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Original article

Borrelia burgdorferi sensu lato-infected *Ixodes ricinus* collected from vegetation near the Arctic Circle

Dag Hvidsten[a,*], Frode Stordal[b], Malin Lager[c], Bjørg Rognerud[b], Bjørn-Erik Kristiansen[d], Andreas Matussek[c], Jeremy Gray[e], Snorre Stuen[f]

[a] Department of Microbiology and Infection Control, University Hospital of North Norway, Sykehusveien 1, 9038 Tromsø, Norway
[b] Department of Geosciences, University of Oslo, P.O. Box 1047 Blindern, 0316 Oslo, Norway
[c] Department of Laboratory Services, Division of Medical Services, County Hospital Ryhov, Sjukhusvägen 1, 55185 Jönköping, Sweden
[d] Research Group of Host–Microbe Interactions, Department of Medical Biology, Faculty of Health Sciences, UiT – The Arctic University of Norway, P.O. Box 6050 Langnes, 9037 Tromsø, Norway
[e] UCD School of Biology and Environmental Science, University College Dublin, Dublin 4, Ireland
[f] Norwegian University of Life Sciences, Department of Production Animal Clinical Sciences, Kyrkjevegen 332/334, 4325 Sandnes, Norway

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ABSTRACT

This is the first study to determine the density of questing *Ixodes ricinus* in northern Norway. It was performed at two sites in Brønnøy, which has been known for its tick permissive habitats for decades and is one of the northernmost habitats with an abundant *I. ricinus* population in the world. From April to November 2011, all stages of host-seeking *I. ricinus* were collected from the two sites. The overall prevalence of nymphs infected with *Borrelia burgdorferi* sensu lato was 21% and that of adult ticks 46%. The rates of the genospecies *Borrelia afzelii*, *Borrelia garinii*, and *Borrelia valaisiana* were similar to findings in most other studies in Scandinavia, with *B. afzelii* by far the most prevalent at 76%. The high *Borrelia*-infection prevalence in ticks from Brønnøy may explain the high incidence rate of reported Lyme borreliosis in the municipality.

1. Introduction

The common tick *Ixodes ricinus*, one of the most important vectors in Europe transmitting infectious agents to humans (Estrada-Peña, 2008), harbours *Borrelia burgdorferi* sensu lato, the causative agent of Lyme borreliosis. *I. ricinus* ticks are expanding their geographic distribution (Gray et al., 2009; Hvidsten et al., 2014; Jaenson and Lindgren, 2011; Jenkins et al., 2012; Jore et al., 2011; Medlock et al., 2013), and tick-borne infections are currently a growing concern in Scandinavia. In the last ten years, approximately 250–350 cases (5–7 per 100,000 population) of systemic infection of Lyme borreliosis have been reported annually to the Norwegian surveillance reporting system for communicable diseases (MSIS, http://www.msis.no). In northern Norway, with 10% of the inhabitants in the country, only an average of five cases has been reported annually. In the municipality of Brønnøy (Fig. 1), 10 cases of Lyme borreliosis were reported to MSIS from 2007 through 2013. These Brønnøy cases account for 29% of all reported cases in region of northern Norway over this period, although the municipality has only 1.6% of the inhabitants in the region. These disease data suggest that Brønnøy has tick-permissive habitats that distinguish it from other parts of northern Norway.

In the 1930s, a study of ticks from farm animals in Norway consisted of the collection of nymphs and adults of *I. ricinus* northwards to Brønnøy (Tambs-Lyche, 1943). Mehl (1983) found nymphal and adult *I. ricinus* ticks by flagging as far as Brønnøy; further north, in addition to flagging, he examined host animals, burrows and bird nests, but was not able to detect ticks other than *Ixodes trianguliceps* and *Ixodes uriae*. Using multiple data sources, Jore et al. (2011) concluded that *I. ricinus* has reached coastal areas as far north as Harstad at approximately 69° N (Fig. 1). In the last decades, *I. ricinus* has been found sporadically in all three counties of northern Norway (Nilssen, 2010). Whether these ticks represent resident populations or transient populations introduced by migratory birds or large mammals, such as deer, remains unknown. Two recent studies collected ticks from pets and found that the density of ticks and the *Borrelia* infection rate in the ticks were much higher in Brønnøy than further north (Hvidsten et al., 2014; Jenkins

* Corresponding author.
E-mail address: dag.hvidsten@gmail.com (D. Hvidsten).

