 1997, "Sustainable tick and tickborne disease control in livestock improvement in developing countries", *Veterinary Parasitology,* vol. 71, no. 2-3, pp. 77-97.

de Castro, J.J. & Newson, R.M. 1993, "Host resistance in cattle tick control", *Parasitology Today,* vol. 9, no. 1, pp. 13-17.

Deem, S.L. 2010, "Thoughts on the threat of Ehrlichia ruminantium infection", *Journal of the American Veterinary Medical Association,* vol. 237, no. 9, pp. 1020.

Deem, S.L., Norval, R.A.I., Yonow, T., Peter, T.F., Mahan, S.M. & Burridge, M.J. 1996, "The epidemiology of heartwater: Establishment and maintenance of endemic stability", *Parasitology Today,* vol. 12, no. 10, pp. 402-405.

Du Plessis, J.L. & Malan, L. 1988, "Susceptibility to heartwater of calves born to non-immune cows.", *Onderstepoort Journal of Veterinary Research,* vol. 55, no. 4, pp. 235-237.

DuPlessis, J.L. & Malan, L. 1987, "The non-specific resistance of cattle to heartwater.", *Onderstepoort Journal of Veterinary Research,* vol. 54, no. 3, pp. 333-336.

Eisler, M.C., Torr, S.J., Coleman, P.G., Machila, N. & Morton, J.F. 2003, "Integrated control of vector-borne diseases of livestock – pyrethroids: panacea or poison?", *Trends in Parasitology,* vol. 19, no. 8, pp. 341-345.

Jonsson, N.N., Bock, R.E., Jorgensen, W.K., Morton, J.M. & Stear, M.J. 2012, "Is endemic stability of tick-borne disease in cattle a useful concept?", *Trends in Parasitology,* vol. 28, no. 3, pp. 85-89.

Kriek, N. 2009, "Mnisi Community Programme (MCP). An integrated interface programme. *Centre for Veterinary Wildlife Studies Faculty of Veterinary Science, UP. Namaacha*

MacIvor, K.M. & Horak, I.G. 1987, "Foot abscess in goats in relation to the seasonal abundance of adult Amblyomma hebraeum and adult Rhipicephalus glabroscutatum (Acari: Ixodidae).", *Journal of the South African Veterinary Association,* vol. 58, no. 3, pp. 113-118.

Mahan, S.M., Peter, T.F., Semu, S.M., Simbi, B.H., Norval, R.A.I. & Barbet, A.F. 1995, "Laboratory reared Amblyomma hebraeum and Amblyomma variegatum ticks differ in their susceptibility to infection with Cowdria ruminantium", *Epidemiology and Infection,* vol. 115, no. 2, pp. 345-353.

Norval, R.A.I., Andrew, H.R. & Yunker, C.E. 1990, "Infection rates with Cowdria ruminantium of nymphs and adults of the bont tick Amblyomma hebraeum collected in the field in Zimbabwe", *Veterinary Parasitology,* vol. 36, no. 3–4, pp. 277-283.

Norvl, R.A.I., Donachie, P.L., Meltzer, M.I., Deem, S.L. & Mahan, S.M. 1995, "The relationship between tick (Amblyomma hebraeum) infestation and immunity to heartwater (Cowdria ruminantium infection) in calves in Zimbabwe", *Veterinary Parasitology,* vol. 58, no. 4, pp. 335-352.

O'Callaghan, C.J., Medley, G.F., Peter, T.F. & Perry, B.D. 1998, "Investigating the epidemiology of heartwater (Cowdria ruminantium infection) by means of a transmission dynamics model", *Parasitology,* vol. 117, no. 1, pp. 49-61.

Perry, B.D. & Young, A.S. 1995, "The past and future roles of epidemiology and economics in the control of tick-borne diseases of livestock in Africa: the case of theileriosis", *Preventive Veterinary Medicine,* vol. 25, no. 2, pp. 107-120.

Peter, T.F., Bryson, N.R., Perry, B.D., O'Callagan, C.J., Medley, G.F., Smith, G.E., Mlambo, G., Horak, I.G., Burridge, M.J. & Mahan, S.M. 1999, "Cowdria ruminantium infection in ticks in the Kruger National Park", *Veterinary Record,* vol. 145, no. 11, pp. 304-307.

Peter, T.F., Burridge, M.J. & Mahan, S.M. 2002, "Ehrlichia ruminantium infection (heartwater) in wild animals", *Trends in Parasitology,* vol. 18, no. 5, pp. 214-218.

