Monitoring the spread of infected *Dermacentor reticulatus* ticks in the Netherlands

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Abstract

The tick *Dermacentor reticulatus* is an important vector of *Babesia canis*, *Babesia caballi* and several other pathogens. Canine babesiosis was first confirmed in the Netherlands in 1985 and in 2004 an outbreak occurred. In 2007, autochthonous populations of *D. reticulatus* were confirmed in the Netherlands, which means that the geographical range of the tick is expanding. A total of 200 adult *D. reticulatus* ticks were collected from the vegetation and dogs in the Netherlands in 2014 and 2015. The locations that were surveyed are St. Philipsland and Bergen. The ticks were screened by polymerase chain reaction (PCR) and Reverse Line Blot (RLB) Hybridization for the presence of *Anaplasma* spp., *Ehrlichia* spp., *Babesia* spp., *Theileria* spp., *Borrelia* spp. and *Rickettsia* spp. The pathogens *Anaplasma phagocytophilum* (1.6%), *Ehrlichia canis* (0.5%), *Theileria equi* (0.5%), *Borrelia valaisana* (0.5%) and *Rickettsia raoultii* (2.6%) were detected in *D. reticulatus* ticks collected from the vegetation, along with *Ehrlichia/Anaplasma* (7.9%), *Theileria/Babesia* (11.6%) and *Babesia* (4.7%) catch-alls. *Babesia canis* and *Babesia caballi* were not detected. It was concluded that *D. reticulatus* is spreading within the Netherlands and that the presence of these pathogens in this tick may suggest a possible role of *D. reticulatus* in the life cycle and transmission of these pathogens in the Netherlands.

Introduction

Ticks are ectoparasites which feed on blood of animals and humans. They are vectors of many pathogenic microorganisms, protozoa, rickettsiae, spirochetes and viruses that are important in both the veterinary and the medical field (Jongejan & Uilenberg, 2004). Diseases such as anaplasmosis, babesiosis, ehrlichiosis, Lyme borreliosis and rickettsiosis can be transmitted by ticks (Nijhof et al., 2007). Globally, ticks are mostly economically important for livestock, but there is also a large impact on public health in the northern hemisphere (Jongejan & Uilenberg, 2004). Only in industrialized countries tick-borne pathogens of pets are of economic importance, but tick-borne pathogens infecting horses form restraints to international sporting events and trade which involve horses (Jongejan & Uilenberg, 2004). In Europe, ticks transmit more pathogens than any arthropod and are therefore the most important vectors of infectious diseases in animals and humans (Jongejan & Uilenberg, 2004; Michelet et al., 2014).

In Europe, the most common tick is *Ixodes ricinus*, also known as the sheep or castor bean tick (Bonnet et al., 2013; Claerebout et al., 2013; Cochez et al., 2012; Karbowiak, 2014; Nijhof et al., 2007). *I. ricinus* ticks can transmit a wide range of pathogens such as *Anaplasma* spp., *Babesia* spp., *Bartonella* spp., *Coxiella burnetii, Ehrlichia* spp., *Francisella tularensis*, Lyme borreliosis etiological agents (*Borrelia* spp.), *Rickettsia* spp. and tick-borne encephalitis virus (TBEV) (Bonnet et al., 2013; Claerebout et al., 2013; Michelet et al., 2014; Nijhof et al., 2007). *Rhipicephalus sanguineus*, the brown dog tick, and *Dermacentor reticulatus*, the European meadow tick or ornate dog tick, are sporadically introduced into Northern Europe and the Netherlands with imported dogs or by dogs travelling from endemic areas (Claerebout et al., 2013; Karbowiak, 2014; Nijhof et al., 2007). *D. reticulatus* is the second most abundant tick species in Europe, and the localization of *D. reticulatus* is limited (Bonnet et al., 2013; Cochez et al., 2012). *R. sanguineus* can transmit *Babesia vogeli*, *Ceropithifilaria* spp., *Ehrlichia canis*, *Hepatozoon canis*, *Rickettsia conorii* and others (Claerebout et al., 2013).

This report is about *D. reticulatus* ticks in the Netherlands; therefore, the focus will be on this tick species.

Dermacentor reticulatus

D. reticulatus is an important vector of *Babesia canis*, *Babesia caballi* and *Theileria equi*, and can also transmit pathogens such as *Anaplasma ovis* and *Rickettsia* spp. *Dermacentor* spp. are also suspected of transmitting several other pathogens such as *A. marginale, B. burgdorferi*, *B. microti*, *C. burnetii, F. tularensis,* and TBEV (Bonnet et al., 2013; Claerebout et al., 2013; Jongejan et al., 2015; Karbowiak, 2014; Nijhof et al., 2007). *D. reticulatus* is the second most important hard tick species in Europe after *I. ricinus* regarding their numbers and impact on the economy (Karbowiak, 2014).

The genus *Dermacentor* includes 33 species with ornate scuta, short palps and eyes, and it usually follows a three-host life cycle (Jongejan & Uilenberg, 2004). *Dermacentor* ticks occur on all continents, with the exception of Australia. Several species of *Dermacentor* such as *D. reticulatus* and *D. marginatus* infest domestic animals and livestock in Eurasia. In North America *D. andersoni* and *D. variabilis* are important. *Dermacentor* spp. do not play an important role in livestock in Africa (Jongejan & Uilenberg, 2004).

Dermacentor ticks are three-host ticks, which means that larvae, nymphs and adults feed on different hosts. Adult ticks feed mostly on wild and domestic animals such as horses, ruminants and dogs and accidentally humans, while larvae and nymphs infect small mammals such as rodents and insectivores, and birds (Bonnet et al., 2013; Karbowiak, 2014; Matjila et al., 2005). *Dermacentor* ticks are not host specific and could therefore infest and transmit several pathogens during their life cycle to several vertebrate hosts, including humans (Estrada-Pena & Jongejan, 1999). The life cycle is in approximately one year complete. *Dermacentor* larvae and nymphs are endophilic, which means that they live in

burrows of rodents and other small mammals, thus limiting the contact that is possible with these stages (Bonnet et al., 2013). *Dermacentor* males are partial blood feeders, which has consequences for the transmission of tick-borne pathogens (Bonnet et al., 2013). *Dermacentor* ticks are mainly active in spring and autumn and they are mostly absent in the summer on the vegetation (Obsomer et al., 2013).

Tick-borne diseases in *D. reticulatus*

The direct consequences of feeding of the tick are stress and damage to the skin, and *D. reticulatus* ticks can transmit a variety of pathogens (see above), which makes the tick epidemiologically important in Europe (Karbowiak, 2014). Particularly in Europe, companion animals such as dogs can acquire and transmit ehrlichiosis and babesiosis by travelling to the Mediterranean region and returning to their home country, which is outside the normal enzootic range of the vector tick (Irwin, 2010; Jongejan & Uilenberg, 2004). It is estimated that every year, 1500 dogs (Estrada-Peña et al., 2004) are imported into the Netherlands. Most of these originate from southern Europe, which is where *D. reticulatus* occurs (Estrada-Peña et al., 2004).

Canine babesiosis was first confirmed in the Netherlands 1985 in five cases (Uilenberg et al., 1985) and in 2004, 23 dogs that had not been outside of the country were confirmed to suffer from babesiosis simultaneously (Matjila et al., 2005). Autochthonous populations of *D. reticulatus* were confirmed in 2007 (Jongejan et al., 2015).

Symptoms of babesiosis in dogs include anorexia, lethargy, pyrexia, pale mucus membranes, dark urine, vomiting, icterus and petechiae (Bodaan et al., 2007; Irwin, 2010; Matjila et al., 2005). The clinical signs in dogs can vary from a mild short-term disease to an acute disease, which is caused by a severe hemolysis, quickly resulting in death (Jongejan et al., 2011). Clinical signs also include splenomegaly, hepatomegaly, anaemia, thrombocytopenia, hemoglobinuria and bilirubinuria (Jongejan et al., 2011; Matjila et al., 2005). Acute renal failure, respiratory failure and/or refractory hypotension may complicate a case of babesiosis (Irwin, 2010).

It has been demonstrated that equine piroplasmosis, caused by *B. caballi*, has emerged in the South-West of the Netherlands. Among 300 horses in that area, two acute clinical *T. equi* cases and subclinical *B. caballi* infections have been diagnosed in resident horses in 2010 with a sero-prevalence of 1.3% (Butler et al., 2012; Jongejan et al., 2015).

The habitats of *D. reticulatus*

The main habitats of *D. reticulatus* are wetlands such as freshwater tidal marshes, natural deciduous forests situated near water, mildly damp open areas with few bushes and trees and vast dormant water areas (Karbowiak, 2014). The tick is most commonly seen in wet forests with river valleys and ravine systems, meadows and lake shores. It seems therefore that the key factor for this tick to thrive is the combination of drying soil and a high level of ground water (Jongejan et al., 2015; Karbowiak, 2014; Nijhof et al., 2007; Obsomer et al., 2013). These locations provide shelter against desiccation and insulation against extreme environmental situations, which make the locations suitable for the survival of ticks (Jongejan et al., 2015; Nijhof et al., 2007). Together with the presence of populations of horses, cattle, sheep and roe deer, these type of locations are ideal for adult ticks to feed on hosts (Jongejan et al., 2015). This ideal environment for *D. reticulatus* is also accomplished when agricultural land is converted into semi natural reserves and where free-ranging animals such as cattle and deer are introduced (Nijhof et al., 2007).

As is the case with *I. ricinus*, the habitat of *D. reticulatus* is not limited to natural areas, but has spread to urban areas. A distinctive feature of *D. reticulatus* is being able to adapt to habitats which have been and are continued to be influenced by humans, such as recreational parks and nature reserves integrating afforestation or merging bushy vegetation (Karbowiak, 2014). However, because of a

limited number of regular observations, the occurrence and activity of ticks in urban areas is challenging to determine.

The geographical distribution of *D. reticulatus*

The geographical distribution pattern of *D. reticulatus* is highly focal and associated with its habitat needs (Jongejan et al., 2015; Karbowiak, 2014). The tick can be found in Northern Africa, Europe, and Western Asia. The geographical distribution ranges from the western Palearctic region, in isolated areas in south-western England and France in the West, to Siberia in Central Asia in the East (Cochez et al., 2012; Estrada-Peña et al., 2004; Jongejan et al., 2015; Karbowiak, 2014). This distribution pattern is limited and localized, but it can be divided into two main areas: Eastern and Western Europe. This phenomenon has not been observed in other tick species (Karbowiak, 2014). The western region consists of an area from France and south-western England to east Germany. In the Netherlands, isolated foci have been reported. In central Europe, *D. reticulatus* is absent. The eastern region includes eastern parts of Poland, Belarus and the European part of Russia and Siberia (Karbowiak, 2014).

This geographical range was relatively stable up until the 1970s and 1980s. Since the 1990s, *D. reticulatus* has been found in previously vacant areas of this tick, this being Central and Northern Europe; it is evident that the western population is spreading to the East. Previously, the French-Belgian border was considered to be the northern boundary of *D. reticulatus* in Western Europe, but several reports have now indicated that the geographical range is expanding (Claerebout et al., 2013; Cochez et al., 2012; Karbowiak, 2014). In the last decade, *D. reticulatus* ticks have been found in the field in the Netherlands, Belgium and Germany (Claerebout et al., 2013; Cochez et al., 2012). Cases of canine babesiosis in Germany, Hungary, Switzerland and the Netherlands where the disease has not been seen previously support the evidence of the expansion of the distribution of *D. reticulatus* (Cochez et al., 2012). There are several potential reasons for the expansion of *D. reticulatus*, mainly being natural factors mostly associated with the climate such as temperature change and changes in rainfall, and human activity such as urbanization, migration, travelling and trade (Irwin, 2010; Karbowiak, 2014).

In this survey, *D. reticulatus* ticks from the Netherlands have been screened by polymerase chain reaction (PCR) and Reverse Line Blot (RLB) Hybridization to detect the presence of several pathogens. The pathogens that were tested for are *Anaplasma* spp., *Ehrlichia* spp., *Babesia* spp., *Theileria* spp., *Borrelia* spp. and *Rickettsia* spp. The ticks that were tested have been collected during field surveys in the Netherlands and through the 'Tickbusters survey' that was set up by the Utrecht Centre for Tick-borne Diseases (UCTD). The aim of this study is to determine the prevalence of *B. canis*, *B. caballi* and other *Babesia* and *Theileria* species in *D. reticulatus* ticks, along with *Anaplasma*, *Ehrlichia*, and *Borrelia* species, and to determine whether there are novel field locations in the Netherlands where *D. reticulatus* exists. Also, the possible prevalence of *D. marginatus* will be investigated.

Materials and Methods

Collection of ticks

Ticks were sent in through the 'Tickbusters survey' to the UCTD by veterinarians who removed the ticks from dogs belonging to their client pet owners or by pet owners themselves. The following data were noted: host, location, date of tick collection, host sex and whether the tick-infested pet had been outside of the Netherlands prior to finding the tick on the animal. The dogs came from (or were walked in previously to obtaining the ticks) Egmond aan Zee (A), Groningen (B) and Heiloo (C) (Figure 1).