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et al., 2012). However, ticks collected from the vegetation are more relevant to the risk of tick bite and disease in humans. Furthermore, analysis of unfed ticks for *B. burgdorferi* s.l. might result in a higher sensitivity of detection compared with analysis of engorged ticks because the blood of cats and dogs may have a borreliacidal effect which would have a negative effect on detection (Hovius et al., 1998; Hvidsten et al., 2014).

The present study aimed to determine *I. ricinus* activity in the municipality with the northernmost known established tick population in Norway. We also aimed to determine the tick density and the prevalence of *B. burgdorferi* s.l. to estimate the risk of *Borrelia* infection. To our knowledge, repeated samplings of *I. ricinus* ticks with the flagging method have not been performed previously so close to the Arctic Circle.

2. Materials and methods

2.1. Study areas

Brønnøy is a municipality (population 7900 in 2015) in the southern part of the northern Norway region (population 480,700 in 2015) (Fig. 1). It is located on the western coastline and consists of both mainland and numerous islands. Host-seeking ticks were collected at two sites, located 4 km northwards and 9 km southwards, respectively, of the city centre of Brønnøysund. The sites were chosen among several others where tick attachment on dogs and humans had been reported. Site 1 (Mosheim, 65°30′ N, 12°14′ E, 10 m above sea level) is a flat recreational area on the mainland often used by the local population. The ground consists of grass and

Fig. 1. Northwestern Europe. Norway: The municipality of Brønnøy includes the city of Brønnøysund (65°28′ N, 12°12′ E), which is located approximately 120 km south of the Arctic Circle (66°33′ N) in the county of Nordland. The region of northern Norway consists of (towards northeast) the counties of Nordland (red), Troms (orange) and Finnmark (yellow). Coordinates: Farsund (58°05′ N, 06°48′ E); Harstad (68°48′ N, 16°32′ E).

heather; partly open but with scattered mixed deciduous trees such as birch (*Betula pubescens*), juniper (*Juniperus communis*) and willow (*Salix* spp.). There are scattered blueberry (*Vaccinium myrtillus*) and bog bilberry (*Vaccinium uliginosum*) bushes; the soil drainage is good and the vegetation litter is moderate. Site 2 (Torget, 65°23′ N, 12°05′ E, 25 m above sea level) is situated close to a well-known tourist path on an island of 16.4 km^2. The sampling area available is a forest located on a gentle slope of a few hundred square metres. The area consists of heterogeneous deciduous trees including grey alder (*Alnus incana*) and rowan (*Sorbus aucuparia*) in addition to the species mentioned at site 1. The ground is covered by fallen branches, dense blueberry and bilberry bushes and is dampened by a very small stream.

Vertebrate hosts at the two sites that feed adult ticks include farm animals, Eurasian elk (*Alces alces*), and roe deer (*Capreolus capreolus*). In addition to large animals, vertebrate hosts that feed immature ticks include the common shrew (*Sorex araneus*), the bank vole (*Myodes glareolus*) and the field vole (*Microtus agrestis*). Water voles (*Arvicola amphibius*) are found on the islands.

2.2. Climate of the study area

To characterise the climatic conditions in Brønnøy, we used a data set developed by the Norwegian Meteorological Institute, based on a time series from 1957. A geostatistical method was applied to observations from a large number of meteorological stations to create air temperature and precipitation maps on a 1 km × 1 km regular grid (Orskaug et al., 2011; Tveito et al., 2005); see further details in Lindholm et al. (2012). According to Köppen's climate zones the climate in Brønnøy is classified as subarctic maritime (designated as *Dfb*) (Peel et al., 2007).

