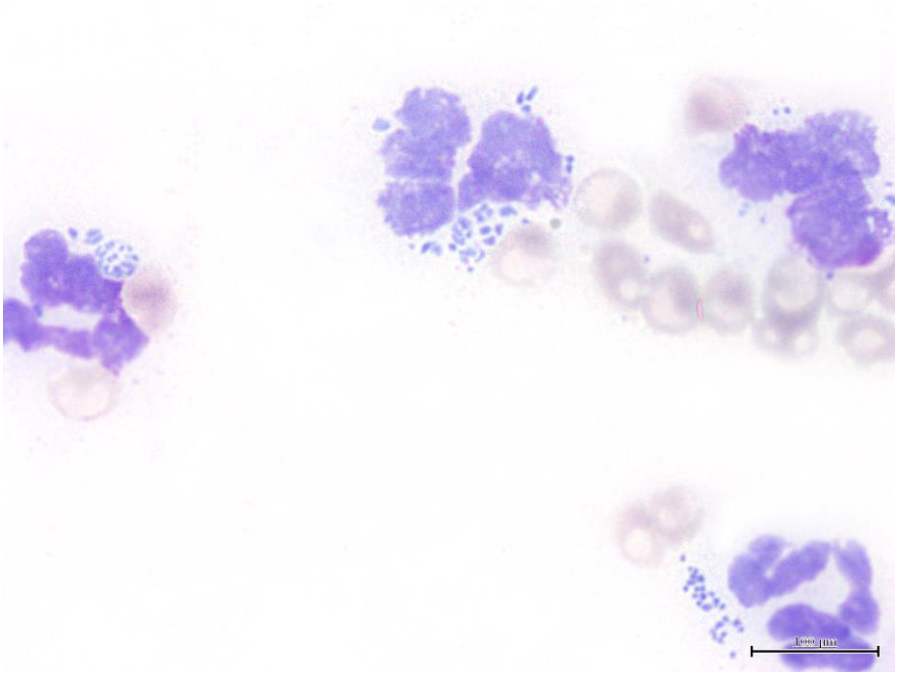
Anaplasma phagocytophilum infection in sheep



Investigating disease outbreaks in the Netherlands and development of an experimental tick transmission model

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Foreword

This master thesis was written as part of my research internship, which I conducted at Utrecht Centre for Tick-borne Diseases (UCTD) in order to achieve my master's degree in Veterinary Medicine at Utrecht University. I preferred to perform my research internship on an infectious disease which also infects humans. Thus I was looking for a research subject on zoonosis. However, I could not have hoped for getting the opportunity to perform my research internship on a subject as interesting and relevant as this study. So I was really satisfied and grateful for getting this opportunity. From September 2014 until December 2014 I took part in this study for a period of 12 weeks, in which I conducted much of the laboratory work and eventually wrote this master thesis.

This whole project started in May 2014, after local veterinarian Reinard Everts reported high mortality (10-15% since 2010) and high morbidity (high fever, locomotion disorders and cachexia) in sheep introduced into the Bargerveen nature reserve. Empiricism over that period showed that therapy with long acting oxytetracycline antibiotics stopped clinical signs and mortality. Initial blood screening seemed to confirm the presumption that Anaplasma phagocytophilum (transmitted by ticks of the genus Ixodes) was at least part of the cause of the observed mortality in sheep. Anaplasma is a zoonosis and literature suggests that it is widespread in Europe, Asia and the US. However, much of this bacterium is still unknown. So the reported outbreak of Anaplasma in Bargerveen was the starting point for further research. Initially a field study was set up with sentinel sheep, which was later followed by a laboratory study in order to study different aspects of the tick-host-pathogen transmission dynamics under controlled conditions. This master thesis shows results of only part of the study. However the experimental tick transmission model (which was developed for this experiment) has already made a promising start, as described in this paper.

This master thesis was written under supervision of prof. dr. Frans Jongejan. I am really grateful for all his support, help and inspiration. I would also like to give special thanks to Gabriel Goderski and Laura Berger for help with the laboratory work and Reinard Everts for collecting data and blood samples in the field study in Bargerveen.

I hope you will enjoy reading this paper just as much, as I did writing it.

Stefan Burgers
Utrecht, 17-12-2014

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Abstract

Background: *Anaplasma phagocytophilum* is the etiologic agent of tick-borne fever (TBF) in sheep and of human granulocytic anaplasmosis (HGA) in humans. *Anaplasma* is transmitted by *Ixodes ricinus* ticks (in Europe), causes a sudden onset of high fever and eventually leads to immunosuppression. This study was initiated, after an outbreak of *Anaplasma* was reported in sheep introduced into the Bargerveen nature reserve in the Netherlands. The study consisted of two parts. A field study with sentinel sheep, in which *Anaplasma* was collected from natural clinical cases in the Bargerveen nature reserve. The second part of the study consisted of the development of an experimental tick transmission model, in order to study the tick-host-pathogen transmission dynamics.
Methods: In the sentinel study, a total of 16 *Anaplasma*-negative sheep were introduced into the Bargerveen nature reserve. Sheep were monitored by full physical examination and blood samples were taken once a week in order to perform both blood smears and PCR/RLB.
In the experimental study, *Anaplasma*-negative sheep kept under laboratory conditions were experimentally inoculated with an *Anaplasma* strain collected in 1988 from sheep on the North Sea Island of Ameland. Sheep were monitored by daily temperature measurement and PCR/RLB for the presence of *Anaplasma*. Ticks, which were subsequently placed on the sheep, were analyzed after they completed their blood meal by PCR/RLB to determine the infection rate of ticks.
Results: In the sentinel study, blood stabilates were made and stored for later use in an experimental model. Analysis of data, gathered from both clinical examination and blood sample analysis, revealed a possible relationship between tick-borne fever on one hand and both lymphadenopathy and a rectal temperature of ≥ 40,5 ºC on the other hand. A specificity of 100% was found for blood smears compared to PCR/RLB, indicating a positive blood smear is a suitable diagnostic tool to confirm the presence of *Anaplasma* in a clinical setting. However a negative blood smear does not rule out the presence of *Anaplasma*, as a sensitivity of 40% was found in this study. After 8 days already 8 out of 16 (50%) introduced sheep were positive for *Anaplasma.* At the end of this fieldstudy, 51 days after introduction, 13 out of 16 (81%) sheep were positive for *Anaplasma*.
In the experimental tick transmission study, a fever peak was observed which started at approximately day 4 after inoculation and lasted for 4 to 6 days. Infection rates in ticks still had to be determined at the time of writing this paper.
Conclusion: A high infection rate of *Anaplasma* was found in newly introduced sheep into the Bargerveen nature reserve. The high infection rate observed in sheep demonstrates that sheep are suitable as sentinel animals for TBF. It is not known whether the high infection rate in the Bargerveen nature reserve is just a local characteristic, or whether the presence of *Anaplasma* is widespread in sheep pastures or nature reserves all over the Netherlands. Further research should give insight into the spread and impact of *Anaplasma* in different nature reserves (and/or sheep pastures) in the Netherlands.
In the experimental model, a self-limiting infection in sheep was observed. The model is suitable for producing infected ticks. This model as well as these ticks can subsequently be used in further studies. Results of these studies could eventually lead to a One-Health approach of *Anaplasma* as an emerging zoonosis.

Keywords: *Anaplasma phagocytophilum*, tick-borne fever (TBF), *Ixodes ricinus*, sheep, zoonosis, One-health, transmission dynamics.

Samenvatting

Achtergrond: *Anaplasma phagocytophilum* is het etiologische agens van Tick Borne Fever (TBF) in schapen en van Humane Granulocytaire Anaplasmose (HGA) bij mensen. *Anaplasma* wordt overgedragen door teken van de soort *Ixodes ricinus* (in Europa), veroorzaakt een plotseling ontstaan van hoge koorts en leidt uiteindelijk tot immunosuppressie. Dit onderzoek is opgezet na melding van een uitbraak van *Anaplasma* bij schapen die nieuw werden geïntroduceerd in het natuurgebied Bargerveen in Nederland. Twee deelonderzoeken werden uitgevoerd. Het eerste onderzoek bestond uit een veldstudie met detectie schapen, waarin *Anaplasma* werd verzameld van natuurlijke klinische gevallen in het natuurgebied Bargerveen. Het tweede deel van het onderzoek bestond uit de ontwikkeling van een experimenteel teken transmissie model, om de teek-gastheer-pathogeen transmissie dynamiek te onderzoeken.
Methoden: In de veldstudie werd een totaal van 16 *Anaplasma*-negatieve schapen geïntroduceerd in het natuurgebied Bargerveen. Schapen werden wekelijks onderworpen aan een volledig lichamelijk onderzoek. Daarnaast werden bloedmonsters afgenomen, om hierop vervolgens bloeduitstrijkjes en PCR/RLB uit te voeren.
In de experimentele studie werden *Anaplasma*-negatieve schapen, gehouden onder laboratorium omstandigheden, experimenteel geïnoculeerd met een *Anaplasma* stam die in 1988 is verzameld van schapen op het Noordzee eiland Ameland. Schapen werden gemonitord door de dagelijkse meting van lichaamstemperatuur en PCR/RLB voor de aanwezigheid van *Anaplasma*. Teken, die vervolgens op de schapen werden geplaatst, werden nadat ze hun bloedmaaltijd hadden voltooid geanalyseerd met PCR/RLB om de infectiegraad van de teken te bepalen.
Resultaten: In de veldstudie werden bloed-stabilates gemaakt en opgeslagen voor latere inoculatie in een experimenteel model. Analyse van de gegevens, verzameld via zowel lichamelijk onderzoek als analyse van bloed monsters, toonde een mogelijke relatie aan tussen TBF en lymphadenopathie en tevens tussen TBF en een rectale temperatuur van ≥ 40,5 ºC. Een specificiteit van 100% werd gevonden voor bloeduitstrijkjes in vergelijking met PCR/RLB, wat aangeeft dat een positief bloeduitstrijkje een geschikt diagnostisch hulpmiddel is om de aanwezigheid van *Anaplasma* aan te tonen in een klinische setting. Dit sluit in een negatief bloeduitstrijkje de aanwezigheid van *Anaplasma* niet uit, gezien een sensitiviteit van 40% werd gevonden in dit onderzoek. Na 8 dagen waren al 8 van de 16 (50%) geïntroduceerde schapen positief voor *Anaplasma.* Aan het einde van de veldstudie, 51 dagen na introductie, waren 13 van de 16 (81%) schapen positief voor *Anaplasma*.
In de experimentele teken-transmissie studie werd een koortspiek waargenomen, die ongeveer 4 dagen na inoculatie begon en vervolgens ongeveer 4 tot 6 dagen aanhield. De resultaten van de infectiegraad van teken waren nog onbekend op het moment dat dit master proefschrift werd afgerond.
Conclusie: Een hoge infectiegraad van *Anaplasma* werd gevonden in schapen die nieuw werden geïntroduceerd in het natuurgebied Bargerveen. De hoge infectiegraad die werd waargenomen in schapen bewijst dat schapen geschikte detectiedieren zijn voor TBF. Niet bekend is of de hoge infectiegraad in het natuurgebied Bargerveen enkel berust op een enkele uitbraak, of dat de aanwezigheid van *Anaplasma* wijd verspreid is in weilanden en natuurgebieden in heel Nederland. Verder onderzoek kan een beter beeld geven van de verspreiding en impact van *Anaplasma* in verschillende natuurgebieden (en/of weilanden) in Nederland.
In het experimentele model werd een zelf-limiterende infectie in schapen waargenomen. Het model is geschikt voor de productie van geïnfecteerde teken. Zowel dit model als deze teken kunnen vervolgens in toekomstige studies gebruikt worden. Resultaten van deze experimenten kunnen uiteindelijk bijdragen aan een One-Health aanpak van *Anaplasma* als opkomende zoönose.

Trefwoorden: *Anaplasma phagocytophilum*, tick borne fever (TBF), *Ixodes ricinus*, schapen, zoönose, One-health, transmissie dynamiek.

1. Introduction

Earlier this year, high mortality (10-15%) and high morbidity (high fever, locomotion disorders and cachexia) was reported in sheep after introducing them into the Bargerveen nature reserve in the north-east of the Netherlands. It was suspected that this observation was due to pathogens transmitted by ticks. Preliminary characterization showed the presence of the bacterium *Anaplasma phagocytophilum*, which was confirmed when further blood samples were tested for the presence of a range of tick-borne pathogens known to be present in the Netherlands (by the use of RLB). This led to the idea to use this nature reserve as a setting for studying and collecting *Anaplasma*.

1.1 Background information about *Anaplasma phagocytophilum*

Anaplasma phagocytophilum (formerly known as *Ehrlichia phagocytophila*, *Ehrlichia equi* and in humans the human granulocytic ehrlichiosis (HGE) agent) is an obligate intracellular, gram-negative bacterium, which has a tropism for phagocytic cells, surviving and replicating in neutrophilic granulocytes. *A. phagocytophilum* is associated with *Ixodes* tick species worldwide, especially on the northern hemisphere (Stuen, Granquist, Silaghi 2013). In Europe, the average *A. phagocytophilum* prevalence in *I. ricinus* (the most common tick in The Netherlands) ranges between 1% and approximately 20% (Stuen, Granquist, Silaghi 2013). *Anaplasma* is known to cause disease (sometimes even resulting in death) in domestic ruminants (sheep and cattle), but also occurs in horses, dogs, cats and in humans (Stuen, Granquist, Silaghi 2013). Thus *A. phagocytophilum* is a zoonosis, causing human granulocytic anaplasmosis (HGA).

Clinical signs in sheep include mainly a sudden onset of high fever. After exposure, sheep develop clinical signs within 14 days and fever lasts for 1 to 2 weeks (Stuen and Longbottom 2011). Tick-borne fever (a term which is exclusively used to describe an infection with *A. phagocytophilum*) is seldom fatal, unless it is complicated by other secondary infections as a result of immunosuppression (Stuen and Longbottom 2011).

A clinical diagnosis can be made based on a combination of a sudden onset of high fever and the typical cytoplasmatic inclusion bodies in especially neutrophilic granulocytes. Light microscopy of blood smears taken in the initial fever period are usually sufficient to confirm the diagnosis by demonstrating the morulae, which represent as blue inclusions (Stuen and Longbottom 2011). This microscopic detection of the inclusions may be difficult and prolonged examination is often required to accurately detect *A. phagocytophilum*, as less than 0,1% of neutrophils may show these morulae (Thomas, Dumler, Carlyon 2009).
For definive diagnosis, laboratory confirmation is required (Woldehiwet 2010). Laboratory methods include immuno-histochemistry of tissue samples and PCR. Serology can also be used to support the diagnosis, for example with indirect immunofluorescent antibody (IFA) test. However, it may not be straightforward to use IFA to diagnose acute infection in sheep, as IFA titers remain persistent for months after the primary *A. phagocytophilum* infection (Stuen and Longbottom 2011). Pathology can also be useful, as an enlarged spleen (up to 4-5 times the normal size) can be regarded as indicative of tick-borne fever (TBF) in sheep (Stuen and Longbottom 2011). Of these diagnostic tests, PCR and IFA provide the highest sensitivity for the diagnosis of *A. phagocytophilum* (Stuen, Granquist, Silaghi 2013). In this study a combination of quick screening for infection by blood smears and definitive diagnosis by PCR/RLB or qPCR are used.

Therapy consists of antibiotics, of which tetracycline or doxycycline are preferred. Prevention of disease in domestic animals is done by reduction of tick infestation by use of chemical acaricides (such as pyrethroids).

1.2 Zoonosis

In humans, clinical signs of infection with *A. phagocytophilum* (known as human granulocytic anaplasmosis (HGA)) range from mild self-limiting febrile illness to fatal infections. Commonly, clinical signs include fever, headache, myalgia and malaise. These initially mild and influenza-like

symptoms likely causes *Anaplasma* to be underdiagnosed. As *Anaplasma* causes immunosuppression, infection with TBF could increase the chance of acquiring secondary infections which complicate recovery. While most human infections probably result in mild clinical signs and symptoms, reports from the US demonstrate that infection with *Anaplasma* results in hospitalization for 36%, ICU admission in 7%, and death in 0.6% of cases (Dumler 2012).

Different ecotypes of *Anaplasma* have been identified, with different enzootic cycles. All human isolates and the vast majority of sheep isolates belong to ecotype I (Jahfari et al. 2014), which also has the broadest range of wildlife hosts. Therefore, it can be assumed that *Anaplasma* found in sheep has zoonotic potential and thus sheep are a relevant model for studying *Anaplasma*. Gathered results in this study might add useful information regarding the One-Health approach of *Anaplasma,* as not only sheep but possibly also humans and wildlife hosts may benefit when the transmission of *Anaplasma* will be effectively disturbed.

1.3 Background information about *Ixodes ricinus*

I. ricinus covers a wide geographic range (Europe and North-Africa) and is involved in the transmission of a large variety of pathogens of medical and veterinary importance. *Ixodes ricinus* serves as a main vector in the transmission of *Anaplasma phagocytophilum.* Besides, it is involved in transmission of *Borrelia burgdorferi* (causing Lyme borreliosis), tick-borne encephalitis virus, *Francisella tularensis* (causing Tularaemia), *Rickettsia Helvetica*, *Rickettsia monacensis*, *Babesia divergens* and *Babesia microti* (responsible for Babesiosis) and Louping ill virus (Jeffries et al. 2014).

The life cycle of *Ixodes ricinus* ticks consists of 4 stages (Figure 1), egg, larva, nymph and adult. Ticks need to have a blood meal in order to molt to the next life stage. A total of three hosts are necessary in order to complete the life cycle, which usually takes 2-3 years.

Larvae hatch from an egg in spring and after a few months are ready to have their first blood meal, usually on a mouse or vole. After 3-4 days of feeding and a 10-20 times increase in body mass, the 6-legged larvae drop to the ground. Several months later, the fed larvae will molt to 8-legged nymphs that usually feeds on the second host in the following year. Nymphs generally feed for 4-5 days on small to medium-sized animals (including humans and sheep) in spring or summer. After feeding as nymphs, they will drop and molt to adult male or female ticks. These adult ticks may already be able to feed on their third and last host in autumn. Adult female ticks feed for 7 days on a large animal (often deer or livestock including sheep, but also humans and dogs), growing in size from 3-5 mm up to 1 cm as she is fully engorged. Adult male ticks stay on the host for longer periods of time in order to mate with females (using pheromones to attract female tick), while taking sporadic small blood meals. The fed female tick will drop off, overwinter and seek conditions favorable for egg production and will remain in this environment for 4-8 weeks before laying about 3000 eggs in early spring.

Ticks on sheep are often found around the mouth, ears and eyelids and around the udder and axillary region. In the off-host periods, *I. ricinus* requires a relative humidity of at least 80%.

