THE ROLE OF BIRDS IN DISSEMINATING TICKS AND TICK-BORNE PATHOGENS

Lenneke Schuurmans (3957950)
Utrecht Centre of Tick-Borne Diseases (UCTD)

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Faculty Supervisor: Prof. Dr. F. Jongejan (F.Jongejan@uu.nl)
Utrecht Centre for Tick-borne Diseases
Utrecht University

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Abstract

Introduction: Every year, birds migrate to and from their wintering sites and by doing so they have the possibility to easily cross barriers such as mountains, desserts and oceans. Ticks can attach to birds when feeding on the ground, and therefore birds can transport ticks and tick-borne pathogens to new areas. This dissemination of ticks by birds can play an important rol because ticks are importants vector of pathogens afftecting both human and animal health.

Materials and Methods: Birds were captured in the Netherlands by a biologist with a special permission for the capture of birds. Ticks of these birds were collected in tubes and finally submitted the UCTD with additional information. After identification, DNA was extracted from these ticks and both PCR and RLB was conducted.

Results: Of a total number of 624 ticks, 621 were identified as *Ixodes ricinus* and the other 3 were identified as *Hyalomma scupense*. *Borrelia garinii, Borellia afzelii, Babesia burgdorferi sensu lato* and *Babesia venatorum* were found in a total 33 samples and thereby, one *Ixodes ricinus* tick was found to be positive for another *Babesia* species as tested for. The ticks were collected from 89 birds of 14 different bird species. All these bird species have different migrating routes and different overwintering sites. Whereas some species travel only to Southern Europe, some species travel all the way to South Africa. The *Marsh Warbler* travels, of the bird species found, the most southwards to South-Africa. Surprisingly, that *Ixodes ricinus* tick positive for an, in all probability, exotic *Babesia*-species and the *Hyalomma scupense* ticks were both found on the *Marsh Warbler*.

Discussion: In order to arrive at better and more complete results in succeeding research, improvement of tick collection is nessecary.

Conclusion: Birds play an important role in the disseminating of ticks and tick-borne pathogens, especially the non-exotics. But nevertheless, exotic ticks and possible exotic pathogens are also found to be disseminated by birds, especially by the *Marsh Warbler.*

Introduction

Bird migration

Every year, birds migrate from their breeding sites to their wintering sites and back (1). Many species can move quickly over long distances, up to thousands of kilometers. They can cross barriers like fences, mountains, desserts and oceans, and move about much faster than other non-winged animals (2,3). Birds can thus visit widely separated areas in different seasons, but they will finally return to the same locations every year. Bird movement occurs continuously, but is most persistent in spring and autumn. The travel routes of birds span almost the entire planet and thus the distribution of birds is continually changing (2).

To understand the ecology and population dynamics of bird species and to find out where individual birds are found throughout the year, the use of metal rings to mark birds individually have been used for more than 100 years. It provides precise information about the location once the bird is found and the information of its ring is recorded (4).

Ticks on birds

Migrating birds (especially passerines) are important hosts for ticks. Due the ability to cross barriers and the speed of movement of birds, they can spread ticks far beyond the home ranges of non-winged animals during migration periods (e.g. mammals and reptiles) (3,5). The prevalence of ticks on birds is influenced by different variables, as the density of ticks on the vegetation, age and sex of individual birds and differences in immunocompetency of those birds (5). Besides, it further varies between years, seasons, locations and different bird species, since ticks are only able to infest birds while feeding on the ground (3). Bird species spending much of their time close to the ground, are in general more heavily infested with ticks. Breeding birds are expected to be exposed to ticks during both foraging and accessing of their nests to care of their chicks. Most larvae and nymphs are found in May and June. In these months, birds are settling in their reproductive territories and thus an increased risk of infestation by ticks is present (5).

Avian mobility and migration, in combination with the fact that ticks infest birds at their stopover sites while resting, may play a role in the dynamics of tick populations and thus potentially increase the dissemination of both ticks and tick-borne pathogens (5). Every spring, millions of birds migrate while potentially carrying equally vast numbers of ticks, possibly infected with tick-borne pathogens, to new areas (6).

Pathogens in ticks

Ticks are members of the phylum Arthropoda and were the first arthropods established as vectors of pathogens (7,8). Now, these arthropods are the most important vectors of pathogens for both humans and animals (9). The tick population in Europe has expanded in its distribution and the incidence of tick-borne diseases has increased (7,9). The increasing tick numbers has a major impact, especially because of the transmission of zoonotic tick-borne pathogens, in both veterinary and public health (7,9).

Ticks and tick-borne pathogens in Europe

The tick family *Ixodidae,* also known as 'hard ticks' due to the scutum on the dorsal site of their body, is in Europe by far the largest and the most important family, both medically and economically. *Ixodes ricinus* is the most abundant tick found in Europe, which is a competent vector for *Borrelia, Anaplasma*, *Babesia*, *Rickettsia* but also for Tick-borne Encephalitis Virus (8). Of these pathogens, *Borrelia burgdorferi* is the most common, which may cause Lyme Borreliosis (10). Nevertheless, the fact that *Ixodes ricinus* is a common species in the Netherland, as mentioned earlier, does not directly implicate that none of these ticks are exotic ones. When ticks are carried during migration to the Netherlands and possibly bring new pathogens, they can be considered to be exotic ticks. Knowledge of the pathogens carried by these ticks will make it possible to differentiate the exotic ticks from the non-exotic ticks.

Exotic ticks and tick-borne pathogens

Exotic ticks are ticks never mentioned to occur in specific places, but do exist in other places. Ticks of the genus *Hyalomma*, for example, are found in southern Europe, Africa and Asia. Some *Hyalomma*-species may carry the Crimean Congo Hemorrhagic Fever Virus, which is until now only reported in Africa, Asia, the Middle-East and Southern-Europe (11). Another pathogen carried by exotic tick is *Ehrlichia ruminantium,* causing heartwater in sub-Saharan Africa. This pathogen is transmitted by ticks of the *Amblyomma* genus, especially *Amblyomma hebraeum* and *Amblyomma variegatum* (12).

Birds transporting pathogens

The transport of tick-borne pathogens by birds can occur in different ways. Besides transporting infected ticks, birds may be infected with a tick-borne pathogen themselves along the way. Subsequently, this pathogen is carried to other locations where parasitizing ticks may be infected, eventually infecting additional hosts and enhancing continuity of the process. Migrating birds, thus, can bring exotic ticks and exotic tick-borne pathogens to new areas. But solely the transportation of ticks, however, does not necessarily result in new foci's of tick-borne diseases, because the survival of ticks depends on the (un)suitability of the climate of the area.

Materials and methods

Bird capture and tick collection

Birds were captured by Bert Winters, a biologist with special permission which is required to utilize nets for the capture of birds. He captures the birds on two locations in North-Holland, namely Wieringerwerf (Noorderdijk 28) and Hippolytushoef (Molenweg 3), using fine-grained mist nets. These nets are almost invisible as long as they are located at a shady place. Birds will fly into these nets and are finally removed from these nets by hand.

The birds are primarily captured to provide them with a ring, but are simultaneously weighted, measured and checked for ticks. If ticks were found, they were removed from the bird with small forceps. Ticks of the same bird were collected in a tube with screwcap and provided with a number. This number was noted in a table with corresponding information about the bird species and date of capturing. The tubes were sent to the UCTD, including the table with additional information.

Tick identification

Ticks were, once received from Bert Winters, identified using the book '*Ticks of domestic animals in the Mediterranean region: A guide to identification of species'* (13). This book provides methods for tick identification, as well as clarifying pictures of different tick species and a variety of tick species' characteristics to further support the identification process. With the use of this book, each tick was identified by examining morphologic characteristics. Appendix A shows all ticks identified in order of date of capture.

DNA extraction, PCR and RLB

After identification, DNA was extracted by using the Nucleo®Spin tissue kit, following the protocol for DNA extraction of the UCTD (Appendix B). In order to lower the total number of DNA extractions, ticks were pooled. Common ticks, such as *Ixodes ricinus*, from the same birds were separated into groups of larvae, nymphs and adults and finally pooled in groups with a maximum of 30 larvae, 20 nymphs or 5 adults. Less common exotic tick species were tested individually.

To examine the DNA samples, both Polymerase Chain Reaction (PCR) and Reverse Line Blotting (RLB) was used, following the protocols of the UCTD (Appendix D and E). These tests were conducted to find out which pathogens were present in the ticks. The membrane used for RLB contained probes for different *Babesia, Theileria, Borrelia* and *Rickettsia* species (Appendix F). In order to detect every positive sample, both PCR and RLB of each DNA sample were done in duplo. Ticks were considered to be infected with a pathogen when this pathogen was found on two of more occasssions.

Ticks tested once positive and once negative were retested again and considered negative when results were negative, or considered positive when results were positive.

Main purpose of the study

The main purpose of this study was to investigate the role of birds in disseminating ticks and tick-borne pathogens of medical and/or veterinary importance.

Results

Results of birds capture and tick identification

The ticks used for this study were collected over a total period of 4 months (17 April till 8 August 2015) from a total of 89 captured birds. Another 4 samples were ticks which are found on humans, such as Bert Winters or one of his co-workers, while bird capturing and 5 samples consist of ticks which are found in Bert his car and thus were distached from the birds after capturing. This results in a total number of 624 ticks, of which 621 were identified as *Ixodes ricinus* and the other 3 were identified as *Hyalomma scupense.* Since *Hyalomma scupense* is not a common tick in the Netherlands, two nymphs and one adult tick, should be are as exotic ticks.

The 621 Ixodes ticks can be subdivided into 126 adult females, 6 adult males, 376 nymphs and 113 larvae. The 3 Hyalomma ticks consisted of 2 nymphs and 1 adult Female. (For overview see table in appendix A). Surprisingly, the adult Hyalomma tick was alive in the collecting tube, after about three months of survival. This tick was found on a *Marsh Warbler (Acrocephalus palustris)* together with two Hyalomma nymphs. Thus, it is likely to suggest that all these three ticks were collected as nymphs, but one of them moulted into an adult tick during the 3 months in the tube.

All the collected ticks were, as mentioned above, from a total amount of 89 birds, 4 humans and 5 samples with non-attached ticks. The 89 birds consisted of 14 different species: *Daw, Blackbird, Song Trush, Marsh Warbler, Great Warbler, Dunnock, European Robin, Eurasian Blackcap, Eurasian Wren, Common Whitethroat, Lesser Whitethroat, Nightingale, Greenfinch* and the *Short-toed treecreeper.* Figure 1 shows the total captured amount of birds per species. Immediately notable is the unequal distribution of the bird species. Some bird species are captured just once or twice, as other species are captured for more than 10 times. The *European Robin* and *Dunnock* are the most captured bird species in this survey.