We have analysed the period 1961–2012. During this period, 2011 was the warmest year, and the spring months (March–April–May) of 2011 were the second warmest (Fig. 2). The yearly average normal temperature of the 30-year normal period 1961–1990 was 4.7 °C at the two sites, increasing by 0.9 °C in the following 20-year period (1991–2010). The mean annual precipitation in Brønnøy was 1849 mm (1981–2010), and the duration of snow cover (>5 cm) at both sites in the decade 2003–2012 was approximately indicated by the range of 50–100 days yearly (http://www.senorge.no). The number of days with average temperature above 5 °C (average of the years 2008–2012) in the areas northwards and southwards of Brønnøy is depicted in Fig. 3, showing that Brønnøy and nearby islands have a relatively warm climate compared to districts inland and further north. Over the period 1981–2010, the length of the growing season (defined as starting when the temperature on five consecutive days exceeds 5 °C, and ends after five consecutive days of temperature below 5 °C) in Brønnøy was 179 days, but increased to 189 days in the decade 2001–2010. As shown above, 2011 was an exceptionally warm year, resulting also in a very long growing season (241 days).

2.3. Tick collection and tick density

Samplings were performed using a white flannel cloth (hereafter called 'flag') of 1.40 m × 0.70 m (approximately 1 m^2). The leading edge of the flag was nailed by drawing-pins to a wooden plank, which was attached to a mop base. A lead chain of 65 g was sewn into the trailing edge of the flag; the chain functioned to keep the flag's surface close to the ground when moving the flag on the ground. The joint of the telescopic handle makes it easy to move it in every direction in the vegetation, also sideways. The collectors moved with the flag stretched before them at a velocity of 0.5–1 m/s, and the flag was inspected on both sides every 10 m. At site 1, the flag was moved in a transect line of 140 m, which covered a surface of 100 m^2; for each sampling, a new direction was taken

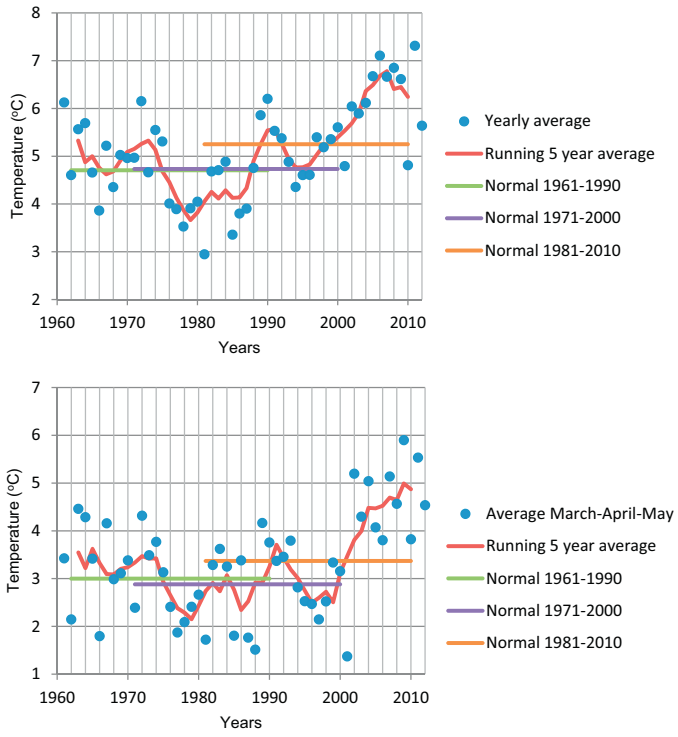

Fig. 2. Average temperature at site 2 (Torget) has changed from 1961 to 2010. The top panel shows the annual average temperature and the bottom panel shows the average spring temperature (March, April and May). The red line represents the 5-year running average and the blue dots represent each year. Average annual and average spring temperature are shown for three 30-year periods: 1961–1990 (green line), 1971–2000 (purple line), and 1981–2010 (orange line).

