From
Dept. of Biology, University of Oslo and
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Thesis:

Dispersal of ticks and tick-borne pathogens by birds

Dynamics of birds' transport of ticks to Norway

Gunnar Hasle, MD
2010

There are three rules for writing the novel. Unfortunately, no one knows what they are.
W. Somerset Maugham; English dramatist & novelist (1874 - 1965)

Title page: Photo: Ola Nordsteien.
Greenfinch, *Carduelis chloris*, caught at Jomfruland bird observatory 1 September 2003. This bird had been ringed and examined three days earlier and had no ticks after release. It carried more than 300 ticks (nymphs and larvae) and was unable to fly. The ornithologists put it in a cage and tried to feed it, but, unfortunately, it was found dead the following day.

Last page: Photo: Gunnar Hasle.
Giant Kingfisher, *Megaceryle maxima*, caught at Ntsinini (S25°36' E30°23'), South Africa. This was one of the most spectacular birds caught in the MEDUNSA (Medical University of South Africa) bird ectoparasite project.

To my wife, Aase, and children: Solveig, Elias, Susanna and Maja.

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ABSTRACT

Hard ticks (Ixodidae) are important disease vectors for humans and animals. In rich and temperate countries, human medicinal aspects are of the most concern to the public, while tick-borne diseases in livestock have immense economic consequences in tropical and subtropical countries. Tick-borne encephalitis is an emerging disease in Norway, and the prevalence of Lyme borreliosis and tick-borne encephalitis has increased in several northern European countries.

Ticks feed on the blood from vertebrate hosts. They have a very limited mobility and are dependent on their hosts for dispersal. This study examines birds' capacity for spreading ticks and tick-borne diseases across long distances and geographical barriers. Four bird observatories were used in the study, including Lista, on the southwestern coast of Norway, and three more eastern coastal islands: Jomfruland, Store Færder and Akeröya. During the spring migrations of 2003 to 2005, 9,768 passerine birds from the four bird observatories were examined for ticks. From these birds, 713 carried a total of 517 larvae and 1,440 nymphs. The highest prevalence of tick infestation was observed in the *Turdus* species and other ground-feeding birds, e.g., the robin (*Erithacus rubecula*) and dunnock (*Prunella modularis*). With the exception of 10 nymphs and 1 larva, the predominant tick species was *Ixodes ricinus*. Seven nymphs of *Hyalomma rufipes* and 1 larva of *Dermacentor* sp. were also found, which must be a new species for Norway. The prevalence of tick infestation and the number of ticks per bird varied with location, year and month. Blackbirds (*Turdus merula*) caught at Lista and Jomfruland, which are localities with many ticks, had more ticks than those from locations with few or no ticks, suggesting local tick recruitment. A similar study in 1965–1970 at Akeröya and Store Færder found ticks on 4.2% of the birds, while this study found infestation of 6.9% at the same localities (*P*<0.001), suggesting an increase in infestations. *Borrelia* spp. was found in 70/513 nymphs (19 *B. afzelii*, 38 *B. garinii*, 2 *B. turdi* and 11 *B. valaisiana*) and in 14/172 larvae (10 *B. garinii*, 1 *B. turdi* and 3 *B. valaisiana*). *B. turdi* is new to Europe, but was previously found in *I. turdi*, a tick species that is a parasite to birds in Japan. Ticks collected from birds of the *Turdus* spp. had a higher prevalence of *Borrelia* spp. than ticks from the other passerine genera. Therefore, the *Turdus* spp. is particularly important. The high prevalence of *Borrelia* may be related to *Borrelia* infection of the birds and transmission of *Borrelia* through co-feeding. Ticks that were co-fed with a *Borrelia*-infected tick had an increased probability of being infected with the same *Borrelia* species. Ticks collected on birds from Lista were less likely to have *Borrelia* than ticks found on birds from the more eastern localities. The prevalence of the different *Borrelia* species in ticks collected from migratory birds may be related to migration routes.

Sera from 306 healthy pastured cows from 24 farms along the southern Norwegian coast were tested for *Babesia divergens* by an immunofluorescence antibody test. This test showed a frequency of around 50% positive in the western part of the coast of West Agder and in the eastern part of East Agder and Telemark, while approximately 5% positive was found in the coastal area between these locations. Bovine babesiosis is a declining disease in Norway, which could be continuously reintroduced by birds.

By using 17 microsatellites of *I. ricinus*, Mendelian laws were used to show that broods of tick larvae had more than one father, which would give the offspring a higher genetic diversity than if there were only one father. This could facilitate the colonisation of a new locality if engorged female ticks were brought to new locations by birds. Even though it is difficult to prove that birds are responsible for new tick species and tick-borne pathogens in new areas, the findings of this study suggest that birds are capable of introducing these ticks, and it is difficult to find an alternative explanation for the emergence of tick-borne encephalitis in Norway.

Dispersal of ticks and tick-borne pathogens by birds

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LIST OF PAPERS.

I.   Røed, K.H., Hasle, G., Midthjell, L., Skretting, G., Leinaas, H.P., 2006. Primer note. Identification and characterization of 17 microsatellite primers for the tick, *Ixodes ricinus*, using enriched genomic libraries. Molecular Ecology Notes. 6, 1165–1167.

II.   Hasle, G., Røed, K.H., Leinaas, H.P., 2008. Multiple Paternity in Ixodes ricinus (Acari: Ixodidae), Assessed by Microsatellite Markers. Journal of Parasitology. 94(2), 345–347.

III.   Hasle, G., Bjune, G.A., Edvardsen, E., Jakobsen, C., Linnehol, B.A., Røer, J.E., Mehl, R.A., Røed, K.H., Pedersen, J.E., Leinaas, H.P., 2009. Transport of ticks by migratory passerine birds to Norway. Journal of Parasitology. 95(6), 1342–1351.

IV.   Hasle, G., Bjune, G.A., Christensson, D., Røed, K.H., Whist, A.C., Leinaas, H.P., 2010. Detection of *Babesia divergens* in southern Norway using an immunofluorescence antibody test in cow sera. Acta Veterinaria Scandinavica. 52(55) doi:10.1186/1751-0147-52-55.

V.   Hasle, G., Bjune, G.A., Midthjell, L., Røed, K.H., Leinaas, H.P., 2010. Transport of *Ixodes ricinus* infected with *Borrelia* species to Norway by northward-migrating passerine birds. Ticks and Tick-borne Diseases. Accepted October 22nd 2010.

ABBREVIATIONS AND DEFINITIONS

*	An asterisk is used as a multiplication sign in this thesis.
AVHRR	Advanced Very High Resolution Radiometer. Space-borne sensors on at least two polar-orbiting satellites under the NOAA.
BLAST	Basic Local Alignment Search Tool. An algorithm for comparing biological sequence information, e.g., DNA sequences. A BLAST database is hosted by the NCBI.
CCHF	Crimean Congo haemorrhagic fever: a serious arboviral hemorrhagic fever, transferred by a wide range of tick species, notably, the *Hyalomma* spp.
CI	Confidence interval. It is a 95 % probability that the true value will be within a 95 % CI, i.e., the range between a value - 1.96 * standard deviation and the value + 1.96 * standard deviation.
DNA	Deoxyribonucleic acid: The material that makes up genes.
ECM	Erythema chronicum migrans: the primary rash seen in Lyme borreliosis.
GLM	Generalized linear model. A linear regression model which is unifying various statistical models, for studying effects and interaction of different variables.
GPS	Global positioning system. A satellite-based navigation system.
IFAT	Indirect immunofluorescence antibody test (see Box III).
IPTG	Isopropyl β-D-1-thiogalactopyranoside. A molecular biology reagent that induces the transcription of the gene coding for β-galactosidase.
IgG	Immunoglobulin G, one of the five classes of antibodies.
LI	Louping ill, also called ovine encephalomyelitis: a serious arboviral infection that primarily affects sheep. It may also cause neurological disease in humans. Phylogenetically, the Louping ill virus is closely related to the tick-borne encephalitis virus.
LIV	Louping ill virus: an arbovirus belonging to the TBEV-complex, transmitted by Ixodid ticks.
NCBI	National Center for Biotechnology Information, a US government-funded institution under the National Institutes of Health.
NDHRS	Norwegian Dairy Herd Recording System. A national database containing individual dairy cow registration on health, diseases, milk quality, meat quality, calving, artificial insemination and culling. Administered by TINE SA (previously TINE BA), which is Norway's largest producer, distributor and exporter of dairy products.

NDVI   Normalised Difference Vegetation Index. Index based on the phenomenon that green plants reflect near infrared rays (NIR) and absorb red light: NDVI= (NIR-red light)/ (NIR+red light). NDVI is normally calculated from satellite data.

NOAA   National Oceanic and Atmospheric Administration. A research institution under the United States' Department of commerce. "Mission: To understand and predict changes in Earth's environment and conserve and manage coastal and marine resources to meet our Nation's economic, social, and environmental needs" (www.noaa.gov).

OHF   Omsk hemorrhagic fever: a serious arboviral hemorrhagic fever, transferred by *Ixodes* and *Dermacentor* spp.

PCR   Polymerase chain reaction: multiplication of gene material from a sample by matching with two primers, forward and reverse. The sequence between the forward and reverse site will be multiplied and can later be sequenced.

RH   Relative humidity. The partial pressure of water vapour in the air expressed as a percentage of the saturated vapour pressure of water at a given temperature.

Sp.   Species (plural spp.). In the Linnaean system a species is ranked below genus and above race/subspecies. A species is usually defined as a group of organisms that can interbreed. In modern bacteriology, where phylogeny is based on DNA, all degrees of relatedness are possible, and the species concept has little meaning.

TBE   Tick-borne encephalitis: a serious arboviral infection, giving meningitis or encephalitis, often with neurological sequelae.

TBEV   Tick-borne encephalitis virus: an arbovirus belonging to the TBEV-complex, i.e., western (W-TBEV), far eastern (FE-TBEV) and Siberian (S-TBEV) subtypes of TBEV, transmitted by Ixodid ticks.

INTRODUCTION

"The need for imaginative thinking and research in the epidemiology of diseases transmitted by arthropods is made manifest by new views of the longevity and host ranges of arthropod-borne viruses, as well as by other biological and medical phenomena. Among these are the intercontinental transport of ticks by migrating birds."

Harry Hoogstraal
(Hoogstraal et al., 1963)

Aristotle (384-322 BC) referred to ticks as "disgusting parasitic animals" (Hillyard, 1996). The attitude among biologists through the 19th and much of the 20th centuries considered parasitism as an inferior way of life (Zimmer, 2000). Modern biologists realise the parasites' immense influence on many ecosystems and are fascinated by the parasites' impressive biological adaptations. Ticks encounter adaptive challenges, such as desiccation, frost, host finding, mate finding, host immunity, hosts' blood coagulation, and dispersal, and they have developed ingenious solutions to all of them. The ticks' dispersal ability may be their most impressive achievement.

Despite a very limited mobility (maximum of 50 m during a lifetime, but mostly less than 5 m for an adult *Ixodes* sp.) (Daniel and Dusbábek, 1994) and geographical barriers, ticks have managed to colonise all places with a suitable climate and appropriate hosts, which means that they have effective ways of dispersing across these barriers. Cervid animals may harbour a large number of ticks and may migrate over large distances; however, when it comes to crossing oceans, deserts, glaciers and mountains, birds are the only capable transport hosts. The purpose of this project is to study the birds' role in spreading ticks and tick-borne diseases.

Health consequences of climate change and an increase in tick-borne diseases in northern Europe are issues of considerable scientific and public concern. A study of tick-borne diseases has to take into account the biology of the ticks and their vectors, in addition to the pathogenicity, host competence for the pathogens and abiotic factors. Medicine may be regarded as "applied zoology". Zoology is among the basic medical sciences, although it plays a modest role in the current medical training programs in Norwegian universities. However, parasitoses, vector borne diseases, zoonoses, many allergic conditions, envenomations and animal attacks are medical conditions where zoological knowledge is required. This Ph.D. project is a collaboration between the Faculty of Mathematics and Natural Sciences, Department of Biology, the Faculty of Medicine, Institute of Health and Society, at the University of Oslo, and the Norwegian School of Veterinary science.

Even though this study examined one way of dispersal (birds), and largely of a single genus of pathogens (*Borrelia*) for one tick species, *Ixodes ricinus* (sheep tick)[1] in the southern part of one small country (Norway), the perspective of this study is general in nature. Hopefully, the knowledge gained from this work will be useful for science and the public by adding to the general understanding of tick biology and ecology. This work may also serve as an inspiration for those who want to work in the interdisciplinary field between zoology and medicine.

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[1] In this text, the Latin names of ticks will be used along with the common English names of their vertebrate hosts. Both names are given the first time.

BACKGROUND

Box I
Some Ixodid ticks of known medical importance
(Peters, 1992; Hillyard, 1996; Estrada-Peña and Jongean, 1999; Walker et al., 2000 and 2003; Jongean and Uilenberg, 2004; Turell, 2007)

Genus/species	Main hosts	Distribution	Associated diseases or pathogens
Ixodes apronophorus	voles (*Microtus* spp.)	Russia	Omsk hemorrhagic fever (OHF)
I. cookei	marmots (*Marmota* spp.)	Canada	Powassan encephalitis
I. granulatus	rodents, leporids	Asia	*Rickettsia conorii*
I. hexagonus	hedgehog	Europe	*B. burgdorferi* s.l., *R. conorii*
I. holocyclus	marsupialls, rodents	Australia	*R. australis*, tick paralysis
I. lividus	sand martin (*Riparia riparia*)	Europe, Russia	FE-TBE
I. marxi	Red Squirrel (*Tamiasciurus hudsonicus*)	USA, Canada	Powassan encephalitis
I. ovatus	rodents, leporids	East Asia	*Rickettsia japonica, Francisella tularensis*
I. pacificus	rodents, deer, birds	Western USA	*B. burgdorferi* s.s.
I. persulcatus	rodents, larger mammals, birds	Russia and Japan	FE-TBE, S-TBE, OHF, *B. burgdorferi* s.l.
I. ricinus	all land-living vertebrates	Europe to western Russia	W-TBE, LI, *R. conorii, B. burgdorferi* s.l., *F. tularensis, Babesia divergens, Babesia microti, Anaplasma phagocytophilum*
I. scapularis	rodents, deer, birds	Eastern USA, Canada	*B. burgdorferi* s.s., *B. microti, A. phagocytophilum*
I. spinipalpis	deer mice (*Peromyscus* spp.)	USA, Canada	Powassan encephalitis
Haemapphysalis concinna	rodents	Europe to Japan	FE-TBE, *R. sibirica*
H. flava	rodents, leporids	Japan	*F. tularensis, R. japonica*
H. japonica douglasi	rodents, birds	Russia, China, Korea	FE-TBE, *R. sibirica*
H. leachi	dog, livestock, birds	Africa	*R. conorii, Coxiella burnetii*
H. leporipalustris	leporids, rodents	USA, Canada	Colorado tick fever (CTF)
H. neumanni	rodents, birds	Russia, China, Korea	FE-TBE
H. punctata	rodents	Europe, NW Africa, SW Asia	*R sibirica*
H. spinigera	rodents, monkey, man, cattle	South India, Sri Lanka	Kyasanur Forest disease (KFD)
Hyalomma aegyptium	tortoises	Africa, Middle East	*R. conorii*
H. anatolicum	birds, small mammals	Europe, Asia, Africa	CCHF
H. asiaticum	jirds (*Meriones* spp.)	Russia	*R. sibirica, C. Burnetii*
H. detritum	large domestic animals	North Africa	CCHF, *C. burnetii*
H. dromedarii	camel, man	Africa	CCHF, *C. burnetii*
H. marginatum	birds, cattle, goats	Russia, Asia, Africa	CCHF, *C. burnetii, R. sibirica*
H. rufipes	birds, cattle, goats	South Europe, Africa	CCHF
H. truncatum	large wild and domestic herbivores	Africa	CCHF, *R. conorii*
Amblyomma americanum	mammals , birds	SW USA	*Ehrlichia chaffensis, R. rickettsii, F. tularensis*
A. cajennense	birds, mammals, man	Mexico, Colombia, Brasil	*R. rickettsii*
A. hebraeum	larger mammals, large birds	S Africa	*R. conorii, R. africae*
A. variegatum	larger mammals	Equatorial Africa	CCHF, *R. conorii, R. africae*
Dermacentor andersoni	leporids, rodents	W USA, Canada	CTF, Powassan encephalitis, *R. rickettsii, F. tularensis*, tick paralysis
D. albipictus	moose, horse, man	USA, Canada	*R. rickettsii*
D. marginatus	rodents, birds, larger mammals	Europe, Russia	OHF, TBE, *C. burnetii, R. Conorii, R. sibirica, R. slovaca, F. tularensis*
D. nuttalli	larger mammals	Russia	*R. sibirica*
D. occidentalis	dog, leporids, rodents	USA	CTF, *R. rickettsii*
D. parumapertus	lepoprids, rodents	USA	CTF, *R. rickettsii, F. tularensis*
D. reticulatus	rodents, birds, dog	Europe, Russia	OHF, TBE, *R. Conorii, R. sibirica, F. tularensis*
D. silvarum	rodents, birds, larger mammals	Europe, Russia, Asia	FE-TBE, *R. sibirica*
D. variabilis	dog, leporids, rodents	USA	*R. rickettsii, F. tularensis*, tick paralysis
Rhipicephalus appendiculatus	larger mammals	Africa	CCHF, *R. conorii*
R. evertsii	larger mammals	Africa	CCHF, *R. conorii*
R. rossicus	cattle	Central Asia	CCHF
R. sanguienus	dog	pan-tropical and -subtropical	*R. rickettsii, R. sibirica, R. conorii*
R. simus	larger mammals	Africa	*R. conorii*

The importance of ticks as disease vectors

Hard ticks (Acari: Ixodidae) have a significant impact on public health and the rural economy in many parts of the world. They are the most important vectors of infectious diseases to domestic animals, and they are only second to mosquitoes as vectors of diseases to humans (Parola and Raoult, 2001; Jongean and Uilenberg, 2004), see Box I.

In humans, tick-borne arboviruses cause neurologic disease and, in some cases, hemorrhagic fever with high mortality. The following are of special interest in Europe: tick-borne encephalitis virus (TBEV: Western, Far Eastern and Siberian subtype, i.e., W-TBEV, FE-TBEV and S-TBEV, respectively), Louping ill virus (LIV), Omsk haemorrhagic fever virus, Powassan virus, Nairovirus, Coltivirus (Eyach virus) and Crimean-Congo haemorrhagic fever (CCHF) virus (Charrel et al. 2004). The serious W-TBE, which is widespread in Central Europe and the Baltic countries, has recently been discovered as causing human infection in Norway and has also been found in *I. ricinus* in Agder, Southern Norway (Skarpaas et al. 2002 and 2006). Favourable climatic conditions for TBEV appear to be present in the coastal parts of Vestfold, Telemark and Agder Counties (Randolph and Rogers, 2000, 2001).

Tick-borne *Rickettsia* fevers (family: Anaplasmataceae, see Figure 1) of varying severities are known in many parts of the world: Rocky Mountain spotted fever, Boutonneuse fever and African tick bite fever (Cowan 2003, Jensenius et al. 2003a and b). Occasionally, *Anaplasma phagocytophilum* may cause serious disease in humans (Kristiansen et al., 2001).

Babesia divergens, which is a cattle disease may cause disease in humans without a functioning spleen (Gorenflot et al., 1998)

Lyme disease, caused by bacteria of the species complex *Borrelia burgdorferi* s. l.,[2] is the most prevalent and widespread vector-borne human infection in the Northern Hemisphere and is a main reason for increased concern about tick-borne pathogens in this part of the world (de Meeûs et al. 2002). Before 1995, all cases of Lyme borreliosis were notifiable in Norway. Since then, only reporting of cases of chronic and disseminated Lyme borreliosis has been mandatory. There has been an increased incidence of borreliosis in Norway since 1995 (Figure 3). In many other European countries, an increase of both borreliosis and TBE was observed between 1990 and 2000 (Randolph, 2001).

In domestic animals, tick-borne diseases have an immense economic importance because of increased mortality along with decreases in milk yields, growth rates, abortion/calving intervals, hide quality and the high cost of preventive measures, including dipping, vaccination, chemotherapy and veterinary costs (Brown, 1997). Heartwater, caused by *Ehrlicia* (*Cowdria*) *ruminatum,* may lead to an overall mortality of about 70% in naïve livestock in endemic areas (Mahan et al. 2001). East coast fever (*Theileria parva*), tropical theileriosis (*T. annulata*), babesiosis/redwater (*Babesia bovis*, *B. major*, *B. bigemina* and *B. divergens*) and anaplasmosis (*A. phagocytophilum*) are major causes of losses in cattle (Brown, 1997). Other species of *Anaplasma*, *Theileria* and *Babesia* attack sheep (Hashemi-Fesharki, 1997) in tropical and subtropical regions. In northern Europe,

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[2] *Borrelia* is a genus of spirochetes. The species complex *Borrelia burgdorferi* sensu lato comprises all of the *Borrelia* species (sometimes referred to as "genospecies") that may cause Lyme borreliosis in humans and also includes species that are phylogenetically related to the human pathogenic species (see Figure 2).

cattle are in some areas commonly attacked by *B. divergens* (Paper IV).

Ticks play an important role in the epidemiology of dermatophilosis (Zaria, 1993) and may cause tick paralysis in humans and animals (Purwar, 2009) by injecting a venom that is similar to botulinum toxin (Grattan-Smith et al., 1997).They may also cause non-paralytic toxicoses (Mans et al., 2004). An adult female of *Ixodes scapularis* (blacklegged tick) consumed 0.51 ml of blood, while *Amblyomma americanum* (lone star tick) consumed 0.81 ml, *Rhipicephalus sanguineus* (brown dog tick) consumed 0.55 ml and *Dermacentor variabilis* (American dog tick) consumed 1.34 ml in an experiment on dogs (Koch and Sauer, 1984). The ticks concentrate the blood two to three times and inject the waste back into their host (Rechav et al., 1994). This mechanism partly explains their efficacy as vectors of diseases. Tick infestation may cause anaemia in hedgehogs (*Erinaceus europaeus*) (Pfäffle et al., 2009), rabbits, large ungulates (Jellison and Kohls, 1938) and certainly in the bird shown on the front cover of this thesis.

Anaplasma phagocytophilum is a common disease pathogen in sheep and was first named "*sjodogg*" in Norway in 1780 (Stuen, 2003). An investigation of red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*) and moose (*Alces alces*) in coastal areas revealed that the majority of the animals in areas where ticks occur had been exposed to this bacterium (Stuen et al. 2002).

Babesia divergens cause redwater disease (in Norwegian: "*blodpiss*") in cattle, which was first scientifically described in Norway in 1901 (Stuen 2003).

In wildlife, a wide range of tick-borne diseases can be observed. Levine (1971) described 71 species of mammalian *Babesia* species, some of them only found in one mammal species. For birds,

at least 13 *Babesia* spp. are described, and likewise, some of these have only been found in one bird species while others seem to be specific to the birds' order or family. (Peirce, 2000). The host-specificity of the *Babesia* species implies that birds may only act as transportes of ticks infected with a mammalian *Babesia* species, not as carriers of the parasite.

The flagellate *Trypanosoma cervi* and the filaria *Dipetalonema rugosicauda* have been found in *I. ricinus* parasitising roe deer (Jeanson and Tälleklint, 1992), but it is not clear if ticks may act as vectors for these parasites, or if they are a dead end. For the *D. rugosicauda* the presence of this filaria in adult males (Aeschlimann et al., 1979) shows a transstadial[3] survival of the parasite, which indicates an adaption to survive in the ticks.

Ticks are important disease vectors in wild and domestic animals and humans. In temperate countries, the human pathogenic aspects of ticks get most of the public attention, while in tropical and subtropical countries, the impact on domestic animals, and the subsequent economic consequences are most important.

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[3] Transstadial survival: from one instar through molting to the next instar. Transovarial survival: from adult females to the offspring.

Tick biology

Phylum
Arthropoda (Arthropods)

Subphylum
Chelicerata (Chelicerata)

Class
Arachnida (Spiders et cetera)

Subclass
Acari (Ticks and mites)

Order
Parasitiformes

Suborder
Ixodida (Ticks)

Family
Ixodidae (Hard ticks):
Prostriata: *Ixodes*
Metastriata: *Amblyomma*, *Dermacentor*, *Haemaphysalis*, *Hyalomma*, *Rhipicephalus*

Family
Argasidae (Soft ticks):
Argas, *Ornithodorus*

Family
Nuttalliellidae, only one species:
Nuttalliella namaqua

Hard ticks (Ixodidae) is a family in the suborder Ixodida (Box II). As opposed to the soft ticks, Argasidae, they have a hard chitin shield (in males: scutum/in females, nymphs and larvae: scutellum). Ixodidae have three mobile instars after hatching from eggs: larva, nymph and adult (Figure 4). In all of the mobile instars, the individual needs to have a blood-meal from a vertebrate host. They feed only one time before moulting to the next instar. Most often this occurs on three different hosts. After engorging, which results in the multiplication of their weight by 80-120 times, the females produce 1000-10,000 eggs

(Hillyard, 1996). Before this study, there were twelve species of hard ticks (Acari: Ixodidae) found in Norway (Mehl 1983; Lillehaug, 2003).

The abundance of questing ticks will vary during the day and from day to day because of responses to changes in the microclimate. Hubálek et al., (2003 a) found a positive correlation between tick activity and temperature, and a negative correlation between tick activity and relative humidity. Abundance will also vary from year to year due to survival through the last year's humidity and temperature. Finally, abundance can vary through decades because of changes in vegetation and availability of host animals.

Biology of the ticks found in this study (Paper III).
Ixodes ricinus (sheep tick) is the only Norwegian tick species that normally act as a vector for diseases in humans and domestic animals. Additionally, it is the only tick species commonly questing on the ground in Norway. This tick is widespread across Europe and is found along the coastal areas of southern Norway, north to Brønnøysund (latitude N65º30'), in the county of Nordland. It is a three-host tick and needs to feed once between each instar, in order for the female to produce eggs. Larvae and nymphs can parasitise mammals of all sizes, along with birds and reptiles. *I. ricinus* seems able to parasitise any bird or mammal species occurring within its habitat and distributional range, but it cannot complete its life cycle on small mammals and birds because the adults need hosts as large as or larger than cats (Jaenson et al., 1994). The blood meal for this tick lasted three to four days for larvae and nymphs in an experiment with great tits (*Parus major*) (Heylen and Matthysen 2010). As adult *I. ricinus* rarely infest passerine birds, there are no data for the duration of feeding of adult *I. ricinus* in birds; however, in a study of rabbits, the mean feeding time was seven days in the first feeding experiment for rabbits that were not immunised against *I. ricinus*. Rabbits typically mount an immune reaction to ticks, leading to a prolonged feeding period. In the

fourth feeding experiment, a mean feeding time of nine days was observed (Bowessidjaou et al., 1976). The duration of one generation will depend on climatic conditions and access to a blood meal, but each instar can survive a maximum of one year. The rate of development can be predicted by an hour-degree model (Randolph 2002). The species is sensitive to desiccation. An adult, unfed female can live for at least three months in 25°C at 95% relative humidity (RH) but will die within 8 days at 70% RH and within 2 days at 0% RH (Daniel and Dusbábek, 1994). These ticks actively regulate water loss by their position in the vegetation. There are seasonal variations in the tick's activity, depending on climate (Lees and Milne 1951), and an experimental study suggested that larvae escape desiccation by becoming quiescent (Randolph and Storey 1999). Another critical factor is freezing (Dautel and Knülle, 1997). The ticks hibernate in winter and will start active questing in the spring when the average five day maximum temperature is 7°C (Perret et al., 2000). In the Norwegian climate, the species can only survive in near-coastal regions, which is typically less than 30 km from the sea. *I. ricinus* is a vector for a wide range of pathogens occurring in Norway, including the *Borrelia burgdorferi* s. l. species complex, *Babesia divergens, Anaplasma phagocytophilum*, *Francisella tularansis*, TBEV and LIV. During the last several decades, *I. ricinus* has been found in new areas in Scandinavia, indicating geographical expansion of the parasite in Nordic latitudes (Lindgren & Gustafson 2001). The distributional range of both ticks and diseases may expand in Norway and other countries as a consequence of climate change and changes in vegetation due to changes in the use of the landscape (Gray et al., 2009). There are currently no publications that confirm an increased distribution of *I. ricinus* in Norway, although veterinarians in inland communities, such as Seljord and Kviteseid in Telemark (personal communication), report ticks on dogs in areas where ticks have not been seen previously.

Ixodes arboricola (tree-hole tick) is associated with birds that nest in holes and caves. No mammals, except for bats, have been recorded as hosts. Its nidiculous habitat requires them to detach from their hosts at night, as opposed to *I. ricinus,* which detach during day. This species may remain attached to the bird host for 20 d (Heylen and Matthysen 2010). It may harbour the TBEV.

Ixodes frontalis (passerine tick) is also associated with bird nests but is not restricted to holes. Martens (*Martes* spp.) and badger (*Meles meles*) may occasionally act as hosts. *I. frontalis* may be a vector for *Coxiella burnetii* and some animal viruses (Hillyard, 1996).

The hard tick *Hyalomma rufipes* (hairy *Hyalomma* or coarse-legged *Hyalomma*), previously named *Hyalomma marginatum rufipes* (Apanaskevich and Horak, 2008), is a two-host tick. The tick enters the first host (bird) as a larva, then feeds and molts to a nymph while being attached to the host. The time on the bird host will be about three weeks. After engorgement at the nymphal stage, the ticks release and molt on the ground into adults before entering the final host, which is typically a large mammal. *H. rufipes* and the related species *H. marginatum* and *H. turanicum* are vectors for CCHF, which is an emerging disease in Turkey. *H. marginatum* has optimal habitat suitability in Spain and North Africa, but occurs as far north as northern France. According to climate predictions, northern France may have the optimal habitat suitability for these ticks by 2050 (Estrada-Peña and Venzal, 2007).

The hard tick *Dermacentor reticulatus* (ornate cow tick) is increasing its distribution in northern Europe. It is a vector for *Babesia canis*, *F. tularensis*, *Rickettsia slovaca* and *C. burnetii* (Dautel et al., 2006). This tick has not been found previously in Scandinavia, although a case of *Babesia canis* in a Norwegian dog that had not been abroad

indicated that the vector may be sporadically brought into Norway, either by travelling dogs or migratory birds. It is also possible that *I. ricinus* may have acted as an occasional vector (Øines et al., 2010). As opposed to *I. ricinus*, *D. reticulatus* thrives in habitats with intense solar radiation. The adults may survive a northern European winter (Dautel and Knülle, 1996), but eggs and larvae cannot. It is uncertain whether nymphs may survive the winter. One possibility is that the temperature sum of the summer season determines the possibility of fulfilling the whole life cycle of *D. reticulatus* during one summer, which may be a requirement for this species to establish in an area (Dautel et al., 2006). Potentially, global warming could alter the length of the season, allowing enough time and warmth for this tick species to survive and multiply in Norway.