Ticks were also collected in several areas in the Netherlands by dragging pieces of flannel cloth through the vegetation. Through the ticks that were submitted by veterinarians, these novel field locations were found. In total, two areas have been dragged: Sint Philipsland (D) and Bergen (E) (Figure 2). The location and date of tick collection were noted. Among the ticks that were submitted through the 'Tickbusters survey', a few ticks were also collected from the vegetation. These locations were Egmond aan Zee (A) and Egmond aan den Hoef (F) (Figure 2).

Figure 1. *Dermacentor reticulatus* ticks collected from dogs from various locations in the Netherlands. The letters correspond to the locations presented in table 5.

Figure 2. *Dermacentor reticulatus* ticks collected from the vegetation from various locations in the Netherlands. The letters correspond to the locations presented in table 5.

The ticks were identified to genus, species level and stage using a stereo microscope and the book published by Estrada-Peña et al. (2004), *Ticks of domestic animals in the Mediterranean region*. The ticks were assigned a unique database ID number and stored in 70% alcohol until further use.

DNA extraction

DNA was extracted from individual ticks using the Nucleospin® Tissue Kit (Art. No. 740952.10/.50/.250, Macherey-Nagel). The tick were disrupted in lysis buffer using the TissueLyser LT (Qiagen, The Netherlands) with 5 mm stainless steel beads according to the instructions of the manufacturer. See Appendix A for the protocol that was used for DNA extraction. The DNA samples were stored at -20°C until used for PCR amplification.

Polymerase Chain Reaction (PCR)

A PCR was performed using a primer for *Anaplasma/Ehrlichia* spp., *Babesia/Theileria* spp. and *Borrelia* spp. (Table 1). *Rickettsia* spp. was also included in these.

Table 1. PCR primers and sequences.

Pathogen	Primer	Sequence	T*m*
Anaplasma/	Ehr-F	5'-GGA ATT CAG AGT TGG ATC MTG GYT CAG -3'	61.0 °C
Ehrlichia	Ehr-R	5'-Biotin-CGG GAT CCC GAG TTT GCC GGG ACT TYT TCT -3'	69.5 °C
Babesia/	RLB-F2	5'-GAC ACA GGG AGG TAG TGA CAA G	57.9 °C
Theileria	RLB-R2	5'-biotin-CTA AGA ATT TCA CCT CTG ACA GT	53.7 °C
Borrelia	Bor-F	5'- ACC ATA GAC TCT TAT TAC TTT GAC CA -3'	60.3 °C
	Bor-R	5'- Biotin-GAG AGT AGG TTA TTG GCC AGG G-3'	65.0 °C

To perform a PCR, a master mix was prepared (Table 2). A master mix contains a buffer, 10 mM dNTPs, a forward primer, a reverse primer, H_2O and DNA polymerase. A positive and a negative control were made for every pathogen that was tested for. A specific PCR was executed in the thermal cycler (Table 3 and 4). See Appendix B for the protocol that was used for PCR amplification. After PCR amplification, the PCR products were stored at 4°C until used for RLB Hybridization.

Table 2. PCR mix.

Total master mix for 1 reaction	
5.0 µl	5x Phire reaction buffer
0.5 µl	10 mM dNTPs
0.5 µl	F primer (20pM/ µl)
0.5 µl	R primer (20pM/ µl)
0.125 µl	2U/ µl Phire Hot Start II DNA polymerase
15.875 µl	H_2O
2.5 µl	DNA

Table 3. *Anaplasma/Ehrlichia* and *Babesia/Theileria* PCR programs, temperature cycle.

Number of cycles	Time	Temperature
1 cycle	30 sec	98 °C
10 cycles	5 sec	98 °C
	5 sec	67 – 57 °C
	7 sec	72 °C
40 cycles	5 sec	98 °C
	5 sec	57 °C
	7 sec	72 °C
1 cycle	1 min	72 °C

Table 4. *Borrelia* PCR program, temperature cycle.

Number of cycles	Time	Temperature
1 cycle	30 sec	98 °C
10 cycles	5 sec	98 °C
	5 sec	60 – 50 °C
	7 sec	72 °C
40 cycles	5 sec	98 °C
	5 sec	52 °C
	7 sec	72 °C
1 cycle	1 min	72 °C

Agarose gel electrophoresis

In order to establish that the PCR was working correctly, an agarose gel electrophoresis with a positive and negative control of the PCR was performed. The UCTD protocol was used and a 1.125% agarose gel was made. The positive and negative control samples were mixed with a 6x DNA loading dye and they were then loaded into the sample wells, together with a 100 bp DNA ladder as a reference. The gel was run for 30 to 45 minutes. After adequate migration, the gel was observed using an UV-illuminator. It could be concluded that the PCR was performed correctly if the positive control sample was visible with a fragment and the negative control sample was not visible and thus showed no amplification, which was the case. A picture of the gel was not taken.

Reverse Line Blot (RLB) Hybridization

RLB Hybridization allows multiple samples to be analyzed simultaneously against multiple probes. The RLB membrane contains species-specific oligonucleotides, which are applied in lines and are covalently linked to the membrane by a 5' terminal aminolinker.

The PCR products were applied to the membrane using a miniblotter in such a way that the direction of the PCR products was perpendicular to the direction of the species-specific oligonucleotides. Two control oligonucleotides, *Ehrlichia* and *Babesia*, were also applied to the membrane. The membrane was thoroughly washed to remove PCR products that had not been bound. Visualization of the hybridized PCR products was achieved using chemiluminescence. Visualization makes use of a biotin label attached to the PCR primer. The biotin label was incubated with a streptavidin ligand conjugated to an enzymatic label, HRP. Afterwards, the blot was incubated with peroxidase substrate, ECL, resulting in a reaction that produces light which can be detected on a suitable film when incubated for 10 minutes (Figure 3). After development of the film, spots appeared where species-specific oligonucleotides and PCR products had hybridized. At that point, the (possible) identity of microorganisms in the sample can be identified (Figure 4) (Isogen Life Science, 2004). See Appendix C for the protocol that was used for RLB Hybridization.

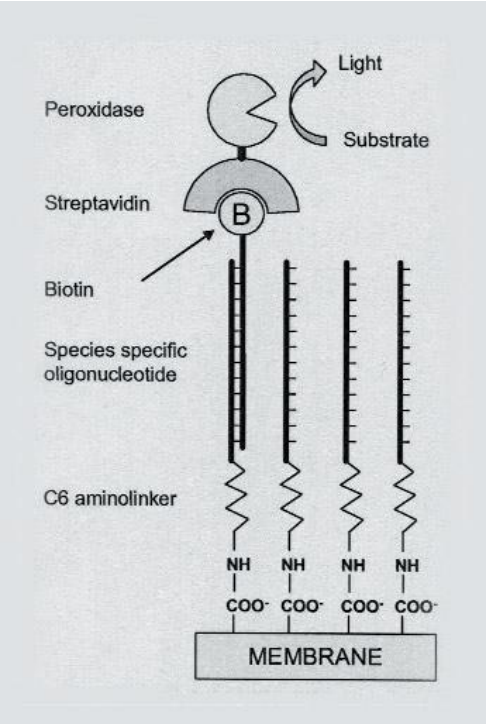

Figure 3. Schematic representation of the hybridization principle (RLB Hybridization, 2004).

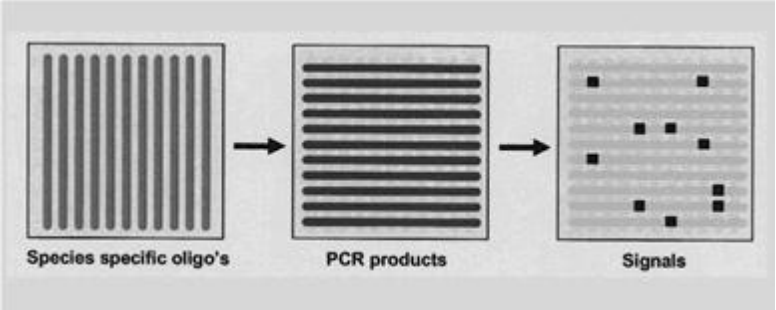

Figure 4. Schematic representation of the RLB assay (RLB Hybridization, 2004).

Results

Collected ticks

A total of 200 adult *D. reticulatus* ticks was collected for the present survey in 2014 and 2015 from several different locations and dogs in the Netherlands. The locations of St. Philipsland (Zeedijk; 51°35'30"N, 4°11'20"E) and Bergen (PWN Noordhollands Duinreservaat, entrance Woudweg; 52°39'20"N, 4°39'25"E) have been surveyed for ticks from the vegetation. The location in St. Philipsland represented a field where beef cattle grazed from April until November. Many *Dermacentor* ticks were found in the tall grass. The field is close to the canal Schelde-Rijn and is separated from the road by a dike (Figure 5 and 6). The location of Bergen was a grazing area for Highland cattle and Konik horses (Figure 7 and 8). *Dermacentor* ticks were found in dune areas where the soil was moist. Most ticks were found in heather and wood small-reed. The area where the most ticks were found is called 'Verbrande Pan'.

Figure 5. St. Philipsland.

Figure 6. St. Philipsland.

Figure 7. Bergen.

Figure 8. Bergen.

No *D. marginatus* ticks were found; all *Dermacentor* ticks were identified as *Dermacentor reticulatus* (Estrada-Peña et al., 2004). 190 ticks were collected alive from the vegetation: 59 males from St. Philipsland in November 2014, 60 females from St. Philipsland in November 2014, sixteen males on 17-3-2015, 27 females on 17-3-2015, fifteen males from Bergen on 20-3-2015, and thirteen females from Bergen on 20-3-2015. Ten ticks were sent in through the 'Tickbusters survey' to the UCTD, which were collected from either vegetation or dogs in 2014 in several locations. The owners of the dogs did not mention a visit outside of the Netherlands. For a complete overview of which ticks were found on what dates and where, see Figure 1 and 2 and Table 5. For an overview of the number of ticks collected from each location, see Table 6.

Table 5. List of the ticks that were researched in the present survey.

Tick number	Detection date	Number of ticks	Host	Stage	Location	Corresponding letter on map (Figure 1 and 2)
34 – 92	Nov-2014	59	Vegetation	Male	St. Philipsland	D
93 - 152	Nov-2014	60	Vegetation	Female	St. Philipsland	D
223 – 238	17-3-2015	16	Vegetation	Male	St. Philipsland	D
239 – 265	17-3-2015	27	Vegetation	Female	St. Philipsland	D
266 – 280	20-3-2015	15	Vegetation	Male	Bergen	E
281 – 293	20-3-2015	13	Vegetation	Female	Bergen	E
1	22-5-2014	1	Vegetation	Female	Egmond aan den Hoef	F
2	29-5-2014	1	Vegetation	Female	Egmond aan den Hoef	F
3	29-5-2014	1	Vegetation	Male	Egmond aan den Hoef	F
4	20-10-2014	1	Dog	Male	Egmond aan Zee	A
5	20-10-2014	1	Dog	Female	Egmond aan Zee	A
6	19-9-2014	1	Dog	Male	Egmond aan Zee	A
7	15-6-2014	1	Vegetation	Female	Egmond aan Zee	A
8	20-4-2014	1	Dog	Female	Egmond aan Zee	A
9	15-4-2014	1	Dog	Female	Groningen	B
10	12-5-2014	1	Dog	Male	Heiloo	C

Table 6. Number of ticks collected per location.

Location	Total number of ticks
St. Philipsland	162
Bergen	28
Egmond aan den Hoef	3
Egmond aan Zee	5
Groningen	1
Heiloo	1

Pathogen detection

This is a summary of the RLB results. For the RLB results, see appendix D.

A total of 200 adult *D. reticulatus* ticks collected from the vegetation and dogs in the Netherlands were screened by PCR and RLB for the presence of the pathogens *Anaplasma* spp., *Ehrlichia* spp., *Babesia* spp., *Theileria* spp., *Borrelia* spp. and *Rickettsia* spp.

Per RLB, 40 ticks were tested because no more were able to fit on the blot. Some RLB tests had to be executed more than once because the positive PCR samples did not test positive the first time. With each RLB, a positive PCR control was added to the membrane. Also, an *Ehrlichia* plasmid control and a *Babesia* plasmid control were added to the membrane in order to establish the correct placement of the film on the document with pathogens.

Ticks from the vegetation

A total of 190 ticks, 90 (47.4%) males and 100 (52.6%) females, was collected from the vegetation of St. Philipsland and Bergen and was screened by RLB for the presence of *Anaplasma* spp., *Ehrlichia* spp., *Babesia* spp., *Theileria* spp., *Borrelia* spp. and *Rickettsia* spp. (Table 7, Figure 9, and Appendix D).