from the same starting point. Although it is preferable to sample ticks per unit area, this is sometimes impossible, as at site 2, which was characterised by fallen branches and dense bushes. Therefore, at site 2 ticks were sampled for 30-min periods, in which sampling time and inspections were included, both varying according to the number of ticks collected. If the weather conditions were suitable (i.e., air temperature ≥7 °C and ≤20 °C; no rain or strong wind), we aimed to sample ticks every second week. Air temperature and relative humidity were measured with Testo 610 (Testo, Egg, Switzerland) at 1 m above ground level.

In June and August 2012, we collected ticks on subsequent days at both sites. To obtain some measure of comparison for the sites, ticks were sampled from squares of 10 m × 10 m at both sites, and on the same days were also sampled for 30-min periods at site 2.

The collected ticks were transferred to 2.5 ml plastic tubes with 1 ml ethanol 70%, and thereafter stored at 4–8 °C until DNA extraction and *Borrelia* PCR (1–10 larvae, one nymph, or one adult tick per tube) were performed. The order in which the tubes were chosen for analysis was random. Tick species and stages were determined using a stereo microscope (20–80×) (Arthur, 1963; Filippova, 1977; Hillyard, 1996).

2.4. Detection and characterisation of Borrelia spp. in I. ricinus

Reverse transcribed total nucleic acid was prepared from ticks as described previously (Jenkins et al., 2012). Reverse transcription increases the sensitivity of the LUX 16S rRNA real-time PCR test used for genus level detection of *Borrelia* spp. 10–100-fold (Wilhelmsson et al., 2010) resulting in a sensitivity of less than one cell per PCR reaction (Wilhelmsson et al., 2013). Ticks containing *Borrelia* spp. were further characterised to determine *Borrelia* genospecies by nested PCR amplification and sequencing of the

