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Original article

The distribution limit of the common tick, *Ixodes ricinus,* and some associated pathogens in north-western Europe

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ABSTRACT

In north-western Europe, the common tick, *Ixodes ricinus*, is widely established, its distribution appears to be increasing and the spread of tick-borne diseases is of increasing concern. The project *'Flått i Nord'* (Ticks in northern Norway) commenced in spring 2009 with the intention of studying the tick's distribution and that of its pathogens in northern Norway. Several methods were used: cloth-dragging, collecting from trapped small mammals, and collecting from pets. Since 2010, the occurrence of ticks in the region of northern Norway was determined directly by cloth-dragging 167 times in 109 separate locations between the latitudes of 64 °N and 70 °N (included seven locations in the northern part of Trøndelag County). The northernmost location of a permanent *I. ricinus* population was found to be Nordøyvågen (66.2204 °N, 12.59 °E) on the Island of Dønna. In a sample of 518 nymphal and adult ticks, the *Borrelia* prevalence collected close to this distribution limit varied but was low (1–15 %) compared with the locations in Trøndelag, south of the study area (15–27 %). Five specimens (1 %) were positive for *Rickettsia helvetica*. The length of the vegetation growing season (GSL) can be used as an approximate index for the presence of established populations of *I. ricinus*. The present study suggests that the threshold GSL for tick establishment is about 170 days, because the median GSL from 1991 to 2015 was 174–184 days at sites with permanent tick populations, showing a clear increase compared with the period 1961–1990. This apparent manifestation of climate change could explain the northward extension of the range of *I. ricinus*.

1. Introduction

The *Ixodes ricinus* species complex comprises certain medically important ticks in the northern hemisphere, including *Ixodes scapularis* and *Ixodes pacificus* in North America and *Ixodes ricinus* and *Ixodes persulcatus* in Eurasia (Gray et al., 2016). The increasing range and abundance of these ticks have attracted worldwide attention. In Norway, the focus has been on the expanding distribution of *I. ricinus* (synonyms: castor bean tick, sheep tick, *skogflått* in Norwegian) in certain areas of the country, which presents emerging public health and veterinary challenges. In the region of northern Norway, infections caused by several microbial pathogens have so far been detected: *Anaplasma phagocytophilum* in cattle, sheep (Stuen et al., 2005) and dogs (Meldal and Stuen, 2012); *Babesia divergens* in cattle (S. Stuen, personal communication); *Candidatus* Neoehrlichia mikurensis in ticks (Larsson et al., 2018). Infection with *Borrelia burgdorferi* sensu lato (s.l.) in humans was indirectly determined by measuring IgG antibody prevalence in the region's blood donors (Hvidsten et al., 2017). In this region, the incidence of tick-borne diseases in humans and animals is much lower than in the southern regions of Norway (http://www.msis.

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no/) (Hvidsten et al., 2017). Although tick-borne encephalitis virus has been detected in ticks along the coastline further north as far as Brønnøysund (Soleng et al., 2018), all cases of human infection with the virus have been contracted only in five counties in southern Norway south of 60 °N (https://www.fhi.no/).

Climate change appears to be the most important cause of the spread of the tick northwards (Gray et al., 2009) and among the most important factors is the temperature change. Mean temperature in Norway has increased during the last decades; in consequence, the snow cover extent is markedly decreased, in particular in spring and north of the latitude of 63 °N (Rizzi et al., 2018). As a result of temperature increases, the growing season length (GSL) is expected to be longer. GSL, defined following specific criteria, is the period (in days) when air temperatures exceed a mean of 5 °C. In Sweden, localities with a GSL of more than 170 days have been associated with the occurrence of *I. ricinus* (Jaenson et al., 2009; Jaenson and Lindgren, 2011).

In the 20th century, a study determined the distribution of *I. ricinus* along the Norwegian western coast up to Brønnøysund (65.5 °N, 12.2 °E) and reported the northernmost location where nymphal and adult *I. ricinus* were collected (Tambs-Lyche, 1943). Surprisingly, Mehl (1983) did not find any *I. ricinus* further north 40 years later. Now, however, *I. ricinus* is regularly reported far outside (i.e. northeast) of the hitherto established distribution limit of *I. ricinus* in Norway, the vast majority as engorged female ticks (Jenkins et al., 2012; Hvidsten et al., 2014). These findings do not confirm the presence of sustainable populations of ticks, which requires the presence of all the active stages (larvae, nymphs and adults) in a locality for at least two consecutive seasons (Anonymous, 1991; Piesman, 1991). Confirming the absence of ticks is much more difficult; the Consensus Conference on Lyme disease (Anonymous, 1991) required both the examination of small mammals for immature ticks, deer or livestock for adult ticks, and cloth-drag sampling *per* locality on three separate days for at least ten person-hours at an appropriate time of year and in favourable weather.