Of those ticks, fifteen (7.9%) tested positive for *Ehrlichia/Anaplasma* catch-all, three (1.6%) tested positive for *Anaplasma phagocytophilum*, one (0.5%) tested positive for *Ehrlichia canis*, 22 (11.6%) tested positive for *Theileria/Babesia* catch-all, nine (4.7%) tested positive for *Babesia* catch-all 1, nine (4.7%) tested positive for *Theileria* catch-all, one (0.5%) tested positive for *Theileria equi*, eight (4.2%) tested positive for *Theileria parva*, one (0.5%) tested positive for *Borrelia valaisana*, two (1.1%) tested positive for *Rickettsia* catch-all, five (1.6%) tested positive for *Rickettsia raoultii* and 152 (80.0%) of all ticks tested negative for *Anaplasma* spp., *Ehrlichia* spp., *Babesia* spp., *Theileria* spp., *Borrelia* spp and *Rickettsia* spp.

Table 7. Pathogens detected in *D. reticulatus* ticks collected from the vegetation in the Netherlands. Note: several ticks tested positive for more than one pathogen, which has consequences for the proportions.

	Number of ticks	**Proportion**
Total number of ticks	190	100.0%
Males	90	47.4%
Females	100	52.6%
***Ehrlichia/Anaplasma* catch-all**	15	7.9%
Anaplasma phagocytophilum	3	1.6%
Ehrlichia canis	1	0.5%
***Theileria/Babesia* catch-all**	22	11.6%
***Babesia* catch-all 1**	9	4.7%
***Theileria* catch-all**	9	4.7%
Theileria equi	1	0.5%
Theileria parva	8	4.2%
Borrelia valaisana	1	0.5%
***Rickettsia* catch-all**	2	1.1%
Rickettsia raoultii	5	2.6%
Negative	152	80.0%

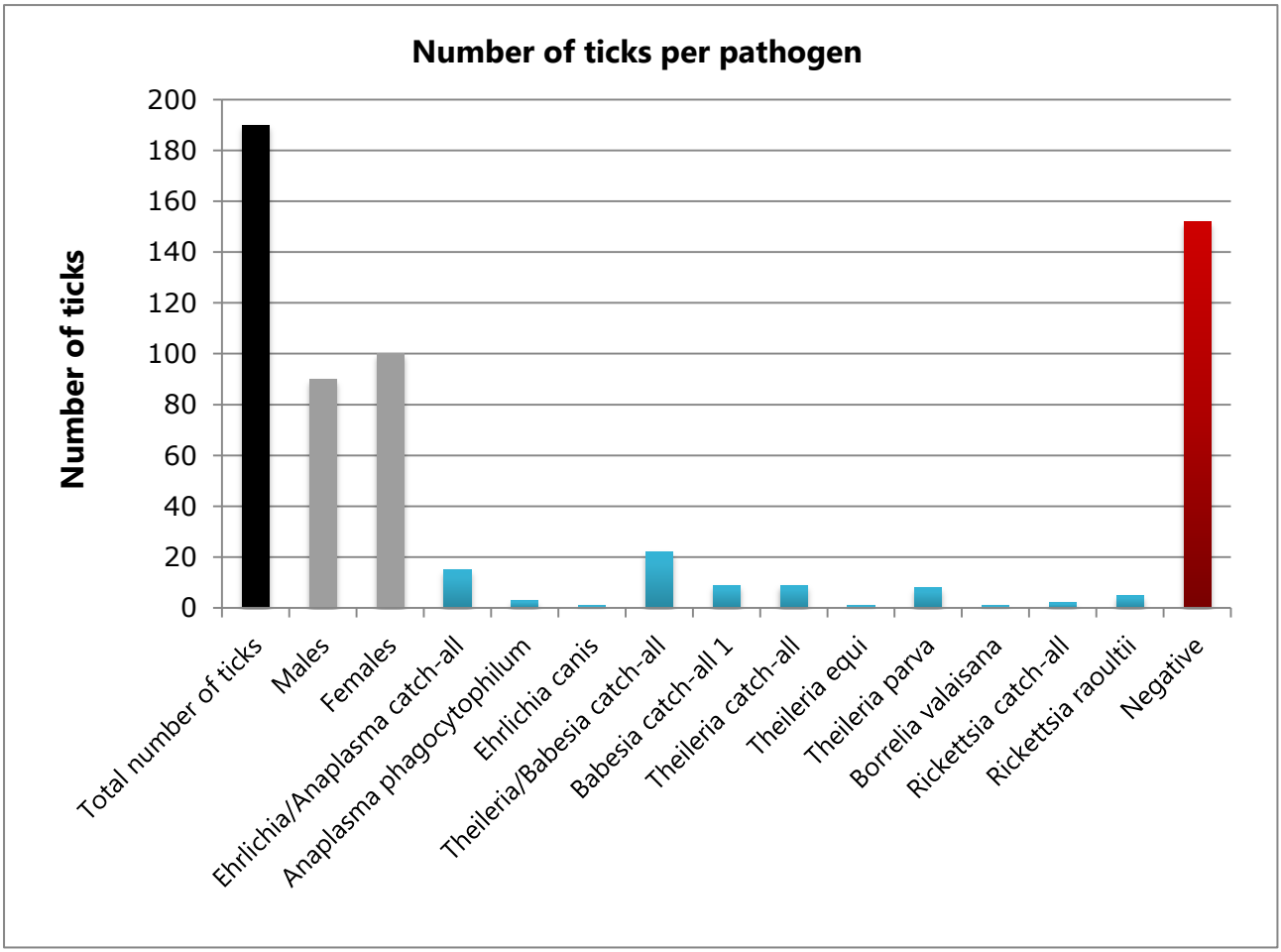

Figure 9. Pathogens detected in *D. reticulatus* ticks collected from the vegetation in the Netherlands. The total number of ticks collected in this survey is represented by the black bar. The proportion of males and females is represented by the grey bars. The blue bars represent numbers of ticks that tested positive for a pathogen. Several ticks tested positive for more than one pathogen, which causes these ticks to be represented more than once in this figure. The number of ticks that tested negative for all of the pathogens that were tested for in this survey is represented by the red bar.

Ticks from St. Philipsland

A total of 162 ticks was collected from the vegetation of St. Philipsland and was screened by RLB for the presence of *Anaplasma* spp., *Ehrlichia* spp., *Babesia* spp., *Theileria* spp., *Borrelia* spp. and *Rickettsia* spp. (Table 8, Figure 10 and Appendix D). In November of 2014, 59 males and 60 females were collected. Originally, 130 females were found at this location on this date, but a random selection was made of the number of ticks to be tested. On 17-3-2015, sixteen males and 27 females were collected. Altogether, 75 (46.3%) males and 87 (53.7%) females were collected. Of those ticks, thirteen (8.0%) tested positive for *Ehrlichia/Anaplasma* catch-all, two (1.2%) tested positive for *Anaplasma phagocytophilum*, one (0.6%) tested positive for *Ehrlichia canis*, 22 (13.6%) tested positive for *Theileria/Babesia* catch-all, nine (5.6%) tested positive for *Babesia* catch-all 1, nine (5.6%) tested positive for *Theileria* catch-all, one (0.6%) tested positive for *Theileria equi,* eight (4.9%) tested positive for *Theileria parva*, one (0.6%) tested positive for *Borrelia valaisana*, two (1.2%) tested positive for *Rickettsia* catch-all, five (3.1%) tested positive for *Rickettsia raoultii* and 117 (72.2%) of all ticks tested negative for *Anaplasma* spp., *Ehrlichia* spp., *Babesia* spp., *Theileria* spp., *Borrelia* spp and *Rickettsia* spp.

Table 8. Pathogens detected in *D. reticulatus* ticks collected from the vegetation of St. Philipsland. Note: several ticks tested positive for more than one pathogen, which has consequences for the proportions.

	Amount	Proportion
Total number of ticks	162	100.0%
Males	75	46.3%
Females	87	53.7%
***Ehrlichia/Anaplasma* catch-all**	13	8.0%
Anaplasma phagocytophilum	2	1.2%
Ehrlichia canis	1	0.6%
***Theileria/Babesia* catch-all**	22	13.6%
***Babesia* catch-all 1**	9	5.6%
***Theileria* catch-all**	9	5.6%
Theileria equi	1	0.6%
Theileria parva	8	4.9%
Borrelia valaisana	1	0.6%
***Rickettsia* catch-all**	2	1.2%
Rickettsia raoultii	5	3.1%
Negative	117	72.2%

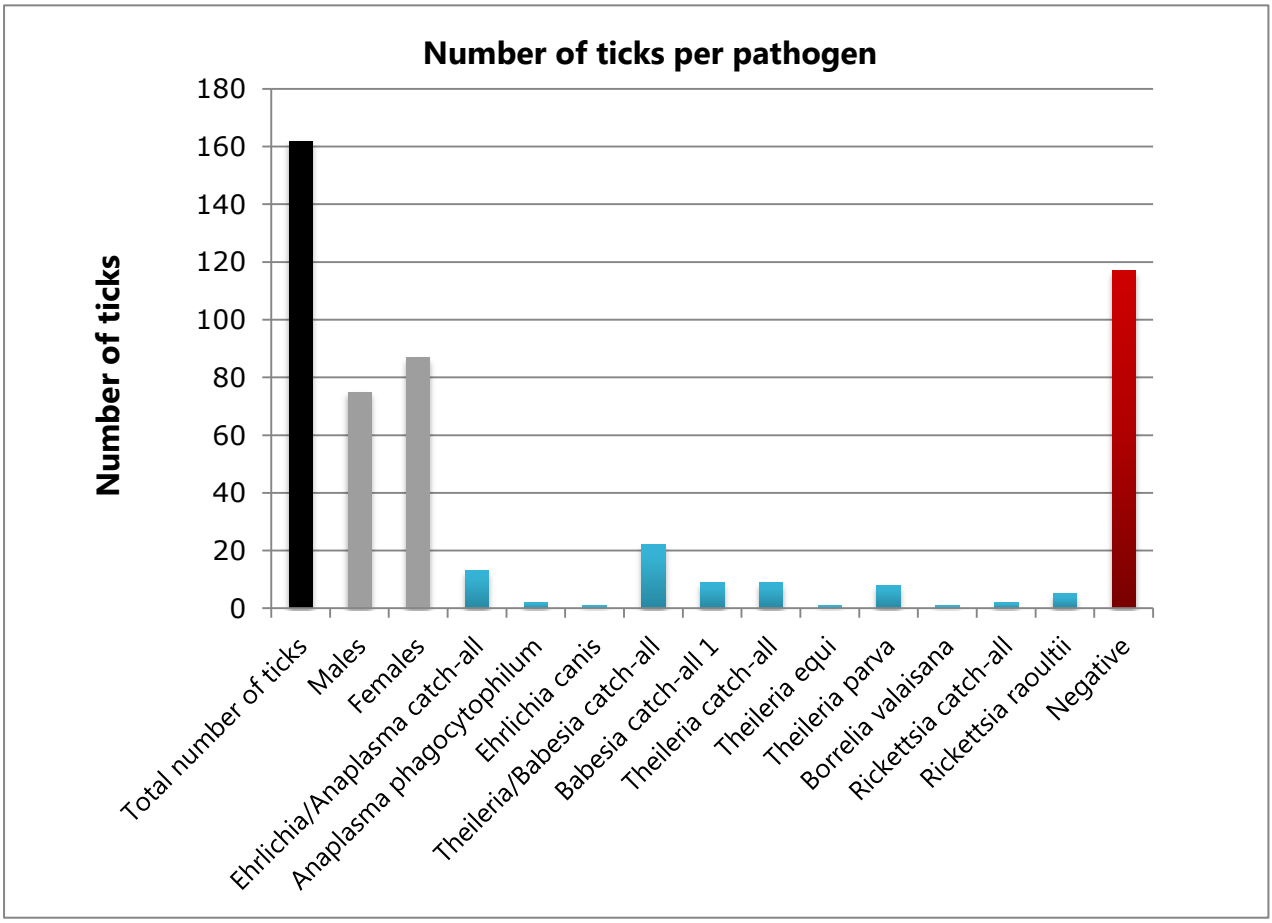

Figure 10. Pathogens detected in *D. reticulatus* ticks collected from the vegetation of St. Philipsland. The total number of ticks collected in this survey is represented by the black bar. The proportion of males and females is represented by the grey bars. The blue bars represent numbers of ticks that tested positive for a pathogen. Several ticks tested positive for more than one pathogen, which causes these ticks to be represented more than once in this figure. The number of ticks that tested negative for all of the pathogens that were tested for in this survey is represented by the red bar.