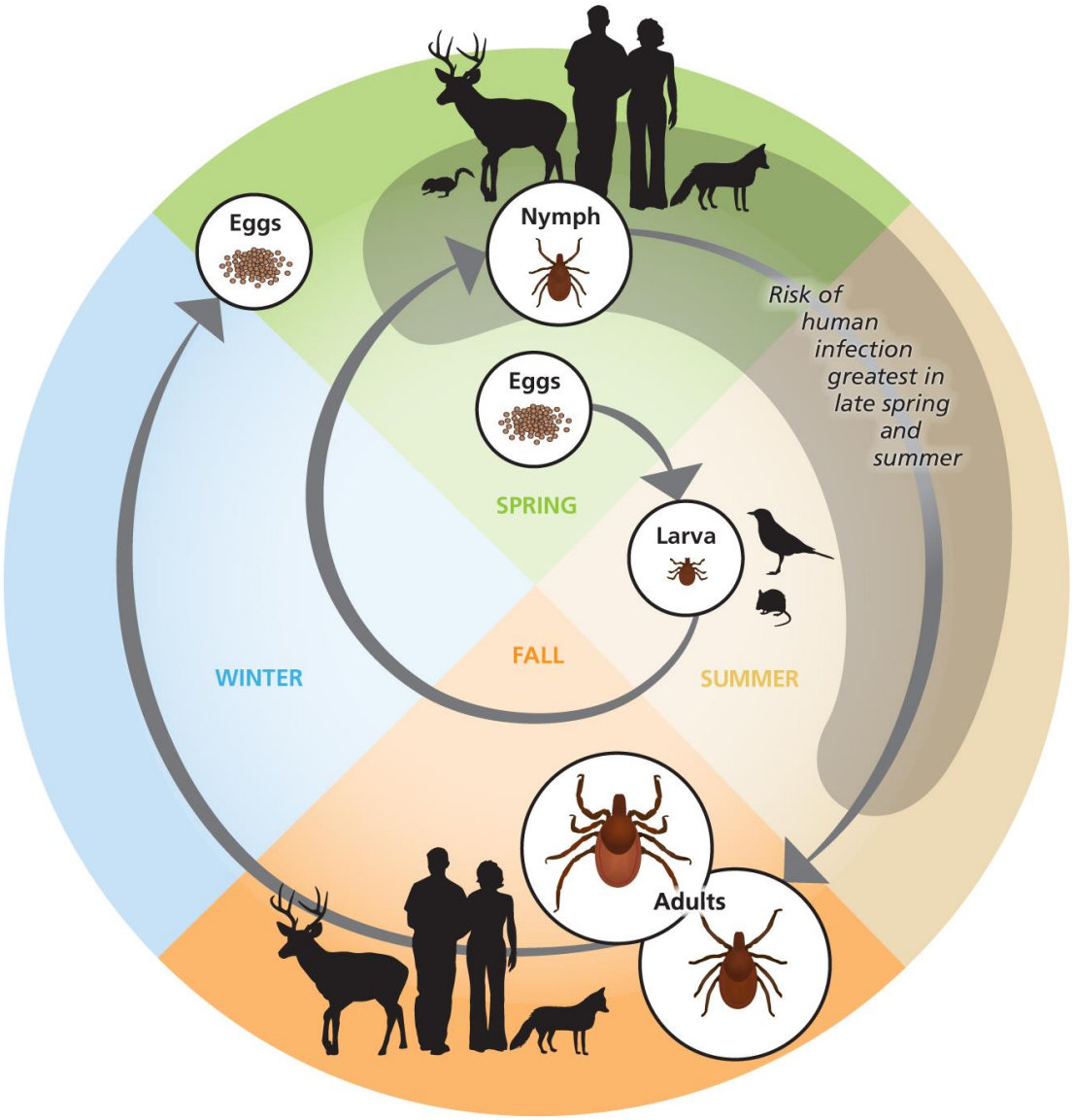

Figure 1. Life cycle of *I. ricinus* ticks (Orent 2013). Completing a life cycle from egg via larva and nymph to adult (which reproduces and takes part in the production of eggs), usually takes 2-3 years.

1.4 Statement of purpose

Purpose of this study was to develop an experimental tick transmission model, in order to eventually determine a strategy to reduce the impact of ticks and tick-borne diseases (specifically of *Anaplasma*) on sheep in the Netherlands. This would not only favor sheep, but possibly also humans and wildlife hosts. So this study could add in the One-Health approach of *Anaplasma* as an emerging zoonosis. Therefore, the first part of the study was set up mainly to collect *Anaplasma* from natural clinical cases in the Bargerveen nature reserve. The second part of this study was set up to be able to further study the interaction between vector (*I. ricinus*), host (sheep of *Ovis aries*), agent (*A. phagocytophilum*) and the environment (Figure 2).

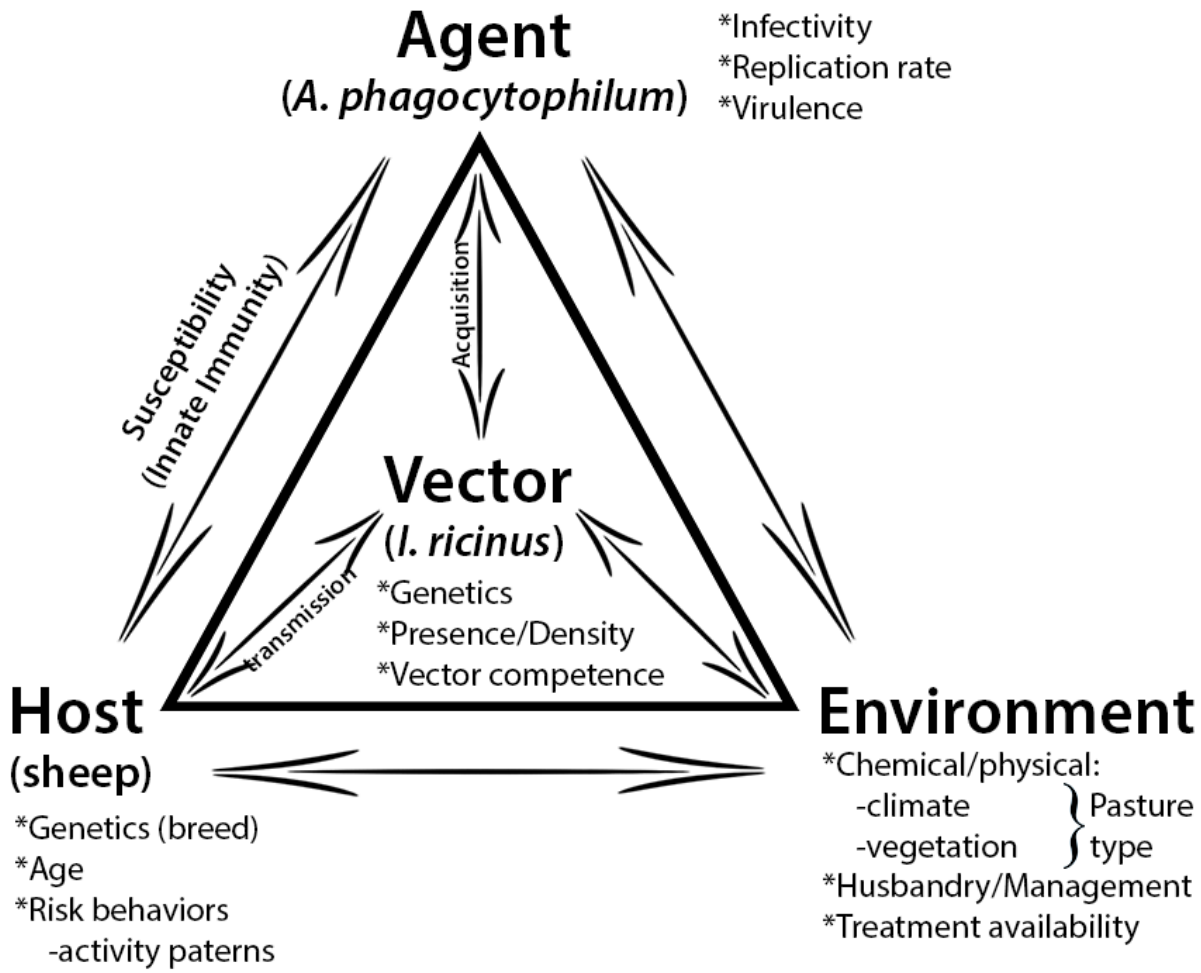

Figure 2. The epidemiologic triangle, including factors influencing each of the components which take part in the tick-host-pathogen transmission dynamics. The epidemiologic triangle can be used to identify areas of potential intervention to reduce infection rate and disease prevalence. Vector competence (which is mentioned in the triangle above) is a complex characteristic which governs a tick's ability to acquire, support the development and transmit a tick-borne disease from one host to another. It influences variations in disease transmission among tick populations, hence affecting disease epidemiology.

1.5 General method of investigation

In order to achieve this purpose, a tick transmission model was developed wherein it is possible to study the interaction between *Ixodes ricinus* ticks, sheep and *A. phagocytophilum*.
As already mentioned before, this study consists of two parts. In the first part, sentinel sheep were introduced into the Bargerveen nature reserve and regularly (on a weekly basis) monitored clinically and blood samples were collected. These blood samples were screened by PCR/RLB for tick-borne diseases known to be present in the Netherlands, including several known *A. phagocytophilum* strains. Blood stabilates were made of some *Anaplasma*-positive blood samples, for later inoculation of *Anaplasma*-negative sheep in an experimental tick transmission model. The second part of this study was an experimental study, in which a tick transmission model was developed to study several variables relevant in the interaction between tick, host and pathogen (*Anaplasma*) under laboratory conditions. The variables being tested are acquisition of *Anaplasma* by Specific Pathogen Free ticks (SPF-ticks), transmission by *Anaplasma*-positive ticks to non-infected sheep and finally the speed of transmission and the concept of co-feeding will be studied.

1.6 Hypotheses and predictions

1.6.1 Sentinel study

In the sentinel study the main purpose was to collect *Anaplasma* from the Bargerveen nature reserve. Therefore new sheep were introduced into the nature reserve. On a weekly basis, sheep were examined physically (including measurement of sheep temperature) by the local veterinarian and blood samples were collected and processed at UCTD by PCR/RLB. We predicted that if Tick-borne Fever (TBF) caused by *Anaplasma phagocytophilum* is the main cause of disease and possibly mortality in sheep introduced into nature reserves in the Netherlands, than it would be possible to detect *Anaplasma* in blood samples collected from sentinel sheep and clinical symptoms (fever) and possibly even mortality related to *Anaplasma* would be encountered in the newly introduced sheep.

Thus for the sentinel study the following hypothesis was tested:

Tick-borne Fever (TBF) caused by *Anaplasma phagocytophilum*, transmitted by *Ixodes ricinus* ticks is the main cause of disease and possibly mortality in sheep introduced into nature reserves in the Netherlands.

Null hypothesis: *Anaplasma phagocytophilum* cannot be detected in sheep introduced into nature reserves in the Netherlands and does not cause clinical signs or even death.

1.6.2 Experimental study

In the experimental study, the main purpose was to further elucidate the interaction between *Ixodes ricinus* ticks, sheep and *A. phagocytophilum* under laboratory conditions. This was tested in consecutive rounds testing several variables which could be relevant in the interaction between tick, host and pathogen.

1.6.2.1 Acquisition

The first round in the experimental study is called the acquisition. Infection rates reported in many studies are higher in adult *Ixodes ricinus* ticks than in nymphs. This may be due to the fact that adult ticks have had an additional (infected) blood meal in comparison to nymphs. Here this possible explanation (based on transstadial transmission) will be called the 'Cumulative theory'. However, as nymphs are larger and take up a larger amount of blood than the smaller larvae, a higher infection rate seen in the adult ticks (resulting from nymphs after molting) may alternatively be due to the intake of a larger (infected) blood meal as nymphs (before molting). This will be called the 'Clearance theory' in this paper, as this supposes ticks clear themselves off all *Anaplasma* in advance of each blood meal. This theory states that the larger volume of blood uptake (by feeding nymphs compared to larvae) represents a higher chance of becoming infected and therefor a higher infection rate. We predicted that if higher infection rates in adult ticks are due to the intake of a larger (infected) blood meal of nymphs in comparison to larvae, than it would be expected that the infection rate of non-infected nymphs which fed on infected sheep (Table 1, nr. 9) have a higher infection rate than non-infected larvae which fed on infected sheep (Table 1, nr. 1).

Thus the following hypothesis was tested:

The higher infection rates in the adult ticks may be due to the intake of a larger (infected) blood meal of nymphs in comparison to larvae.

Null hypothesis: No difference is seen in infection rate of SPF-larvae which fed on infected sheep (Table 1, nr. 1) compared to SPF-nymphs which fed on infected sheep (Table 1, nr. 9).

1.6.2.2 Transmission

The second round in the experimental study is called the transmission. In this part the transmission of *Anaplasma* from tick to sheep is tested. We predicted that if nymphs infected as larvae transmit all the *Anaplasma* organisms during feeding as nymphs, than it would be expected that the infection rate in infected nymphs which fed on non-infected sheep (Table 1, nr. 5) is lower (or even null) compared to non-infected larvae which fed on infected sheep (Table 1, nr. 1).

Thus the following hypothesis was tested:

Ixodes nymphs infected as larvae transmit all the *Anaplasma* organisms during feeding as nymphs.

Null hypothesis: Nymphs infected as larvae transmit not all *Anaplasma* organisms and therefore are not all negative for *Anaplasma* after feeding of on non-infected sheep (Table 1, nr. 5).

1.6.2.3 Speed of transmission

The second round in this experimental study is called the speed of transmission. Recent studies on the transmission dynamics of *Ehrlichia canis* by *Rhipicephalus sanguineus* ticks demonstrated that *Ehrlichia* is already transmitted by ticks feeding on dogs for as short as 3 hours (Fourie et al. 2013). In this experiment, the speed of transmission of *A. phagocytophilum* will be monitored. We predicted that if the speed of transmission of *Anaplasma* from infected ticks to non-infected sheep takes less than 36-48 hours, than it would be expected that sheep become *Anaplasma* positive after less than 36 hours of tick attachment.

Thus the following hypothesis was tested:

The speed of transmission of *Anaplasma phagocytophilum* is much faster than previous studies have indicated (36-48 hours).

Null hypothesis: Transmission of *A. phagocytophilum* from infected *I. ricinus* ticks to sheep takes at least 36 hours of tick attachment.

1.6.2.4 Co-feeding

The third round in this experimental study is called co-feeding. Co-feeding was demonstrated to occur in tick-borne encephalitis virus (Randolph 2011), *Borrelia* (Pérez et al. 2011; Pérez et al. 2012) and *Rickettsia conorii* (Zemtsova et al. 2010) and occurs when non-infected ticks feed closely together with infected ticks and acquire the infection directly from the tick feeding lesion rather than through the uptake of infected blood of a febrile sheep reacting to these parasites. We predicted that if *Anaplasma* can be transmitted from infected ticks to non-infected ticks by co-feeding, than it would be expected that when attaching both infected and non-infected ticks to non-infected sheep at least part of the non-infected ticks would become positive for *Anaplasma*. Co-feeding could have implications for epidemiology of *Anaplasma*, as this would allow ticks to acquire infection with *Anaplasma* even if the host build up immunity against this parasite.

Thus the following hypothesis was tested:

Transmission of *Anaplasma phagocytophilum* can occur through co-feeding ticks.

Null hypothesis: no co-feeding occurs in *I. ricinus* ticks for *Anaplasma*, therefore none of the non-infected ticks used in this experiment will become positive after feeding on non-infected sheep together with infected ticks.

1.7 What to expect in this paper

For the period of 12 weeks of research internship, there was not enough time to conduct all experiments and test all hypotheses stated above. However some interesting results were already gathered. The rest of this paper gives more information on the experiments conducted to test the hypotheses and gives an overview of some interesting results gathered during my research internship.

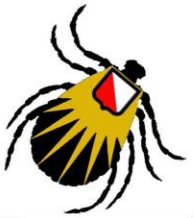

Table 1: All possible scenarios of ticks feeding on infected or non-infected sheep in all 3 different life stages. Expected outcome after the last given blood meal (represented in this table with an X) is given per scenario and per theory. The rows marked in purple are used in the experimental design of both the acquisition and transmission experiment.

	Nr.	Experiment (in which the given scenario is used)	**Larvae** feeding on:		**Nymphs** feeding on:		**Adults** feeding on:		Nr. Of infected blood meals	**Infection rate of ticks:**	
			Infected sheep	Non-infected sheep	Infected sheep	Non-infected sheep	Infected sheep	Non-infected sheep		**Clearance theory** (based on volume of blood uptake) last blood meal as: larvae=low, nymph = medium, adult = high	**Cumulative theory** (based on nr. of blood meals) 0=negative, 1=low, 2=medium and 3=high (1, 2 and 3 are expected to be positive in an experimental setting)
SPF larvae	**1**	**Acquisition**	**X**						**1**	**Low**	**Low**
	2	Transmission			X				2	Medium	Medium
	3	-					X		3	High	High
	4	Transmission AND Co-feeding						X	2	Negative	Medium
	5	**Transmission**				**X**			**1**	**Negative**	**Low**
	6	-					X		2	High	Medium
	7	Transmission						X	1	Negative	Low
	8	-		X					0	Negative	Negative
	9	**Acquisition**			**X**				**1**	**Medium**	**Low**
	10	-					X		2	High	Medium
	11	Speed of transmission						X	1	Negative	Low
	12	Co-feeding				X			0	Negative	Negative
	13	-					X		1	High	Low
	14	-						X	0	Negative	Negative

Some additional explanation of the different theories used in the table above:
*Cumulative theory: in nature the cumulative theory would represent a larger chance of being infected as single tick if more blood meals are taken (and thus the tick is in a higher life stage). However, in this experimental setting in which the infection state of the sheep on which ticks feed is known, it is expected that all ticks are positive that fed at least one time on an infected sheep. The cumulative theory states that a higher infection rate is found in adult ticks due to having had an additional blood meal. The higher the number of blood meals, the higher the chance of the tick becoming infected.

The clearance theory is what is stated in our hypothesis, that all *Anaplasma* organisms are transmitted to the host before a new blood volume is taken. So before each blood meal the tick clears itself of *Anaplasma* and a larger volume of blood uptake (by feeding nymphs compared to larvae) represents a higher chance of becoming infected and therefore a higher infection rate.

The difference between these two theories is in the fact that for the clearance theory we expect that, when the last blood meal is on a non-infected sheep, the ticks will all be negative (except for the contribution of co-feeding, which is why this has to be tested in an experimental setting). Whereas in the cumulative theory ticks maintain their infection rate even if the last host was a non-infected sheep. In a natural setting, where infected and non-infected animals are mixed together and the feeding history of a tick is not known, generally the more hosts fed on the higher the infection rate.

2. Material and method

In this study information is gathered from sheep (physical examination, blood sample analysis and in some cases pathology) and from ticks removed from sheep. This section involves the experimental setup and laboratory procedures used, in order to obtain the results.

2.1 Experimental procedures

Here experimental procedures used to obtain the results are described. These experimental procedures includes physical examination of the sheep, blood smears, DNA extraction blood, DNA extraction ticks, PCR, RLB, qPCR, sequencing and Pathology.
All blood samples and ticks were processed in the laboratory of UCTD. In order to obtain information about tick-borne pathogens (including but not limited to *Anaplasma*) present in the sheep's blood or ticks the process of DNA-extraction, followed by polymerase chain reaction (PCR) and reverse line blotting (RLB) or qPCR was performed. All laboratory techniques used in this study are described below.