Vector and host competence

Vector competence is the vector's ability to transmit a disease. Ticks are effective transmitters of disease. The pathogens take advantage of the ticks' need for getting rid of excess fluid to ingest as many nutrients as possible. The ticks use their salivary glands for this purpose. The ticks also need to impair the hosts' immunity, but this immunosuppression may also adventitiously impair the immunity to the pathogens and facilitate their transmission. Furthermore, the tick saliva has anti-platelet, anti-coagulant and anti-vasoconstrictory properties. Conceivably, some of these pharmacologically active compounds could prove useful in medicine (Bowman et al., 1997). Some tick-borne pathogens are transmitted via the tick faeces (Parola and Raoult, 2001). For a tick to act as a vector, the pathogen must be able to live and multiply within the ticks. Estrada-Peña and Jongean (1999) have reviewed the vector capacity of approximately 12 soft ticks and over 20 hard ticks that commonly attack humans. The soft ticks are vectors for the

relapsing fever group of *Borrelia* species, one *Borrelia* species for each tick species, while members of the *Ixodes* species of the northern hemisphere that commonly attack humans are vectors for the *B. burgdorferi* s. l., species complex. Among these species, the same *Borrelia* spp. may infect different tick species. The other genera of hard ticks, such as *Amblyomma*, *Dermacentor*, *Haemaphysalis*, *Hyalomma* and *Rhipicephalus* are not vectors of *Borrelia*. *I. ricinus* has the capacity to harbour a very wide range of pathogens, including *Borrelia*, *Babesia*, *Anaplasma*, *Rickettsia* and TBEV.

Vertebrate host competence includes the ability to act as a reservoir for a pathogen. There are differences in host competence for the hosts of different pathogens. For the *Borrelia* spp., this difference is possibly due to differences in resistance to host complements. *B. garinii* infects birds and rodents (depending of the ribotype[4] of *B. garinii*), while *B. valaisiana* normally only infect birds. *B. afzelii* infects rodents, and *B. burgdorferi* s.s. may infect birds and most mammal species, except deer (Kurtenbach et al., 2002). A Swiss study showed that *B. burgdorferi* s.s. was most commonly isolated in squirrels (*Sciurus vulgaris*) (Humair and Gern, 1998). When double infections of two *Borrelia* species occur in *I. ricinus*, the combination of *B. garinii* and *B. valaisiana* is much more common than *B. garinii* and *B. afzelii* (Kurtenbach et al., 2001). These observations reflect the host competence of birds and mammals. For example, infection from a double-infected bird is more common than consecutive infection by two classes of vertebrate hosts. Among birds, it seems that the *Turdus* species are especially prone to infection by *B. garinii* and *B. valaisiana* (Humair and Gern, 1998; Humair, 2002). The prevalence of *Borrelia* spp. will be influenced by the availability of hosts. If the larvae and nymphs of *I. ricinus* feed on cervids instead of small rodents, the consequence may be a high abundance of ticks, but a low incidence of borreliosis, as

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[4]Fingerprinting of genomic DNA restriction fragments that contain DNA coding for ribosomal RNA

seen in *I. ricinus* that had fed on roe deer (Jaenson and Tälleklint, 1992).

For *A. phagocytophilum*, the infectious agent of human granulocytic ehrlichiosis, a wide range of mammals (ungulates, rodents, lagomorphs and carnivores) and birds may act as hosts (de la Fuente et al., 2005).

The *Babesia* species, on the other hand, are much more host specific (Levine 1971; Peirce, 2000). Only two species are known to attack humans, *B. divergens* and *B. microti*. In Europe, *B. divergens* is only observed in people without a functioning spleen or that may be immunocompromised in other ways. *B. microti* has been a concern in USA, where it is known to cause subclinical to lethal effects in previously healthy individuals (Gorenflot et al., 1998, Krause, 2002). This difference may be due to differences in awareness and availability of tests (Hunfeldt et al., 2002).

Csángo et al. (2004) found TBEV-antibodies in 16.4% of dogs in a veterinary practice in Arendal, Norway, indicating that a lot of subclinical cases had occurred. TBEV may cause a serious meningoencephalitis in dogs (Weissenböck et al., 1997). TBE infections are normally subclinical in ungulates (Šikutová, 2009). Unpasteurised goat milk is an alternative way for humans to get TBE (Balogh et al., 2010), indicating that viremia occurs, and antibodies of TBE are commonly found in goat, sheep and cow serum in TBE-endemic areas (Šikutová, 2009). However, viremia is usually a transient phenomenon in arboviral infections, and the stable reservoir of TBE is the tick itself, which transfers the virus from nymphs to larvae when cofeeding on small rodents, especially the bank vole (*Clethrionomus glareolus*) and field mice (*Apodemus flavicollis* and *A. sylvaticus*). This transmission is not dependant on viremia (Labuda et al., 1993).

All human tick-borne diseases are zoonoses (Jongean and Uilenberg, 2004), and humans are normally a dead-end for further transmission, as the infections normally are of short duration. Therefore, TBEV could not remain in nature if humans were the only host. Understanding tick-borne diseases requires knowledge in zoology and veterinary medicine.

The ticks' vertebrate hosts

At least 700 of the about 800 existing tick species have some degree of host specificity. Vertebrates that congregate for resting or nesting, and returns to nests and burrows are typically parasitised by host specific ticks, while wandering vertebrates, with large home ranges, and which do not congregate or nest in burrows and holes have ticks with moderate or low specificity, or have ticks with atypical life cycles (i.e. one or two hosts). Among the 212 *Ixodes* species 40 are strictly bird parasites for all stages. Even ticks with a high host specificity may bite humans in special situations, for example when hunters slaughter an animal (Hoogstraal and Aeschlimann, 1982) or when bird ringers visit a sea-bird colony. The host specificity has been partly attributed to a sampling bias, and to ecological specificity (Klompen et al., 1996).

From a medical point of view (i.e., tick-borne zoonoses), ticks that feed on all sorts of vertbrates would be most important. The *Ixodes* species that are vectors for Lyme borreliosis and TBE (*I. ricinus*, *I. persulcatus* and *I. scapularis*) are generalists and do often bite humans (Parola and Raoult, 2001). These species quest in open air, i.e. not mainly within animal dwellings or colonies.

As larger mammals are necessary to complete the life cycle of *I. ricinus* (Jaenson et al., 1994) and they can harbour a much higher number of ticks of all stages than is seen on birds (www.flaattogflue.no; Paper III), it seems obvious that larger mammals from a quantitative point of view are more important for the tick populations than passerine birds are. The cervid animals

would also be important for transport of ticks, as red deer, roe deer and moose, altogether counting about 100,000 individuals (Andersen et al., 2010), have yearly migratory ranges of 200, 100 and 50-60 kilometers repectively (Reimers, 1990). Although these animals may reach coastal island by swimming, transport by birds would be needed to cross oceans. Birds are commonly parasitised by the immature stages of *I. ricinus*, and can easily cross geographical barriers, and they occur in enormous numbers (Table 1).

Bird Migration

Bird observatories, mainly manned by volunteers, monitor the bird migration by observation and ringing. Recovered rings, on dead or recaught birds, give data for the birds' movements. The Lista bird observatory is situated on a promontory of the southwestern Norwegian coast. The Store Færder and Akeröya bird observatories are situated near the inlet of the Oslo fjord, about 20 km from each other, while Jomfruland is about 60 km SW of Store Færder (Figure 1 in Paper III).

There are between 30,000,000 and 85,000,000 migratory passerine[5] birds in Norway (Table 1). During spring (northward) migration most of the species, such as the robin (*Erithacus rubecula*), redstart (*Phoenicurus phoenicurus*), song thrush (*Turdus philomelos*) and willow warbler (*Phylloscopus trochilus*), follow a western pathway along the Atlantic coast. Birds heading for Sweden follow a more eastern parallel northeastern route (Fransson and Hall-Karlsson, 2008). Therefore, few of the species that follow the Atlantic coast migrate via Sweden. Norwegian blackbirds (*Turdus merula*)

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[5] Passerines are birds belonging to the order Passeriformes. In Northern Europe, all passerines belong to the suborder Passeri (oscines). Passerine birds are also called "perching birds" or "song birds" (including crows). Only passerine birds are included in this project.

mainly winter on the British Isles and may migrate directly over the North Sea or via continental West Europe (Wernham et al., 2002). The *Sylvia* warblers migrate broadly across the Alps and may either cross the Skagerrak Sea via Denmark or follow the Swedish coast (Bakken et al., 2006). A few species have an eastern migratory route, such as the white wagtail (*Motacilla alba*), which may migrate through Denmark or Sweden, and the bluethroat (*Luscinia svecica*), which has a strictly eastern route. The Norwegian redpoll (*Carduelis flammea*) may spend the winter from England to Russia, but it seems that the nominate subspecies, *flammea*, migrate to the east, while the subspecies *cabaret* migrate to the west (Bakken et al., 2006). In this study, the ratio of *flammea*/*cabaret* was 4/183 at Lista and 57/115 at the three more eastern bird observatories.

However, most birds arriving at the four bird observatories included in this study have to cross at least 112 km of open water. Therefore, these four bird observatories offer a unique opportunity to study the transport of ticks across a geographical barrier. A typical day distance during migration may range from 40 km/d (blackbird) to 90 km/d (redstart) (Bakken et al., 2006). However, if needed, the birds can traverse much larger distances. Blackbirds that winter on the British Isles have to cross at least 500 km over the North Sea. At a flight speed of 47 km/h (Meinertzhagen, 1955), it takes more than ten hours of non-stop flying to reach Lista on the other side.

The three eastern study locations are situated north of Jutland (see map, i.e., Paper III, Figure 1). Migratory passerine birds will follow land when possible, to rest and eat. Therefore, most of the birds arriving at Jomfruland, Store Færder and Akeröya will have to come via Jutland. There would be a negative selection for heading for Lista, compared to heading north when leaving Jutland, as there is a risk of not finding land at all, but still, many birds that are ringed at Jutland and continental Europe are recovered at Lista (Bakken et al. 2006). This could be

explained by a change in the true track[6] as a cause of wind.

The areas where the bird observatories are situated are overrepresented with respect to the number of birds captured for ringing or control (Bakken et al. 2006). There are no hard data (e.g., radar surveillance) confirming that these areas represent migration corridors or are the result of a sampling bias. However, these four bird observatories (as well as Mølen bird observatory, between Jomfruland and Store Færder) are situated where birdwatchers have experienced a rich influx of birds, which indicates a higher than average bird migration at Lista and across the area from Jomfruland to Akerøya.

Seasonal bird migration, between breeding habitats in the summer and the non-breeding season in the lower latitudes, even across the Equator, occurs in all temperate regions of the northern and southern hemisphere (Palaearctic and Nearctic regions) (Hayes, 1995).

Apart from the regular seasonal migration, major invasions of birds may occur due to searches for food. A notable example is the invasions of the Siberian spotted nutcracker (*Nucifraga caryocatactes macrorhyncos*) in northern Europe (Svensson et al., 2004). In subtropical and tropical regions, birds may migrate outside the breeding season in response to rainy seasons and to food availability in different altitudes. Such intratropical migrants, for example the Red-billed quelea (*Quelea quelea*), may migrate in huge flocks over large distances, especially in East Africa (Ward and Jones, 1977; Sinclair et al., 2002). Even mainly sedentary birds move in response to the need for food, and movements of short distances may have significance for tick dispersal. Birds can easily cross fences, e.g. between cattle pastures and wildlife

reserves, and in a situation where insecticides are used to control ticks and resistance develops, an interchange of ticks from farm to farm could, conceivably, facilitate the spreading of insecticide resistance.

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[6] The true track is the path over the ground, as opposed to heading, which is the flight direction.

AIMS OF THE STUDY

It is a well-known fact that birds may be parasitised by ticks and those migratory birds, consequently, may bring individual ticks to new localities. However, there are no updated, Norwegian regional-specific data on this issue.

The main purpose of this study was to obtain updated quantitative Norwegian data for the birds' capacity to disperse ticks and tick-borne pathogens. As there are many bird species involved, a large number of birds had to be examined to make calculations for each species. The data were sampled over several years, over several locations, to see the effect these variations had on the import of ticks.

The hypothesis was that this transport will be dependent on the birds' migration routes and species. Therefore, data from other countries could not be used as proxy data for prevalences in Norway, but the foreign data would be valuable for comparison with the Norwegian data, which also could be compared with historical data.

Additionally, this study explored the possibility that the ticks could acquire the pathogens during feeding on the birds, either by cofeeding or from a systemically infected bird.

The present data on this possible dispersal mechanism of ticks and tick-borne pathogens will contribute to the understanding of the general pattern of transport of ticks and tick-borne diseases by birds in temperate regions all over the world.

This a study of the birds' impact on the ticks' dipersal, using quantitative methods, but the object of the study is mainly qualitative: is it possible that birds are capable of spreading ticks and tick-borne pahogens to new areas, and under which conditions is it possible?

LITERATURE ABOUT TICKS ON MIGRATORY BIRDS

General literature on bird migration (Alerstam, 1990), bird taxonomy (Svensson et al., 2004), Norwegian bird populations and their migration habits (Gjershaug et al., 1994; Bakken et al., 2006), as well as literature specific for Swedish and British migratory birds (Fransson and Hall-Karlsson, 2008; Wernham et al., 2006) and on the general biology and taxonomy of ticks (Hillyard 1996; Morel and Perez, 1977 a and b) is a necessary foundation for this study.

The literature search was performed with Google Scholar (http://scholar.google.no/), through the network of the University of Oslo. Google Scholar has many advanced filtering functions, such as time period, author, language, journal, words and phrases (also "not" a certain word), including the ability to recognise and propose near matches, detect available full-text versions, and citation management via programs such as EndNote®.

Previous studies of ticks found on birds are summarised in Table 4. Before this project was initiated, there was ample documentation of birds' capability to act as hosts for ticks, including ticks that are infected with pathogens. The prevalence of tick infestation on the birds shows a 40-fold variation between the different studies. The different bird species' feeding habits (i.e., ground feeding or not) (Mehl et al., 1984; Hasle et al., 2009), age of the bird (Scharf 2004) and season (Hoogstraal et al., 1963; Olsén et al., 1995 a) influence the prevalence of tick infestation. Only one large study on ticks on birds in Norway has previously been done (Mehl et al., 1984). By studying migratory birds from the same location as was previously done, it was possible to make a historical comparison. Hoogstraal et al. (1961 and 1963) provided distribution maps of summer and winter habitats of the most important bird species. The studies from Canada, USA, Russia and Switzerland looked at birds that were migrating over land that could have picked up ticks anywhere. Likewise, most of the Swedish migratory birds (Olsén et al., 1995 a; Comstedt et al., 2006) pass through overlapping migration routes over the Danish island Zealand, although the birds collected on Ottenby (on the southern tip of Gotland) and Bornholm most likely would have crossed the Baltic Sea from Germany. The study of the more western Norwegian bird observatories permitted comparison with the more eastern mogratoru routes covered by the Swedish studies, both concerning tick prevalence and *Borrelia* infection of the ticks. Therefore, although several studies have examined ticks on birds, few previous studies have explicitly addressed the intercontinental transport of ticks.

DESIGNING A STUDY OF TICKS IMPORTED BY BIRDS

An important methodological challenge in this study has been the question: "How can it be ascertained that a bird is newly arrived?" If a bird is examined for the presence of ticks in an area where ticks are abundant on the ground, this is a crucial issue, as there would be a risk of finding ticks that were not imported, but rather contracted locally. There are different approaches to this question. The bird ringers' statement that a bird is newly arrived could not be used for this purpose, although the statement would most often probably be true. The number of migrating birds observed and caught at the bird observatories is much higher than the number that could be sustained at these locations; therefore, a majority of birds caught in the nets would be caught during passage of the locality. Furthermore, each bird species has a peak arrival time, which varies from year to year. Most of the birds caught before or during the peak arrival time could be regarded as newly arrived; however, some of the newly arrived birds would need a few days rest after crossing the ocean, leaving ample possibilities to contract ticks. This could especially be a problem at Lista, where many of the arriving birds have crossed 500 km of the North Sea. If several birds of one species were caught the same day at a locality during peak arrival times, this could indicate a migrating flock; however, not all species migrate in flocks.

Several methods were examined to solve this problem, including biometric data, wind trajectories and genetic markers.

Biometric data

The hypothesis was that newly arrived birds would have used their fat reserves during the ocean crossing and could thereby be identified by measuring their weight/length or by visual evaluation. Robins that were caught were scored according to a visual fat score from zero to eight (Kaiser, 1993). The fat scores were compared for birds that were caught during and after peak arrival in addition to birds that the bird ringers identified as "newly arrived", and recaught birds, i.e. definitely not newly arrived. The most conservative criterion for identifying newly arrived birds would be to observe a bird that was caught before or during the peak arrival time. For this group, there was a fat score of 1 in 15 out of 66 robins (22.7%, 95% CI: 13.3-34.7%), compared to 0 out of 27 (95% CI: 0.0-12.8%) recaught robins. For fat scores 3 and 4, there were no significant differences between the two categories (Table 2). Although there was a difference between these groups, all fat scores were represented; therefore, the fat score could not be used to identify newly arrived birds. Furthermore, based on the ringers' experience, some recaught birds even lost weight the first day after arrival (personal communication, Jan Erik Røer, Lista bird observatory) possibly because they were too exhausted to seek food.

The robins with a low fat score of 1 or 2 were significantly more often infested by ticks than the ones with a high fat score, possibly reflecting that birds with a better general condition were protected against tick infestation (Table 3). This may imply that the risk of contracting ticks is highest when the birds have just arrived, and that the risk of finding locally acquired tick would be higher on a bird with a low fat index than on a well-fed bird.

Wind trajectories

The hypothesis was that the birds would migrate when wind conditions were favourable. Small passerine birds have a flight speed of about 30-40 km/hr and would be expected to take advantage of tail winds when possible. It would be reasonable to assume that birds caught on days with favourable winds would be more likely to have newly arrived than ones caught on days when crossing the sea would imply flying against the wind. A wind trajectory is a drawing on a map of the historic path of the air at a certain location. The position of the

air is plotted for certain times and at a defined altitude. The map shows where the air at a certain locality had been each hour before a certain time (see Figure 5). Conceivably, the wind trajectories could indicate the region of departure for each bird caught. For example, a western trajectory could indicate that the birds arriving at Lista had crossed the North Sea, while a southeastern trajectory would indicate that the birds had arrived from Jutland.

The Norwegian meteorological institute provided wind trajectories for 100 m and 500 m above the earth's surface for spring 2003. The data included trajectories for the 18 hrs before 0700 h local time before April 13, 0600 h April 13 to May 4 and 0500 h after May 5, at the four test locations. The Skagerrak, Kattegat and North Sea, Denmark and southern Norway were subdivided into areas, and each of the days' wind trajectories at 100 and 500 m for 6 and 12 hours before 0700 h were scored according to these areas.

Unexpectedly, there was no correlation between the number of birds arriving per day and favourable wind trajectories; however, in single cases, it could seem helpful. For instance, on April 12, 2003 there was a 30-km/h SW wind in the North Sea, and the wind trajectory for Lista started at the coast of Scotland. Nine blackbirds and one song thrush arrived that morning. These birds had probably crossed the North Sea during the night. In other cases, the wind trajectories were different at 100 and 500 m, and sometimes the bird observatories received many birds despite wind trajectories from the northeast. This observation can be explained by the fact that small passerine birds can fly much higher than 500 m (Alerstam 1993) and possibly may be able to find tail winds. On clear days, birds may correct their heading, when the wind direction leads the birds away from their destination, while on cloudy days, the true track may be much more influenced by winds

(personal communication, Thomas Alerstam); however, this type of analysis would require data on clearness for all hours and all possible routes. Therefore, additional wind trajectories for other altitudes would hardly be helpful. In conclusion, the wind trajectories were not sufficient for determining newly arrived birds or where they came from.

Genetic markers

The idea was that it could be possible to find genetic markers that could determine the origin of a certain tick individual. If a tick had genetic alleles common in England and rare in continental Europe or Norway, this would strongly indicate that the tick was imported from England. Furthermore, a comparison of the frequency of exotic alleles in ticks in coastal regions in Norway with ticks from more inland localities could allow quantifying the impact of the import of ticks across the sea.

Seventeen microsatellite markers for *I. ricinus* were made (Paper I) to study population genetics. Ticks were collected by flagging in England, Holland, Denmark, Sweden, Germany, Poland, the Czech Republic and Jomfruland in Norway, and DNA was extracted from about 20 ticks from each of the mentioned localities, as well as ticks from deer from Hitra in Norway. The ticks were examined for the presence of the different microsatellite alleles. Unfortunately, the microsatellites appeared to have too many null-alleles to be suitable for population genetics software, and simple cluster analysis did not reveal any region-specific alleles. Therefore, the microsatellites did not solve the problem of proving that ticks were imported.

The microsatellite markers were still useful by proving that multiple paternities occur in *I. ricinus*, which may be important for the ticks' ability to colonise new areas by a few founder animals, as is discussed in Paper II.

Are the ticks imported or not?

There was no 100% reliable method to determine if a tick found on a bird was imported. For the analyses in Paper III, the ticks found on migratory birds that were not excluded because they had been caught earlier the same year, were juveniles, or had a brooding patch, were included in the study. Except for the ticks found on blackbirds in Jomfruland late in the season, the vast majority of ticks would be imported. The order of magnitude of the import by ticks, the different bird species' relative contribution to the tick import and the effects of locality, month and year could be assessed, even if a few of the ticks were acquired locally. For the analyses in Paper V, a more conservative approach was pursued to avoid wrongly concluding that pathogens found in locally acquired ticks were imported by birds. Ticks collected from birds on Store Færder and Akerøya, where almost no local ticks were present, were considered as imported, while only engorged ticks on birds that arrived before or during the peak arrival time for each year and locality were included in the pathogen analysis. However, even at Store Færder, where the ticks had to be imported to the island, the ticks could in some cases have been transported from locations along the Agder coast, and not across the Skagerrak. This could, in turn, influence the prevalence of *Borrelia* in the ticks found on the birds, as *Borrelia* spp. are common in questing ticks at the Agder coast (Jenkins et al., 2001; Lundsett 2004).

ETHICAL ISSUES

This is a descriptive study of birds' role in transporting ticks, and no intervention effects were postulated or studied. The birds were caught with nets (Figure 6) during the migration, and therefore, permission from the National Board of Animal Experimentation was needed. They approved the field sampling. Bird ringing is considered a minor trauma for the birds that were studied. Normally, the birds remain calm during release from the nets and later handling (transport in a cotton bag, measuring, ringing, examination and picking off ticks) (Figures 7, 8 and 9). Occasionally, birds are found dead in the nets, but these are rare accidents. The nets were examined at least once per hour, and they were closed during rain. If a net is left open during raining, the birds will become wet and may die from cooling. Bird ringers are not likely to cause the death of birds because they like birds, promote bird conservation, and do not want to discredit bird ringing. The handling time resulting from the tick project was only increased by about one minute for each bird (i.e., less when no ticks were found, and more when many ticks were found). The removal of the ticks did not seem to affect the birds adversely and was probably beneficial by removing the parasites. Every year, the number of birds caught was reported to the National Board of Animal Experimentation.

Many ticks had to be killed and were placed alive in 70% ethanol. If they have a capacity of suffering, it was in any case a suffering of short time.

For the blood sampling of the cows, an oral communication with the secretary of the National Board of Animal Experimentation was sufficient, as blood sampling is not considered an intervention that needs to be treated as an animal experimentation issue. All the farmers that were asked to participate in this study gave their consent. During the sampling, the farmers provided dressing to avoid bringing pathogens from one herd to another. The cows' skin was washed with 70% ethanol with klorhexidin, and disposable needles were used (Figure 10). The sampling was performed by the author, Gunnar Hasle, with help from the farmers. No employees were involved in this potentially risky procedure. In the publication, the farms were anonymised to avoid violating confidentiality, although the participating farmers may recognise their own farms' position on the survey map of an approximate scale of 1:1,500,000.

Apart from the blood sampling from the cows, no risks were imposed on the participants in the study as a result of this project. Bird ringers have a potential risk of contracting pathogens from the birds, and they followed procedures to minimise this risk. All handling of the ticks was done by clockmakers' forceps. These forceps were blunted to avoid penetrating the skin of the birds and the bird ringers.

MATERIALS AND METHODS

Tick genetics: Making new microsatellites (Paper I)

Twenty adult ticks were collected by flagging in localities in southern Norway. DNA was extracted by the spin column technique using a DNeasy tissue kit (Qiagen). Procedure: the specimens were crushed/grinded (in this case, using the tip of a Pasteur pipette to severe the ticks' cuticula). After lysis with a protease, the specimens were transferred to a spin column with a membrane that binds to the DNA. The membrane was washed twice by a buffer in a centrifuge. After washing, the DNA was detached from the membrane by an elution buffer and spinned down into vials. The DNA from all the ticks was pooled together.

Microsatellites were isolated by the method described by Hamilton (1999). After digesting the DNA into fragments of 200 to 1000 base pairs, the DNA was ligated to double-stranded SNX linkers for "tagging" the fragments. To select fragments that contained repeated sequences, the DNA-fragments were hybridised with a biotinylated oligonucleotide probe (in this case only one probe: $(GT)_{15}$, not the whole range of different probes that were used by Hamilton) and captured on streptavidin-coated magnetic beads, as biotin strongly binds to streptavidin. DNA that was not hybridised was washed away using a magnet. The enriched fragments were ligated into a plasmid vector (*Xba*I digested pBluescriptIIKS(+) vector, Stratagene), which also carry the property of ampicillin-resistance, and the vector was transformed into E. coli cells (XL1-Blue MRF'cells, Stratagene). The cells were cultivated on LA plates (Luria-Bertani medium supplemented with 1.5% agar and 50 µg of ampicillin per ml), the ampicillin was added to the agarose medium to select the plasmid-containing cells. As not all of the cells with plasmids would contain inserts of the DNA fragment, the cells had to be screened for inserts. This was done by X-gal and IPTG, which is included in the medium: X-gal is a galactose analogue which is broken down to a blue-coloured substance by β- galactosidase. The E. coli produce a β-galactosidase with a carboxy-terminal, which is inactive, and the plasmid contain the so-called Lasz gene which in the presence of IPTG produces its own inactive β-galactosidases. When the two inactive enzymes are combined, they become active. Plasmids with DNA-inserts do not produce the β- galactosidase gene. The result is that the colonies with inserts appear white, while the remaining colonies appear blue. Positive clones were picked directly from the agarose plates denaturated at 95°C for 10 minutes and amplified by using T7 and M13 reverse primers. Polymerase chain reaction (PCR) products of 300-800 bp were sequenced (see Box IV). Primers were designed from the sequences using Oligo software.

Tick genetics: Study of tick family groups (Paper II)

Three adult female, fully engorged *I. ricinus*, each with an attached, copulating male were collected from cows on the island Hille, near Mandal, Norway. They were allowed to complete copulation and were incubated at room temperature in a humid chamber through oviposition and hatching of larvae. DNA was extracted from the males, females and 20 larvae from each female by using the spin column technique, and PCR by using the new microsatellites was performed. Paternity of the attached male and the minimum number of fathers involved in each family was determined by Mendelian laws.

Collection of ticks from birds (Paper III)

Northward migrating passerine birds were caught with mist nets at four bird observatories along the southern Norwegian coast: Lista, Jomfruland, Store Færder and Akerøya (Figure 1, Paper III). The birds were examined for ticks around the beak, eyes and ear-openings by using head-mounted magnifying glasses (Figure 8). The ticks

were picked off by a blunted clockmakers' forceps and placed in 70% pure ethanol, along with a label with date, bird species and serial number. One vial was used for each bird. The ticks were later examined with a Nikon stereo-microscope, with maximum 50x magnification, for the identification of species, instar and degree of engorgement.

Data for the date, bird observatory, serial number of each bird, ring number (for unique identification of the individual bird host), bird species, sex and age of the bird (when possible to determine), tick species, tick instar and degree of engorgement were recorded in a database using Microsoft Office Excel.

SPSS version 15 was used to make cross-tabulation of bird species and tick infestation (i.e., prevalence of infestation and number of nymphs per bird) and of a reduced species list by year and location, and to construct panel graphs with one standard error to compare the tick infestations according to year, month and location, with blackbirds treated separately.

The effects of area, year and month on observations of the number of nymphs per bird were tested using a generalized linear model (GLM) (McCullagh and Nelder, 1989) using a negative binomial distribution with a dispersion parameter set at 8. Interaction effects between year and area, month and area, and month and year were compared. A comparison of means was also performed with the GLM (log link and Quasi-Poisson distribution family) using R statistical software (R Development Core Team, 2008).

To compare the frequency of infestation with previous data from two of the four locations a Chi square test was performed by using SPSS.

Collection of blood samples from cows and detection of *Babesia divergens* antibodies (Paper IV)

> Box III
> **IFAT: Indirect immunofluorescence antibody test**
> Different dilutions of sera are incubated on slides with antigen. The antibodies will bind to the antigen and are made visual by a flouresceine-conjugated anti-antibody. The slides are read in a microscope using ultraviolet light. Positive samples are identified by showing a fluorescence that is more intense than in the negative control.

With the help of local agricultural authorities, farmers who pastured their dairy cows in woodlands along the southern Norwegian coast, from Lista to Vestfold, were identified. The farmers got an information letter (Appendix I) and a form, where they were asked if ticks were a problem on the farms, if they accepted blood sampling, and they were asked to locate their farm on the map (Appendix II).

The exact positions of the farms were later detected with a GPS navigation device, and entered into the Google Earth to show the locations on a satellite image. The positions were later drawn into a blind-map provided by Cappelen Damm as (Figure 1, Paper IV).

During and after the sampling, the farmers were also asked if they had seen cases of redwater among their cows. Twenty-three farms, scattered as much as possible along the coast, were included. Blood samples were taken from the tail, both EDTA and full blood for serum. The full blood samples were centrifuged, separated and frozen within 48 hrs. The sera were examined for *B. divergens* IgG antibodies by using indirect immunofluorescence antibody test (IFAT) (see Box III). An experienced microscopist read the slides blindly, and the intensity of

the fluorescence and the highest dilution giving fluorescence were recorded.

The results were compared with statements from the farmers and recordings in the Norwegian Dairy Herd Recording System (NDHRS).

Exact confidence intervals for binomial proportions were calculated using the principles introduced by Clopper and Pearson and implemented in R (R Development Core Team, 2008).

Detection of *Borrelia spp.* in ticks (Paper V)

Box IV
Species identification by sequencing
The PCR-product is annealed with a mixture of deoxynucleoide triphosphates (dATP, dGTP, dCTP and dTTP) and dideoxynucleotide triphosphates (ddATP, ddGTP, ddCTP, and ddTTP) that are labelled with different fluorescent dyes. The different dideoxynucleotide triphosphates act as chain terminators, which results in synthesis of DNA fragments of different lengths with a labelled nucleotide at the end, which can be separated in a capillary gel. A sensor at the end of the gel detects the fluoroescence as a rise and fall in light of different wavelengths as they appear, representing the different nucleutides. The sequence of the DNA can thereby be deduced. The sequences are sent to the Basic Local Alignment Search Tool (BLAST) –database, hosted by the National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi), where the sequences are aligned with known DNA-sequences. The best match gives the species identification.