Petney, T.N., Horak, I.G. & Rechav, Y. 1987, "The ecology of the African vectors of heartwater, with particular reference to Amblyomma hebraeum and Amblyomma variegatum.", *Onderstepoort Journal of Veterinary Research,* vol. 54, no. 3, pp. 381-395.

Provost, A. & Bezuidenhout, J.D. 1987, "The historical background and global importance of heartwater.", *Onderstepoort Journal of Veterinary Research*, vol. 54, no. 3, pp. 165-169.

Schwalbach, L.M.J., Greyling, J.P.C. & David, M. 2003, "The efficacy of a 10% aqueous Neem (Azadirachta indica) seed extract for tick control in Small East African and Toggenburg female goat kids in Tanzania", *South African Journal of Animal Sciences,* vol. 33, no. 2, pp. 83-88.

Steen, van der F. 2013 "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." *Faculty of Veterinary Medicine, Utrecht University*

Stuen, S. & Longbottom, D. 2011, "Treatment and Control of Chlamydial and Rickettsial Infections in Sheep and Goats", *Veterinary Clinics of North America - Food Animal Practice,* vol. 27, no. 1, pp. 213-233.

Utrecht Centre for Tick-borne Diseases (UCTD). 2013, *UCTD Protocols. The Netherlands*

Utrecht Centre for Tick-borne Diseases (UCTD). 2014, *UCTD Protocols. The Netherlands*

Vachiéry, N., Raliniaina, M., Stachurski, F., Adakal, H., Molia, S., Lefrançois, T. & Martinez, D. 2006, "Understanding the mechanisms of transmission of Ehrlichia ruminantium and its influence on the structure of pathogen populations in the field", *Annals of the New York Academy of Sciences;* vol. 1078, pp. 495-497

Wesonga, F.D., Mukolwe, S.W. & Grootenhuis, J. 2001, "Transmission of Cowdria ruminantium by Amblyomma gemma from infected African buffalo (Syncerus caffer) and eland (Taurotragus oryx) to sheep", *Tropical Animal Health and Production,* vol. 33, no. 5, pp. 379-390.

http://web.up.ac.za/default.asp?ipkCategoryID=16376. The Mnisi Community Programme; University of Pretoria. *Visited on 16-11-2013*

9. APPENDIX A

9.1 Questionnaire Survey

UTRECHT CENTRE FOR TICK-BORNE DISEASES (UCTD)
FAO REFERENCE CENTRE FOR TICKS AND TICK-BORNE DISEASES
Tick collections from goats

UCTD/14/F05/1
05 Feb 2014

TICK COLLECTION FROM GOATS FORM

Sampling date	
Village	
GPS household	
Name owner	
Total number of goats	
Number of sampled goats	
Number of collected ticks	
ID tick tube(s)	
Additional pictures ID	

Does the owner perform any tick control?	
What kind of tick control is performed?	
How often does the owner perform tick control?	
How many goats (suddenly) died last year?	
Has the owner seen pedaling movements just before goats died?	
Has the owner seen lameness/infected feet?	
How is the animal treated and how often?	
Would the owner be interested in new tick control methods?	

Form filled in:

by _____ on _____ _____

Signature

Comments:

DEPARTMENT OF VETERINARY TROPICAL DISEASES (DVTD)
FACULTY OF VETERINARY SCIENCE

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

10. APPENDIX B

10.1 DNA Extraction

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

- Wear gloves and use filtertip pippettips.