2.1.1 Physical examination by the local veterinarian and collection of blood

All sheep in this study were followed regularly by physical examination. Measurement of temperature was regarded as the most valuable information, as the main clinical sign of TBF is a sudden onset of high fever. Temperature was measured rectally and fever was defined as a body temperature of 40 degrees centigrade or above. Normal sheep temperature ranges from 38,5 until about 40,0 degrees centigrade. As sheep are easily stressed, temperature is probably influenced by former proceedings performed on the sheep. In order to reduce the impact of stress on temperature results, especially in the experimental study, the same protocol was used each time data were collected. Starting with temperature measurement.

Sheep in the sentinel study were clinically examined immediately before introduction into the Bargerveen nature reserve. From then, physical examination was performed on a weekly basis until either the sheep died or were taken out of the nature reserve because of a critical clinical situation (which was to the assessment of the local veterinarian). Results of physical examination were noted on the physical examination score sheet, which is included in the appendix. Immediately after physical examination of the sheep, blood samples (10-mL volume) were collected by jugular vein puncture with needle and vacutainer tubes containing EDTA anticoagulant and send by post to the UCTD in a transport safe sealed bag suitable for biological substances category B (UN3373) within a bubble wrap envelope for further processing.

Sheep in the experimental study were subjected to daily temperature measurement from day 0 (inoculation) until day 14 or until fever was over. Temperature data were tracked and clinical abnormalities in behavior were written down in a logbook. Blood samples were collected by jugular vein puncture in tubes containing EDTA anticoagulant after disinfection of the skin overlaying the jugular vein by ethanol. Blood tubes were transported to the UCTD laboratory in a transport safe sealed bag suitable for biological substances category B (UN3373) within a lockable plastic container. Blood samples collected in order to screen for the presence of tick-borne diseases (day -7 and day 0) were processed according to DNA extraction, PCR and RLB hybridization. Blood samples collected in order to detect if sheep were (still) infected with *Anaplasma* were processed according to either DNA extraction, PCR and RLB hybridization or DNA extraction and qPCR. The latter of this is only possible in the experimental tick transmission model study and after screening for the presence of other tick-borne pathogens on day -7 and day 0, as qPCR only detects if *Anaplasma* is present.

2.1.2 Blood smears

Immediately after arrival of the blood at UCTD, a blood smear was made in a laminar flow cabinet in order to quickly screen for the presence of *Anaplasma*. After drying for about 1 hour microscopy slides were stained with 'kwik diff stain kit' and examined under a microscope at 1000x for at least 5 minutes for presence of morulae (micro-colonies of *Anaplasma*) in neutrophil granulocytes, which represent as blue (basophilic) inclusions in the cytoplasm (Figure 3).

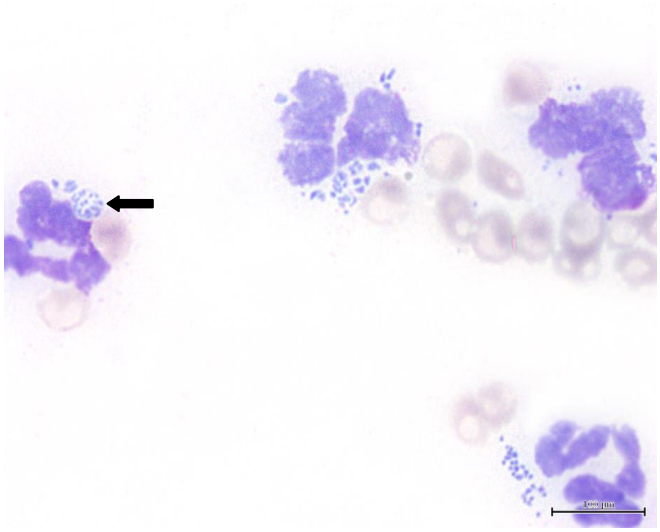

Figure 3. Photo of a blood smear, showing both erythrocytes (in red coloration) and neutrophil granulocytes (purple nucleus surrounded by a light-gray colored cytoplasm). Black arrow points at a cytoplasmic inclusion body of *Anaplasma phagocytophylum* (morulae) inside a neutrophil granulocyte.

2.1.3 DNA extraction of blood

DNA was extracted from each blood sample, in order to gather results concerning the pathogens present (by means of subsequent PCR and RLB hybridization). DNA extraction was performed using the Nucleospin Tissue Kit (Machery-Nagel), of which the protocol is included in the appendix. The process of DNA extraction involves lysis of all blood cells, after which the DNA is separated from the other particles using different kinds of buffers. The DNA that was obtained was amplified by means of PCR, to ensure enough DNA is available for detection during RLB hybridization. A protocol of DNA extraction from blood is included in the appendix.

2.1.4 DNA extraction of ticks or sheep tissue samples

The process of DNA extraction from ticks or sheep tissue samples is practically the same as from blood. However, some additional steps are necessary in order to lyse all tissue cells. A protocol of DNA extraction of ticks/sheep tissue is added in the appendix.

2.1.5 PCR

Extracted DNA was amplified by means of PCR. PCR is a process whereby specific selected strands of DNA are amplified (Figure 4). Using a specific set of primers complementary to a certain part of the pathogen's DNA (target sequence), ensures only the pathogen's DNA is amplified. Thereby increasing the amount of a specific part of the pathogen's DNA (target sequence) and thus the sensitivity and the specificity of the subsequent RLB outcome. In this study, primers complementary to the following pathogens were used: *Anaplasma spp.*, *Ehrlichia spp.*, *Babesia spp., Theileria spp.* and *Borrelia spp.* These pathogens were selected because these are all tick-borne pathogens known to be present in The Netherlands.

Because of their relative similarity to one another concerning the PCR-protocol, primers of *Anaplasma* and *Ehrlichia* were put together as well as *Babesia* and *Theileria*. For each of the primer

sets (a forward and a reverse primer) one set of mastermix was made, in order to be able to execute the PCR. Thus a total of three mastermixes were made each time PCR was performed; *Anaplasma/Ehrlichia*, *Babesia/Theileria* and *Borrelia*. Besides the forward and reverse primer, the mastermix also contained water, 5x Phire reaction buffer, 10 mM dNTPs and 2U/µl Phire hot start II DNA polymerase. The mastermix was then added to eppendorf tubes together with the extracted DNA samples. In all reactions, positive and negative controls were used. Finally the eppendorf tubes were placed in the PCR machine and the settings were adjusted in order to ensure maximum DNA replication. The protocol used to perform PCR is added in the appendix.

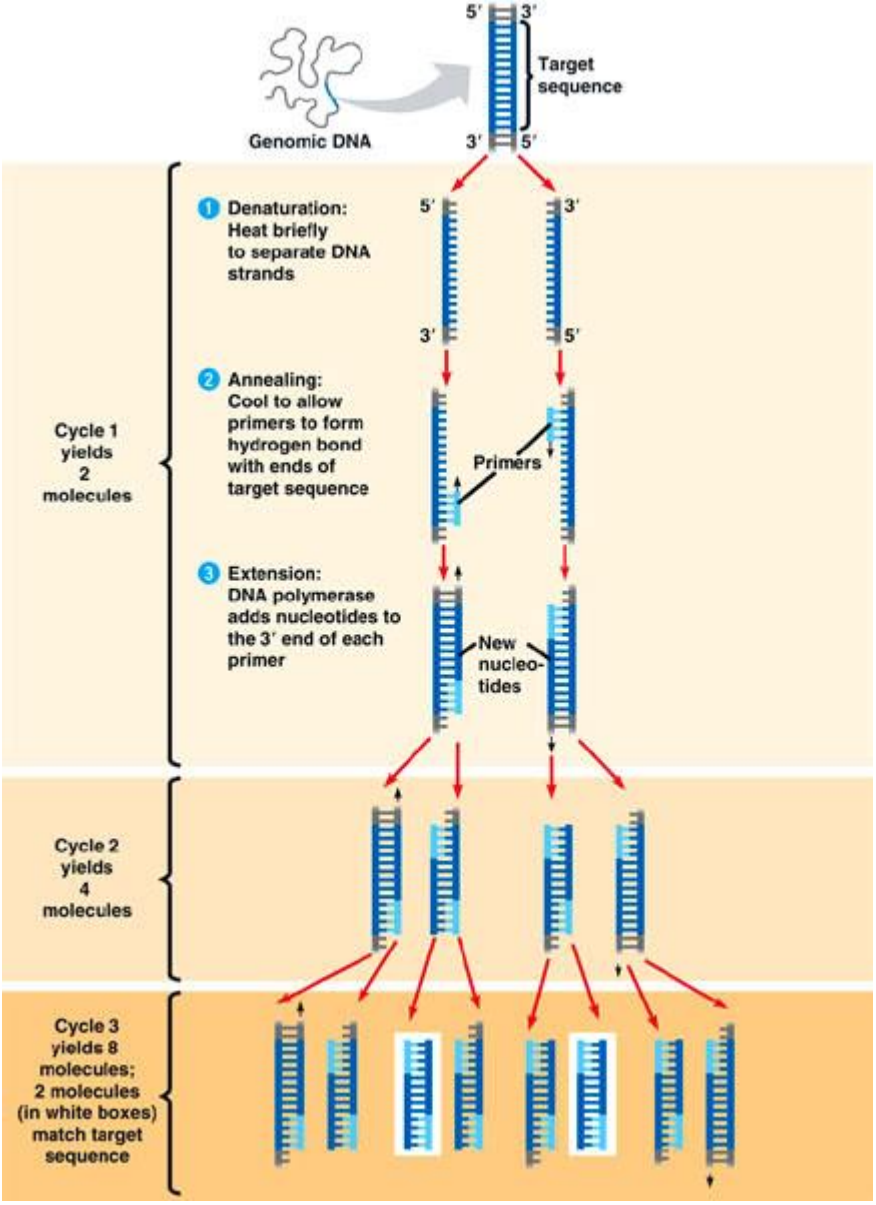

Figure 4. Schematic representation of consecutive PCR steps in order to amplify a target DNA sequence (Ablett et al. 2014).

2.1.6 RLB hybridization

Reverse line blotting (RLB) hybridization is a process in which target DNA strands amplified by PCR are introduced onto a membrane containing covalently-bound probes complementary to the amplified DNA sequences of the specific pathogens (Figure 5). So if the amplified DNA is complementary to one of the probes on the membrane, it will attach to the probe. These amplified

DNA strands are labeled with biotin. Streptavidin labeled with peroxidase binds to the biotin label. The peroxidase consequently catalysis a reaction with the ECL reagents. This chemical reaction results in luminol, a substance in ECL reagent 2, becoming oxidized and producing light. A light-sensitive film is subsequently placed on top of the membrane in order to visualize the binding of the PCR products, after developing of the film. A protocol of the RLB procedure is included in the appendix. The membrane is then washed for reuse. A protocol of the membrane stripping procedure is also included in the appendix.

The RLB used in this study contains probes for detection of the following the tick-borne pathogens: *Anaplasma spp.*, *Ehrlichia spp.*, *Babesia spp., Theileria spp.* and *Borrelia spp* (Figure 6). Probes used for detection of *A. phagocytophilum* are shown in Table 2. Besides the species-specific probes, *catch-all* probes are included on the membrane in order to screen more general for the presence of pathogens. These *catch-all* probes consist of highly conserved parts of the DNA. The RLB membrane used in this study contained catch-all probes for *Ehrlichia/Anaplasma*, *Theileria/Babesia*, *Babesia*, *Theileria* and *Rickettsia*.

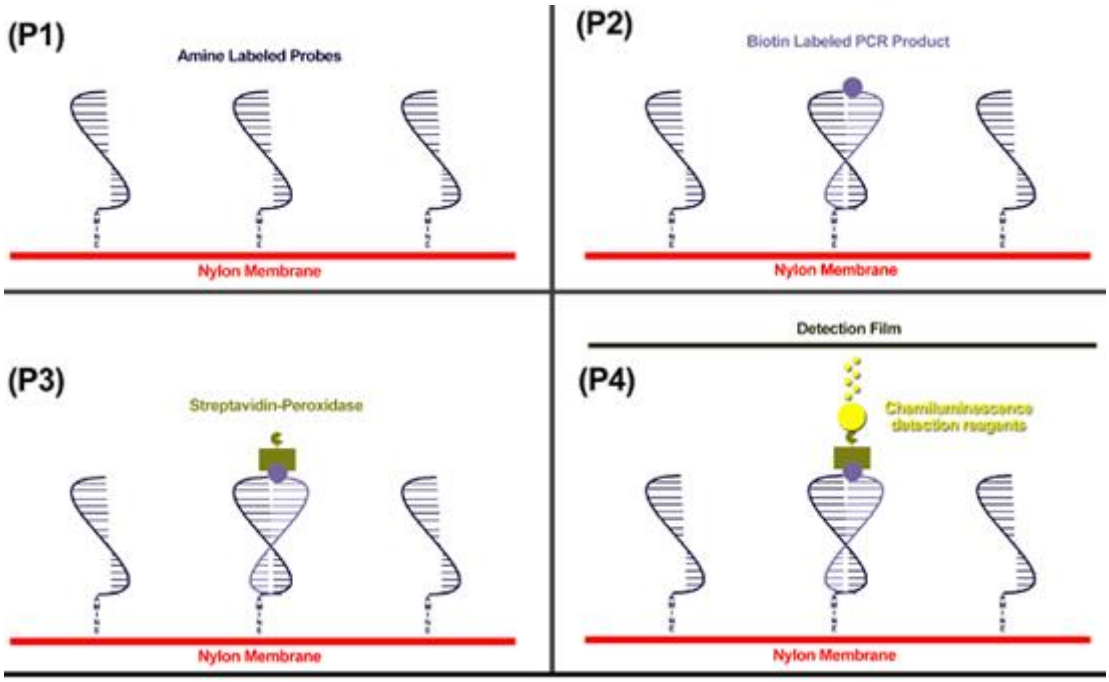

Figure 5. Schematic representation of subsequent RLB hybridization steps (O'Sullivan et al. 2011). (P1) Amine labeled probes are bound covalently to a nylon membrane. (P2) PCR products labeled with biotin bind to the complementary probe. (P3) Streptavidin, labeled with peroxidase, is incubated with the membrane and binds to the biotin label. (P4) Peroxidase catalysis a reaction in the ECL detection reagents, producing light to which is a light-sensitive film is exposed in order to visualize the regions on the membrane where PCR products attached to the probes. The membrane is then washed for re-use.

Table 2: Primers used for the detection of *A. phagocytophilum*. Four different primers were used on the RLB. Nucleotides that deviate from the other DNA sequences are marked in red.

Pathogen	Primer DNA-sequences
Anaplasma phagocytophilum 1	TTG CTA TAA AGA ATA ATT AGT GG
Anaplasma phagocytophilum 3	TTG CTA TGA AGA ATA ATT AGT GG
Anaplasma phagocytophilum 5	TTG CTA TAA AGA ATA GTT AGT GG
Anaplasma phagocytophilum 7	TTG CTA TAG AGA ATA GTT AGT GG

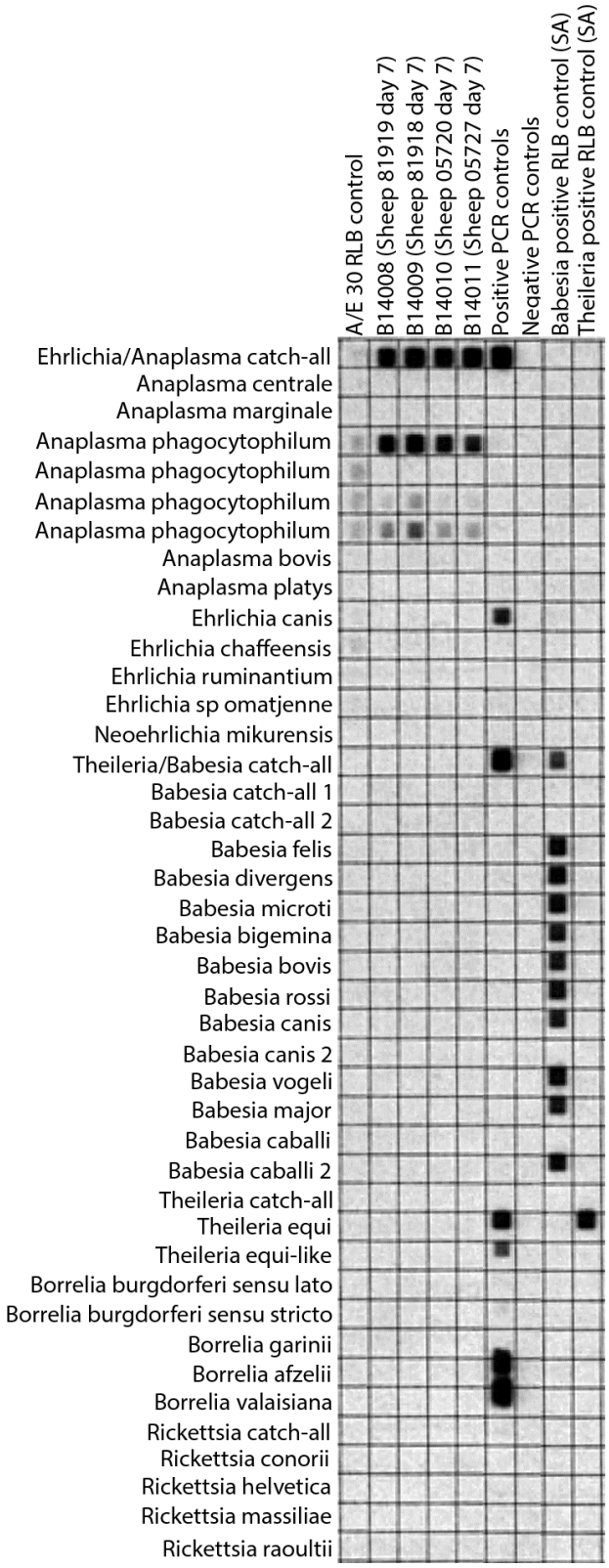

Figure 6. RLB outcome example (this outcome was performed in the second controlled introduction in the sentinel study).

2.1.7 qPCR

Real-time PCR or quantitative PCR (qPCR) is a technique whereby DNA amplification (by means of PCR) and detection are combined into a single step. Advantage of this method is that it is quicker in comparison with amplification by PCR followed by RLB hybridization. However, qPCR only screens for the presence of *Anaplasma*. Other tick-borne pathogens are not detected by qPCR. Therefore this technique is only suitable for the detection of *Anaplasma* in ticks or sheep blood collected in the experimental tick transmission model study after ticks or sheep were proven negative of other tick-borne pathogens (by RLB hybridization on female ticks producing the eggs (as no transovarial transmission is known to occur) and on blood samples collected at day -7 and day 0). Other advantage of qPCR is that it allows for a quantification of this *Anaplasma* infection.