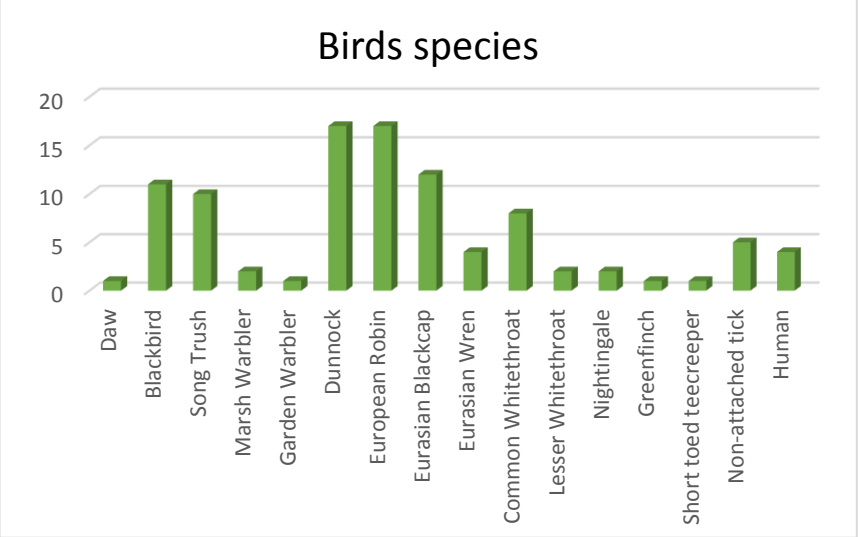

Figure 1; total number of birds per species

Figure 2 shows the division of the total number of captured birds per species per month. April and May are combined to 1 month, as only two dates of these two months are included. Noticeable is

that, by far, the most birds are captured in June. Some species, as the *Garden Warbler, Common Whitethroat, Lesser Whitethroat, Marsh Warbler Nightingale* and the *Greenfinch*, are only found specifically in June. The *daw* is only captured in April/May and the *Short-toed treecreeper* is only found in July. The finding of *Eurasian Wrens,* turned out to be only in June and August. Species as the *Blackbird, European Robin, Eurasian Blackcap, Dunnock* and the *Song trush* were birds most captured birds, in (almost) every month.

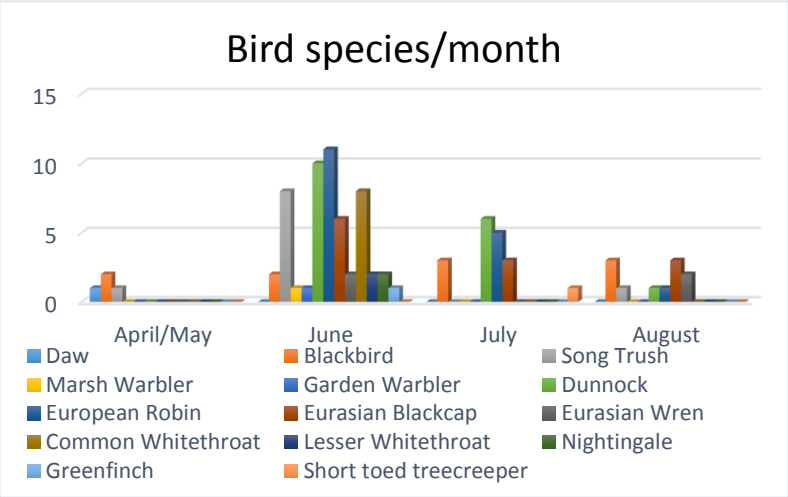

Figure 2; Total number of birds per species per month

Even more interesting is, which bird species is the most tick-infested species and when this occurred. Figure 3 shows the total number of ticks collected per bird species per month. Immediately striking is June, which represents 138 ticks. This is, the *Song Trush,* the most captured bird species in June. Other striking results are those of the *Dunnock* and *European Robin* in both June and July, the *Common whitethroat* in June and the bars which present the *Blackbird* as they show almost similar heights.

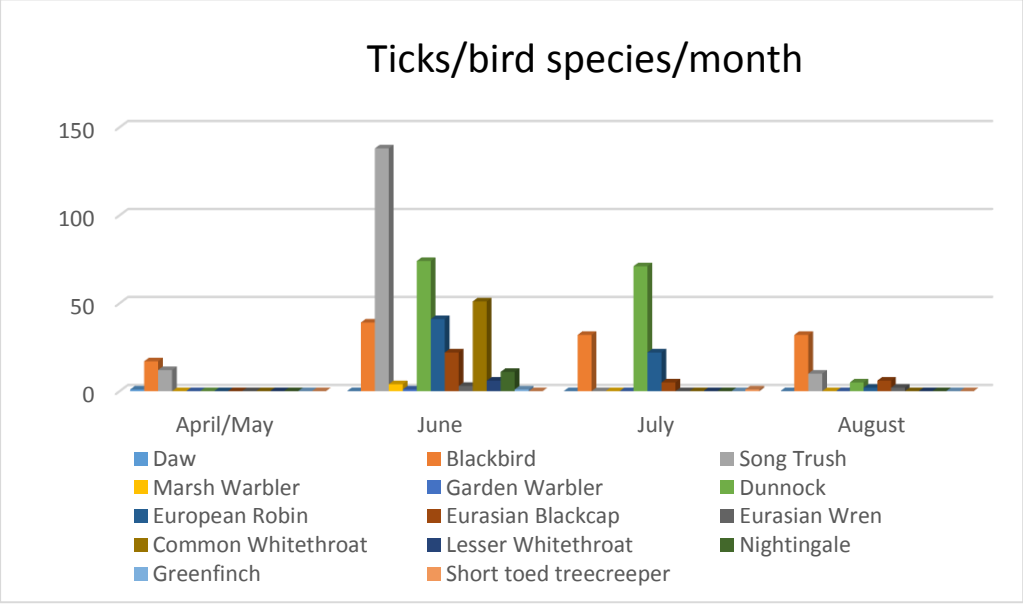

Figure 3; total number of ticks per bird species per month

In order to get a better overview of most infested bird species per month it is necessary to establish a link between the total number of birds per species every month and the total of ticks of that bird species per month. This will provide an overview of the average number of ticks per bird per species per month, which is a combination of the information of figure 2 and figure 3.

These average numbers are shown in figure 4. Eye-catching is the *Blackbird* in June, which is, in contrast to figure 3, higher than the *Song thrush's*. Furthermore, the *Blackbird* appears to have a high average tick number every month, as the *Song Trush* appears to have this every month except July and the *Dunnock* all months from June. Other species, as *Marsh Warbler, Common Whitethroat* and the *Lesser Whitethroat*, have an average of about 6 or less ticks per bird dependent of the months they were captured.

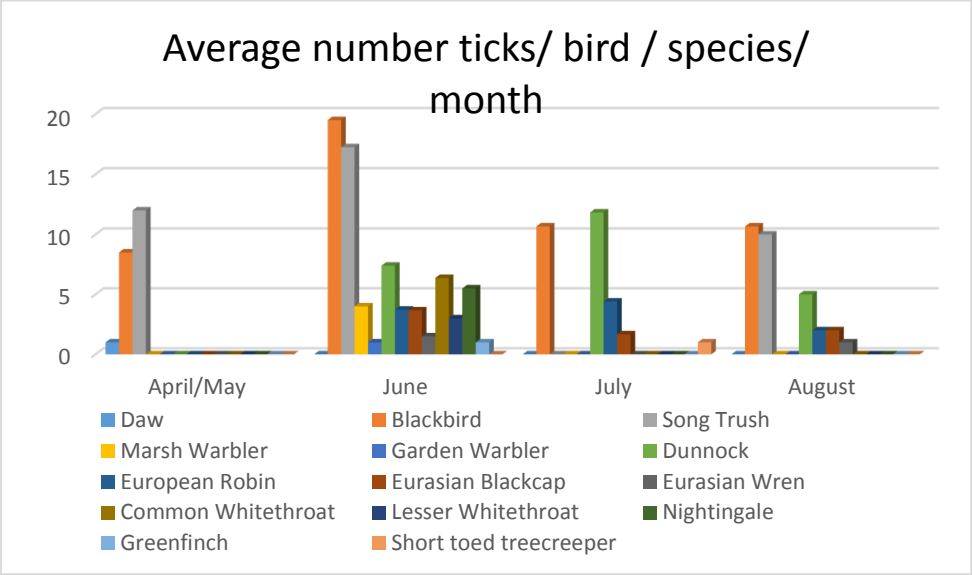

Figure 4; the average number of ticks per bird per bird species per month

Travel routes

In order to get an overview of the wintering sites of birds and thus the possibility of bringing exotic pathogens to the Netherlands, is necessary to better understand bird's travel routes. The lines which represents bird's migration routes are shown in figure 5.

Daw: this species travels around continental Europe, depending of its origin. Daws with their origin in the Netherlands stay there in winter, but Scandinavian and East- European daws travel to the Netherlands in winter (14).

Blackbird: this species travels around continental Europe, Great-Britain and Ireland. Dutch blackbirds stay in the Netherlands all year, but blackbirds from more northwards countries travel to the South or West to their wintering sites (14).

Song Trush: this species travels around continental Europe and over the North Sea towards England and Ireland. The biggest part of the Dutch Song trush population travels toward England and France in winter (14).

Marsh Warbler: this species migrate in winter towards Africa, across the East African Pathway. This long journey occurs in two stages. Firstly, they migrate to North-East Africa and after an interruption of 2-3 months they resume their journey to the final wintering grounds, as South-Africa, Kenia, Tanzania and Zambia (14,15).

Garden Warbler & Nightingale: this species travels South-West across France and the Iberian Peninsula to their wintering sites in Tropic Africa. The passage from Europe to Africa occurs around the street of Gibraltar (14).

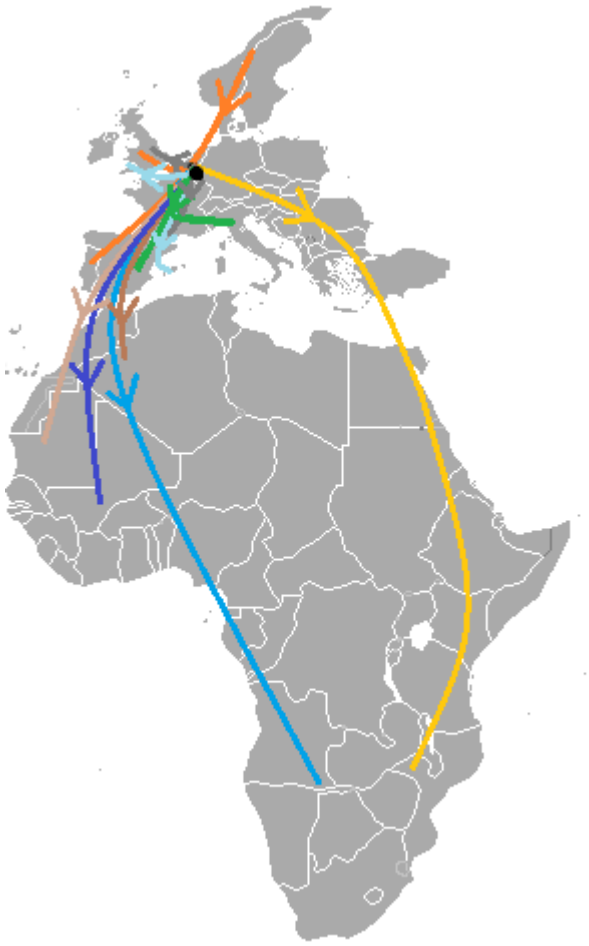

Figure 5: the lines in this figure represents the migrate routes of different bird species. The Daw, Eurasian Wren and Short-toed treecreeper are not shown, as there is no data or no migration. Orange lines: Blackbird, Grey lines: Song Trush, Yellow line: Marsh Warbler, Ocean blue line: Garden Warbler and Nightingale, Green lines: Dunnock and European Robin, Brown line: Eurasian Blackcap, Beige line: Common Whitethroat, Dark blue line: Lesser Whitethroat, Light blue lines: Greenfinch

Dunnock & European Robin: these species travels around continental Europe. In wintertime, they stay in the Netherlands or migrate more to southwards Europe (14).