1. Clean workspace with sodium hypochloride.
2. Turn on a water bath at 56°C.
3. Take the proteinase K solution from the freezer and store at 4°C.
4. Wash the ticks in a sonofication bath with demineralized water for up to 30 seconds.
5. Put the ticks, with cleaned forceps, in 1.5ml tubes with 70% ethanol and vortex for several seconds.
6. Wash the forceps in 70% ethanol followed by washing in demineralized water after each tick.
7. Take the ticks from the tubes and let it dry on a clean tissue paper and place the dried ticks in a sterile 2ml tube with 180µl T1 lysis buffer.
8. Freeze the samples at -80°C for 15 minutes.
9. Add a 5 or 7mm (depending on tick size) metal bead to the frozen samples.
10. Disrupt the ticks in the TissueLyser LT at 50 oscillations per second for 3 minutes.
11. Briefly spin down the tubes. **1000x g maximum!**
12. Add 25µl proteinase K and vortex.
13. Prelyse the samples at 56°C in a water bath for 3 hours and vortex every hour.
14. **During the incubation**; empty and clean the sonification bath.
15. **During the last incubation hour**; turn on the heating block at 70°C and preheat the BE.
16. Briefly spin down the tubes. 1000x g maximum!
17. Add 200µl B3 buffer and vortex.
18. Incubate the tubes at 70°C for 15 minutes.
19. Briefly spin down the tubes. 1000x g maximum!
20. Add 210µl 96% ethanol, vortex and briefly spin down the tubes. 1000x g maximum!
21. Transfer the supernatant to new sterile 1.5ml tubes. (Tick parts are allowed to be transferred.)
22. Centrifuge the tubes at 11,000x g for 2 minutes.
23. Transfer the supernatant to spin columns. Avoid pipetting tick parts, as it can block the spin column.
24. Centrifuge the columns at 11,000x g for 1 minute. Discard the flow through.
25. Add 500µl BW buffer and centrifuge the columns at 11,000x g for 1 minute. Discard the flow through.
26. Add 600µl B5 buffer and centrifuge the columns at 11,000x g for 1 minute. Discard the flow through.
27. Centrifuge the columns at 11,000x g for 1 minute.
28. Place the spin columns in sterile 1.5ml tubes. Label the tubes accordingly.
29. Add100µl preheated BE buffer directly on the membrane of the spin columns and incubate at room temperature for 1 minute.
30. Centrifuge the columns at 11,000x g for 1 minute. Discard the spin columns.
31. Store the DNA samples at 4°C for use within the next few days or store at -20°C for long term preservation.
32. Turn off all equipment and clean working space with sodium hypochloride.

10.2 PCR RLB procedure

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

- Wear (green) gloves and use filtertip pippettips.
- 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
5.875 µl	PCR grade H2O

End volume of every PCR individual sample is: 25 µl

1. Put DNA samples a (few) day(s) before the PCR at 4°C.
2. Turn on the DNA workstations in the clean room and the dirty room.
3. Clean workspace in both DNA workstations with sodium hypochloride.
4. Label the PCR and Eppendorf tubes and put them in the DNA workstation in the clean room.
5. Turn on the UV-light in both DNA workstations for 20 minutes.
6. **During the UV-light**; thaw the PCR reagents at room temperature, except the polymerase.
7. Prepare the PCR mix in the Eppendorf tube(s). Multiply the reagent volumes by the number of samples plus 10% of the number of samples: 40 DNA samples + 1 PCR control = 41 + 10% = 45 samples.
8. Pipet the master mix gently up and down to mix well.
9. Pipet 22,5µl master mix to each PCR tube and add the leftover mix to an additional tube which will be the negative PCR control.
10. Close the PCR tubes and remove them from the workstation, clean the workspace with sodium hypochloride and turn on the UV-light for 20 minutes.
11. Take the closed PCR tubes to the dirty room and place them in the workstation.
12. Vortex the DNA samples, spin them down briefly at 11,000x g and place them in the workstation.
13. Add 2.5µl DNA sample to the corresponding PCR tube.
14. Add 2.5µl of the positive control (, corresponding to the PCR to be performed,) to the positive PCR control tube.
15. Vortex and spin down briefly.
16. Clean the workstation with sodium hypochloide and turn on the UV-light for 20 minutes.
17. Run the corresponding PCR program
18. Store the PCR products at 4°C for use within the next few days or store at -20°C for long term preservation.
19. Turn off both DNA workstations after the UV-light is switched off.

10.3 Agarose gel electrophoresis

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

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.I
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).

10.4 Reverse Line Blot (RLB) hybridization

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

- Wear gloves and use non-filter pipet tips.
- Strictly follow the one-way route: Clean room → Dirty room → PCR room