2.1.8 Sequencing

Sequencing is a process in which the nucleotide sequence is determined of a given DNA fragment. In this study the DNA sequence of the *Anaplasma* strain used for inoculation of sheep will be determined, in order to be able to compare it's DNA sequence in relation to DNA sequences of *Anaplasma* studied in other publications. However this sequencing still had to be done at the moment of finishing this master thesis.

2.1.9 Pathology

It was agreed with the supervising local veterinarian in the sentinel study that all sheep that died during the experiment would be necropsied. During the experiment, three of the sheep that met the inclusion criteria at start of the study died. Two died within the study period and one died a few days after ending the experiment. Necropsy was only performed on two sheep; one that died within the study period and one that died a few days after the end of this study.
If sheep in the experimental tick transmission study would die, then necropsy would be performed. However under laboratory conditions *Anaplasma* was well under control and none of the experimental sheep died during the experiment.

2.2 Sentinel study

This section describes the experimental design of the field study in which sentinel sheep were introduced into the Bargerveen nature reserve.

2.2.1 Study area

This study was conducted in the Bargerveen Nature Reserve located in the province of Drenthe in the north-east of the Netherlands (Figure 7, Figure 8), covering a total area of about 21 km^2 at a latitude of $52^0$68' north and a longitude of $7^0$02' east. Average altitude is about 18 m above sea level. The vegetation consists mainly of large peat areas, heather and small lakes, with rainfall of 800 mm/year (quite evenly spread over the year) and an average annual temperature of about 9 ºC (with average month temperatures ranging from 1 ºC to 16 ºC). The farmer who participated in this sentinel study houses a herd of at least 200 sheep in this nature reserve, which are kept within the nature reserve from May to November. More farmers have sheep stationed within this nature reserve.

2.2.2 Study population

The study population consists of a total of 16 sheep of various breeds, which were introduced into the study area in three consecutive controlled introductions of respectively 10, 4 and 2 sheep. Sheep were bought form a sheep farm in Zuidlaren and all sheep were born and kept within 20-40 km distance from the Bargerveen nature reserve. Sheep were only included in the study if they met all inclusion criteria. Inclusion criteria used, were a negative PCR/RLB for all (tick-borne) pathogens according to the RLB used and a good clinical condition assessed by the supervising veterinarian at the moment of introduction. In total 6 sheep, 2 sheep per controlled introduction, were excluded from the sentinel study due to returning a positive RLB for at least one of the tick-borne diseases. The remaining sheep population that met all inclusion criteria consisted of 16 sheep. Samples were collected between July 2014 and November 2014.

2.2.3 Experimental design

As the main purpose of this sentinel study was to collect *Anaplasma* from the Bargerveen nature reserve, sheep negative for *Anaplasma* or other tick-borne diseases were introduced into the Bargerveen nature reserve. Three controlled introductions under strict veterinary supervision followed.

The local veterinarian was asked to perform full physical examination and collect sterile EDTA blood samples of the sheep on a weekly basis (for a maximum period of 8 weeks), in order to be able to collect *Anaplasma* and to monitor infection rate of sheep over time. Physical examination and blood sampling were also performed immediately before introduction into the Bargerveen nature reserve, in order to check whether the sheep met all inclusion criteria. As already mentioned before, a sample of the score sheet which was used for gathering clinical data is included in the appendix.

Supervising veterinarians were also instructed to inform the author if intervention was needed in order to save the sheep's lives or if sheep suddenly died. Initially, sentinel animals were not treated in order to monitor events under the local prevailing conditions. However TBF is known to render the host susceptibility to secondary infections. Therefore sheep were kept under strict veterinary supervision in order to be able to quickly intervene, if necessary to save the sheep's lives. The need to intervene was monitored by clinical impression and by measuring the presence of fever, in combination with a positive blood smear or PCR/RLB. Several times, the decision to intervene was made solely based on clinical condition of a sheep. Waiting for the laboratory results would cause unnecessary discomfort for the sheep, because of the high likelihood of TBF after the first results started to appear. Intervention consisted of removing the sheep out of the nature reserve followed by treatment with long acting oxytetracycline. If mortality occurred in sheep, necropsy was performed at GD Deventer.

Blood samples were collected on a weekly basis and were send to UCTD in a transport safe sealed bag suitable for biological substances category B (UN3373) within a bubble wrap envelope for further processing of data by performing blood smears, DNA extraction, PCR and RLB hybridization. Some of the blood samples were, when RLB hybridization was proven to be positive for *Anaplasma*, used to make a blood stabilate (stored in liquid nitrogen) for later use in an experimental tick transmission model.

Results were tracked per sample and were later processed per sheep and per controlled introduction. RLB hybridization was performed on all blood samples and results were plotted as cumulative infection with *Anaplasma,* in order to follow the course of infection rate in sheep. As on every blood sample both a blood smear and RLB hybridization was performed, outcomes of these two diagnostic techniques could be compared. No statistical analysis was performed on the gathered data. However, data were analyzed in order to detect trends visible in the collected data.

Figure 7. Sheep in the Bargerveen Nature Reserve.

Figure 8. Map of the Netherlands. The red sheep represents the location of the Bargerveen nature reserve in the province of Drenthe.

2.3 Experimental tick transmission model

This section describes the experimental tick transmission model, in which relevant variables in the tick-host-pathogen transmission dynamics were studied under controlled laboratory conditions.

2.3.1 Study area

This study was conducted under controlled laboratory conditions in the stables at the Department of Farm Animal Health (DFAH) of the Faculty of Veterinary Medicine at Utrecht University.

Biosecurity
As *Anaplasma* is a zoonosis, a strict biosecurity protocol was used to prevent ticks from escaping out of the experimental setup in order to minimize the chance for people (and other farm animals within the building) to get into direct contact with these ticks. Therefore physical separation of the sheep (from the rest of the Farm Animal Health Department) and several physical barriers were put into place.

Barriers implemented in order to minimize the risk for people working within the boundaries of the experimental setup, included wearing of hand gloves and a disposable coverall. The border of the experimental setup consisted of a gutter filled with a solution of Delladet 1% and a changing room. The changing room served as an extra physical barrier as it was the only point of entrance or exit to the experimental setting, in which boots were changed and the disposable coverall was put on (at arrival) or off (at departure).

In preparation of this experiment, a tick mortality experiment with Delladet was conducted in order to determine the effect of different Delladet concentrations on tick mortality at different time intervals after submergence of ticks into the solution. This tick mortality experiment was performed with the purpose of evaluating the potential of Delladet as a reliable physical barrier in order to prevent ticks from escaping out of the experimental setting.
In this experiment it was found that immersion of ticks into a solution of Delladet had a positive effect on mortality compared to immersion into tap water. Variations between Delladet concentrations seemed of minor importance concerning mortality rate. Remarkable and relevant finding during the experiment, was that ticks placed in a test tube with a Delladet solution sank immediately to the bottom and were not able to get back to the surface. This effect was probably due to the diminished surface tension caused by the disinfectant Delladet. On the other hand, ticks in the control tubes were able to crawl out of the water.
Based on the results of this tick mortality experiment in different Delladet concentrations, it was recommended to use a concentration of 1% Delladet, as higher concentrations seemed to have no additional effect on the mortality rate. A more extensive description of this experiment and a protocol for filling the gutter with Delladet (which was used by the animal caretakers that took care of the experimental sheep) is included in the appendix.

2.3.2 Study population

Tick (vector)
Ticks used during this experiment were *I. ricinus* and all hatched from eggs as SPF-larvae. SPF-status of larvae was tested for each batch by performing RLB on the adult female tick which laid the eggs. Although it is known that no transovarial transmission of *A. phagocytophilum* occurs, some other tick-borne pathogens are known to be transmitted vertically from adult female tick to eggs. Therefore larvae were only marked SPF if their mother was negative for all tick-borne pathogens according to the RLB used. Depending on the experiment conducted, ticks of larval, nymph or adult stage were placed on infected- or non-infected sheep.

Host
Each experimental round consisted of a maximum of 4 sheep. For each experimental round 4 new sheep were obtained from the Department of Farm Animal Health and only became part of the study population if they met the inclusion criteria. Sheep were introduced within the containment of the Farm Animal Health Department 7 days before actual start of the experiment (day -7). This was done in order for the sheep to acclimate and to prevent possible infection by ticks with tick-borne diseases in the waiting time between day -7 and start of the experiment. At day -7 also

blood samples were collected, to screen if sheep met the inclusion criteria. Inclusion criteria used were a negative PCR/RLB for all tick-borne pathogens present on our RLB (on the blood sample taken at day -7) and good clinical condition at start of the experiment. Sheep that did not match the inclusion criteria, were removed out of the experimental setting. Therefore, each experimental round consisted of a maximum of 4 *Anaplasma* negative sheep.

Adult Texel sheep born and bred in The Netherlands were used. They were fed hay and pelleted feed concentrate with water available ad libitum. Each experimental round received a serial number, starting with M1. Table 3 shows the number of sheep that met the inclusion criteria per experimental round conducted.

Table 3. Overview of number of sheep that met the inclusion criteria per experimental round.

Experimental round	Nr. of sheep included	Tested scenario (Table 1)
M1	3	Nr. 1
M2	4	Nr. 9

Pathogen
In this study the *Anaplasma* stabilate number GU 196 collected on 13-06-1988 from sheep on the North Sea Island of Ameland, the Netherlands was used (Jongejan et al. 1989; van Miert et al. 1984). All isolates were stored in liquid nitrogen as infected blood stabilates, and were cryoprotected with 10% dimethylsulphoxide (DMSO) before being used. The DNA will be sequenced in order to be able to compare the *Anaplasma* type used in this study with other publications.

2.3.3 Experimental design

Main purpose of the experimental study was to further elucidate the interaction between *Ixodes ricinus* ticks, sheep and *A. phagocytophilum* under controlled laboratory conditions. This was tested in consecutive rounds testing several variables which could be relevant in the tick-host-pathogen transmission dynamics. Depending on the experiment conducted, infection rate was measured in ticks or transmission of infection to sheep was monitored.

Sheep were or were not experimentally inoculated with a specific strain of *Anaplasma,* depending on the experiment. Inoculation was done by injection of 2-mL of thawed stabilate (infected sheep blood) in the jugular vein, which was proven positive for (only) *Anaplasma* by RLB. Bags made of cloth were attached dorsally to the back of the sheep in two patches, one cranial and one caudal (Figure 9). Special glue was used. Depending on the experiment, ticks of specific life stages and infected or not-infected were put on the shaved sheep skin within these bags made of cloth. The bags were closed by a rope. Ticks were harvested on a daily basis, after which ticks were put in the incubator with a temperature of 24 degrees centigrade and humidity of 95% in order to provide the right circumstances for the ticks to molt to the next life stage. This molting takes approximately 4-6 weeks, depending on tick stage.
All sheep were monitored daily by rectal temperature measurement. If fever was measured, defined as a temperature above 40 degrees centigrade, a sterile EDTA blood sample of about 10mL was collected in order to monitor for the presence of *Anaplasma* (via blood smear and PCR/RLB or qPCR).
After 14 days or after fever had subsided in all sheep, the experiment was ended. The last remaining ticks were removed with a forceps, tick bags were removed, skin damage was treated for 3 consecutive days with Acederm® woundspray and sheep were treated with tick repellent (Tectonik®) and one injection of 500 mg oxytetracycline in order to clean experimental sheep from infection.

Only acquisition was tested, but no results concerning infection rates in ticks were available at moment of finishing this master thesis. Results will be tracked per sample and will later be processed per sheep and per experimental round. By comparing outcomes of different scenarios, the stated hypotheses will be tested. No control group was used and data were not processed single-blinded.

Transmission, speed of transmission and co-feeding were not performed during the period I performed my research internship. But these experiments will be conducted in this experimental tick transmission model and therefore are still described below.

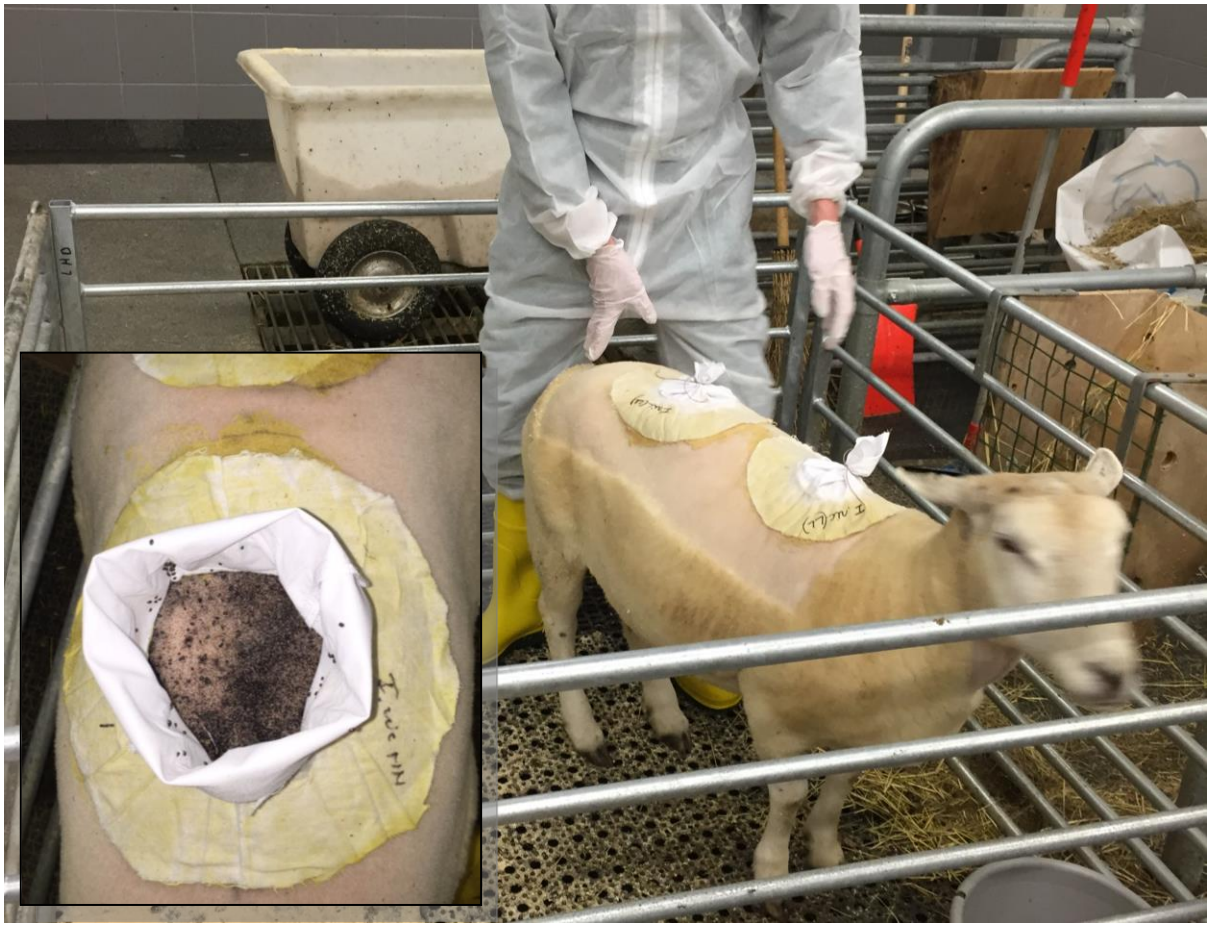

Figure 9. Sheep with two patches, wherein ticks are released during the febrile period. The picture on the left shows an opened patch with nymphs after their blood meal.

2.3.3.1 Acquisition

In order to test whether higher infection rates in adult ticks are due to the intake of a larger (infected) blood meal of (non-infected) nymphs in comparison to (non-infected) larvae, SPF larvae (M1) and non-infected nymphs (M2) were placed on experimentally infected sheep in two different experimental rounds (Figure 10). After feeding, all ticks were removed and after molting to the next tick life stage infection rate of ticks will be determined by performing qPCR on larvae and nymphs. Resulting infection rates will be compared in order to analyze whether non-infected nymphs which fed on infected sheep (Table 1, nr. 9) have a higher infection rate than non-infected larvae which fed on infected sheep (Table 1, nr. 1).

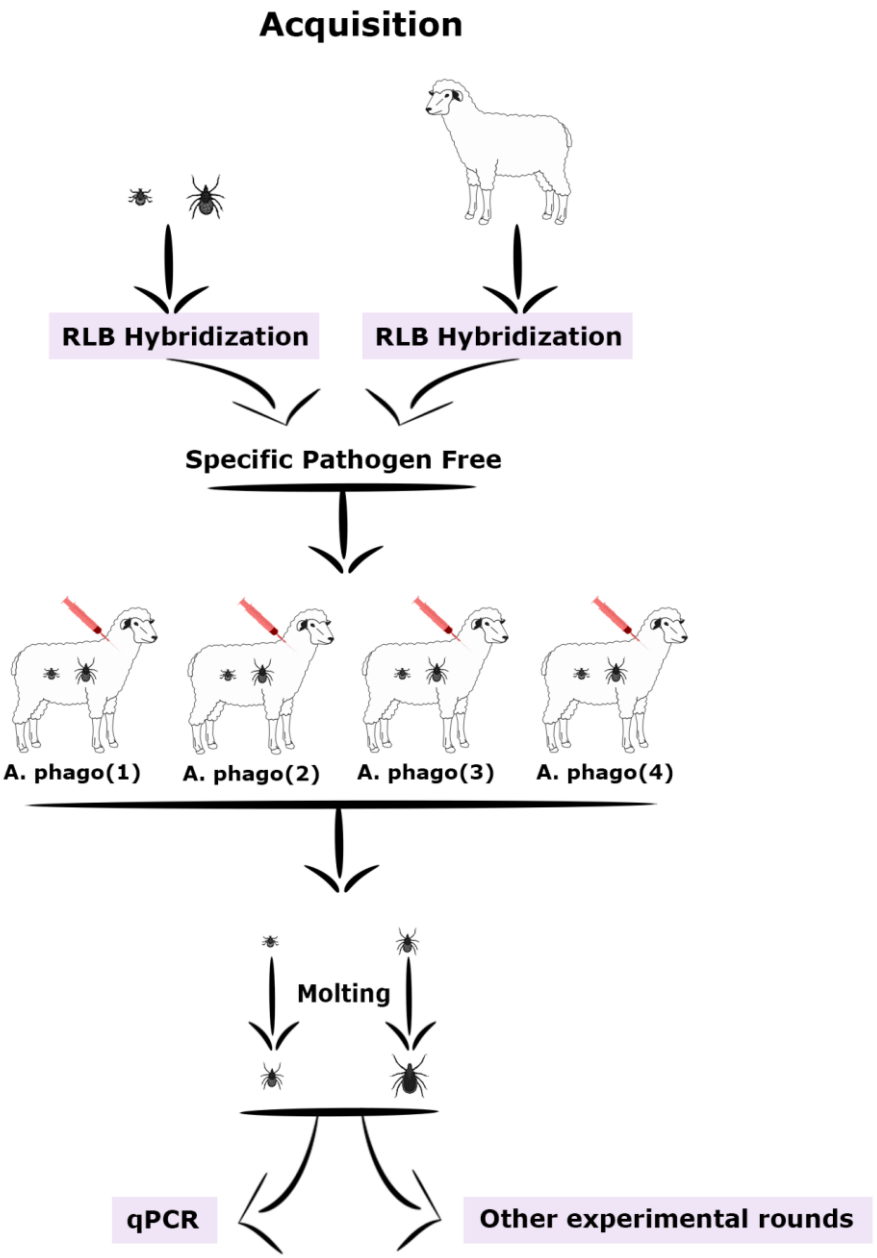

Figure 10. Flowchart of acquisition of *Anaplasma* by tick larvae compared to tick nymphs. Infection rates between these tick stages will be compared, in order to test whether higher infection rates in adult ticks are due to the intake of a larger (infected) blood meal of nymphs in comparison to larvae.