To reduce the risk of examining Norwegian ticks that were wrongly believed to be imported by birds, a conservative approach had to be employed. For Akerøya and Store Færder bird observatories, the ticks were unlikely acquired at the locations, as flagging revealed that there were almost no questing ticks at these locations. From Jomfruland (Figure 11) and Lista,

where questing ticks were abundant, only ticks that were engorged and from birds caught before or during peak arrival times for each year at the given locality were examined.

DNA was extracted from the ticks by using the spin column technique (for nymphs: as described above; for the tiny DNA-amounts of larvae: E.Z.N.A Micro Elute® Genomic DNA KIT, OMEGA Bio-Tek, Norcross, GA, USA, the procedure is the same for both kits).

PCR with a general *Borrelia* fla B Outer primer, (Forward: AAR-GAA-TTG-GCA-GTT-CAA-TC; Reverse: GCA-TTT-TCW-ATT-TTA-GCA-AGT-GAT-G) (Clark 2005) for detection of *Borrelia* spp. was used. Positive samples were sequenced by dye terminator sequencing as developed by Smith et al. (1986) (see Box IV).

Crosstabulations of *Borrelia* spp. and locality and *Borrelia* spp. and *Turdus* spp. versus other bird species, were calculated by the use of SPSS version 15. Exact confidence intervals were calculated as in Paper IV. For statistical comparison of the different selections of the sample, a two-sided Fisher's exact test, using SPSS, version 15 was performed.

To test of the effect of Lista versus the other localities a logistic regression (GLM), implemented in R was used. Possible differences between years and ticks from *Turdus* vs. non-*Turdus* spp. were taken into account. Additionally, the interaction of *Turdus* and cofeeding and the effects of engorgement and cofeeding on the prevalence of different Borrelia spp. were tested with a GLM.

SYNOPSIS

Paper I: Primer note. Identification and characterisation of 17 microsatellite primers for the tick, *Ixodes ricinus*, using enriched genomic libraries.

> Box V
> **The Hardy-Weinberg principle**
> Following the Mendelian laws, the frequency of genotypes can be calculated if the frequencies of different alleles are known. The frequency of homozygotes for one allele is the square of the frequency of that allele, and the frequency of a heterozygote is the frequency of one allele multiplied with the frequency of the other. Microsatellites are dominant genes, and gene pairs with one allele and a null allele will phenotypically appear as homozygous, and therefore, a deviation of the Hardy-Weinberg principle will be observed

The aim of this project was to establish new tools for doing studies on the population genetics of *I. ricinus* and related tick species.

Seventeen polymorphic microsatellites (see Table 1, Paper I) were characterised among 24 individuals of *I. ricinus* sampled from an island in the Oslo fjord region. The number of observed alleles ranged from 2 to 17, and the observed heterozygosities were between 0.10 and 0.83. Analysis of family materials gives evidence of non-Mendelian inheritance, or deviation from the Hardy-Weinberg's equilibrium (see Box V), for several of the characterised loci, which could be explained by the presence of null alleles.

Paper II: Multiple paternity in *Ixodes ricinus* (Acari: Ixodidae), assessed by microsatellite markers.

It has previously been shown that ticks mate several times. However, it has not been shown that there was more than one father successfully siring each brood of eggs. For most larvae, the attached males could be excluded as possible sire, and in the three tested families, at least two of the three females mated successfully with more than one male. This finding suggests that multiple paternity is a common reproductive strategy in *I. ricinus*, which may have consequences for the ticks' dispersal success by increasing the genetic diversity in broods from single females colonising new sites.

Paper III: Transport of ticks by migratory passerine birds to Norway.

A total of 9,768 passerine birds from 4 bird observatories along the southern Norwegian coast, including the areas of Akeröya, Jomfruland, Store Færder and Lista, was examined during the spring migration in 2003, 2004 and 2005. Altogether, 713 birds carried a total of 517 larvae and 1,440 nymphs. The highest prevalence of tick infestation was observed in the *Turdus* spp., robin, redstart and dunnock (*Prunella modularis*). The degree of tick infestation varied during each season, between localities, and from year to year. Blackbirds caught in localities with many ticks had greater infestation than those from localities with few or no ticks, suggesting local tick recruitment. A similar study performed during 1965–1970 (Mehl et al., 1984) involving two of the bird observatories in the present study found ticks on 4.2% of birds, as opposed to 6.9% at the same localities in this study (P<0.001). Additionally, there were higher prevalences of ticks on the birds than have been found in Swedish bird observatories (Olsén et al., 1995 a; Comstedt et al., 2006), i.e., 7.3% in this study, versus 3% in both the Swedish studies.

With the exception of ten nymphs and one larva, the predominant tick species was *I. ricinus*. Seven nymphs of *Hyalomma rufipes* and one larva of *Dermacentor* sp. were also found. The only *Dermacentor* sp, previously found in Norway is an imported American species, *D. albipictus* (winther tick). None of the European species of *Dermacentor*, i.e., *D. reticulatus* and *D. marginatus*, have

previously been found in Norway, and therefore the imported larva had to be new to Norway. *H. rufipes* and *D. reticulatus* are important disease vectors. Two nidiculous species (i.e., two *I. arboricola* and one *I. frontalis)* were also found, but these species are endemic in Norway, although rare, and have hardly any medical or veterinary importance.

Paper IV: Detection of *Babesia divergens* in southern Norway using an immunofluorescence antibody test in cow sera.

To study the distribution of *Babesia divergens* (Apicomplexa: Piroplasmida), sera from 306 healthy pasturing cows from 24 farms along the southern Norwegian coast were tested by using an IgG IFAT. The results of this test showed that 27% of the sera were positive for *B. divergens* antibodies. The sensitivity of this test for detecting the presence of *B. divergens* was much higher in the study area than the sensitivity obtained by relying on the reporting of babesiosis in the Norwegian Dairy Herd Recording System (NDHRS) and by farmers' observations of redwater. Antibody testing of pasturing cows would, therefore, appear to present a convenient and reliable method for detecting *B. divergens* over extensive areas. The fraction of antibody-positive sera that were detected showed a two-humped distribution, with a high fraction of positives being found in municipalities in the western and eastern parts of the study area, while the municipalities between these areas had few or no positive serum samples.

Paper V: Transport of *Ixodes ricinus* infected with *Borrelia* species to Norway by northward-migrating passerine birds.

Borrelia spp. was found in 70 of 513 examined nymphs (19 *B. afzelii*, 38 *B. garinii*, 2 *B. turdi* and 11 *B. valaisiana*) and in 14 of 172 examined larvae (10 *B. garinii*, 1 *B. turdi* and 3 B. *valaisiana*). Ticks collected from the *Turdus* spp. had a higher prevalence of *Borrelia* spp. than ticks from the other passerine genera. Ticks collected on birds of the genus *Turdus* from the south-western locality Lista had less *B. afzelii* and *B. garinii* than from the other more eastern localities. Ticks that are cofeeding (Figure 12) with a *Borrelia*-infected tick have an increased probability of being infected with the same *Borrelia* sp.

GENERAL DISCUSSION

Hubálek (2004) distinguished between three ways birds may play a role as spreaders of a pathogen: 1. biological carriers (i.e., the pathogen multiplies in the birds), 2. mechanical carriers (i.e., the pathogen survives on the skin and feather or a passage in the intestinal tract), and 3. carriers of infected hematophagous ectoparasites, as is typically seen with the transport of ticks. Hubálek also suggested two additional mechanisms, one where the infected birds may transmit a tick-borne disease to the ticks, and the alternative where ticks may transmit pathogens to each other by cofeeding, as the results in Paper V suggest. In cases where birds are infected with a tick-borne disease, the continued spreading of the disease to new ticks through the season may be more important than the transport of infected ticks during migration. Birds may be persistent carriers of *Borrelia* (Richter et al., 2000, Gylfe et al., 2000) and *Anaplasma* (de la Fuente et al., 2005). In addition to the tick-borne pathogens there are several viral, bacterial and fungal pathogens and one helminth that may potentially attack humans and be carried by birds, although there are few cases where transmission actually has been proven, such as West Nile virus, St. Louis encephalitis virus, Western equine encephalitis virus, Influenza A virus, *Chlamydia psittaci*, vero cytotoxin producing *E. coli*, *Salmonella enterica typhimurium*, *Mycobacterium avium*, *Cryptococcus neoformans* and the bird schistosomiasis that results in swimmers' itch (Hubálek, 2004; Tsiodrias et al., 2008). However, these pathogens and the wide range of parasites of other animals where birds are hosts are not the subject of this thesis.

Transport of ticks to new areas by birds.

It is difficult to prove that birds actually have caused the dispersal of ticks. In northern Europe, dogs carrying ticks can reach any area by transport on land, or by airplanes and boats. What we can prove is that birds are capable of seeding ticks, and thereby tick-borne pathogens, to areas where climate and vegetation are suitable for the ticks, and vertebrate vectors for all tick stages are available. Single individuals of *I. ricinus* are occasionally found on dogs in Finnmark county in Norway (species confirmed by the National Institute of Public Health, Norway), which is at least 400 km from the nearest locality of a viable *I. ricinus* population. As the climate is not suitable for the persistence of *I. ricinus* in Finnmark, these individuals must have been brought there at immature stages by a host that can move this distance within four days (i.e., birds or transported pets). To found a new population, introduced ticks will have to mate. *I. ricinus* can find each other on the vertebrate hosts (Milne, 1950), and this makes mating possible even when ticks are scarce. The accidental finding of nymphs or adults in a new place for a tick species is not proof that the distribution range of a tick has expanded. The finding of questing larvae on different spots would be the definite sign that a population is established.

This project provides new quantitative data on the birds' potential for transporting ticks and tick-borne pathogens to Norway, by collecting northward migrating passerine birds, examining them for the infestation by ticks, identifying the ticks species and instars and determining the occurrence of *Borrelia* in the ticks. A rough estimate of the amount of ticks transported to Norway by the northward migrating bird would be 30-85 million birds * 0.15 nymphs per bird = 4 – 13 million nymphs per year. Correspondingly, with 0.05 larvae per bird, the birds may transport 1.5-4 million larvae of *I. ricinus*. The imported larvae would only make a small contribution to the Norwegian tick pool, as there is about 90% mortality from larva to nymph (Randolph, 1998). A more realistic estimate could be made by multiplying the estimated populations (Table 1) of each species with the number of nymphs per bird as is given in Table 1 of Paper III, which gives a result in the same

order of magnitude (3-10 million imported nymphs per year). However, these are estimates with high uncertainties, and most of the uncertainties are due to the estimates of bird populations. For example, nobody knows if the population of blackbird is closer to 200,000 or 2,000,000. Therefore, the uncertainty inherent in the method of sampling ticks from birds, with the risk that some of the ticks were acquired locally, will have a small impact on these calculations. Although the import of tick nymphs can be counted in millions, the number must be small compared to the number of ticks that actually exist in Norway. Therefore, the import of ticks by birds would not have any mentionable effect on the population dynamics of an already existing population. In a long-term perspective, however, it could have an impact on the population genetics in places where migratory birds rest after crossing the Skagerrak. Unfortunately, the genetics tools that were developed for this purpose (Paper I) could not discriminate foreign from indigenous tick genes. As an *I. ricinus* female lays about 2000 eggs (Randolph 1998), the multiplication potential is enormous. If an imported tick brings genes that are in some way advantageous for the ticks' survival, transport by birds might nevertheless have an impact on the gene pool of the population.

From a quantitative point of view, other factors will be much more important than the birds' import of ticks and tick-borne pathogens. The abundance of ticks in nature varies naturally from year to year, from site to site and with vegetation type (Stafford et al., 1995; Hubálek et al., 2003 b; Jouda et al., 2003; Wielinga et al., 2006). Vegetation type changes with changes in human use of the nature, and over the longer term, with climate. Cervid animals (moose, roe deer and red deer) have had a vastly increasing population in Norway over the last 50 years (Andersen et al., 2010). The distribution range of roe deer and

red deer is still increasing, and mild winters may cause even more roe deer to survive. Apparently, the significance of cervid animals has increased dramatically since Thambs-Lyche made his great study on bovine babesiosis in the 1930s. Thambs-Lyche wrote: "roe deer hardly play any major role in the spreading of ticks" and: "ticks have never been found on red deer" (Thambs-Lyche, 1943, pp 530 and 531). Now, hunters regularly find large numbers of ticks on these animals (http://www.flattogflue.no/). Ostfeld et al. (1998 and 2001) showed an interaction between masting of oak trees and the number of white-footed mice (*Peromyscus leucopus*), the behaviour of white-tailed deer (*Odocoileus virginianus*), who feed on acorns when available, and gypsy moths (*Lymantria dispar*). These moths feed on oak leaves and influence masting, while parasitoids regulate the population of gypsy moths, together with the white-footed mouse, which feed on the moths' larvae and pupae. They found that the number of acorns influenced the number of mice, which in turn influenced the number of *I. scapularis* ticks (Ostfeld et al., 1998 and 2001). The indirect effect acorns had on the tick population was stronger than the effect of rainfall and temperature found by Jones and Kitron (2000). The incidence of acute human cases of borreliosis is positively correlated with the abundance of questing ticks (Stafford et al., 1998). This illustrates that the epidemiology of tick-borne diseases has to be viewed in a wide ecological framework, where climate is only one of the factors taken into account. Distribution ranges seem to be largely limited by vegetation and climate conditions, except in strictly host specific ticks, like *Amblyomma rhinocerotis*, which is limited by the distribution of its rhinoceros hosts (Cumming, 1999). Vegetation can be studied with remote sensing (Randolph, 2000), using satellite data through the Normalised Difference Vegetation Index (NDVI), which basically measures the absorption and reflection of red light and near infrared light to discriminate different vegetation types, such as scrub and grassland, from forest. These data are obtained through the Advanced Very High Resolution Radiometer

(AVHRR) of the National Oceanic and Atmospheric Administration (NOAA). Such data can be used to assess the onset and end of the growth season (Beck et al., 2007). Estrada-Peña et al. (2001) combined vegetation data and data of temperature and rainfall to produce a map predicting the distribution of *I. ricinus* at a world-map scale. However, no one knows exactly what the predicting climate factors are for the distribution of each tick species. Thambs-Lyche (1943) proposed the year isotherm of 5°C, which correlates well with the distribution of *I. ricinus* in Norway. As ticks have a limited ability to survive low temperatures, an isoline[7] of days below a certain temperature below zero during the winter could be a possible parameter for the distribution of a tick species in a temperate country, although the thickness of the snow cover would influence the temperature on the ground. Contact with ice severely influences the ticks' mortality during low temperature (Dautel and Knülle, 1997), and no account is taken of this in a pure temperature model. An isoline for the number of days when the summer temperature is high enough for the ticks to be active would be equally biologically meaningful. These parameters will represent correlations, as the distribution of a tick species is determined by the microclimate and a complex web of biotic factors. Factors other than dispersal ability will limit the geographic distribution range of a generalist ticks species, and birds can only have an influence when a geographic barrier is present, preventing the much more effective spread by larger mammals. This will also be the case for the birds' possible influence on the distribution of a tick-borne pathogen.

We have shown that a non-endemic tick species may be brought to Norway by birds. If one in 10,000 birds bring a larva of *D. reticulatus*, 30-85 million birds may bring 3000-8500 individuals of this species to Norway every year. These larvae will have to survive a high interstadial mortality rate to become adults, and a male and a female would have to find each other. This seems unlikely, but ticks imported to Norway are not randomly spread. Birds arriving at a small coastal island may rest long enough after crossing the ocean for the tick to drop off. Small rodents, which are hosts for the nymphs, are abundant on such islands. After moulting to the adult stage, the tick may enter one of the few sheep pasturing on the same island, which may already harbour a mate, and could succeed in producing a hatch of eggs. At present, the growth season is probably too short and the temperatures too low for *D. reticulatus* to establish in Norway. If climate changes, this species may survive in a Norwegian locality. So far it has been difficult to relate climate changes to changes in tick distribution, as changes in vegetation may well be caused by changes in agricultural practises. However, phenology based on satellite data indicates that the growth season in Norway increased more than four weeks from 1982-1999 (http://project.itek.norut.no/phenology/).

There has been some public concern over the eastern species *I. persulcatus* (Taiga tick) coming to Norway. *I. persulcatus* is a vector for the same pathogens as *I. ricinus* and is also a vector for the FE-TBEV and S-TBEV subtypes of TBEV, which are thought to be more serious than the W-TBEV (Lundkvist et al., 2001; Golovljova et al., 2004). Reports indicate a mortality of 1-2% for W-TBEV and may be as high as 20-40% for FE-TBEV, although doubt has been raised that a bias in diagnosis may explain this difference (Heinz et al., 2007). The S-TBEV may cause a chronic course of TBE in Siberia, but not in the Baltic countries (Gritsun et al., 2003; Randolph, 2008). The biology of *I. persulcatus* is very similar to that of *I. ricinus*. Both are not nidiculous (exophilic) and quest in the vegetation (Alekseev, 2000). Like *I. ricinus*, it parasitises mammals and

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[7] A line on a map connecting places registering the same amount or ratio of some geographical or meteorological phenomenon or phenomena.

birds (Nuttal and Labuda, 1994). This is a tick that may survive in the Norwegian climate. The distribution range includes areas with a very harsh climate (e.g., around the Baikal Sea), where winter temperatures reach -40°C. This indicates a much better cold-hardiness than is seen for *I. ricinus*. Additionally, the summers are dry in Siberia, and the ticks do not quest during summer (personal experience: six hours of flagging in a perfect tick biotope at the Baikal sea in august 2006, yielded no ticks). A tick that can survive any Norwegian winter temperature and aestivate during the driest summers would probably be able to live in areas in Norway where *I. ricinus* cannot survive.

On a large-scale map there are large areas of overlap in Eastern Europe where these two species occur, but within this area there are different microclimatic conditions where the two species live separately (Lindgren and Jaenson, 2006). There is no experimental evidence of crossing *I. ricinus* and *I. persulcatus*, but an experiment with *I. persulcatus* and the related species *I. scapularis* (previous *I. dammini*) showed that they were perfectly capable of mating, but the offspring was sterile (Oliver et al., 1993). In a situation where two related species may hybridise and all of the offspring are sterile, the reproductive success of the least abundant species will suffer much more than the most abundant. The distribution of *I. ricinus* and *I. persulcatus* in Eastern Europe fits with the hypothesis that these two species may mate together and produce sterile offspring. The small number of *I. persulcatus* that possibly could be brought to Norway by birds would probably not survive in an area occupied by *I. ricinus*. On the other hand, if *I. persulcatus* is introduced to a place with a climate where *I. ricinus* cannot survive, it could establish a population. However, this would most likely occur through Sweden. One bird species, the bluethroat (*Luscinia svecica*), is a common bird in mountainous regions

and has an eastern migratory route through areas where *I. persulcatus* occur (Fransson and Hall-Karlsson, 2008). This species could, theoretically, transport these ticks across the Baltic Sea. If *I. persulcatus* establish in Sweden, it could easily be spread by cervids to Norway, through continuous areas with a climate too harsh for *I. ricinus.* Although difficult to prove, one case suggests that birds have been responsible for seeding new tick species and a tick-borne pathogen into an area: *I. persulcatus* is seen in Kokkola (N63º50' E23º07'), Finland, several hundred kilometres from the known western distribution range of this species. There have even been human cases with S-TBEV in the same area, which has not been found other places in Finland (Jääskeläinen et al., 2006). This is a seemingly discontinuous distribution, although the authors cannot rule out the possibility that an unnoticed, continuous distribution is present.

Paper II showed that one single tick female gives birth to eggs that have more than one father. Therefore, the genetic variation in the offspring may be sufficient for one single founder animal to found a population. However, there are few tick species that use birds as hosts for the adult instar. Normally, this is only the case for the nidiculous species specialised in parasitising birds, e.g., *I. arboricola* and *I. frontalis.* In this project, an adult *I. ricinus* was found on the feathers of a long-eared owl (*Asio otus*) caught at Store Færder. Additionally, a fully engorged female was found on a seriously wounded redstart at Jomfruland. Unfortunately this specimen was destroyed by mould before species identification was performed. In a study in Sweden 7 adult *I. ricinus* were collected from 13260 birds (Comstedt et al., 2006), proving that birds occasionally may carry adult *I. ricinus*.

Although birds are possible transport vectors for new tick species, vertebrate hosts of the size of cats and larger would be much more effective, as these animals normally harbour adult ticks. Norwegian veterinaries got a serious reminder of this when a mustang (*Equus* sp.) from USA was imported in 2001, and 15 adult *Dermacentor albipictus* were

discovered on the horse four months after it had arrived to Norway (Lillehaug, 2003). *D. albipictus* is a one-host species that live as far north as Canada, in climatic conditions very similar to the Norwegian climate. They parasitise moose and other cervids. Fortunately, the protective measures that were executed seem to have prevented the establishment of this species in Norway, which had never previously been seen in Europe.

The international trade of animals implies a risk of introducing exotic ticks to new areas. For example, *Hyalomma aegypticum* and at least nine species of *Amblyomma* have been found on reptiles imported to Poland (Nowak, 2010). In most cases the ticks will not find a suitable habitat, but one notable exception is the dog tick, *Rhipicephalus sanguineus*, which is native to the Old World, but has been introduced to the New World, probably by dogs (Aguiar et al., 2007). This species can survive in kennels and is now spread globally between N50° and S35° (Walker et al., 2003). If a potential vertebrate hosts of *I. persulcatus* were imported from Siberia to a Norwegian mountain district *I. persulcatus* could be introduced.

H. rufipes (previously *H. marginatum rufipes*) is a tick that is regularly found by bird ringers in Norway, Sweden and Finland (Brinck et al. 1965; Mehl et al., 1984; Olsén et al., 1995 a; Paper III). As this species has an optimal climate around the Mediterranean (Estrada-Peña and Venzal, 2007) it seems obvious that it cannot establish in Norway. However, a northward moving of climate zones may make this species a more common guest, with a faint risk of transferring CHF to someone. *H. rufipes* species parasitises birds in the immature stages. Humans usually contract CHF by slaughtering cattle, which get the virus from adult *Hyalomma* spp. (Ergönyl, 2006).

Spreading of tick-borne pathogens by birds.

As for the seeding of new tick species, it is difficult to prove that the spreading of tick-borne diseases actually has been caused by birds, although the genetic similarities of *Borrelia* specimens found on the sea-bird tick *Ixodes uriae* on islands in both the northern and southern hemisphere (Olsén et al., 1993; 1995 b) strongly suggests that this type of transport has occurred. If a new pathogen comes with a tick, an outbreak may be initiated. One infected bite is probably sufficient to transfer a tick-borne disease in a susceptible host. This is shown experimentally for *Borrelia burgdorferi* and *Babesia microti* with *I. scapularis* in white-footed mice (*Peromyscus leucopus*) (Mather 1990), and it is seen by ample clinical experience with human erythema chronicum migrans (ECM). It can also be deducted from the epidemiology of TBE, as the prevalence of TBEV in questing ticks is rarely more than 5% (Süss et al., 2002), and humans normally get one or a few tick bites at a time. A fraction of ticks transported across the sea will bring pathogens, and a fraction of these will find a susceptible host. If it is an animal host, it is likely that the host at the same time will be bitten by local ticks, which then may contract the pathogen.

Borrelia spp. are abundant in ticks in Norway. One study showed a prevalence of 16.2% in nymphs and 15.4% in adult males and females (Jenkins et al., 2001). There are several studies showing transport of *Borrelia*-infected ticks by birds (Table 4). Although the quantitative impact of *Borrelia* brought by birds would be minute, the birds could potentially have an impact on disease dynamics in humans, domestic animals and wildlife by spreading new strains of an already present pathogen, and, as is shown in Paper V, new *Borrelia* spp. to new areas. Strangely, there is no positive correlation between tick abundance and prevalence of *Borrelia* in the ticks (Randolph 2001; Jouda et al., 2004). This should have been expected if ticks transmit the *Borrelia* to each other through cofeeding, but a high density of ticks may be seen in areas with many roe deer,

which may cancel a possible effect of cofeeding because they are not competent hosts for *Borrelia* (Jaenson and Tälleklint, 1992). Understanding the prevalence and distribution of tick-borne pathogens require detailed knowledge of host-tick-pathogen interactions. The prevalence of the different *Borrelia* spp. would be expected to vary in a local scale, depending on which vertebrate host is most important for sustaining an enzootic cycle of *Borrelia.* For example, in England, a high proportion of *I. ricinus* nymphs feed on pheasants (*Phasanius colchicus*), which can be infected by *B. garinii* and *B. valaisiana*, but not *B. afzelii,* thereby partly blocking the circulation of *B. afzelii* (Randolph, 2000). In Norway, two studies of flagged ticks showed that *B. afzelii* was the most prevalent *Borrelia* species in Norway (Jenkins et al., 2001, Lundsett 2004). There is no register of *Borrelia* species in human cases of Lyme borreliosis, but notification based on clinical symptoms 1995-2004 showed that 71.0% presented with neuroborreliosis, 21.8% with arthritis and only 5% with acrodermatitis atrophicans (Nygård et al., 2005). As *B. garinii* is associated with neuroborreliosis, *B. burgdorferi s.s.* with Lyme arthritis and *B. afzelii* with acrodermatitis atrophicans (van Dam et al., 1993; Balmelli and Piffaretti, 1995; Ryffel et al., 1999) it seems to be a discrepancy between clinical manifestations and *Borrelia* species in questing ticks in Norway, which could be a result of a bias in which cases are diagnosed, but it could also represent a higher virulence of *B. garinii*. Birds, especially the *Turdus* spp. have high vector competence for *B. garinii.* Small rodents may also harbour *B. garinii*, but not all ribotypes (Kurtenbach et al., 2002), and are favourable hosts for *B. afzelii* (Humair et al., 1995). The high fraction of neuroborreliosis suggests that the *Turdus* spp. play a major role in the epidemiology of clinical Lyme borreliosis in Norway. There were no ticks infected with *B. afzelii* on nymphs collected from birds at Lista (Paper V),

where a high proportion of northward migrating birds come from the British Isles. Although this was not a statistically significant difference, when comparing this result with ticks from birds caught at the more eastern localities, it is consistent with a main influx of ticks from England.

The Anaplasmataceae is a family of gram negative, obligate intracellular bacteria, comprising important pathogen genera, including several tick-borne species, such as *Ehrlichia ruminatum* and *Anaplasma phagocytophilum*. Many of the *Rickettsia* spp., in the important spotted fever group are tick-borne, as showed in Box I. There are no data for *Rickettsia* spp. in *I. ricinus* in Norway, but a Swedish study found *R. helvetica* in 16% of ticks collected from mammal hosts, although no rickettsial disease is endemic in Sweden (Nilsson et al., 1999). A recent study found that 11.3% of ticks collected from migratory birds in Sweden were infected with Rickettsial agents, mostly *R. helvetica* (Elfving et al., 2010). R. *Helvetica,* as well as *R. monacencis* and *R. slovaca,* which have also been found in *I. ricinus* (Simser et al., 2002), belong to the spotted fever group. Considering the wide range of pathogens that may be transmitted by *I. ricinus,* these findings strongly suggest that the pathogenic *Rickettsia* spp. may be transmitted by *I. ricinus.* Curiously, Hayes et al. (1980) found rickettsiae in spermatogonia, spermatocytes and maturing spermatids of *I. ricinus* and suggested that a veneric transmission of pathogens from adult males to females may occur. If this is true, it could also be true for other tick-borne pathogens.

A. phagocytophilum is a widespread pathogen (Dumler et al., 2005). Lundsett (2004) found *A. phagocytophilum* in 17.8% of ticks collected by flagging in Norway. Björsdorff et al. (2001) found *A. phagocytophilum* in 8.0% of *I. ricinus* nymphs from Swedish migratory birds, Alekseev et al., 2001) found the same pathogen in 14% in *I. ricinus* in Kaliningrad and Ogden et al. (2008) found *A. phagocytophilum* in 0.4% of *I. scapularis* nymphs in Canada. In connection with this project, 511 nymphs collected from

migratory birds were examined with PCR by the same method as used by Lundsett and found *A. phagocytophilum* in 13.1%. The findings were not confirmed by sequencing and were not published. This pathogen is established in most areas where *I. ricinus* is prevalent (Stuen and Bergström, 2001), and cervid animals represent a much more effective means of spreading ticks on the mainland and to coastal islands than birds; therefore, it seems reasonable to assume that the birds' contribution to spreading this ubiquitous pathogen has little biological significance, unless subgroups of different virulence exist.

The situation is completely different for *B. divergens*, which has become rare in some parts of the Norwegian coast (Paper IV). Possible explanations for this decline are changes in the use of pastures, and effective chemotherapy. The possibility that birds may reintroduce this parasite must be taken into account. A PCR with a primer specific for the *B. divergens* group (Lundsett, 2004) was performed on 511 nymphs from northward migratory birds, and *B. divergens* was found in 1.2% of them (unpublished results). Other *Babesia* spp., e.g., *B. microti* and *B. bovis,* occur in Europe (Meer-Scherrer et al., 2004) but have not been found in Norway. *B. canis* has recently been found in Norway for the first time (Øines et al., 2010). These *Babesia* spp. have not been included in this study. For *B. canis*, which has *Dermacentor reticulatus* as its main vector, the finding of a *Dermacentor* larva on a willow warbler (*Phylloscopus trochilus*) at Akeröya (Paper III) is of special interest. In Paper IV, the occurrence of antibodies against the bovine pathogen *B. divergens* in pasturing cattle along the southern Norwegian coast was examined. This pathogen is now a limited problem within its distribution range in southern Norway, but has the potential of re-emergence if letting cows pasture in the woods is recommended. A high fraction of cows (median about 50%) in the

western part of West Agder and in the eastern part of East Agder and in Telemark had antibodies against *B. divergens*, as opposed to a median of about 5% positives in the area between (Figure 2 in Paper IV). The areas where the fraction of *Babesia*-positive cows is high fit with the areas where the bird observatories are situated. Bird migration corridors could explain the high prevalence of *B. divergens* in cows in these areas, but there are no scientifically controlled data confirming that there are more migratory birds in these areas.