Eurasian Blackcap: this species travel South-West to their wintering sites in countries of the West part of the Mediterranean Area, as Spain, Morocco and Algeria (14).

Eurasian Wren: this species lives in all countries of Europe, except in the most Northwards countries. In winter, this species stay in their origin countries and thus there is no migration route (14).

Figure 6; the dark brown area represents the Sahel region in Africa

Common Whitethroat & Lesser Whitethroat: this species travels South-West, across France and the Iberian Peninsula to their wintering sites in Africa. The most important wintering site of the Common Whitethroat is the West-side of the Sahel in Africa (see Figure 6), whether the South-side of this region the wintering side for the Lesser Whitethroat is (14).

Greenfinch: only some of these species travels in winter to France, the Iberian Peninsula or, even more less, to Great Britain. Most greenfinches stay in their country of origin in winter (14).

Short-toed treecreeper: of this species, no data is known about their traveling and migrating is known.

Positive samples

In order to test all ticks with RLB after PCR the ticks were pooled. All *Ixodes ricinus* ticks of the same bird were separated into groups of larvae, nymphs and adults and finally pooled in groups with a maximum of 30 larvae, 20 nymphs or 5 adults. All *Hyalomma scupense* ticks were tested individually. Finally it resulted in total number of 163 samples, of which 3 samples consisted of Hyalomma ticks (1 sample with an adult tick and 2 samples with nymphs) and 160 samples of Ixodes ticks (61 samples with adult ticks, 66 samples which nymph ticks, 33 samples with larvae). The overview of the pools is shown in Appendix C.

All the samples, consisting of these pooled ticks, were tested with RLB after PCR for different species of *Babesia, Theileria, Borrelia* and *Rickettsia*. As mentioned earlier, samples must be tested twice positive with RLB to consider is as a positive. All samples tested positive once were tested once again in order to confirm. All these, exactly 65, samples are shown in the table in figure 7. The samples found positive in this extra testing, are colored green and thus these samples are confirmed positive.

Figure 7: Once tested positive samples, tested once extra. Green lines indicates that this sample is tested positive in the extra test. Underlined pathogens means that the sample is tested positive for that pathogen (only when two of more pathogens were a Possibility to test positive)

Sample No	Content	Pathogen
8	1 Adult- Ixodes ricinus	*B. garinii*
20	1 Adult- Ixodes ricinus	*B. garinii*
21	2 Adults- Ixodes ricinus	*B. garinii*
23 (was also tested positive for *B. garinii)*	4 Adults- Ixodes ricinus	Babesia catch-all
33	2 Larvae- Ixodes ricinus	*B. burgdorferi sensu lato*
35	3 Larvae- Ixodes ricinus	*T. parva*
41	1 Adult- Ixodes ricinus	*B. venatorum*
43	8 Nymphs- Ixodes ricinus	*B. afzelii*
44	1 Adult- Ixodes ricinus	*B. afzelii*
46	8 Nymphs- Ixodes ricinus	*B. burgdorferi sensu lato*
47	2 Adults- Ixodes ricinus	*B. afzelii*
51	7 Nymphs- Ixodes ricinus	*B. afzelii*
53 (was also tested positive for *B. venatorum)*	1 Nymph- Ixodes ricinus	*B. burgdorferi sensu lato*
55	1 Adult- Ixodes ricinus	*B. burgdorferi sensu lato*
57	6 Nymphs- Ixodes ricinus	*B. venatorum*
58	2 Adults- Ixodes ricinus	*B. venatorum* and *B. afzelii*
63	3 Nymphs- Ixodes ricinus	*B. garinii*
65	2 Nymphs- Ixodes ricinus	Theileria catch-all and Babesia catch-all
66	2 Nymphs- Ixodes ricinus	Theileria catch-all
68	4 Larvae- Ixodes ricinus	Theileria catch-all
69	1 Larvae- Ixodes ricinus	*Theileria equi-like* and *B. garinii*
77	1 Nymph- Ixodes ricinus	*B. burgdorferi sensu lato*
80 (was also tested positive for *B. venatorum)*	9 Nymphs- Ixodes ricinus	*B. afzelli*
81	3 Larvae- Ixodes ricinus	*B. venatorum*
83	1 Nymph- Ixodes ricinus	Theileria catch-all
84	1 Adult- Ixodes ricinus	*T. annulata*
85	1 Nymph- Ixodes ricinus	*B. rossi*
89	5 Adults- Ixodes ricinus	*B. garinii*
90	5 Adults- Ixodes ricinus	*B. garinii* and Theileria catch-all
92	1 Larvae- Ixodes ricinus	*B. garinii* and Theileria catch-all
93	14 Nymphs- Ixodes ricinus	*B. venatorum* and Theileria catch-all
96	1 Adult- Ixodes ricinus	Theileria catch-all
98	5 Adults- Ixodes ricinus	*B. venatorum*
99	9 Nymphs- Ixodes ricinus	Theileria catch-all
100	1 Larvae- Ixodes ricinus	*B. garinii*
101	8 Adults- Ixodes ricinus	*B. garinii*
104	1 Adult- Ixodes ricinus	Babesia catch-all
105	1 Adult- Ixodes ricinus	Babesia catch-all, Theileria catch-all and *B. afzelii*
109	1 Nymph- Ixodes ricinus	Theileria catch-all, Babesia catch-all and *B. rossi*
110	8 Nymphs- Ixodes ricinus	Babesia catch-all
113 (was also tested positive for *B. garinii)*	1 Adult- Ixodes ricinus	Theileria/Babesia catch-all
115 (was also tested positive for *B. venatorum)*	14 Nymphs- Ixodes ricinus	*B. burgdorferi sensu lato*
116	15 Nymphs- Ixodes ricinus	Babesia catch-all
117	1 Larvae- Ixodes ricinus	*B. rossi*
120	3 Larvae- Ixodes ricinus	*B. venatorum*
121	1 Adult- Ixodes ricinus	*B. burgdorferi sensu lato*
123	2 Adults- Ixodes ricinus	Theileria catch-all
126	3 Nymphs- Ixodes ricinus	Theileria catch-all
131	14 Nymphs- Ixodes ricinus	*B. garinii*
132	7 Larvae- Ixodes ricinus	*B. garinii*
134	12 Nymphs- Ixodes ricinus	*B. venatorum*
136	1 Adult- Ixodes ricinus	Theileria catch-all
137	1 Nymph- Ixodes ricinus	Theileria catch-all
138	3 Larvae- Ixodes ricinus	Theileria catch-all
140	1 Nymph- Ixodes ricinus	Theileria catch-all
141	1 Nymph- Ixodes ricinus	Theileria catch-all
142	5 Adults- Ixodes ricinus	*B. garinii*
143	5 Nymphs- Ixodes ricinus	*B. garinii*
146	1 Nymph- Ixodes ricinus	Theileria catch-all
149	1 Nymph- Ixodes ricinus	Theileria catch-all and *B. garinii*
151	5 Nymphs- Ixodes ricinus	*B. garinii*
155	5 Nymphs- Ixodes ricinus	*B. rossi*
156	1 Adult- Ixodes ricinus	Theileria catch-all

158	3 Adults- Ixodes ricinus	*B. garinii*
163	1 Adult- Hyalomma scupense	*B. major* and *B. bigemia*

Finally, 34 out of 163 samples were tested positive twice and thus 129 samples are considered to be negative. An overview of these positive samples is given in figure 8 below. As the hyalomma ticks are not mentioned in table 8, they were all found to be negative.

Figure 8: Positive samples

Sample No	Content	Pathogen
21	2 Adults- Ixodes ricinus	*B. garinii*
23	1 Larvae- Ixodes ricinus	*B. garinii*
32	3 Adults- Ixodes ricinus	*B. garinii*
33	2 Larvae- Ixodes ricinus	*B. burgdorferi sensu lato*
41	1 Adult- Ixodes ricinus	*B. venatorum*
43	8 Nymphs- Ixodes ricinus	*B. afzelii*
44	1 Adult- Ixodes ricinus	*B. afzelii*
51	7 Nymphs- Ixodes ricinus	*B. afzelii*
53	1 Nymph- Ixodes ricinus	*B. venatorum* and *B. burgdorferi sensu lato*
55	1 Adult- Ixodes ricinus	*B. burgdorferi sensu lato*
63	3 Nymphs- Ixodes ricinus	*B. garinii*
76	1 Adult- Ixodes ricinus	Babesia catch-all
80	9 Nymphs- Ixodes ricinus	*B. venatorum*
90	5 Adults- Ixodes ricinus	*B. garinii*
92	1 Larvae- Ixodes ricinus	*B. garinii*
95	1 Adult- Ixodes ricinus	*B. garinii*
100	1 Larvae- Ixodes ricinus	*B. garinii*
101	8 Adults- Ixodes ricinus	*B. garinii*
105	1 Adult- Ixodes ricinus	*B. afzelii*
113	1 Adult- Ixodes ricinus	*B. garinii*
115	14 Nymphs- Ixodes ricinus	*B. venatorum* and *B. burgdorferi sensu lato*
121	1 Adult- Ixodes ricinus	*B. burgdorferi sensu lato*
131	14 Nymphs- Ixodes ricinus	*B. garinii*
132	7 Larvae- Ixodes ricinus	*B. garinii*
137	1 Nymph- Ixodes ricinus	*B. afzelii*
140	1 Nymph- Ixodes ricinus	*B. garinii*
144	14 Larvae- Ixodes ricinus	*B. garinii*
149	1 Nymph- Ixodes ricinus	*B. garinii*
150	1 Adult – Ixodes ricinus	*B. garinii*
156	1 Adult- Ixodes ricinus	*B. garinii*
157	1 Larvae- Ixodes ricinus	*B. garinii*
158	3 Adult- Ixodes ricinus	*B. garinii*
159	3 Adult- Ixodes ricinus	*B. garinii*
160	2 Nymphs- Ixodes ricinus	*B. garinii*

The total number of positive samples, only consist of *Ixodes ricinus* ticks. These samples can be classified into groups, based on the found pathogen. These groups can be further divided into 3 groups based on the stage of the tick (adult, nymph or larvae). The groups are shown in figure 9, in which the total number of every pathogen is given.