Ticks from Bergen

A total of 28 ticks, 15 (53.6%) males and 13 (46.4%) females, was collected from the vegetation of Bergen and was screened by RLB for the presence of *Anaplasma* spp., *Ehrlichia* spp., *Babesia* spp., *Theileria* spp., *Borrelia* spp. and *Rickettsia* spp. (Table 9, Figure 11 and Appendix D). Of those ticks, two (7.1%) tested positive for *Ehrlichia/Anaplasma* catch-all, one (3.6%) tested positive for *Anaplasma phagocytophilum* and 26 (92.9%) of all ticks tested negative for *Anaplasma* spp., *Ehrlichia* spp., *Babesia* spp., *Theileria* spp., *Borrelia* spp and *Rickettsia* spp.

Table 9. Pathogens detected in *D. reticulatus* ticks collected from the vegetation of Bergen. Note: several ticks tested positive for more than one pathogen, which has consequences for the proportions.

	Number of ticks	Proportion
Total number of ticks	28	100.0%
Males	15	53.6%
Females	13	46.4%
***Ehrlichia/Anaplasma* catch-all**	2	7.1%
Anaplasma phagocytophilum	1	3.6%
Negative	26	92.9%

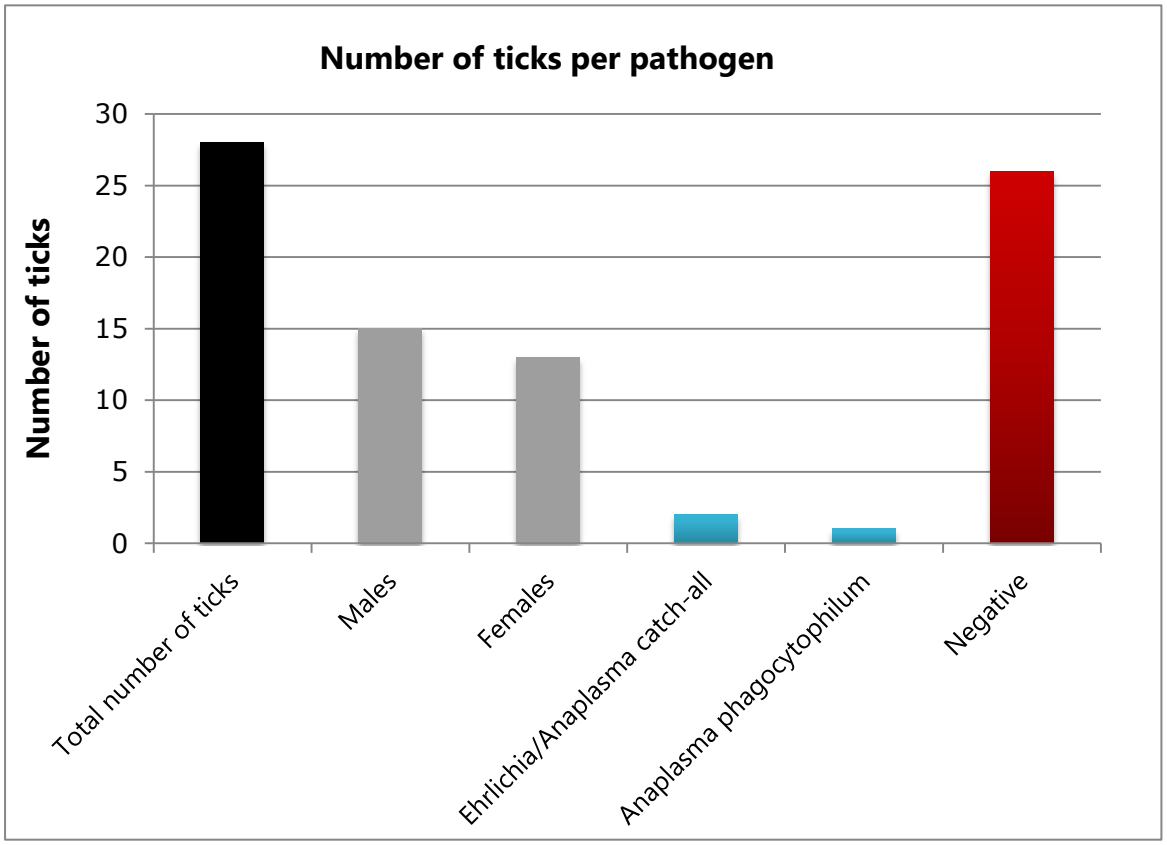

Figure 11. Pathogens detected in *D. reticulatus* ticks collected from the vegetation of Bergen. The total number of ticks collected in this survey is represented by the black bar. The proportion of males and females is represented by the grey bars. The blue bars represent numbers of ticks that tested positive for a pathogen. Several ticks tested positive for more than one pathogen, which causes these ticks to be represented more than once in this figure. The number of ticks that tested negative for all of the pathogens that were tested for in this survey is represented by the red bar.

Ticks sent in through the 'Tickbusters' survey

A total of ten ticks were collected from dogs and the vegetation from several locations and on different dates (Table 5). One tick (tick number 2) was a female collected from the vegetation of Egmond aan den Hoef on May 29th 2014. It tested positive for *Theileria* catch-all. The rest of the ticks tested negative for *Anaplasma* spp., *Ehrlichia* spp., *Babesia* spp., *Theileria* spp., *Borrelia* spp. and *Rickettsia* spp.

Discussion

In this study, the results are reported of a survey in which *D. reticulatus* ticks collected from several locations and dogs in the Netherlands were screened for pathogens that they may harbour, in order to establish the prevalence of tick-borne pathogens in *D. reticulatus*. This tick species was chosen because it is the second most important tick in Europe and in the Netherlands, and because the habitat of *D. reticulatus* seems to be expanding (Bonnet et al., 2013; Claerebout et al., 2013; Cochez et al., 2012; Karbowiak, 2014). The aim of this study was initially to determine the prevalence of *B. canis* and *B. caballi*, but other pathogens were also included in the survey.

In the last few decades, *D. reticulatus* ticks have been found in several places where they did not occur before, including the Netherlands and Germany. Previously, the French-Belgian border was considered to be the northern boundary of *D. reticulatus* in Western Europe (Cochez et al., 2012; Karbowiak, 2014). During the last two decades, the occurrence of autochthonous cases of canine babesiosis in Belgium suggests that *D. reticulatus* could be indigenous in this country (Claerebout et al., 2013). In Belgium, low numbers of *D. reticulatus* were previously found on dogs (Claerebout et al., 2013). However, it was unclear whether these ticks were part of indigenous populations or were imported from another country after travelling abroad. Therefore, several locations were researched and *D. reticulatus* was found, confirming the presence of this tick (Cochez et al., 2012). Questing populations of *D. reticulatus* have recently been found by flagging in the Netherlands (Nijhof et al., 2007). The tick has also been documented in Germany, Austria, Poland, western Switzerland and Belgium (Cochez et al., 2012; Matjila et al., 2005).

In 2008-2009, a survey was conducted to investigate the presence of several different tick species collected from cats and dogs in Belgium, along with the pathogens that they carried (Claerebout et al., 2013). In this study, *I. ricinus* was found to be the most common tick species infesting companion animals, followed by *I. hexagonus*, which was similar to other studies in North-Western Europe. Besides *I. ricinus* and *I. hexagonus*, modest numbers of *R. sanguineus* and *D. reticulatus* were found on dogs. All *R. sanguineus* ticks were considered to be imported as they were collected from dogs with a travelling history. Most *D. reticulatus* ticks were also collected from dogs with a travelling history, but one particular dog had never been outside Belgium. From this dog, *Dermacentor* ticks were repeatedly sampled. The presence of questing *D. reticulatus* ticks in the area where the dog was walked on a daily basis was confirmed by flagging. This was the first indigenous population of *D. reticulatus* in Belgium. Further investigation confirmed the presence of at least four other populations of *D. reticulatus* in Belgium (Claerebout et al., 2013).

During the spring and autumn of 2004, 23 cases of autochthonous canine babesiosis occurred in the Netherlands in dogs who had never left the country (Nijhof et al., 2007). The presence of *B. canis* was confirmed in the ticks that were found on these dogs (Matjila et al., 2005). Up until this point, no autochthonous population of *D. reticulatus* had been recorded in the Netherlands.

Factors such as climate change, altered human activity, increases in host population density such as in cervids, landscape use and movement and transport of animals carrying ticks may explain the divided distribution from before the 1990s and also the current distribution, which is expanding (Cochez et al., 2012; Karbowiak, 2014; Nijhof et al., 2007). The same goes for the increase in incidence of canine tick-borne diseases, which is due mostly because of changes in ecology, climate change, human behavior and other factors (Jongejan et al., 2011).

The East-West division of the *D. reticulatus* populations relates to the climate in Europe. The climate in Poland is a transitional area that lies between continental and oceanic climates; this is where dry air from the continent meets moist air from the Atlantic (Jongejan et al., 2015; Karbowiak, 2014). This influences the vegetable cover and the animals associated with it. There is also a transitional zone

between the flora and fauna of these two areas, which limits the range of many species of plants and animals in the centre of Poland. Therefore, the centre marks the eastern of western limit of the distribution (Karbowiak, 2014).

Another potential hypothesis concerns the biology of *D. reticulatus* and the changing climatic conditions. The tick exists in open areas where there are large fluctuations in weather, and thus in temperature, humidity and insulation levels. Ranging temperatures of the soil limit oviposition and egg development, and changes in i.e. humidity restrict the reproductive period to one or two months (Karbowiak, 2014). Therefore, the occurrence or absence of *D. reticulatus* ticks is very dependent on the indigenous climate. The divided area of occurrence can thus be explained with the presence of five climate regions in Europe (Karbowiak, 2014). It seems that the absence of *D. reticulatus* in eastern Germany and western Poland corresponds with a region for the cold season.

Most of the hypotheses for the expansion of the geographical range of *D. reticulatus* include global warming. *D. reticulatus* is especially susceptible to climatic factors. Their threshold temperature and humidity for activity are relatively low, the spring activity of the tick begins soon after the snow disappears at 2-4°C, and one may find active ticks in January and February (Karbowiak, 2014). The warmer climate makes overwintering of young developmental stages easier and lengthens the period of activity of adults. It is thus possible for the ticks to spread to new areas (Karbowiak, 2014).

Human activities such as urbanization, irrigation, deforestation and grazing are also able to change the dynamics and geographical distribution of species, including vectors such as ticks. Agriculture and farming especially have an influence on the expansion of *D. reticulatus* ticks. In the 1980s and 1990s, a reform in politics in central Europe caused changes in local reforestations, agriculture and a reduction in pesticides that were used and the number of cattle held. Some of these may be favourable for the spreading of this tick (Karbowiak, 2014).

Tourism and trade also plays a role. Dogs travelling with their owners from one country to another and transportation of large animals for farming or trade are well-known factors to contribute (Irwin, 2010; Karbowiak, 2014)

Management of protected areas, nature reserves and recreational areas in towns and parks ensures a larger population of wild large mammals and birds. This also ensures living conditions for species of plants and invertebrates, including ticks, because the right microclimate and habitats of hosts such as ruminants are created (Jongejan et al., 2015; Karbowiak, 2014). Also, environmental corridors are created to expand the living areas of many animals, and this includes ticks. St. Philipsland has been visited before and apparently harbours permanent local populations of *D.* reticulatus since 2005 (Jongejan et al., 2015). Cattle in St. Philipsland has been found to be infested with large numbers of adult *D. reticulatus* ticks, which demonstrates that this location provides good environmental conditions for *D. reticulatus* to inhabit permanent residency in the Netherlands (Jongejan et al., 2015). This is the reason why St. Philipsland was researched again in the present survey. Bergen was also chosen because *D. reticulatus* ticks had previously been found in this location. Egmond aan Zee was a location where *D. reticulatus* has also been previously collected from the vegetation (Jongejan et al., 2015).

The first five autochthonous cases of babesiosis in the Netherlands were reported in 1985, which were associated with *D. reticulatus* ticks (Uilenberg et al., 1985). In the spring and autumn of 2004, outbreaks of autochthonous canine babesiosis occurred simultaneously in two different areas in the Netherlands, in Arnhem and in The Hague, which affected 23 dogs (Jongejan et al., 2015; Matjila et al., 2005). The dogs had not been outside the Netherlands. Of the 23 dogs, nineteen animals recovered and four dogs died. From three of the dogs, adult *D. reticulatus* ticks were collected. At the time, there were no ticks found in the vegetation where these dogs were walked, but in 2007 the presence of

autochthonous populations of *D. reticulatus* ticks in the Netherlands was confirmed (Jongejan et al., 2015).

D. reticulatus has been introduced to and has come from endemic areas in Europe from dogs travelling, and the tick can sustain a *Babesia* infection for several generations (Jongejan et al., 2015). This may lead to autochthonous cases of babesiosis in non-endemic countries, as has been reported previously in the Netherlands, Belgium, Germany, Norway and Switzerland (Jongejan et al., 2015; Matjila et al., 2005).

These data suggest that the geographical range of *D. reticulatus* is expanding and that prevalences of *Babesia* spp. occur more often than before.