2.3.3.2 Transmission

To test whether *Ixodes ricinus* nymphs infected as larvae transmit all the *Anaplasma* organisms during feeding as nymphs, specific pathogen free (SPF) larvae are fed on experimentally inoculated sheep (Figure 11). After feeding, all larvae are removed and incubated under 24 degrees centigrade and humidity of 95% in order for them to molt to nymphs. Infection rate of larvae is determined by qPCR on part of these ticks. Other part of these ticks molted to nymphs,

will subsequently be attached to non-infected sheep. After removal and incubation, during which molting to adults takes place, infection rate in adult ticks will be measured by qPCR. The hypothesis will be tested by comparison of infection rate in non-infected larvae which fed on infected sheep with ticks that had the same background and were subsequently fed as nymphs on non-infected sheep.

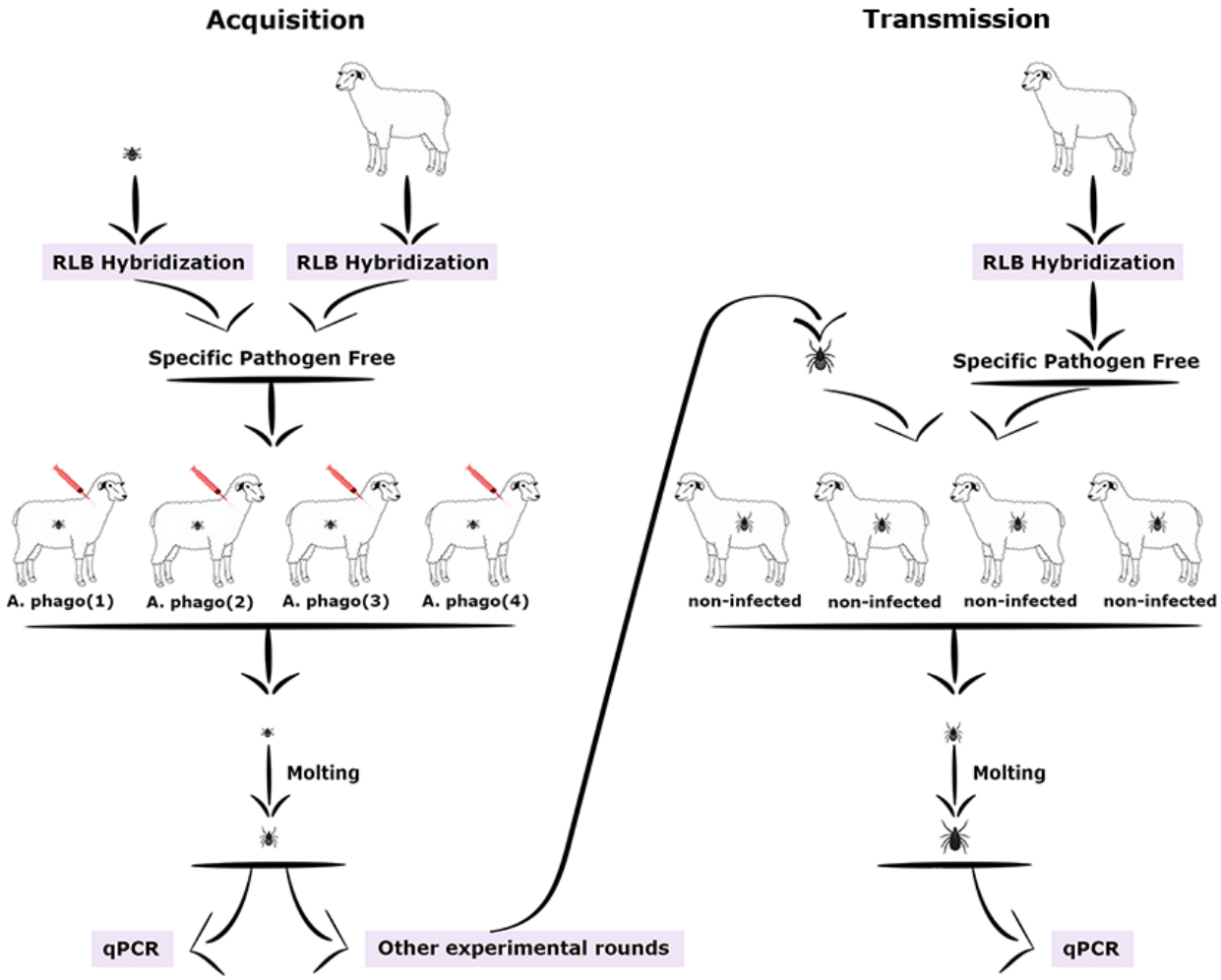

Figure 11. Flowchart of transmission (after acquisition) of *Anaplasma.* In the acquisition, larvae are fed on infected sheep, after which infection rate is determined by qPCR. Consequently these larvae are fed on non-infected sheep to analyze whether all *Anaplasma* organisms are transmitted during feeding (thereby clearing themselves from infection when feeding on a non-infected sheep).

2.3.3.3 Speed of transmission

In this experiment the speed of transmission of *Anaplasma* from infected ticks to non-infected sheep will be tested (Figure 12). Therefore adult ticks that are fed on infected sheep as (larvae and) nymphs are placed on non-infected sheep for different time intervals (respectively 3, 6, 12 and 24 hours) after which sheep will be followed for having fever and becoming positive for *Anaplasma* by qPCR.

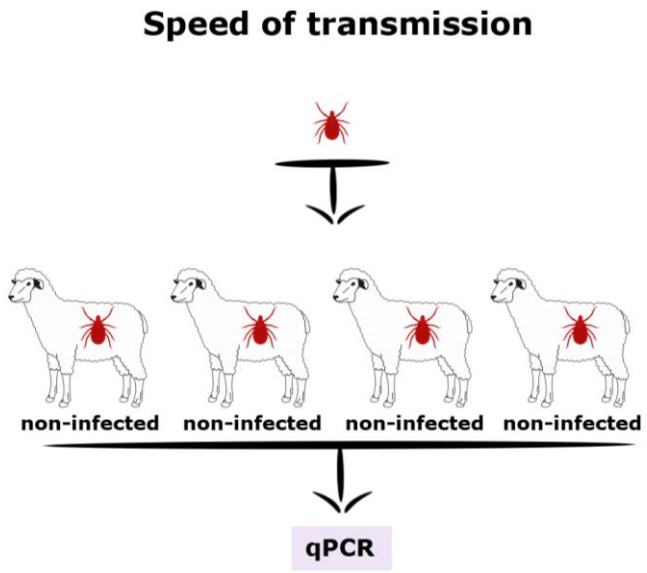

Figure 12. Flowchart of speed of transmission. Adult infected ticks will be allowed to attach to non-infected sheep for different time intervals (3, 6, 12 and 24 hours) after which sheep will be followed for becoming *Anaplasma* positive.

2.3.3.4 Co-feeding

The concept of co-feeding will be tested for occurrence and to what extend it occurs. Therefore non-infected nymphs will be placed on non-infected sheep together with infected adult ticks for a certain periods (Figure 13). After which ticks will be collected and infection rate of both experimental tick groups will be determined by qPCR. Sheep will also be monitored for becoming *Anaplasma* positive. (This monitoring of sheep is practically the same experiment as the speed of transmission experiment described above. Combining of these two experiments will give all results needed to test both hypotheses at the same time.)

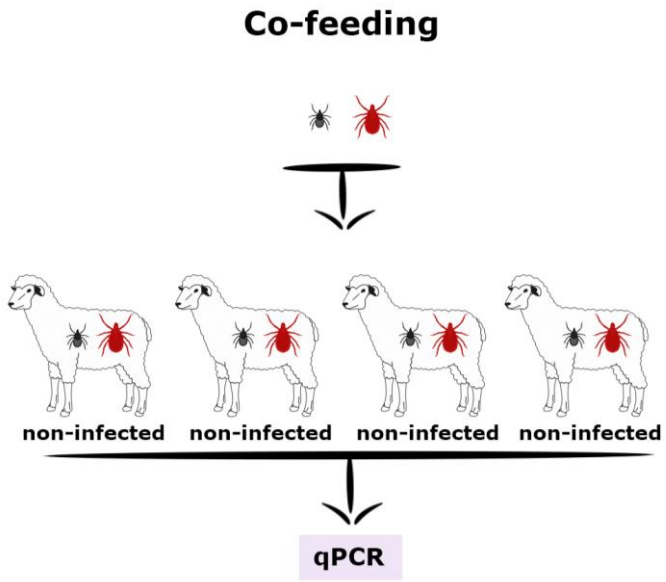

Figure 13. Flowchart of co-feeding. Non-infected nymphs together with infected adult ticks will be placed on non-infected sheep. It will be monitored whether non-infected nymphs acquired infection with *Anaplasma* (when at the same time sheep were not positive) by performing qPCR on them.

3. Results

3.1 Sentinel study

In this experiment the tested hypothesis is that TBF (caused *by Anaplasma phagocytophilum* and transmitted by *Ixodes ricinus* ticks) is the main cause of disease and possibly mortality in sheep introduced into nature reserves in the Netherlands. After introduction into the Bargerveen nature reserve, sheep were monitored by full physical examination and blood samples were collected regularly. Off all 64 blood samples analyzed, 20 (31%) were RLB positive.

Physical examination

Analysis of physical examination data revealed that lymphadenopathy (defined as several enlarged lymph nodes on at least one data collection moment) was scored at 14 different sampling moments. Of all blood samples taken from sheep with 2 or more enlarged lymph nodes, 7 out of 14 (50%) were positive for *Anaplasma* by RLB. These samples were collected in 10 different sheep. In sampling moments where 3 or more enlarged lymph nodes were scored, even 3 out of 4 (75%) of samples were positive for *Anaplasma*.

Of all 26 sampling moments in which a rectal temperature higher than or equal to 40 ºC was measured, 10 (38 %) sheep were RLB positive. In case temperature reached 40,5 ºC or above, 7 out of 14 (50%) samples were RLB positive and when of a temperature over 41 ºC was measured 4 out of 4 (100%) blood samples were RLB positive. For comparison, in 26 out of a total of 64 (41%) blood samples, both RLB was positive and a rectal temperature of ≥ 40 ºC was measured. In case the rectal temperature was ≥ 40,5 ºC, 14 out of a total 64 (22%) blood samples were positive and when the temperature was ≥ 41 ºC, it was found that 4 out of a total 64 (6%) blood samples were positive.

Scabby ears was reported by the local veterinarian at 7 sampling moments and in 6 different sheep. RLB was positive for *Anaplasma* in 2 out of 7 (29%) sampling moments in which scabby ears were scored.

Off all 45 blood samples collected from 12 different ewe sheep, 16 samples were RLB positive (36%). Off all 19 blood samples collected from 4 different lambs, 4 were RLB positive (21%). All other data collected by clinical examination were noted too few to draw any conclusions, and consequently were not included in this results section.

Ticks collected from sheep

In total 6 ticks were collected from 3 different sheep (2 ticks on day 0 and 4 ticks on day 12) all in the second controlled introduction. Off all these collected ticks, respectively 0 and 2 ticks were proven positive for *Anaplasma* and all were adult female ticks of species *I. ricinus*.

Blood smears compared to RLB hybridization

The outcome of blood smears was compared with the RLB hybridization outcome as golden standard (Table 4). A total of 64 blood samples were collected from 16 sheep at several moments during the period of the study. All blood samples were processed by both blood smears and RLB hybridization. In 8 out of 64 blood smears evaluated, clear examples of morulae within neutrophil granulocytes were observed and thus these samples were scored positive for *Anaplasma*. In all 8 of these positive blood smears the RLB was positive for *Anaplasma*.

Off the 20 blood samples which were tested positive for *Anaplasma* by RLB, 8 were scored positive by microscopic evaluation of blood smears. Thus a sensitivity of 40% was scored for blood smears as diagnostic tool. All 44 RLB negative blood samples were also scored negative by microscopic evaluation of blood smears.

Infection rate of sheep over time

Evaluation of infection status of sheep with *Anaplasma* was performed based upon RLB hybridization. Results were plotted as cumulative infection with *Anaplasma,* in order to follow the course of infection rate in sheep over time. As soon as 8 days after introduction, already 8 out of 16 (50%) sheep were positive for *Anaplasma* (Figure 14). At 28 days past introduction, 11 out of 16 (69%) sheep were positive and after 51 days 13 out of 16 (81%) sheep were positive for *Anaplasma*.

These 16 sheep were introduced in 3 consecutive controlled introductions. At the end of the first controlled introduction (day 51), 7 out of 10 (70%) sheep were positive for *Anaplasma* (Table 5). The second and third controlled introduction was ended at respectively 12 and 14 days after introduction and resulted in 4 out of 4 (100%) sheep respectively 2 out of 2 (100%) sheep being infected with *Anaplasma* (Table 6, Table 7). The duration of the second and third controlled introduction was considerably shorter compared to the first controlled introduction. According to the supervising veterinarian, in both these second and third controlled introductions the clinical condition of the introduced sheep was too poor in order to continue in the experiment without any intervention. Therefore, the second and third controlled introduction were ended earlier than initially planned.

RLB on 64 blood samples which were part of this study, returned positive outcomes for *A. phagocytophilum*, *Babesia venatorum* and *Borrelia species* (Figure 15). A total of 4 *Ehrlichia/Anaplasma catch-all's* were detected. These *catch-all's* are probes of a highly conserved part of the *Ehrlichia* and *Anaplasma* DNA. As no hybridization to any of the species specific RLB probes was detected, the species and type could not be classified any further.

Mortality occurred in 3 sheep included in this sentinel study. All of the sheep that died were part of the first controlled introduction. In two of these sheep, necropsy was performed.
Sheep 81912 died within the study period and was found dead on day 51 after introduction. The macroscopic and microscopic results of necropsy on ewe sheep 81912 were cachexia, hydrothorax, hydropericardium, pulmonary edema and signs of liver fluke. McMaster egg counting technique was performed on small intestine content and showed 1750 *Trichostrongylus*/*Strongylus* eggs. This sheep showed signs of haemonchosis (caused by *Haemonchus contortus*), distomatosis (caused by *Fasciola Hepatica*) and catarrhal pneumonia within the left diaphragmatic lobe. Screening of liver and lung by RLB, revealed the lung was positive for *A. phagocytophilum*.
Sheep 82228 actually died a few days after ending of this study. The macroscopic results of necropsy on tup lamb 82228 were a little hyperemia of the intestinal wall and few content within the small intestine. Microscopy of lung tissue showed dispersed lung edema and the small intestine showed a lot of gram-negative rods, including many *Clostridium spp*. This sheep probably died of enterotoxaemia caused by *Clostridium perfringens*. One other sheep died within the study period, but no necropsy was performed.

Several blood stabilates were made from blood collected from two different sheep and were stored in liquid nitrogen for later use. All other sheep were removed from the Bargerveen Nature Reserve to another pasture after the controlled introduction was ended and were treated with long acting oxytetracycline. All recovered well from treatment.

Table 4. Comparing the outcome of blood smears with RLB hybridization as Golden Standard.

		Condition (as determined by 'Golden Standard = RLB outcome')			
	Total population	*Condition positive*	*Condition negative*		
Test outcome (blood smear)	*Test outcome positive*	8 (True positive)	0 (False positive)	8	Positive predictive value = 100% (8/8)
	Test outcome negative	12 (False negative)	44 (True negative)	56	Negative predictive value = 79% (44/56)
		20	44	**64**	
		Sensitivity 40% (8/20)	Specificity 100% (44/44)		

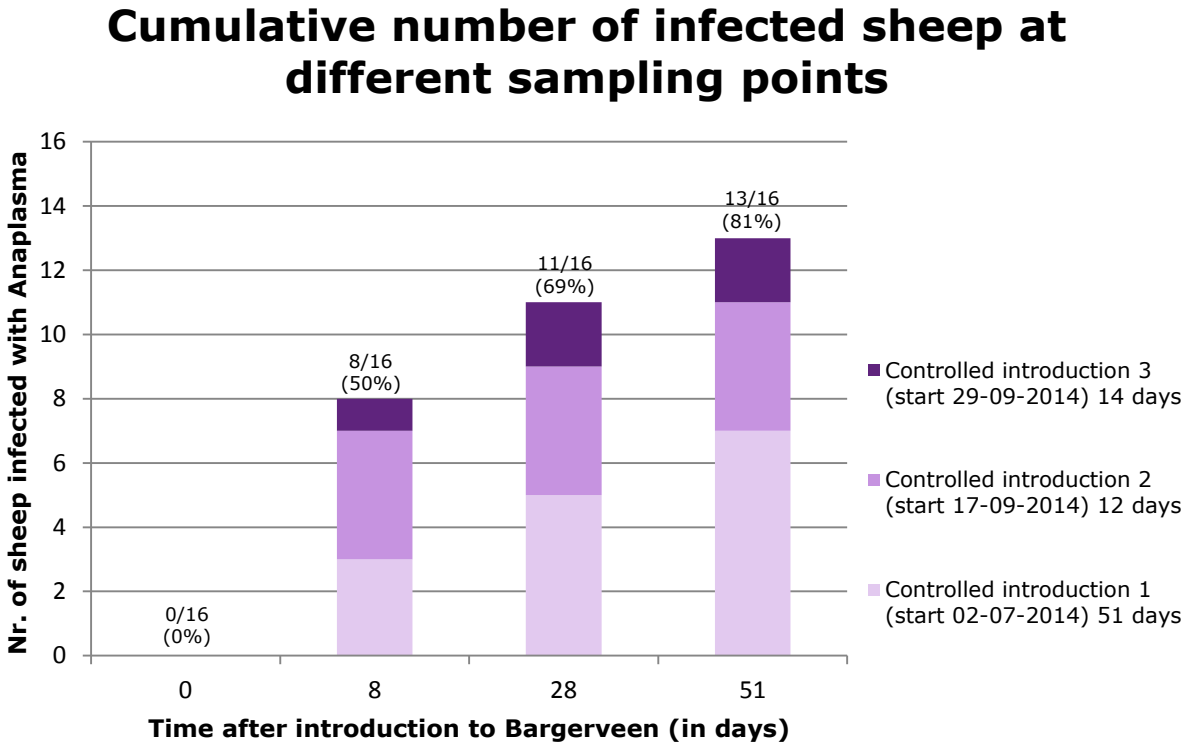

Figure 14. Cumulative infection rate of sheep with *Anaplasma* at different sampling moments after introduction to the Bargerveen nature reserve. Only 8 days after introduction, already 8 out of 16 (50%) sheep were proven infected with *Anaplasma* by PCR/RLB. At the end of the study 13 out of 16 (81%) sheep were positive for *Anaplasma*.