Although an important issue, the ticks in this study were not examined for TBEV. Four of the bird observatories in the study are situated on islands, and freezing and transport of frozen material for RNA-PCR would be difficult to carry out. Additionally preservation in 70% ethanol is a good method for keeping the ticks for later species identification, which could not be performed at the bird observatories. However, a Swedish group have examined ticks for TBEV (Waldenström et al., 2007) and they found TBEV in 2/529 larvae and 4/409 nymphs. TBE is an emerging disease in Norway; the first case was reported in 1998 (Skarpaas et al., 2002; 2006). The distribution is discontinuous with the rest of the distribution area of TBE in Europe. Between one and four million *Sylvia* warblers migrate to Norway each spring and bring ticks (Paper III). Many of these migrate across TBE-endemic areas in Central Europe before arriving in Norway. To bring TBEV-infected ticks from Central Europe to the endemic areas in Norway the birds have to cross at least 400 km of land and at least 112 km of open sea. This is barely possible: Although the maximum speed for the garden warbler, *Sylvia borin*, is 291 km per day, the mean speed of migration is 50 km per day, and *I. ricinus* larvae and nymphs remain on the host for just 3-4 days. A total of 835 northward migrating *Sylvia* warblers from 2003 and 2005 were examined, and 38 larvae (0.046/bird) and 63 nymphs (0.075/bird) of *I. ricinus* were found feeding on the birds. These species would, be expected to carry 45,000-180,000 larvae and 75,000-300,000 nymphs to Norway every year, but just a

small fraction of these would be transported from TBE-endemic areas. TBE is maintained in nature by a fragile enzootic transmission cycle (Randolph and Rogers, 2000), when larvae contract the virus by co-feeding with TBE-infected nymphs on small rodents. The transovarial transmission of TBEV is low: only about 2.4-7.8% of larvae born from an infected female are themselves infected (Danielová and Holubová, 1991; Randolph, 2008). Therefore, questing larvae of *I. ricinus* rarely carry TBEV. Considering the large number of larvae imported, there is a possibility that birds can import TBEV-infected tick larvae, which, after moulting to nymphs, could spread the virus further to local larva. Furthermore, infected nymphs imported to Norway could mature to the adult stage, and by transovarial transmission give birth to TBEV-infected larvae. Therefore, it is a small possibility that birds could spread TBEV, and it is difficult to imagine any other way the virus could have reached the southern Norwegian coast. Although the enzootic transmission cyclus is fragile, a climate model predicting the distribution of TBE (Randolph 2001) indicated favourable conditions at the Agder coast. Therefore, in principle, one single TBEV-infected tick could introduce the disease. The next question would be: why has this not happened before? TBE may well have been unrecognised in Norway for many years, and it may have died out because of the fragile enzootic transmission cyclus and been reintroduced by birds, or it may have been introduced during the last decades because TBEV is increasing in Europe. Traavik et al. (1978) reported TBEV in ticks from Western Norway, but this may have been LIV, as the identification was made by immunological methods. Traavik (1979) also found TBEV-antibodies in 19.6% of humans living in areas where *I. ricinus* occurs in Western Norway, but this may be due to cross-reacting antibodies. One Norwegian woman (a patient of Gunnar Hasle) had serous meningitis when she

lived in Risør (at the coast of East Agder, Norway) in 1987. At the time she had not been in any known area where TBE occurs, but Risør is at the coast of Aust Agder, near areas where TBE has later been found, and where LI has not been reported. She was examined at Ullevål University hospital for sequelae and was tested for TBE-antibodies in 2001. TBEV IgG was strongly positive. She had not had any other meningitis or encephalitis since 1987, and had not been in any area where TBE is known to occur. This may be the first case of TBE in Norway, although the first officially notified case was in 1998. The role of birds in the epidemiology of TBE is still unknown. It has been isolated from *I. uriae* and tissue from guillemot (*Uria aalge*) in the Murmansk area (Chastel 1988). LI, which is closely related to TBE and may cause disease in humans (Davidson et al., 1991), has been found in Western Norway (Stuen 1996). Nucleotide sequence analyses indicate that LIV has been recently transported to Norway from the British Isles (Gao et al., 11993).)

An alternative explanation to transport by birds is that TBEV has existed in the Scandinavian peninsula since Sweden and Denmark were connected 8-9,000 years ago (Björck, 1995) and that the virus has lived unrecognised in a zoonotic cycle. Phylogenetic analyses indicate time points of divergence of W-TBEV and FE-TBEV of 400 and 600 years before present (Zanotto, 1996; Haglund, 2000), which indicates a much more recent exchange of genetic material across the sea. The TBEV strain found in Norway is phylogenetically closely related to the strains found in northern continental Europe (Skarpaas et al., 2006).

This study has not proven that birds have introduced TBEV to Norway, but it can be concluded that it is possible that such transport has happened and that this is the most parsimonious explanation for the existence of TBEV north of the Skagerrak Sea.

Originally, the blood sampling from dairy cows (Paper IV) was intended to be a study of mapping the distribution of TBEV by

using cow sera, and to see if this distribution could be related to bird migration. It proved difficult to determine if antibody reactivity was really due to TBEV and not cross-reacting antibodies, and it was decided to use the material to examine for *Babesia divergens* instead (therefore, TBE was the issue in the original information sheet that was distributed to the farmers, and in the project description to the local agricultural authorities, see Appendix I and III).

CONCLUSIONS

It was previously known that migratory birds could harbour infected ticks. However, previous data could not be directly generalised to Norwegian birds, as migratory routes are different. The unique advantage of this study was the possibility to study the transport of ticks on birds that were presumed to have crossed an oceanic barrier of at least 112 km. The study revealed great differences in different bird species' capacity of transporting ticks, and that birds that feed on the ground, especially the *Turdus* spp., are particularly prone to carry ticks, as is found in previous studies in other countries. The finding of a larva of the genus *Dermacentor* on a willow warbler (*Phylloscopus trochilus*) at the Akerøya bird observatory represents a species not previously found in Norway. The only *Dermacentor* species that is common in northern Europe is *D. reticulatus*, a species that has not been previously found in Norway. This shows that a new tick species may be transported across the sea. The study also revealed two species of *Borrelia* that are "new", i.e., previously undiscovered, in Norway: *B. valaisiana* and *B. turdi*. The latter is an Asiatic species that is new for Europe, and it was found on northward migrating birds on Akerøya and Store Færder. These cases illustrate the birds' potential for importing new tick species and diseases. The prevalence of *Borrelia* in ticks caught on migratory birds in this study could be related to different migratory routes of Norwegian and Swedish birds. The study also showed that both infection of the ticks by infected bird hosts and by cofeeding with infected ticks may play a role in spreading infected ticks to new localities. Ticks carried by the *Turdus* spp. have more *Borrelia garinii* and *B. valaisiana* than ticks from other bird species, which adds to the importance of the *Turdus* spp.

The new microsatellites that were made for studying tick genetics were used to prove that several fathers may sire a brood of eggs from an *I. ricinus* mother. This may imply that if a bird occasionally brings an adult female tick to a new place with a suitable climate, the genetic variation in the tick's offspring would be sufficient to establish a new population.

This project, and ample evidence from other studies, supports the statement that birds have the potential of spreading ticks and tickborne diseases. The emergence of TBE in Norway is most probably a result of transport by migrating birds.

A probable increase in the amount of ticks on migratory birds was suggested by comparing the new data with Mehl et al.'s data (1984), and this adds to the knowledge about the consequences from changes in human uses of nature and climate change.

Paper IV shows that antibody testing of cows' sera is a feasible way of detecting the parasite *Babesia divergens* in an area, and this study showed that this parasite is abundant in two areas along the southern Norwegian coast, which are near the regions where the bird observatories are located. It is not known if the distribution of *B. divergens* can be causally related to possible bird migration corridors, but it is possible that birds may seed this parasite, and it seems obvious that eradication would depend on the eradication in all places where migrating tick hosts may pass.

The answer to the qualitative part of the aims of this study would be: Seeding of ticks to new areas and establishment of new tick populations is possible, provided the climate is suitable and hosts that can harbour adult ticks are present. Seeding of new tick-borne pathogens is possible when competent hosts are present. The quantitative part implies great uncertainties; at best orders of magnitude for transport of ticks by birds can be given.

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FIGURES AND TABLES.

Figure 1. Unrooted phylogenetic tree of the family Anaplasmataceae. From: Dumler et al., 2005, with permission.
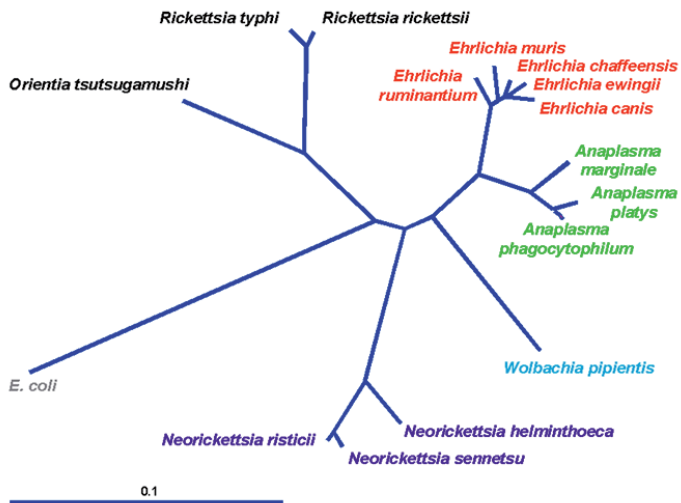
Figure 2. Unrooted phylogenetic tree of the *Borrelia burgdorferi*, sensu lato species complex. From Rudenko et al., 2009, with permission.
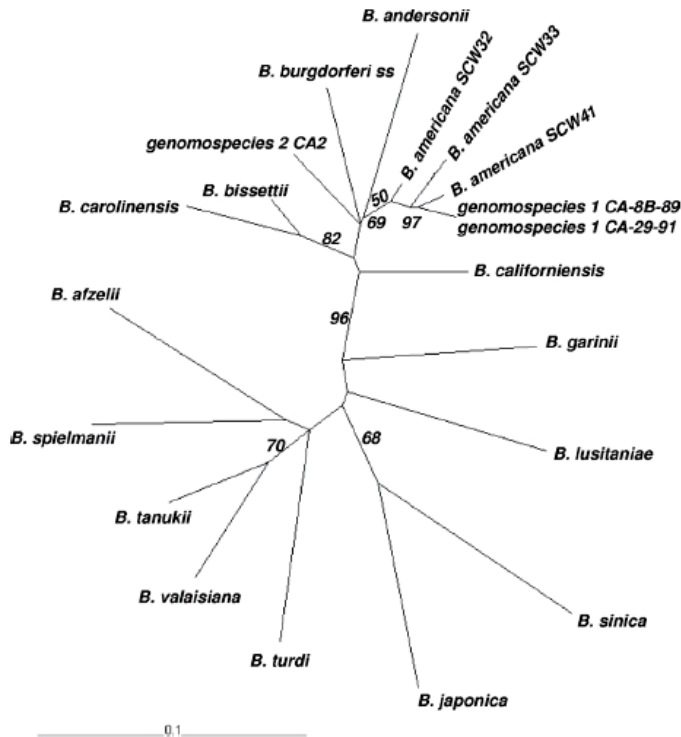

FIG. 1. Species phylogeny based on concatenated sequences of five genomic loci of control *Borrelia* species available from databases and obtained in this study. Maximum-likelihood settings were estimated using ModelTest. The maximum-likelihood phylogenetic tree was inferred using the best-fit evolutionary model GTR+Γ+I (with gamma distribution in four categories) for the *B. burgdorferi* data set in PhyML 3.0. Branch support was calculated by bootstrap analysis (500 replicates for molecular data set). The tree is unrooted. *Borrelia* species presented in Fig. 1 are as follows: *Borrelia burgdorferi* B31, *Borrelia afzelii* VS461, *Borrelia andersonii* 21133, *Borrelia bissettii* DN127, *Borrelia californiensis* CA443, *Borrelia carolinensis* SCW22, *Borrelia garinii* 20047, *Borrelia japonica* HO14, *Borrelia lusitaniae* PotiB2, *Borrelia sinica* CMN3, *Borrelia spielmanii* A14S, *Borrelia tanukii* HK501, *Borrelia turdi* Ya501, *Borrelia valaisiana* VS116, genomospecies 1 strain CA-8B-89, genomospecies 1 strain CA-29-91, genomospecies 2 strain CA-2, *Borrelia americana* strain SCW-41[T], *Borrelia americana* strain SCW-32, and *Borrelia americana* strain SCW-33. Bar, number of nucleotide substitutions per site. ss, sensu stricto.

Figure 3. Notified cases of chronic and disseminated Lyme borreliosis in Norway.
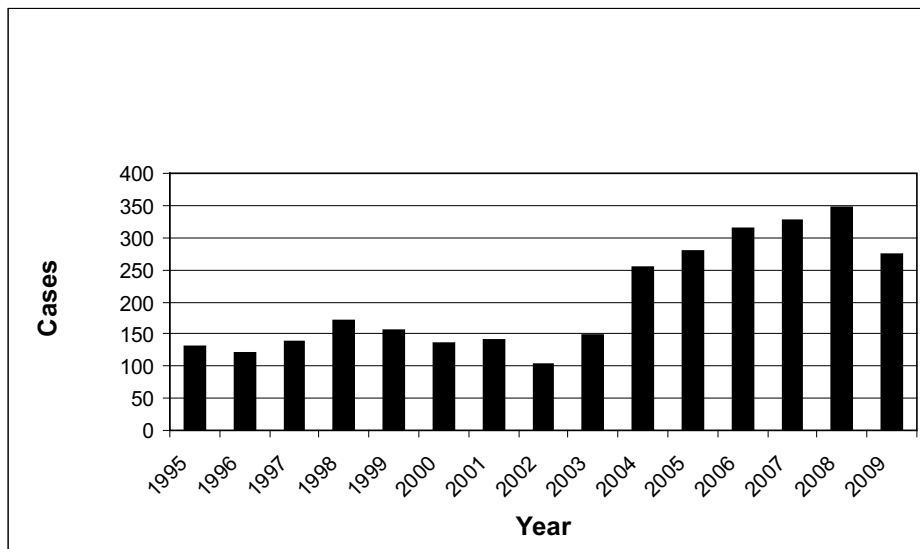Source: National Institute of public health http://www.msis.no/

Figure 4. The mobile instars of *Ixodes ricinus*: Larva, nymph, adult male, adult female and engorged female.

Figure 5. Example of a wind trajectory for the four included bird observatories (from West to East:) Lista, Jomfruland, Store Færder and Akerøya, with markings for where the air at 100 meters above ground was estimated to be each hour before 0500 UTC (i.e., 0700 h, Central European summer time) April 3rd 2003. Arrows give the wind directions; Long "feathers" represents 10 knots (=18,52 km/h), short "feathers" 5 knots, at 0500 UTC. Northward migrating birds would have strong head winds this night, and small passerine bird woulg not have been able to cross Skagerrak 100 meters above ground.
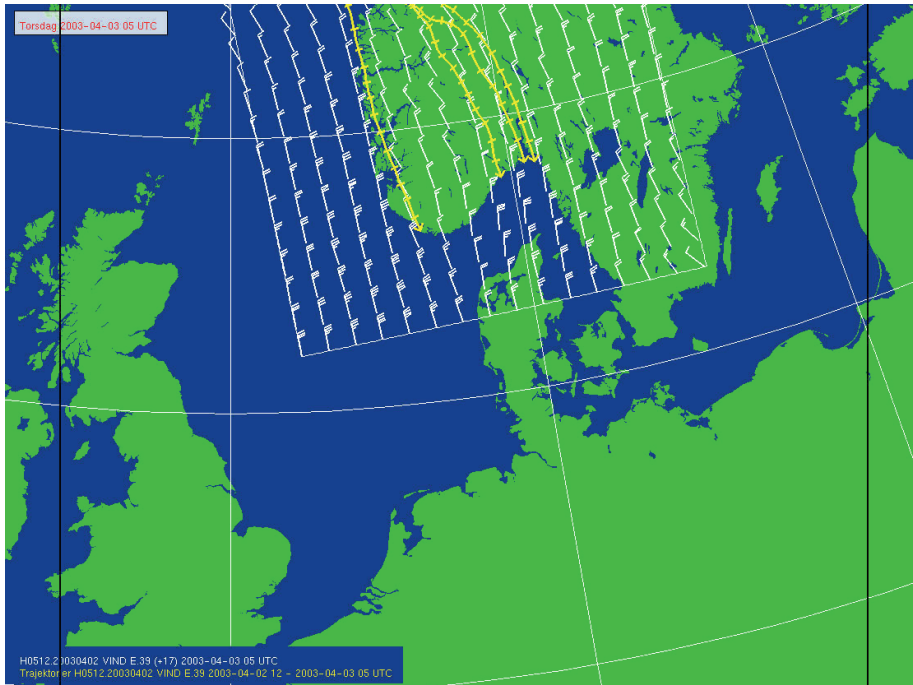
Figure 6. Japanese mist net.

Figure 7. A female red-backed shrike (*Lanius collurio*) is mildly protesing while being released from the net.

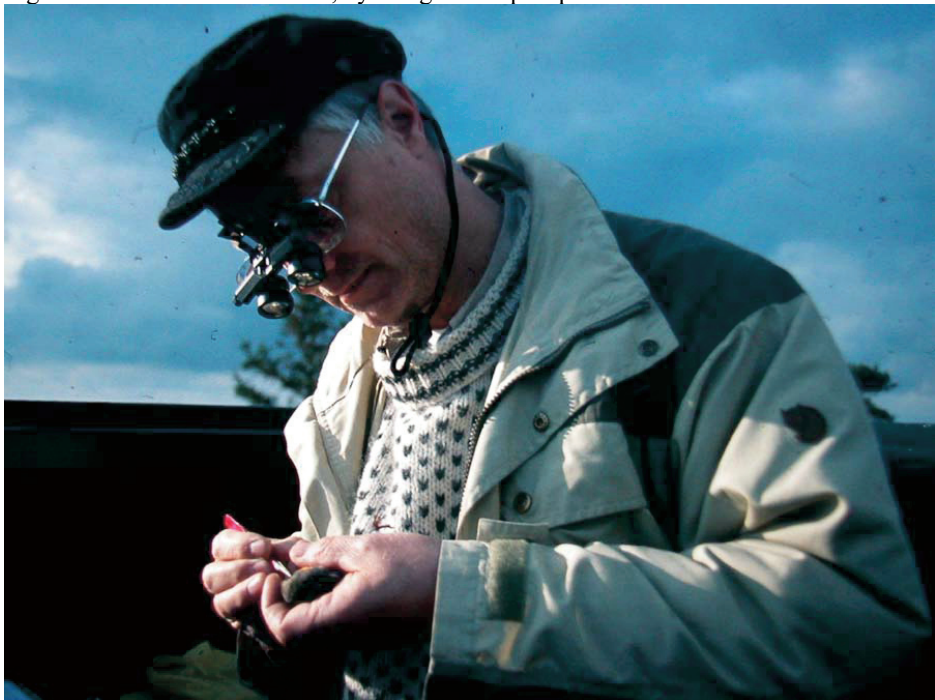Figure 8. Examination of a bird, by using telescopic spectacles.

Figure 9. Removing a fully engorged *Ixodes ricinus* larva from a female blackbird, *Turdus merula*.

Figure 10. Blood sampling from the tail vein of a dairy cow.

Figure 11. A perfect biotope for *Ixodes ricinus*: A coastal island, Jomfruland, with mild winters and humid air through the summer. Oak and hazelnut trees give plenty of storable food for small rodents, and leaves for protection. Adult ticks feed and mate on pasturing cows.

Figure 12. Four *Ixodes ricinus* nymphs cofeeding on the eyelid of a female blackbird, *Turdus merula*.

Table 1. Estimated numbers of Norwegian passerine migratory birds, compiled from Gjershaug et al., 1994.

Latin	English	Norwegian	Low estimate	High estimate
Acrocephalus schoenbaenus	Sedge Warbler	sivsanger	20000	200000
Acrocephalus scirpaceus	Reed Warbler	rørsanger	2000	20000
Alauda arvensis	Skylark	sanglerke	200000	1000000
Anthus cervinus	Red-throated pipit	lappipelerke	10000	40000
Anthus pratensis	Meadow Pipit	heipipelerke	2000000	4000000
Anthus spinoletta	Water Pipit	skjærpipelerke	100000	400000
Anthus trivialis	Tree Pipit	trepipelerke	2000000	4000000
Carduelis cannabina	Linnet	tornirisk	20000	30000
Carduelis flammea	Redpoll	gråsisik	200000	4000000
Carduelis flavirostris	Twite	bergirisk	200000	1000000
Carduelis spinus	Siskin	grønnsisik	200000	2000000
Delichon urbica	House martin	taksvale	400000	1000000
Emberiza schoeniclus	Reed Bunting	sivspurv	1000000	2000000
Erithacus rubecula	Robin	rødstrupe	1000000	3000000
Fidecula hypoleuca	Pied Flycatcher	s/h fluesnapper	400000	2000000
Fringilla coelebs	Chaffinch	bokfink	2000000	3000000
Fringilla montifringilla	Brambling	bjørkefink	2000000	4000000
Hippolais icterina	Icterine Warbler	gulsanger	100000	600000
Hirundo rustica	Swallow	låvesvale	200000	800000
Lanius collurio	Red-backed Shrike	tornskate	10000	20000
Lanius exubitor	Great Grey Shrike	varsler	10000	20000
Luscinia svecica	Bluethroat	blåstrupe	1000000	200000
Motacilla alba	White Wagtail	linerle	200000	1000000
Motacilla flava	Yellow wagtail	gulerle	200000	1000000
Muscicapa striata	Spotted flycatcher	gråfluesnapper	200000	1000000
Oenanthe oenanthe	Wheatear	steinskvett	1000000	2000000
Phoenicurus phoenicurus	Redstart	rødstjert	100000	1000000
Phylloscopus collybita	Chiffchaff	gransanger	200000	1000000
Phylloscopus sibilatrix	Wood Warbler	bøksanger	2000	20000
Phylloscopus trochilus	Willow Warbler	løvsanger	4000000	20000000
Prunella modularis	Dunnock	jernspurv	1000000	3000000
Pyrrhula pyrrhula	Bullfinch	dompap	20000	100000
Regulus regulus	Goldcrest	fuglekonge	1000000	2000000
Riparia riparia	Sand Martin	sandsvale	200000	500000
Saxicola rubetra	Whinchat	buskskvett	1000000	600000
Sturnus vulgaris	Starling	stær	400000	1000000
Sylvia atricapilla	Blackcap	munk	400000	1400000
Sylvia borin	Garden Warbler	hagesanger	400000	1400000
Sylvia communis	Whitethroat	tornsanger	100000	600000
Sylvia curruca	Lesser Whitethroat	møller	200000	600000
Troglodytes troglodytes	Wren	gjerdesmett	200000	1000000
Turdus iliacus	Redwing	rødvingetrost	2000000	3000000
Turdus merula	Blackbird	svarttrost	200000	2000000
Turdus philomelos	Song Thrush	måltrost	1000000	2000000
Turdus pilaris	Fieldfare	gråtrost	2000000	6000000
Turdus torquatus	Ring Ouzel	ringtrost	20000	200000
Other passerine species			6000	20000
Total			29120000	85770000

Table 2. Crosstabulation of visually evalated fat score, i.e., 1= lowest fat deposit, versus arrival at the peak arrival time for robins, birds caught late in the season, birds that were evulated as being "newly arrived" by the bird ringers and recaught birds, i.e., documented not newly arrived.

Robins, *Erithacus rubecula* at Lista, 2003.				
Fat score	Peak	After 22.april	"Newly arrived"	Recaught
1	15	5	20	0
2	22	16	57	11
3	28	17	42	15
4	1	1	1	1
Total	66	39	120	27

Table 3. Robins at Lista, tick prevalence versus visually evaluated fat score

Fat score	N birds	Birds with ticks	% (95%CI)
1-2	96	18	18.6(11.5-28.0)
3-4	69	4	5.8(1.6-14.2)

Table 4. Previous studies on ticks on birds (studies smaller than 700 examined birds are not included) . Categories: N=Northward migrating (sampling during spring); S=Southward migrating (sampling during fall); R=Resident birds (sampling when birds are not migrating)

	Country	Catergory	N birds	% infested	Pathogen detection
Alekseev et al., 2001	Russia	N+S	1606	6.8	*A. phagocytophilum* and *Borrelia* spp.
Björsdorff et al., 2001	Sweden	N+S	3054	2.4	*A. phagocytophilum*
Comstedt et al., 2006[8]	Sweden	N+S	13260	3.3	*Borrelia* spp.
Elfving et al., 2010	Sweden	N+S	13260	3.3	*Rickettsia* spp.
Hoogstraal et al.,1963 [9]	Egypt	S	31434	3.3	No
Ishiguro et al., 2000	Japan	S	1733	10.0	*Borrelia* spp.
Mehl et al., 1984	Norway	N	3943	4.2	No
Nicholls and Callister, 1996	USA		4256	9.4	*Borrelia* spp.
Nuorteva and Hoogstraal, 1963	Finland	N	2619	2.2	No
Ogden et al., 2008	Canada	N	39095	0.4	*A. phagocytophilum* and *Borrelia* spp.
Olsén et al., 1995	Sweden and Denmark	N	10575	3.0	*Borrelia* spp.
Olsén et al., 1995	Sweden and Denmark	S	12423	1.2	*Borrelia* spp.
Poupon et al., 2006	Switzerland	N+S+R	1270	15.5	*Borrelia* spp.
Scharf 2004	USA	R	1423	16.2	No
Smith et al., 1996	USA	N	11324	1.2	*Borrelia* spp.
Smith et al., 1996	USA	S	8607	0.2	*Borrelia* spp.
Stafford et al., 1995	USA	R	5297	15.2	*Borrelia* spp.
Waldenström et al., 2007	Sweden	N+S	13260	3.3	TBEV
Wiesbrod and Johnson 1989	USA	N+S	5131	1.1	*Borrelia* spp.

[8] Comstedt et al., 2006, Waldenström et al., 2007 and Elfving et al., 2010 are based on the same material.

[9] Hoogstraal et al. also examined northward migrating birds (Hoogstraal et al., 1961). They found 1025 immature ticks, but the total number of birds is not given.

APPENDICES

Reiseklinikken

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Oslo, *Date*

Undersøkelse av kuer med hensyn til skogflåttencefalitt (TBE) på Sørlandskysten

Undetegnede holder på med doktorgradsstudium om fuglers betydning for spredning av flått og flåttbårne sykdommer. I denne sammenheng ønsker vi å kartlegge utbredelsen av skogflåttencefalitt på Sørlandskysten. Skogflåttencefalitt er en alvorlig sykdom, som er et stort helseproblem i Sverige, Finnland, Baltikum og Sentral-Europa. Det er først i løpet av de siste årene denne sykdommen har dukket opp i Norge, se tabell i vedlagte prosjektbeskrivelse.

Sykdommen overføres til mennesker med flåttbitt, men kan også overføres ved å drikke upasteurisert geitemelk, og kanskje kumelk.

Kuer er velegnet til å finne ut om det finnes smitte blant den lokale flåtten, da de i løpet av sitt liv vil ha hatt mange hundre flått på seg, og én smittet flått sannsynligvis er nok til at kua blir smittet. Det vi undersøker er om kuene har antistoffer, det vil si om kuene har vært utsatt for smitte. De vil da være immune, og ikke smittefarlige. Om vi finner at kuene i et område har vært utsatt for smitte vil det være grunn til å være nøye med å forebygge flåttbitt (og kanskje vaksinere mennesker som bor i området?), og det vil ikke være tilrådelig å drikke upasteurisert melk fra kuer som beiter i det samme området. Kuene ser ikke ut til å bli syke av TBE-virus. Ingen karantenetiltak er påkrevet om vi finner TBE-antistoffer hos noen av kuene.

Det jeg lurte på var om jeg kan få lov til å komme på gården din og ta blodprøver av kuene dine. Blodprøvene tas fra undersiden av halen, og det ser ikke ut til å være plagsomt for kuene. Jeg kommer uten assistent, så jeg vil trenge litt hjelp til å holde halen oppe.

Jeg vil også trenge en liste over kuenes nummer/navn og alder og at du tegner inn beiteområdet på et kart som jeg tar med.

Jeg driver dette prosjektet uten støtte fra det offentlige, så jeg kan dessverre ikke tilby noe vederlag for bryet. Du vil kort tid etter innsamlingen få tilbakemelding om resultatet for dine kuer, resultatene vil senere bli publisert. Jeg tar sikte på å undersøke henimot 1000 kuer i løpet av de tre første ukene i april.

Jeg vil i første omgang be om at du fyller ut vedlagte spørreskjema, og returnerer det til meg så snart som mulig.

Med vennlig hilsen

Gunnar Hasle

Spørreskjema til kubønder på sørlandskysten

Skjemaet bes returnert i vedlagte ferdigfrankerte konvolutt så snart som mulig til:

Lege Gunnar Hasle
Reiseklinikken
St Olavs plass 3
0165 Oslo

Name; Place

Har du kuer på utmarksbeite?

I hvilket tidsrom av året går kuene på utmarksbeite?

Har kuene dine mye flått?

Har du merket en tydelig økning de siste ti årene?

Får jeg lov til å komme å ta blodprøver av kuene dine?

Hvis ja, vennligst marker på kartet nedenfor så nøyaktig som mulig hvor gården din ligger:

Til Landsbrukssjefene i Aust- og Vest-Agder Fylkeskommuner.

Prosjektbeskrivelse for innsamling av blodprøver fra kuer på Sørlandskysten.

Skogflåttencefalitt (TBE) er en ny sykdom i Norge.

```
TBE i Norge, meldt MSIS:
1995 1996 1997 1998 1999 2000 2001 2002 2003 2004
0    0    0    1    1    2    1    2    1    4
Smittested 1994-2004:
Norge 12 (Tromøya  2, Mandal 3, Lyngdal 1, Grimstad 1, Søgne 1,
Aust-Agder 1, Farsund 1, Songdal (Vest-Agder) 1,  Kristiansand
```

TBE-smitte opptrer svært lokalt, og det er av stor betydning å få kartlagt hvor smitten finnes.

Smitten vedlikeholdes i terrenget ved at flått smitter hverandre når de suger blod av smågnagere. Dyr og mennesker som blir bitt av smittede flått kan bli smittet av sykdommen.

Antistoff mot smitten kan påvises i blodet i mange år etter en infeksjon.

Peter Csángó og medarbeidere (1) fant antistoffer mot skogflåttencefalittvirus (TBEV) hos 52(16,4%) av 317 hunder som hadde fått tatt blodprøve hos veterinær Ellef Blakstad i Arendal.

Kuer er velegnet til å undersøke om et område er infisert av TBEV, da de ofte får mange flåttbitt, og vil derved ha stor sannsynlighet for å fange opp smitten.

Undertegnede har allerede gjort en undersøkelse av 28 kuer på Jomfruland, hvor vi ikke kunne påvise smitte.

Planlagt prosjekt.
Jeg ønsker å undersøke kuer som har gått på utmarksbeite langs kysten fra Farsund til Arendal, både de stedene hvor TBE forekommer og mest mulig jevnt fordelt de øvrige stedene. Det må være kuer som beiter i skog og skogkanter, og som er betydelig belastet med flått.

Jeg har snakket med sekretæren i Forsøksdyrutvalget, Espen Engh, som sier at det ikke trengs søknad til forsøksdyrutvalget for å ta blodprøver. Prøvetagningen forutsetter selvfølgelig at bøndene gir tillatelse.

Blodprøvene skal analyseres av undertegnede, på laboratoriet ved sykehuset i Kristiansand.

Resultatene skal publiseres i et vitenskapelig tidsskrift som et ledd i min medisinske doktorgrad. Hver enkelt kubonde får tilbakemelding om funn på sine kuer.