Previously, in a survey by Jongejan et al. (2015), a percentage of 1.64% for *B. canis* has been reported in *D. reticulatus* ticks in four different field locations in the Netherlands (Dintelse Gorzen, Rozenburg, Slikken van de Heen and St. Philipsland) (Jongejan et al., 2015). Two ticks (0.23%) tested positive for *B. caballi*; these ticks were found in the vegetation in the Dintelse Gorzen in the Netherlands and in De Panne in Belgium. None of the ticks that were collected from animals or humans were infected with any known *Babesia* or *Theileria* species (Jongejan et al., 2015). This was the first information reported of indigenous field ticks infected with *B. canis* and *B. caballi* in the Netherlands and Belgium (Jongejan et al., 2015).

In the present study, 22 ticks (11.6%) collected from the vegetation tested positive for *Theileria/Babesia* catch-all, nine ticks (4.7%) tested positive for *Babesia* catch-all 1, nine ticks (4.7%) tested positive for *Theileria* catch-all, one tick (0.5%) tested positive for *T. equi* and eight ticks (4.2%) tested positive for *T. parva*. One out of three ticks collected from the vegetation of Egmond aan den Hoef tested positive for *Theileria* catch-all. Even though the positive PCR sample in the RLB of 16-4-2015 from numbers 74-113 did not test positive, several samples on the blot did test positive for *Theileria/Babesia* catch-all and *Babesia* catch-all 1; therefore, it was assumed that this RLB was carried out correctly.

However, none of the ticks tested positive for *B. canis*, *B. caballi* or other specific *Babesia* pathogens (see appendix D). A possible explanation is that the total number of ticks, collected from vegetation and hosts was too small.

The natural infection rate of *B. canis* in *D. reticulatus* ranges from 1% to 3.6% (Rar et al., 2005). Other studies report infection rates of *B. canis* in *D. reticulatus* ticks from 0% (Germany) to 2.3% (south-western Slovakia) and even 14.7% (eastern Slovakia) (Jongejan et al., 2015). The low infection rate lowers the chances for dogs or other animals to pick up infected *D. reticulatus* ticks. This may be an explanation for the lack of additional clinical cases reported in the Netherlands and Belgium (Jongejan et al., 2015).

In the study by Nijhof et al. (2007), ticks that had been submitted by veterinarians and the general public between July 2005 and October 2006 were analyzed (Nijhof et al., 2007). *D. reticulatus* was found in several locations in the Netherlands for the first time. In this study, *B. canis* was also not detected in any of the *D. reticulatus* ticks found in the study. This in turn is in contrast with infection rates that were reported in a study in Slovakia and Western Siberia. These infection rates ranged from 1% to 3.6% (Nijhof et al., 2007). A potential explanation was that no dogs were allowed into (some of) the locations where *D. reticulatus* is located, thereby reducing the chances of dogs carrying *B. canis* and introducing the infection into the tick population (Nijhof et al., 2007).

For prevention, dogs can be vaccinated against *B. canis*, but considering the local distribution of *D. reticulatus* and the low prevalence of *B. canis* in the Netherlands, this does not seem to be necessary

for the time being for dogs that do not travel to endemic areas (Bodaan et al., 2007). However, veterinarians should be alert when a tick bite is mentioned from a dog owner together with one or more of the symptoms of babesiosis, particularly during or immediately after the activity peak of *D. reticulatus*, which is in the spring and autumn (Bodaan et al., 2007). Dogs with babesiosis can be treated with a supportive treatment and a specific treatment, such as imidocarb dipropionate (Imizol®) (Irwin, 2010; Matjila et al., 2005). It has been reported that sterilization of infection by treatment with imidocarb establishes a higher susceptibility to re-infection. Also, untreated animals have more resistance to homologous challenge (Matjila et al., 2005). Vaccinating animals would therefore be the preferred preventive measure to be taken in an endemic area. Vaccination would not prevent infection but it would adequately reduce the clinical symptoms (Matjila et al., 2005).

In the present survey, 11.6% of the ticks collected from the vegetation tested positive for *Theileria/Babesia* catch-all, 4,7% of the ticks tested positive for *Theileria* catch-all and 4.7% of the ticks tested positive for *Babesia* catch-all. Some of the *Theileria/Babesia* and *Theileria* catch-alls can be explained by subsequent results for *T. parva*, which is discussed later on in the discussion. This still means that there were other ticks positive for the catch-alls. This may be because the specific pathogen that the tick carried was not included in the blot, or that this is a pathogen not previously known.

One tick (0.5%) tested positive for *T. equi*. *T. equi* can cause equine piroplasmosis, which is a disease with a great economic impact on horses, since only 10% of the world's horse population resides in areas that are free from *T. equi* and *B. caballi* (Butler et al., 2012; Jongejan & Uilenberg, 2004). Clinical signs include pale mucus membranes, pyrexia, ataxia, haematuria and thrombocytopenia (Adaszek et al., 2011). The disease is endemic in many tropical and subtropical areas, but areas with a moderate climate can also be affected when horses are exposed to ticks. Factors that promote the spreading of ticks that carry piroplasmosis are transportation of horses and habitat changes because of climate change (Butler et al., 2012). The Netherlands were considered to be free of autochthonous equine piroplasmosis in 2010, but Butler et al. (2012) revealed horses that tested positive for *T. equi* and *B. caballi* (Butler et al., 2012). *D. reticulatus* is an important vector of *T. equi*. However, the prevalence of *T. equi* in ticks this study is small and in the Netherlands and Belgium, the prevalence and incidence of equine piroplasmosis is not well known. Therefore, this should be further investigated.

Despite the fact that no specific *Babesia* pathogens were found in this study, other interesting findings were documented.

Fifteen ticks (7.9%) tested positive for *Ehrlichia/Anaplasma* catch-all, three ticks (1.6%) tested positive for *A. phagocytophilum* and one tick (0.5%) tested positive for *E. canis*. The fact that there are more catch-all signals than there are specific pathogen signals, means that either the ticks carried pathogens that were not represented on the blot membranes, or that they were pathogens that were previously unknown.

The only recognized vectors of *A. phagocytophilum* are *Ixodes* spp. (Chomel, 2011; Little, 2010). Records of *D.* reticulatus ticks infected with *A. phagocytophilum* are rare and the ability of this tick to transmit this pathogen is not yet fully understood (Karbowiak et al., 2014). There are studies that have demonstrated low prevalences of *A. phagocytophilum* in *D. reticulatus* ticks and there are studies that did not detect the pathogen in this tick species.

The study by Bonnet et al. (2013) hypothesized that *Dermacentor* ticks may be able to transmit *A. phagocytophilum,* among other pathogens. However, none of the *D. reticulatus* ticks collected in that study were positive for *A. phagocytophilum* (Bonnet et al., 2013). In the study by Jahfari et al. (2014), *A. phagocytophilum* was not found in 59 *D. reticulatus* ticks in vegetation of Europe (Belgium and the Netherlands) either (Jahfari et al., 2014). Also, Richter et al. (2012) tested 283 *D. reticulatus* ticks

collected from the vegetation in Berlin, Germany, but none of them tested positive for *A. phagocytophilum* (Richter et al., 2013). In a study by Tijsse-Klasen et al. (2013), none of the 61 *D. reticulatus* ticks collected from the vegetation of England and Wales tested positive for *A. phagocytophilum* (Tijsse-Klasen et al., 2013).

A. phagocytophilum, which can cause tick-borne fever in ruminants and granulocytic anaplasmosis in humans, dogs, cats and horses, has recently been found in *D. reticulatus* ticks in the vegetation in Lithuania and in ticks in Chernobyl (Jongejan et al., 2015; Karbowiak et al., 2014). In Belgium, the pathogen was found in ticks that were collected from a red deer (Wirtgen et al., 2011). In a study by Szekeres et al. (2015), in Southern Hungary *D. reticulatus* ticks (among others) were collected from the vegetation, and two out of 64 *D. reticulatus* ticks tested positive for *A. phagocytophilum* (3.1%) (Szekeres et al., 2015). This percentage is similar to the percentage found in the present study. *D. reticulatus* has proven vector competence for *A. marginale*, and these ticks infected with *A. marginale* have recently been found in France (Jongejan et al., 2015). However, vector competence for *A. phagocytophilum* has not been proven yet.

Ehrlichia canis, *Ehrlichia chaffeensis*, *Ehrlichia ewingii* or a co-infection with these and other tick-borne pathogens can cause canine ehrlichiosis (Little, 2010). *E. canis* is important worldwide and is responsible for a life-threatening disease. Dogs are a reservoir for this pathogen and are also the main host for the primary vector tick *R. sanguineus* (Chomel, 2011; Little, 2010). *D. variabilis,* which is a North American species of the genus *Dermacentor*, has also been shown to experimentally transmit *E. canis* (Aktas et al., 2015; Hornok et al., 2013; Little, 2010). In a study by Hornok et al. (2013) on tick species on carnivorous hosts in Central Europe, thirteen *D. marginatus* nymphs were reported to carry *E. canis* (31%). None of the four *D. reticulatus* nymphs carried *E. canis*. To their knowledge, it was the first time *E. canis* had been reported in *D. marginatus* nymphs (Hornok et al., 2013).

To the best of knowledge, *D. reticulatus* has previously not been known to either carry or transmit *E. canis*. This is therefore something that possibly needs to be further investigated.

In the present study, one tick (0.5%) tested positive for *B. valaisana*, two ticks (1.1%) tested positive for *Rickettsia* catch-all and five ticks (2.6%) tested positive for *R. raoultii*.

A prevalence of below 0.5% in questing *D. reticulatus* ticks has been reported for *Borrelia* spp. (Schreiber et al., 2014). A recent study failed to detect *Borrelia* spp. in *D. reticulatus* ticks in Berlin (Schreiber et al., 2014). This is similar to the prevalence of *Borrelia* spp. found in the present study (0.5%), which is accounted to *B. valaisana*.

R. raoultii belongs to the spotted fever group rickettsiae. It is suspected to cause tick-borne lymphadenopathy (TIBOLA) in humans (Nijhof et al., 2007; Reye et al., 2013). *R. raoultii* has previously been detected in many European countries, including the Netherlands (Jongejan et al., 2015; Nijhof et al., 2007). In the study of Nijhof et al. (2007), 33 *D.* reticulatus ticks found in the vegetation tested positive for *R. raoultii* (Nijhof et al., 2007). Reye et al. (2013) found a prevalence of 22.6% of *R. raoultii* in questing *D. reticulatus* ticks in Belarus (Reye et al., 2013). This was comparable to a prevalence of 22.3% that was reported in Slovakia (Reye et al., 2013). In Germany, a prevalence of 30% for *R. raoulti* has been reported (Schreiber et al., 2014). However, these prevalences are not similar to the prevalence found in the present study (2.6%). This may be because the total number of ticks was not high enough. In the study by Schreiber et al. (2014), only the genospecies *R. raoultii* was found in *D. reticulatus* out of all *Rickettsia* spp. (Schreiber et al., 2014). The same was found in the present study.

Originally, 130 females were found at St. Philipsland in November 2014, but a random selection of 60 ticks was made of the number of ticks to be tested. This may have had consequences for the prevalence of the pathogens that were found, but because a random selection has been made and

because the total number of ticks is of a reasonable size, the results can still be interpreted. The rest of the ticks can of course still be tested later on.

Any of the findings in the present study may be accidental and the total number of sampled ticks, which could be too low, may have an influence on the findings. Also, when a pathogen is recorded in a tick, it may come from a recent blood meal, and it does not necessarily mean that this tick species is a capable vector (Obsomer et al., 2013).

On the RLB blot, spots appear where species specific oligonucleotides and PCR products have hybridized. The interpretation of these spots is subjective, therefore the prevalences of the pathogens mentioned in this survey may be different than reported.

On the first blot, which was performed on 13-4-2015, eight ticks tested positive for *T. parva*. This pathogen, which causes East Coast fever, does not normally occur in Europe, but does occur in eastern and southern Africa (Laisser et al., 2014). It was therefore concluded that a lab contamination had occurred, and that the *T. parva* results in this study should be neglected. This also means that because of this, several samples could have tested positive for *Theileria* catch-all, and possibly for *Theileria/Babesia* catch-all too. This should be taken into account when these results are considered. After it was concluded that the lab was possibly contaminated, a thorough lab cleaning session was scheduled. All of the opened and/or replaceable equipment and supplies were thrown away and replaced, every surface and item including the floor was cleaned with sodium hypochloride, and all the lab coats were washed. After the cleaning, no more samples tested positive for *T. parva*.

As seen in Appendix D, on some of the blots, the RLB controls of *Ehrlichia* and *Babesia* are not lit up as strong as on others. This is because halfway through this survey, the RLB controls had to be diluted because of a shortage. Nevertheless, the RLB controls were still visible and could thus still be used. Also, in several of the blots it can be seen that some of the positive PCR controls did not test positive, as they should. This happened several times on different occasions and with all controls of *Ehrlichia/Anaplasma* spp., *Babesia/Theileria* spp. and *Borrelia* spp. This posed a problem because as a result, no samples from that blot tested positive at all. Several PCRs and RLBs were then performed again and some of them did work in the end and could therefore be used, but overall the issue of the positive PCR controls that did not test positive persisted. An agarose gel electrophoresis was then performed to test whether the PCR and the PCR primers were working correctly, and this was the case. It was also checked whether the tests were carried out according to the protocol. After this, most of positive PCR controls tested positive on the RLB.

