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Original article

Screening for multiple tick-borne pathogens in *Ixodes ricinus* ticks from birds in Denmark during spring and autumn migration seasons

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ABSTRACT

Presently, it is uncertain to what extent seasonal migrating birds contribute to the introduction of ticks and tick-associated pathogens in Denmark. To quantify this phenomenon, we captured birds during the spring and autumn migration at three field sites in Denmark and screened them for ticks. Bird-derived ticks were identified to tick species and screened for 37 tick-borne pathogens using real-time PCR. Overall, 807 birds, representing 44 bird species, were captured and examined for ticks during the spring (292 birds) and autumn migrations (515 birds). 10.7% of the birds harboured a total of 179 *Ixodes ricinus* ticks (38 ticks in spring and 141 in the autumn) with a mean infestation intensity of 2.1 ticks per bird. The European robin (*Erithacus rubecula*), the common blackbird (*Turdus merula*), and the common redstart (*Phoenicurus phoenicurus*) had the highest infestation intensities. 60.9% of the ticks were PCR-positive for at least one tick-borne pathogen. *Borrelia* DNA was found in 36.9% of the ticks. The *Borrelia* species detected were *B. spielmanii* (15.1%), *B. valaisiana* (13.4%), *B. garinii* (12.3%), *B. burgdorferi* s.s. (2.2%), *B. miyamotoi* (1.1%), and *B. afzelii* (0.6%). In addition, 10.6% and 1.7% of the samples were PCR-positive for spotted fever group *rickettsiae* and *Candidatus* Neoehrlichia mikurensis. All of the tick-borne pathogens that we found in the present study are known to occur in Danish forest populations of *I. ricinus*. Our study indicates that migrating birds can transport ticks and their pathogens from neighboring countries to Denmark including sites in Denmark without a sustainable tick population. Thus, a tick-borne pathogen affecting human or animal health emerging at one location in Europe can rapidly be introduced to other countries by migrating birds. These movements are beyond national veterinary control. The current globalization, climatic and environmental changes affect the potential for introduction and establishment of ticks and tick-borne pathogens in Northern Europe. It is therefore important to quantify the risk for rapid spread and long distance exchange of tick-borne pathogens in Europe.

1. Introduction

Each year, hundreds of millions of birds pass through Scandinavia during their spring and autumn migrations (Olsén et al., 1995; Waldenström et al., 2007). Some of these migratory birds carry ticks, which remain attached to the birds for several days during the blood feeding (Olsén et al., 1995; Waldenström et al., 2007). A study in Sweden estimated that spring and autumn migratory birds carry 6.8 million ticks and 4.7 million ticks, respectively (Olsén et al., 1995). Over one quarter of ticks (26.6%) are infected with tick-borne pathogens such as *Borrelia burgdorferi* sensu lato (s.l.) (Olsén et al., 1995), which include the etiological agents of Lyme borreliosis in humans. As these birds are able to migrate across geographical and man-made barriers and evade veterinary control, they can disperse ticks and tick-borne pathogens over large distances, potentially introducing exotic tick-associated pathogens to new habitats (Hasle, 2013; Ogden et al., 2009). This is relevant, since milder climate and environmental changes may create opportunities for the establishment of new tick vector species, as well as new tick-borne pathogens (Gray et al., 2009). Migrating birds' capacity to spread exotic tick species has already been documented in previous Scandinavian studies where, for example, the species *Ixodes persulcatus* was identified for the first time in Scandinavia (Olsén et al., 1995) and *Dermacentor* sp. for the first time in Norway (Hasle et al., 2009). Consequently, it is important to quantify how arthropod vectors and vector-borne pathogens are imported by birds into Denmark from neighboring countries, particularly since climate and

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environmental change, combined with rapidly increasing globalization, may introduce new tick species and emerging tick-borne pathogens into Europe (Kilpatrick and Randolph, 2012). From the site of introduction, birds may rapidly disperse these ticks and tick-borne pathogens over large areas.

Members of the *B. burgdorferi* s.l. complex are among the most important tick-borne pathogens associated with *I. ricinus*. From this complex, at least five genospecies, *B. afzelii*, *B. burgdorferi* s.s., B. garinii, *B. spielmanii* and *B. bavariensis* are known to cause Lyme borreliosis in humans (Rizzoli et al., 2011; Rudenko et al., 2011). *Anaplasma phagocytophilum* is the cause of human granulocytic anaplasmosis (HGA) (Dumler et al., 2007) and *Rickettsia helvetica* may cause illness characterized by mild influenza-like symptoms (Parola et al., 2005). *Candidatus* Neoehrlichia mikurensis is an emerging zoonotic pathogen, which mainly affects immunocompromised persons (Grankvist et al., 2014).