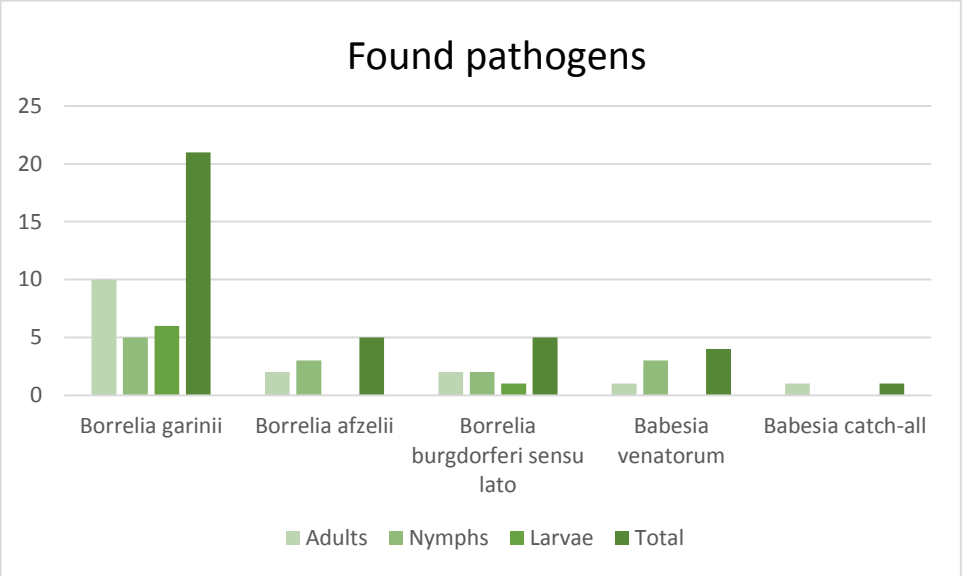

Figure 9; Found pathogens classified in groups based on the tick's stage

The total of 34 positives samples were also distributed over 16 adult ticks, 13 nymphs and 6 larvae. The total number of Ixodes tick samples, as mentioned before, consist of 61 samples of adult ticks, 66 samples of nymphs and 33 of larvae. This means that 26% of the adult ticks was positive for one of the pathogens, whereas 20% of the nymphs and 18% of the larvae was.

Of those 34 positive samples, 21 were positive for *B. garinii*, 5 for *B. afzelii,* 5 for *B. burgdorferi sensu lato*, 4 for *Bab. venatorum (EU1)* and 1 sample is found positive on the Babesia catch-all. *B. garinii* and *B. burgdorferi sensu lato* are found in both adults, nymphs and larvae. *B. afzelii* and *Bab. venatorum* are only found in adults and nymphs and finally, the Babesia catch-all is only found in 1 adult tick.

Discussion

Tick collection

Bert Winters is the only person who submitted ticks to the lab and thus all ticks used for this study are collected from birds which are captured at one of his two locations (Wieringerwerf and Hippolytushoef). To investigate the role of birds in the disseminating of ticks and tick-borne pathogens and thus get a better overview of this role representing the Netherlands, it is necessary to collect ticks of birds from more places around the Netherlands.

In this study, ticks collected in April, May, June, July and August were used. As mentioned earlier, April and May are combined as of both months only some samples are used. In August, only samples of the first half part of this month are used. In both June and July, a lot of samples is used scattered around the whole month, and thus these months give the most reliable results.

As the nets were located in the bushes and trees, both birds with and without a ring were captured. Only the birds without a ring are taken into the car to ring them and thus only those birds are checked for the presence of ticks. This finally results in collected ticks from only the new ringed birds, while the ringed birds may also carry ticks. In order to arrive at better results in succeeding research, ticks should be collected from all birds captured.

The ticks were collected from birds using forceps, which is never cleaned in between. With the removal of ticks, squeezing a tick is not unusual and thus tick DNA is sticked at these forceps. Contamination of ticks with other tick DNA is thus possible due to the use of this forceps. Nevertheless, ticks are washed in an ultrasonic water bath before DNA extraction, but it cannot be excluded if the tick DNA is not completely removed with this washing step. And thus, the use of these forceps may potentially lead to false-positive results.

Reverse Line Blotting(RLB)

Initially, the ticks were tested on *Anaplasma/Ehrlichia, Babesia/Theileria* and *Borrelia/Rickettsia* by PCR and RLB. But unfortunately it turns out to test only *Babesia/Theileria* and *Borrelia/Rickettsia*, due to contamination of the laboratories with an *Anaplasma/Ehrlichia*-positive RLB-control. This RLB-control consist of plasmids of *Anaplasma centrale, Anaplasma marginale, Ehrlichia* canis and *Ehrlichia ruminantium.* Due to the contamination with these plasmids, almost every DNA samples turns out to be positive on all these pathogens. Secondly, positives of *Anaplasma/Ehrlichia* can cause cross-reaction with *Rickettsia,* which causes false-positives for *Rickettsia.* After several total clean-ups of the lab with a natriumchloride-solution, the contamination was not eliminated. Finally, the decision was made to stop testing for *Anaplasma/Ehrlichia* in this study, to provide at least reliable results of the presence of *Babesia, Theileria, Borrelia* and *Rickettsia.*

While testing, it became clear that every RLB-batch of the same samples turned out completely different. Samples which were found to be positive for a pathogen on the first batch, turned out to be negative for that pathogen on the next batch. Perhaps, this could be a result of testing with pooled ticks, which are often small and thus contain only a little DNA. DNA extraction results in 100 microliter containing the DNA of the pooled ticks. To perform a PCR, 2,5 microliter of the DNA is used. Logically, when the total 100 microliter contains only a little DNA, there is a possibility that in the used 2,5 microliter is no or just a part of the total DNA. Which leads to false-negative results.

Unfortunately, the positive PCR controls were not always present what could be an indication for an unsuccessful PCR. But, some batches showed positive samples for the pathogens of which the PCR control was not present. This means the PCR was (partly) successful, otherwise the samples would all be negative too.

The poor reproductivity of the batches, as mentioned in above, made it difficult to make an informed decision about the positivity of the samples. Finally, the decision was made to consider the presence of positive samples on a batch without the presence of the positive PCR control as positive. All twice positive tested samples are considered positive and samples tested only once positive are tested again. When these samples turn out to be positive again, they are considered to be positive too.

Due to the contamination in the lab and the absence of positive PCR controls on the RLB batches, different numbers of batches are made of every set of samples. Ideally, every set has at least 2 batches with positive controls to ensure the positivity or negativity. This has succeeded for every set of samples, except for the third. Of the third not any batch had the full presence of all PCR controls. With the decision to consider positives on a batch without controls as positive samples and in order to limit time needed, the lack of a good batch of the third set of samples was accepted.

One of the two last RLB batches, which was made in order to make a distinction between real positive and negative samples, had no positive PCR control for babesia. Since no time was left to test these samples over, this lack of control was accepted.

Pathogens found

In order to better understand the pathogens found, is it necessary to obtain some knowledge about the different pathogens and their topographic prevalence.

- *B. burgdorferi sensu lato* is the collective name of a Borrelia genospecies complex. Thus, the positivity of samples for *B. burgdorferi sensu lato* means that the sample is positive for one of these genospieces. As the 5 positive tested samples were not found to be positive for both *B. garinii, B. burgdorferi sensu stricto* or *B. afzelii*, these samples were positive for one of the other Borrelia genospecies of the complex (as *B. valaisiana* or *B. lusitaniae*) (16).
- *Borrelia garinii* and *Borrelia afzelii* are spirochetes of the B. burgdorferi sensu lato-complex, as at least 3 other Borrelia-species are in Europe (16). *B. garinii* is, of all the species of this

complex, the most found species in Europe. *B. afzelii* is also a common species in Europe, but in slightly smaller numbers than *B. garinii* (17). The principal vector of these species is *Ixodes ricinus* and infection can cause lyme borreliosis, what is the most tick-borne disease in the northern hemisphere. These two species of the complex are found to be pathogenic to humans too (16).

- *Babesia venatorum* was first characterized in 2003 and has been recovered in *I. ricinus* from roe deer, which are the main host of this species, in Slovenia and France. It becomes a more common pathogen in Europe and has also been discovered in domestic animals in the Netherlands (18). This babesia species is a zoonotic one and thus can cause human babesiosis (19)
- The positive babesia catch-all means the sample is positive for a babesia species, other than tested for with the RLB. As we do not know which species it is, it is not possible to determine where this pathogen originates.

Non-exotic ticks can carry exotic pathogens when they are transported from other countries where those exotic pathogens prevalence. On the other hand, non-exotic pathogens can be carried from the Netherlands to other countries where this pathogen is considered to be exotic. In order to better understand the dissemination of these found tick-borne pathogens, it is necessary to link the found pathogens with the birds where the ticks are collected from.

The samples which were positive for *B. garinii* are collected from different birds. Of these samples, 8 out of 21 were collected from 6 Song Trushes, 7 samples from 4 Blackbirds, 3 samples of an Eurasian Blackap 1 sample of a Lesser Whitethroat and finally two of these samples were a non-attached sample and one of an human. The 5 *B. afzelii* positive samples were collected from 3 Common Whitethroats, a Dunnock and a Greenfinch. The samples positive for *B. burgdorferi sensu lato* are collected from a Song Trush, 2 Common Whitethroats, a Dunnock and a Blackbird. Samples positive for *Bab. venatorum* are collected from a Eurasian Blackcap, a Common Whitethroat, and 2 Dunnocks. The Babesia catch-all is from a Marsh Warbler. An overview of the pathogens linked with the birds in showed in figure 10.

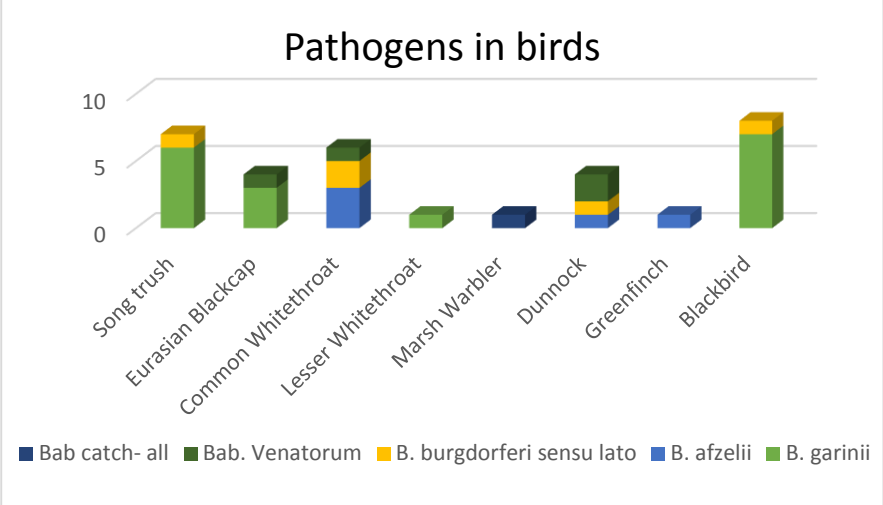

Figure 10; overview of pathogens presence in the ticks per bird species

The first remarkable fact is that no pathogens are found in *Daws, Garden Warbler, European Robins, Eurasian Wrens, Nightingales* and *Short-toed treecreepers.*

The *Song Trush, Blackbird, Greenfinch* and *Dunnock* stay, as mentioned earlier, in Europe in winter. These bird species carry only 'European pathogens' and as they never leave Europe, it is plausible to say that these bird species would not bring exotic pathogens to the Netherlands or introduce Netherland's ticks in new regions.

The *Eurasian Blackcap, Common Whitethroat* and *Lesser Whitethroat* migrate in winter to the West-part of Africa. Only *Bab. venatorum* and Borrelia-species are found in ticks on these bird species. These birds have the possibility to bring exotic pathogens to Europe in their travel back from their wintering site, but no exotic pathogen wass found. The possibility to introduce European ticks in new areas exist.