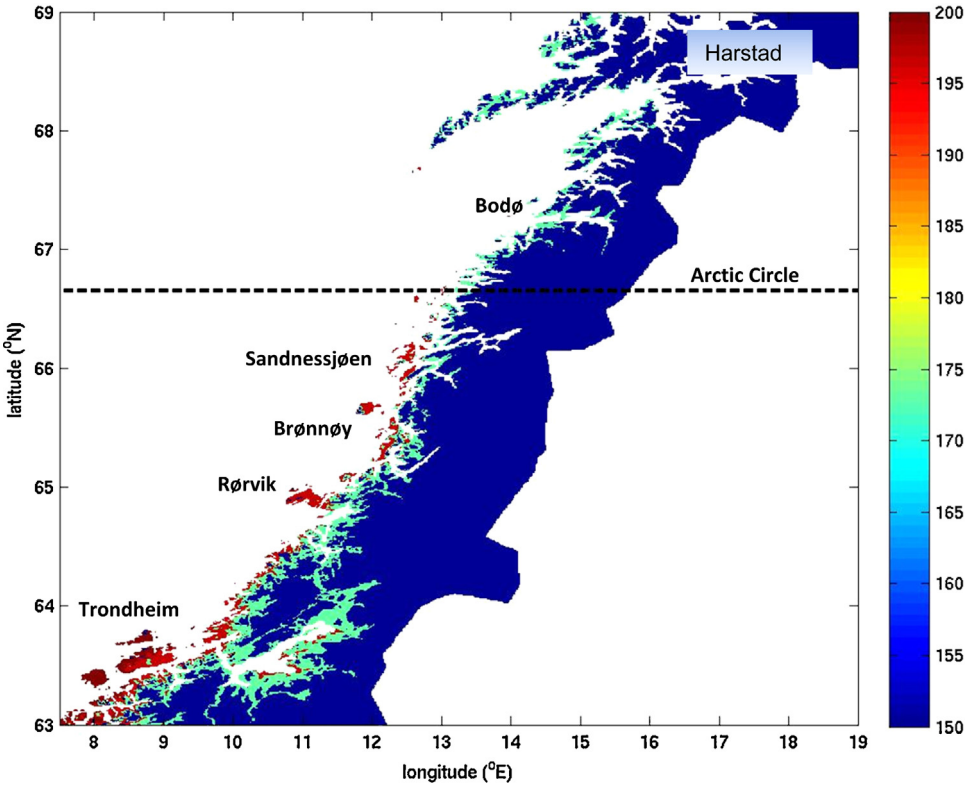

Fig. 3. Colour sketch map of the surrounding region of Brønnøy. The colour column (150–200 days) displays average number of days above 5 °C (average of the years 2008–2012).

5S–23S as well as 16S–23S intergenic spacer regions, following Postic et al. (1994) and Bunikis et al. (2004). Sequencing was performed by GATC Biotech (GATC Biotech AG, Konstanz, Germany). Chromatograms were analysed using the RipSeq web application (iSentio, Bergen, Norway), which allows species determination in samples containing up to three species simultaneously (Kommedal et al., 2009). In ticks where characterisation to *Borrelia* genospecies level failed, a second real-time PCR test targeting *flaB* was done to confirm *Borrelia* DNA presence (Jenkins et al., 2012).

2.5. Statistical analysis

The density of ticks was determined as the number of ticks collected by one person per 100 m^2 or per 30 min. Peak tick density in the 2011 season was determined as the site's highest number of ticks by one person per 100 m^2 or per 30 min. The difference in *B. burgdorferi* s.l. prevalence for nymphal and adult ticks, separately at site 1 and site 2, were calculated with Fisher's exact test (http://graphpad.com). We also did the same calculations with nymphs at site 1 vs. site 2, and with adults at site 1 vs. site 2. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Numbers of ticks

In 2011, 582 *I. ricinus* ticks (116 larvae, 319 nymphs and 147 adults) were collected from 19 April to 12 November (207 days). No other tick species was found. At site 1, a total of 139 ticks were collected on seven visits (from 27 June to 14 October) out of 10. At site 2, 443 ticks were collected on all 10 sampling occasions. The median number of days between samplings at site 1 and 2 was 23 and 21, respectively (at both sites, range 13–35). Peak nymphal density at site 1 was 10 nymphs/100 m^2 on 20 June, and at site 2, it was 53 nymphs/30 min on 6 July. Small numbers of larvae were found at both sites, and although there were insufficient data to reliably assess seasonality, it was evident that the peaks, such as they were, occurred in June and August/September.

In June 2012, tick numbers (nymphs/adults) collected from 10 m × 10 m squares at site 1 and 2 were 3/3 and 25/26, respectively, and in August 2012, 8/0 and 30/15, respectively. The time-based method (30-min periods) was used at site 2 on the same days, and tick numbers (nymphs/adults) were 41/17 in June and 17/33 in August.

3.2. The prevalence and diversity of Borrelia burgdorferi sensu lato

Since, as expected, *Borrelia* was not detected in any of the larvae; the following calculations apply to nymphs and adult ticks (Table 1). In total, 135 (29%) of the ticks contained *Borrelia* spp. as detected by real-time PCR; site 1, 40% (32/81) and site 2, 27% (103/385). Genospecies were determined in 97% (131/135) of the ticks. *Borrelia afzelii* was detected in 76% (102/135), *Borrelia garinii* in 18% (24/135) and *Borrelia valaisiana* in 4% (5/135) (Table 1). There was a significant difference in *Borrelia* prevalence between all ticks at site 1 (40%, 32/81) and all ticks at site 2 (27%, 103/385) ($P = 0.03$). The difference in the *Borrelia* prevalence between nymphs (21%, 67/319) and adults (46%, 68/147) at both sites and at site 2 (20%, 55/278, and 45%, 48/107, respectively), was significant ($P < 0.001$). There were no other significant differences in *Borrelia*-infection prevalence (nymphs or adults at site 1 vs. site 2; nymphs vs. adults at site 1).