Table 5. First controlled introduction. Most extreme and abnormal values found at physical examination, microscopy of blood smear, RLB hybridization and final outcome per sheep in the first controlled introduction.

Gender	Animal nr.	Highest Temp.	Clinical signs	Blood smear	PCR/RLB	Final outcome
Ewe	43738	41,3		+	A. phago	Alive
Ewe	44945	41,8	Mildly scabby ears and nose	+	A. phago	Alive
Ewe	14352	39,7	Lymphadenopathy	-	A. phago + Borrelia afzelii	Alive
Ewe	45277	40,0		+	A. phago	Alive
Ewe	81912	40,7		+	E/A catch all	Died (found on day 51: necropsy was performed)
Ewe	5934	40,9	Mildly scabby ears and nose	-	A. phago	Alive
Ewe lamb	82229	40,7		+	A. phago	Alive
Ewe	82250	41,2	Lymphadenopathy	-	A. phago	Alive

lamb						
Tup lamb	82132	40,5	Heavy scabby ears	-	-	Died (found on day 51)
Tup lamb	82228	40,6		-	E/A catch all + Babesia venatorum	Alive (but died few days after end of study: necropsy was performed)

Table 6. Second controlled introduction.

Gender	Animal nr.	Highest Temp.	Clinical signs	Blood smear	PCR/RLB	Final outcome
Ewe	81918	40,9		+	A. phago + Babesia venatorum	Alive
Ewe	81919	39,7		-	A. phago	Alive
Ewe	5727	40,7	Very dull	-	A. phago	Alive
Ewe	5720	39,2	Dull	-	A. phago	Alive

Table 7. Third controlled introduction.

Gender	Animal nr.	Highest Temp	Clinical signs	Blood smear	PCR/RLB	Final outcome
Ewe	81910	41,2	Mildly scabby ears and lymphadenopathy	+	A. phago	Alive
Ewe	72823	40,3	Dull and Mildly scabby ears	+	A. phago	Alive

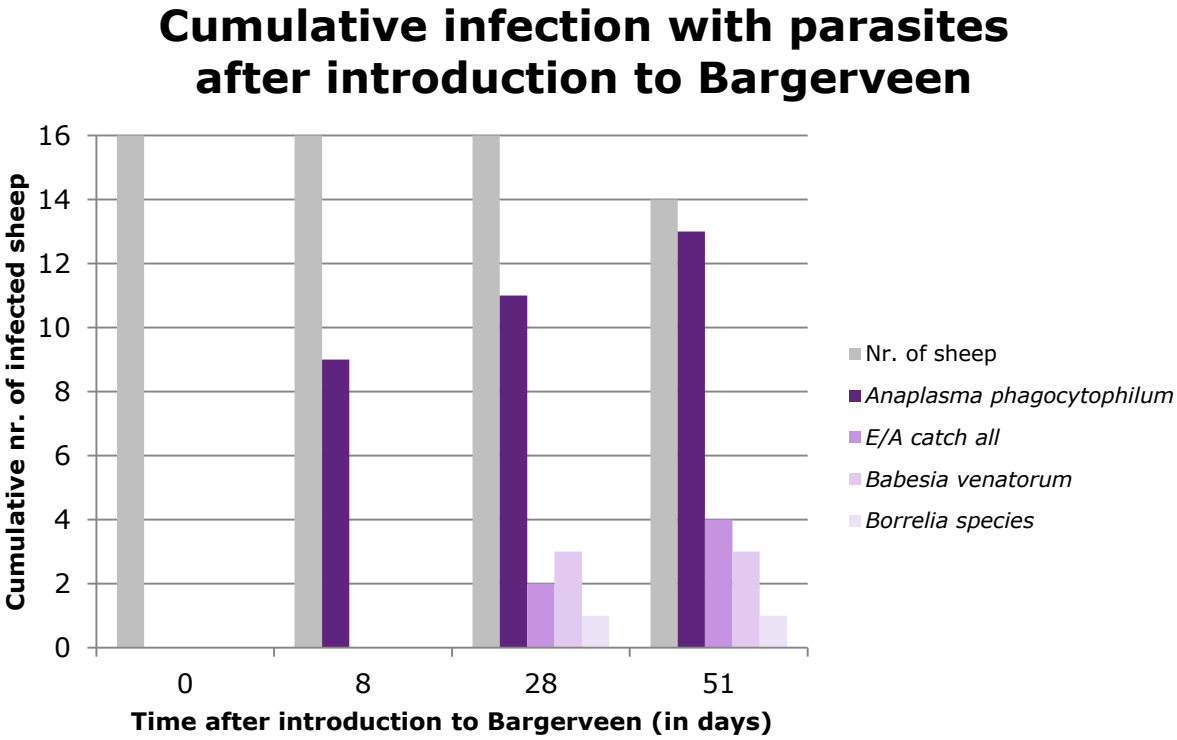

Figure 15. Tick borne pathogens detected by RLB hybridization on blood samples collected on several days after introduction into the Bargerveen nature reserve. At the last measuring point, 2 sheep which were not infected with *Anaplasma* (one measuring point earlier at day 28) were dead and thus no blood sample was taken. E/A catch all is *Ehrlichia/Anaplasma catch all* and is a less specific probe compared to the *Anaplasma* probe, which is more specific.

3.2 Experimental tick transmission study

Aim of this experimental study, was to further study several variables which could be relevant in the interaction between *Ixodes ricinus* ticks, sheep and *A. phagocytophilum* under controlled laboratory conditions. This would be tested in consecutive rounds, testing acquisition, transmission, speed of transmission and co-feeding. As time was restrictive, not all experimental rounds could be executed before finishing this paper. However two experimental rounds were executed with the goal to test the acquisition hypothesis. In the first experimental round (M1), larvae were fed on infected sheep. In the second experimental round (M2), nymphs were fed on infected sheep. Results that were gathered during these two rounds are described below. However, results concerning tick infection rates were not available at the moment of finishing this paper.

After inoculation, sheep were monitored by rectal temperature measurement. Results of the course of temperature, show a febrile period which started at approximately day 4 after inoculation, peaked at day 5 or 6 after inoculation and lasted for approximately 4 to 6 days (Figure 16, Figure 17). RLB results of blood samples taken within the febrile period were all positive.
In study round M1, non-infected larvae were placed on infected sheep. Those larvae experienced big losses. A lot less engorged larvae were collected than were initially placed onto the sheep. Incubation of larvae in order to allow them to molt to nymphs also resulted in big losses. However

this might be due to the fact that alive ticks were not separated for several days of feces and dead ticks before they went in the incubator.
In study round M2, non-infected nymphs were placed on infected sheep. The resulting number of living nymphs collected was much better compared to the larvae in study round M1. Besides, much less ticks were lost after molting. But this may be due to the fact that ticks were separated from feces and dead ticks soon after collection.

Tick infection rate results will follow and were not available at the moment of finishing this master thesis.

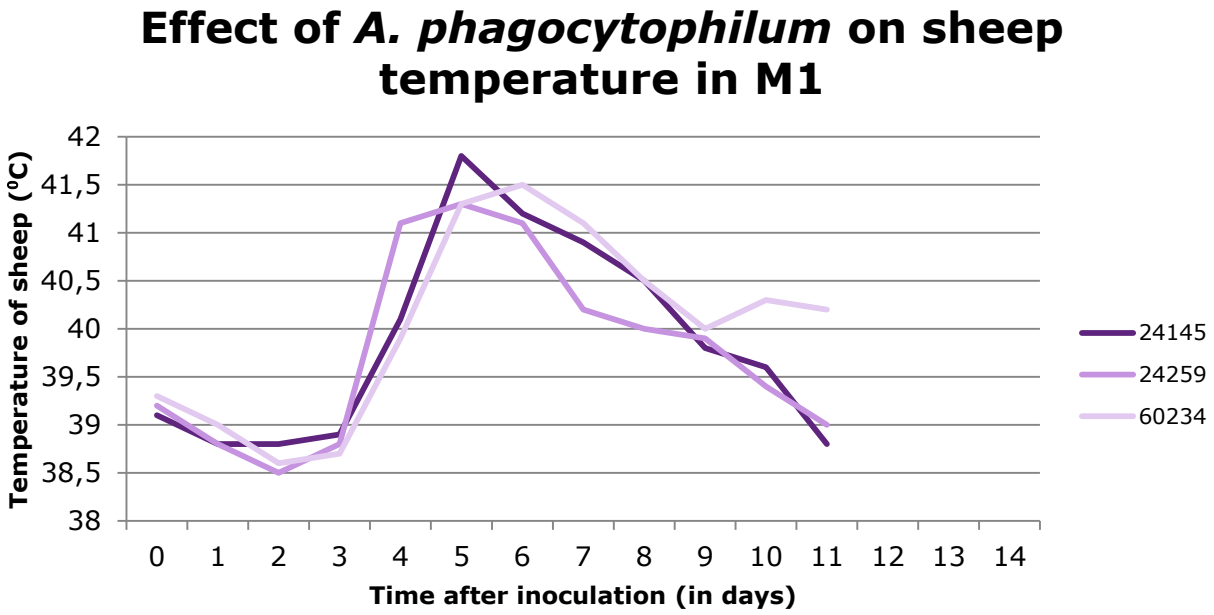

Figure 16. Effect of *Anaplasma* on sheep temperature in the first experimental round (M1). Febrile period lasted from day 4 until approximately day 8 after inoculation (with a peak at day 5).

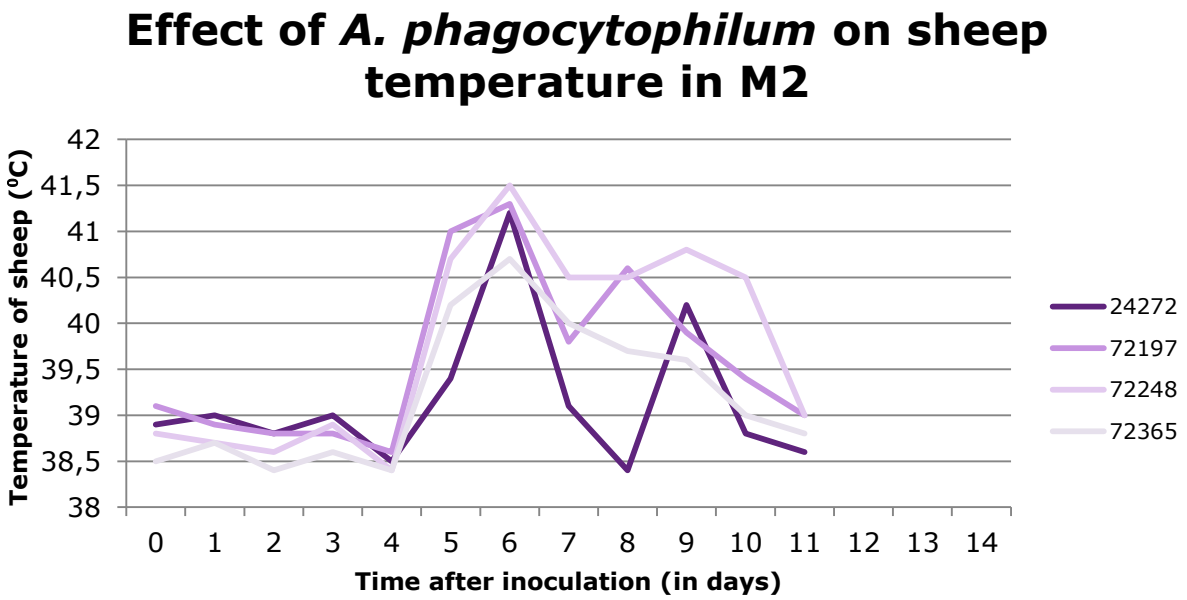

Figure 17. Effect of *Anaplasma* on sheep temperature in the second experimental round (M2). Febrile period started on day 5 after inoculation, but from there a more erratic course is observed with peaks and valleys. Temperature reaches baseline in all 4 sheep at day 11.

4. Discussion

Goal of this study was to develop an experimental tick transmission model in order to eventually determine a strategy to reduce the impact of ticks and tick-borne diseases (especially of *Anaplasma*) on sheep in the Netherlands.

4.1 Sentinel study

Main purpose of this study was to collect *Anaplasma* from natural clinical cases in the Bargerveen nature reserve. Therefore, *Anaplasma*-negative sheep were introduced into this nature reserve. Sheep were also monitored for infection rate and clinical course of disease. We predicted that if Tick-borne Fever (TBF) caused by *Anaplasma phagocytophilum* is the main cause of disease and possibly mortality in sheep introduced into nature reserves in the Netherlands, than it would be possible to detect *Anaplasma* in blood samples collected from sentinel sheep and clinical symptoms (fever) and possibly even mortality related to *Anaplasma* would be encountered in the newly introduced sheep.

Analysis of data collected by physical examination, revealed a suspect association between the number of enlarged lymph nodes and the infection state of a sheep. Of all samples taken from sheep with 2 or more enlarged lymph nodes, 7 out of 14 (50%) were positive for *Anaplasma*. In sheep with three or more enlarged lymph nodes, 75% (3 out of 4) of blood samples was positive for *Anaplasma*. While of all 64 blood samples analyzed, only 20 (31%) were RLB positive. The increase in the positive predictive value of lymphadenopathy for TBF as more lymph nodes were enlarged, makes it probable that there is a correlation between 2 or more enlarged lymph nodes (in case of a clinical suspicion for *Anaplasma*) and the presence of *Anaplasma*. This finding is in line with other articles describing that (mild) lymphadenopathy can be seen in TBF (Carrade et al. 2009; Reppert et al. 2013). Statistical analysis on data collected from experimentally inoculated sheep kept under laboratory conditions should be performed, in order to determine if there really is a correlation between TBF and lymphadenopathy.

Analysis of rectal temperature data collected, revealed that in case a minimal temperature of 40 ºC, 40,5 ºC or 41 ºC was found, blood samples were RLB positive in respectively 10 out of 26 (38%), 7 out of 14 (50%) or 4 out of 4 (100%). Whereas the portion of RLB positive blood samples related to the total number of analyzed blood samples, reveals 26 out of 64 (41%) in case of a minimal temperature of 40 ºC, 14 out of 64 (22%) in case of a minimal temperature of 40,5 ºC and 4 out of 64 (6%) in case of a minimal temperature of 41 ºC. The increase in positive predictive value in case of a minimal temperature of both 40,5 ºC or 41 ºC (50% respectively 100%) related to the average occurrence of fever in these groups in relation to the total number of blood samples evaluated (22% respectively 6%), makes it probable that there is a correlation (in case of a clinical suspicion for *Anaplasma*) between fever of more than 40,5 and the presence of *Anaplasma*.
In 2 out of 7 (29%) sampling moments where scabby ears were scored, blood samples were RLB positive for *Anaplasma*. The infection rate of samples collected from sheep with scabby ears were thus comparable to the overall infection rate in all collected blood samples in this study of 31% (20 out of 64). Although the sample size of sheep with scabby ears was really small, there seems to be no clear relation between scabby ears and the presence of *Anaplasma*.
There seems to be no clear relation between age and susceptibility for *Anaplasma* based upon this results, as the percentage of RLB positive samples of ewe respectively lamb (36% respectively 21%) does not deviate much of the overall 31% of RLB positive samples found in all 64 blood samples analyzed.

Of the 6 ticks collected from three different sheep, 2 ticks were infected with *Anaplasma*. Although this is a too small number to draw conclusions, *Anaplasma* was detected in ticks. To gather more insight in the infection rate of ticks present in study area, more ticks should be collected. Thereby discriminating between tick collected from sheep and ticks collected from vegetation. Comparing these two distinct groups by their relative infection rate, could yield more information regarding

the role of sheep as reservoir for Anaplasma compared to other possible wildlife hosts in this nature reserve.
All 6 collected ticks were adult female *I. ricinus* ticks. I. These ticks were all collected in mid- to late September. As in late summer or the beginning of fall adult ticks are the most common life stage (Figure 1), these data regarding tick stage correspond to the life cycle of *I. ricinus* ticks.

In all 8 blood samples with positive blood smears, RLB was also positive for *Anaplasma*. Thus, a positive predictive value of 100% was found for blood smears compared to RLB hybridization as Golden Standard. However, it should be mentioned that the positive predictive value is not intrinsic to a test and is also dependent on the prevalence of *Anaplasma* in the samples analyzed. The prevalence of *Anaplasma* may be higher in this newly introduced population (which was *Anaplasma* negative before introduction) compared to sheep which are on a pasture for a longer time. Those sheep may already have built up resistance due to earlier challenges with *Anaplasma* infected ticks. Furthermore, this study area was not randomly chosen for this experiment. However it was chosen based upon a clinical suspicion of a TBF outbreak in sheep on a larger scale. Therefore, the Bargerveen nature reserve may be a nature reserve which contains a more pathogenic strain or has a higher prevalence of *Anaplasma* compared to other sheep pastures. Repeating of this research in other nature reserves or sheep pastures may yield more information about the presence of (different strains of) *Anaplasma* in nature reserves in the Netherlands, infection rates and whether this Bargerveen area encountered an isolated outbreak of *Anaplasma* or if this is a problem on a much larger scale.

As expected, the number of samples detected positive for *A. phagocytophilum* by PCR/RLB were much larger than those obtained through microscopic examination of blood smears. A sensitivity of 40% and specificity of 100% was found, when comparing blood smear outcomes with PCR/RLB outcomes assumed as Golden Standard.
Blood smears thus have a low sensitivity, indicating a negative blood smear does not necessarily mean the blood sample is not infected with *Anaplasma*. Amorim et al. 2014 also found a low sensitivity of microscopic evaluation of blood smears compared to PCR for the erythrocytic parasites *A. marginale* and *Babesia spp.* Explaining this low sensitivity with a lack on detection of positive animals in the early or chronic stages of infection, when the circulating number of parasites is low.

As a high specificity (100%) of blood smears is observed in this experiment, positive blood smears are a useful diagnostic tool for confirming the presence of *Anaplasma* in a clinical setting. Blood smears rarely give a positive result in RLB negative sheep. So in case of a positive blood smear result, there is a high probability of the presence of *Anaplasma* and the blood sample can be regarded as positive. As performing blood smears takes few time, is inexpensive and positive results are reliable, blood smears are suitable as diagnostic tool in a clinical setting for confirming the presence of *Anaplasma*. These results are in line with the accepted knowledge that blood smears can be a useful and above all easy to use diagnostic tool to screen for the presence of *Anaplasma* in a clinical setting. Positive blood smears can be assumed to be really positive. However a negative blood smear outcome does not rule out the presence of *Anaplasma*. Thus PCR (followed by RLB hybridization) stays the Golden Standard as diagnostic tool for detection of *Anaplasma*.