The *Marsh Warbler* travels in winter to the exotic South-part of Africa. A *Ixodes ricinus* tick of this bird species was found positive for the Babesia catch-all, which could mean that this bird had brought an exotic Babesia species to the Netherlands in the travel back home from Africa. It is also possible that this bird species bring Dutch's ticks and pathogens to new areas in Africa.

It is striking that, on the *Marsh Warbler*, both Hyalomma ticks and a tick with the Babesia catch-all has been found. Only two samples of this bird species are found in this study and both samples consist interesting material as the exotic Hyalomma ticks and the possible exotic babesia species in an Ixodes tick.

Conclusion

Birds play an important role in the disseminating of ticks and tick-borne pathogens, especially the non-exotics. But nevertheless, exotic ticks and possible exotic pathogens are also found to be disseminated by birds, especially by the *Marsh Warbler.*

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Appendix A: Tick identification

Date of collection	Number on the collection tube	Bird spieces	serial number	Genus	Species	Stage	Sex
17/04/2015	182	Daw	0	Ixodes	Ricinus	Adult	Female
31/05/2015	183	Blackbird	1	Ixodes	ricinus	Nymph	
			2	Ixodes	ricinus	Nymph	
			3	Ixodes	ricinus	Nymph	
			4	Ixodes	ricinus	Nymph	
			5	Ixodes	ricinus	Nymph	
			6	Ixodes	ricinus	Nymph	
			7	Ixodes	ricinus	Nymph	
			8	Ixodes	ricinus	Nymph	
			9	Ixodes	ricinus	Nymph	
			10	Ixodes	ricinus	Nymph	
			11	Ixodes	ricinus	Nymph	
			12	Ixodes	ricinus	Nymph	
			13	Ixodes	ricinus	Nymph	
	184	Song Trush	14	Ixodes	ricinus	Nymph	
			15	Ixodes	ricinus	Nymph	
			16	Ixodes	ricinus	Nymph	
			17	Ixodes	ricinus	Nymph	
			18	Ixodes	ricinus	Nymph	
			19	Ixodes	ricinus	Nymph	
			20	Ixodes	ricinus	Nymph	
			21	Ixodes	ricinus	Nymph	
			22	Ixodes	ricinus	Nymph	
			23	Ixodes	ricinus	Nymph	
			24	Ixodes	ricinus	Nymph	

			25	Ixodes	ricinus	Nymph	
	185	Blackbird	26	Ixodes	ricinus	Adult	Female
			27	Ixodes	ricinus	Nymph	
			28	Ixodes	ricinus	Adult	Female
			29	Ixodes	ricinus	Adult	Female
06-06-2015	186	Blackbird	30	Ixodes	ricinus	Nymph	
			31	Ixodes	ricinus	Nymph	
			32	Ixodes	ricinus	Nymph	
			33	Ixodes	ricinus	Larvae	
			34	Ixodes	ricinus	Nymph	
			35	Ixodes	ricinus	Larvae	
			36	Ixodes	ricinus	Nymph	
			37	Ixodes	ricinus	Nymph	
			38	Ixodes	ricinus	Larvae	
			39	Ixodes	ricinus	Nymph	
			40	Ixodes	ricinus	Larvae	
			41	Ixodes	ricinus	Adult	female
			42	Ixodes	ricinus	Nymph	
			43	Ixodes	ricinus	Nymph	
			44	Ixodes	ricinus	Larvae	
			45	Ixodes	ricinus	Larvae	
			46	Ixodes	ricinus	Larvae	
			47	Ixodes	ricinus	Larvae	
			49	Ixodes	ricinus	Larvae	
			50	Ixodes	ricinus	Larvae	
	187	Blackbird	51	Ixodes	ricinus	Nymph	
			52	Ixodes	ricinus	Nymph	
	188	Marsh Warbler	53	Hyalomma	scupense	Adult	Female
			54	Hyalomma		Nymph	

			55	Hyalomma		Nymph	
08-06-2015	189	Dunnock	56	Ixodes	ricinus	Nymph	
			57	Ixodes	ricinus	Nymph	
			58	Ixodes	ricinus	Nymph	
			59	Ixodes	ricinus	Nymph	
			60	Ixodes	ricinus	Nymph	
	190	Dunnock	61	Ixodes	ricinus	Nymph	
			62	Ixodes	ricinus	Nymph	
			63	Ixodes	ricinus	Nymph	
			64	Ixodes	ricinus	Nymph	
			65	Ixodes	ricinus	Nymph	
			66	Ixodes	ricinus	Nymph	
			67	Ixodes	ricinus	Nymph	
	191	Song Trush	68	Ixodes	ricinus	Larvae	
			69	Ixodes	ricinus	Nymph	
			70	Ixodes	ricinus	Nymph	
			71	Ixodes	ricinus	Nymph	
			72	Ixodes	ricinus	Nymph	
			73	Ixodes	ricinus	Nymph	
			74	Ixodes	ricinus	Nymph	
			75	Ixodes	ricinus	Larvae	
			76	Ixodes	ricinus	Larvae	
			77	Ixodes	ricinus	Larvae	
	192	Blackbird	78	Ixodes	ricinus	Nymph	
			79	Ixodes	ricinus	Nymph	
			80	Ixodes	ricinus	Nymph	
			81	Ixodes	ricinus	Nymph	
			82	Ixodes	ricinus	Adult	Female
			83	Ixodes	ricinus	Nymph	
			84	Ixodes	ricinus	Nymph	

			85	Ixodes	ricinus	Adult	Female
			86	Ixodes	ricinus	Nymph	
			87	Ixodes	ricinus	Nymph	
			88	Ixodes	ricinus	Nymph	
			89	Ixodes	ricinus	Nymph	
			90	Ixodes	ricinus	Nymph	
			91	Ixodes	ricinus	Adult	Female
			92	Ixodes	ricinus	Adult	Female
			93	Ixodes	ricinus	Nymph	
	193	Song Trush	94	Ixodes	ricinus	Nymph	
	194	Human	95	Ixodes	ricinus	Adult	Male
13-06-2015	195	European Robin	96	Ixodes	ricinus	Adult	Female
			97	Ixodes	ricinus	Larvae	
	196	Eurasian Blackcap	98	Ixodes	ricinus	Adult	Female
	197	Song Trush	99	Ixodes	ricinus	Adult	Female
			100	Ixodes	ricinus	Adult	Female
			101	Ixodes	ricinus	Nymph	
			102	Ixodes	ricinus	Nymph	
			103	Ixodes	ricinus	Larvae	
	198	Song Trush	104	Ixodes	ricinus	Nymph	
			105	Ixodes	ricinus	Adult	Female
			106	Ixodes	ricinus	Adult	Female
			107	Ixodes	ricinus	Adult	Female
			108	Ixodes	ricinus	Nymph	
			109	Ixodes	ricinus	Nymph	
			110	Ixodes	ricinus	Nymph	
			111	Ixodes	ricinus	Nymph	
			112	Ixodes	ricinus	Nymph	
			113	Ixodes	ricinus	Adult	Female

			114	Ixodes	ricinus	Adult	Female
			115	Ixodes	ricinus	Adult	Female
			116	Ixodes	ricinus	Nymph	
			117	Ixodes	ricinus	Adult	Female
			118	Ixodes	ricinus	Nymph	
			119	Ixodes	ricinus	Nymph	
			120	Ixodes	ricinus	Nymph	
			121	Ixodes	ricinus	Nymph	
			122	Ixodes	ricinus	Nymph	
			123	Ixodes	ricinus	Nymph	
14-06-2015	199	Eurasian Wren	124	Ixodes	ricinus	Nymph	
			125	Ixodes	ricinus	Nymph	
	200	Blackbird	126	Ixodes	ricinus	Nymph	
15-06-2015	201	Song Trush	127	Ixodes	ricinus	Nymph	
			128	Ixodes	ricinus	Nymph	
			129	Ixodes	ricinus	Nymph	
			130	Ixodes	ricinus	Nymph	
			131	Ixodes	ricinus	Adult	Female
			132	Ixodes	ricinus	Adult	Female
			133	Ixodes	ricinus	Adult	Female
			134	Ixodes	ricinus	Nymph	
			135	Ixodes	ricinus	Nymph	
			136	Ixodes	ricinus	Nymph	
			137	Ixodes	ricinus	Nymph	
			138	Ixodes	ricinus	Nymph	
			139	Ixodes	ricinus	Nymph	
			140	Ixodes	ricinus	Nymph	
			141	Ixodes	ricinus	Nymph	
			142	Ixodes	ricinus	Adult	Female
			143	Ixodes	ricinus	Nymph	

			144	Ixodes	ricinus	Adult	Female
			145	Ixodes	ricinus	Nymph	
			146	Ixodes	ricinus	Adult	Female
			147	Ixodes	ricinus	Adult	Female
			148	Ixodes	ricinus	Nymph	
			149	Ixodes	ricinus	Nymph	
			150	Ixodes	ricinus	Nymph	
			151	Ixodes	ricinus	Nymph	
			152	Ixodes	ricinus	Nymph	
			153	Ixodes	ricinus	Nymph	
			154	Ixodes	ricinus	Nymph	
			155	Ixodes	ricinus	Nymph	
			156	Ixodes	ricinus	Nymph	
			157	Ixodes	ricinus	Larvae	
			158	Ixodes	rcinus	Larvae	
			159	Ixodes	ricinus	Nymph	
			160	Ixodes	ricinus	Nymph	
	202	Dunnock	161	Ixodes	ricinus	Nymph	
			162	Ixodes	ricinus	Nymph	
			163	Ixodes	ricinus	Larvae	
			164	Ixodes	ricinus	Nymph	
			165	Ixodes	ricinus	Nymph	
			166	Ixodes	ricinus	Larvae	
			167	Ixodes	ricnus	Larvae	
			168	Ixodes	ricinus	Nymoh	
			169	Ixodes	ricinus	Nymph	
	203	Dunnock	170	Ixodes	ricinus	Nymph	
			171	Ixodes	ricinus	Adult	Female
			172	Ixodes	ricinus	Nymph	
			173	Ixodes	ricinus	Nymph	

			174	Ixodes	ricinus	Larvae	
			175	Ixodes	ricinus	Nymph	
			176	Ixodes	ricinus	Nymph	
			177	Ixodes	ricinus	Larvae	
			178	Ixodes	ricinus	Nymph	
	204	Eurasian Blackcap	179	Ixodes	ricinus	Adult	Female
	205	Eurasian Blackcap	180	Ixodes	ricinus	Nymph	
			181	Ixodes	ricinus	Nymph	
			182	Ixodes	ricinus	Nymph	
			183	Ixodes	ricinus	Nymph	
			184	Ixodes	ricinus	Nymph	
			185	Ixodes	ricinus	Nymph	
			186	Ixodes	ricinus	Nymph	
			187	Ixodes	ricinus	Nymph	
			188	Ixodes	ricinus	Nymph	
			189	Ixodes	ricinus	Nymph	
			190	Ixodes	ricinus	Nymph	
			191	Ixodes	ricinus	Nymph	
			192	Ixodes	ricinus	Nymph	
			193	Ixodes	ricinus	Nymph	
			194	Ixodes	ricinus	Adult	Female
	207	Human	195	Ixodes	ricinus	Adult	Female
20-06-2015	208	Common whitethroat	196	Ixodes	ricinus	Nymph	
			197	Ixodes	ricinus	Nymph	
			198	Ixodes	ricinus	Nymph	
			199	Ixodes	ricinus	Nymph	
			200	Ixodes	ricinus	Nymph	
			201	Ixodes	ricinus	Nymph	
			202	Ixodes	ricinus	Nymph	