4. Discussion

This study complements an earlier, more extensive one in northern Norway in which ticks (almost all adults) were collected from

Table 1
Borrelia genospecies in nymphal and adult *Ixodes ricinus*; sampled from two sites in Brønnøy, northern Norway.

Site[a]	Stage[b]	No. ticks	*Borrelia* positive, total		*B. afzelii*		*B. garinii*		*B. valaisiana*		*Borrelia* sp., ND[c]	
		n	*n*	%	*n*	% of *Borrelia* positive	*n*	% of *Borrelia* positive	*n*	% of *Borrelia* positive	*n*	% of *Borrelia* positive
1	N	41	12	29	12	100	0	0	0	0	0	0
2	N	278	55	20	49	89	3	5	2	4	1	2
1+2	N	319	67	21	61	91	3	4	2	3	1	1
1	A	40	20	50	14	70	5	25	0	0	1	5
2	A	107	48	45	27	56	16	33	3	6	2	4
1+2	A	147	68	46	41	60	21	31	3	4	3	4
1+2	A+N	466	135	29%	102	76%	24	18%	5	4%	4	3%

[a] The *Borrelia* prevalence in all ticks at site 1 (40%) was significantly different from all ticks at site 2 (27%) ($P = 0.03$).
[b] N, nymphs; A, adult ticks; *Borrelia* species was not found in larvae ($n = 116$).
[c] ND; *Borrelia* genospecies not identified.

pets, mainly dogs. In that study, *I. ricinus* appeared to be especially prevalent in Brønnøy and its adjacent municipalities and were infected with at least three *B. burgdorferi* s.l. genospecies. The present study focused entirely on Brønnøy and was designed to provide data on tick activity at two different sites in the municipality, and also on the risk to public health expressed as the relative abundance of unfed ticks collected from the vegetation and the frequency of their infection with *B. burgdorferi* s.l. Collecting ticks directly from the vegetation by flagging, rather than from hosts, permits a more direct measure locally of human tick bite risk, especially since, in contrast to collections of adult ticks from dogs and cats, nymphs are the most common stage obtained by flagging and are responsible for the vast majority of human tick bite (Maiwald et al., 1998; Robertson et al., 2000). As in the previous study (Hvidsten et al., 2014), *B. afzelii* was the most common genospecies; the infection rates with *B. burgdorferi* s.l. were higher than reported in other studies in Norway (Jenkins et al., 2001; Paulauskas et al., 2008; Soleng and Kjelland, 2013) except for Farsund (58°05′ N, 06°48′ E) in the southernmost part of Norway where the prevalence was 31.3% in 2007 (Kjelland et al., 2010).

In the present study, it was necessary to collect ticks using two different methods, area-based and time-based. While it is possible, in general, to establish a relationship between the two methods (Gray et al., 1998), but this is influenced by tick density and vegetation structure, and it should be noted that the time-based approach is usually more variable than the area-based method. The similar area-based sampling (i.e., 10 m × 10 m) at the two sites resulted in 5–6 times as many ticks being captured at site 2 than at site 1. The reasons for the higher tick numbers at site 2 are not clear, because the climate is similar and large hosts such as roe deer and Eurasian elk are present at both sites. However, more trees and the denser undergrowth at site 2 provide a more favourable micro-climate for the ticks. The data from both sites provides ample evidence of a significant risk of tick bite, with numbers sampled reaching levels matching those recorded further south (Jenkins et al., 2001; Kjelland, 2011; Lindström and Jaenson, 2003).