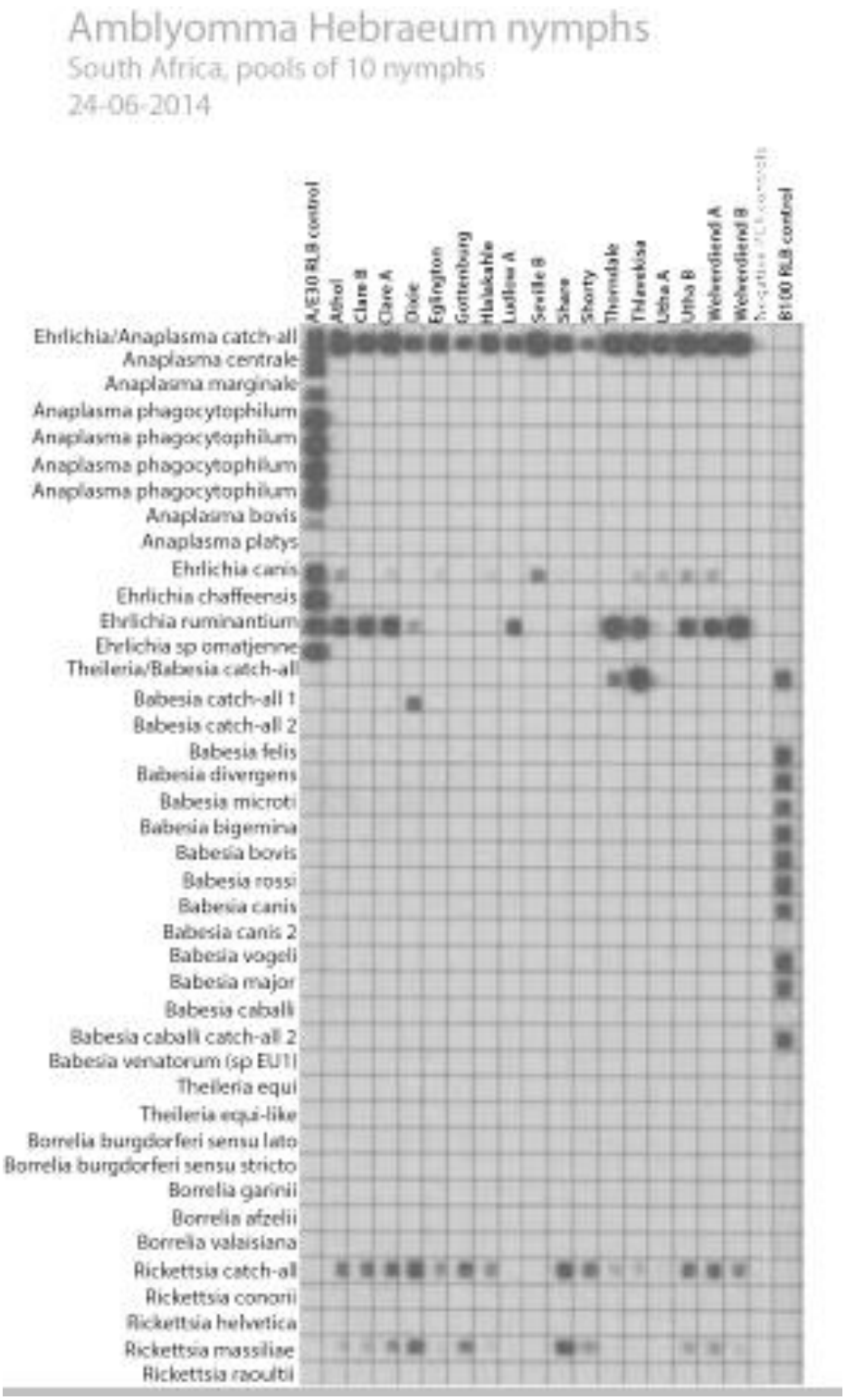
1. Turn on the heating block at 100°C.
2. Turn on the hybridization oven at 42°C. Preheat 50ml 2x SSPE/0.5% SDS solution in the hybridization oven at the same time.
3. Turn on the water bath at 50°C. Preheat in the water bath the 2x SSPE/0.5% SDS solution at the same time.
4. Clean working space with 70% ethanol.
5. Combine and dilute the PCR products, per DNA sample, in a 1,5 ml eppendorf tube. Use of every PCR sample 10µl 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 150µl 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 (150µl), 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.
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 downthe 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. C
21. Change the waterbath temperature to 80°C and place the 1% SDS solution inside the waterbath.
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 10ml ECL (5ml ECL1 + 5ml 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 with 20 mM EDTA at 4 °C until stripping.
30. Turn off all equipment and clean working space

11. APPENDIX C

11.1 RLB results, *Amblyomma hebraeum* nymphs

Note that Eglington in this blotter is the same village as Hluvakani.
Utha B and Welverdiend A are not included in the results, since these villages were mixed during the DNA-extraction.



11.2 RLB results, *Amblyomma hebraeum* adults

Note that Eglington in this blotter is the same village as Hluvakani.