			203	Ixodes	ricinus	Nymph	
	209	Common whitethroat	204	Ixodes	ricinus	Adult	Female
	210	European Robin	205	Ixodes	ricinus	Adult	Female
			206	Ixodes	ricinus	Nymph	
			207	Ixodes	ricinus	Nymph	
			208	Ixodes	ricinus	Nymph	
			209	Ixodes	ricinus	Nymph	
			210	Ixodes	ricinus	Nymph	
			211	Ixodes	ricinus	Nymph	
			212	Ixodes	ricinus	Nymph	
			213	Ixodes	ricinus	Nymph	
	211	Common whitethroat	214	Ixodes	ricinus	Adult	Female
			215	Ixodes	ricinus	Adult	Female
			216	Ixodes	ricinus	Larvae	
			217	Ixodes	ricinus	Nymph	
			218	Ixodes	ricinus	Nymph	
			219	Ixodes	ricinus	Nymph	
	212	Lesser Whitethroat	220	Ixodes	ricinus	Nymph	
			221	Ixodes	ricinus	Nymph	
			222	Ixodes	ricinus	Nymph	
	213	Common Whitethroat	223	Ixodes	ricinus	Nymph	
			224	Ixodes	ricinus	Nymph	
			225	Ixodes	ricinus	Nymph	
			226	Ixodes	ricinus	Nymph	
			227	Ixodes	ricinus	Nymph	
			228	Ixodes	ricinus	Adult	Female
			229	Ixodes	ricinus	Nymph	
			230	Ixodes	ricinus	Nymph	
			231	Ixodes	ricinus	Nymph	

	214	Common Whitethroat	232	Ixodes	ricinus	Nymph	
			233	Ixodes	ricinus	Adult	Female
			234	Ixodes	ricinus	Adult	Female
			235	Ixodes	ricinus	Adult	Female
	215	Common Whitethroat	236	Ixodes	ricinus	Adult	Female
			237	Ixodes	ricinus	Nymph	
			238	Ixodes	ricinus	Nymph	
			239	Ixodes	ricinus	Nymph	
	216	Common Whitethroat	240	Ixodes	ricinus	Nymph	
			241	Ixodes	ricinus	Adult	Female
			242	Ixodes	ricinus	Adult	Female
			243	Ixodes	ricinus	Nymh	
			244	Ixodes	ricinus	Nymph	
			245	Ixodes	ricinus	Larvae	
			246	Ixodes	ricinus	Larvae	
			247	Ixodes	ricinus	Nymph	
			248	Ixodes	ricinus	Larvae	
			249	Ixodes	ricinus	Nymph	
			250	Ixodes	ricinus	Nymph	
	217	Common Whitethroat	251	Ixodes	ricinus	Nymph	
			252	Ixodes	ricinus	Adult	Female
			253	Ixodes	ricinus	Adult	Female
			254	Ixodes	ricinus	Adult	Female
			255	Ixodes	ricinus	Adult	Female
			256	Ixodes	ricinus	Adult	Female
			257	Ixodes	ricinus	Larvae	
			258	Ixodes	ricinus	Adult	Female
	218	Lesser Whitethroat	259	Ixodes	ricinus	Nymph	
			260	Ixodes	ricinus	Nymh	

			261	Ixodes	ricinus	Nymph	
21-06-2015	219	Eurasian Blackcap	262	Ixodes	ricinus	Adult	Female
	220	Dunnock	263	Ixodes	ricinus	Nymph	
			264	Ixodes	ricinus	Nymph	
	221	Dunnock	265	Ixodes	ricinus	Nymph	
			266	Ixodes	ricinus	Nymph	
	222	European Robin	267	Ixodes	ricinus	Larvae	
			268	Ixodes	ricinus	Larvae	
			269	Ixodes	ricinus	Larvae	
			270	Ixodes	ricinus	Larvae	
			271	Ixodes	ricinus	Larvae	
			272	Ixodes	ricinus	Larvae	
			273	Ixodes	ricinus	Larvae	
			274	Ixodes	ricinus	Larvae	
			275	Ixodes	ricinus	Larvae	
	223	European Robin	276	Ixodes	ricinus	Larvae	
			277	Ixodes	ricinus	Larvae	
			278	Ixodes	ricinus	Larvae	
			279	Ixodes	ricinus	Larvae	
	224	Eurasian Blackcap	280	Ixodes	ricinus	Larvae	
			281	Ixodes	ricinus	Nymph	
			282	Ixodes	ricinus	Nymph	
	225	European Robin	283	Ixodes	ricinus	Adult	Female
	226	European Robin	284	Ixodes	ricinus	Larvae	
			285	Ixodes	ricinus	Adult	Female
	227	European Robin	286	Ixodes	ricinus	Nymph	
			287	Ixodes	ricinus	Nymph	
	228	European Robin	288	Ixodes	ricinus	Larvae	

			289	Ixodes	ricinus	Larvae	
			290	Ixodes	ricinus	Adult	Female
	229	Non-attached tick	291	Ixodes	ricinus	Nymph	
24-06-2015	230	Marsh Warbler	292	Ixodes	ricinus	Adult	Female
	231	Garden Warbler	-geen teek erin				
	232	Nightingale	293	Ixodes	ricinus	Adult	Female
	233	Dunnock	294	Ixodes	ricinus	Nymph	
			295	Ixodes	ricinus	Nymph	
			296	Ixodes	ricinus	Nymph	
			297	Ixodes	ricinus	Nymph	
			298	Ixodes	ricinus	Nymph	
			299	Ixodes	ricinus	Nymph	
			300	Ixodes	ricinus	Nymph	
			301	Ixodes	ricinus	Nymph	
			302	Ixodes	ricinus	Larvae	
			303	Ixodes	ricinus	Nymph	
			304	Ixodes	ricinus	Larvae	
			305	Ixodes	ricinus	Larvae	
	234	Non-attached tick	306	Ixodes	ricinus	Adult	Female
	235	Dunnock	307	Ixodes	ricinus	Nymph	
	236	European Robin	308	Ixodes	ricinus	Adult	Female
			309	Ixodes	ricinus	Nymph	
	237	European Robin	310	Ixodes	ricinus	Larvae	
			311	Ixodes	ricinus	Larvae	
			312	Ixodes	ricinus	Larvae	
			313	Ixodes	ricinus	Larvae	
			314	Ixodes	ricinus	Larvae	

			315	Ixodes	ricinus	Larvae	
	238	Eurasian Blackcap	316	Ixodes	ricinus	Adult	Female
	239	Song trush	317	Ixodes	ricinus	Adult	Female
			318	Ixodes	ricinus	Nymph	
			319	Ixodes	ricinus	Adult	Female
			320	Ixodes	ricinus	Adult	Female
			321	Ixodes	ricinus	Adult	Female
			322	Ixodes	ricinus	Adult	Female
			323	Ixodes	ricinus	Adult	Female
			324	Ixodes	ricinus	Nymph	
			325	Ixodes	ricinus	Adult	Female
			326	Ixodes	ricinus	Adult	Female
			327	Ixodes	ricinus	Adult	Female
			328	Ixodes	ricinus	Adult	Female
			329	Ixodes	ricinus	Adult	Female
			330	Ixodes	ricinus	Adult	Female
			331	Ixodes	ricinus	Adult	Female
			332	Ixodes	ricinus	Nymph	
			333	Ixodes	ricinus	Nymph	
			334	Ixodes	ricinus	Adult	Female
			335	Ixodes	ricinus	Adult	Female
			336	Ixodes	ricinus	Nymph	
			337	Ixodes	ricinus	Nymph	
			338	Ixodes	ricinus	Nymph	
			339	Ixodes	ricinus	Nymph	
			340	Ixodes	ricinus	Nymph	
			341	Ixodes	ricinus	Nymph	
			342	Ixodes	ricinus	Nymph	
			343	Ixodes	ricinus	Larvae	
			344	Ixodes	ricinus	Nymph	

	240	Dunnock	345	Ixodes	ricinus	Nymph	
			346	Ixodes	ricinus	Nymph	
			347	Ixodes	ricinus	Nymph	
			348	Ixodes	ricinus	Nymph	
			349	Ixodes	ricinus	Nymph	
			350	Ixodes	ricinus	Nymph	
			351	Ixodes	ricinus	Nymph	
			352	Ixodes	ricinus	Nymph	
			353	Ixodes	ricinus	Nymph	
			354	Ixodes	ricinus	Nymph	
			355	Ixodes	ricinus	Nymph	
			356	Ixodes	ricinus	Nymph	
			357	Ixodes	ricinus	Nymph	
			358	Ixodes	ricinus	Nymph	
			359	Ixodes	ricinus	Nymph	
			360	Ixodes	ricinus	Nymph	
			361	Ixodes	ricinus	Nymph	
			362	Ixodes	ricinus	Nymph	
			363	Ixodes	ricinus	Nymph	
			364	Ixodes	ricinus	Nymph	
			365	Ixodes	ricinus	Nymph	
			366	Ixodes	ricinus	Nymph	
			367	Ixodes	ricinus	Nymph	
			368	Ixodes	ricinus	Nymph	
			369	Ixodes	ricinus	Nymph	
			370	Ixodes	ricinus	Nymph	
			371	Ixodes	ricinus	Nymph	
	241	Human	372	Ixodes	ricinus	Adult	Male
26-06-2015	242	Eurasian Wren	373	Ixodes	ricinus	Adult	Female
	243	Nightingale	374	Ixodes	ricinus	Larvae	

			375	Ixodes	ricinus	Larvae	
			376	Ixodes	ricinus	Larvae	
			377	Ixodes	ricinus	Larvae	
			378	Ixodes	ricinus	Larvae	
			379	Ixodes	ricinus	Larvae	
			380	Ixodes	ricinus	Larvae	
			381	Ixodes	ricinus	Larvae	
			382	Ixodes	ricinus	Larvae	
			383	Ixodes	ricinus	Larvae	
	244	Song Trush	384	Ixodes	ricinus	Adult	Female
			385	Ixodes	ricinus	Nymph	
			386	Ixodes	ricinus	Nymph	
			387	Ixodes	ricinus	Adult	Female
			388	Ixodes	ricinus	Nymph	
			389	Ixodes	ricinus	Adult	Female
			390	Ixodes	ricinus	Nymph	
			391	Ixodes	ricinus	Nymph	
			392	Ixodes	ricinus	Nymph	
			393	Ixodes	ricinus	Nymph	
			394	Ixodes	ricinus	Adult	Female
			395	Ixodes	ricinus	Adult	Female
			396	Ixodes	ricinus	Nymh	
			397	Ixodes	ricinus	Larvae	
			398	Ixodes	ricinus	Nymph	
	245	Song Trush	399	Ixodes	ricinus	Adult	Female
			400	Ixodes	ricinus	Adult	Female
			401	Ixodes	ricinus	Nymph	
			402	Ixodes	ricinus	Nymph	
			403	Ixodes	ricinus	Nymph	
			404	Ixodes	ricinus	Nymph	