There are ample data supporting that birds are significant dispersers of tick-borne pathogens in Europe (Comstedt et al., 2006; Elfving et al., 2010; Hasle, 2013; Taragel'ová et al., 2008). Furthermore, some migrating bird species breed in urban parks and private gardens in Denmark. These species may therefore introduce infected ticks to areas like urban parks and suburban gardens that are otherwise considered free of ticks because they lack a sufficient density of large mammals to sustain a tick population.

There are several European studies of ticks on migrating birds (Capligina et al., 2014; Comstedt et al., 2006; Elfving et al., 2010; Hasle, 2013; Hasle et al., 2009; Hornok et al., 2014; Lommano et al., 2014; Palomar et al., 2012; Poupon et al., 2006; Waldenström et al., 2007). However, surveys for ticks and their microorganisms on the migrating bird populations flying in and out of Denmark are still scarce (Olsén et al., 1995). Tick-borne pathogens such as *B. burgdorferi* s.l., *B. miyamotoi, R. helvetica*, *A. phagocytophilum*, *Candidatus* Neoehrlichia mikurensis, *Babesia divergens* and *B. venatorum* are already present in the *I. ricinus* populations of Denmark (Fertner et al., 2012; Kantsø et al., 2010; Michelet et al., 2014; Skarphédinsson et al., 2007; Svendsen et al., 2009). The importance of migratory birds in dispersing tick-borne pathogens in Denmark including parks and suburban gardens is presently unclear. Furthermore, it is uncertain if exotic tick-borne pathogens are present in tick vectors on migratory Danish birds.

The purpose of this study was to quantify the tick load on Danish migratory bird species and to determine the diversity of tick species and the prevalence of tick-associated pathogens being imported into Denmark by birds. We collected ticks from migratory birds that were captured during the spring and autumn migration in Denmark. We determined the species identity of the ticks and screened them for 37 tick-borne pathogens simultaneously using a recently developed high-throughput real-time PCR assay (Michelet et al., 2014).

2. Materials and methods

2.1. Tick collection

Birds were caught in mist nets during routine bird-catching operations of ringing migrant birds. Ticks were collected from birds in the spring and autumn migrations of 2016 at three coastal bird ringing stations in southern Denmark. During the spring migration, the birds were collected at Gedser Bird Observatory (54.56, 11.96) and the Råhede bird ringing site (8.65, 55.27) (between 14-05-2016 and 10-06-2016). During the autumn migration, birds were collected from the Keldsnor (54.73, 10.71) and Gedser Bird observatories (between 01-09-2016 and 07-11-2016). Not all captured birds were examined for ticks on each collection day to avoid having excessive numbers of individuals of the same bird species from the same day and because the capacity for examining birds was limited.

Each bird was classified to species and given a unique identification number. The head and neck region of each bird was examined for 5–10 s. Ticks were removed from birds with forceps and placed into plastic vials containing 70% ethanol. The collected ticks were labeled with a unique identification number and their developmental stage was determined under a dissection microscope.

2.2. DNA extraction and real-time PCR screening of tick-borne pathogens

Ticks were homogenized in a Qiagen tissuelyser II (Qiagen, Hilden, Germany) for 2 min at 20 Hz (using a 5 mm steel bead (Qiagen, Hilden, Germany) and 50 µl of PBS). After homogenization, we used the following extraction procedure: 175 µl incubation buffer (D290) (Promega, Wisconsin, USA), 175 µl lysis buffer (MC501) (Promega, Wisconsin, USA) and 30 µl Proteinase K (20 mg/ml) (Promega Wisconsin, USA) were added to the lysed samples which were incubated at 56 °C for 2 h. After incubation, additional 300 µl of lysis buffer (MC501) was added. The remaining part of the DNA extraction was performed on a Maxwell® 16 Instrument (Promega Wisconsin, USA) according to the manufactures instructions.

For the screening of tick-borne pathogens, we applied a high-throughput real-time PCR assay (Michelet et al., 2014) assay (Michelet et al., 2014), which is adapted to the BioMark microfluidic real-time PCR system (Fluidigm, CA, USA). With this assay, we were able to simultaneously perform the identification of 5 tick species and the detection of 37 tick-borne pathogens listed in Table 1 (Michelet et al., 2014).