1. Csángó, P. A.; Blakstad, E.; Kirtz, G. C.; Pedersen, J. E.; Czettel, B. (2004): Tick-borne encephalitis in southern Norway. Emerg Infect Dis. 10(3):533-4

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II

MULTIPLE PATERNITY IN *IXODES RICINUS* (ACARI: IXODIDAE), ASSESSED BY MICROSATELLITE MARKERS

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ABSTRACT: This investigation examines multiple paternity in *Ixodes ricinus* (Acari: Ixodidae). Previous studies have shown that multiple mating occurs in this tick, but this is the first evaluation of multiple paternity. Three family groups were examined by a panel of polymorphic microsatellite loci; all ticks were bred from wild-collected engorged females with a copulating male attached. For most larvae, the attached males could be excluded as possible sire, and in the 3 tested families, at least 2 of 3 females mated successfully with more than 1 male. This finding suggests that multiple paternity is a common reproductive strategy in *I. ricinus*, which may have consequences for the ticks' dispersal success by increasing the genetic diversity in broods from single females colonizing new sites.

Mating patterns have received much attention in the study of the reproductive strategies of arthropods (e.g., Barnard, 2004; Alcock, 2005). Multiple paternity will increase the offspring's genetic diversity. This may have a positive effect on founding success by reducing the risk of local, stochastic extinction by inbreeding and by improving tolerance to environmental variability (e.g., Saccheri et al., 1998; Frankham et al., 2004). In Europe, *Ixodes ricinus* show clear signs of increasing abundance and expansion along the northern part of its range (Lindgren and Gustafson, 2001), and increasing attention has been paid to this species, notably as a vector of the pathogenic bacteria *Borrelia burgdorferi* s.l. and tick-borne encephalitis (Randolph, 2001). Several studies exist on the mating patterns of ixodid ticks (Bouman et al., 1999; Kiszewski et al., 2001; Zemek et al.. 2002), but multiple paternity in *I. ricinus* has not been studied specifically. In an observational study of *I. ricinus*, repeated mating was observed both in males (maximum 4 times) and females (maximum 2 times) (Graf, 1978). Multiple mating is known to occur in many genera of hard and soft ticks (Oliver, 1974), also in *Ixodes scapularis* (Yuval and Spielman, 1990) and *Ixodes uriae* (McCoy and Tirard, 2002). However, multiple mating does not necessarily mean multiple paternity. A more detailed study of the related species *I. uriae* (McCoy and Tirard, 2002) showed multiple paternity in 2 of 7 family groups tested by use of microsatellite analyses. To test whether this is also the case in *I. ricinus*, we analyzed 3 family groups, i.e., the female, the last mating male, and a selected number of hatching offspring for a panel of polymorphic microsatellite loci.

MATERIALS AND METHODS

Three fully engorged female *I. ricinus* with a copulating male attached were collected from cows on the island of Hille in West Agder, Norway. After completing copulation in a glass vial, the males were removed and stored in 70% ethanol. The engorged females were incubated at room temperature until oviposition, and the eggs were incubated further until hatching of larvae took place. When the larvae emerged, they and the female were also transferred to 70% ethanol. Fifteen larvae in 2 family groups and 18 larvae in 1 group were examined. In using larvae instead of eggs, we assumed all larvae to be diploids, i.e., no homozygosity due to unfertilized eggs (see McCoy and Tirard, 2002).

The specimens were crushed using the tip of a glass rod and DNA

was isolated using DNeasy® Tissue kit (QIAGEN, Valencia, California). DNA was amplified in polymerase chain reaction (PCR) with 10 different *I. ricinus*-specific microsatellite primers (IRN-4, IRN-7, IRN-8, IRN-12, IRN-14, IRN-15, IRN-17, IRN-30, IRN-31, and IRN-34; Røed et al., 2006). In our analysis of paternity, we used only the 3 loci where both the mother and the attached male were in most cases scored as heterozygote, i.e., IRN-14, IRN-15, and IRN-34. The forward primers were end-labeled with fluorescence, and the PCR was performed on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, California). Each PCR contained 10-ml reaction mixtures, 20–40 ng of genomic template DNA, 50 mM KCl, 1.5 mM MgCl, 10 mM Tris-HCl, 0.2 mM dNTP, and 0.5 U of *Taq* polymerase (QIAGEN). Thermocycling parameters after denaturation at 94 C for 5 min were 30 cycles of 95 C for 1 min, annealing temperature (cf. Røed et al., 2006) for 30 sec, followed by extension at 72 C for 1 min. The last polymerization step was extended to 10 min. PCR products were added to buffer containing formamide and 5-carboxytetramethylrhodamine–labeled standard (Applied Biosystems), and electrophoresed using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

In contrast to the microsatellites used by McCoy and Tirard (2000, 2002) in *I. uriae*, microsatellites of *I. ricinus* are often associated with genotypes deviating from Hardy–Weinberg equilibrium by heterozygote deficiency and cases of nonamplified products (cf. de Meeûs et al., 2002; Røed et al., 2006). Such genotypic errors are usually related to null alleles (e.g., Dakin and Avise, 2004) and allelic dropout (e.g., Wattier et al., 1998), both of which may introduce substantial errors into empirical assessments of species mating systems by scoring individuals erroneously as homozygotes (Blouin, 2003; Dakin and Avise, 2004). Therefore, it is necessary to account for the possibility that some alleles might be present without being scored and that some apparent homozygotes may be due to null alleles. We adopted this conservative approach to avoid overestimating multiple paternity, although it could lead to an underestimation.

RESULTS

In all families, there were several loci for which the mother and her larvae were scored as different homozygotes (Table I). This suggests the frequent presence of null or nonamplified alleles. In all cases where the mothers were scored as heterozygous, 1 of the maternal alleles was detected in all larvae (except for some larvae where no product was amplified at certain loci). For family 1, there were 4 different alleles in IRN-14 of which 1 did not occur in the copulating pair. This is consistent with 1 additional sire being involved. A previous male with IRN-14 104/108, IRN15 101/112, and IRN-34 114/116 could have sired all the offspring. Regardless, the attached male could be excluded as possible sire for 5 of the 15 larvae (Table I). In family 2, at least 2 sires were involved, as there were 3 nonmaternal alleles both in IRN-14 and IRN-15. The attached male could not have sired any of the tested offspring. Three larvae showed no amplified allele in locus IRN-14, whereas the mother was

TABLE I. Alleles scored in 3 polymorphic microsatellite loci in 3 family groups of *I. ricinus*. Alleles excluding the attached male as sire are marked in bold.

DNA-nr	IRN-14		IRN-15		IRN-34	
Mother 1	110	112	101	101	97	97
Attached male 1	104	106	112	112	114	116
Larva 1.1	**108**	110	101	101	97	97
Larva 1.2	**108**	110	101	101	97	97
Larva 1.3	104	110	101	101	97	114
Larva 1.4	**108**	110	101	101	97	97
Larva 1.5	104	112	101	101	97	114
Larva 1.6	104	112	101	112	97	114
Larva 1.7	**108**	112	101	101	97	116
Larva 1.8	**108**	110	101	101	97	114
Larva 1.9	**108**	110	101	112	97	114
Larva 1.10	**108**	110	101	112	97	116
Larva 1.11	**108**	112	101	112	97	116
Larva 1.12	**108**	112	101	101	97	116
Larva 1.13	104	110	101	101	97	114
Larva 1.14	**108**	110	101	101	97	116
Larva 1.15	104	110	101	101	97	114
Mother 2	112	122	97	99	114	114
Attached male 2	106	154	97	101	114	120
Larva 2.1	112	**130**	**83**	97	0	0
Larva 2.2	122	122	**83**	97	110	110
Larva 2.3	112	122	**83**	99	114	114
Larva 2.4	122	122	97	**112**	110	110
Larva 2.5	0	0	**83**	99	116	116
Larva 2.6	112	122	**83**	99	110	114
Larva 2.7	122	**130**	**83**	97	116	116
Larva 2.8	122	122	**83**	99	114	116
Larva 2.9	112	**130**	**83**	99	110	110
Larva 2.10	112	122	99	**112**	114	114
Larva 2.11	0	0	97	**112**	114	116
Larva 2.12	112	**130**	**83**	97	114	116
Larva 2.13	**108**	122	97	101	0	0
Larva 2.14	122	**130**	**83**	99	116	116
Larva 2.15	122	**130**	0	0	116	116
Larva 2.16	122	122	**83**	99	0	0
Larva 2.17	112	122	97	**112**	114	116
Larva 2.18	0	0	97	**112**	114	116
Mother 3	108	108	83	114	116	124
Attached male 3	108	110	83	101	97	114
Larva 3.1	108	110	83	101	124	124
Larva 3.2	108	120	83	101	124	**142**
Larva 3.3	108	120	83	114	116	116
Larva 3.4	108	164	114	114	114	116
Larva 3.5	108	120	83	114	116	116
Larva 3.6	108	120	83	114	124	**142**
Larva 3.7	108	120	83	101	124	124
Larva 3.8	108	120	83	114	116	116
Larva 3.9	108	164	83	83	114	116
Larva 3.10	108	120	101	114	116	**142**
Larva 3.11	108	120	101	114	124	124
Larva 3.12	108	108	83	101	116	116
Larva 3.13	108	148	83	114	124	124
Larva 3.14	108	120	83	83	124	124
Larva 3.15	108	128	83	114	116	116

heterozygous. This inconsistency may be due to low amplifiability. Some microsatellites may be more difficult than others to amplify. Furthermore, in tick larvae there are very small amounts of DNA. In family 3, 6 different alleles of IRN-14 were noted in the larvae. Even if there were a nonamplified maternal allele in the mother, at least 3 fathers would be necessary to sire this brood.

DISCUSSION

Even though we analyzed a limited number of larvae in each family group and applied a highly conservative criterion for accepting additional fathers, we have shown that at least 2 of 3 females studied mated successfully with more than 1 male. Seemingly, the copulating males contributed little, if anything, to the genes of the larvae tested. As *I. ricinus*, females lay about 2,000 eggs (Randolph, 1998), several more successful conceptions might have taken place in addition to those observed in our analyzed material. In a similar study of *I. uriae*, McCoy and Tirard (2002) found multiple paternity (in 2 of 7 family groups), and they discussed this in relation to climate variability of its habitat and adaptation to host responses. The latter argument seems particularly relevant for *I. ricinus*, which can parasitize virtually any mammal and bird living in their habitat. However, the fact that 2 species with a different biology show multiple paternity suggests that this may be a more general characteristic of ticks, not necessarily explained by special adaptations in each species. This view is supported by the fact that multiple mating is common in Ixodidae (Oliver, 1974), although data on actual multiple paternity are lacking. Genetic studies of family groups of other tick species would be needed to determine whether *I. uriae* and *I. ricinus* are unique in this respect.

In its northern distribution range, such as in Norway, *I. ricinus* are accidentally found in new locations, probably brought there by birds, because birds are known to carry ticks (e.g., Mehl et al., 1984). Multiple paternity, as found by McCoy and Tirard (2002) and the present study, may have considerable consequences for the ability of engorged females to colonize new sites, and for improving the overall fitness by producing offspring with variable genotypes (McCoy and Tirard, 2002). With multiple paternity, 1, or very few, females could be in a position to produce offspring with sufficient genetic diversity to form a founder population.

Multiple mating may also be important in increasing the likelihood of the sexual transmission of pathogens between ticks, a mechanism that has been experimentally demonstrated for both tick-borne encephalitis virus (Chunikhin et al., 1983) and *Borrelia* sp. (Alekseev et al., 1999). Furthermore, the spotted fever group, Rickettsiae, has been detected in immature spermatozoa of *I. ricinus* (Hayes et al., 1980), suggesting that this class of pathogens may also be transmitted sexually. Because *I. ricinus* copulates several times, this may partly explain the much higher prevalence of tick-borne encephalitis virus found in engorged ticks than in specimens collected by flagging in endemic areas (Asokliene, 2004; Suss et al., 2004).

Thus, multiple mating may be of importance to the probability of a tick acting as a disease vector. If multiple paternity improves the ability of ticks to colonize new habitats and to

resist the immune responses of hosts, it will indirectly be important for disease dynamics.

ACKNOWLEDGMENTS

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III

TRANSPORT OF TICKS BY MIGRATORY PASSERINE BIRDS TO NORWAY

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ABSTRACT: Ticks can be transported over large distances and across geographical barriers by avian hosts. During the spring migrations of 2003 to 2005, 9,768 passerine birds from 4 bird observatories along the southern coastline of Norway were examined for ticks. Altogether, 713 birds carried a total of 517 larvae and 1,440 nymphs. The highest prevalence of tick infestation was observed in thrushes and dunnock (*Prunella modularis*). The degree of tick infestation varied during each season, between localities, and from year to year. Blackbirds (*Turdus merula*) caught in localities with many ticks had greater infestation than those from localities with few or no ticks, suggesting local tick recruitment. A similar study performed during 1965–1970 involving 2 of the bird observatories in the present study found ticks on 4.2% of birds, while we found infestation of 6.9% at the same localities ($P < 0.001$). With the exception of 10 nymphs and 1 larva, the predominant tick was *Ixodes ricinus*. Seven nymphs of *Hyalomma rufipes* and 1 larva of *Dermacentor* sp. were also found. No species of *Dermacentor* had previously been found in Norway.

There has been a slight northward and westward extension of the distribution of the sheep tick *Ixodes ricinus* (Acari: Ixodidae) along the northern margin of the population distribution (Lindgren et al., 2000). Between 1990 and 2000, the number of reported cases of tick-borne diseases such as borreliosis and tick-borne encephalitis (TBE) increased in northern Europe (Randolph, 2001; Bröker and Gniel, 2003). TBE is an emerging disease in Norway (Skarpaas et al., 2006). Norway forms part of the northern border of the distribution of *I. ricinus*, which is found as far north as N 65°30′ in coastal areas (Mehl, 1983). During the 1930s, veterinarians practicing in southern Norway recorded *I. ricinus* only from near-coastal regions (Thambs-Lyche, 1943). In recent years, however, it has been regularly observed up to 80 km from the coast. It has been suggested that this development is a combination of climate change and altered agricultural practices, with extensive areas of previous pastureland being overgrown (Gray et al., 2009). There is medical and public concern that the expansion of this species and its associated tick-borne pathogens will continue in Norway. Similar changes have been predicted for the closely related species *Ixodes scapularis* in North America (Ogden et al., 2008).

As is typical for ixodid ticks, *I. ricinus* is a 3-host tick with 4 developmental stages, i.e., egg, larva, nymph, and adult. A single blood meal from each host is required for the tick to molt from each molde stage to the next and for the adult female to produce eggs. Larvae and nymphs may feed on mammals and birds of all sizes, and even reptiles. Adults require medium- to large-sized mammal hosts (Jaensson, 1994).

Ixodes ricinus has low mobility and is, therefore, dispersed by its host species. Large mammals, in particular, cervids, may be heavily infested with ticks; therefore, they have a high impact on short- and medium-range transport. Birds, on the other hand, may transport ticks over long distances and across geographical barriers. It is well known that ticks may be transported by migratory passerine birds (Hoogstraal et al., 1961, 1963; Mehl et al., 1984; Olsén et al., 1995; Smith et al., 1996; Ishiguro et al., 2000; Alekseev et al., 2001).

Ixodes ricinus is a vector for a wide range of pathogens of humans and other animals, i.e., arboviruses such as Louping ill virus and Western TBE virus, *Borrelia* spp., *Babesia* spp., *Anaplasma* (*Ehrlichia*) spp., *Rickettsia* spp. (Estrada-Peña and Jongejan, 1999), and *Franciscella tularensis* (Brantsæther et al., 1998). These pathogens have different geographical distributions; however, changes in climate and agricultural practices may be changing the suitability of tick habitats. This could have implications for their emergence and further expansion along the northern part of their distribution range, including Norway. Pathogens such as *Borrelia*, *Anaplasma*, and TBE virus have been found on ticks collected from birds in Sweden (Olsén et al., 1995; Bjöersdorff et al., 2001; Waldenström et al., 2007), highlighting the importance of a better understanding of birds as vectors for tick transport in general. In Norway, most migratory birds cross the Skagerrak, Kattegat, or North Sea when returning from their wintering areas each spring. This migration is monitored systematically at a number of bird observatories along the coast, which also offer a valuable opportunity to study general aspects of the long-distance transport of ticks. Most of the migratory passerine birds in Norway follow a western pathway along the Atlantic coast of continental Europe from their wintering areas in southern Europe or Africa. Blackbirds (*Turdus merula*) mainly winter on the British Isles, while the *Sylvia* spp. migrate broadly over the Alps; a few species, such as pied wagtail (*Motacilla alba*), redpoll (*Carduelis flammea*), and bluethroat (*Luscinia svecica*), even migrate east of the central European mountain ranges (Bakken et al., 2006).

In the present study, we utilized the established bird observatories along the southern coast of Norway to obtain data on the transport of ticks by birds across an extensive geographical barrier represented by the Kattegat, Skagerrak, and North Sea. Since species with different migratory routes may encounter ticks and pathogens from different parts of Europe, we chose to cover the whole coast from the Swedish border to the southwestern tip of Norway to track as much of the spring migration as possible. Our objective was to improve the understanding of the potential

role of migrant passerine birds in the present expansive dynamics of ticks and tick-borne pathogens in northern Europe.

By quantifying the prevalence of ticks on birds arriving at these bird observatories during spring migration, we addressed the following questions. First, which bird species are most important for this transportation? Second, does the pattern of tick infestation of migratory birds vary between localities and year, and over the course of the spring? If so, is this due to spatial and temporal variation within species or to different species composition in the localities? Finally, does a comparison of our data with previous data indicate an increase in tick transport by birds?

MATERIALS AND METHODS

Four bird observatories covering the main migratory routes participated in this study from 2003 to 2005, i.e., Akerøya, Store Færder, Jomfruland, and Lista (Fig. 1), which accounted for 2,385, 2,231, 2,588, and 2,564 birds, respectively. Akerøya and Store Færder are rocky islands with a few sheep. Flagging of ticks was performed using a 50 × 80 cm white towel mounted on a stick (Green, 1931). It was moved at a speed of about 1 m/sec. The towel was inspected and ticks were collected after every 2–3 sec of contact with the vegetation. For the maximum yield of ticks, we chose partly shadowed areas when searching. Ticks are rare on Akerøya and Store Færder (Akerøya, for example, yielded 5 ticks in 6 hr of flagging, while Store Færder yielded no ticks after 2 hr of flagging). Jomfruland is a much more fertile island with deciduous (large numbers of oak and hazelnut trees) and coniferous forests, scrub, and pastureland grazed by cattle. Ticks are abundant on Jomfruland, and more than 100 ticks were collected per hour of flagging. Lista is on the mainland, with coniferous forest, wetlands, and pastureland in the vicinity of the bird observatory. Ticks are common, with 40 ticks collected per hour of flagging in a wooded area. Despite the availability of hosts (sheep and rodents), the number of ticks on Akerøya and Store Færder is very low, likely due to short vegetation, with few habitats suitable for the ticks.

Birds were examined for ticks during the spring (northward) migration from the middle of March or beginning of April (depending on the procedures of the bird observatories) to the end of May. Birds were caught for ringing with mist nets and were routinely examined for ticks around the eyes, beak, and ear openings, where the majority of ticks are found (Mehl et al., 1984). The ticks were picked off with tweezers and placed in 70% ethanol for subsequent examination. For each bird examined, the date, ring number, species, sex, age, and number of ticks found were recorded. The ticks were examined using a stereomicroscope for species identification.

Only passerine birds were included in this study. Individuals that had been examined earlier in the same season, as well as birds with brooding patches and juveniles, were excluded from the study as these were evidently not new migrants. Both nymphs and larvae were collected; however, we focused on nymphs, as they are more important than larvae as pathogen vectors. Due to high interstadial mortality (Randolph, 1998), nymphs would also be far more effective than larvae as potential founding animals in a situation of range expansion.

Previous studies of tick transport by birds have not explored the possibility that ticks might be acquired locally. We looked at differences between localities with no, few, and many local ticks and compared the infestation of migratory birds in order to sort out the impact of local tick populations.

The effects of area, year, and month on observations of the number of nymphs per bird were tested using a generalized linear model (GLM) (McCullagh and Nelder, 1989). Interaction effects between year and area, month and area, and month and year were compared. The number of nymphs per bird can be expected to follow a Poisson-type distribution, indicating a log link function for the GLM. Examination of the mean number of nymphs and the variance of the distribution by area revealed high overdispersion. Therefore, a negative binomial distribution with a dispersion parameter set at 8 was chosen, using SPSS version 15 (SPSS; Chicago, Illinois).

A comparison of means was also performed with the GLM (log link and quasi-Poisson distribution family), using R statistical software (R Development Core Team, 2008). To compare the frequency of infestation
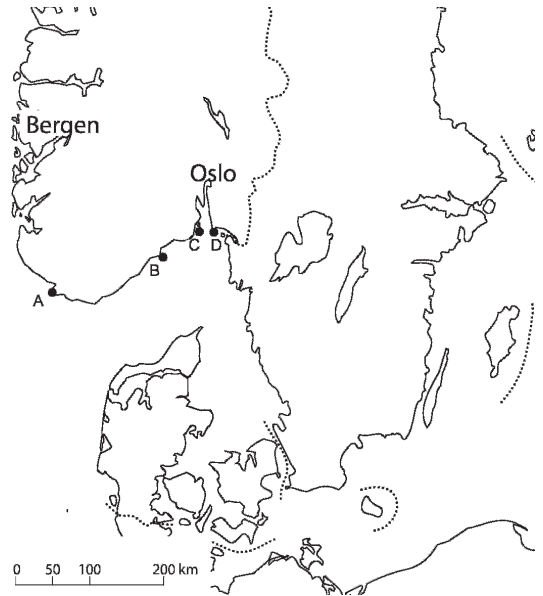
FIGURE 1. Bird observatories participating in the project. (A) Lista, (B) Jomfruland, (C) Store Færder, and (D) Akerøya. The westerly location of Lista makes it a more likely reception point for birds from the British Isles than the other bird observatories. The vast majority of migratory birds that migrate along the Atlantic coast must pass Jutland, Denmark. Akerøya may also receive some birds migrating along the Swedish coast.

with previous data from 2 of the 4 locations, we performed a chi-square test.

Graphs of the mean number of nymphs per bird with bars of 1 standard error (1 SE) were constructed using SPSS version 15. To illustrate the impact of blackbirds, which had much higher infestation rates than other bird species, they were treated separately.

The ticks were only handled with tweezers that were heated with a flame after each use; accordingly, the participants in the study were at no risk of contracting pathogens from the ticks. The National Board of Animal Experimentation approved the field collecting and handling of the birds.

RESULTS

The importance of the different bird species

This study included 9,768 birds, of which 713 (7.3%) carried ticks. In total, we collected 517 larvae and 1,440 nymphs. There were large variations in the contributions of the different bird species to the number of ticks collected due to differences in both tick prevalence and the number of each bird species caught (Table I). Of 65 different bird species examined, 23 were represented by at least 50 individuals. We found 84.4% of the nymphs and 74.5% of larvae on 6 bird species, i.e, blackbird, dunnock (*Prunella modularis*), redstart (*Phoenicurus phoenicurus*), robin (*Erithacus rubecula*), song thrush (*Turdus philomelos*), and willow warbler (*Phylloscopus trochilus*). The thrush family (Turdidae) included 25.3% of the birds caught, but 63.0% of the tick-infested birds in our sample, and they hosted 78.8% of all the nymphs collected. The large thrushes often carried many ticks on a single host. Blackbirds alone accounted for 54.2% of all nymphs

TABLE I. Total number of tick nymphs and larvae on birds represented with at least 50 individuals in our sample.

		% With ticks	Larvae	Nymphs	Birds	Nymphs per bird
Turdus merula	Blackbird	31.5	102	781	543	1.44
Erithacus rubecula	Robin	12.7	182	156	1,263	0.12
Turdus philomelos	Song thrush	25.1	22	97	187	0.52
Prunella modularis	Dunnock	15.7	12	91	198	0.46
Phoenicurus phoenicurus	Redstart	20.3	22	48	207	0.23
Phylloscopus trochilus	Willow warbler	2.1	45	42	3,383	0.01
Fringilla coelebs	Chaffinch	10.6	18	26	198	0.13
Sylvia curruca	Lesser whitethroat	6.1	4	23	329	0.07
Sylvia atricapilla	Blackcap	6.6	29	20	363	0.06
Sylvia communis	Whitethroat	13.3	5	20	143	0.14
Regulus regulus	Goldcrest	2.0	14	12	244	0.05
Carduelis chloris	Greenfinch	5.3	6	12	187	0.06
Carduelis flammealcabaret	Redpoll	2.8	7	8	316	0.03
Oenanthe oenanthe	Wheatear	4.7	0	8	107	0.07
Carduelis cannabina	Linnet	4.8	2	7	126	0.06
Sylvia borin	Garden warbler	1.5	0	6	339	0.02
Phylloscopus collybita	Chiffchaff	2.2	7	5	549	0.01
Troglodytes troglodytes	Wren	9.3	11	5	54	0.09
Anthus pratensis	Meadow pipit	4.3	3	4	164	0.02
Turdus pilaris	Fieldfare	3.6	0	2	56	0.04
Fidecula hypoleuca	Pied flycatcher	1.1	2	1	94	0.01
Motacilla alba	Pied wagtail	1.5	0	1	67	0.01
Hippolais icterina	Icterine warbler	0.0	0	0	66	0.00
Carduelis spinus	Sisikin	0.0	0	0	50	0.00
Other species, <50 individuals		7.5	24	65	535	0.11
Total		7.3	517	1,440	9,768	0.15

collected. On Jomfruland, 11 of the blackbirds caught had more than 15 ticks. Among the small thrushes, robins were a very common species and possessed a fairly high number of nymphs per bird (Table I). Apart from the thrushes, dunnock and chaffinch (*Fringilla coelebs*) also showed a high to moderately high degree of infestation. Willow warblers had very few ticks, but owing to their high abundance, they ranked sixth in total number of nymphs collected per species (Table I). The *Sylvia* sp. warblers were fewer in number, but exhibited somewhat higher infestation levels than the 2 *Phylloscopus* spp. In contrast, ticks associated with some very common birds were present in low numbers. Tree pipit (*Anthus trivialis*) was represented by only 11 birds in our sample, but carried 9 larvae and 2 nymphs, while large numbers of the species were seen and heard flying over the Akerøya bird observatory without stopping.

Spatial and temporal variation

The distribution of numbers of different bird species and numbers of nymphs in different localities, years, and times of capture are summarized in Tables II and III. The number of ticks per bird varied significantly between year, site, and season (Figs. 2, 3). There were also significant interactions between site and year, and month and year, but not between site and month. The lack of significance in the last interaction indicates that similar patterns in monthly variation were observed at all bird observatories (Fig. 3; Table IV).

With 0.260 nymphs per bird, Jomfruland generally had a higher number of nymphs per host than the other localities, compared with Akerøya at 0.089, Lista at 0.140, and Store Færder at 0.087

($P < 0.001$). This was mainly related to infestations on blackbirds at Jomfruland, with 4.49 nymphs per bird (n = 105) (Tables II, V; Figs. 4, 5). By comparison, there were 0.71 nymphs per blackbird (n = 438) at the other observatories ($P < 0.001$).

The number of nymphs per bird was unusually high in 2004 at Jomfruland and Store Færder (Fig. 2). The blackbird's dominant role as a tick carrier caused the high prevalence at Jomfruland in 2004 and was most likely related to local recruitment (see below). At Store Færder we also saw a peak in 2004, but this was not related to blackbirds, as can be seen when blackbirds are excluded from the dataset (Fig. 5). That year at the Store Færder site, there was an unusually high number of robins and dunnock, with a high number of nymphs per bird, and a low number of willow warblers with very few nymphs (Tables I, II). This was not the case at the other bird observatories. Therefore, the peak at Jomfruland in 2004 was caused by changes in prevalence within a single host (blackbirds), while at Store Færder, variations were caused by numeric dominance of different species.

For all localities, the total number of nymphs per bird caught was lower in May than in April (Fig. 3), due to the arrival of large numbers of willow warblers, with few ticks. In fact, 2,939 of the 5,937 (49%) birds caught in May were willow warblers. All species had a comparatively low number of ticks in March (Table III), and for the 3 species that contributed most to our sample, there was also an increase from April to May. Blackbirds had 1.4 nymphs per bird (n = 233) in April and 4.2 nymphs per bird (n = 100) in May ($P < 0.001$); song thrushes had 0.4 nymphs per bird (n = 141) in April and 1.2 nymphs per bird (n = 37) in May ($P = 0.004$); and robins had 0.11 nymphs per bird (n = 1,036) in April and 0.29 nymphs per bird (n = 134) in May ($P < 0.001$). Very few

TABLE II. The number of birds and tick nymphs caught each year at the different bird observatories in this study (see text). The bird species on which most nymphs were found are presented separately (*Sylvia* spp. are grouped together).

	2003		2004		2005		Total	
	Birds	Nymphs	Birds	Nymphs	Birds	Nymphs	Birds	Nymphs
Lista								
Erithacus rubecula	137	20	90	5	179	15	406	40
Fringilla coelebs	17	1	51	2	49	2	117	5
Phoenicurus phoenicurus	11	0	30	4	10	3	51	7
Phylloscopus trochilus	66	1	218	3	143	0	427	4
Prunella modularis	20	3	33	36	20	7	73	46
Sylvia spp.	69	2	90	1	66	2	225	5
Turdus merula	44	97	40	31	107	63	191	191
Turdus philomelos	11	17	11	2	40	2	62	21
Other species	296	17	274	2	442	22	1,012	41
Total	671	158	837	86	1,056	116	2,564	360
Jomfruland								
E. rubecula	112	8	20	10	66	1	198	19
F. coelebs	16	17	4	0	17	3	37	20
P. phoenicurus	11	5	4	0	16	7	31	12
P. trochilus	172	0	469	19	676	5	1,317	24
P. modularis	31	4	1	5	6	8	38	17
Sylvia spp.	239	23	70	11	126	16	435	50
T. merula	40	134	18	234	47	103	105	471
T. philomelos	10	9	0	0	5	6	15	15
Other species	136	16	42	4	234	26	412	46
Total	767	216	628	283	1,193	175	2,588	674
Store Færder								
E. rubecula	28	3	233	41	65	2	326	46
F. coelebs	1	0	14	0	22	0	37	0
P. phoenicurus	20	4	22	6	12	0	54	10
P. trochilus	349	1	54	1	340	1	743	3
P. modularis	3	1	47	22	14	0	64	23
Sylvia spp.	210	2	72	4	82	1	364	7
T. merula	14	20	46	24	75	15	135	59
T. philomelos	3	3	18	17	12	2	33	22
Other species	111	6	146	15	218	2	475	23
Total	739	40	652	130	840	23	2,231	193
Akerøya								
E. rubecula	93	16	76	19	164	16	333	51
F. coelebs	1	0	0	0	6	1	7	1
P. phoenicurus	9	3	36	10	26	6	71	19
P. trochilus	86	2	404	5	406	4	896	11
P. modularis	2	0	7	3	14	2	23	5
Sylvia spp.	46	2	56	2	49	3	151	7
T. merula	11	6	28	12	73	42	112	60
T. philomelos	8	1	16	10	53	28	77	39
Other species	124	5	252	5	339	10	715	20
Total	380	35	875	66	1,130	112	2,385	213

ticks were collected in March, i.e., 4 larvae and 39 nymphs. In April and May, almost one fourth of the ticks were larvae (Table III).