			405	Ixodes	ricinus	Adult	Female
			406	Ixodes	ricinus	Adult	Female
			407	Ixodes	ricinus	Adult	Female
			408	Ixodes	ricinus	Nymph	
			409	Ixodes	ricinus	Adult	Female
			410	Ixodes	ricinus	Nymph	
			411	Ixodes	ricinus	Adult	Female
			412	Ixodes	ricinus	Nymph	
			413	Ixodes	ricinus	Adult	Female
			414	Ixodes	ricinus	Nymph	
			415	Ixodes	ricinus	Nymph	
			416	Ixodes	ricinus	Nymph	
			417	Ixodes	ricinus	Nymph	
			418	Ixodes	ricinus	Nymph	
			419	Ixodes	ricinus	Nymph	
			420	Ixodes	ricinus	Nymph	
			421	Ixodes	ricinus	Nymph	
			422	Ixodes	ricinus	Nymph	
			423	Ixodes	ricinus	Nymph	
			424	Ixodes	ricinus	Nymph	
	246	Dunnock	425	Ixodes	ricinus	Nymph	
	247	Human	426	Ixodes	ricinus	Adult	Female
27-06-2015	248	European Greenfinch	427	Ixodes	ricinus	Adult	Female
29-06-2015	249	European Robin	428	Ixodes	ricinus	Adult	Female
			429	Ixodes	ricinus	Larvae	
03-07-2015	250	Short-toed treecreeper	430	Ixodes	ricinus	Adult	Female
	251	Eurasian Blackcap	431	Ixodes	ricinus	Nymph	
	252	Dunnock	432	Ixodes	ricinus	Nymph	
			433	Ixodes	ricinus	Nymph	

			434	Ixodes	ricinus	Nymph	
			435	Ixodes	ricinus	Nymph	
			436	Ixodes	ricinus	Nymph	
			437	Ixodes	ricinus	Nymph	
			438	Ixodes	ricinus	Larvae	
			439	Ixodes	ricinus	Nymph	
			440	Ixodes	ricinus	Nymph	
	253	Dunnock	441	Ixodes	ricinus	Nymph	
	254	Non-attached tick	442	Ixodes	ricinus	Adult	Female
06-07-2015	255	Dunnock	443	Ixodes	ricinus	Larvae	
			444	Ixodes	ricinus	Nymph	
			445	Ixodes	ricinus	Nymph	
			446	Ixodes	ricinus	Nymph	
			447	Ixodes	ricinus	Nymph	
			448	Ixodes	ricinus	Nymph	
			449	Ixodes	ricinus	Nymph	
			450	Ixodes	ricinus	Nymph	
			451	Ixodes	ricinus	Larvae	
			452	Ixodes	ricinus	Nymph	
			453	Ixodes	ricinus	Nymph	
			454	Ixodes	ricinus	Nymph	
			455	Ixodes	ricinus	Nymph	
			456	Ixodes	ricinus	Nymph	
			457	Ixodes	ricinus	Nymph	
			458	Ixodes	ricinus	Nymph	
			459	Ixodes	ricinus	Larvae	
	256	Dunnock	460	Ixodes	ricinus	Nymph	
			461	Ixodes	ricinus	Nymph	
			462	Ixodes	ricinus	Nymph	

			463	Ixodes	ricinus	Nymph	
			464	Ixodes	ricinus	Nymph	
			465	Ixodes	ricinus	Nymph	
			466	Ixodes	ricinus	Nymph	
			467	Ixodes	ricinus	Nymph	
			468	Ixodes	ricinus	Nymph	
			469	Ixodes	ricinus	Nymph	
			470	Ixodes	ricinus	Nymph	
			471	Ixodes	ricinus	Nymph	
			472	Ixodes	ricinus	Nymph	
			473	Ixodes	ricinus	Nymph	
			474	Ixodes	ricinus	Nymph	
			475	Ixodes	ricinus	Larvea	
	257	Eurasian Blackcap	476	Ixodes	ricinus	Adult	Female
	258	European Robin	477	Ixodes	ricinus	Nymph	
			478	Ixodes	ricinus	Larvae	
			479	Ixodes	ricinus	Larvae	
			480	Ixodes	ricinus	Larvae	
	259	Blackbird	481	Ixodes	ricinus	Adult	Female
	260	Non-attached tick	482	Ixodes	ricinus	Adult	Female
			483	Ixodes	ricinus	Adult	Male
	261	Eurasian Blackcap	484	Ixodes	ricinus	Adult	Female
			485	Ixodes	ricinus	Nymph	
			485a	Ixodes	ricinus	Adult	Female
	262	European Robin	486	Ixodes	ricinus	Larvae	
			489	Ixodes	ricinus	Larvae	
			490	Ixodes	ricinus	Nymph	
			491	Ixodes	ricinus	Larvae	

			492	Ixodes	ricinus	Nymph	
			493	Ixodes	ricinus	Larvae	
			494	Ixodes	ricinus	Nymph	
			495	Ixodes	ricinus	Larvae	
	263	European Robin	496	Ixodes	ricinus	Nymph	
	264	European Robin	497	Ixodes	ricinus	Adult	Female
			498	Ixodes	ricinus	Larvae	
			499	Ixodes	ricinus	Nymph	
			500	Ixodes	ricinus	Nymph	
			501	Ixodes	ricinus	Adult	Female
			502	Ixodes	ricinus	Adult	Female
	265	Blackbird	503	Ixodes	ricinus	Nymph	
			504	Ixodes	ricinus	Larvae	
			505	Ixodes	ricinus	Adult	Female
			506	Ixodes	ricinus	Nymph	
			507	Ixodes	ricinus	Adult	Female
			508	Ixodes	ricinus	Adult	Female
			509	Ixodes	ricinus	Adult	Female
			510	Ixodes	ricinus	Adult	Female
			511	Ixodes	ricinus	Adult	Female
			512	Ixodes	ricinus	Adult	Female
			513	Ixodes	ricinus	Larvae	
			514	Ixodes	ricinus	Larvae	
			515	Ixodes	ricinus	Nymph	
			516	Ixodes	ricinus	Nymph	
			517	Ixodes	ricinus	Adult	Female
			518	Ixodes	ricinus	Nymph	
			519	Ixodes	ricinus	Larvae	
			520	Ixodes	ricinus	Adult	Female

			521	Ixodes	ricinus	Nymph	
			522	Ixodes	ricinus	Nymph	
			523	Ixodes	ricinus	Larvae	
			524	Ixodes	ricinus	Nymph	
			525	Ixodes	ricinus	Nymph	
			526	Ixodes	ricinus	Larvae	
			527	Ixodes	ricinus	Nymph	
			528	Ixodes	ricinus	Nymph	
			529	Ixodes	ricinus	Larvae	
			530	Ixodes	ricinus	Nymph	
			531	Ixodes	ricinus	Nymph	
			532	Ixodes	ricinus	Nymph	
	266	Dunnock	533	Ixodes	ricinus	Nymph	
			534	Ixodes	ricinus	Nymph	
			535	Ixodes	ricinus	Nymph	
			536	Ixodes	ricinus	Adult	Female
			537	Ixodes	ricinus	Nymph	
			538	Ixodes	ricinus	Nymph	
			539	Ixodes	ricinus	Nymph	
			540	Ixodes	ricinus	Nymph	
			541	Ixodes	ricinus	Nymph	
			542	Ixodes	ricinus	Nymph	
			543	Ixodes	ricinus	Nymph	
			544	Ixodes	ricinus	Nymph	
			545	Ixodes	ricinus	Nymph	
			546	Ixodes	ricinus	Nymph	
			547	Ixodes	ricinus	Nymph	
			548	Ixodes	ricinus	Nymph	
			549	Ixodes	ricinus	Nymph	
			550	Ixodes	ricinus	Nymph	

			551	Ixodes	ricinus	Nymph	
			552	Ixodes	ricinus	Nymph	
			553	Ixodes	ricinus	Nymph	
			554	Ixodes	ricinus	Nymph	
			555	Ixodes	ricinus	Nymph	
			556	Ixodes	ricinus	Nymph	
			557	Ixodes	ricinus	Larvae	
			558	Ixodes	ricinus	Larvae	
	267	Dunnock	559	Ixodes	ricinus	Nymph	
	268	European Robin	560	Ixodes	ricinus	Larvae	
			561	Ixodes	ricinus	Larvae	
			562	Ixodes	ricinus	Larvae	
13-07-2015	269	Non-attached tick	563	Ixodes	ricinus	Adult	Female
			564	Ixodes	ricinus	Adult	Male
			565	Ixodes	ricinus	Adult	Male
			566	Ixodes	ricinus	Adult	Male
	270	Blackbird	567	Ixodes	ricinus	Nymph	
03-08-2015	271	Eurasian wren	568	Ixodes	ricinus	Nymph	
	272	Blackbird	569	Ixodes	ricinus	Adult	Female
			570	Ixodes	ricinus	Adult	Female
			571	Ixodes	ricinus	Adult	Female
			572	Ixodes	ricinus	Adult	Female
			573	Ixodes	ricinus	Nymph	
			574	Ixodes	ricinus	Larvae	
			575	Ixodes	ricinus	Adult	Female
			576	Ixodes	ricinus	Nymph	
			577	Ixodes	ricinus	Nymph	
			578	Ixodes	ricinus	Nymph	

			579	Ixodes	ricinus	Larvae	
			580	Ixodes	ricinus	Larvae	
			581	Ixodes	ricinus	Larvae	
			582	Ixodes	ricinus	Larvae	
			583	Ixodes	ricinus	Larvae	
			584	Ixodes	ricinus	Larvae	
			585	Ixodes	ricinus	Larvae	
			586	Ixodes	ricinus	Larvae	
			587	Ixodes	ricinus	Larvae	
			588	Ixodes	ricinus	Larvae	
			589	Ixodes	ricinus	Larvae	
			590	Ixodes	ricinus	Larvae	
			591	Ixodes	ricinus	Larvae	
			592	Ixodes	ricinus	Nymph	
	273	Eurasian Blackcap	593	Ixodes	ricinus	Adult	Female
			594	Ixodes	ricinus	Nymph	
05-08-2015	274	Eurasian wren	595	Ixodes	ricinus	Larvae	
	275	Eurasian Blackcap	596	Ixodes	ricinus	Adult	Female
			597	Ixodes	ricinus	Nymph	
	276	Song Trush	598	Ixodes	ricinus	Adult	Female
			599	Ixodes	ricinus	Nymph	
			600	Ixodes	ricinus	Nymph	
			601	Ixodes	ricinus	Nymph	
			602	Ixodes	ricinus	Larvae	
			603	Ixodes	ricinus	Nymph	
			604	Ixodes	ricinus	Nymph	
			605	Ixodes	ricinus	Larvae	
			606	Ixodes	ricinus	Larvae	
			607	Ixodes	ricinus	Larvae	

08-08-2015	277	European Robin	608	Ixodes	ricinus	Larvae	
			609	Ixodes	ricinus	Adult	Female
	278	Dunnock	610	Ixodes	ricinus	Nymph	
			611	Ixodes	ricinus	Nymph	
			612	Ixodes	ricinus	Nymph	
			613	Ixodes	ricinus	Nymph	
			614	Ixodes	ricinus	Nymph	
	279	Eurasian Blackcap	615	Ixodes	ricinus	Adult	Female
			616	Ixodes	ricinus	Larvae	
	280	Blackbird	617	Ixodes	ricinus	Adult	Female
			618	Ixodes	ricinus	Adult	Female
			619	Ixodes	ricinus	Adult	Female
			620	Ixodes	ricinus	Adult	Female
			621	Ixodes	ricinus	Adult	Female
			622	Ixodes	ricinus	Adult	Female
			623	Ixodes	ricinus	Nymph	
			624	Ixodes	ricinus	Nymph	

Appendix B: DNA extraction protocol

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.	