Initially, more ticks that were sent in through the ´Tickbusters survey´ were planned to be tested in this survey, including more ticks from dogs and also horses. These samples were being tested after 12-5-2015, but they tested positive for many catch-alls and pathogens. These were deemed to be false results due to a contamination or defective equipment or supplies which were used in the PCR procedure. This occurred on several blots, before action was being taken to find the issue. First, every surface and all of the equipment and all of the labs were once more thoroughly cleaned with sodium hypochloride. All of the opened and/or replaceable equipment and supplies were thrown away and replaced. The lab coats were cleaned as well. New supplies such as buffers were made and supplies such as PCR primers and polymerase were also replaced. Certain steps in the protocols were being rewritten to ensure a better clean-up, such as cleaning the RLB lab with sodium hypochloride instead of ethanol. From this point onwards, only some of the newer RLB membranes were used.

New PCR controls were made by performing a DNA extraction, PCR and RLB on several blood samples that were known to be positive for pathogens such as *E. chaffeensis, E. canis, E. ruminantium*, *A. centrale*, *A. marginale*, *B. burgdorferi*, *B. bigemina*, *B. canis*, *B. canis canis*, *B. major*, *B. vogeli, T.*

annulata. and *T. parva*. Despite all of these measures, the results continued to test positive for many catch-alls and pathogens.

Then, an external professional was asked her opinion on the matter, and several actions took place. The lab coats were washed with chloride and were then distributed throughout the different labs where they would stay, door closers were installed and walking routes were designed in order to minimize the contamination between the different labs. Also, a different polymerase was tested, because the polymerase that was used before was possibly not specific enough. A point was made that the membranes could be replaced in the future if these methods did not work in order to obtain correct results, because they could be damaged.

Unfortunately, at the end of the present study, these methods were still put into place, making the testing of more ticks not possible. This is also the reason that no negative PCR controls were tested, because these were planned to be tested towards the end of the study.

Conclusion

It is clear that changing ecosystems, increasing numbers of hosts in nature reserves and travelling all have an influence on the geographical range of *Dermacentor reticulatus*. In the locations visited in this survey, *D. reticulatus* outnumbered *Ixodes ricinus*. Several pathogens including *Anaplasma phagocytophilum* (1.6%), *Ehrlichia canis* (0.5%), *Theileria equi* (0.5%), *Borrelia valaisana* (0.5%) and *Rickettsia raoultii* (2.6%), and *Ehrlichia/Anaplasma* (7.9%), *Theileria/Babesia* (11.6%) and *Babesia* (4.7%) catch-alls were detected in *D. reticulatus* from the vegetation, which may suggest a possible role of *D. reticulatus* in the life cycle and transmission of these pathogens in the Netherlands. *Babesia canis* and *Babesia caballi* were not detected. However, experiments will need to demonstrate the vector competence of *D. reticulatus*, as the presence of a pathogen in a tick does not necessarily mean that the tick can transmit the pathogen to susceptible hosts. Nevertheless, the information in the present study is useful for epidemiological studies of tick-borne pathogens in the Netherlands and to try and prevent risks associated with pathogen transmission by *D. reticulatus* to animals and humans. *D. reticulatus* populations should be monitored in more detail and other pathogens should be studied in the future, as the list in this study is not complete. Subsequently, there is a need for effective preventive measures in dogs and other animals to control ticks. In the future, it will depend on the localization of the tick as to which policy to use for the control of babesiosis. Tick prevention such as collars or spot-on treatment should also be considered.

Acknowledgements

The author would like to thank prof. F. Jongejan for his advice and support. The author would also like to thank L. Berger and G. Goderski for their help and advice.

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Appendix A: Protocol for DNA extraction from ticks.

UTRECHT CENTRE FOR TICK-BORNE DISEASES (UCTD)
FAO REFERENCE CENTRE FOR TICKS AND TICK-BORNE DISEASES

DNA EXTRACTION FROM TICKS

Sample description	
Number of samples	

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 hypochlorite.	
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.	

UTRECHT CENTRE FOR TICK-BORNE DISEASES (UCTD)

FAO REFERENCE CENTRE FOR TICKS AND TICK-BORNE DISEASES

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 hypochlorite.	

DNA extraction done:

by _____ on _____ _____

Signature

Comments:

Appendix B: Protocol for the PCR RLB procedure.

UTRECHT CENTRE FOR TICK-BORNE DISEASES (UCTD)
FAO REFERENCE CENTRE FOR TICKS AND TICK-BORNE DISEASES

PCR RLB PROCEDURE

Sample description	
Number of samples	

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.		

UTRECHT CENTRE FOR TICK-BORNE DISEASES (UCTD)

FAO REFERENCE CENTRE FOR TICKS AND TICK-BORNE DISEASES

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.		

PCR done:

by _____ on _____ _____

Signature

Comments:

Appendix C: Protocol for the RLB Hybridization procedure.

UTRECHT CENTRE FOR TICK-BORNE DISEASES (UCTD)

FAO REFERENCE CENTRE FOR TICKS AND TICK-BORNE DISEASES

REVERSE LINE BLOT HYBRIDIZATION PROCEDURE

Sample description	
Number of samples	
Membrane 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.	

UTRECHT CENTRE FOR TICK-BORNE DISEASES (UCTD)

FAO REFERENCE CENTRE FOR TICKS AND TICK-BORNE DISEASES

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	Remove the foil and wash the membrane **twice** with preheated 1% SDS at 80°C for 30 minutes under gentle shaking.		
30	Wash the membrane with 20mM EDTA at room temperature for 15 minutes under gentle shaking.		
31	Store the membrane in a seal bag with 20mM EDTA at 4°C.		
32	Turn off all equipment and clean workspace.		

RLB hybridization done:

by _____ on _____ _____

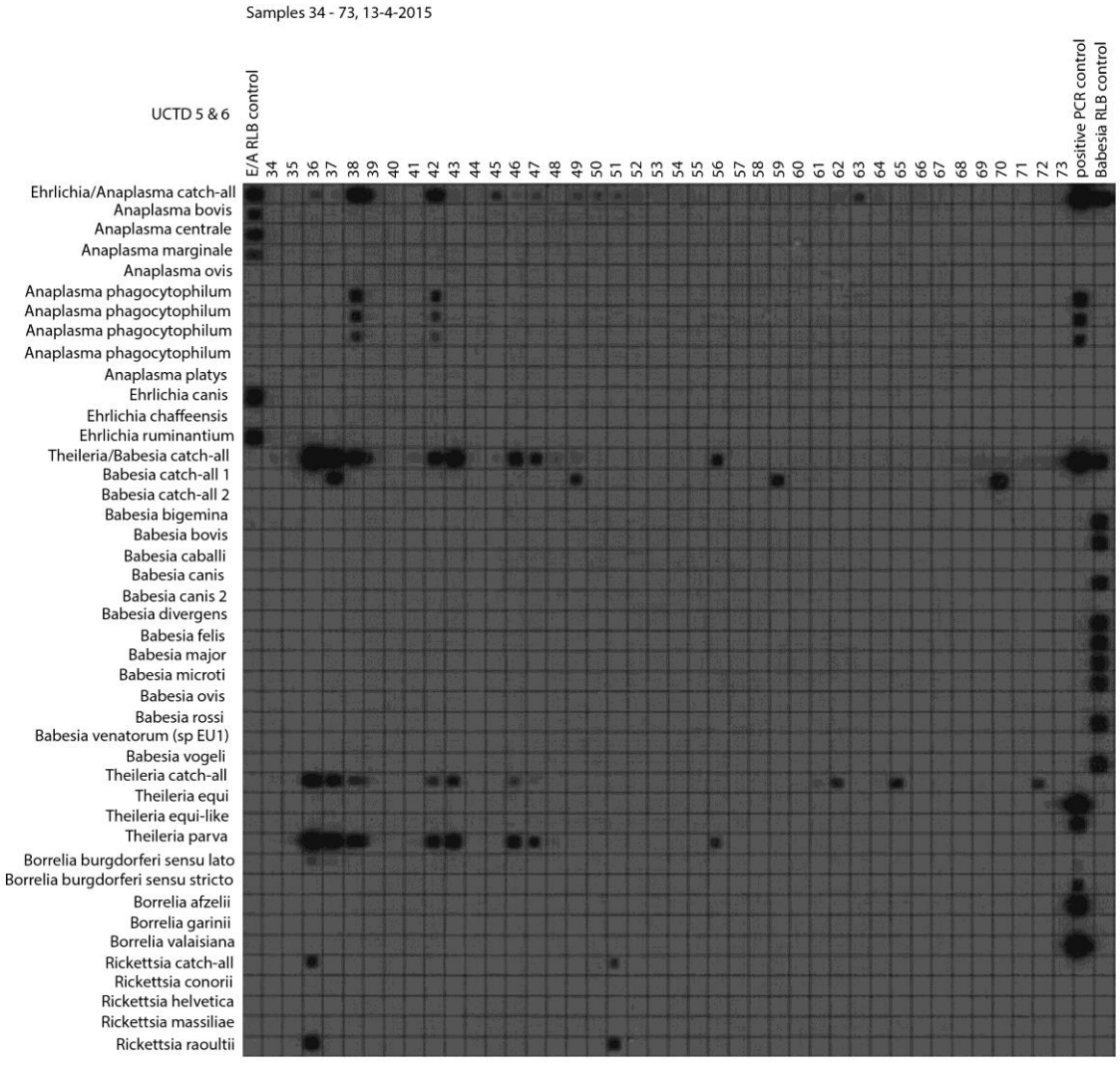
Signature

Comments:

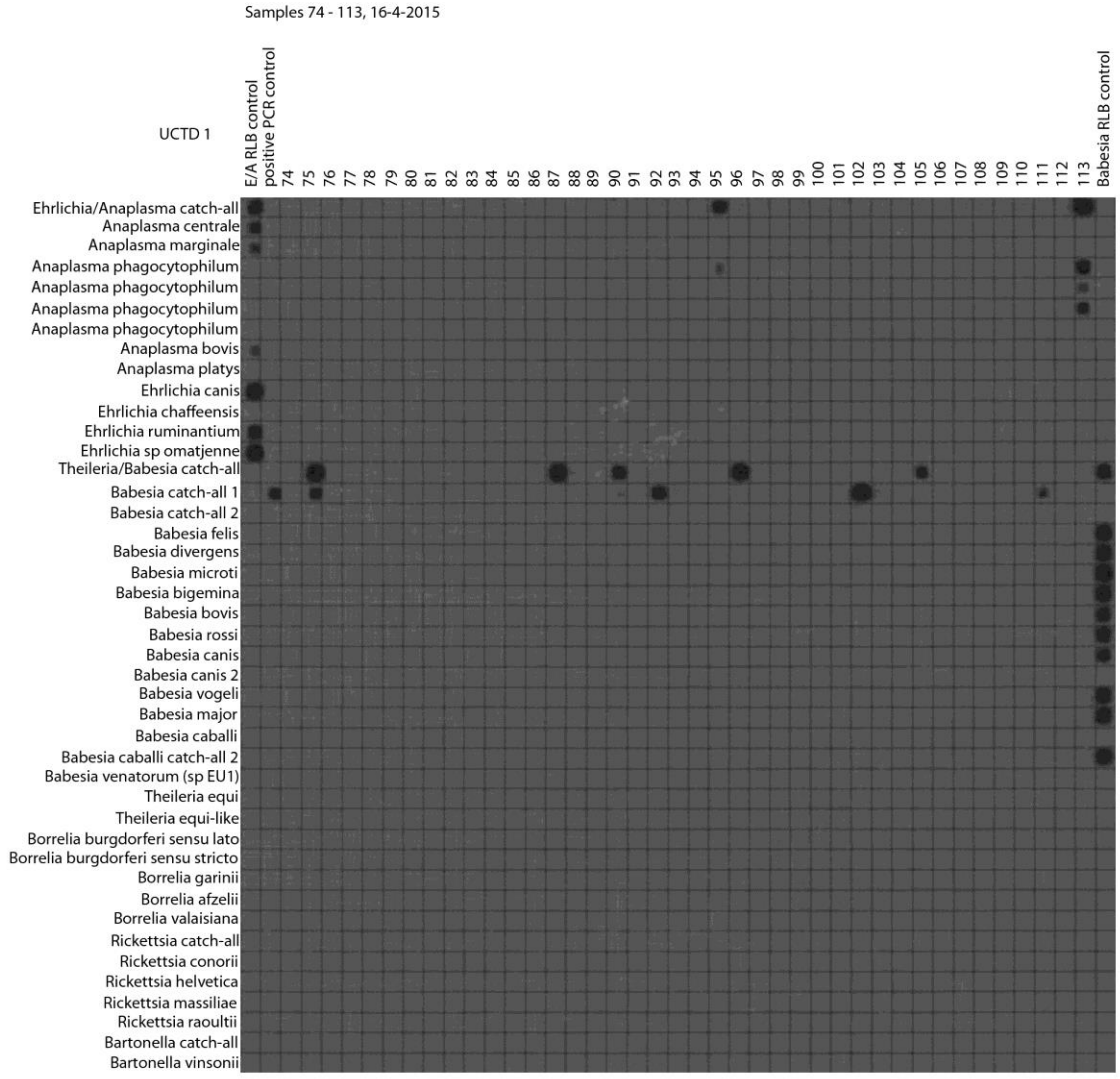
Appendix D: RLB results.