Local recruitment of ticks

Blackbirds had far more ticks at Jomfruland than at Akerøya and Store Færder, while Lista showed an intermediate number, corresponding with the abundance of local ticks. There was no difference in recruitment between the localities in March, but at Jomfruland and Lista, there was a marked increase in ticks from March to May (Fig. 6). This increase after the main immigration season was most likely due to local recruitment, since the increase was seen at localities where ticks were abundant locally and not at sites that were virtually free of ticks. The observed increase in tick prevalence through the spring in robins (Table III) was significant not only at Jomfruland (0.05 nymphs per bird in April vs. 0.39 in May, $P < 0.001$), but also on Store Færder (0.10 in April vs. 0.45 in May, $P < 0.001$), where there are very few local ticks. The increase in ticks on robins at Store Færder indicates that the increase through the spring was caused in part by an increase in ticks on arriving birds. Robins arrived in waves, and when several

TABLE III. The number of birds and tick nymphs collected each month. The bird species in which the most nymphs were found are presented separately (*Sylvia* spp. are grouped together).

	March			April			May			Total		
	Birds	L	N	Birds	L	N	Birds	L	N	Birds	L	N
Erithacus rubecula	93	0	4	1,036	115	113	134	67	39	1,263	182	156
Fringilla coelebs	32	0	1	114	0	18	52	18	7	198	18	26
Phoenicurus phoenicurus	0	0	0	25	0	5	182	22	43	207	22	48
Phylloscopus trochilus	0	0	0	444	1	5	2,939	44	37	3,383	45	42
Prunella modularis	10	0	0	175	8	86	13	4	5	198	12	91
Sylvia spp.	0	0	0	71	15	2	1,104	23	67	1,175	38	69
Turdus merula	210	4	32	233	54	331	100	44	418	543	102	781
Turdus philomelos	9	0	0	141	15	53	37	7	44	187	22	97
Other	146	0	2	1,092	40	62	1,376	36	66	2,614	76	130
Total	500	4	39	3,331	248	675	5,937	265	726	9,768	517	1,440

L, larvae; N, nymphs.

individuals were caught on the same day at a given locality, they were almost certainly new migrants. Among 359 robins, when 20 or more were caught in a single day, we found 0.14 nymphs per bird, which is similar to the abundance of all robins in our sample (0.12), indicating that single robins captured in our study represent newly arrived birds.

Comparison with previous data

We found a prevalence of 6.9% (n = 5,242) at Akerøya and Store Færder from 2003 to 2005, which is higher than the corresponding prevalence of 4.2% (n = 3,943) found by Mehl et al. (1984) from 1965 to 1970 (chi-square = 30.22, $P < 0.001$). Comparing the 2 datasets with regard to some of the important

FIGURE 2. Yearly variation in the mean number ± SE of tick nymphs per bird caught during the spring migration (March to May) at 4 sites on the coast of southern Norway from 2003 to 2005. (**A**) Lista, (**B**) Jomfruland, (**C**) Store Færder, and (**D**) Akerøya.

Bird observatory
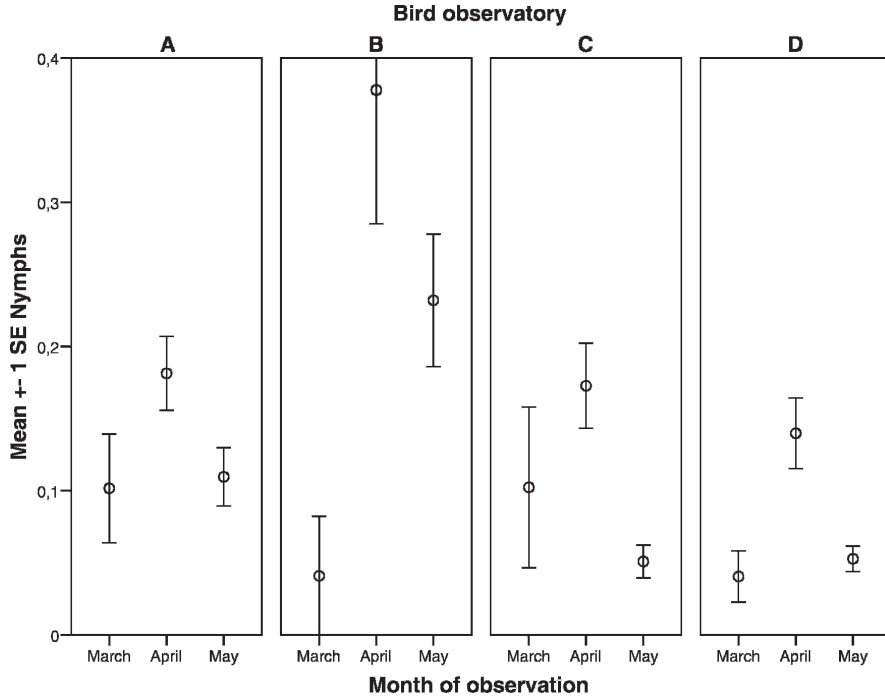
FIGURE 3.   The mean number ± SE of tick nymphs per bird caught at 4 sites on the coast of southern Norway from 2003 to 2005. Monthly variation during spring. (A) Lista, (B) Jomfruland, (C) Store Færder, and (D) Akerøya.

passerines, there was a highly significant increase in tick prevalence, but not in all species. For willow warblers, there was an apparent decrease (Table VI).

Tick species other than *Ixodes ricinus*

In addition to *I. ricinus*, we also found a few individuals of 4 other tick species, i.e., 7 fully engorged nymphs of *Hyalomma rufipes* were found on 6 birds, i.e., a garden warbler (*Sylvia borin*), a redstart (*P. phoenicurus*), a reed warbler (*Acrocephalus scirpaceus*), a wheatear (*Oenanthe oenanthe*), a whitethroat (*Sylvia communis*), and 2 individuals of thrush nightingale (*Luscinia luscinia*), which are host species that winter in Africa. We found 2 nymphs of *Ixodes arboricola* (on a robin and a song thrush) and 1 *I. frontalis* (on a

blackcap *Sylvia atricapilla*). One larva of *Dermacentor* sp. was found on a willow warbler caught at Akerøya.

DISCUSSION

Our finding that the members of the thrush family and dunnock have more ticks than average for the whole bird sample is consistent with the findings of Mehl et al. (1984) and Olsén et al. (1995). These avian species feed on the ground. The willow warbler is regarded as the most common bird species in Norway. In spite of low tick prevalence, it nonetheless contributes significantly to the transoceanic transport of these parasites. The number of each bird species caught during our study does not reflect each bird's overall abundance. For example, birds that were underrepresented in our sample, such as fieldfare (*Turdus pilaris*) and tree pipit, may be far more important than our numbers indicate. The impact of each species on the import of ticks must, therefore, be evaluated by combining information about each bird species' abundance with the extent of their infestation by ticks. Since between 20 million and 70 million passerine birds migrate to Norway each spring (Gjershaug et al., 1994), many hundreds of thousands, probably millions, of tick nymphs are also brought to Norway across the sea every year. Compared to the enormous numbers of ticks already present in nature, the number of ticks transported by birds is small; therefore, the transport of ticks by birds will have little impact

TABLE IV. Tests of site, month, and year for effects on tick nymphs per bird. Generalized linear model. Type III sum of squares.

	Wald chi-square	df	Sig.
(Intercept)	1651.261	1	0.000
Site	159.799	3	0.000
Month	58.000	1	0.000
Year	52.725	2	0.000
Site-year interaction	81.036	6	0.000
Site-month interaction	0.998	3	0.802
Month-year interaction	15.795	2	0.000

TABLE V. Effects of locality on the mean number of tick nymphs per bird from March to May. The results from the Jomfruland bird observatory are used as a reference. P values <0.05 are marked with bold type.

	Lista	P	Jomfruland	Store Færder	P	Akerøya	P
Erithacus rubecula	0.099	0.941	0.096	0.141	0.272	0.153	0.177
Fringilla coelebs	0.043	**<0.001**	0.541	0	0.993	0.143	0.315
Phoenicurus phoenicurus	0.137	0.088	0.387	0.185	0.177	0.268	0.431
Phylloscopus trochilus	0.009	0.237	0.018	0.004	**0.018**	0.012	0.298
Prunella modularis	0.63	0.627	0.447	0.359	0.783	0.217	0.568
Sylvia spp.	0.022	**0.002**	0.115	0.019	**<0.001**	0.046	0.051
Turdus merula	1	**<0.001**	4.486	0.437	**<0.001**	0.536	**<0.001**
Turdus philomelos	0.339	0.128	1	0.667	0.564	0.506	0.286
Other species	0.041	**0.001**	0.112	0.048	**0.026**	0.028	**<0.001**
Total	0.14	**<0.001**	0.26	0.087	**<0.001**	0.089	**<0.001**

on the local population dynamics where ticks are already established. The introduced ticks, however, may influence the genetic diversity of ticks along the bird migration routes. Furthermore, pathogens are brought along with the ticks collected on the birds (data not shown), and there is a possibility that they may be established in new areas. Theoretically, if new areas become hospitable to ticks, the distribution range of tick species may be enlarged by migratory birds spreading ticks over long distances and across geographical barriers.

Natural yearly variation must be considered when analyzing long-term patterns, e.g., those due to climate change. Therefore, in studying the transport of ticks by birds, it is important to consider variation during the season and between localities and year (Figs. 2, 3; Tables II, III). Variation between years is most likely due to stochastic meteorological variations at the last stop before arrival; for example, temperature and humidity influence tick questing (Schulze et al., 2001; Hubálek, 2003), while wind and bad weather (Richardson, 1978; Weber et al., 1998) influence migration.

We did not find any consistent variation in tick prevalence or abundance among the different host species between the different localities, apart from blackbirds at Jomfruland. The localities are

FIGURE 4. Yearly variation in the mean number ± SE of tick nymphs per blackbird (*Turdus merula*) caught at 4 sites on the coast of southern Norway from 2003 to 2005. (**A**) Lista, (**B**) Jomfruland, (**C**) Store Færder, and (**D**) Akerøya.
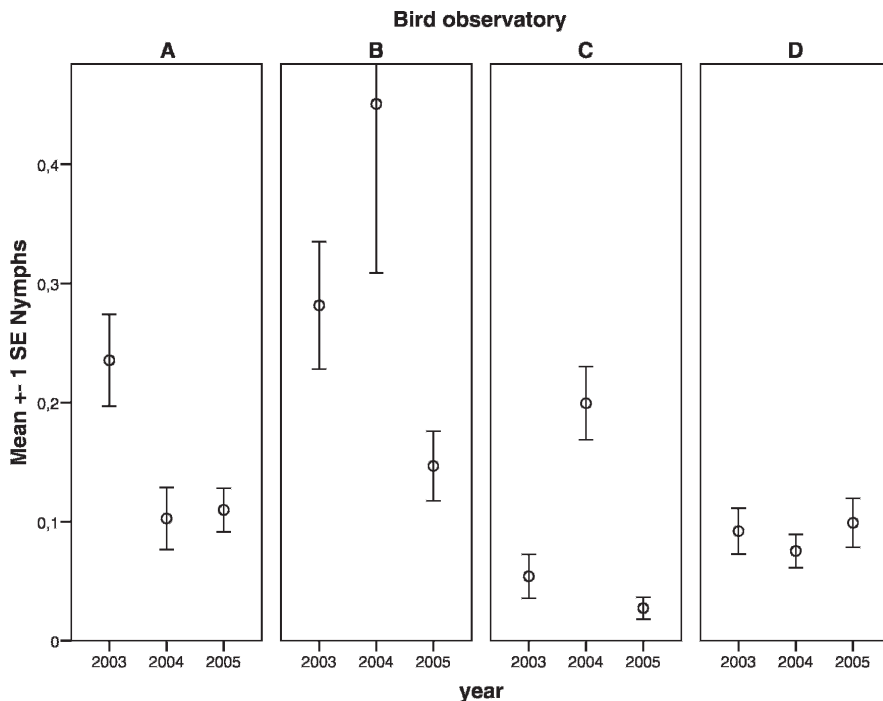
Bird observatory
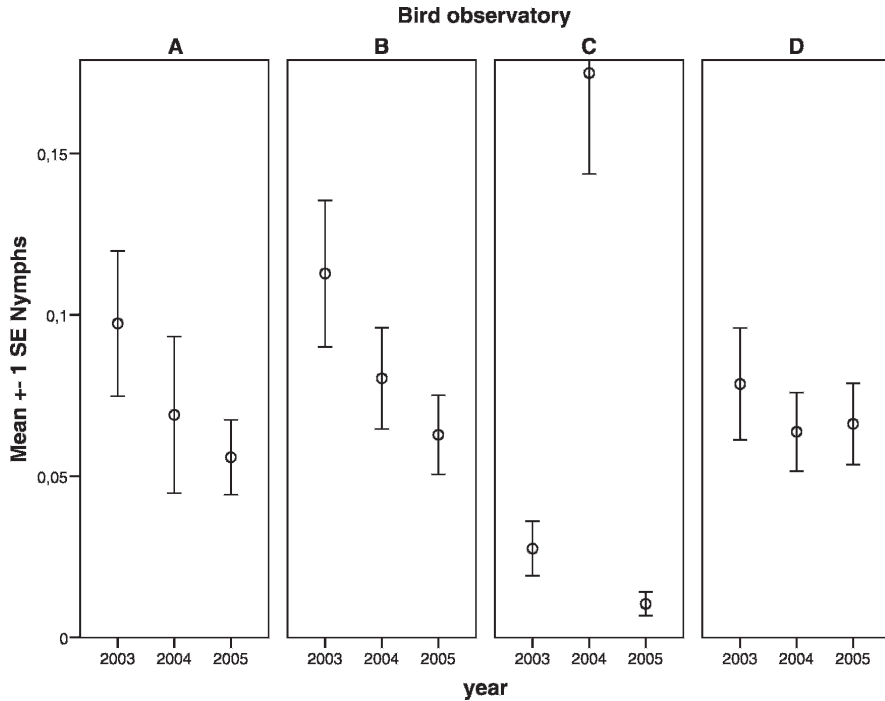
FIGURE 5.   Yearly variation in the mean number ± SE of tick nymphs per bird caught during the spring migration (March to May) at 4 sites on the coast of southern Norway from 2003 to 2005. Blackbirds (*Turdus merula*) are excluded. (**A**) Lista, (**B**) Jomfruland, (**C**) Store Færder, and (**D**) Akerøya.

so close to each other that migration routes overlap. We found a higher prevalence of ticks, however, than Olsén et al. (1995) found in spring migration in Sweden, i.e., in total, 7.3% versus 3.0%; blackbirds, 31.5% versus 27.7%; robins, 12.7% versus 3.7%; song thrushes, 25.1% versus 7.4%; dunnock, 15.7% versus 5.8%; redstarts, 20.3% versus 7.4%; and willow warblers, 2.1% versus 0.6%. These differences between Norwegian and Swedish migratory birds are likely a reflection of different tick abundances along different migration routes, but could also be due to fluctuations in tick populations, given that the material in the Swedish study was all sampled during a single year, 1991.

The low prevalence in March probably reflects low temperature and, correspondingly, low questing activity of the ticks in the U.K. and continental Europe. *Ixodes ricinus* nymphs exhibit increasing questing from March to May (Randolph et al., 2000). The increase in the number of ticks per bird through the spring observed in robins was most likely due to increased tick questing in the locations from which they migrated, since an increase of this nature was observed even at Store Færder, where very few local ticks are present.

There were significantly more ticks per bird at Jomfruland than at Lista, Akerøya, and Store Færder (Table V). This can be

TABLE VI.   Comparison of the tick prevalence found in the present study (2003–2005) and a study by Mehl et al. (1984) conducted from 1965 to 1970.

Bird species	2003–2005			1965–1970			P
	n Infested	n Birds	% Infested	n Infested	n birds	% Infested	
Erithacus rubecula	100	663	15.1	44	627	7.0	<0.001
Phoenicurus phoenicurus	29	128	22.7	32	288	11.1	0.004
Phylloscopus trochilus	27	1,672	1.6	17	505	3.4	0.019*
Prunella modularis	12	88	13.6	11	89	12.4	NS
Sylvia spp.	18	551	3.3	14	579	2.4	NS
Turdus merula	45	248	18.1	12	262	4.6	<0.001
Turdus philomelos	35	110	31.8	8	97	8.2	<0.001
Total	363	5,242	6.9	164	3,943	4.2	<0.001

* Lower prevalence in the present study.
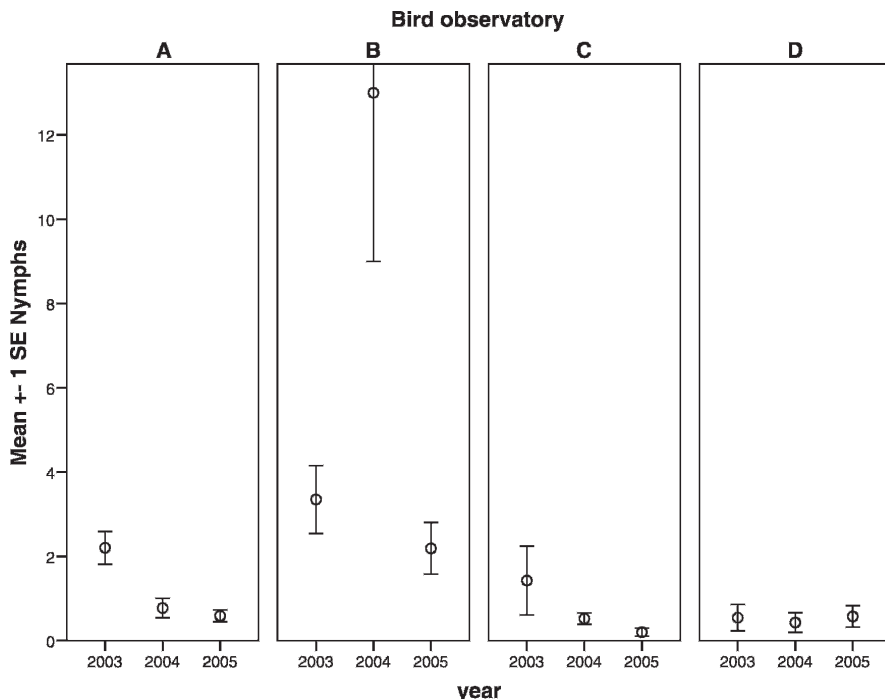
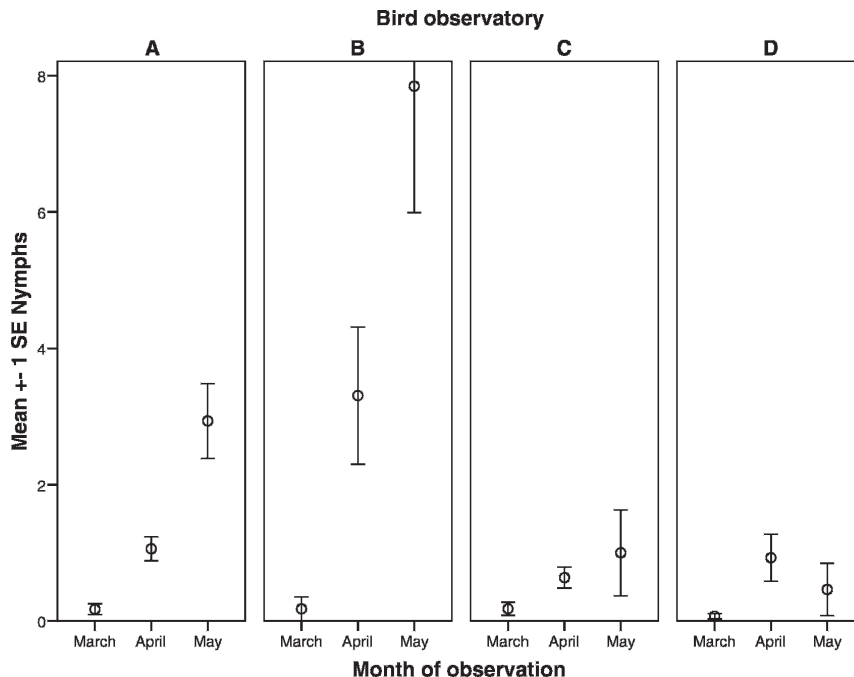FIGURE 6.   The mean number ± SE of tick nymphs per blackbird (*Turdus merula*) caught at 4 sites on the coast of southern Norway from 2003 to 2005. Monthly variation during spring. (**A**) Lista, (**B**) Jomfruland, (**C**) Store Færder, and (**D**) Akerøya. Jomfruland and Lista are localities with many local ticks.

explained by local infestation at Jomfruland, where ticks are abundant. Tick recruitment after arrival in Norway represents a challenge in terms of interpreting the data. This is seen most clearly for blackbirds, as individuals caught late in the season at Jomfruland have a heavy tick burden (Fig. 6). Since several of the birds caught late in the season are residents, an estimation of the tick prevalence will overestimate the importance of a species as a long-distance vector for ticks if the birds are collected in a locality with many resident ticks. Data from a locality where ticks are abundant should, therefore, not be interpreted as representative of the prevalence of imported ticks on birds. However, the tick recruitment at, for example, Jomfruland, can still be used to assess the relative tendency of different species to acquire ticks.

Individuals that arrive late in the season are probably more heavily infested from their departure location compared with those who arrive early. Therefore, by only considering ticks collected during the peak of arrival as imported, we may underestimate the import of ticks. Since there are almost no ticks on the islands of Akerøya and Store Færder, examination of birds caught in these localities mainly reflects imported ticks, even when caught after peak arrival.

To document the importance of pathogens, a more conservative approach is needed. Blackbirds caught in large flocks early in the season (March and beginning of April), and other species caught during peak arrival times, can be considered to be imported with a high degree of certainty and are, thus, suitable for analyses of the

transport of tick-borne pathogens. However, since the large thrushes have an especially high tendency to acquire ticks locally, only individuals caught in virtually tick-free localities such as Akerøya and Store Færder can reliably be considered as carrying imported ticks.

The present study and the study conducted by Mehl et al. (1984) from 1965 to 1970 were performed using the same methods and in the same localities. Mehl et al. (1984), however, did not perform continuous sampling through the spring, and our results thus are not directly comparable. Nonetheless, the increase in tick prevalence suggests a real increase in the transport of ticks on northward migrating birds during the last 30–40 yr. One reason for this increase could be climate changes in Europe, which have led to a significant mean advancement of spring events by 2.3 days per decade (Parmesan and Yohe, 2003). These phenological changes would likely also have led to an earlier increase in tick activity during the spring and, therefore, increased tick infestation on migratory birds. Earlier bird migration in spring as a response to increased temperatures has also been observed in several studies (Gienapp et al., 2007; Rubolini et al., 2007).

The finding of exotic tick species such as *Dermacentor* sp. and *H. rufipes* demonstrates the potential of birds to transport new tick species across geographical barriers. *Dermacentor reticulatus* has an increasing distribution range in Germany, although not in the most northern regions (Dautel et al., 2006). It is a vector for *Rickettsia sibirica* and of Omsk hemorrhagic fever (Estrada-Peña

and Jongejan, 1999). Global warming may move the distribution range of the univoltine *D. reticulatus* northward, and the species could cross the sea via migratory birds. *Hyalomma rufipes* is a 2-host tick, i.e., each has its larval and nymphal stage on the same host. It is commonly found on birds in Africa, but does not occur north of the Mediterranean region. Since it may remain attached to the same host for up to 28 days (Knight et al. 1978), this tick species may be transported by birds over vast distances. At present, however, *H. rufipes* cannot be established in a Norwegian climate. It is a vector of Crimean-Congo hemorrhagic fever (CCHF), and given that the CCHF virus is transmitted transovarially (Ergönül, 2006), unprotected handling of these ticks could entail a risk of contracting CCHF.

ACKNOWLEDGMENTS

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IV

AVS **ACTA VETERINARIA SCANDINAVICA**

RESEARCH   **Open Access**

Detection of *Babesia divergens* in southern Norway by using an immunofluorescence antibody test in cow sera

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Abstract

Background: The incidence of bovine babesiosis, caused by *Babesia divergens* (Apicomplexa: Piroplasmida) has decreased markedly since the 1930 s, but may re-emerge as a consequence of climate change and changes in legislation and pasturing practices. This is a potentially serious disease, with both economical and animal welfare consequences. Therefore, there is a need to survey the distribution of *B. divergens*.

Methods: We tested sera from 306 healthy pastured cows from 24 farms along the southern Norwegian coast by using an indirect immunofluorescence IgG antibody test (IFAT). Fractions of seropositive cows were compared by calculating 95% CI.

Results: The results of this test showed that 27% of the sera were positive for *B. divergens* antibodies. The fraction of antibody-positive sera that we detected showed a two-humped distribution, with a high fraction of positives being found in municipalities in the western and eastern parts of the study area, while the municipalities between these areas had few or no positive serum samples.

Conclusions: Neither the farmers' observations nor the Norwegian Dairy Herd Recording System give an adequate picture of the distribution of bovine babesiosis. Serological testing of cows by using IFAT is a convenient way of screening for the presence of *B. divergens* in an area.

Background

Though the incidence of bovine babesiosis is low in Norway, these pathogens have immense economic importance throughout the world, with the highest prevalence being found in the tropics [1]. The costs associated with this infection are associated with mortality, ill-thrift, abortions, loss of milk and meat production as well as with measures taken to control its spread [2]. *Babesia divergens* is the main cause of bovine babesiosis in northern Europe [3], although *B. major*, occurs in southeast England, Holland and the Friesian Islands in Germany [4]. *Babesia* species are intraerythrocytic protozoa that cause fever, haemoglobinuria (redwater) and anaemia in cattle that are exposed to the parasite as adults. Calves are relatively resistant to *B. divergens* [5,6] and exhibit mild or no effects of the disease, while

infected adults may have a high mortality [7,8]. *Babesia* spp. can cause serious infections in humans who do not have a functioning spleen or who are immunocompromised as a result of immunosuppressive drugs, malignancy or HIV-infection [9]. The only case of human *B. divergens* diagnosed in Norway is a splenectomised veterinarian in Western Norway in 2007 (personal communication, Kristine Mørch, Haukeland University Hospital).

Cattle are the only natural vertebrate host for *B. divergens*. Reindeer and gerbils, and splenectomised individuals of other species may be infected experimentally. Sheep, wild cervids and rodents that occur in the area where it is distributed are all considered to be resistant to *B. divergens* [3]. However, this issue is controversial, as new studies indicate that roe deer and red deer may be infected by *B. divergens* [10,11]. The vector of *B. divergens* in Western Europe is *Ixodes ricinus* (Acari: Ixodidae) [3], which can parasitise a wide range of vertebrates [12]. Vertebrate hosts may act as vehicles for

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spreading *Babesia*-infected ticks, though only adult females of *I. ricinus* can become infected with *B. divergens* from cattle [13]. Transovarial and transstadial transmission of *B. divergens* occur in *I. ricinus* [14], and the infection can last for at least two generations [13]. Thus, these ticks may also represent a reservoir of the parasites, though only a small percentage of the larvae from the infected females usually carry the pathogen [13]. Each female of *I. ricinus* produces approximately 2,000 eggs [15], so there will be a correspondingly high mortality from one stage to the next in a stable tick population. Supposing a maximum 3 years generation time of *I. ricinus* and a maximum of three generations of parasite survival through transovarial transmission, the pathogen would, therefore, be expected to gradually disappear within a decade in areas where there are no vertebrate hosts present to transmit the infection to the ticks. After recovering from acute babesiosis, cattle may sustain a low level of parasitaemia for at least two years, which may be followed by the development of immunity to the parasite, without any detectable parasites in the blood [16]. Opsonising antibodies play an important role in protecting hosts against *B. divergens* infection, but the acquired immunity is not dependent on circulating antibodies, and *in vitro* tests have demonstrated a role of T-lymphocytes in protection against the disease. Antibody levels generally fall below the level of detection within six months after treatment [2]. The long-lasting host-parasite interaction results in the cattle acting as an effective reservoir of the parasites [17].

In Norway, the law does not mandate obligatory notification of bovine babesiosis, and no systematic study on the distribution of this parasite has been undertaken since the work of Thambs-Lyche from 1933-1940 where 1388 cases per year were reported [18]. One way of estimating the number of cases of this infection that exist today is by looking at sales of imidocarb, a veterinary medicine used to treat bovine babesiosis. Approximately 300 vials of 1200 mg imidocarb are sold per year in Norway (Bjørn Loe, Schering-Plough, personal communication), and this amount would be sufficient for treatment of a maximum of 600 individuals. Alternatively, data recorded at the Norwegian Dairy Herd Recording System (NDHRS) can be examined, since every cow in Norway is assigned an individual Cow Health Card on which all diseases are recorded by veterinarians or farmers and then reported to the NDHRS. This system has been in operation nationally since 1975 [19], and the health code and date of all disease treatment events are maintained in a central database. From 1996-2008, 121 cases of bovine babesiosis were reported in the NDHRS per year. Thus, both of these estimation methods indicate that the incidence of bovine babesiosis in Norway has fallen markedly since the 1930 s. This decrease

coincides with, and may be explained by, a marked decrease in pasturing of cattle. In 1938, almost all of the 1.3 million cattle population in Norway were pastured regularly, whereas only 220,000 of the present 920,000 cattle population are pastured during the summer [20,21]. A decrease in bovine babesiosis has also been documented in Ireland. Gray et al. suggested that this might be due to a combination of several factors, such as an increase in average farm size and destruction of ticks' habitat by increased sheep pasturing. On the other hand, they suggested that the rate of clinical disease is low in western Ireland because of enzootic stability, i.e., the herds are naturally immune [22].

Bovine babesiosis is regarded as a limited problem in Norway, being confined to coastal areas north to southern Nordland county [23]. However, there may be a locally elevated risk of contracting babesiosis, which might be an argument against importing adult cows from inland localities where redwater does not occur and that, therefore, will not harbour any acquired immunity to the disease. In addition, changes in climate and pasturing practices could also lead to an increase in the incidence and distribution of bovine babesiosis. As the distributional range of ticks in Scandinavia expands [24], bovine babesiosis may be introduced into areas where livestock do not have a natural immunity to infection. We have no sound scientific data in support of an expansion of tick distribution in Norway, although this has been documented in Sweden [24]. Moreover, since 2004 all tie-stalled cattle in Norway have been required to be pastured for a minimum of 8 weeks during the summer [25], and this same legislation will also apply to cows in free-stalls by 2013, which could lead to an increase in bovine babesiosis. Because of these changes an updated map of the distribution of this parasite is needed for the purpose of better management. The distribution of *B. divergens* could be mapped by testing for the presence of the pathogen in ticks using PCR. Lundsett [26] tested 439 flagged ticks along the southern Norwegian coast and found only one tick that was positive for *B. divergens* using this method. Radzijevskaja [27] found no *B. divergens* in 91 ticks (16 adults, 75 nymphs) collected in Jomfruland, where we found that redwater is perceived to be a problem by farmers. Thus, testing ticks for *B. divergens* directly is both laborious and costly, and would require analysis of a very large number of ticks.

The aim of this study was to use a well-established indirect immunofluorescent antibody test (IFAT) to detect the presence of *B. divergens* antibodies in blood sera [28], and to evaluate this method as a means of mapping the distribution of the pathogen by comparing our results with information obtained either through reporting through the NDHRS or by interviewing the farmers.

Materials and methods

The study area consisted of farms with cows that were pastured in wooded areas within the previously established distribution of bovine babesiosis [29]. Twenty-four farms scattered along most of the southern Norwegian coast west of the Oslo Fjord (Figure 1) were included in the study. Farms using hillside or wooded areas for pasturing were identified with the help of local agricultural authorities. None of the farmers who were asked to participate in the study refused. All the farmers confirmed that ticks occur on their farms, and the cattle were pastured on the property. All of the 306 cows included in the study were healthy and at least one year old when tested. On one farm (Farm 23), all the cows had been purchased one year prior to the study from various inland localities and had been pastured for just one season at this farm. *I. ricinus* is distributed mainly near the coast in this part of Norway. The study
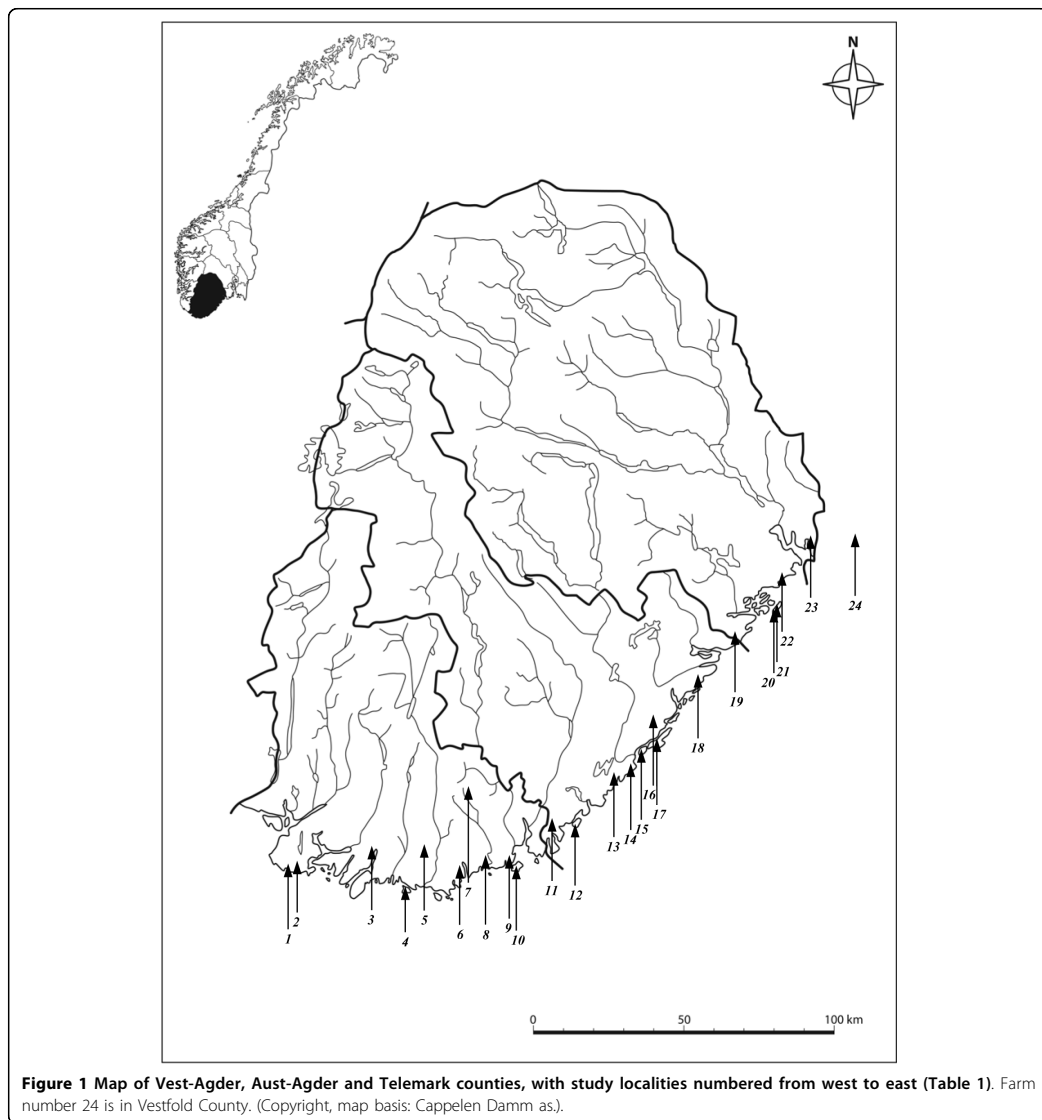
Figure 1 Map of Vest-Agder, Aust-Agder and Telemark counties, with study localities numbered from west to east (Table 1). Farm number 24 is in Vestfold County. (Copyright, map basis: Cappelen Damm as.).

included one inland farm approximately 30 kilometres from the sea (Farm 7) that was included because human Lyme borreliosis had been reported in this municipality, thus indicating the presence of ticks, according to the Norwegian Surveillance System for Communicable Diseases (MSIS) [30]. Blood samples were collected in May 2004 on farms 20 and 21, and samples were collected from all other sites in October and November 2005. The blood samples were stored at 4°C within a few hours after collection, and the serum portion of the samples was separated and frozen within 72 hours.

All of the sera were tested using an indirect immuno-fluorescent antibody test (IFAT) [28] for IgG as described by Christensson [31,32], and Christensson and Moren [33] with the following modifications: Antigen was prepared in 2002 from blood of a calf infected with *Babesia divergens* with approx. 10% infected erythrocytes as described by Christensson [32]. The antiserum used was was FITC conjugated rabbit anti bovine IgG, produced by ICN Cappel, code 55280, lot 03683, diluted at 1/200 to give comparable readings with control sera used by Christensson and Morén [33]. Control sera were obtained from calves used for vaccine production in the year 2001 drawn before infection and four weeks after having showed acute parsitaemia. Negative control serum showed no or uncertain reaction at a dilution of 1/20 or higher. The positive control sera had an end-point titre of 1/1280-1/2560. For each day of reading IFAT-slides a negative control at 1/40 and a positive control at 1/40, 1/160 and 1/1280 were included. As the purpose of the test was to identify seropositive/seronegative animals sera were read at dilutions at1/40 and 1/160. Slides were read blindly and scored by Christensson as having uncertain (+), positive (++) or strongly positive immunofluorescence (+++), at dilutions of 1:40 and 1:160. To minimise the risk of false positives, only sera with a minimum +++ score at a dilution of 1:40 were counted as positive.

Farmers were interviewed to determine if there had been cases of redwater on their farms and if they had experienced redwater in cows that were imported to the farm. Data on the cases of babesiosis in the included farms were obtained from the NDHRS.

To test the suitability of using PCR on full blood, we chose samples for a pilot study from four farms where redwater was common, according to the local farmers, and DNA from 100 μl from 20 samples of frozen EDTA-blood, and 25 samples of 100 μl blood clot, frozen after spinning and removal of the serum, were isolated in a spin-column, using DNeasy Blood & Tissue Kit (Qiagen), and eluted to 200 μl, according to the manufacturer's protocol. The isolation of DNA contained a lysis step and washing. Five μl of the eluate was run in *B. divergens* real-time PCR for 40 cycles with

primers BdiF, BdiR and BdiT. The PCR was performed by Telelab (Skien, Norway), using an in-house method, as described by Lundsett [26]. The laboratory used a synthetic amplicon with the sequence of *B. divergens*, serially diluted in human DNA as a positive control. The reaction mix and human DNA was used as a negative control. The observed cutoff for detection was 30 *B. divergens* DNA copies, i.e. 15 to 30 individual cells, depending of whether they are asexual, diploid cells or sexual, haploid cells.

Exact confidence intervals for binomial proportions were calculated using the principles introduced by Clopper and Pearson [34] and implemented in R (R Development Core Team, 2008).

Results

Of the 306 sera that we tested, 84 (27%) had positive IFAT results. A high percentage of these positive results were found in the western and eastern ranges of the study area, and a much lower rate of positive test results was found in the middle range of the study area (Table 1; Figure 2). Farm 23 had 3 positive test results among

Table 1 Municipality of the test localities in Figure 1 and test results of indirect immunofluorescence antibody tests (IFAT) for *Babesia divergens*.

Farm	Municipality	Neg	Pos[1]	N	% pos
1	Farsund	6	5	11	45
2	Farsund	6	9	15	60
3	Lyngdal	6	2	8	25
4	Mandal	0	9	9	100
5	Mandal	5	7	12	58
6	Søgne	2	7	9	78
7	Songdalen	13	1	14	7
8	Søgne	3	1	4	25
9	Kristiansand	9	0	9	0
10	Kristiansand	3	0	3	0
11	Lillesand	16	2	18	11
12	Lillesand	8	0	8	0
13	Grimstad	29	2	31	6
14	Grimstad	19	0	19	0
15	Arendal	10	0	10	0
16	Arendal	14	1	15	7
17	Arendal	6	4	10	40
18	Risør	4	13	17	76
19	Kragerø	11	1	12	8
20	Kragerø	6	12	18	67
21	Kragerø	2	5	7	71
22	Bamble	12	0	12	0
23	Porsgrunn	13	3	16	19
24	Larvik	19	0	19	0
Total		222	84	306	27

1. IFAT IgG titres scored as 1:40 (+++) or higher are defined as positive.

Figure 2 Fraction of cows positive for *Babesia divergens* IFAT IgG antibodies at a titre of 1:40 (+++) or higher in 24 different farms along the southern Norwegian coast, arranged form west to east. Error bars: 95% confidence intervals.
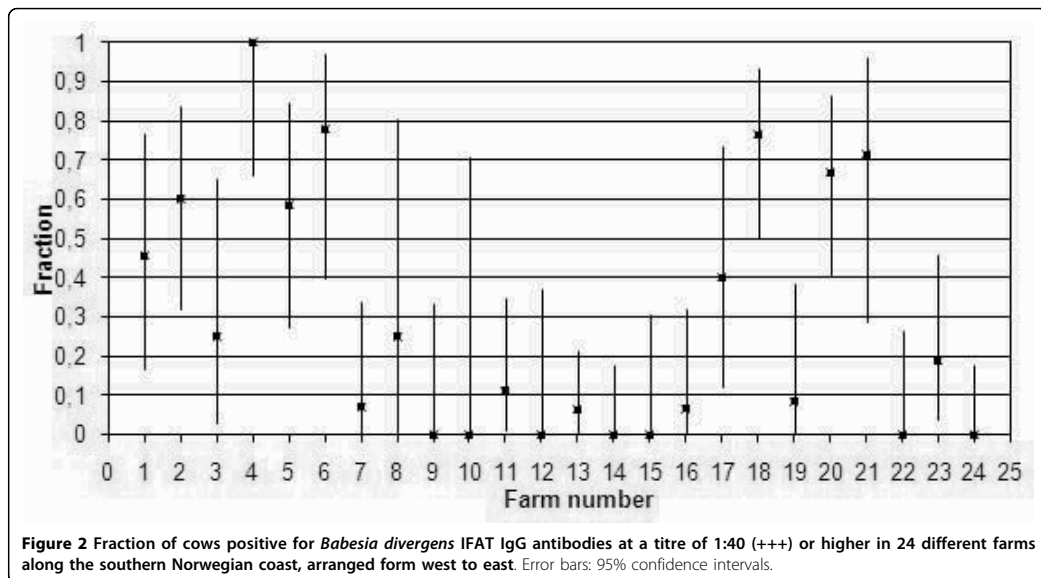
the 16 cows that had been imported from inland localities one year before the study, indicating that there is a substantial risk of babesiosis in their present locality. The presence of *B. divergens* was confirmed by IFAT in a total of 17 of the 24 farms we tested. Farmers had observed redwater in only ten of the farms where *B. divergens* was detected, and only four of these cases of redwater had been recorded by the NDHRS (Figure 3). All of the cows on one of the farms in the study were *B. divergens*-antibody positive, though the owner had never seen any cases of redwater. We detected *B. divergens* antibodies in 17 of the 25 cows that we tested on Jomfruland, where Radzijevskaja [27] found no infected ticks.

The PCR pilot study gave no positive results.

Discussion

In Norway and Sweden the only cattle *Babesia* reported is *B. divergens* [35,36]. With regard to this and the strong reaction to the antigen used we assume that the seropositive animals were/had been infected with the species *Babesia divergens*. Our results demonstrate that testing of cattle for seropositivity to *B. divergens* is a far better method for mapping the distribution of this pathogen than using indirect methods, such as interviewing farmers or relying on the NDHRS. When it presents clinically, redwater is easily recognizable by farmers and veterinarians, and because prompt treatment is usually required to prevent deleterious effects of the disease, veterinarians often treat the disease without

performing any laboratory tests. There are few data available on the attack rate of bovine *B. divergens* infections. Our data indicate that there are many subclinical cases of *B. divergens* infection, which is in agreement with previous studies on outbreaks [7,37] and in stable infected herds [38]. An extensive study of *B. divergens* seroprevalence was conducted in Northern Ireland, showing an overall seroprevalence of 31,8%[39], i.e., close to the overall seroprevalence in our limited material. A second study carried out in Northern Ireland [40] found consistent estimates when comparing results from a farm survey, a veterinary practise survey and seroprevalence data, with an estimated clinical incidence of 0,26% per year. The number of cases in the Agder counties, according to the NHDRS, is 18.4 cases per year in a population of ca. 10400 dairy cows (Statistics Norway, http://www.ssb.no/emner/10/04/10/jt1999/tab-2001-04-03-07.html, Jordbrukstelling 1999), which would give an incidence of 0.18% per year. Our results indicate an incomplete registration of cases of redwater in the NHDRS, possibly because veterinarians are not always consulted e. g. during the dry period, in mild cases of redwater, or that the farmers fail to observe redwater while the cows are out at pasture. The farms that we included in our study were not randomly selected, but were chosen because the pastures were in wooded areas, and were situated near the coast in the distribution area of *I. ricinus* in Norway. They would therefore be expected to have more babesiosis than average farms in the same counties.

Farm	IFAT IgG positive	Noticed	Notified
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Figure 3 Comparison of three sources of information for the occurrence of babesiosis on the farms in this study. IFAT IgG positive: At least one cow positive for IFAT *Babesia divergens* IgG. Noticed: Farmers' statement that redwater occurs in cows on the farm or is detected when adult cattle are imported to the farm. Notified: Clinical cases registered on the diary cow health cards, compiled by the Norwegian Dairy Herd Recording System from 1996-2008.

Because cows are parasitised by hundreds of ticks in the course of a season, and a single bite from an infected tick is sufficient for transmission of *Babesia*, [41] cows are likely to contract *B. divergens* if it is present in their pasturing areas. The screening of cows for *B. divergens* infection would therefore be expected to be a sensitive method for detecting the presence of the parasite in a locality, if testing is performed at a time of the year when *Babesia*-antibodies are at the highest. Serum samples that we collected on Jomfruland in May were not directly comparable to those that we collected in October and November, as the May samples could either contain persistent antibodies from the previous year, or there might be early infections from the same year. The mean temperature April 1st-15th was 5.3°C, and no temperatures of below 0°C were recorded (The Norwegian Meteorological Institute), which means that tick questing may well have occurred during this period. With an incubation time of 1-3 weeks [3], seroconversions during May 2004 would be expected to occur. As we tested only once for each locality we did not demonstrate the seasonal and yearly variation of antibodies described by l'Hostis et al. [38]. Further studies are needed to decide which month would be optimal for detecting the presence of *B. divergens* in a locality along the Southern Norwegian coast. However, ticks are still parasitizing the cows in October and November and these months would therefore be expected to be a good choice for detecting *B. divergens* antibodies.

The sensitivity of serologic testing for detecting *B. divergens* will depend on the cut-off level that is set for a positive score on the test. At a cut-off level of 1:40 (++) the sensitivity and specificity of an individual antibody test are reported to be 100% and 97%, respectively [32]. Setting the cut-off value at this level would, therefore, likely result in the detection of a few false positives due to non-specific cross reactivity. This problem is illustrated by our results on Farm 24, where only one cow was found to be positive at the detection level of 1:40 (++), and there were no positive tests at more stringent detection levels. This result could represent either a false positive or a low titre in a cow that was infected a long time ago. Because the aim of this study was to be able to detect the present occurrence of *B. divergens* at a particular locality, a high sensitivity for detecting the pathogen on a given farm is desirable, and the number of cows tested is crucial. By testing a median of twelve cows per locality, we were able to achieve a much higher sensitivity for detecting *B. divergens* on a given farm than farmers' observations and the existing NDHRS can provide. At all the farms where samples with 1/40(+++) were detected there were also samples positive at 1/160, indicating that these are real positives. Therefore, by setting a cut-off level of 1:40 (+++) for defining a case of seropositivity for *B. divergens*, antibody testing should result in a specificity of nearly 100%, unless cross-reacting *Babesia* spp. are occurring and, consequently, the risk of falsely concluding that *B. divergens* occurs on a farm will be small. The related species *B. capreoli* cause babesiosis in roe deer and red deer [42], and roe deer may also be infected by the newly discovered *Babesia* sp. EU1 [43]. These parasites cannot be serologically distinguished from *B. divergens*. They cannot give clinical infection in cattle, but there is a possibility that a subclinical infection may cause seroconversion [44], although Schmid et al. [45] found no seropositive cows in an area in which ticks positive for

these two non-bovine *Babesia* species were found. It is therefore unlikely that these *Babesia* species would influence the number of seropositive cows in this study significantly. There are no published studies on these *Babesia* species in Norway, but a Swedish study suggested that babesiosis caused by *B. capreoli* is very rare in Sweden [46].

An alternative to antibody testing is to test directly for the presence of the pathogen in cattle blood samples. Calder et al. [47] found an approximately 80% sensitivity for detecting *Babesia bovis* by PCR in steers, up to 300 days after experimental infection. The method these investigators used required a concentration step involving ultracentrifugation of haemolysed blood. We considered this to be too laborious a method to be useful as a field assay. We did attempt direct PCR to detect *B. divergens* without performing the concentration step in 30 samples from areas where we found the highest incidence of *B. divergens* by IFAT, but none of these samples were found to be *B. divergens*-positive by this method. Cultivation of *Babesia* in cell culture, which enables detection of *Babesia* at a level of 10 parasites per 1 ml of blood [48], is another possibility for mapping the distribution of this parasite, but it is not feasible to use this method when sampling is being carried out in scattered locations. For our purposes, therefore, we found that antibody screening was a much more convenient method for assessment of the occurrence of *B. divergens* in a locality than any of the other methods that are available for detecting this pathogen. Gerbil-derived antigen is found to be equally specific to *B. divergens* obtained from cattle [49], and could be a cheaper alternative in future studies.

In the communities on the coast of southern Norway where cows are pastured, the animals are confined to the farms on which they are kept. Consequently, testing cows for the presence of *B. divergens* infection should provide results that are specific to a given locality, as opposed to performing serological testing on other hosts of tick-borne pathogens, such as wildlife, dogs or humans. Because *B. divergence* is unlikely to survive for more than a decade in regions where cattle are not pasturing and cattle is the only host for *B. divergens* at the Southern coast of Norway, testing cow sera appears to be an effective method for mapping *B. divergens* over the area of distribution of *I. ricinus*. The same is not the case if using cattle as sentinel animals for serological testing for other tick-borne pathogens, such as *Anaplasma*, *Borrelia* or the TBE virus, that infect a wider range of hosts.

Malandrin et al. [48] found a drop in IFAT antibody titre from 320, 320 and 1280 to 80, 80 and 320 respectively in samples from three cows taken 6 and 9 months after acute babesiosis, indicating an antibody duration of more than a year, but much shorter than the cows' lifespan. Sahibi et al. [50] found no significant cumulative effect of cow age on the presence of *Babesia*-antibodies. This is consistent with a short duration of antibodies in the bloodstream after infection, meaning that detection of antibodies indicates a recent infection, as is illustrated by the seasonal variation of *B. divergens*-antibodies that was found by l'Hostis et al. [38], indicating repeated infections during the season. This implies that the lifetime risk of acquiring bovine babesiosis is higher than the current rate of infection that was determined in the study we present here.

Our IFAT data indicate that there are two areas along the southern Norwegian coast in which bovine babesiosis is highly endemic, consisting of one western area (Lista-Mandal) and one eastern area (Kroger-Risør) (Figure 1, Table 1). This uneven distribution was not reported by Thambs-Lyche in a study carried out along the same part of coastal Norway [29]. For other *Babesia* species, it has been shown that reduction of the incidence of tick bites can bring the reproduction rate of the parasite below 1, indicating that it could be possible to eradicate the parasite [41,51]. Our results indicate that, in the area from Sandaled to Arundel, which is within the distribution area of *I. ricinus* and is an area where cattle are pastured in a natural setting, *B. divergens* occurs at very low frequencies or not at all. In fact, the disease associated with this pathogen has virtually disappeared since the 1930 s, when Thambs-Lyche reported babesiosis in this area. This seems promising with regard to the possibility of eradicating this disease. An attempt to eradicate the disease would require the implementation of control measures over its entire distribution because wild hosts can spread infected ticks. Cervid animals are the most important hosts for adult ticks [52]. Red deer, roe deer and moose have yearly migratory ranges of 200, 100 and 50-60 kilometres respectively [53], and Cervid animals, therefore, have the potential for transporting large numbers of ticks over long distances. Furthermore, birds can transport ticks across geographical barriers. In a recent study, 7.3% of northward migratory passerine birds were found to carry one or more ticks [54], so, in a situation where cows are pastured in an area that is free of *B. divergens*, or where there is an unstable population of the pathogen, *B. divergens* could conceivably be introduced by birds.

Conclusions

At present, bovine babesiosis is a limited animal health problem in Norway. The most obvious possible cause of the decline in incidence since the 1930 s is changes in the use of pastures. Changes in legislation leading to increased use of wood pasturing may reverse the decline

in incidence, and we may also see a climate-related increase. An increased incidence of *B. divergens* in cattle could have important economic and animal welfare consequences, and further studies are needed to evaluate whether it would be cost effective to implement preventive measures against the spread of this pathogen. Antibody testing of pastured cows is a simple way of mapping the distribution of the pathogen.

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Authors' contributions
GH prepared the fieldwork, interviewed the farmers, performed all the blood sampling and wrote the main part of the paper. GB, KHR and HPL provided valuable and significant contributions to the writing of the paper. DC headed the laboratory work, and performed all the microscopy of the slides in the immunofluorescence antibody test. Furthermore, he contributed significantly to the writing of the paper. ACW contributed with data from the Norwegian Dairy Herd Recording System, and also contributed significantly to the writing of the paper. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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Title: Transport of Ixodes ricinus infected with Borrelia species to Norway by northward-migrating passerine birds.

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Abstract: Birds are capable of transporting ticks and, consequently, tick-borne pathogens over long distances and across geographical barriers such as oceans and deserts. The purpose of this study was to assess the prevalence of Borrelia spp. in ticks transported by birds by using PCR .
A total of 9,768 northward-migrating passerine birds was examined for ticks at four bird observatories along the southern Norwegian coast during their spring migration in 2003-2005. Two of the bird observatories were located on islands where flagging revealed very few or no ticks (Akerøya and Store Færder), while the other two were located in areas with established dense tick populations: an island, Jomfruland (>100 ticks per hour of flagging) and a mainland locality, Lista (40 ticks in one hour of flagging). Borrelia spp. were found in 70 (13.6%) of 513 examined Ixodes ricinus nymphs (19 B. afzelii, 38 B. garinii, two B. turdi and 11 B. valaisiana) and in 14 (8.1%) of 172 examined I. ricinus larvae (ten B. garinii, one B. turdi and three B. valaisiana). This report is the first to identify B. turdi in Europe. Ticks collected from birds of the Turdus spp. (T. merula, T. philomelos and T. iliacus), had a higher prevalence of Borrelia spp. than ticks from the other passerine genera. Ticks that were co-feeding with a Borrelia-infected tick had an increased probability of being infected with the same Borrelia species. Ticks collected on birds from the south-western locality Lista were less likely to have Borrelia than ticks found on birds from the other, more eastern localities. The Turdus spp. are particularly important, both because they carry many ticks per bird and because ticks carried by these species have a higher prevalence of Borrelia. This higher prevalence may be related to Borrelia infection of the birds or transmission of Borrelia through cofeeding. The prevalence of the different Borrelia species in ticks collected from migratory birds can be related to migration routes.

Response to Reviewers:

Transport of *Ixodes ricinus* infected with *Borrelia* species to Norway by northward-migrating passerine birds.

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Abstract

Birds are capable of transporting ticks and, consequently, tick-borne pathogens over long distances and across geographical barriers such as oceans and deserts. The purpose of this study was to assess the prevalence of *Borrelia* spp. in ticks transported by birds by using PCR .

A total of 9,768 northward-migrating passerine birds was examined for ticks at four bird observatories along the southern Norwegian coast during their spring migration in 2003-2005. Two of the bird observatories were located on islands where flagging revealed very few or no ticks (Akerøya and Store Færder), while the other two were located in areas with established dense tick populations: an island, Jomfruland (>100 ticks per hour of flagging) and a mainland locality, Lista (40 ticks in one hour of flagging). *Borrelia* spp. were found in 70 (13.6%) of 513 examined *Ixodes ricinus* nymphs (19 *B. afzelii*, 38 *B. garinii*, two *B. turdi* and 11 *B. valaisiana*) and in 14 (8.1%) of 172 examined *I. ricinus* larvae (ten *B. garinii*, one *B. turdi* and three *B. valaisiana*). This report is the first to identify *B. turdi* in Europe. Ticks collected from birds of the *Turdus* spp. (*T. merula*, *T. philomelos* and *T. iliacus*), had a higher prevalence of *Borrelia* spp. than ticks from the other passerine genera. Ticks that were co-feeding with a *Borrelia*-infected tick had an increased probability of being infected with the same *Borrelia* species. Ticks collected on birds from the south-western locality Lista were less likely to have *Borrelia* than ticks found on birds from the other, more eastern localities. The *Turdus* spp. are particularly important, both because they carry many ticks per bird and because ticks carried by these species have a higher prevalence of *Borrelia*. This higher prevalence may be related to *Borrelia* infection of the birds or transmission of *Borrelia* through cofeeding. The prevalence of the different *Borrelia* species in ticks collected from migratory birds can be related to migration routes.

Keywords: Migratory birds, tick-borne pathogens, *Ixodes ricinus*, dispersal, *Borrelia*, *cofeeding*.

Introduction

Norway is in the northern distributional range of *Ixodes ricinus*. This species is the vector for a wide range of pathogens of humans and animals, including *Borrelia* spp., *Babesia* spp., *Anaplasma* (*Ehrlichia*) spp., *Rickettsia* spp., *Francisella tularensis* and arboviruses such as Louping ill virus (LIV) and tick-borne encephalitis-virus (TBEV) (Estrada-Peña and Jongejan, 1999; Brantsæter et al., 1998). The *Borrelia burgdorferi* s. l. species complex (causing Lyme borreliosis in humans) is found widely in Europe (Parola and Raoult, 2001), with several genospecies differing in geographic range even within northern Europe (see Rauter and Hartung, 2005; Vennestrøm 2008): Only *B. garinii* is common in all northern European countries. Most common in Denmark, Germany and Poland is *B. afzelii* (constituting 35-50% of the isolates), which, however, is rare in the United Kingdom. *B. burgdorferi* s. s., is rare in Denmark and in the UK , but accounts for about 15-30% of the isolates in Germany and Poland. In contrast, *B. valaisiana* appears to be rare in Germany and Poland, and more common in the UK and Denmark. In Norway, two studies have been done to analyse *Borrelia* spp. in questing ticks with species specific PCR primers, and obtaining quite different results: Jenkins et al. (2001) found 10.6% positive for *B. afzelii*, 0.3% *B. garinii* and 2.6% *B. burgdorferi* s.s. (N=341)., while Lundsett (2004) found 1.6, 1.1 and 0.5%, of the three species respectively (N=439).

In is of great interest to distinguish between the different genospecies, as they may lead to different disease manifestations in humans. *B. burgdorferi s.s.* is associated with Lyme arthritis, *B. garinii* with neuroborreliosis and *B. afzelii* with acrodermatitis atrophicans (van Dam et al., 1993; Balmelli and Piffaretti, 1995; Ryffel et al., 1999). This association is, however, not absolute (Picken et al., 1998). The role of *B. valaisiana* as a pathogen is not yet clearly established (Baranton and de Martino, 2009). The incidence of chronic and disseminated Lyme borreliosis has increased in Norway from 100-170 cases per year between 1995 and 2003 to 250-350 per year between 2004 and 2009 (data available from the Norwegian Institute of Public Health, www.msis.no), and an increase of borreliosis was observed in many other European countries between 1990 and 2000 (Randolph, 2001). The observed increase in borreliosis may partly be due to increased awareness, but at least in Norway, testing for *Borrelia* sp. has been routine in neurological departments during recent decades. There has not been any increase in the sensitivity of the test methods routinely used until the last 1-2 years, indicating that there has been a real increase in the incidence.

Non-human hosts show differences in host competence for the different *Borrelia* spp (see Kurtenbach et al., 2002;Humair et al., 1998; Hániková et al., 2003).

Ticks have very little mobility (Korch, 1994), but they may be transported over long distances by their vertebrate hosts during feeding. In particular, bird hosts may efficiently transport ticks across geographical barriers such as oceans and deserts (Hoogstraal et al., 1961 and 1963; Mehl et al., 1984; Olsén et al., 1995; Smith et al., 1996; Ishiguro et al., 2000; Poupon et al., 2006; Hasle et al., 2009 a and b). *Borrelia* spp. have been found in ticks collected from birds (Olsén et al., 1993 and 1995; Comstedt et al., 2000; Ishiguro et al., 2000; Poupon et al., 2006; Ogden at al., 2008).

The purpose of this study was to investigate the prevalence of the different *Borrelia* species, identified using DNA sequencing, in ticks collected from passerine birds arriving on the Norwegian coast during their spring migration to illustrate the potential for the spread of tick-borne pathogens across the sea, and to determine whether some

bird taxa contribute to the spread of ticks more significantly than others. In addition, we wanted to explore the significance of locality, and thereby differences in migratory routes, by comparing the western locality Lista with three more eastern localities on the southern Norwegian coast. By this approach we addressed the question whether the *Borrelia* prevalence in ticks found on migratory birds would be influenced by the *Borrelia* prevalence at localities the migratory birds passed during recent days.