The interval of monitoring the sheep was not as consequent as it ideally should be. No data were collected between day 28 and day 51, leading to a gab in data of 23 days. In order to more accurately monitor the progress of the infection rate, data should be collected at least once a week. Sheep infected just after day 28 could long have been recovered from infection at day 51, possibly leading to an underdiagnosis of *Anaplasma* in this study. Therefore it can be assumed that the real infection rate at the end of this study was even higher than the 81% which was found in this study. Smaller intervals between collection of blood samples could yield more detailed information regarding infection rates in newly introduced sheep over time.

Over the course of this experiment, blood samples returned positive outcomes for *A. phagocytophilum*, *Babesia venatorum*, and possibly also *Borrelia* species. This is in line with the knowledge of tick-borne parasites known to occur in sheep.
In several blood samples, the RLB showed a positive catchall but no positive result for any of the *A. phagocytophilum* types used. This could be caused by other types of *Anaplasma* or *Ehrlichia*

which are not on the membrane (and possibly even unknown) or there could be a mutation in the DNA sequence complementary to the probe used on the RLB membrane. Therefore, sequencing of at least one of these samples would be indicated. This DNA-sequence could then be compared (blasted) to other known *Anaplasma* types, the probes used by RLB, and possibly even to other probes available for RLB in order to be able to pool the *A. phagocytophilum* type(s) active in the Bargerveen Nature reserves.

Mortality was also observed in 3 out of 16 introduced sheep, however necropsy on two of these sheep could not explicitly link the cause of death with *Anaplasma*. Infection with *Anaplasma* might have led to immunosuppression, which could have resulted in sheep 81912 to die of gastrointestinal parasites and cause sheep 82228 to die of enterotoxaemia (caused by *Clostridium perfringens*). For further studies sending in all sheep that die during the study for necropsy, with a specific questing for looking for *Anaplasma phagocytophilum,* and RLB on tissue samples could help to determine if occurred mortality is or is not related to infection with *Anaplasma*.

Blood of infected sheep was collected and stabilates were made from blood samples collected from 2 different sheep. Stabilates were stored in liquid nitrogen for later use to inoculate sheep in an experimental setting under controlled laboratory conditions. Thereby providing the possibility to further study this *Anaplasma* strain without infection pressure of other parasites in the field, which might complicate the observed results. Enabling us to specifically test certain parts in the transmission dynamics of *Anaplasma*.

Already 50% (8 out of 16) of sheep were infected with *Anaplasma* 8 days after introduction. Increasing to 81% (13 out of 16) sheep at the end of the study 51 days after introduction. Because of the high infection rate found in sheep in this study, sheep are suitable sentinel animals for TBF. As sheep in nature reserves are longtime and highly exposed to ticks and are sensitive to pick up *Anaplasma* (which according to other literature is mostly of the same ecotype which causes clinical cases in humans (Jahfari et al. 2014)), sheep in nature reserves may be useful detectors to follow infection rates of specific nature reserves over time. Based upon these results, it would be possible to assign a certain risk factor to a nature reserve for people recreating in it. TBF in Dutch nature reserves requires further investigation, in particular the livestock-wildlife-human interface.

It is not known whether the assumed high infection rate found in the sentinel study is due to a large number of ticks, a high infection rate of ticks or for example a more infectious strain of *Anaplasma* in that study area. However, this high infection pressure makes the Bargerveen nature reserve suitable for further field studies with *Anaplasma*. These field studies could eventually help to decrease the impact of tick-borne diseases in sheep.

Our results confirm the hypothesis that *Anaplasma* is a main cause of disease (and possibly mortality) in newly introduced sheep into the Bargerveen nature reserve. By disproving the null hypothesis, it will be concluded that there are grounds for believing that the hypothesis is true. The null-hypothesis was rejected, as *A. phagocytophilum* was found in samples of sheep after introduction into the nature reserve, clinical signs were observed which could fit to TBF and mortality was observed which could be the result of complicated secondary infections due to immunosuppression caused by TBF. Enlarged lymph nodes seems to correlate with infection with *Anaplasma*, with a higher correlation if more lymph nodes were enlarged. And a temperature of ≥ 40,5 ºC seems to correlate with infection with *Anaplasma* in this study. Thus based upon these results, it will be concluded that *Anaplasma* is a main cause of disease (and possibly mortality) in newly introduced sheep into the Bargerveen nature reserve.

4.2 Experimental tick transmission model

Main purpose of this experimental study, was to further elucidate the tick-host-pathogen transmission dynamics. Therefore, a tick transmission model was developed, wherein *Anaplasma* negative sheep were inoculated with an *Anaplasma* strain and monitored by rectal temperature measurement and blood sample collection for detecting the presence of *Anaplasma* by PCR/RLB. In order to confirm or reject the hypothesis, infection rate in ticks and/or transmission of infection to sheep has to be analyzed. Several different experiments were described; acquisition, transmission,

speed of transmission and co-feeding. Not all experiments were conducted. Only the acquisition experiment was performed before this master thesis was finished. Apart from analysis of temperature recordings, data concerning infection rates in the acquisition study were not available at the moment of finishing this master thesis.

Results of the course of temperature show a febrile period, which started at approximately day 4 after inoculation, peaked at day 5 or 6 after inoculation and lasted for approximately 4 to 6 days. *Anaplasma* thus induces a self-limiting infection in sheep.

In the two experimental rounds executed, the experimental tick transmission model was tested and seems to work well. Ticks of both larval and nymph stages fed on sheep, though bigger losses were observed in the larval stages. So this tick transmission model is suitable for testing the transmission dynamics of *Anaplasma* by ticks of all life stages.

5. Follow up

This master thesis only contains part of the results of the experimental setup which was described in this paper. However more interesting results may follow as this experiment progresses.

This transmission model could result in more fundamental (epidemiologic) knowledge concerning the transmission of *Anaplasma* to hosts. This knowledge may then be used as a basis to effectively reduce the impact of ticks and tick-borne diseases on not only sheep, but also humans and other wildlife hosts. A One-Health approach may thus be the solution to effectively combat the emerging disease of tick-borne fever.

The experimental tick transmission model developed in this study may not only be suitable for studying transmission dynamics of *Anaplasma* by *Ixodes ricinus* ticks, but other pathogens or tick species can be introduced into this model as well.

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8. Appendix

In this appendix, some supportive documents used in this study and mentioned in the text are included. The index below shows an overview of the appendices attached to this master thesis.

8.1 Physical examination score sheet

Collection date: **……**
Instruction: **1. Results of overall impression and physical examination can be filled in per sheep, in the table below.**
2. Please collect EDTA blood samples from all sheep in a sterile manner.
(2 samples per sheep, 3 samples if temperature reached 40,0 ºC or above)

					Lymph nodes*					Mucous membranes						
		Breath rate	Pulse rate	Rectal Temperature	Mandibular	Retropharyngeal	Prescapular	Subiliac	Turgor	Skin disorders? (like thickening or flaking skin)	Conjunctival	Scleral	Ticks present**	BCS (… out of 3)	Remarks and overall impression	Removed from Bargerveen?
Sheep numbers	……									Yes/No			Yes/No			
	……									Yes/No			Yes/No			
	……									Yes/No			Yes/No			
	……									Yes/No			Yes/No			

* Lnn only have to be scored as L (left), R (right), or B (both) if enlaged.
** Please score if ticks are present.

8.2 DNA EXTRACTION FROM BLOOD PROCEDURE

Room	
Number of samples	
Sample description	

Heating block ID	
Centrifuge ID	

Wear gloves and use filter pipet tips

Strictly follow the one-way route: Clean room → Dirty room → PCR room

		Done
1	Clean workspace with sodium hypochloride.	
2	Take the proteinase K solution from the freezer and store at 4°C.	
3	Turn on the heating block at 70°C and preheat the BE buffer.	
4	Vortex the blood samples and add 200µl of each to sterile 1.5ml tubes.	
5	Add 25µl proteinase K.	
6	Add 200µl B3 buffer and vortex vigorously (10-20 seconds).	
7	Incubate the tubes at room temperature for 5 minutes.	
8	Incubate the tubes at 70°C for 15 minutes. (The samples should turn brown/black. If this does not happen, extend incubation time up to 30 minutes and vortex vigorously several times.)	
9	Briefly spin down the tubes.	
10	Add 210µl 96% ethanol, vortex and briefly spin down the tubes.	
11	Transfer the supernatant to spin columns.	
12	Centrifuge the columns at 11,000x g for 1 minute. Discard the flow through.	
13	Add 500µl BW buffer and centrifuge the columns at 11,000x g for 1 minute. Discard the flow through.	
14	Add 600µl B5 buffer and centrifuge the columns at 11,000x g for 1 minute. Discard the flow through.	
15	Centrifuge the columns at 11,000x g for 1 minute.	
16	Place the spin columns in sterile 1.5ml tubes. **Label the tubes accordingly**.	

17	Add 100µl preheated BE buffer directly on the membrane of the spin columns and incubate at room temperature for 1 minute.	
18	Centrifuge the columns at 11,000x g for 1 minute. Discard the spin columns.	
19	Store the DNA samples at 4°C for use within the next few days or store at -20°C for long term preservation.	
20	Turn off all equipment and clean working space with sodium hypochloride.	

8.3 DNA EXTRACTION FROM TICKS

Room	
Number of samples	
Sample description	

Water bath ID	
Sonification bath ID	
TissueLyser LT ID	
Heating block ID	
Centrifuge ID	

Wear gloves and use filter pipet tips

Strictly follow the one-way route: Clean room → Dirty room → PCR room

		Done
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 buffer.	
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	Add 100µl 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.	

8.4 PCR RLB PROCEDURE

Rooms		
Number of samples		
Sample description		

Workstation 1 ID	
Workstation 2 ID	
PCR machine ID	

Wear (green) gloves and use filter pipet tips

Strictly follow the one-way route: Clean room → Dirty room → PCR room

Primers:	***Anaplasma* *Ehrlichia***	***Babesia* *Theileria***	***Borrelia***	***Rickettsia***	**Other:**

Reagent	**1x**	**Number of samples + 10%**	

PCR grade H_2O	15.875µl	
5x Phire reaction buffer	5.0µl	
10mM dNTPs	0.5µl	
Forward primer (20pmol/µl)	0.5µl	
Reverse primer (20pmol/µl)	0.5µl	
2U/µl Phire Hot Start II DNA polymerase	0.125µl	

		Done	
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.		

8.5 REVERSE LINE BLOT HYBRIDIZATION PROCEDURE

Room	
Number of samples	
Sample description	

Heating block ID	
Water bath ID	
Hybridization oven ID	
Shaker ID	
Membrane ID	
Blotter ID	

Wear gloves and use non-filter pipet tips

Strictly follow the one-way route: Clean room → Dirty room → PCR room

		Done
1	Clean workspace with 70% ethanol.	
2	Turn on a heating block at 100°C.	
3	Turn on the hybridization oven at 42°C en preheat 50ml 2x SSPE/0.5% SDS solution.	
4	Turn on the water bath at 50°C en preheat the bottle with 2x SSPE/0.5% SDS solution.	
5	Combine and dilute the PCR products per DNA sample in a 1.5ml tube. Take 10µl of every PCR product and add 2x SSPE/0.1% SDS to a final volume of 160µl. (10µl *Anaplasma/Ehrlichia* PCR + 10µl *Babesia*/*Theileria* PCR + 140µl 2x SSPE/0.1% SDS.)	
6	Take 10µl of the RLB positive controls and add 150µl 2x SSPE/0.1% SDS to a 1.5ml tube,	
7	Denature the diluted PCR samples and controls at 100°C for 10 minutes.	
8	**During the denaturation step**; wash the membrane at room temperature with 2X 2SSPE/0.1% SDS for 5 minutes under gentle shaking and fill a bucket with ice.	
9	Immediately transfer the samples in order on ice after the denaturation.	
10	Prepare the miniblotter by placing the membrane on the lanes, with the line pattern of the membrane perpendicular to the lanes of the blotter. Place de support cushion on the membrane followed by the other half of the blotter. Turn the blotter right-side up without moving the membrane and turn the screws hand-tight,	
11	Remove residual fluid in the slots by aspiration.	
12	Briefly spin down the tubes at 4°C and place them back on ice in order.	
13	Fill the slots with the samples (150µl) and fill the first, last and other empty slots with 2x SSPE/0.1% SDS. **Avoid air bubbles.**	

14	Hybridize the blotter at 42°C for 60 minutes in the hybridization oven without shaking.		
15	Remove the samples by aspiration.		
16	Dissemble the blotter and remove the membrane from the blotter.		
17	Wash the membrane **twice** with preheated 2x SSPE/0.5% SDS at 50°C for 10 minutes under gentle shaking.		
18	**During the washing step**; clean the blotter and the support cushion.		
19	Incubate the membrane with 50ml 2x SSPE/0.5% SDS + 5µl streptavidin at 42°C for 30 minutes in the hybridization oven under gentle shaking. **Discard the streptavidin solution in a tube and into the bio-waste bin. Do not pour it in the sink.**		
20	**During the streptavidin hybridization**; change the water bath temperature to 42°C and preheat the bottle with 2x SSPE/0.5% SDS solution. Keep the lid open.		
21	Wash the membrane **twice** with preheated 2x SSPE/0.5% SDS solution at 42°C for 10 minutes under gentle shaking.		
22	Change the water bath temperature to 80°C and preheat the bottle with 1% SDS solution.		
23	Wash the membrane **twice** with 2x SSPE at room temperature for 5 minutes, under gentle shaking.		
24	**During the washing step**; prepare the foil and film cassette and check if the developing machine is on (5th floor).		
25	Add 10ml ECL (5ml ECL1 + 5ml ECL2) to the membrane and gently shake by hand until the whole membrane is covered. **Discard the ECL in a tube and into the bio-waste bin. Do not pour it in the sink.**		
26	Cover the membrane in foil and place it in the film cassette. **Avoid air bubbles.**		
27	Go to the dark room and expose a film to the membrane for 10 minutes.		
28	Develop the film with the developing machine.		
29	Strip the membrane or store it in a seal bag with 20mM EDTA at 4°C until stripping.		
30	Turn off all equipment and clean workspace.		

8.6 MEMBRANE STRIPPING PROCEDURE

Room	

Water bath ID	
Shaker ID	
Membrane ID	

Wear gloves

Strictly follow the one-way route: Clean room → Dirty room → PCR room

		Done
1	Turn on the water bath at 80°C and preheat the bottle with 1% SDS.	
2	Wash the membrane **twice** with preheated 1% SDS at 80°C for 30 minutes under gentle shaking.	
3	Wash the membrane with 20mM EDTA at room temperature for 15 minutes under gentle shaking.	
4	Store the membrane in a seal bag with 20mM EDTA at 4°C.	

8.7 Delladet tick mortality experiment

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Experiment conducted: 8-9-2014

8.7.1 Introduction

For a study with the zoonotic disease tick-borne fever (TBF), caused by *Anaplasma phagocytophilum* (an obligate intracellular, gram-negative bacterium), a group of sheep is going to be experimentally infected with this bacterium. Subsequently, specific pathogen-free (SPF) ticks will be placed on the sheep. This allows these ticks to infect themselves with *Anaplasma*, while having a blood meal. Ticks will be collected and examined for infection rate of *Anaplasma* after completing a blood meal on the experimentally infected sheep.

This study will be conducted at the Department of Farm Animal Health, as part of the Faculty of Veterinary Medicine of Utrecht University. Ticks, used in this experiment, are (after contact with the sheep) potentially infected with *A. phagocytophilum* and should be handled as such. To prevent the infected ticks from escaping out of the experimental setup, a gutter is used to serve as a physical barrier. This gutter is filled with tap water. However, since the stables are cleaned with Delladet, the question arose if filling the gutter with a Delladet solution would provide a better physical barrier in order to completely prevent any ticks from escaping. Delladet is a common disinfectant and consists of at least 6 components, of which alkyldimethyl benzylammonium chloride (ADBAC) is the basic component. ADBAC is a surfactant and thus lowers the surface tension. For this experiment we measured the mortality of ticks in several concentrations of Delladet compared to water. The goal of this experiment was to test if Delladet solutions give a higher mortality rate of ticks than only tap water.

8.7.2 Material and method

For this experiment 10 tubes were used; 1 tube without water, 3 tubes with only tap water (negative controls) and 6 tubes containing different concentrations of Delladet in tap water (Figure 18). In the product description of Delladet (Hugh Crane, 2014) a working concentration of 1%-2% is recommended for disinfection. The maximum concentration mentioned by the manufacturer is 9,1%. Therefore, we decided to test with a Delladet concentration of 0,5%, 1%, 1,5%, 2%, 2,5% and 3% in this experiment.

For each of these 10 tubes, 10 ticks (5 males and 5 females) of the species *Rhipicephalus turanicus* ticks were used. These *R. turanicus* ticks were used in this experiment, as they are known for their mobility. Therefore making scoring of mortality easier, which was defined as no observable mobility seen even after being pushed with a forceps.
Ticks were submerged in the fluid within the tube and were put on the bottom of the tube. At a given time, all ticks were collected in a petri dish with filter paper at the bottom. The number of dead ticks were scored. This was done by first letting them rest for a few minutes, which was followed by blowing hot breath (CO_2) over the petri dish. This stimulated these ticks to become active. Ticks that moved their legs, were scored as alive. After scoring all the petri dishes, ticks were separated based upon being alive or not. All ticks that were still alive, were returned into the solution wherein they were submerged before. Ticks scored dead, were excluded out of the experimental group. The procedure described above was one scoring round. A few subsequent scoring rounds were needed in order for all the ticks in the highest concentrations of Delladet to die. Cumulative mortality in different solutions and concentrations was scored and processed.

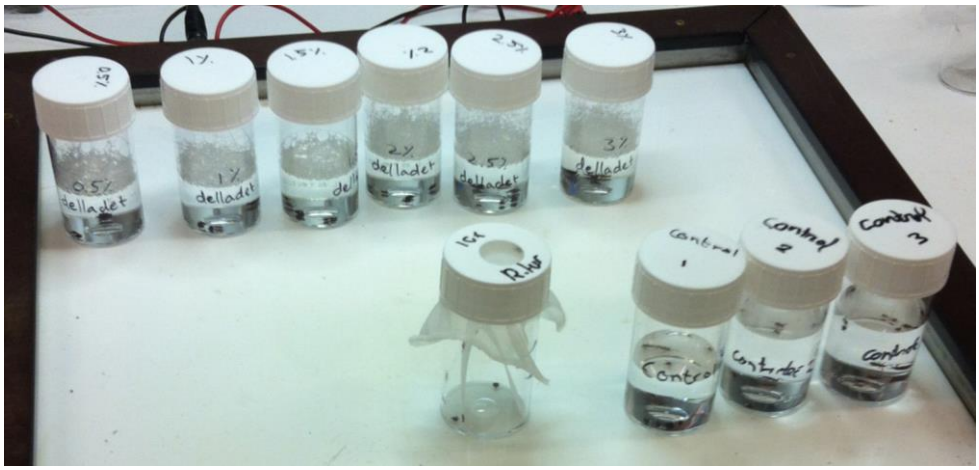

Figure 18. Experimental setup: 10 tubes; 1 without water (bottom-left), 3 tubes with only tab water (bottom right) and 6 tubes containing different Delladet concentrations (top).