We used the TaqMan PreAmp Master Mix (Applied Biosystems, CA, USA) for DNA pre-amplification, according to the manufacturer's instructions. Equal volumes (200 nM final each) of all primers (except for the ones targeting tick DNA) were combined. Pre-amplification PCR was performed in a final volume of 5.1 µl consisting of 2.5 µl TaqMan PreAmp Master Mix (Applied Biosystems, CA, USA), 1.3 µl pooled primer mix and 1.3 µl DNA. The pre-amplification program was one cycle at 95 °C for 10 min, 14 cycles at 95 °C for 15 s and 4 min at 60 °C. Finally, the samples were diluted 1:5 and stored at −20 °C until use.

Pre-amplified DNA from the ticks was run on the BioMark™ real-time PCR system (Fluidigm, CA, USA), using 48.48 dynamic arrays (Fluidigm, CA, USA). For the amplifications we used 6-carboxyfluorescein (FAM) - and black hole quencher (BHQ1) - labeled TaqMan probes (Eurofins, Denmark) with TaqMan Gene expression master mix according the manufacturer's instructions (Applied Biosystems, CA, USA). The real-time PCR data were analyzed using the Fluidigm real-time PCR analysis software to obtain crossing point (CP) values. A negative water control (NTC) was included for each sample. An *Escherichia coli*-specific assay with spiked-in *E. coli* DNA was used as a control for internal inhibition.

Table 1
Tick-borne pathogens and tick species included in the real-time PCR assay.

Genus	Species
Borrelia	*Borrelia* spp. *B. garinii*, *B. afzelii*, *B. spielmanii*, *B. valaisiana*, *B. lusitaniae*, *B. miyamotoi*, *B. bissettiae*, *B. burgdorferi* sensu stricto
Anaplasma	*A. phagocytophilum*, *A. marginale*, *A. platys*, *A. ovis*, *A. centrale*
Ehrlichia	*E. canis*, *E. ruminantium*, *E. chaffeensis* *Candidatus* Neoehrlichia mikurensis
Rickettsia	The spotted fever group (SFG) *rickettsiae*, *R. helvetica*, *R. conorii*, *R. slovaca*, *R. massiliae*
Francisella	*F. tularensis*
Coxiella	*C. burnetii*
Babesia	*B. divergens*, *B. microti*, *B. canis*, *B. venatorum*, *B. caballi*, *B. vogeli*, *B. bovis*, *B. bigemina*, *B. major*, *B. ovis*
Bartonella	*B. henselae*, *B. quintana*
Theileria	*T. equi*, *T. annulata*
Ixodes	*I. ricinus*, *I. persulcatus*, *I. hexagonus*
Dermacentor	*D. reticulatus*, *D. marginatus*

Table 2
Percentage of birds infested with *I. ricinus* ticks and intensity of tick infestation on migratory birds collected during the spring and autumn migration seasons.

Bird species[#]	No. infested/examined birds		No. of ticks (larvae:nymphs)		Infestation intensity (collected ticks/infected birds)	
	Spring	*Autumn*	*Spring*	*Autumn*	*Spring*	*Autumn*
Sub-Saharan wintering grounds						
Acrocephalus palustris (Marsh warbler)	2/8		3 (1:2)		1.5	
Acrocephalus scirpaceus (Eurasian reed warbler)	0/44	1/14		1 (0:1)		1
Anthus trivialis (Tree pipit)*		2/3		3 (1:2)		1.5
Hippolais icterina (Icterine warbler)	3/35	0/1	3 (0:3)		1	
Luscinia luscinia (Thrush nightingale)*	1/1		1 (1:0)		1	
Muscicapa striata (Spotted flycatcher)	0/5	1/3		1 (1:0)		1
Phoenicurus phoenicurus (Common redstart)	5/11	0/7	15 (6:9)		3	
Phylloscopus trochilus (Willow warbler)	0/34	1/14		3 (3:0)		3
Sylvia communis (Common whitethroat)	2/37	0/3	3 (1:2)		1.5	
Sylvia curruca (Lesser whitethroat)	1/11	2/22	1 (1:0)	5 (0:5)	1	2.5
South European wintering grounds						
Erithacus rubecula (European robin)*	3/5	40/175	9 (8:1)	77 (52:25)	3	1.93
Prunella modularis (Dunnock)*	1/8	0/21	3 (0:3)		3	
Sylvia atricapilla (Eurasian blackcap)	0/8	4/51		5 (4:1)		1.25
Turdus philomelos (Song thrush)*	0/1	1/6		1 (0:1)		1
North European wintering grounds						
Troglodytes troglodytes (Eurasian wren)*		1/8		1 (1:0)		1
Turdus merula (Common blackbird)*		9/31		38 (13:25)		4.22
Non-migratory/resident						
Parus major (Great tit)		2/5		2 (2:0)		2
Passer montanus (Eurasian tree sparrow)*	0/1	2/4		3 (0:3)		1.5
Pyrrhula pyrrhula (Bullfinch)		1/4		1 (0:1)		1

* Ground foraging birds. [#]Only bird species from which ticks were retrieved are included in the list.

2.3. PCR amplification and sequencing

We were concerned that the ticks that tested positive for *Babesia vogeli* in the Fluidigm real-time PCR were due to cross-reactivity with other pathogen species as this species has not previous been found in ticks in Denmark. Tick DNA extractions that tested positive for *B. vogeli* in the Fluidigm real-time PCR were therefore sequenced using the following protocol. Universal Babesia–Theileria–Hepatozoon primers, BTH-1 F (5´-cctgmgaracggctaccacatct-3´) and BTH-1R (5´-ttgcgaccatactccccca-´3) (Criado-Fornelio et al., 2003), were used to amplify a 750-bp fragment of the 18S rRNA gene from these samples. This 750-bp fragment was subsequently used as a template for a nested PCR with primers GF2 (5´-gtcttgtaattggaatgatgg-3´) and GR2 (5´ccaaagactttgatttctctc-3´) (Zintl et al., 2011). The PCR reactions were performed in a 50 µl reaction mix, which contained 5 µl of 10 × Gold Buffer (Applied Biosystems, CA, USA), 1.5 mM $MgCl_2$ (Applied Biosystems, CA, USA), 0.4 µM of each primer (Eurofins, Vejen, Denmark), 2.5 units of GoldTaq DNA polymerase (5 U/µl) (Applied Biosystems, CA, USA), 0.2 mM dNTP and 2 µl of template DNA. Using a T3 thermocycler (Biometra, Göttingen, Germany), the amplification conditions for the first PCR were as previously described by Criado-Fornelio et al. (2003), while the second PCR was performed according to Lyp et al. (2016) (Łyp et al., 2016).

PCR products were purified using a MiniElute PCR purification kit (Qiagen, Hilden, Germany) and sequenced with the GF2/GR2 primers by cycle sequencing on an ABI 3130 genetic analyzer (Applied Biosystems, CA, USA) and using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, CA, USA) according to the manufacturer's instructions. The sequences were assembled in BioNumerics, version 7.1 (Applied-Math, Sint-Martens-Latem, Belgium).

To confirm the identities of a subset of the results of the Fluidigm real-time PCR analysis, fragments of the flagellin (*Borrelia*-specific primers), groEL (targeting *Candidatus* Neoehrlichia mikurensis), and 5S–23S rRNA intergenic spacer (targeting *R. helvetica* and SFG *rickettsiae*) genes were amplified from eight tick DNA extractions by conventional PCR and Sanger sequenced by using previously described methods (Hodžić et al., 2015; Jado et al., 2006; Sato et al., 1997).

The *R. helvetica* sequences obtained in this investigation have been deposited in GenBank with accession numbers MH549707 to MH549709. The *Candidatus* Neoehrlichia mikurensis sequence has accession number MH593876 and the *Borrelia* sequences have been deposited in GenBank with accession numbers MH593877 to MH593880.

2.4. Statistics

Comparisons of proportions were done by calculating p-values using either Fisher's exact test or the Chi-Square test depending on the sample size using the Statistical Analysis Software (SAS), SAS Institute Inc. Cary, NC.

3. Results

3.1. Tick infestations

We captured 807 individual birds belonging to 44 different species. We sampled 292 birds (31 different species) in the spring and 515 birds (31 different species) in the autumn. In total, 179 feeding ticks were collected from 86 infested individual birds. Of these ticks, 95 (53%) were larvae and 84 (47%) were nymphs. From the spring and autumn migrations, 38 and 141 ticks were collected, respectively. Most of the ticks were semi-engorged to almost fully engorged with blood. Overall, 10.7% (86/807) of the sampled birds harbored ticks and the average infestation intensity for the subset of infested birds was 2.1 ticks per bird. However, there were large differences in infestation intensity between species and the maximum infestation intensity was 14 ticks per infested bird. All the tick samples were positive for *I. ricinus* in the real-time PCR assay.

Of the 44 different bird species that were captured, 19 species carried ticks (Table 2). All the collected spring and autumn migratory bird species are listed in Tables A and B, respectively, of the supplementary material. Among the ten bird species represented by > 10 individuals, the proportion infested with ticks varied significantly between the species both in spring (Fisher's Exact Test $p < 0.0001$; $n = 226$; $DF = 8$) and in autumn (Chi-Square $p < 0.0001$; $n = 436$; $DF = 8$). In spite of the relatively high number of specimens examined, very few ticks were retrieved from the Eurasian reed warbler (*Acrocephalus scirpaceus*), the icterine warbler (*Hippolais icterina*), the willow warbler (*Phylloscopus trochilus*), the dunnock (*Prunella modularis*), the

Table 3
Prevalence of tick-borne pathogens detected in the bird-associated *I. ricinus* ticks.

	Spring (%)			Autumn (%)		
	Larvae	Nymphs	Total	Larvae	Nymphs	Total
Pathogen species						
***Borrelia* spp.**	39 (7/18)	35 (7/20)	37 (14/38)	31 (24/77)	44 (28/64)	37 (52/141)
***B. burgdorferi* s.s.**	6 (1/18)	5 (1/20)	5 (2/38)	0	3 (2/64)	1.4 (2/141)
B. afzelii	0	0	0	0	2 (1/64)	0.7 (1/141)
B. garinii	0	5 (1/20)	3 (1/38)	9 (7/77)	22 (14/64)	14.9 (21/141)
B. spielmanii	11 (2/18)	15 (3/20)	13 (5/38)	12 (9/77)	20 (13/64)	15.6 (22/141)
B. valaisiana	6 (1/18)	0	3 (1/38)	10 (8/77)	23 (15/64)	16.3 (23/141)
B. miyamotoi	0	10 (2/20)	5 (2/38)	0	0	0
SFG rickettsiae*	11 (2/18)	15 (3/20)	13 (5/38)	10 (8/77)	9 (6/64)	9.9 (14/141)
R. helvetica	11 (2/18)	5 (1/20)	8 (3/38)	9 (7/77)	5 (3/64)	7.1 (10/141)
***Candidatus* N. mikurensis**	0	0	0	0	5 (3/64)	2.1 (3/141)

* SFG: Spotted fever group.

Eurasian blackcap (*Sylvia atricapilla*), the common whitethroat (*Sylvia communis*) and the lesser whitethroat (*Sylvia curruca*) (Table 2). The three remaining bird species, the European robin (*Erithacus rubecula*), the common blackbird (*Turdus merula*) and the common redstart (*Phoenicurus phoenicurus*), accounted for only 28.4% of the examined birds but carried 77.2% of all the collected *I. ricinus* ticks (Table 2).

3.2. Tick-borne pathogens

The 179 *I. ricinus* ticks were screened for 37 tick-borne pathogens (Table 1). 60.9% (109/179) of these ticks were infected with at least one tick-borne pathogen. The results of the real-time PCR are summarized in Table 3. A PCR run was considered valid when all NTCs were negative, all samples were positive for *I. ricinus* DNA (which served both to confirm the tested species and as a DNA extraction control), and all *E-coli* controls were positive (served as internal inhibition controls for each sample). Eight of the tick DNA samples were also PCR amplified and sequenced. BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi) confirmed that the identities of the sequences were in agreement with the real-time PCR results.

There was no significant difference (Fisher's exact test; $p > 0.005$) between the percentage of infected nymphs (64.7%) and larvae (56.8%).

The most common pathogens in the PCR-positive samples were *Borrelia* spp., which were detected in 36.9% (66/179) of the ticks, representing six different *Borrelia* species. *B. spielmanii* was the most common species (15.1%; 27/179), followed by *B. valaisiana* (13.4%; 24/179) and *B. garinii* (12.3%; 22/179), whereas *B. burgdorferi* s.s. (2.2%; 4/179), *B. miyamotoi* (1.1%; 2/179) and *B. afzelii* (0.6%; 1/179) were less abundant. Spotted fever group (SFG) rickettsiae (primarily *R. helvetica*) were seen in 10.6% (19/179) of the samples and *Candidatus* Neoehrlichia mikurensis in 1.7% of the samples (3/179). None of the ticks were PCR-positive for *A. phagocytophilum* or any of the other tick-borne pathogens included in the real-time PCR assay.

3.3. Co-infections

Ticks infected with two tick-borne pathogens constituted 17.3% (31/1179) of those examined (29% and 71% of co-infected ticks were larvae and nymphs, respectively). Most co-infected ticks contained different *Borrelia* genospecies (22/31), with *B. garinii* and *B. valaisiana* as the most common combination. Co-infections also occurred between *Borrelia* spp. and rickettsiae spp. (9/31) and between *Borrelia* spp. and *Candidatus* Neoehrlichia mikurensis (2/31). The common blackbird carried most of the co-infected ticks (for details, see Table C in the supplementary information).

3.4. Non-specific amplification with B. vogeli primers

Ten of the ticks were PCR-positive for *B. vogeli,* a parasite that has not previously been detected in Denmark. Since the *Babesia* primers could cross-react with other blood parasites, the *B. vogeli*-positive samples were further tested using conventional PCR and Sanger sequencing. According to BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi), the sequences were 99% identical to the 18S rRNA gene of a genus within the phylum Apicomplexa, *Isospora* sp., which is an internal parasite of birds (Yang et al., 2017).

4. Discussion

In the epidemiology of vector-borne zoonoses, avian mobility is of particular concern, since it represents an uncontrollable source of vector and pathogen exchange. It is therefore important to quantify how these birds support the circulation and spread of vector-borne diseases affecting humans, livestock, pets and wildlife. Prior to this study, information concerning the introduction of tick-borne pathogens to Denmark by migratory birds was limited, as the last study on the subject dates to 1995 and includes only one Danish location on the far eastern island of Bornholm (Olsén et al., 1995).

The infestation intensity or tick load of 2.1 ticks per infested bird was comparable to studies in nearby countries such as Latvia, Germany, Switzerland and Sweden, where tick loads ranged from 1.92 to 2.58 ticks per infested bird (Capligina et al., 2014; Lommano et al., 2014; Waldenström et al., 2007).

It is well established that the tick load of birds is correlated with feeding time on the ground (Hasle, 2013). We found highly variable tick loads among bird species but given the small sample sizes, the differences can only be considered indicative of trends. Common blackbirds and European robins are both ground-feeders, and had the highest tick loads. These observations are in agreement with previous studies where the common blackbird (Hasle et al., 2009; Lommano et al., 2014; Olsén et al., 1995) and the European robin (Hasle et al., 2009; Lommano et al., 2014) likewise were among the bird species with the highest mean infestation intensity. Common blackbird and European robin populations include both local residents and short-distance migrants, and these two bird species could introduce ticks and their pathogens from nearby European countries or from (robins only) South European countries (Adriaensen and Dhondt, 1990; Ashmole, 1962; Bønløkke et al., 2006). However, only 9 out of 124 ticks were collected from the European robin in the spring migration (and none from the common blackbird). Consequently, a much larger sample size of spring-migrating birds and their ticks is needed to be able to conclude anything about the significance of the European robin and the common blackbird in the import of ticks and their pathogens to Denmark. The higher tick infestations rates in autumn compared to spring may be

partly explained by the high forest cover in Southern Scandinavia. This dominating land cover may potentially expose the autumn migrating birds to ticks while resting and feeding on their way south towards Denmark with Sweden having a forest cover of 67% (https://ec.europa.eu/eurostat). However differences in the abundance of questing ticks between spring and autumn may also have contributed to the higher infestations rates in autumn as abundance of larvae and nymphs change rapidly in early spring with nymphs appearing before larvae (Schulz et al., 2014). As observed by others (Olsén et al., 1995), common redstarts in spring also had high tick loads. The common redstart is a long-distance migrating bird that winters in the Sahel zone of Africa (Kristensen et al., 2013). Common redstarts could import ticks with exotic pathogens from South or West Europe to Denmark in the spring, where it uses a direct route to northern Europe via the Iberian Peninsula (Kristensen et al., 2013).

In Norway, Hasle et al. (2009) found that less than 0.1% of migrating birds were infested exotic ticks such as *Dermacentor* sp. and *Hyalomma rufipes* (Hasle et al., 2009). Considering the small sample size of our study (n = 292 birds in the spring migration), it is therefore not surprising that we found only one tick species, *I. ricinus*, on the captured birds. The same result was observed in large-scale Scandinavian studies of migrating birds and is most likely caused by the detachment of exotic tick species at stopover sites in southern and central Europe (Comstedt et al., 2006; Olsén et al., 1995).

In total, 60.9% of the 179 ticks were PCR-positive for one or more tick-borne pathogens, with *B. burgdorferi* s.l. and *rickettsiae* as the most common agents. The prevalence of SFG *rickettsiae* in the present study (10.6%) was similar to other European studies, where it ranged between 11.3%–17.6% in bird-associated ticks (Elfving et al., 2010; Palomar et al., 2012) and between 13%–14.3% in Danish field-collected ticks (Svendsen et al., 2009).

While birds are reservoir hosts for *B. garinii*, *B. valaisiana* and *B. burgdorferi* s.s. (Gryczyńska and Welc-Falęciak, 2016), the main reservoir hosts of *B. spielmanii* are rodents (Richter et al., 2011). Therefore, it was somewhat unexpected to find 11 larvae PCR-positive for *B. spielmanii*. Since 5 of the 11 larvae were from birds that also carried *B. spielmanii*-infected nymphs, this observation might be explained by co-feeding transmission of the bacteria (Belli et al., 2017; Gern and Rais, 1996; Voordouw, 2015). For example, a recent study by Heylen et al. (2017) showed that rodent-adapted genospecies such as *B. afzelii* could use co-feeding transmission to infect larval ticks feeding in close proximity to *B. afzelii*-infected nymphs (Heylen et al., 2017). The remaining 6 larvae were from birds without *B. spielmanii*-infected nymphs, but they may have dropped off before sampling. *B. spielmanii*-infected ticks have previously been identified from birds, although in relatively low numbers (Gryczyńska and Welc-Falęciak, 2016; Heylen et al., 2014).

The prevalence of *B. spielmanii* was highest in ticks from the European robin and the common redstart. *B. spielmanii* was detected for the first time in Denmark in 2014, with an estimated prevalence between 0.95% and 1.78% (Michelet et al., 2014). Therefore, the high prevalence of *B. spielmanii* in the present study (15.0%) is surprising. The most common genospecies in the common blackbird were *B. garinii* and *B. valaisiana*. This result was not surprising, since the common blackbird plays a central role in the ecology of these two *Borrelia* species in Europe (Hanincová et al., 2003; Humair et al., 1998; Norte et al., 2013; Taragel'ová et al., 2008).

We did not identify any exotic tick-borne pathogens from the bird-associated ticks. However, we did find samples that tested positive for bacterial species such as *B. miyamotoi* and *Candidatus* Neoehrlichia mikurensis, which have only recently been observed in Denmark. *B. miyamotoi* was discovered for the first time, with low prevalence (0.2% to 1.3%), in Danish field-collected ticks in 2014 (Michelet et al., 2014). *B. miyamotoi* was first discovered in Japan in 1995 (Fukunaga et al., 1995) but has since been found in *I. ricinus* throughout central and northern Europe, with prevalences varying between 0.5% and 5% (Krause et al., 2015; Telford et al., 2015; Wagemakers et al., 2017; Wilhelmsson et al., 2013). This *Borrelia* species belongs to the relapsing fever spirochete group, but causes a nonspecific febrile illness often misdiagnosed as acute Lyme disease without a rash (Telford et al., 2015). The reservoir species of *B. miyamotoi* is not yet resolved but this bacterium has been detected in blood or ticks removed from passerine or galliform birds (Hamer et al., 2012; Lommano et al., 2014; Wagemakers et al., 2017). Rodents are also important reservoir hosts for *B. miyamotoi* (Burri et al., 2014; Krause et al., 2015).

Candidatus Neoehrlichia mikurensis was first identified in Denmark by PCR in 2011 (Fertner et al., 2012). This emerging pathogen has a rodent reservoir and can cause systemic inflammatory infections in immune-compromised individuals (Silaghi et al., 2016). We found an overall prevalence of 1.6% (3/180) of *Candidatus* Neoehrlichia mikurensis in the bird-associated ticks. So far, this tick-borne pathogen has been detected in at least 16 European countries, with overall prevalence rates ranging from < 1% to > 20% (Silaghi et al., 2016). In Denmark, the prevalence of this pathogen in field-collected *I. ricinus* ticks is low (from 0.2% to 2.6%) (Fertner et al., 2012; Michelet et al., 2014) compared to nearby countries such as Sweden and Germany, where a prevalence up to 12.5% has been reported (Silaghi et al., 2016).

Both *B. miyamotoi* and *Candidatus* Neoehrlichia mikurensis may be recently introduced and emerging tick-borne diseases in Europe. Our results indicate that once introduced to a site in Europe, migratory birds can spread these tick-borne pathogens to other areas. Thus, the recent discovery of *B. miyamotoi* and *Candidatus* Neoehrlichia mikurensis in tick populations in Denmark could result from introductions elsewhere in Europe, or they may have previously been overlooked in Denmark.

Although *A. phagocytophilum* is frequently seen in Danish field-collected ticks, none of the bird-derived ticks were PCR-positive for this bacterium. This observation is not surprising, since there is no evidence that birds can transmit *A. phagocytophilum* to ticks (Hasle, 2013) and small rodents and roe deer are considered to be the main reservoir for this tick-borne pathogen (Jensen et al., 2017; Skarphédinsson et al., 2005; Víchová et al., 2014).

I. ricinus is not a vector of *Isospora* since this parasite is passed between birds by fecal-oral transmission of oocysts (Reece et al., 2017). Consequently, the samples PCR-positive for *B. vogeli* are the products of primer/probe cross-reactions with DNA from ticks engorged with blood from *Isospora*-positive birds. This illustrates that PCR results from blood-filled ticks must be interpreted with extra care, as internal blood parasites not vectored by ticks can be accidently amplified.

5. Conclusions

The common blackbird and the European robin were the bird species most frequently infested with *I. ricinus* ticks, and had the highest prevalence of tick-borne pathogens. These two ground-dwelling bird species thrive in many habitats including urban areas. Even though the abundance of ticks in parks and gardens are low, the probability of a tick biting human hosts may be higher in an urban habitat than in rural areas (Rizzoli et al., 2011). Therefore, the continuous low-level introduction of infected ticks by migratory birds to gardens and urban green areas, which may otherwise not be able to sustain a viable tick population, may constitute an unrecognized zoonotic risk to humans (Rizzoli et al., 2014). Annual introduction of ticks by migrating birds into ten provinces not able to sustain a viable tick population has also been demonstrated to occur in Canada, and it has been suggested that these constitute a risk of human infections with *Borrelia* (Ogden et al., 2009). In Denmark only 14% of the land surface is covered with different forest types that all sustain *I. ricinus* endemic populations. Spring and autumn introductions of ticks by migrating garden birds to e.g. parks and gardens in urban and suburban areas may therefore dramatically increase the area of risk of tick bites. New investigations are needed to shed light on the ecological drivers of tick density and the prevalence of tick-borne pathogens in Danish urban and suburban

areas.

Border control, EU-legislation and veterinary measures effectively limit the spread of many animal infections in Europe, but wild, migratory animals evade these control measures. The observed infestation rate of 2.1 ticks per infested migrating bird supports previous observations that migrating birds may be a significant factor in the dispersal of zoonotically important and emerging tick-borne pathogens within and between European countries. At present, Denmark appears to be free from many of the tick species and tick-borne pathogens found in southern Europe and also free from tick species found further north in Fennoscandia (*Haemaphysalis punctata* and *I. persulcatus*). Migrating birds may occasionally introduce exotic ticks and tick-borne pathogens from southern Europe into Denmark but the existing climate and environment does not allow them to establish themselves. In case of habitat changes in Denmark, e.g. due to climate change or the introduction of new suitable host species, this constant circulation of pathogens between countries may rapidly spread any *I. ricinus*-borne pathogens in Europe to new suitable sites in Denmark. Furthermore, the same mechanism may rapidly spread exotic tick-borne pathogens to Denmark once they are introduced to a site in Europe.

Conflict of interest

The authors declare no conflicts of interest.

Ethical statement

Catching and handling the birds was approved by the Copenhagen Bird Ringing Centre (J. Nr. SN 302-009) under permission from the Danish Forest and Nature Agency. Only birds already caught as a part of the ongoing national ringing of birds were selected for examination and removal of ticks which was done simultaneously with the ringing of the birds.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ttbdis.2019.01.007.

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