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.	

by _____ on _____ _____

Signature

Comments:

Appendix C: Pooled groups DNA extraction

Extraction	Number	Bird Number	Tick Species	Tick Stage	Number of ticks		corresponding numbers
1	1	182	I ricinus	Adult	1		0
	2	183	I ricinus	Nymph	13		1 t/m 13
	3	184	I ricinus	Nymph	12		14 t/m 25
	4	185	I ricinus	Adult	3		26,28,29
	5	185	I ricinus	Nymph	1		27
	6	186	I ricinus	Nymph	9		30,31,32,34,36, 37,39,42,43
	7	186	I ricinus	Larvae			33,35,38,40,44, 45,46,47,49,50
	8	186	I ricinus	Adult	1		41
	9	187	I ricinus	Nymph	2		51, 52
	10	189	I ricinus	Nymph	5		56 t/m 60
	11	190	I ricinus	Nymph	7		61 t/m 67
	12	191	I ricinus	Larvae	4		68, 75, 76, 77
	13	191	I ricinus	Nymph	6		69 t/m 74
	14	192	I ricinus	Nymph	12		78, 79, 80, 81, 83, 84, 86, 87, 88, 89, 90, 93
	15	192	I ricinus	Adult	4		82, 85, 91, 92
	16	193	I ricinus	Nymph	1		94
	17	194	I ricinus	Adult	1		95
	18	195	I ricinus	Adult	1		96
	19	195	I ricinus	Larvae	1		97
	20	196	I ricinus	Adult	1		98
2	21	197	I ricinus	Adult	2		99, 100
	22	197	I ricinus	Nymph	2		101, 102
	23	197	I ricinus	Larvae	1		103
	24	198	I ricinus	Nymph	13		104, 108, 109, 110, 111, 112, 116, 118, 119, 120, 121, 122, 123
	23	198	I ricinus	Adult	4		105, 106, 107, 113
	26	198	I ricins	Adult	3		114, 115, 117
	27	199	I ricinus	Nymph	2		124, 125
	28	200	I ricinus	Nymph	1		126
	29	201	I ricinus	Nymph	12		127, 128, 129, 130, 134, 135, 136, 137, 138, 139, 140, 141
	30	201	I ricinus	Nymph	13		143, 145, 148, 149, 150, 151, 152, 153, 154,

							155, 156, 159, 160
	31	201	I ricinus	Adult	4		131, 132, 133, 142
	32	201	I ricinus	Adult	3		144, 146, 147
	33	201	I ricinus	Larvae	2		157, 158
	34	202	I ricinus	Nymph	6		161, 162, 164, 165, 168, 169
	35	202	I ricinus	Larvae	3		163, 166, 167
	36	203	I ricinus	Adult	1		171
	37	203	I ricinus	Nymph	6		170, 172, 173, 175, 176, 178
	38	203	I ricinus	Larvae	2		174, 177
	39	204	I ricinus	Adult	1		179
	40	205	I ricinus	Nymph	14		180 t/m 193
3	41	205	I ricinus	Adult	1		194
	42	207	I ricinus	Adult	1		195
	43	208	I ricinus	Nymph	8		196 t/m 203
	44	209	I ricinus	Adult	1		204
	45	210	I ricinus	Adult	1		205
	46	210	I ricinus	Nymph	8		206 t/m 213
	47	211	I ricinus	Adult	2		214, 215
	48	211	I ricinus	Larvae	1		216
	49	211	I ricinus	Nymph	3		217, 218, 219
	50	212	I ricinus	Nymph	3		220, 221, 222
	51	213	I ricinus	Nymph	7		223, 224, 225, 226, 227, 229, 230, 231
	52	213	I ricinus	Adult	1		228
	53	214	I ricinus	Nymph	1		232
	54	214	I ricinus	Adult	3		233, 234, 235
	55	215	I ricinus	Adult	1		236
	56	215	I ricinus	Nymph	3		237, 238, 239
	57	216	I ricinus	Nymph	6		240, 243, 244, 247, 249, 250
	58	216	I ricinus	Adult	2		241, 242
	59	216	I ricinus	Larvae	3		245, 246, 248
	60	217	I ricinus	Nymph	1		251
	61	217	I ricinus	Adult	6		252, 253, 254, 255, 256, 258
	62	217	I ricinus	Larvae	1		257
	63	218	I ricinus	Nymph	3		259, 260, 261
	64	219	I ricinus	Adult	1		262
	65	220	I ricinus	Nymph	2		263, 264
	66	221	I ricinus	Nymph	2		265, 266
	67	222	I ricinus	Larvae	9		267, 268, 269, 270, 271, 272, 273, 274, 275

	68	223	I ricinus	Larvae	4		276, 277, 278, 279
	69	224	I ricinus	Larvae	1		280
	70	224	I ricinus	Nymph	2		281, 282
	71	225	I ricinus	Adult	1		283
	72	226	I ricinus	Larvae	1		284
	73	226	I ricinus	Adult	1		285
	74	227	I ricinus	Nymph	2		286, 287
	75	228	I ricinus	Larvae	2		288, 289
	76	228	I ricinus	Adult	1		290
	77	229	I ricinus	Nymph	1		291
	78	230	I ricinus	Adult	1		292
	79	232	I ricinus	Adult	1		293
	80	233	I ricinus	Nymph	9		294, 295, 296, 297, 298, 299, 300, 301, 303
4	81	233	I ricinus	Larvae	3		302, 304, 305
	82	234	I ricinus	Adult	1		306
	83	235	I ricinus	Nymph	1		307
	84	236	I ricinus	Adult	1		308
	85	236	I ricinus	Nymph	1		309
	86	237	I ricinus	Larvae	6		310 t/m 315
	87	238	I ricinus	Adult	1		316
	88	239	I ricinus	Adult	5		317, 319, 320, 321, 322
	89	239	I ricinus	Adult	5		323, 325, 326, 327, 328
	90	239	I ricinus	Adult	5		329, 330, 331, 334, 335
	91	239	I ricinus	Nymph	12		318, 324, 332, 333, 336, 337, 338, 339, 340, 341, 342, 344
	92	239	I ricinus	Larvae	1		343
	93	240	I ricinus	Nymph	14		345 t/m 358
	94	240	I ricinus	Nymph	13		359 t/m 371
	95	241	I ricinus	Adult	1		372
	96	242	I ricinus	Adult	1		373
	97	243	I ricinus	Larvae	10		374 t/m 383
	98	244	I ricinus	Adult	5		384, 387, 389, 394, 395
	99	244	I ricinus	Nymph	9		385, 386, 388, 390, 391, 392, 393, 396, 398
	100	244	I ricinus	Larvae	1		397
	101	245	I ricinus	Adult	8		399, 400, 405, 406, 407, 409, 411, 413

	102	245	I ricinus	Nymph	18		401, 402, 403, 404, 408, 410, 412, 414, 415, 416, 417, 418, 419, 420, 421, 422, 423, 424
	103	246	I ricinus	Nymph	1		425
	104	247	I ricinus	Adult	1		426
	105	248	I ricinus	Adult	1		427
	106	249	I ricinus	Adult	1		428
	107	249	I ricinus	Larvae	1		429
	108	250	I ricinus	Adult	1		430
	109	251	I ricinus	Nymph	1		431
	110	252	I ricinus	Nymph	8		432, 433, 434, 435, 436, 437, 439, 440
	111	252	I ricinus	Larvae	1		438
	112	253	I ricinus	Nymph	1		441
	113	254	I ricinus	Adult	1		442
	114	255	I ricinus	Larvae	3		443, 451, 459
	115	255	I ricinus	Nymph	14		444, 445, 446, 447, 448, 449, 450, 452, 453, 454, 455, 456, 457, 458
	116	256	I ricinus	Nymph	15		460 t/ 474
	117	256	I ricinus	Larvae	1		475
	118	257	I ricinus	Adult	1		476
	119	258	I ricinus	Nymph	1		477
	120	258	I ricinus	Larvae	3		478, 479, 480
5	121	259	I ricinus	Adult	1		481
	122	260	I ricinus	Adult	2		482, 483
	123	261	I ricinus	Adult	2		484, 485a
	124	261	I ricinus	Nymph	1		485
	125	262	I ricinus	Larvae	5		486, 489, 491, 493, 495
	126	262	I ricinus	Nymph	3		490, 492, 494
	127	263	I ricinus	Nymph	1		496
	128	264	I ricinus	Adult	3		497, 501, 502
	129	264	I ricinus	Nymph	2		499, 500
	130	264	I ricinus	Nymph	1		498
	131	265	I ricinus	Nymph	14		503, 506, 515, 516, 518, 521, 522, 524, 525, 527, 528, 530, 531, 532

	132	265	I ricinus	Larvae	7		504, 513, 514, 519, 523, 526, 529
	133	265	I ricinus	Adult	9		505, 507, 508, 509, 510, 511, 512, 517, 520
	134	266	I ricinus	Nymph	12		533, 534, 535, 537, 538, 539,3 540, 541, 542, 543, 544, 545,
	135	266	I ricinus	Nymph	13		546, 547, 548, 549, 550, 551, 552, 553, 554, 555, 556, 557, 558
	136	266	I ricinus	Adult	1		536
	137	267	I ricinus	Nymph	1		559
	138	268	I ricinus	Larvae	3		560, 561, 562
	139	269	I ricinus	Adult	4		563, 564, 565, 566
	140	270	I ricinus	Nymph	1		567
	141	271	I ricinus	Nymph	1		568
	142	272	I ricinus	Adult	5		569, 570, 571, 572, 575
	143	272	I ricinus	Nymph	5		573, 576, 577, 578, 592
	144	272	I ricinus	Larvae	14		574, 579, 580, 581, 582, 583, 584, 585, 586, 587, 588, 589, 590, 591
	145	273	I ricinus	Adult	1		593
	146	273	I ricinus	Nymph	1		594
	147	274	I ricinus	Larvae	1		595
	148	275	I ricinus	Adult	1		596
	149	275	I ricinus	Nymph	1		597
	150	276	I ricinus	Adult	1		598
	151	276	I ricinus	Nymph	5		599, 600, 601, 603, 604
	152	276	I ricinus	Larvae	4		602, 605, 606, 607
	153	277	I ricinus	Larvae	1		608
	154	277	I ricinus	Adult	1		609
	155	278	I ricinus	Nymph	5		610 t/m 614
	156	279	I ricinus	Adult	1		615
	157	279	I ricinus	Larvae	1		616
	158	280	I ricinus	Adult	3		617, 618, 619
	159	280	I ricinus	Adult	3		620, 621, 622

	160	280	I ricinus	Nymph	2		623, 624
	161	188	H scupense	Nymph	1		55
	162	188	H scupense	Nymph	1		54
	163	188	H scupense	Adult	1		53

Appendix D: PCR amplification protocol

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.	

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 E: RLB protocol

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.	

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: