Role of birds in the biology of Lyme disease Borrelia

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Antibodies against *B. burgdorferi* s.l. were detected in people frequently bitten by *I. uriae*. Thus the marine enzootic *Borrelia* cycle may be a risk for humans, either by direct transfer of the spirochete from *I. uriae* or via introduction of *Borrelia* into a terrestrial enzootic *Borrelia* cycle.

In order to investigate the role of passerine (Passeriformes) birds as amplification hosts in the terrestrial *Borrelia* cycle, experimental infections of canary finches (*Serinus canaria*) and redwing thrushes (*Turdus iliacus*) were carried out. The result showed that *B. burgdorferi* s.l. can persist for several months in passerine birds and the infection in redwing thrushes can be reactivated in response to migration. Thus, birds may be more infectious to ticks during their migration and therefore important long-range disseminators of *B. burgdorferi* s.l.

Migration in birds is associated with elevated stress hormones that in turn can cause reactivation of latent infections. Lyme disease in humans could perhaps be activated when the immune response is modulated by stress. Herein I describe a patient with a stress activated latent *Borrelia* infection, which supports this hypothesis.

The seabird tick *I. uriae* has a circumpolar distribution in both the northern and southern hemispheres and in this study identical *B. garinii* flagellin gene (*flaB*) sequences were detected in *I. uriae* from these hemispheres, indicating a transequatorial transport of *B. garinii*. Parsimony analysis of *I. uriae* ITS2 and 16S rDNA sequences suggested that northern and southern *I. uriae* might be reproductively separated. Therefore passive transport of infected ticks between the polar regions is unlikely and instead seabirds probably carry an active *Borrelia* infection during their migration.

In conclusion, this work shows that migrating seabirds and passerine birds probably are important for the long-range dispersal of *B. burgdorferi* s.l., and that this mechanism of dispersal could be important for the distribution of human Lyme disease.

Key words: Lyme disease, birds, *Borrelia*, *I. uriae*, infection reactivation, stress, bipolar distribution, transequatorial transport

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LIST OF ABBREVIATIONS AND TERMS

B. burgdorferi s.l.	*Borrelia burgdorferi* sensu lato
B. burgdorferi s.s.	*Borrelia burgdorferi* sensu stricto
bp	base pair
ELISA	enzyme-linked immunosorbent assay
EM	erythema migrans
FlaB	flagellin, flagellar core protein
HSV-1	herpes simplex virus type 1
ITS2	internal transcribed spacer 2, located between nuclear 5.8S and 28S rRNA genes in eucaryotic cells
kbp	kilo basepairs
kDa	kilo Dalton
Mbp	Mega basepairs
Osp	outer surface protein
PCR	polymerase chain reaction
PFGE	pulse-field gel electrophoresis
rDNA	DNA encoding rRNA genes
RFLP	restriction fragment length polymorphism
rRNA	ribosomal RNA
spirochetemia	spirochetes in the blood of an infected host

PAPERS IN THIS THESIS

This thesis is based on the following articles and manuscripts that are referred to in the text by their Roman numerals (I-VI).

I. Gylfe, Å., Olsen, B., Straševičius, D., Marti Ras, N., Weihe, P., Noppa, L., Östberg, Y., Baranton, G., Bergström, S., 1999. Isolation of Lyme disease *Borrelia* from puffins (*Fratercula arctica*) and seabird ticks (*Ixodes uriae*) on the Faeroe Islands. J. Clin. Microbiol. 37:890-896.

II. Olsen B., Gylfe, Å., Bergström, S., 1996. Canary finches (*Serinus canaria*) as an avian infection model for Lyme borreliosis. Microb. Pathog. 20:319-324.

III. Gylfe, Å., Bergström, S., Lundström, J., Olsen, B., 2000. Reactivation of *Borrelia* infection in birds. Nature 403:724-725.

IV. Gylfe, Å., Wahlgren, M., Fahlén, L., Bergström, S., 2001. Stress-activated latent Lyme disease. Submitted.

V. Olsen, B., Duffy, D. C., Jaenson, T. G. T., Gylfe, Å., Bonnedahl, J., Bergström, S., 1995. Transhemispheric exchange of Lyme disease spirochetes by seabirds. J. Clin. Microbiol. 33:3270-3274.

VI. Gylfe, Å., Yabuki, M., Drotz, M., Bergström, S., Fukunaga, M., Olsen, B., 2001. Phylogeographic relationships of *Ixodes uriae* (Acari: Ixodidae) and their significance to transequatorial dispersal of *Borrelia garinii.* Submitted.

INTRODUCTION

Lyme disease is a vector borne zoonosis caused by spirochetes in the group *Borrelia burgdorferi* sensu lato (s.l.) (Wang et al., 1999c). Hard ticks (Acari: Ixodidae) serve as vectors and small rodents are the main reservoirs, although several mammals and birds are competent reservoirs for *B. burgdorferi* s.l. Since birds fly and travel long distances during their migration, they are excellent vehicles for various ectoparasites and microorganisms. This thesis describes the role of birds in Lyme disease *Borrelia* biology in general and *Borrelia* ecology and epidemiology in particular.

Lyme disease

The first manifestation of Lyme disease is often erythema migrans (EM), a skin rash growing radially from the site of the tick bite (Weber and Burgdorfer, 1993). EM is often subclinical but can be associated with flu like symptoms such as fever, muscle pain and headache. Untreated, the infection may disseminate and cause various neurological symptoms including meningitis, arthritis or in rare cases myocarditis. In severe cases, Lyme disease can become chronic and resistant to treatment, with neurological disorders, arthritis or atrophy of the skin.

Diagnosis

The diagnosis of Lyme disease must be based on clinical symptoms and a history of exposure to ticks (Stanek et al., 1996). The ultimate confirmation is cultivation of *Borrelia* spirochetes from a biopsy specimen. It is however, difficult to perform, with a success rate of 0-70% depending on the type of specimen inoculated and stage of the disease (Wilske and Preac-Mursic, 1993). As the number of spirochetes in tissues is low, direct observation of spirochetes in samples is rarely possible but PCR amplification of *Borrelia* DNA is useful

and sometimes performed in clinical diagnosis (Lebech et al., 2000; Priem et al., 1997).

Serology is commonly used to support the diagnosis although a large proportion of patients do not develop antibodies against *Borrelia*, especially in early Lyme disease (Aguero-Rosenfeld et al., 1996). Enzyme-linked immunosorbent assay (ELISA) is the most common method used, but there are problems with false positives, due to e.g. other spirochetal infections, rheumatoid arthritis or Epstein-Barr virus infection (Magnarelli, 1995). Immunoblot is regarded as more specific and is often used together with a sensitive ELISA (Dressler et al., 1993; Ledue et al., 1996). Interpretation of the results depends on the strain used and the source of antigens (Hauser et al., 1998; Hauser et al., 1997) as well as on the immunological background of the population in the particular geographical area.

Causative agent

Spirochetes within the genus *Borrelia* can be divided into different pathogenic groups: Lyme disease *Borrelia*, relapsing fever *Borrelia*, and the animal spirochetosis agents *B. anserina* and *B. coriaceae* (Sonenshine, 1993).

Borrelia burgdorferi sensu lato

The causative agents of Lyme disease are *B. burgdorferi* s.l., a group consisting of the ten species, i.e. *B. burgdorferi* sensu stricto (s.s.), *B. afzelii*, *B. garinii*, *B. japonica*, *B. andersonii*, *B. valaisiana*, *B. lusitaniae*, *B. bissettii*, *B. tanukii*, *B. turdi*, and several unnamed variants (Baranton et al., 1992; Canica et al., 1993; Fukunaga et al., 1996a; Kawabata et al., 1993; Le Fleche et al., 1997; Marconi et al., 1995; Postic et al., 1998; Wang et al., 1997). *B. burgdorferi* s.s., *B. afzelii*, and *B. garinii* are the currently known human pathogens but there are also unknown types of *B. burgdorferi* s.l. isolated from Lyme disease patients (Picken et al., 1996; Strle et al., 1997; Wang et al., 1999b).

Spirochetes are long, thin, helical bacteria, with multiple bipolar endoflagella that make them highly motile. *B. burgdorferi* s.l. is up to 30 µm long, with a diameter of 0.2-0.5 µm (Barbour and Hayes, 1986; Goldstein et al., 1996) and can easily be viewed in a dark field or phase contrast microscope. Cultivation is possible at 20-37°C in rabbit serum supplemented BSKII that is a rich and complex medium (Barbour, 1984).

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Figure 1. *B. burgdorferi* s.l. stained by indirect immunofluorescence using the flagellin (FlaB) antibody H9724. (Photo: Björn Olsen)

B. burgdorferi s.l. show chemotaxis (Shi et al., 1998) and are especially motile in viscous medium (Kimsey and Spielman, 1990). In the periplasmic space 7-11 flagella attached to the poles are wrapped around the cell cylinder, giving the bacterium its characteristic flat wave shape (Barbour and Hayes, 1986; Goldstein et al., 1994; Motaleb et al., 2000). The outer membrane is fluid with an unusually high lipoprotein content (Brandt et al., 1990; Fraser et al., 1997). Several lipoproteins, notably the outer surface proteins OspA-C, show a differential pattern of expression in ticks and vertebrate hosts (Seshu and Skare, 2000). The functions of OspA and OspC have been thoroughly studied and have been shown to be important in the tick. OspA is expressed by the spirochete in the unfed tick's midgut and mediates adhesion to the midgut epithelium (Pal et al., 2000). OspC expression is induced 36-48 hours into the blood meal and probably mediates the escape of spirochete from the tick midgut via the

hemolymph to the salivary glands, from where it enters the new vertebrate host (Ohnishi et al., 2001; Schwan and Piesman, 2000; Schwan et al., 1995). Antibodies against OspA in the blood of vertebrate hosts kill spirochetes in the tick midgut before dispersal to the salivary glands, and thereby block transmission to the vertebrate host (de Silva et al., 1996; de Silva et al., 1999). Antibodes against OspC are also efficient, probably blocking the transmission by preventing migration of spirochetes to the salivary glands (Gilmore and Piesman, 2000). Recombinant OspA is an efficient vaccine against Lyme disease (Sigal et al., 1998; Steere et al., 1998) and immunizations with OspC confer protective immunity in animal studies (Gilmore et al., 1996). The use of these vaccines may however be limited by the apparent variability of these proteins (Wilske et al., 1996).

Genome and genetics

Most of the genome of the *B. burgdorferi* s.s. strain B31 was published in 1997 (Fraser et al., 1997) and it was completed in 2000 (Casjens et al., 2000). The genome size is 1.52 Mbp; 910 kbp on the linear chromosome, and 610 kbp divided on 12 linear and 9 circular plasmids. The G+C content is low, only 28.6% on the chromosome. No biosynthetic pathways and few virulence genes have been identified. The plasmids mainly contain unique *Borrelia* genes with unknown function, 14.5% of the plasmid genes being putative lipoprotein genes that may be involved in host-parasite interactions. Some plasmids have a high fraction of pseudogenes, possibly reflecting an ongoing rapid evolution. Plasmid content differs between strains and loss of plasmids has been associated with loss of infectivity (Casjens et al., 2000).

Insertional inactivation of genes has been performed in *B. burgdorferi* s.s. There are however numerous obstacles and infectious low passage strains are particularly difficult to transform (Tilly et al., 2000).

Typing methods

Both phenotypic and genotypic typing methods have been applied to the several hundreds of *B. burgdorferi* s.l. strains isolated. Among the phenotypic methods, serotyping using monoclonal antibodies against OspA (Wilske et al., 1993) and OspC (Wilske et al., 1995) is the simplest and most commonly performed (Wang et al., 1999c). Solely determining the protein profile on SDS-PAGE is not sufficiently reliable.

Genotypic methods are not only applicable to cultivated strains but also to PCR amplified DNA. PCR-based methods include species specific PCR, randomly amplified polymorphic DNA (RAPD) fingerprinting or arbitrary primed (AP-) PCR, Restriction Fragment Length Polymorphism of PCR amplified fragments (PCR-RFLP) and nucleotide sequencing (Wang et al., 1999c). PCR-RFLP of the intergenic spacer between the duplicated 23S and 5S rRNA genes in *B. burgdorferi* s.l. is particularly useful (Postic et al., 1994). Methods requiring cultivation of the spirochetes are for example ribotyping by hybridisation with rDNA directed probes to RFLP generated fragments of genomic DNA, pulse field gel electrophoresis (PFGE), and the labour-intensive DNA-DNA reassociation analyses. Many methods have high congruence but differing resolution at the species and subspecies levels (Wang et al., 1999c).

Taxonomy

B. burgdorferi s.l. taxonomy is based on whole genome DNA-DNA reassociation analyses. The general rule in bacterial taxonomy, that less than 70% similarity between genomes constitute different species, applies to *B. burgdorferi* s.l. (Postic et al., 1994; Wayne et al., 1987).

Sequence analysis of the 16S rRNA gene and the flagellin gene (*flaB*) supply data representative for the whole genome (Fukunaga et al., 1996b; Wang et al., 1997). These sequences are very conserved, and particularly the 16S rRNA gene sequences in *B. burgdorferi* s.l are highly homologous, indicating a

recent speciation (Le Fleche et al., 1997). Other gene sequences, for example *p66* (Jonas Bunikis, personal communication), or the *hbb* gene (Valsangiacomo et al., 1997) are more variable and may contain more phylogenetic information. The outer surface protein genes *ospA* and *ospC* are less reliable in phylogeny as they are highly variable (Theisen et al., 1995; Wang et al., 2000) and subject to lateral gene transfer (Dykhuizen et al., 1993; Jauris-Heipke et al., 1995; Livey et al., 1995; Rosa et al., 1992; Wang et al., 1999a). However, it is usually possible to correctly determine the species from *ospA* and *ospC* sequences (Wang et al., 2000).

Molecular and epidemiological methods have been used to study the evolutionary history of *B. burgdorferi* s.l. species. *B. garinii* is the most heterogeneous *B. burgdorferi* s.l. species and also has the widest geographic distribution, indicating that it may be the most ancient species (Marti Ras et al., 1997). Furthermore, it is believed that *B. burgdorferi* s.s. evolved in North America because of the greater heterogeneity of strains observed there compared to Europe (Foretz et al., 1997; Marti Ras et al., 1997). Migrating birds may be important in the evolution of *B. burgdorferi* s.l. species if they serve as vehicles for these spirochetes. Once introduced into a new region the spirochete may further evolve to adapt to new reservoir hosts, ticks and ecological niches.

Biogeography and biodiversity

Lyme disease is endemic in large parts of Europe, Asia and North America. *B. burgdorferi* s.s. (Johnson et al., 1984), *B. bissettii* (Postic et al., 1998), and *B. andersonii* (Marconi et al., 1995) are the species present in the USA. *B. burgdorferi* s.s. is also found in western Europe but the most common species in Euroasia are *B. afzelii,* and *B. garinii* (Hubálek and Halouzka, 1997; Li et al., 1998). *B. valaisiana* is frequently detected in Ireland, Great Britain and the Netherlands and has also been isolated in central Europe (Hubálek and Halouzka, 1997; Kurtenbach et al., 1998b). *B. lusitaniae* predominates in

Portugal and Tunisia, and is rarely encountered in central and eastern Europe (De Michelis et al., 2000; Le Fleche et al., 1997). *B. bissettii* like strains were also isolated from patients in Slovenia, indicating that this species occur outside North America and may be pathogenic to humans (Picken et al., 1996; Strle et al., 1997). *B. japonica*, *B. tanukii*, and *B. turdi* are restricted to Japan (Fukunaga et al., 1996a; Kawabata et al., 1993; Masuzawa et al., 1996). There are no confirmed cases of Lyme disease in the southern hemisphere apart from cases where it may have been acquired in Europe (Hudson et al., 1998). This may be a reflection of the lack of competent vector ticks for humans.

Different *B. burgdorferi* s.l. species may have different disease panorama (Wang et al., 1999c), and within a species some types of strains are more prone to cause disseminated infection (Marconi et al., 1999; Seinost et al., 1999; Wormser et al., 1999). Frequencies of strain types often differ when isolates from ticks and patients from the same area are compared (Seinost et al., 1999). Notably, culturing is a bias since it may select for strains that grow well in BSKII medium and not reflect the natural diversity of strains (Liveris et al., 1999; Norris et al., 1997). The virulence factors causing differences in pathogenicity between species and strains need to be further investigated.

Vectors

Vector competence is demonstrated by the ability of the vector to acquire the infection through a blood meal, retain it after moulting and transmit the pathogen to a new host. Ticks (Acari) are vectors for *Borrelia* spirochetes; relapsing fever *Borrelia* is transmitted by soft ticks (Argasidae) and Lyme disease *Borrelia* by hard ticks (Ixodidae) (Sonenshine, 1993). Ticks are arthropods that have several life stages and each transformation requires a blood meal from a vertebrate host. Ixodid ticks undergo three life stages, larva, nymph and adult in 1-3 years depending on the climate (Sonenshine, 1993). These ticks

also transmit other microbial pathogens such as *Ehrlichia, Babesia, Rickettsiae, Francisella* and several viruses (Sonenshine, 1993).

The Ixodes ricinus complex

Ixodid ticks in the *Ixodes ricinus* complex are the predominant vectors of *B. burgdorferi* s.l. (Burgdorfer et al., 1991) and feed on a broad range of vertebrate hosts (Sonenshine, 1993). Larva mainly feed on small rodents but also on birds and lizards. Nymphs use the same hosts as larvae and, in addition, larger mammals. The adult female needs approximately 0.7 ml of blood to be able to lay eggs. Therefore, larger mammals such as deer and rabbits are important for tick reproduction (Sonenshine, 1993). *Borrelia* can persist in the tick through the different life stages, but transovarial transmission to larvae is rare and the main source of infection is vertebrate blood (Matuschka et al., 1992a; Sonenshine, 1993).

The infection rates of different tick populations vary between 1-70% (De Michelis et al., 2000; Gustafson, 1994; paper V). In Europe, *I. ricinus* transmit *B. afzelii, B. garinii, B. burgdorferi* s.s., *B. valaisiana,* and *B. lusitaniae* (Hubálek and Halouzka, 1997). In Asia, *I. persulcatus* transmit *B. afzelii* and *B. garinii* (Li et al., 1998; Nakao et al., 1994). In North America, *I. scapularis* and *I. pacificus* transmit both *B. burgdorferi* s.s. and *B. bissettii* (Postic et al., 1998).

Vector competence of other Ixodes ticks for B. burgdorferi s.l.

Some ticks are solely associated with a single *Borrelia* species and a single vertebrate host. In the USA, *I. dentatus* parasitising cottontail rabbits (*Sylvilagus floridanus*) transmit *B. andersonii* (Marconi et al., 1995; Anderson et al., 1989). In Japan, *I. ovatus* transmit *B. japonica, I. tanuki* transmit *B. tanukii,* and *I. turdus* transmit *B. turdi* (Fukunaga et al., 1996a; Kawabata et al., 1993).

B. burgdorferi s.l. has been isolated from numerous tick species, but many of them have not been shown to be competent vectors of this spirochete. Some ticks may have a restricted host range or geographic distribution that make them less important for human Lyme disease. For example, *I. spinipalis* transmit *B. bissettii* and *B. burgdorferi* s.s. (Postic et al., 1998) in the deserts of Colorado and New Mexico, but is due to the dry conditions restricted to rodent burrows (Dolan et al., 1997; Maupin et al., 1994).

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Figure 2. *I. uriae*, engorged nymph and female (Photo: Björn Olsen)

Ixodes uriae

The seabird tick *Ixodes uriae* has a unique circumpolar distribution in the polar, subpolar and bordering temperate regions of the northern and southern hemispheres, with no known resident population closer to the equator (Arthur,

1963; Chastel, 1988). The bipolar distribution is thought to reflect dispersal by migrating seabirds (Zumpt, 1952), because *I. uriae* is restricted to seabird colonies and parasites a broad range of seabirds including several species undertaking long migrations (Arthur, 1963; Mehl and Traavik, 1983). Given the opportunity, *I. uriae* also attacks mammals including humans (Arthur, 1963; Eley, 1977; Mehl and Traavik, 1983), but the survival is reduced after a mammalian blood meal (Nuttall, 1913). Several viruses, pathogenic to seabirds, have been isolated from *I. uriae* (Chastel, 1988). *B. garinii* has been found to cycle between *I. uriae* and seabirds in a "marine" enzootic *Borrelia* cycle (Bunikis et al., 1996; Olsen et al., 1993).

Reservoir hosts

Mammalian reservoirs for B. burgdorferi s.l.

Reservoir competence is best assessed by xenodiagnosis, i.e. determining the infection rate of laboratory reared pathogen-free ticks after feeding on the vertebrate host. The reservoir host must remain infectious for ticks for a long period of time (Gern et al., 1998) or alternatively be re-infected frequently. To be an important reservoir host, the animal has to be common and abundantly infested with immature stages of ticks (Mather et al., 1989b).

The most important reservoir hosts for *B. burgdorferi* s.l. are small rodents, in Europe e.g. various *Apodemus* mice, edible dormouse (*Glis glis*), and bank vole (*Clethrinomys glareolus* (Gern et al., 1998; Matuschka et al., 1992b). In North America the white-footed mouse (*Peromyscus leucopus*) (Anderson, 1991) has long-term active infections and readily transfer *Borrelia* spirochetes to uninfected ticks (Donahue et al., 1987). Also larger mammals, such as hares (*Lepus*), squirrels (*Sciurus*) and hedgehog (*Erinaceus europaeus*) serve as reservoir hosts in Europe (Gern et al., 1998). However, transmission of spirochetes to uninfected ticks can also occur via co-feeding of infected and

uninfected ticks on a non-reservoir competent vertebrate host (Ogden et al., 1997; Randolph et al., 1996).

Birds as reservoirs for B. burgdorferi s.l.

Birds may be important in dispersal of microorganisms (Palmgren et al., 1997) and ectoparasites (Hoogstraal et al., 1963) since they are highly mobile and many species undertake long migrations. Migratory terrestrial and marine birds may transport *B. burgdorferi* s.l. by carrying infected ticks long distances, or possibly by carrying the infection itself (Anderson and Magnarelli, 1984; Olsen et al., 1995). Some seabird species perform long-distance migrations, even migrations between the Arctic and Antarctic areas (del Hoyo et al., 1992). If migrating birds also carry an active infection within their bodies, this may be a more efficient way to transport pathogenic microorganisms, since many ticks can attach and become infected in the area of arrival.

B. burgdorferi s.l. has been isolated from blood, tissues and engorged ticks collected from several terrestrial bird species (Anderson et al., 1986; Anderson and Magnarelli, 1984; Humair et al., 1998; Ishiguro et al., 2000; McLean et al., 1993; Miyamoto et al., 1997; Nakao et al., 1994; Olsen et al., 1995; Smith et al., 1996; Stafford et al., 1995). Reservoir competence of American robins (*Turdus migratoruis*) (Richter et al., 2000), and pheasants (*Phasianus colchicus*) was recently demonstrated (Kurtenbach et al., 1998a). European Blackbirds (*Turdus merula*) are probably also competent reservoirs for *B. burgdorferi* s.l. (Humair et al., 1998) although, in another study, ticks lost their *B. burgdorferi* s.l. infection in the course of feeding on blackbirds and failed to infect them (Matuschka and Spielman, 1992). These results however, were based on a limited number of birds and therefore need verification. American robins, pheasants and blackbirds are common ground feeding birds, heavily infested with ticks, and therefore potentially important amplification hosts for *Borrelia.* Although feeding success of larvae on pheasants is impaired,

these birds are probably important in maintaining an enzootic cycle of *B. valaisiana* and *B. garinii* (Kurtenbach et al., 1998b). In some ecosystems, birds may even be the most important reservoir hosts for *B. burgdorferi* s.l. (Battaly and Fish, 1993; Gray et al., 2000; Kurtenbach et al., 1998b; Wright et al., 2000).

However, the importance of birds in Lyme disease epidemiology is still a matter of debate. For example, gray catbirds (*Dumetella carolinensis*) are heavily infested with ticks but are not competent reservoirs for *B. burgdorferi* s.l. (Mather et al., 1989a) and, in several studies, experimentally infected birds showed much shorter periods of infectivity to ticks compared to mice (Kurtenbach et al., 1998a; Piesman et al., 1996; Richter et al., 2000). Since *B. burgdorferi s.l.* has a growth optimum at 34°-37°C *in vitro* (Barbour, 1984), the high mean body temperature of passerine birds (Passeriformes), ~40°C (Welty and Baptista, 1988), was thought to be inconsistent with a role as *B. burgdorferi* s.l. amplification hosts. On the other hand, the body temperature of birds varies both temporally and spatially. For example, the skin and air sacs are important in regulating the body temperature and may have lower temperatures than internal organs (Welty and Baptista, 1988). *B. burgdorferi* s.l. has been successfully isolated from naturally infected blackbirds by aspiration of subcutaneously injected BSKII (Humair et al., 1998), and *B. garinii* was more frequently isolated from skin than from other organs in experimentally infected Japanese quail (*Coturnix coturnix japonica*) (Isogai et al., 1994). An interesting fact is that among the three different *B. burgdorferi* s.l. species causing Lyme disease, *B. garinii* seems to have the highest temperature growth optimum (Hubálek et al., 1998). *B. garinii* and *B. valaisiana* are commonly detected in ticks feeding on birds in Europe (Humair et al., 1998; Kurtenbach et al., 1998b) and, in contrast to *B. afzelii*, tolerate pheasant complement, indicating that these species are well adapted to birds (Kurtenbach et al., 1998c). In fact, *B. valaisiana* has not been isolated from rodents in Europe and birds are the only suggested reservoirs for this species (Kurtenbach et al., 1998b). However, in

Europe, *B. burgdorferi* s.s. and *B. afzelii* have also been isolated from larval ticks feeding on birds (Olsen et al., 1995) and in North America, *B. burgdorferi* s.s. was frequently detected in ticks feeding on birds (Smith et al., 1996).

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Figure 3. The marine enzootic *Borrelia* cycle. (Drawing: Björn Olsen)

Some seabirds, e.g. petrels (Procellariidae) and penguins (Spheniscidae) have body temperatures similar to mammals, around 36-38°C (Warham, 1990; Williams, 1995) and may be more suitable hosts for *B. burgdorferi* s.l. spirochetes. On a mammal-free island, *B. garinii* was isolated from *I. uriae* and *B. burgdorferi* s.l. DNA was amplified from the foot-web of a tick infested razorbill (*Alca torda*) (Olsen et al., 1993). In contrast to the terrestrial enzootic cycles of *B. garinii,* the circulation of these borreliae seems to be primarily restricted to colonial seabirds and their tick (Fig. 3). No vertebrate hosts other than seabirds seem to be involved in maintaining the marine enzootic *Borrelia* cycle (Bunikis et al., 1996; Olsen et al., 1993). The importance of seabirds and terrestrial birds as reservoirs for *B. burgdorferi* s.l. and the associated risks for human Lyme disease remains to be determined.

Experimental infections with *B. burgdorferi* s.l.

Mouse models

Several animal models for Lyme disease have been developed but the most commonly used is the mouse (*Mus musculus*). Mice are natural reservoirs that carry long-term active infections with some symptoms resembling human Lyme disease. Lyme arthritis in particular has been extensively investigated in inbred mouse strains (Weis et al., 1999). Mouse models have been used to study for example tissue tropism, immune response, vaccine candidates, differential protein expression, immune evasion, and mechanisms of persistence. *B. burgdorferi* s.l. reside in skin, urinary bladder, heart muscle, joints, kidney, liver, spleen and blood of the infected mouse for months, but the densities are low making detection difficult (Barthold et al., 1993). Mechanisms of persistence involve immune evasion by antigenic variation (Zhang et al., 1997; Zhang and Norris, 1998).

The choice of infecting strain is important. In mice the *B. burgdorferi* s.s. strain N40 is particularly prone to cause arthritis but it may be difficult to find a *B. garinii* strain infectious to mice (personal observations). Animals are usually infected by a subcutaneous injection of cultivated spirochetes. More laborious, but certainly more natural, is infection through infestation with infected ticks. The immune response in syringe-infected animals is for example different from that of naturally infected ones (Kurtenbach et al., 1994; Randolph and Nuttall, 1994) and accelerated infectivity to ticks has been observed in natural infection (Kurtenbach et al., 1998a; Shih and Liu, 1996). Spirochetes delivered by a tick are prepared for infection by upregulation of expression of several *Borrelia* proteins during tick feeding and, in addition, tick saliva contains substances that may be advantageous for the spirochete when establishing an infection (Nuttall, 1999). Diagnosis of infected animals is preferentially performed using pathogen-free ticks (xenodiagnosis) instead of tissue culturing or PCR, thereby

demonstrating not only the presence of *Borrelia* DNA in the host but also the ability of the host to infect ticks.

Bird models

Japanese quail (Isogai et al., 1994) and bobwhite quail (*Colinus virginianus*) (Bishop et al., 1994) have been experimentally infected by subcutaneous inoculation of *B. garinii* and *B. burgdorferi* s.s. cultures respectively. Spirochetes were subsequently detected in skin and several internal organs up to two months after infection, and one blood isolate was obtained from a bobwhite quail. There were no pathological findings except for focal skin lesions with inflammatory infiltrate and spirochetes in some of the birds. All infected birds in these studies developed antibodies against *B. burgdorferi* s.l. (Bishop et al., 1994; Isogai et al., 1994).

American robin (Richter et al., 2000), blackbird (Matuschka and Spielman, 1992), chicken (*Gallus gallus*) (Piesman et al., 1996), and pheasant (Kurtenbach et al., 1998a) have been experimentally infected by *B. burgdorferi* s.l. infected ticks and subject to repeated xenodiagnosis. Infectivity to ticks was observed in all of these bird species except blackbirds, but the duration and efficiency of infectivity differed. American robins were highly infectious to xenodiagnostic ticks the first two months after infection, infecting up to 92% of infested ticks. Thereafter infectivity waned and disappeared 4 months later. Reinfection with the same strain was successful and almost the same degree of infectivity to xenodiagnostic ticks was observed during the second infection. Pheasants on the other hand were less infectious to ticks; around one fourth of xenodiagnostic ticks became infected, but the duration of infection was similar, up to ten weeks. In chicken, infection was successful in one-week-old chicks but infectivity to ticks lasted only three weeks. Three-week-old chicks were less susceptible to infection; infectivity was lower and lasted even shorter. It is not clear why blackbirds could not be experimentally infected although reservoir

competence has been demonstrated for this bird species. These experiments must be repeated involving more individuals and perhaps different strains of *B. burgdorferi* s.l.

The main objectives of these studies have been to clarify the reservoir competence and thus the role of birds in the enzootic *Borrelia* cycles. Generally, the duration of infectivity to ticks or spirochetemia was short compared to mice (Donahue et al., 1987), but viable spirochetes or DNA persisted for several months in different internal organs. Very few pathological findings and symptoms of disease have been observed. Thus, *B. burgdorferi* s.l. infection in birds appears to be mainly asymptomatic.

Reactivation of latent infections

Latent infections are mainly associated with viruses, but can be established by members of all classes of microorgansims. Herpes simplex virus type 1 (HSV-1) is a very common virus causing cold sores mainly in the lips and the oral cavity. During its latent phase, the virus hides in the trigeminal ganglion and upon activation HSV-1 is transported through the trigeminal nerve to the site of original inoculation causing a cold sore. Reactivation of HSV-1 can be triggered by UV- irradiation, menstruation, glucocorticoid therapy, psychological stress, nerve trauma and other factors (Chang, 1971). The classical examples of latent bacterial infections are *Mycobacterium tuberculosis* and the agent of syphilis, *Treponema pallidum*, that can persist in the host in dormant stages for decades. Reactivation can occur when the host's immune system is suppressed by for example chemotherapy or other infections (Mackowiak, 1984). In Lyme disease, there is often a latent phase between the early localised and disseminated stages of disease (Weber and Burgdorfer, 1993). The *Borrelia* spirochetes can persist for long periods of time in the host, but it is not known whether there are any triggers to activate the infection.

In response to increased physical or psychological demands, the mammalian and avian body releases stress hormones such as glucocorticoids and noradrenaline. The stress response mediates reallocation of energy resources to the musculature and nervous system, while for example the immune system is down regulated (Apanius, 1998), which in turn may lead to reactivation of latent infections (Mackowiak, 1984). For example, disruption of the social hierarchy in male mice elicited psychological stress and induced reactivation of latent HSV-1 infection (Padgett et al., 1998). In birds, the basal levels of stress hormones, especially corticosteroides, are elevated during migration (Holberton et al., 1996) indicating that immunocompetence in migrating birds may be impaired and latent infections may be activated.

AIMS

- To isolate and characterise *B. burgdorferi* s.l. from seabirds and the seabird tick *I. uriae*.
- To investigate if humans exposed to infected *I. uriae* are at risk of suffering Lyme disease.
- To study the importance of passerine birds as amplification hosts for *Borrelia*.
- To analyse if a latent *Borrelia* infection can be activated by various stress conditions.
- To examine if migrating birds disperse *B. burgdorferi* s.l. over long distances.

RESULTS AND DISCUSSION

Isolation and characterisation of Lyme disease *Borrelia* from puffins (*Fratercula arctica*) and seabird ticks (*Ixodes uriae*) (paper I)

A marine enzootic *Borrelia* cycle maintained by auks (Alcidae) and their tick *I. uriae* was previously proposed (Olsen et al., 1993), and the spirochetes were subsequently typed as *B. garinii* (Bunikis et al., 1996). However, *Borrelia* had not been cultivated from the proposed reservoir hosts and the strains isolated from *I. uriae* had novel ribotypes and OspC serotypes indicating that they were different from known *B. garinii* strains (Bunikis et al., 1996).

Figure 4. Puffins (*Fratercula arctica*). (Photo: Åsa Gylfe)

Cultivation of *B. burgdorferi* s.l. from birds is difficult, in part due to the difficulties in sampling sufficient amounts of blood. On the Faeroe Islands, puffins (*Fratercula arctica*) (Fig. 4) are traditionally hunted for their meat and feathers. The hunting technique, which was used to catch the birds in the present study, is old and involves catching the birds in nets (Fig. 5). We collected blood from the haematoma that formed in the neck region of puffins killed by cervical dislocation.

Two *Borrelia* isolates were obtained from 102 puffin blood samples and subject to thorough characterisation. Previously isolated strains from *I. uriae* from the same area (paper V) were also included in the study. All strains were shown to be *B. garinii* following two types of RFLP analyses and nucleotide

sequencing of partial 16S rRNA gene and *ospC* sequences. The antigens FlaB, OspA, OspB and OspC were expressed by all strains and recognized by *B. burgdorferi* s.l. specific antibodies, and the rRNA genes were organised in the same way as in all human pathogenic species of *B. burgdorferi* s.l. Indeed, these were not atypical *Borrelia* strains, but had a lot in common with human pathogenic *B. garinii*.

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Figure 5. Puffin hunting on Nólsoy. (Photo: Darius Straševičius)

B. garinii isolated from seabirds and seabird ticks, have _ospC_ alleles associated with human disseminated Lyme disease

Disseminated Lyme disease has been associated with defined alleles of the *ospC* gene (Marconi et al., 1999; Seinost et al., 1999; Wormser et al., 1999). This observation may reflect a clonal origin of infectious strains and the alleles may perhaps be used to identify human pathogenic isolates (Baranton et al., 2001).

Probes G1, G2 and G3, targeting *B. garinii ospC* alleles found in strains causing disseminated infection, were designed based on information from sequences in the EMBL nucleotide sequence database and published literature

(Baranton et al., 2001) (Table 1.). The occurrence of these alleles in *B. garinii* strains isolated from seabirds (puffins) and the seabird tick *I. uriae* was determined. Seven out of eight investigated strains hybridised with probe G1 (Table 2), the *ospC* allele found in strains M57 (Livey et al., 1995) and SL10 (Karlsson et al., 1990) isolated from human CSF, and strain N34 isolated from *I. ricinus* (Wilske et al., 1995).

Occasionally, OspC is horizontally transferred between strains and species (Dykhuizen et al., 1993; Livey et al., 1995; Rosa et al., 1992; Wang et al., 1999a). An additional support for a clonal relationship between the seabird-associated strains reacting with probe G1 and the strain N34 is that all have OspA serotype 6 (Bunikis et al., 1996; Wilske et al., 1995) (strains M57, SL10, Far03 and Far04 have not been serotyped). N34 was isolated in Germany, M57 in the Czech Republic, and SL10 in Sweden. Although the marine and terrestrial *Borrelia* cycles are probably separated, there may be contact zones with mixed avifauna and overlapping distribution of tick species. In these areas, an exchange of marine and terrestrial *Borrelia* may occur. For example, similar *B. garinii* strains were isolated from *I. uriae* and *I. ricinus* in the same archipelago in the Bothnian Gulf of the Baltic Sea (Bunikis et al., 1996) and a local neuroborreliosis patient had antibodies reacting strongly with the *I. ricinus* isolate (Bergström et al., 1992).

Here I demonstrate the presence of *ospC* alleles associated with human disseminated Lyme disease in 7/8 *B. garinii* strains isolated from seabirds and *I. uriae*. This finding supports the idea that there may be exchange between different ecological niches of *B. garinii* and a risk that humans in contact with seabirds contract highly virulent *B. garinii*.

TABLE 1. Primers used for PCR amplification and Southern hybridisation of the *ospC* gene.

	Primer	Sequence (5´ to 3´)	Tempera-ture
PCR	ospC10	GA(GA)GCTTTG(AGC)T(GTC)TCATCTATAG	50°C
	ospC9	G(AT)(TC)TTTAAAATAGCT(TG)(TC)TTTTG	annealing
Hybridization	G1	AGTTGCTGCTGCTACTGATGATCAT	58°C
	G2	GTAAAGAAATTGCAAAGGTGAAGGAA	59.5°C
	G3	AAATGATGGTACTTTAGATAACGAA	50.5°C

TABLE 2. *B. garinii* strains used in this study and results from Southern hybridisation of PCR amplified *ospC* fragments.

	B. garinii isolate	Biological source	Geographic origin	Hybridized with probe
Strains used as controls	Far02	*I. uriae*	Faeroe Islands	G1
	Far03	Puffin blood	Faeroe Islands	G1
	DK27	Human skin (EM)	Denmark	G2
	DK29	Human skin (EM)	Denmark	G2
	DK32	Human skin (EM)	Denmark	G2
	DK6	Human CSF	Denmark	G3
Tested strains	Far01	*I. uriae*	Faeroe Islands	G1
	Far04	Puffin blood	Faeroe Islands	G1
	Fis01	*I. uriae*	Iceland	G1
	Mal01	*I. uriae*	Sweden	G1
	Mal02	*I. uriae*	Sweden	G1
	IUB18	*I. uriae*	Sweden	None

Methods

Dense *Borrelia* cultures was centrifuged, the bacterial pellets dissolved in distilled water, boiled for 2 minutes and thereafter used as templates for PCR amplification. An approximately 280 bp fragment of the *ospC* gene was PCR amplified using the primers ospC10 and ospC9 (35 cycles of 94°C for 30 s, 50°C for 60 s, 72°C for 60 s under previously described conditions (paper I)). The PCR product was diluted 3:100 in distilled water and dot-blotted (GIBCO BRL) onto a Hybond N nylon membrane (Amersham Pharmacia Biotech). The membrane was thereafter incubated 60 s on Whatman 3MM paper soaked in 0.5M NaOH, and 60 s on dry 3MM paper. The procedure was repeated twice and followed by three cycles of 60 s incubation on 3MM paper soaked in 1M Tris-HCl pH 7.5, and 60 s on 2xSSC soaked paper. After cross linking, the membrane was pre-hybridized in a hybridization solution (5 X Denhardt, 5 X SSC, 1% SDS and 1mg/ml salmon sperm DNA) for 2-3 hours and subsequently hybridized at the same temperature in fresh hybridization solution with 22.5 ng 5´Cy-5 labeled probe (MWG biotech) for at least 6 hours. Washing was performed at the hybridization temperature with two 15 min washing steps in 4 X SSC, 0.1% SDS followed by two 15 min washing steps in 2X SSC, 0.1% SDS. The results were visualized using a Storm 850 (Molecular Dynamics).

Serological investigation of humans frequently bitten by *I. uriae* (paper I)

Although *B. garinii* in seabirds and seabird ticks show similarities to human pathogenic strains, it is not known if *I. uriae* can transfer the infection to humans. On the small island Nólsoy, Faeroe Islands, people, sheep and rodents live in close contact with seabirds and *I. uriae*. There is no resident population of *I. ricinus* and no known domestic cases of Lyme disease on the Faeroe Islands. However, *I. uriae* occasionally bite people and we could detect significantly higher levels of antibodies against *B. burgdorferi* s.l. among 81 residents involved in the puffin hunting compared to a Swedish control group not exposed to ticks (Fig. 6). Three of the puffin hunters with a positive reaction in ELISA were confirmed seropositive by immunoblot analysis. Two recalled having been bitten by *I. uriae,* but none had experienced any symptoms of disease. They may have acquired subclinical Lyme disease from *I. uriae* or may have been exposed to *Borrelia* antigens from *I. uriae* bites.

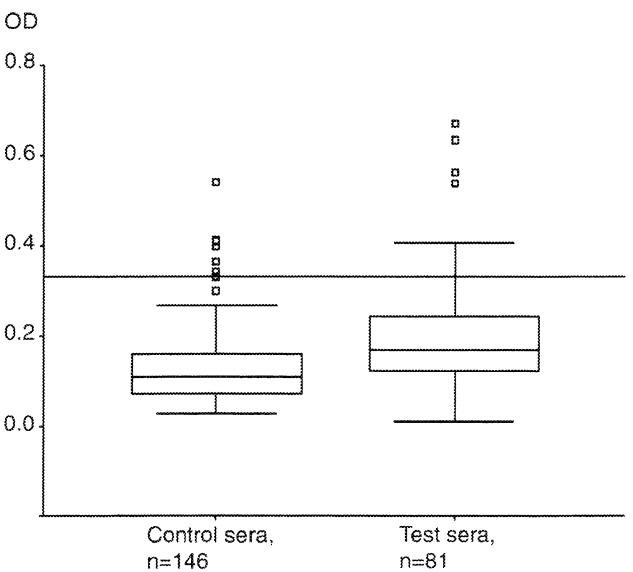

Figure 6. Serology of puffin hunters (test sera) from the Faeroe Islands, whole-cell ELISA OD values. Boxes indicate OD values within the first and third quartiles and the median is shown as a horizontal line in each box. Error bars and squares define extreme values and outliers respectively. The reference line crossing the figure indicates the cut-off level for positive sera.

I. uriae can infest various mammals, but are mammals involved in the enzootic *Borrelia* cycle at Nólsoy? Mice (*Mus musculus*) and cats (*Felis domesticus*) live in the puffin colony and feral sheep (*Ovis aries*) graze in the close vicinity. Only three mice were caught and none of them were infested with ticks. Different internal organs from these mice were inoculated into BSK II medium, but no spirochetes could be detected. The local veterinarian investigated sheep before slaughter in early October, but no ticks were detected and blood cultures were negative for *Borrelia*. Sheep are not competent reservoirs for *B. burgdorferi* s.l., but co-feeding transmission can occur when infected and uninfected ticks feed simultaneously on the same animal (Ogden et al., 1997). Ticks were not abundant in the outskirts of the puffin colony where the sheep grazed and tick activity was probably reduced in October due to lower temperatures and departure of puffins. Due to the limited sample size, data do not allow conclusions to be drawn regarding whether mammals play a role in the enzootic cycle of *Borrelia* on Nólsoy. The mammals studied at Nólsoy may be infected with *Borrelia* but they occur in low numbers compared to the 10 000 puffins. In addition, survival of *I. uriae* is reduced when feeding on hosts other than seabirds (Nuttall, 1913), further limiting the importance of mammals in the local enzootic *Borrelia* cycle.

Canary finches (*Serinus canaria*) as an avian infection model for Lyme borreliosis (paper II)

An avian infection model is required for studies of pathogenicity and the biology of *Borrelia* in birds. To test if canary finches (*Serinus canaria*) could serve this purpose and to investigate if passerine birds could function as reservoir hosts for Lyme disease spirochetes, we infected 8 canary finches with *B. burgdorferi* s.s. It was possible to establish a short-term spirochetemia (up to three weeks), detectable by IFA staining of blood smears and PCR amplification of *B. burgdorferi* s.l. specific DNA. Reinfection with the same strain 9 weeks

later resulted in an even shorter spirochetemia (one week) but *B. burgdorferi* s.l. DNA could be PCR amplified from liver specimens from all infected birds three months after initial infection (one month after the re-infection). Skin, kidney, spleen and lung tissue from some of the birds also contained *B. burgdorferi* s.l. DNA. Although detection of plasmid encoded *Borrelia* DNA does not assure the presence of viable spirochetes, this finding indicates a long-term persistence of spirochetes in the host. These results are similar to other experimental infections in birds, where a brief episode of infectivity to ticks (Piesman et al., 1996), and longer persistence of spirochetes have been observed (Bishop et al., 1994; Isogai et al., 1994). In some studies, birds were infectious to ticks more than ten weeks (Kurtenbach et al., 1998a; Richter et al., 2000), but this is still short compared mice that stay infectious for more than six months (Donahue et al., 1987). There were no signs of disease in the infected canary finches except short-term diarrhoea that did not lead to weight loss. Except for a local skin lesion at the site of inoculation, pathology in response to *B. burgdorferi* s.l. infection has not been demonstrated (Bishop et al., 1994; Isogai et al., 1994). If ticks can feed repeatedly on canary finches without impaired feeding success, this may be a good model to study various aspects of *Borrelia* biology in birds.

Reactivation of *Borrelia* infection in birds (paper III)

The fact that spirochetes persist in birds for long periods of time gave rise to the question "Can the infection be reactivated?" One approach to studying the capability of transmission of Lyme disease spirochetes would be to expose the infected host to stress, thereby attempting to impair host defence and induce spirochetemia. We hypothesised that migration might have such an effect and if so, a mechanism to facilitate bacterial spread over wide geographic distances would be elucidated. To test this hypothesis experimentally, we used redwing thrushes (*Turdus iliacus*), a migratory species frequently infested by ticks (Olsen et al., 1995). To induce migratory behaviour, we decreased the

photoperiod from 12 to 3.5 hours in one room (hereafter called the migratory room), while keeping it constant at 12 hours in another room, (hereafter called the control room). Reduction of the photoperiod is one important inducer of migration, others are change in temperature, shortage of food and the positions of stars (Berthold, 1996). Migratory restlessness or increased nocturnal activity in caged nocturnal migrants is a behaviour that is frequently used to investigate migration in birds (Alerstam, 1990).

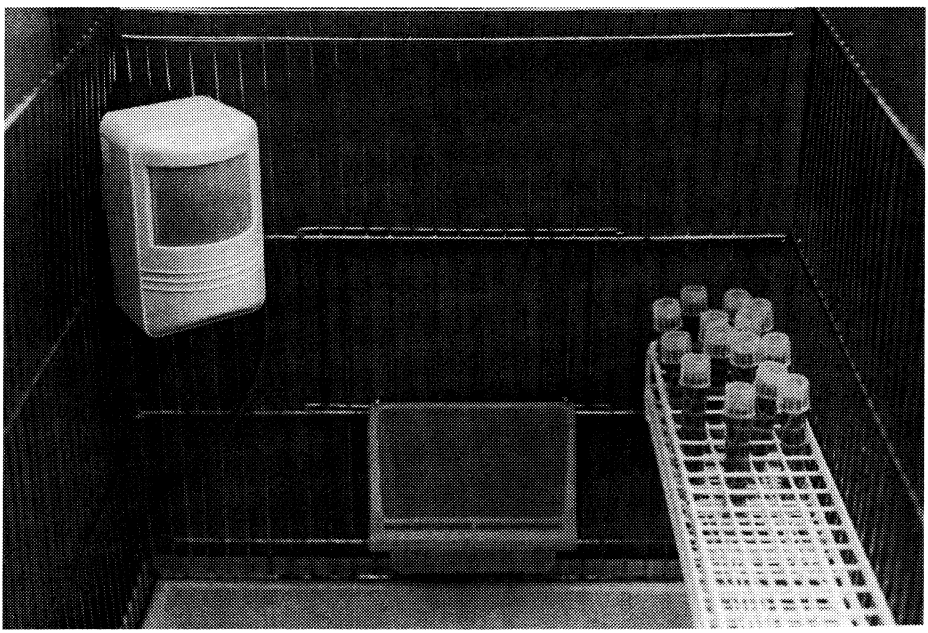

Figure 7. Redwing thrushes were held in individual cages. In each cage, an infrared sensor was installed that registered movements, hopping as well as wing whirring and fluttering. The sensor signals were recorded by a computer program AC97 (JoAC electronics, Lund, Sweden) 24 hours/day, from day 0 until day 83. Once a week, each bird's body mass was measured and blood samples were drawn from the right jugular vein for culturing. In addition, 100 µl of either BSK II medium or PBS was injected subcutaneously into the pectoral or abdominal region, and immediately aspirated and inoculated into BSK II medium for culturing. Body mass was measured and blood and subcutaneous aspirates were taken on days 8, 15, 29, 36, 49, 64, 71, 79, 87 and 93. Body mass was also measured on days 23 and 43, and subcutaneous aspirates taken on day 43. Growth of spirochetes was monitored weekly by direct microscopy. All inoculates were passed to fresh complete BSK II supplied with 9% chicken serum (Life Technologies). To confirm identity to the infecting strain, all obtained cultures were subject to sequencing of an *ospA* gene fragment amplified by the primers AI1 (5´-GCAAAATGTTAGCAGCCTTGAT-3´) and BJ5 (5´-CTGTGTATTCAAGTCTGGTTCC-3´) (Moter et al., 1994) as previously described (paper I).

In the migratory room, eight birds were infected with *B. garinii* and two birds served as uninfected controls, while six infected and three uninfected birds were held in the control room. During days 0-50 there was no significant difference in median nocturnal activity between the two rooms. Around days 42-54, however, when the photoperiod decreased from 7.3 to 5.3 hours, four of the birds in the migratory room increased their nocturnal movements from approximately 60% to 90% of the total dark period. The other six birds in the migratory room increased their nocturnal activity gradually over a longer period of time, whereas the nocturnal activity of birds in the control room did not change during the experiment. Day 50 was set as the onset of migratory restlessness, due to the elevated median nocturnal activity in the migratory room after that day (Fig. 8a). Migratory restlessness was confirmed in the migratory room by a significantly higher nocturnal activity compared to the control room during days 51-83 (Fig. 8a). The migratory response obtained in this study coincides well with the timing of migration in wild birds (Enquist and Pettersson, 1986).

All birds gained weight during the first part of the experiment (days 8-49) as is typical of pre-migratory fat deposition (Alerstam, 1990). The body mass remained high in the control room during days 64-93 while birds in the migratory room consumed their fat reserves, probably as an effect of the increased nocturnal activity, resulting in a significant difference between the birds in the two rooms during days 64-93 (Fig. 8b).

From 5 (n = 8) infected birds of the migratory room, viable spirochetes were detected by direct microscopy in a total of 11 (n = 48) BSKII inoculates originating from blood (4, n = 24) and subcutaneous aspirate (7, n = 24). All positive cultures originated from inoculations between days 71 and 87. No spirochetes were found in the 103 inoculates sampled from these birds before day 71. Spirochetes were neither detected in the 106 inoculates from the six infected birds in the control room, nor in the 83 inoculates from the uninfected

control birds, sampled during days 8-87. The proportion of culture positive birds in the migratory room (5 out of 8) was significantly different from that of the control room (0 out of 6) (Fig. 8c).

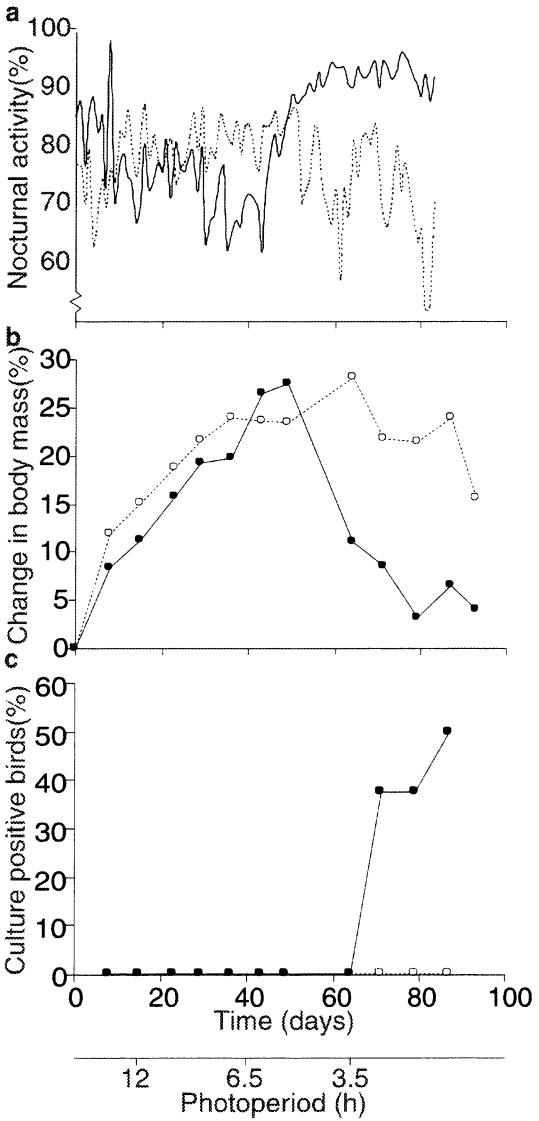

Figure 8. Solid line/filled squares = migration room; dashed line/open squares = control room. Photoperiod in the migration room is indicated at the bottom of the figure. **(a)** Nocturnal activity was defined as the proportion of the dark period when the sensor was signalling and calculated for each night. During days 51 to 83, nocturnal activity was significantly (Mann-Whitney U-test, P = 0.001) higher in the migratory room (median = 91.9%, 1st quartile = 88.7%, 3rd quartile = 95.5%) than in the control room (median = 80.5%, 1st quartile = 66.5%, 3rd quartile = 82.9%),
(b) Median increase in body mass compared to initial body mass during days 0-49 in both rooms was 18.5%, 1st quartile = 9.9%, 3rd quartile = 26.9%. During days 64-93 birds in the migratory room consumed their fat reserves (median increase of body mass compared to initial body mass = 4.9%, 1st quartile = 0.4%, 3rd quartile = 10.1%) while birds in the control room remained fat (median increase of body mass compared to initial body mass = 18.3%, 1st quartile = 14.0%, 3rd quartile = 32.8%), resulting in a significant difference between the two rooms (Mann-Whitney U-test P = 0.008). **(c)** *B. garinii* was isolated from 5/8 infected birds in the migratory room but not from any of the 6 infected birds in the control room, which was a significant difference (Pearson χ^2 = 5.833, 1 df, P = 0.031). Less than two inoculates per sampling occasion became contaminated, except for inoculates from day 93 which were all discarded due to contamination.
SPSS 9.0 for MS Windows (SPSS Inc.) was used for the statistical analysis. All P values were based on two-sided tests of significance. P values of 0.05 or less were considered to indicate statistical significance.

Seven out of eleven cultures positive for viable spirochetes were isolated from subcutaneous aspirate suggesting that this method may be preferable to

blood culture for isolation of *B. burgdorferi* s.l. from birds. All inoculates were subsequently transferred to fresh BSK II medium containing rabbit serum or chicken serum, and viable spirochetes could only be detected in culture medium supplemented with chicken serum. Thus, chicken serum may be a better supplement to BSKII for isolation of spirochetes from birds. All cultivated isolates were identical to that of the infecting strain, confirmed by sequencing of a variable part of the *ospA* gene.

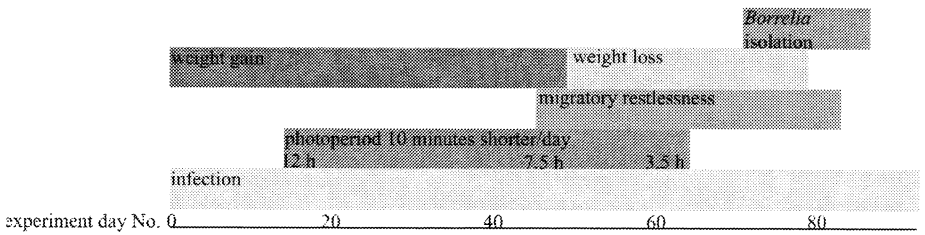

Figure 9. Experimental timeline. Ten cages were placed in the migratory room and 9 cages in the control room. After one week of adaptation, 8/10 and 6/9 birds respectively, were infected on day 0 with 10^4 spirochetes of a low passage *B. garinii* strain, NBS16 (Bergström et al., 1992), by a 0.1 ml subcutaneous injection in the right pectoral region. Uninfected control birds were inoculated with sterile BSKII medium. On day 12, migratory induction started in the migratory room by shortening the photoperiod 5 minutes each morning and evening. On day 64, we terminated this decrease in daylight and the photoperiod was kept at 3.5 hours until the end of the experiment, day 93. The photoperiod in the control room was kept at 12:12 hours (light:dark) until day 93.

The basal levels of stress hormones, especially corticosteroides, are elevated in birds during migration (Holberton et al., 1996). It is known that defense mechanisms against infection and the activity of the immune system in birds, as well as in mammals, can be modulated by stress, such as strenuous exercise (Apanius, 1998; Råberg et al., 1998). The effect of hormonal regulation of the immune system, particularly increased circulating levels of glucocorticoids, may also affect immunocompetence (Besedovsky and del Rey, 1996), which may reactivate latent infections (Mackowiak, 1984).

To refuel, migratory birds stay and forage at stopover sites along their routes (DeGraaf and Rappole, 1995). At these locations, where many birds

are feeding and resting, ticks may attach, and later detach along the migration routes or in the breeding and wintering areas. New foci of tick-borne diseases may be established in this way (Anderson et al., 1986; Mehl et al., 1984), which might explain why *B. burgdorferi* s.l. isolates with identical gene sequences are found at locations separated by geographical barriers such as mountains and seas (Bunikis et al., 1996; paper V). This study provides evidence in favour of an additional, more efficient mechanism for long distance transport of *B. burgdorferi* s.l. These results show that a migratory passerine bird can carry a latent *Borrelia* infection for several months, and that migratory restlessness functions as an activator of the latent infection. These findings increase the understanding of the basic mechanisms of Lyme disease epidemiology. It also elucidates the mechanisms by which important vector-borne, bird-associated microorganisms spread around the world.

Activation of latent Lyme disease (paper IV)

Latent *Borrelia* infection in redwing thrushes can be activated by migratory behaviour that is associated with stress. Can human latent Lyme disease be activated in response to stress modulations of the immune system? *Borrelia* spirochetes can persist for years in Lyme disease patients (Weber and Burgdorfer, 1993) and there is usually a symptom-free phase of latency between the early localised and disseminated stages that may be caused by restraints of the immune system or slow growth of the spirochetes (MacDonald et al., 1990).

A young woman was bitten by a tick when she visited an area in southern Sweden endemic for Lyme disease. This happened in July and she was not exposed to ticks thereafter. Four months later, during a stressful period, she developed an erythema migrans at the site of the previous tick-bite. Concurrent with the erythema she had cold sores on her lips. HSV-1 infection can be reactivated by stress, thus the cold sores may be viewed as an indicator of a stress modulated immune response that may have caused the activation of her

Lyme disease infection. Sequencing of an *ospA* fragment amplified from the tick showed that the infecting strain was *B. afzelii.* Serum from the patient reacted with OspC from the *B. afzelii* strain ACA1 but not with OspC from *B. garinii* or *B. burgdorferi* s.s. indicating that she had been infected with *B. afzelii* and strengthening the causal relation between the tick bite and the Lyme disease infection. This case report indicates that stress modulation of the immune response can activate latent early Lyme disease.

Transequatorial exchange of *B. garinii* by seabirds (papers V and VI)

I. uriae has an exceptionally wide geographical distribution. Transport by seabirds is believed to be the reason for the circumpolar occurrence in both the northern and southern hemispheres (Zumpt, 1952). We wanted to investigate whether *B. burgdorferi* s.l. could be found in *I. uriae* collected at different seabird colonies from both polar regions. Engorged and unfed *I. uriae* were collected at seabird colonies in both the northern and southern hemispheres (Fig. 10) and sent by mail to our lab. The tick idiosoma was dissected and analysed by phase contrast microscopy, IFA, and PCR amplification of *B. burgdorferi* s.l. specific DNA. Due to the long transports, many ticks were dead on arrival, and therefore could only be subjected to PCR analysis.

B. burgdorferi s.l. DNA was amplified from ticks from Alaska, Iceland, Faeroe Islands and Sweden in the northern hemisphere and from New Zealand, and Crozet Islands in the southern hemisphere. A variable 156 bp fragment of the *B. burgdorferi* s.l. *flaB* gene was sequenced from selected ticks, and all sequences obtained showed a high degree of homology to *B. garinii*, suggesting that *B. garinii* occur worldwide in *I. uriae*. Interestingly, identical *flaB* sequences were identified in ticks from New Zealand, Crozet Islands, and Alaska (paper V, Fig. 2, sequence1), from Crozet Islands, Faeroe Islands, and Iceland (paper V, Fig. 2, sequence 4), and from Faeroe Islands and Sweden (paper V, Fig. 2, sequence 3). These data indicate a transequatorial as well as a

circumpolar transport of *B. garinii* by seabirds, either as an active infection in the bird or within ticks attached to the bird.

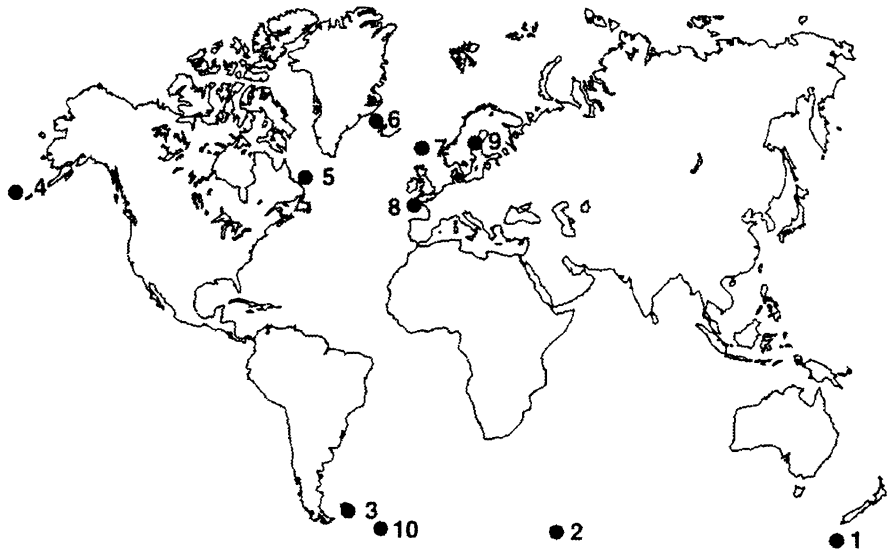

Figure 10. Collection sites of *I. uriae*, paper V and VI. 1, Campbell Island, New Zealand; 2, Crozet Islands; 3, West Point, Falkland Islands; 4, Egg and St. Lazaria Islands, Alaska; 5, Gannet Island, Canada; 6, Flatey Island, Iceland, 7, Nólsoy, Faeroe Islands; 8, Cape Sizun, France; 9, Bonden, Sweden; 10, Bird Island, South Georgia.

I. uriae feed on a wide range of seabirds and, in this study, *B. garinii* could be detected in ticks feeding on auks, fork-tailed storm petrel (*Oceanodroma furcata*), black-browed albatross (*Diomedeida melanophris*) and king penguin (*Aptenodytes patagonicus*). *B. burgdorferi* s.l. DNA has also been detected in *I. uriae* engorged on Kittywake (*Rissa tridactyla*), Gannet (*Sula bassana*), the passerine crossbill (*Loxia curvirostra*), and humans (Hubbard et al., 1998). King penguins at Crozet Islands have antibodies against *B. burgdorferi* s.l., further indicating that seabirds in the southern hemisphere are exposed to this spirochete (Gauthier-Clerc et al., 1999). There are no confirmed cases of Lyme disease with unequivocal origin in the southern hemisphere (Hudson et al., 1998). Since *I. uriae* is restricted to seabird colonies, the transfer

of Lyme disease to humans by this tick would be limited even if it were possible.

If *I. uriae* is transported between the northern and southern hemispheres we would expect that they share the same gene pool. To address this question, *I. uriae* from Sweden, Faeroe Islands, New Zeeland and Crozet Islands were subject to nucleotide sequencing of mitochondrial 16S rRNA and internal transcribed spacer 2 (ITS2) that is located between the nuclear 5.8S and 28S rRNA genes. Parsimony analysis of the combined sequence data indicated that *I. uriae* from the northern and southern hemispheres might be reproductively separated. However, only 4-5 ticks from each site were sequenced from a limited number of sites. To further investigate this finding by the methodology of population genetics (McCoy and Boulinier, 1999), 50-60 ticks from each site could be subjected to determination of a more informative sequence or micro satellites (McCoy and Tirard, 2000).

I. uriae attach to their seabird host for approximately one week (Barton et al., 1995) while migration between Arctic and Antarctic areas takes at least one month (del Hoyo et al., 1992). Since the distribution of *I. uriae* is restricted to the Polar Regions and seabirds rarely go ashore during migration (del Hoyo et al., 1992), a continual attachment and detachment of *I. uriae* at stopover sites is unlikely. However, transport of *I. uriae* within each hemisphere is a more likely event that also may be investigated by population genetics methods.

In conclusion, we have indications of transequatorial transport of *B. garinii* by migratory seabirds. The vector, *I. uriae*, is less likely to be transported this way, as indicated by sequence data from this tick. Birds infectious to ticks are probably more efficient in spreading *B. burgdorferi* s.l. than the unlikely event of introduction of a few infected ticks. Seabirds may thus be important in long distance dispersal of *B. burgdorferi* s.l.

CONCLUSIONS

- *B. garinii* was isolated from puffins and the seabird tick *I. uriae*. *B. garinii* DNA was detected in *I. uriae* in both the northern and southern hemispheres.
- Almost all (7/8) *B. garinii* strains isolated from seabirds and seabird ticks had an *ospC* allele associated with *Borrelia* causing disseminated Lyme disease. Antibodies against *B. burgdorferi* s.l. were detected in people exposed to *I. uriae* bites. Thus the marine enzootic *Borrelia* cycle may be a risk for humans, either by direct transfer from *I. uriae* or via introduction of *Borrelia* into a terrestrial enzootic cycle.
- *B. burgdorferi* s.l. can persist for several months in passerine birds and the infection can be activated in response to migration. Thus, birds may be more infectious to ticks during migration and important in disseminating *B. burgdorferi* s.l.
- Latent *B. burgdorferi* s.l. infections in birds and humans may be activated in response to stress.
- Identical *B. garinii flaB* gene sequences were found in *I. uriae* from the northern and southern hemispheres indicating a transequatorial transport of *B. garinii*. Since northern and southern *I. uriae* may be reproductively separated, passive transport of infected ticks is unlikely and instead seabirds probably carry an active *Borrelia* infection during their migration.

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