Tick numbers	Location	Date of collection
34 - 152	St. Philipsland (vegetation)	November 2014
223 – 265	St. Philipsland (vegetation)	17-3-2015
266 – 293	Bergen (vegetation)	20-3-2015
1 – 3	Egmond aan den Hoef (vegetation)	22-5-2014, 29-5-2014
4 – 6, 8	Egmond aan Zee (dog)	20-10-2014, 19-9-2014, 20-4-2014
7	Egmond aan Zee (vegetation)	15-6-2014
9	Groningen (dog)	15-4-2014
10	Heiloo (dog)	12-5-2014

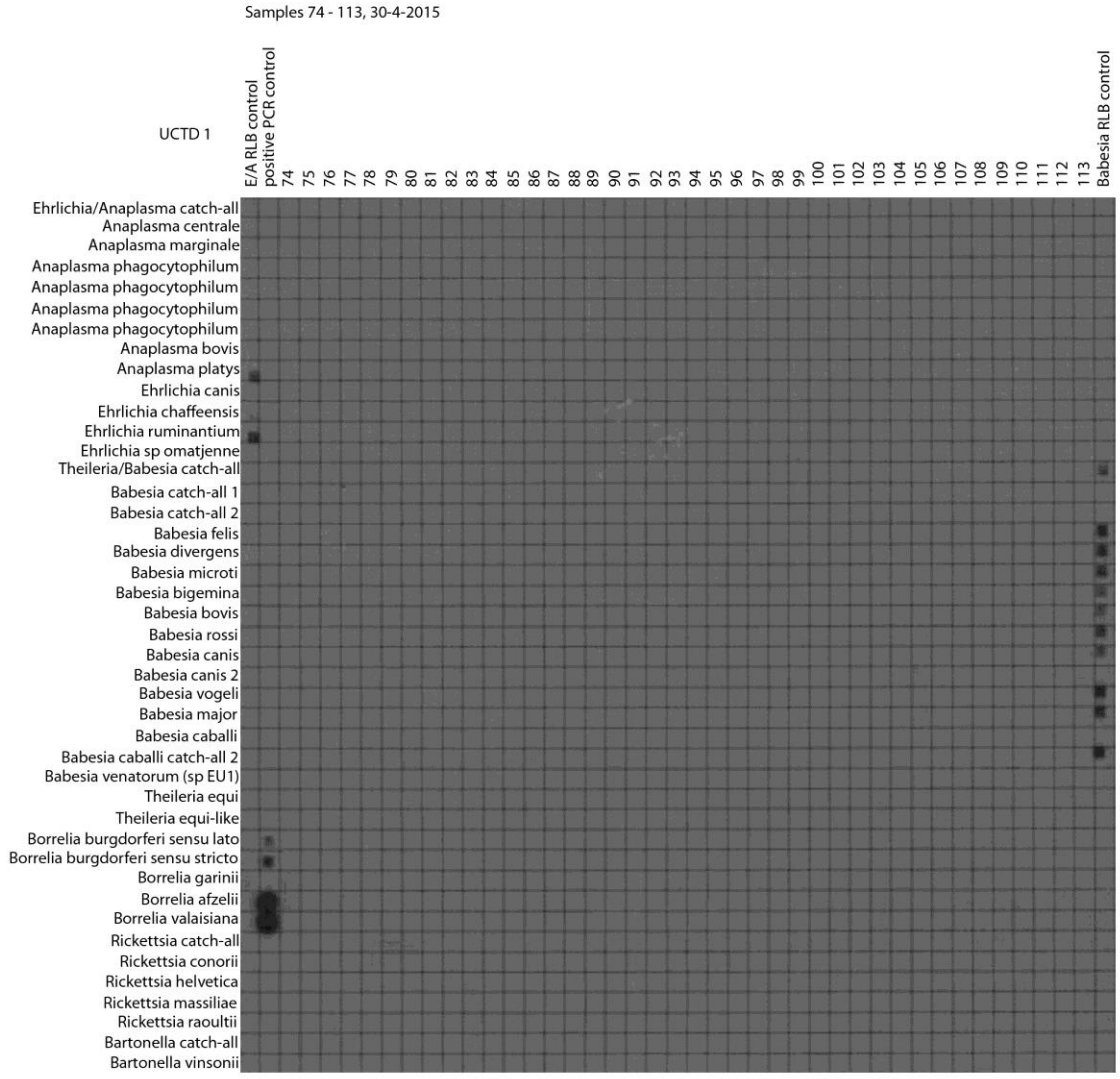
RLB 13-4-2015, St. Philipsland, collected on November 2014 (tick numbers 34 – 73)



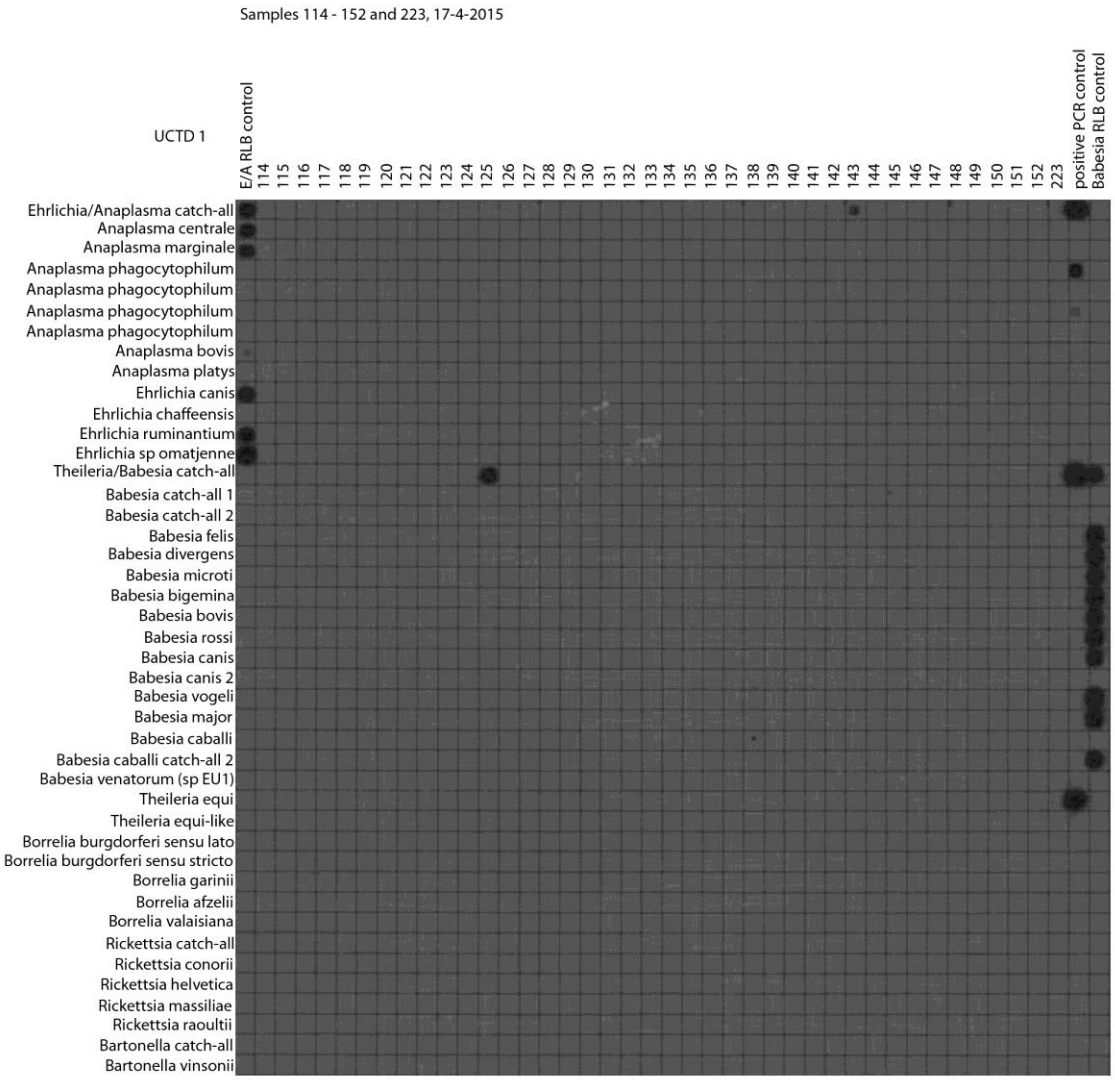
RLB 16-4-2015, St. Philipsland, collected on November 2014 (tick numbers 74 – 113)



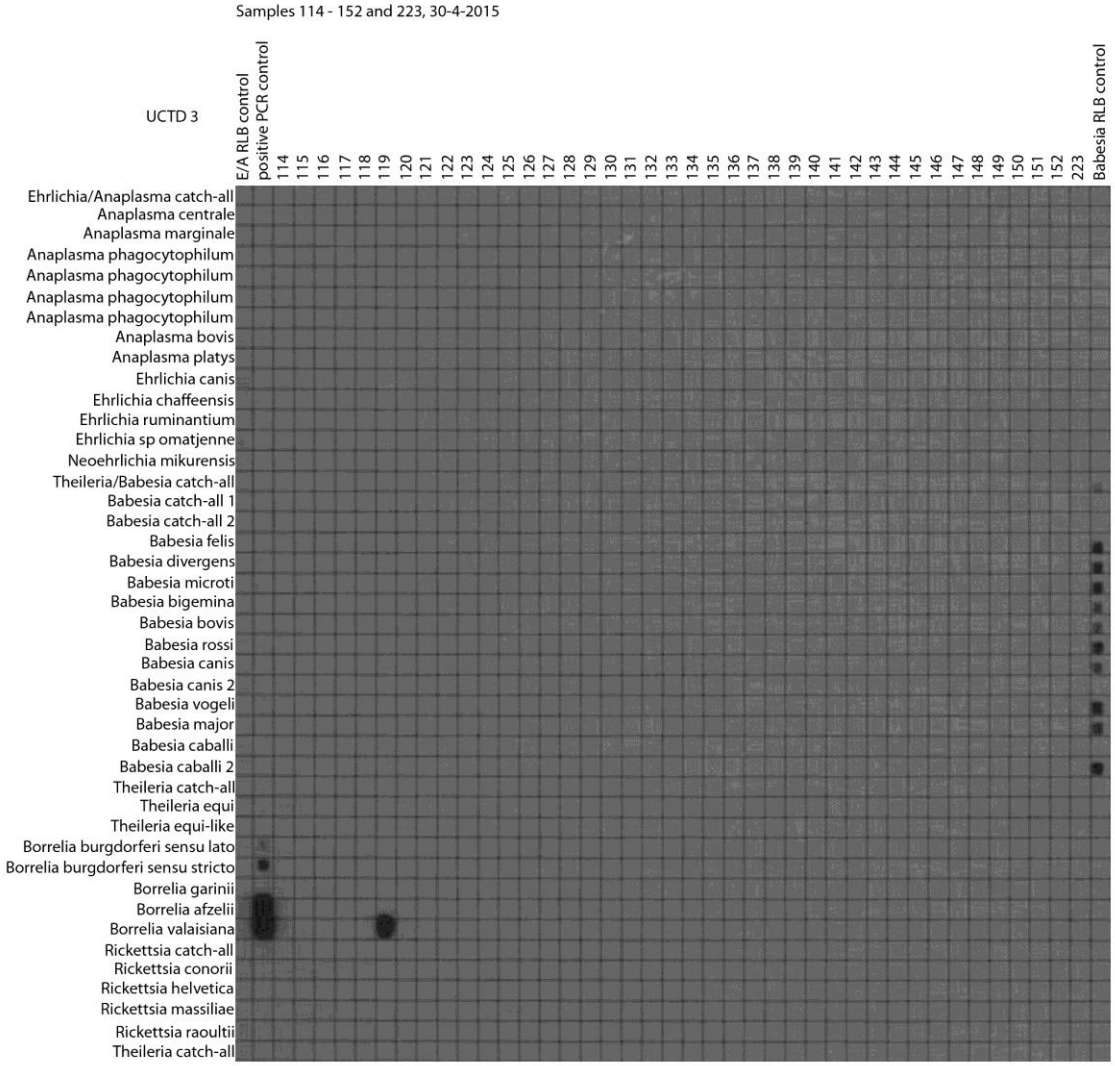
RLB 30-4-2015, St. Philipsland, collected on November 2014 (tick numbers 74 – 113)