Materials and methods

The four Norwegian bird observatories along the southern Norwegian coast were used in the study included Lista, a promontory on the south-western coast, and three islands located at the south-eastern coast of Norway (listed in order from west to east), Jomfruland, Store Færder and Akerøya. Akerøya and Store Færder have very few local ticks, whereas ticks are common on Jomfruland (>100 ticks per hour of flagging) and at Lista (40 ticks after one hour of flagging). A total of 9,768 northward migrating passerine birds was caught with mist nets during their spring migrations in 2003 - 2005 (Hasle et al., 2009 a). The birds were examined for ticks around the eyes, beak and ear openings. The ticks were picked off with tweezers and stored in 70% ethanol for later examination, using a separate vial for each bird. The ticks were examined by a stereomicroscope with a 50-X maximum magnification for species identification and estimation of feeding status. For species identification, we used the illustrations and taxonomic keys of Morel and Perez (1977 a, b). Only *I. ricinus* was included in the study, as only eleven ticks other than *I. ricinus* were found (Hasle et al., 2009 a). We defined nymphs that were flat or slightly thickened but with no increased distance (i.e., <0.66 mm) from the posterior margin of the scutum to the posterior margin of the abdomen, as unengorged. Nymphs and larvae that were thickened and with a prolonged abdomen were defined as engorged. All nymphs collected from birds on Akerøya and Store Færder were examined for pathogens. From Jomfruland and Lista, which have dense local tick populations, we only analysed engorged tick nymphs collected from birds caught before or during the peak arrival time for each bird species. These nymphs could not have been acquired locally during the last day, as the size of *I. ricinus* nymphs does not increase significantly during the first 24 hours of feeding (Gray et al., 2005). This selection was done to minimise the risk of including ticks that had been acquired after their arrival in Norway. We had only 8 unengorged (flat) larvae in the material from Akerøya and Store Færder, which we found to be too few for any sensible analysis. Only fully engorged larvae were included in the analyses. Crushed specimens were not examined, unless most of the contents were still present. Altogether, 513 nymphs and 172 larvae were examined.

The specimens were crushed with the tip of a glass rod, and DNA was isolated by a spin-column technique using the DNeasy Blood & Tissue Kit (Qiagen) for the nymphs and E.Z.N.A Micro Elute® Genomic DNA KIT (OMEGA Bio-Tek) for the larvae, according to the manufacturer's protocols. The isolation of DNA essentially consisted of lytic, washing and elution steps. Engorged and unengorged ticks were treated equally. To avoid inter-specimen contamination, the ticks were handled only with tweezers, which were flame-sterilised after each use, both during field collection and during the laboratory procedure.
We used a general *Borrelia* fla B outer primer, F and R (Clark et al., 2005). The PCR mixture consisted of 2.5 µl DNA isolate, 2.5 mM dNTP, 2.5 µl 10x Qiagen buffer,

1.25µl primer at a concentration of 10 pmol/µl, 0.125 µl Hot Star Taq DNA polymerase (Qiagen) and a volume of sterile distilled water to bring the total volume to 22.5 µl. After heat activation at 95°C for 15 minutes, the PCR protocol was run for 40 cycles: denaturation at 94°C for 30 seconds, annealing at 52°C for 45 seconds and extension at 72°C for 60 seconds. A final 10-minute extension step was performed at 72°C. Before sequencing, the PCR-products were purified by incubating 5 µl PCR and 2 µl Exosap-IT for 15' at 37°C and inactivating the Exosap-IT by heating it to 80°C for 15 minutes. All PCR experiments were conducted in a dedicated facility with separate rooms for reagent preparation, sample preparation, PCR setup and post-PCR analysis, and there was a one-way flow of samples and materials. Regular hypochlorite and/or UV decontamination was performed.

All of the *Borrelia*-positive samples were sequenced to identify different species. Sequencing was performed on a 3100 Genetic Analyzer (Applied Biosystems) using 1.5 µl PCR-product, 1.5 µl Big Dye Terminator version 1.1 (Applied Biosystems), 1.5 µl sequencing buffer, provided by the manufacturer, 3.3 pmol *Borrelia* fla B outer primer, and sterile distilled water to bring the total volume to 10 µl. Some of the samples sequenced with the reverse primer were also sequenced with the forward primer, producing the same result. The sequences were processed with MEGA 4 and aligned in Basic Local Alignment Search Tool (BLAST), National Centre for Biotechnology Information (NCBI). When a ≥ 99% match with a selected sequence of the PCR-products could be obtained, the sequence was recorded as species identification.

Exact confidence intervals for the binomial proportions were calculated using the principles described by Clopper and Pearson (1934), implemented in R (R Development Core Team, 2008). For statistical comparison of the different selections of the sample, we performed a two-sided Fisher's exact test, using SPSS, version 15.

Lista appeared to differ from the more eastern bird observatories in terms of prevalence of *Borrelia* spp. For a more rigorous test of this, we used logistic regression (Generalised Linear Model, GLM, McCullagh and Nelder, 1989), implemented in R. When a binomial distribution was used, there was no significant over- or underdispersion. Possible differences between years and ticks from *Turdus* vs. non-*Turdus* species were taken into account. Ticks may possibly transfer *Borrelia* to each other while feeding close to each other on the same bird, and this could confound the results. Therefore, in cases where ticks were cofeeding with ticks that harboured the same *Borrelia* sp., we included only one tick in this analysis to correct for a possible effect of cofeeding.

For studying the effect of cofeeding, we performed logistic regressions of each of the *Borrelia* sp. Differences between *Turdus* and non-*Turdus* species and engorgement, which in itself could influence the prevalence of *Borrelia*, were taken into account, and also the interaction between cofeeding and *Turdus* spp. Model selection was performed by the use of the AIC. Possible effects of the ticks' instar were also tested.

Because the ticks were handled with tweezers, the participants of the study were not at risk for contracting pathogens from the ticks. The National Board of Animal Experimentation approved the field collecting and handling of the birds.

Results

We detected *Borrelia* in 70 (13.6%) of 513 nymphs and in 14 (8.1%) of 172 larvae. No ticks carried more than one *Borrelia* species. *B. garinii* was the most common species, followed by *B. afzelii*, *B. valaisiana* and a species which have never previously been found in Europe, *B. turdi* (Table 1).

Nymphs and larvae collected from birds from the Lista bird observatory had a lower prevalence of *Borrelia* spp. than the three more Eastern bird observatories, Akerøya, Jomfruland and Store Færder, at 6.1% versus 13.3%, respectively (two-sided Fisher's exact test, P=0.046). Ticks from birds on Store Færder had a significantly higher prevalence of *Borrelia* sp. (17.1%) than did ticks from birds at the other observatories (9.8%) (two-sided Fisher's exact test, P=0.007), which can partly be ascribed to a cluster of six *Borrelia*-positive ticks in a flock of blackbirds (*Turdus merula*) and song thrush (*T. philomelos*) caught on April 16, 2003.

Most of the ticks were collected from ground feeding passerine birds, such as the *Turdus* species, robins (*Erithacus rubecula*), redstarts (*Phoenicurus phoenicurus*) and dunnocks (*Prunella modularis*) (Table 2). Of the 330 birds with tick nymphs included in this study, 47 (55%) of 86 birds of the genus *Turdus* had more than one nymph, compared to only 59 (24%) of the 244 birds of other genera (Two-sided Fisher's exact test, P< 0.001). Strikingly, ticks from *Turdus* spp. had a much higher prevalence of *B. garinii* and *B. valaisiana* than those collected from other bird genera (two-sided Fisher's exact test, P<0.001). This difference was not observed for *B. afzelii* (Table 2 and 3).

In the logistic regression model, which included all species, there was a significantly negative effect of Lista (P=0.023) and a significantly positive effect of *Turdus* (P=1.16*10^{-7}) (Table 4). The difference between Lista and the other localities become more evident when only the *Turdus* species were included in the model (odds ratio 0.248, P=0.0393), and in a model with non-*Turdus* species only, there was no significant effect of Lista. In a similar test for each of the *Borrelia* species, we found significant effects of *Turdus*, Lista and year for *B. garinii* and of *Turdus* and year for *B. valaisiana*. There were no significant effects for *B. afzelii*, and for *B. turdi*, the data were insufficient.

Because all the examined ticks were found around the beak, eyelids and ear openings, ticks on the same bird were cofeeding near each other. The logistic regression models showed a significant positive effect of cofeeding with the same species for *B. afzelii* and *B. valaisiana* (Table 5). The interaction between cofeeding and *Turdus* spp. downregulates the combined effect of cofeeding and *Turdus* spp. The odds ratio (OR) of *Turdus* alone is 30.3, and combined with cofeeding, but without interaction it is 30.3*760= 23,000 . Including the interaction, the resulting OR becomes 30.3*760*0.00180=41.3, which is only a small increase from OR 30.3 for *B. valaisiana*. When "*Turdus* species" was excluded as a variable from the analyses, cofeeding was found to be nearly significant for *B. garinii* as well (P=0.0674). Engorgement was found to have a negative effect for *B. afzelii* and a positive effect for *B. valaisiana*. For *B. turdi*, we found no significant effects, which was most likely because of the small number of ticks infected with this pathogen. There was no effect of the ticks' instar, i.e., nymphs versus larvae in the cofeeding model.

Discussion

There will always be a certain degree of uncertainty whether a tick found on any migrating bird collected in a net was actually transported from southern localities across the sea rather than acquired locally (Hasle et al., 2009 a). In this study, we employed a very conservative approach by choosing only engorged nymphs caught before or during the peak arrival time of each migratory bird species from the localities where ticks occur. However, if birds remained near the bird observatory for several days after arrival, then local ticks could have had time to become engorged. This would especially be expected to be a problem at Lista, where many of the birds will have to rest after crossing the North Sea. In any case, it is reasonable to assume that the examined ticks were *mainly* imported.

The *Borrelia* spp. prevalence of 13.7% found in our study is significantly lower than that found in a study of Swedish northward migrating birds (Olsén et al., 1995). Olsén et al. reported that 36% of 479 nymphs were positive for the *Borrelia fla* gene and 34% positive with the o*spA* gene using PCR analysis. The prevalence of pathogens in ticks collected from recently arriving migratory birds would be expected largely to reflect the prevalence in host-seeking ticks at recent stop-overs along the migration route traversed over the previous three or four days. This may explain the different results for *Borrelia* prevalence in our study and that of Olsén et al., 1995. The main direction of bird migration in Europe is SW 230° (Bruderer, 1997). Birds captured at the Swedish coast follow a more easterly route during their northward migration, mainly through the Danish island Zealand (Fransson and Hall-Karlsson, 2008), than birds migrating to the Norwegian coast which mainly pass along the western coast of Europe, including Jutland. Birds arriving at Lista, especially blackbirds, may migrate directly from the British isles (Bakken et al., 2006), which implies that they have to cross at least 500 km of open sea. A meta-analysis (Rauter and Hartung, 2005) indicates a *Borrelia* prevalence in questing nymphs of 1.5% in Great Britain, 7.2% in Denmark, 8.0% in western Germany, 8.3% in eastern Germany and 9.0% in Poland. An extensive survey across Denmark gave an average of 5% (Jensen et al. 2000), whereas the eastern Danish island Zealand had an average of 15.5% (Vennestrøm et al., 2008). Consequently, one would expect a lower prevalence of *Borrelia* spp. in ticks from birds caught on Lista than those caught on the more eastern localities along the Norwegian coast, as we found. Also, the lower prevalence of *Borrelia* in our study compared to the Swedish study (Olsén et al., 1995) indicates that the birds caught in Norway are bringing ticks from areas where the prevalence of *Borrelia* in ticks is lower than in the more Eastern parts of continental Europe. The prevalence of *Borrelia* was higher than in the reports of questing nymphs (Rauter and Hartung, 2005), both in our study and in the study reported by Olsén et al., indicating that the ticks also acquire *Borrelia* while feeding on the birds.

Interestingly, the *Borrelia* species also varied in their distributional pattern in ticks from different localities (Table 1). The negative effect of Lista was only statistically significant in the GLM for *B. garinii*, although no ticks infected with *B. afzelii* were caught at Lista. This fits well with the low prevalence of these species in ticks on the British Isles. Further studies are needed to determine if the distribution of the *Borrelia* genospecies along the migratory routes can explain this pattern. In our study, nymphs that were collected from the island Store Færder had significantly more *Borrelia* sp. than nymphs collected on Akerøya, an island that is only 20 km east of Store Færder but on the other side of the Oslo fjord. We do not have a good explanation to this; it may be attributed to an accidental clustering of *Borrelia*-infected ticks.

The number of *Borrelia*-infected ticks imported by birds will be small compared with the number of *Borrelia*-infected ticks already present on the ground. However, we cannot exclude the possibility that birds may facilitate the import of foreign *Borrelia* strains and genospecies with different virulence (Purser and Norris, 2000) or immunological properties (Nadelman and Wormser, 2007). Such an occurrence could impact the risk of human reinfection. In this study, we found two *Borrelia* species, *B. valaisiana* and *B. turdi,* which have not been found previously in Norway. *B. valaisiana* was found at all the four bird observatories. Of *B. turdi*, one was found at Akerøya and two at Store Færder. The finding of new species illustrates the birds' potential to import new pathogens. *B. valaisiana* may possibly cause serious infections in humans (Diza et al., 2004) and may also be underdiagnosed because of low sensitivity in conventional antibody assays. There are no studies which have explicitly tested the sensitivity for *B. valaisiana* of the ELISA-kits for *Borrelia* IgM and IgG currently used in Norway (Personal communication with representatives for Diasorin, Oxoid and Siemens Healthcare). However, an immunoblot study with antigen prepared from *B. burgdorferi* s.s., *B. garinii*, *B afzelii* and *B. valaisiana* showed crossreactivity in several of the epitopes (Ryffel et al., 1999). *B. valaisiana* was the most numerous species in the Lista material. *B. turdi* (originally referred to as *Borrelia* Ya501, Fukunaga et al. 1996) has previously found only been found in Japan, in *I. turdus*, a tick parasitizing birds in Japan. *B. turdi* is not known to infect humans (Baranton and Martino, 2009). The identification of this species was based on sequencing, with a match of 99% in BLAST, with no other near matches. The so far extremely patchy observation of this species is difficult to explain. Very likely it has a wider distributional range, but we cannot exclude the possibility that this is in fact a previously unknown *Borrelia* species.

A review by Hubálek and Halouzka (1998), showed average prevalences of *Borrelia* in *I. ricinus* throughout Europe of 1.9%, 10.8% and 17.4% in questing larvae, nymphs and adults respectively. The infections in the questing larvae indicate a substantial transovarial transmission of *Borrelia* spp. in *I. ricinus*, more than reported for *I. scapularis* (Magnarelli et al., 1987; Patrican, 1997) and *I. persulcatus* (Nefedova, 2004) . The prevalence of *Borrelia* in the larvae in our study is so much higher than the average in questing larvae that one may suspect that the *Borrelia* in most cases was acquired during feeding. *B. garinii*, *B. lusitania*, and *B. valaisiana* have been previously found infecting birds, but *B. afzelii* has not (Nakao et al., 1994; Olsén et al., 1993; Kurtenbach, 2002; Háninková et al., 2003; Poupon et al. 2006). This finding is consistent with the fact that we found *B. afzelii* only in the nymphs, which may have acquired them from a previous host. However, a recent study found *B. afzelii* in 11 out of 56 examined *I. ricinus* larvae collected from robin and *Turdus* spp. (Franke et al., 2010). In addition, previous studies have occasionally found *B. afzelii* in *I. ricinus* larvae collected from birds (Olsén et al., 1993; Poupon et al. 2006). This could indicate that the birds may be infected with *B. afzelii,* but transovarial transmission of *B. afzelii* and cofeeding with *B. afzelii*-carrying nymphs may also explain these findings.

The *Turdus* spp. are known to have a higher tick infestation rate than other passerine birds (Mehl et al., 1984; Olsén et al., 1995; Ischiguro et al., 2000; Hasle et al., 2009 a). This higher rate may be due to their habit of spending much time on the ground in search for food, which increases the risk/likelihood for acquiring ticks (Mehl et al., 1984, Hasle et al., 2009 a and b). Therefore, the *Turdus* species appear to be more important than other passerine genera in transporting infected ticks. In addition, the ticks from these birds had a higher prevalence of *B. garinii* and *B. valaisiana* than non-*Turdus* species.

An obvious explanation would be if the *Turdus* species are more competent hosts for *Borrelia* than other passerines, but the observation may also be explained by differences in migratory routes, variations in pathogen prevalence in ticks among habitats used by different bird species, or possibly by horizontal transmission through co-feeding. Our finding that *Turdus* sp. had no effect on the prevalence of *B. afzelii* in the logistic regression indicates that this *Borrelia* species does not infect the *Turdus* species, and thus suggesting that those found on ticks from *Turdus* species have been acquired from a previous host or through cofeeding with other ticks carrying *B. afzelii*. Cofeeding with an infected tick appears to increase the prevalence of *B. afzelii* and *B. valaisiana.* For *B. turdi*, we had too little data to make any conclusions. However, our analyses cannot distinguish between infection caused by direct transmission between cofeeding ticks and infection of the ticks acquired from an infected bird. As the *Turdus* species tend to harbour many ticks (Hasle et al., 2009a), cofeeding will covariate with feeding on a *Turdus* species. For *B. garinii,* the effect of cofeeding was non-significant, but this could be due to the correlation between *Turdus* spp. and cofeeding with infected ticks (the strong effect of *Turdus* indicates that *B. garinii* primarily infects this genus)*.* Non-systemic transmission of *Borrelia* species has been shown in mice (Gern and Rais, 1996). Such a mechanism of transmission to birds has been suggested by different authors (Randolph et al., 1996; Poupon et al., 2006). This mechanism seems likely because, in our experience, ticks cofeeding on birds are densely clustered around the beak or on the eyelids. As *B. afzelii* has not been found infecting birds, our results might indicate direct transmission by cofeeding.

Our finding of a lower prevalence of *B. afzelii* in engorged compared to unengorged ticks may be explained by an inhibitory effect of the pathogen during feeding on the birds (Matuschka and Spielman, 1992). Although possible inhibitory substances from the blood should be eliminated by the lysis and washing steps during DNA isolation, we cannot rule out the possibility that the ingested blood in the ticks had an inhibitory effect on the PCR-analysis (Al-Soud and Rådström, 2001). If this was the case, then our results may show an artificially low prevalence of the *Borrelia* species in the engorged specimens and, consequently, an underestimation of the birds' role in infecting the ticks with *Borrelia*.

The finding of infected ticks on birds during spring migration illustrates an important potential for the spread of tick-borne pathogens. Our findings suggest three mechanisms of the birds' role in spreading *Borrelia*-infected ticks. First, passive transport of previously infected nymphs, and transovarially infected larvae may occur. Second, infected migratory birds may infect the ticks, which are then dropped off in a new location. In support of this mechanism, Gylfe et al. (2000) found a reactivation of *B. garinii* infection in experimentally infected redwings (*Turdus iliacus*) during migratory restlessness, indicating an increased risk of transmitting *Borrelia* to the tick vector during migration. If the migratory birds are chronic carriers of tick-borne diseases, then they may continue to transfer them to ticks that parasitize them after arrival. Sampling of blood or tissue from the birds would provide more information of this issue. Third, the tick vectors may transfer *Borrelia* species between each other through cofeeding while being transported.

The distribution range of *I. ricinus* is expanding further north and further inland in Scandinavia (Lindgren et al., 2000; Jore et al., 2009). In previous studies of birds

carrying infected ticks, the travel distances have not been evaluated. In our study, the ticks were collected from birds in which the majority were judged to have passed over Kattegat, Skagerrak and the North Sea the same night (Hasle et al., 2009 a), which means a non-stop flight of at least 112 km. The introduction of only one or a few infected ticks may be sufficient to establish the pathogen in a new area if a tick population is already present. This study, combined with data tick-infestation frequencies on birds (Hasle et al., 2009 a) and population estimates of the different migratory bird species (Gjershaug et al., 1994), provides data for estimating the magnitude of the transport of pathogens by birds.

Acknowledgements

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Oslo, October 15th 2010

Dear Jochen Süss

Thanks for your e-mail of October 12th 2010, and the enclosed referee's comments. Hopefully we have now resolved the problems with the third version of manuscript, and we look forward to hearing from you.

Our responses to the referees' comments are written with *Italics* in the text below.

Yours sincerely

Gunnar Hasle

Ms. Ref. No.: TTBDIS-D-09-00029
Title: Transport of *Ixodes ricinus* infected with *Borrelia* species to Norway by northward-migrating passerine birds.

(1) "A total of..." always takes the singular form of the verb, not the plural as you have it in a few places. For example, "A total of 1,000 ticks was collected: is the correct format.
Now the two cases of "A total of…was" are corrected.

(2) Abstract and Results - give percentage of ticks in parentheses that were infected with spirochetes. For example, 10(10%) of 100 ticks.
The percentages are now added, as required.

(3) Abstract: Consider mentioning what Turdus spp. you evaluted.
*We have now inserted (*T. merula, T. philomelos *and* T. iliacus*).*
For T. pilaris *(one nymph, infected with* B. garinii*) and* T. torquatus *(nine uninfected nymphs) we don't have sufficient data.*

(4) Mention what tick species (I. ricinus) you evaluated in abstract too - not just in title.
The species name is now inserted before the number of nymphs and larvae, respectively.

We also added the tick species in the tables.

(5) Please add pagination throughout text.
This is now done.

(6) Materials and Methods, page 1, line 57: Change "(Clark 2005)" to "(Clark et al. 2005)"
This is now corrected

(7) Discussion, page 3, line 2/3: "This The" - please correct.
This is now corrected

(8) Table 1 title: provide locality information and dates -
Tables must always be written to stand alone.
New title:
Table 1. Percent of Borrelia-*positive* Ixodes ricinus *as
determined by PCR analysis collected from northward migrating
passerine birds at four different bird observatories along the
southern Norwegian coast during 2003-2005. In parentheses: 95%
confidence interval.*

(9) Table 2 title: could be improved as well. Please eliminate
the word "crosstabulation" and recast the title.
New title:
Table 2. Borrelia-*spp. as determined by PCR analysis found in
nymphs and larvae of* Ixodes ricinus *collected from northward
migrating passerine birds at four different bird observatories
along the southern Norwegian coast: Akerøya, Jomfruland, Lista
and Store Færder, during 2003-2005.*

(10) Table 5: Title is too long and unwieldly; suggest recast.
Also, in body of table, it is unclear if you are talking about
larval or nymphal ticks.
*The new version has a short title, but an explanation that still
is long. This is a complicated issue, and we believe that this
new text makes it understandable:*
Table 5. Effect of cofeeding on Borrelia *infection in* Ixodes
ricinus *(nymphs and larvae). The influence of cofeeding with a
tick infected with the same* Borrelia *species is given as odds
ratios with 95% confidence intervals. A logistic regression (GLM)
was performed for each of the four* Borrelia *species. The
covariates in the model are listed in the left column. Different
effects of nymphs and larvae were also tested, but not found
significant for any* Borrelia *species. The prevalences for the
case that the included variables are zero (=intercept) are given
in parentheses after the* Borrelia *species names. In cases marked
N.S. the variable was excluded during the the model selection
process due to lack of statistical significance. The ticks were
collected from northward migrating birds at the southern
Norwegian coast 2003-2005.*

*The phrase "Model selection was performed by the use of the AIC."
is moved to Material and Methods.*

To explain, we had to insert this in the result chapter:

The interaction between cofeeding and Turdus *spp. downregulates the combined effect of cofeeding and* Turdus *spp. The OR of* Turdus *alone is 30.3, and combined with cofeeding, but without interaction it is 30.3*760= 23,000 . Including the interaction, the resulting OR becomes 30.3*760*0.00180=41.3, which is only a small increase from OR 30.3 for* B. valaisiana.

In addition, in the discussion:
For B. garinii*, the effect of cofeeding was non-significant, but this could be due to the correlation between* Turdus *spp. and cofeeding with infected ticks (the strong effect of* Turdus *indicates that* B. garinii *primarily infects this genus).*

Also, some edting has been done in the table to separate clearly OR, confidence intervals and P-value.

Table

Table 1. Percent of *Borrelia*-positive *Ixodes ricinus* as determined by PCR analysis collected from northward migrating passerine birds at four different bird observatories along the southern Norwegian coast during 2003-2005. In parentheses: 95% confidence interval.

Observatory	N	*Borrelia afzelii*	*B. garinii*	*B. turdi*	*B. valaisiana*	All *Borrelia* spp.
Nymphs:						
Akerøya	177	3.4(1.2-7.2)	5.1(2.3-9.4)	0.6(0-3.1)	0.6(0-3.1)	9.6(5.7-14.9)
Jomfruland	95	3.1(0.6-9.0)	9.4(4.4-17.2)	0(0-3.8)	1.0(0-5.7)	13.7(7.5-22.3)
Lista	80	0(0-4.5)	2.5(0.3-8.8)	0(0-4.5)	5.0(1.4-12.3)	7.5(2.8-15.6)
Store Færder	161	6.2(3.0-11.1)	11.2(6.8-17.1)	0.6(0-3.4)	3.1(1.0-7.1)	21.1(15.1-28.2)
All nymphs	513	3.7(2.2-5.7)	7.4(5.3-10.0)	0.4(0-1.4)	2.1(1.0-3.8)	13.6(10.8-16.9)
Larvae:						
Akerøya	58	0(0-6.2)	6.9(1.9-16.7)	0(0-6.2)	5.2(1.1-14.4)	12.1(5.0-23.3)
Jomfruland	23	0(0-14.8)	4.3(0.1-22.0)	0(0-14.8)	0(0-14.8)	4.3(0.1-22.0)
Lista	18	0(0-18.5)	0(0-18.5)	0(0-18.5)	0(0-18.5)	0(0-18.5)
Store Færder	73	0(0-4.9)	6.8(2,3-15.3)	1.3(0-7.4)	0(0-4.9)	8.2(3.1-17.0)
All larvae	172	0(0-2.1)	5.8(2.8-10.4)	0.4(0-3.2)	2.1(0-3.2)	8.1(4.5-13.3)

Table

Table 2. *Borrelia*-spp. as determined by PCR analysis found in nymphs and larvae of *Ixodes ricinus* collected from northward migrating passerine birds at four different bird observatories along the southern Norwegian coast: Akerøya, Jomfruland, Lista and Store Færder, during 2003-2005.

Bird host	N	*B. afzelii*	*B. garinii*	*B. turdi*	*B. valaisiana*
Nymphs:					
Acrocephalus palustris	1	0	0	0	0
Acrocephalus scirpaceus	1	0	0	0	0
Anthus pratensis	4	0	1	0	0
Carduelis cabaret	5	1	0	0	0
Carduelis cannabina	7	0	1	0	0
Carduelis chloris	4	0	0	0	0
Carpodacus erythrinus	7	0	1	0	0
Erithacus rubecula	112	2	4	0	0
Fidecula hypoleuca	1	0	0	0	0
Fringilla coelebs	1	0	0	0	0
Lanius collurio	2	0	0	0	0
Luscinia luscinia	4	0	0	0	0
Luscinia svecica	2	0	0	0	0
Motacilla alba	2	0	0	0	0
Oenanthe oenanthe	2	0	0	0	0
Parus major	2	0	0	0	0
Phoenicurus phoenicurus	38	1	1	0	0
Phylloscopus collybita	3	0	0	0	0
Phylloscopus trochiloides	1	0	0	0	0
Phylloscopus trochilus	36	2	2	1	1
Prunella modularis	36	3	1	1	2
Pyrrhula pyrhula	1	0	0	0	0
Regulus regulus	6	0	0	0	0
Saxicola rubetra	2	1	0	0	0
Sitta europeae	1	0	1	0	0
Sylvia atricapilla	14	0	0	0	0
Sylvia borin	2	0	0	0	0
Sylvia communis	7	0	1	0	0
Sylvia curruca	22	2	0	0	0
Troglodytes troglodytes	2	0	0	0	0
Turdus iliacus	21	0	5	0	3
Turdus merula	98	4	15	0	4
Turdus philomelos	55	3	4	0	0
Turdus pilaris	1	0	1	0	0
Turdus torquatus	9	0	0	0	1
Willow Warbler	1	0	0	0	0
Sum of positive nymphs	513	19	38	2	11
Larvae:					
Acrocephalus palustris	1	0	0	0	0
Acrocephalus scirpaceus	2	0	0	0	0
Anthus pratensis	1	0	0	0	0
Emberiza schoeniclus	1	0	0	0	0
Erithacus rubecula	77	0	3	0	0
Luscinia luscinia	5	0	0	0	0
Phoenicurus phoenicurus	16	0	1	0	0
Phylloscopus collybita	2	0	0	0	0
Phylloscopus trochilus	11	0	1	0	0
Prunella modularis	1	0	0	0	0
Sylvia atricapilla	16	0	0	0	0
Sylvia communis	3	0	0	0	0
Sylvia curruca	2	0	0	0	0
Troglodytes troglodytes	10	0	2	0	0
Turdus iliacus	3	0	1	0	0
Turdus merula	8	0	1	0	2
Turdus philomelos	13	0	1	1	1
Sum of positive larvae	172	0	10	1	3

Table

Table 3. Percentage of the four *Borrelia* species found in the study in *Ixodes ricinus* collected from the *Turdus* species compared with other passerine birds. In parentheses: 95% confidence intervals.

	N ticks tested	Borrelia afzelii	B. garinii	B. turdi	B. valaisiana
Turdus species	208	3.4(1.4-6.8)	13.5(9.1-18.9)	0.5(0-2.7)	5.3(2.7-9.3)
Non-Turdus species	477	2.5(1.3-4.4)	4.2(2.6-6.4)	0.4(0-1.5)	0.6(0.1-1.8)
χ^2 2-sided Fisher's exact test		*P*=0.613	*P*<0.001	*P*=1.000	*P*<0.001

Table 4. Logistic regression (GLM) for the effect of Lista, *Turdus* sp. and year on all the *Borrelia* spp. in *Ixodes ricinus* collected from northward migrating passerine birds. Odds ratios are calculated in relation to the intercept, i.e., "Not *Turdus* sp.", "Not Lista" and "Year 2003". In parentheses: 95% confidence intervals.

	Odds ratio	*P*
Turdus sp.	4.284(2.501-7.339)	$1.16 * 10^{-7}$
Lista	0.325(0.124-0.853)	0.023
Year 2004	0.482(0.252-0.922)	0.027
Year 2005	0.373(0.186-0.748)	0.005

Table

Table 5. Effect of cofeeding on *Borrelia* infection in *Ixodes ricinus* (nymphs and larvae). The influence of cofeeding with a tick infected with the same *Borrelia* species is given as odds ratios with 95% confidence intervals. A logistic regression (GLM) was performed for each of the four *Borrelia* species. The covariates in the model are listed in the left column. Different effects of nymphs and larvae were also tested, but not found significant for any *Borrelia* species. The prevalences for the case that the included variables are zero (=intercept) are given in parentheses after the *Borrelia* species names. In cases marked N.S. the variable was excluded during the the model selection process due to lack of statistical significance. The ticks were collected from northward migrating birds at the southern Norwegian coast 2003–2005.

	Cofeeding with:			
	B. afzelii (0.0518)	*B. garinii* (0.0419)	*B. turdi*	*B. valaisiana* ($3.8457*10^{-4}$)
Cofeeding	3.876 (1.0160-9.3832) P=0.0468	N.S.	N.S.	759.758 (34.20-16877) P=$2.76*10^{-5}$
Engorged	0.24005 (0.08999-0.66436) P=0.00436	N.S.	N.S.	6.8415 (0.8618-54.31) P=0.0688
Turdus species	N.S.	3.544 (1.9523-6.4713) P=$3.35*10^{-5}$	N.S.	30.30 (3.7939-241.9) P=0.001288
Cofeeding* *Turdus* spp.	N.S.	N.S.	N.S.	0.001795 ($5.46*10^{-5}$-0.059) P=0.000388