We found that tick questing continued for longer at site 2 (207 days) than at site 1. In general, the questing activity may have been unusually long in the 2011 season, correlating with the long growing season that year. Tick activity is known to be positively correlated with high temperatures in the spring months (Perret et al., 2000), and in 2011 the average temperature in March–May (5.5 °C) was the second highest in the period 1961–2012, which may have been influenced the tick questing behaviour (Fig. 2). Temperature is one of the most important cues for tick activation (Belozerov, 1982; Gray, 1991), but the exact temperature threshold for questing tick activity is unknown in many places. A recent study comparing the questing activity of *I. ricinus* populations from different latitudes in United Kingdom and France, found that at lower temperatures, ticks of northern populations showed 20%–30% more questing activity than those of southern populations (Gilbert et al., 2014). In Brønnøy, the mean annual temperature is low (4.7 °C) and therefore the local tick population may be adapted to quest at low temperatures.

Although sampling was not frequent enough to accurately assess seasonal activity, the presence and peaks of the various tick stages (data not shown) seemed to accord well with the findings of most other studies on *I. ricinus* in that nymphs and adults appear in spring and are active until late autumn, with a decline in activity in late July and August before a slight rise in September, while the larvae tend to peak in early summer and early autumn (Dobson and Randolph, 2011; Gray, 1991). However, more detailed studies on seasonal activity are required, particularly since the influence of photoperiod which is known to regulate seasonal activity (Belozerov, 1982), may be particularly important at this northern latitude. In the Arctic Circle, the day length of 24 h daylight at summer solstice changes to polar night at the winter solstice. In the sunlit part of the year, the nearly constant change in day length in Brønnøy is approximately 7 min per day (increasing before and decreasing after the summer solstice), which is considerably longer than the peak value of day length change seen at 56° N (e.g., Edinburgh; Moscow) and 49° N (e.g., Paris; Volgograd), which is 4.8 and 3.7 min, respectively. It is interesting to note that probably no other tick population is influenced by a stronger photoperiod signal than the ticks close to the Arctic Circle, but as yet no studies on diapause in this particular tick population have been undertaken.

Borrelia spp. were detected in 40% and 27% of the ticks (nymphs and adults) from site 1 and 2, respectively. The overall proportion of nymphs containing *Borrelia* spp. was 21% and that of adult ticks 46%. In a previous study from 2010 to 2011, when ticks were collected from pets, a similar *Borrelia*-infection prevalence of 42% was found in the unfed adult ticks from Brønnøy and adjacent municipalities (Hvidsten et al., 2014).

In a previous study from Brønnøy (Soleng and Kjelland, 2013), the *Borrelia*-infection prevalence was 11% and 33% in nymphs and adults, respectively. The proportion of *Borrelia*-infected ticks carrying *B. afzelii* was 95%, which is higher than in the present study (76%). In the previous study from northern Norway (Hvidsten et al., 2014), most of the ticks were adults collected from pets, and the prevalence of *B. afzelii* in nymphs and adults was 65%. The even higher prevalence of *B. afzelii* in nymphs alone in the present study (91%) indicates that they were mainly infected by rodents because *B. afzelii* is known to have a predilection for these hosts (Hanincova et al., 2003).

In conclusion, this study recorded the presence of all active stages of *I. ricinus* at two different sites in Brønnøy. The questing activity period lasted seven months (19 April–12 November), and

the peak density of 53 nymphs per 30-min sampling period was high considering that the study site is only 130 km south of the Arctic Circle. The high *Borrelia*-infection prevalence in ticks from recreational areas may explain the high incidence of reported Lyme borreliosis in the municipality compared with surrounding areas, and the discernible risk of disease that this study reports, should alert the local health authorities. Further studies are needed on the risk of disease transmission and also on seasonal tick activity, including the role of photoperiod, in the northernmost tick populations.

Conflict of interest

All authors state no conflict of interest.

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