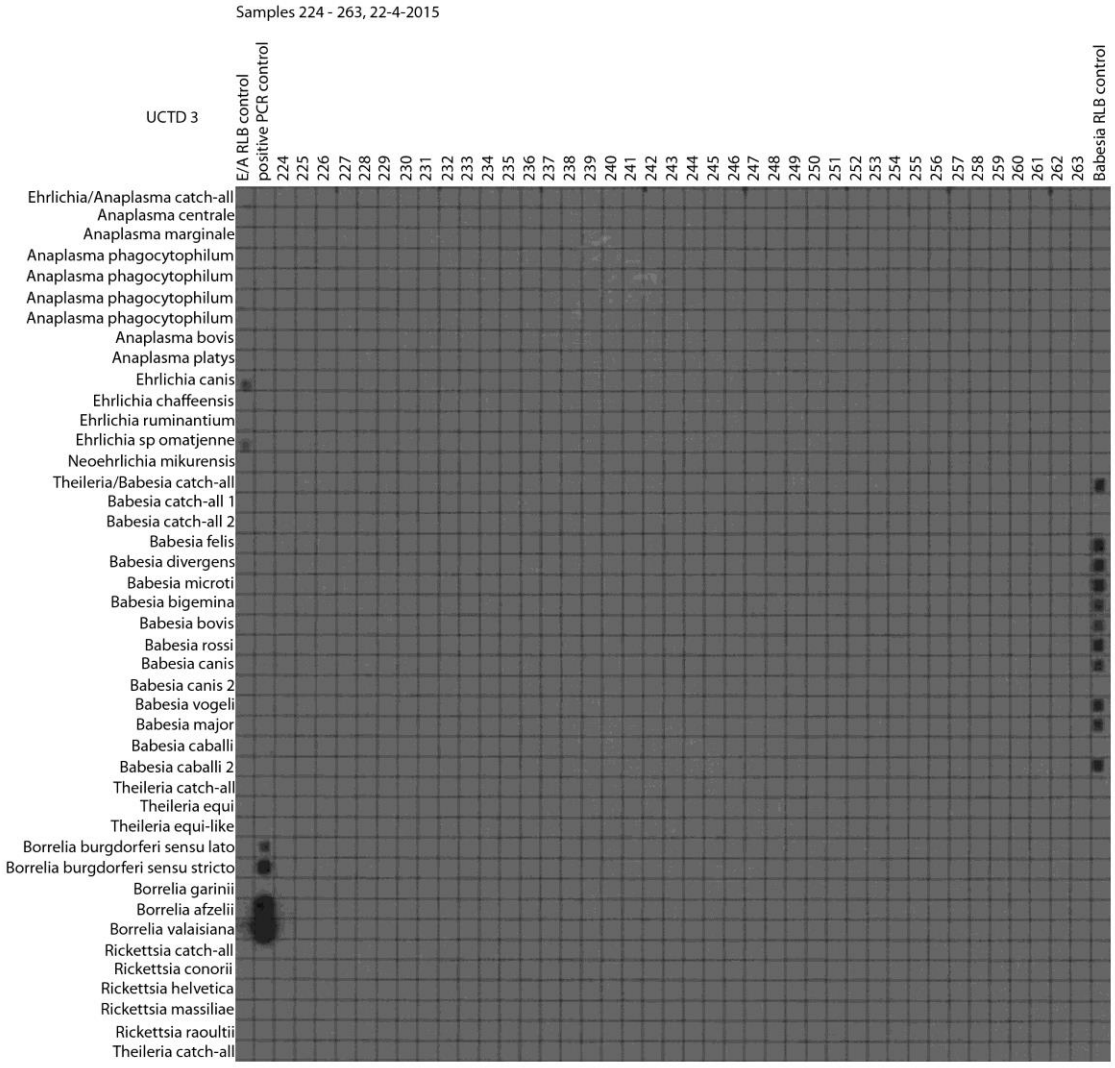
RLB 17-4-2015, St. Philipsland, collected on November 2014 (tick numbers 114 – 152) and St. Philipsland, collected on 17-3-2014 (tick number 223)



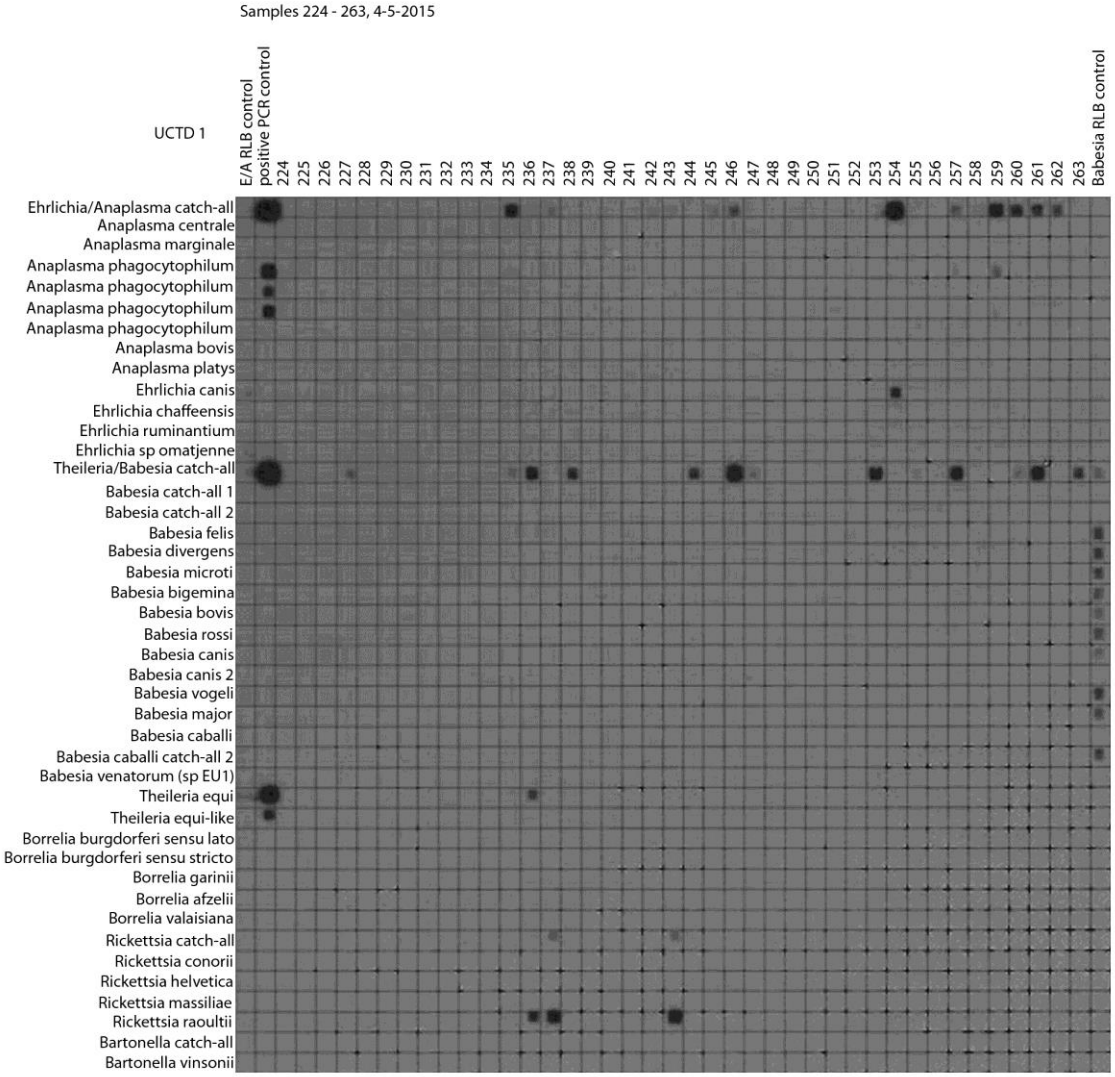
RLB 30-4-2015, St. Philipsland, collected on November 2014 (tick numbers 114 – 152) and St. Philipsland, collected on 17-3-2014 (tick number 223)



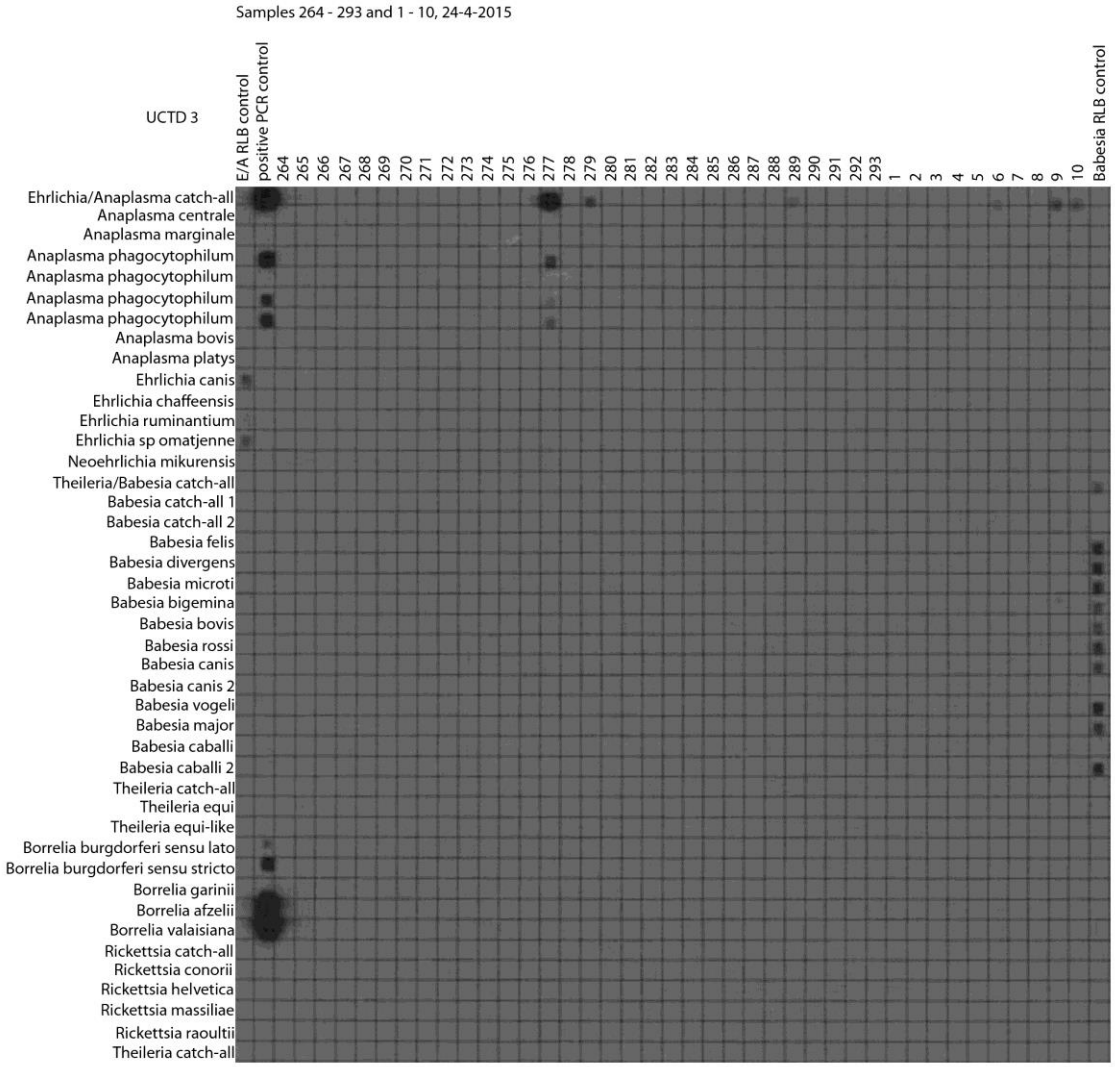
RLB 22-4-2015, St. Philipsland, collected on 17-3-2014 (tick numbers 224 - 263)



RLB 4-5-2015, St. Philipsland, collected on 17-3-2014 (tick numbers 224 - 263)



RLB 24-4-2015, Bergen, collected on 17-3-2014 (tick numbers 264 and 265), and Bergen, collected on 20-3-2014 (tick numbers 266 – 293), and ticks sent in through Tickbusters (numbers 1 – 10)



RLB 12-5-2015, St. Philipsland, collected on 17-3-2014 (tick numbers 264 and 265), and Bergen, collected on 20-3-2014 (tick numbers 266 – 293), and ticks sent in through Tickbusters (numbers 1 – 10)