8.7.3 Results

During execution of the experiment it was observed that ticks submerged in Delladet, immediately sank to the bottom of the tube and were not able to move away from the bottom (Figure 19). Whereas ticks in the control tubes with only water, were able to climb out of the water. Ticks in the tube without water, were even more mobile than ticks submerged in each of the tubs filled with fluids.

At the end of each round of submersion and before the ticks were collected out of the testing tubes for scoring of mortality, the number ticks that had climbed out of the water were counted and scored. At the end of the first round in testing tube C1, C2 and C3 respectively 0, 7 and 2 ticks had climbed out of the water. At the end of the second round in C1, C2 and C3 respectively 0, 5 and 3 ticks were out of the water. And finally at the end of the third round in C1, C2 and C3 respectively 0, 1 and 0 ticks were out of the water. In contrast, none of the ticks in the Delladet concentrations were able to move away from the bottom of the tube.

After the ticks were put on petri dishes to score their survival, ticks submerged in Delladet were considerably less active than control ticks submerged in only water. This difference in activity of ticks submerged in water compared to ticks submerged in Delladet was even clear in ticks in the control tubes which stayed constantly submerged under the water surface.

Mortality
All of the ticks in the control group without water were still alive at the end of the experiment (Figure 20). Of the three control groups submerged in only tap water, at the end of the experiment 4 out of 30 ticks were dead (mortality rate of 13,3% after 5h15m). The results of the testing tubes with Delladet concentrations show a remarkably higher mortality than in the control groups with just tap water. Even in the lowest 0,5% concentration of Delladet, 50% of ticks was dead after 2h28m of exposure. After 5h15m the experiment was ended and by then 57 out of 60 ticks were death (mortality rate of 95% after 5h15m).

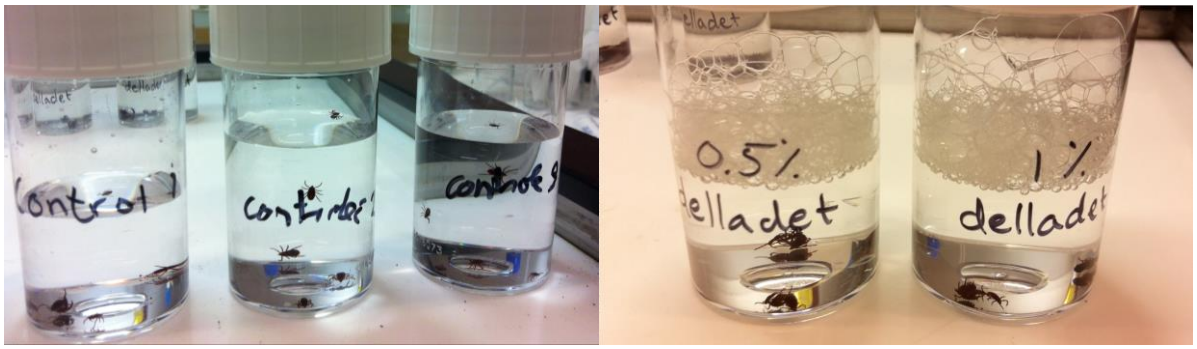

Figure 19. Ticks in control tubes with tap water were able to climb on the tube walls and out of the water (left), whereas ticks in different Delladet concentrations immediately sank to the bottom of the tube and were not able to move away from the bottom.

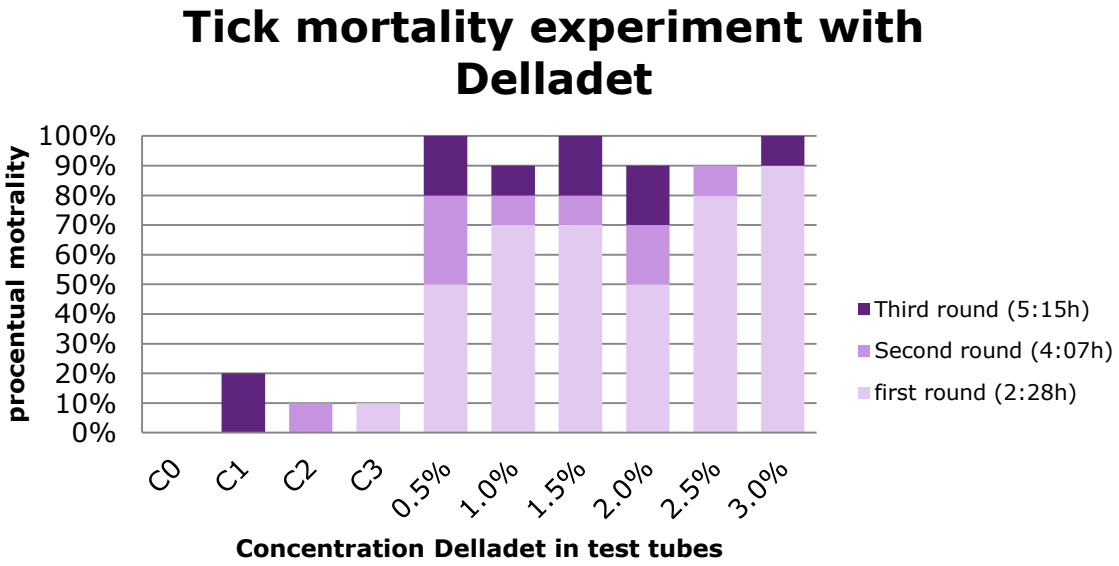

Figure 20. Cumulative mortality of ticks in air (C0), in water (C1, C2 and C3) and in different concentrations of Delladet (0,5%, 1%, 1,5%, 2%, 2,5% and 3%). In a total of 3 consecutive rounds, the mortality was scored at the given time of exposure. After 5h:15m the experiment was ended as only 3 out of 60 ticks in the Delladet concentrations were still alive. The mortality in concentrations of Delladet is obviously higher than in the controls. Variation between the used concentrations of Delladet seems of minor importance.

8.7.4 Discussion

Remarkable observation during execution of this experiment was that ticks sank immediately to the bottom when placed in tubes with a Delladet solution. Ticks in these tubes were also not able to move away from the bottom of the tube. This sinking of the ticks is probably the result of a diminished surface tension caused by a surfactant of which the Delladet solution consists. Soap could thus have a similar effect, but was not tested in this experiment.

Additional advantage of Delladet is it's disinfectant properties. Most forms of micro-organisms, including gram-positive and gram-negative bacteria and yeasts will be killed by this disinfectant. This could mean that ticks infected with *A. phagocytophilum* could be cleaned of infection after contact with the Delladet solution in the gutter. However this has not been examined.

Mortality
Based on the results after 2h28m of submersion, there seems to be an increasing mortality observed in the higher concentrations of Delladet. This concentration dependent mortality is only seen after the first round. After the second and third round the cumulative mortality seems to be evenly distributed over the different concentrations of Delladet. To determine if there is a relation between concentration of Delladet and mortality, further examination is necessary with a higher number of ticks, more consecutive rounds with a lower time-interval between scoring mortality. Apart from the currently used concentrations, higher Delladet concentrations of up to 9% could also be used.

8.7.5 Conclusion

Immersion of ticks into a solution of Delladet, has a positive effect on the mortality rate of ticks compared to immersion of ticks in tap water. Variation between concentrations of Delladet seems of minor importance, concerning mortality rate.

Remarkable observation during execution of this experiment was that ticks placed in a testing tube with a Delladet solution, immediately sank to the bottom of the tube. Besides, they were not able to get from the bottom of the tube. This sinking of ticks was probably due to the diminished surface tension of the disinfectant Delladet.

Delladet thus seems to prevent escaped ticks (possibly infected with *Anaplasma*) that arrive into the gutter from escaping out of the gutter, thereby preventing the escape of these ticks out of the experimental setup. Based on the results of this experiment, a concentration of Delladet of 1% is recommended. As a higher concentration of Delladet seems to have no additional effect on the mortality rate.

8.7.6 References

Hugh Crane (2014) Delladet VS2. Hugh Crane, Cleaning Equipement Limited: http://hughcrane.co.uk/media/product/data-sheets/03HC2503GP.pdf, visited on 9-9-2014.

8.8 Protocol: filling Delladet into gutter of experimental setup

DEC: 2013.II.08.087

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In order to prevent ticks (escaped from the patches made of cloth) from escaping out of the experimental setup, a physical barrier consisting of a gutter filled with Delladet is used.

Take the following steps:

1. Remove all water from the gutter by taking out the plug.
2. Cleanse the gutter of visible dirt.
3. Put the plug back again.
4. Fill the gutter completely with water.
5. Put on plastic gloves (to prevent direct skin contact with possible Delladet residues).
6. Add 3 bottles containing 1 Liter of Delladet (with the blue cap) into the gutter filled with tap water. Divide the content of the 3 bottles equally over the total length of the gutter. Delladet solution will mix out of itself and some bubbles will form on the surface.

Time path:

During the experiment, *each Monday and Thursday* the complete content of the gutter has to be removed, rinsed and filled again with Delladet according to the steps described above. Table below is used to sign off, when this is done.

Date	Remove content out of the gutter	Fill the gutter again according to the steps described above
09-10-2014 (thursday)		
13-10-2014 (monday)		
16-10-2014 (thursday)		
20-10-2014 (monday)		
23-10-2014 (thursday)		

Tick-borne Fever outbreaks in sheep introduced into nature reserves in the Netherlands:

Anaplasma phagocytophilum re-emerging

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Introduction

Anaplasma phagocytophilum is a gram-negative bacterium which causes Tick-Borne Fever (TBF) in a range of animals, including sheep, cattle (Siebinga & Jongejan, 2000), horses, dogs, cats and also humans (Stuen et al., 2013). The aim of this study was to isolate *A. phagocytophilum* from natural clinical cases by using sentinel sheep.

Figure 1: Sheep in the Bargerveen nature reserve.

Methodology

- 16 sheep were introduced into the Bargerveen Nature Reserve in the north-east of the Netherlands, in three controlled introductions.
- Strictly supervised by the local veterinarian (full clinical examination and regular collection of blood samples).
- Blood samples were collected for microscopic evaluation of blood smears and DNA extraction, PCR and RLB hybridization. They were screened for *Anaplasma*, *Ehrlichia*, *Babesia*, *Theileria* and *Borrelia* species.

Results

- 8/16 sheep (50%) were infected with *Anaplasma* 8 days after introduction (Fig. 2).
- End of study: 13/16 (81%) sheep were infected with *Anaplasma* (Fig. 2).
- 3/16 sheep (19%) died in the field.

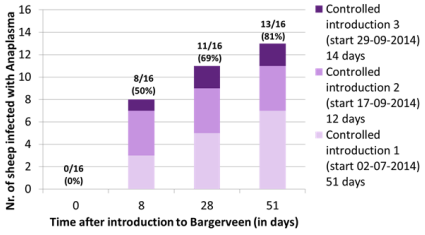

Figure 2: Cumulative number of infected sheep at different sampling points in the sentinel study.

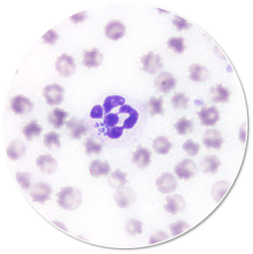

Figure 3: Microscopic picture of Anaplasma phagocytophilum inside a neutrophilic granulocyte.

Table 1: First controlled introduction:

Gender	Animal nr.	Highest Temp.	Clinical signs	Blood smear	PCR/RLB	Final outcome
Ewe	43738	41,3		+	A. phago	Alive
Ewe	44945	41,8	Mildly scabby ears and nose	+	A. phago	Alive
Ewe	14352	39,7	Lymphadenopathy	-	A. phago + B. afzelii	Alive
Ewe	45277	40,0		+	A. phago	Alive
Ewe	81912	40,7		+	E/A catch all	Died (found on day 51)
Ewe	5934	40,9	Mildly scabby ears and nose	-	A. phago	Alive
Ewe lamb	82229	40,7		+	A. phago	Alive
Ewe lamb	82250	41,2	Lymphadenopathy	-	A. phago	Alive
Tup lamb	82132	40,5	Heavy scabby ears	-	-	Died (found on day 51)
Tup lamb	82228	40,6		-	E/A catch all + B. venatorum	Alive (but died few days after end of study: necropsy was done)

Table 2: Second controlled introduction:

Gender	Animal nr.	Highest Temp.	Clinical signs	Blood smear	PCR/RLB	Final outcome
Ewe	81918	40,9		+	A. phago + B. venatorum	Alive
Ewe	81919	39,7		-	A. phago	Alive
Ewe	5727	40,7	Very dull	-	A. phago	Alive
Ewe	5720	39,2	Dull	-	A. phago	Alive

Table 3: Third controlled introduction:

Gender	Animal nr.	Highest Temp	Clinical signs	Blood smear	PCR/RLB	Final outcome
Ewe	81910	41,2	Mildly scabby ears and lymphadenopathy	+	A. phago	Alive
Ewe	72823	40,3	Dull and Mildly scabby ears	+	A. phago	Alive

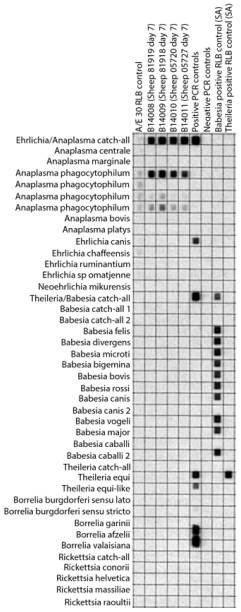

Figure 4: RLB outcome from the second controlled introduction, collected on the 7th day after introduction.

Conclusions

- These results demonstrate that *Anaplasma* is a main cause of disease (and possibly mortality) in newly introduced sheep into Bargerveen Nature Reserve.
- Sheep are suitable sentinel animals for TBF.
- TBF in Dutch nature reserves requires further investigation, in particular the livestock-wildlife- human interface.

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We would like to thank Piet Ursem and Carola Heijnen from Staatsbosbeheer for financial support and Harry Klingenberg and Harrie Imming for cooperation and assistance with sampling of the sheep.

References

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Experimental tick transmission studies in sheep with Anaplasma phagocytophilum

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Introduction

Anaplasma phagocytophilum, transmitted by ticks of the genus *Ixodes,* was first described in Scotland as the agent of Tick-borne Fever in sheep (Stuen et al., 2013). The bacterium has now been reported in a broad range of animals, including small ruminants, cattle, horses, dogs, cats, deer and also humans. *A. phagocytophilum* is widespread in Europe, the U.S., as well as in Asia (Stuen et al., 2013). Here, we study *A. phagocytophilum* in an experimental tick transmission model using sheep as a natural host. Aim of this study is to determine the relative importance of transstadial transmission (vertical) versus co-feeding (horizontal) in *Ixodes ricinus* and other tick species.

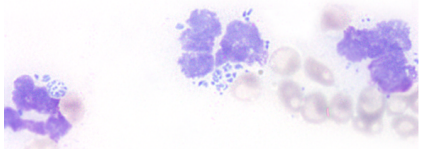

Figure 1: Anaplasma phagocytophilum inside neutrophilic granulocytes in a stained blood smear from sheep.

Methodology

- A strict biosecurity protocol is implemented at the tick-proof facility located at the Department of Farm Animal Health, including water/Delladet barriers and special disposable clothing.
- A broad range of *A. phagocytophilum* isolates is available for testing.
- *Ixodes ricinus* and other tick species are available from specific pathogen-free colonies maintained at the acaridarium of UCTD.
- HL60 cells are in place for isolating of *A. phagocytophilum* in cell culture.
- Blood samples are collected for blood smear examination as well as for DNA extraction, PCR/RLB as well as qPCR.
- *In vitro* feeding of *Ixodes* ticks on silicone membranes is available for laboratory studies with infected ticks as recently done for ticks infected with *Ehrlichia canis* (Fourie et al., 2013).

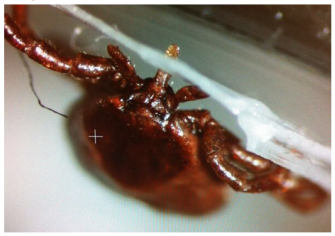

Figure 2: Ixodes tick feeding through a silicone membrane.

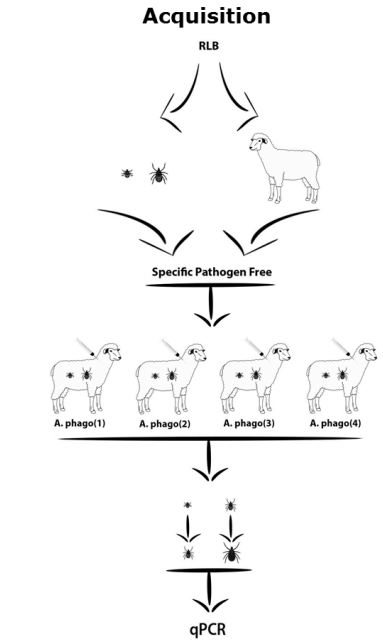

Figure 3: Experimental design for acquisition by ticks

Figure 4: Sheep with two patches wherein ticks are released during the febrile period.

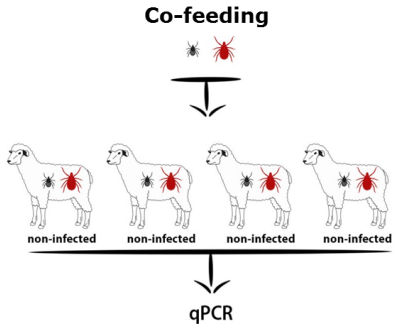

Figure 5: Experimental design for co-feeding.

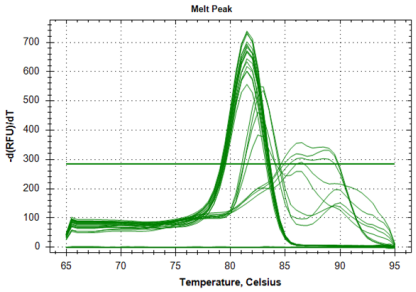

Figure 6: qPCR based on the MSP4 gene of Anaplasma.

Results

- *A. phagocytophilum* induces a self-limiting infection in sheep, characterized by high fever and a high rickettsaemia in neutrophilic granulocytes.
- The model is suitable for producing infected ticks for subsequent *in vitro* laboratory studies.

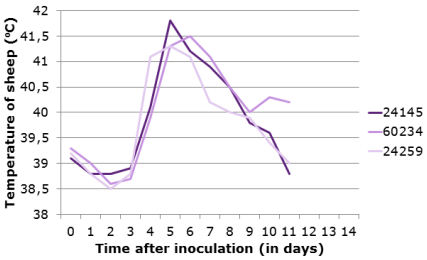

Figure 7: Febrile response to A. phagocytophilum in sheep.

Conclusions

- Finally, feeding of *I. ricinus* nymphs on *Anaplasma*-infected sheep has created the opportunity to study a natural tick-host-pathogen relationship under controlled laboratory conditions.

References

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