Our project, *'Flått i Nord'* (Ticks in northern Norway) commenced in 2009, and its main purpose was to determine the presence of permanent *I. ricinus* populations and the prevalence of human pathogens in the ticks in the northern Norway region (the study area). To identify localities with permanent tick populations, the project complied with the criteria agreed on at the Consensus Conference on Lyme disease (Anonymous, 1991), consisting of the collecting of all three life stages in two successive years. The exact localization of *I. ricinus* occurrence is important knowledge for health authorities, local hospitals and doctors and is more reliable than identifying endemic municipalities with two or more locally acquired cases of Lyme borreliosis because the geographical location of tick attachments in humans or animals is often unknown or uncertain (Diuk-Wasser et al., 2012).

Several articles from *'Flått i Nord'* covering the period 2009–2017 determined the occurrence of various pathogens in *I. ricinus* (Jenkins et al., 2012; Hvidsten et al., 2014; Henningsson et al., 2015; Hvidsten et al., 2015, 2017; Larsson et al., 2018). The results presented here are based on field research in the years 2010–2018, with the main objective being determination of the current distribution limit of *I. ricinus,* with particular reference to climate change, by means of cloth-dragging, capturing small mammals and collecting ticks from pets, and also to provide a climate-based explanation, the GSL, for the data.

2. Materials and methods

2.1. Climate and habitat

The region of northern Norway consists of three counties, Nordland, Troms and Finnmark, covering 113,000 km^2, which is 35 % of the mainland country area. Approximately 9.2 % (486,000) of the Norwegian population live in this region. The region spans more than 6° of latitude, and most of it is north of the Arctic Circle. The harshness of the northern climate is strongly alleviated by the North Atlantic Drift (The Gulf Stream) (Rizzi et al., 2018) and in most parts of northern Norway, the climate can be classified as subarctic (Köppen-Geiger designation as Dfc) (Peel et al., 2007). However, the areas around the coastal cities of Brønnøysund and Sandnessjøen are different from those of the adjacent areas in Norway (except the southern coast), with a relatively warm, humid climate (Köppen-Geiger designation as Dfb) (Peel et al., 2007).

In most sampling areas, the dominant tree species are birch (*Betula* spp.), willow (*Salix* spp.), rowan (*Sorbus aucuparia*), juniper (*Juniperus communis*), Norway spruce (*Picea abies*), Scots pine (*Pinus sylvestris)*, aspen (*Populus tremula*) and grey alder (*Alnus incana*). Few broad-leaf trees grow in the region, but the world's northernmost growing sites of three tree species are in three coastal municipalities in the study area (Kristiansen, 1982): linden (*Tilia cordata*) in Brønnøy, elm (*Ulmus glabra*) in Beiarn, and hazel (*Corylus avellana*) in Steigen (Lid and Lid, 2005). However, the distribution of *I. ricinus* does not follow the occurrence of black alder (*Alnus glutinosa*) as reported in Sweden (Jaenson and Lindgren, 2011) since the northernmost finding of this tree species in Norway is in Trøndelag (64.75 °N).

As far as possible, sampling was conducted where there was evidence of large vertebrate hosts that are essential for the maintenance of tick populations. These include the Eurasian elk, *Alces alces,* which inhabits much of northern Norway, and roe deer, *Capreolus capreolus,* which are mainly present in the southern part of the region (65–66 °N). On the island of Dønna, roe deer have been present since the 1980s, and they have been hunted since 1994. During the years 2009–18, the number of roe deer killed by hunters in Nordland was highest in Dønna (142 *per* year on average) and nearly twice that of Brønnøy, the municipality with the second highest number (https://www.hjorteviltregisteret.no/). North of Dønna, roe deer is not common, and hunting is carried out only in a few municipalities. Semi-domesticated reindeer, *Rangifer tarandus,* are abundant in Finnmark and in parts of Troms and Nordland. However, with some exceptions, the habitats of reindeer and *I. ricinus* do not coincide, and reindeer do not seem to be important for the maintenance of ticks. Populations of other large and medium-sized potential host animals, such as various mammalian carnivores, have a scattered presence in the study area. Medium-sized mammalian predators include the red fox (*Vulpes vulpes*) and a few mustelids. The most numerous species of small mammals in this region are the field vole (*Microtus agrestis),* bank vole (*Myodes glareolus)* and shrews (*Sorex araneus)*. All three are very common and widely distributed. In addition, since there have been many reports of ticks on domestic pets, sampling was conducted in some local fields indicated by pet owners.

2.2. Tick sampling

The evidence for tick presence was obtained in several ways: i) by ticks collected by cloth-dragging; ii) by examination of pets with the help of local veterinarians; iii) from records of ticks sent by post; and iv) from ticks on trapped rodents and shrews. The ticks were analysed for *B. burgdorferi* s.l. and *Rickettsia* spp. The geographical coordinates of locations were recorded with a Garmin Oregon 450 GPS navigator and are shown as decimal degrees (DD), which expresses latitude and longitude geographic coordinates as decimal fractions. Generally, sampling expeditions were carried out between mid-July and the end of September.

Tick sampling was performed: i) by dragging a white flannel cloth of 1.40 m × 0.70 m (approximately 1 m^2), as described before (Hvidsten et al., 2015); or ii) by moving a terry towel on a stick back and forth over the vegetation ("flagging") (Kjelland et al., 2010). Cloth-dragging was performed in the vast majority of excursions (hereafter, both procedures are referred to as cloth-dragging). The time was measured from start to end of the sampling period. From 2017, if it was a likely *I. ricinus* habitat, we sampled transects for 5 m before examining the cloth; thus each transect covered an area of 5 m x 0.7 m (3.5 m^2). If the

location was an unlikely *I. ricinus* habitat, the transect was 15 m (15 m x 0.7 m [10.5 m^2]). In 2010–16, air temperature was measured with Testo 610 (Testo, Egg, Switzerland), at 1 m above ground level. From 2017, the instrument was placed on the ground to measure temperature. In addition to sampling in Nordland and Troms, seven locations in three coastal municipalities in Trøndelag, adjacent to and south of Nordland, were examined by cloth-dragging for comparison purposes (but only in one season).

The collected ticks were counted and transferred to 2.5 ml plastic tubes with 1 ml of 70 % ethanol, and thereafter stored at 4–8° C until total nucleic acid extraction, cDNA synthesis and PCR analysis for *Borrelia* and *Rickettsia* were performed. Samples for analysis consisted of 1–10 larvae, one nymph, or one adult tick *per* tube.

Ticks provided by local veterinarians were collected as described previously (Hvidsten et al., 2014). Pet owners brought ticks to veterinarians who placed the ticks in 2.5 ml plastic tubes, as described above. In 2013, ticks from pets were collected at six veterinary practices in the cities of Narvik, Harstad, Finnsnes, and Tromsø, and in 2015, at six practices in the archipelagos of Lofoten (Leknes and Svolvær) and Vesterålen (Stokmarknes, Sortland, Bø, and Andenes). In 2015, ticks collected from local pets were also received from a farmer on the island of Dønna (Dønnes Farm). The study area of the ticks obtained from pets was restricted to northern Norway (Trøndelag was not included).

Ticks on rodents and shrews were collected using "multi-capture" Ugglan type 2 live-traps (Grahnab, Gnosjö, Sweden). The animals were euthanized by means of a cotton pad with ether in a closed, transparent plastic bag. After no more than three days at ambient temperature, the bag and content were frozen at −20 °C until examination. The rodents and shrews were identified to species and examined for ticks macroscopically in the laboratory. The plastic bag was washed twice with water, shaken for 30–60 s and the contents filtered. Ticks on the filter were then placed in a tube of 2.5 ml with 70 % ethanol and examined microscopically.

2.3. Analysis of ticks

Collected ticks were analysed by PCR for *Borrelia* spp. and *Rickettsia* spp. infection. Criteria for acceptance of specimens from pets were: i) a likely origin of the tick within the study area (northern Norway region), residence of pets within the study area for at least 10 days before collection; ii) anatomical characteristics consistent with *I. ricinus;* and iii) no signs of decay. All collected ticks were examined by stereomicroscope (20–80 x). Tick species and stages were determined according to relevant literature (Arthur, 1963; Filippova, 1977; Hillyard, 1996), paying special attention to the small differences between *I. ricinus* and *I. persulcatus* nymphs and adults, and between larval *I. ricinus* and *I. trianguliceps.* The possibility that some ticks might have been *Ixodes inopinatus* was not considered because of the apparent more southern distribution of this species (Estrada-Peña et al., 2014), although *I. inopinatus* has since been found in northern Germany (Hauck et al., 2019).

2.4. PCR analyses

Reverse transcribed total nucleic acid from the ticks was extracted, purified and isolated automatically using physical and chemical methods, as previously described (Jenkins et al., 2012). All cDNA samples from the ticks were individually assayed using a Light Upon eXtension (LUX) real-time PCR targeting the *16S rRNA* gene of *Borrelia* spp. (Wilhelmsson et al., 2010). 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). cDNA as a type of template also shows higher analytical sensitivity for *16S rRNA* PCR assays compared to PCR assays targeting *flaB* and *ospA* (Lager et al., 2017); the difference in sensitivity between the PCR assays could be due to the higher abundance of *16S* ribosomal RNA. Ticks containing *Borrelia* spp. were further characterized to determine *Borrelia* genospecies by nested PCR amplification and sequencing of the intergenic spacer 5S–23S as well as the intergenic spacer 16S–23S (Postic et al., 1994; 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). Where characterization to *Borrelia* genospecies level failed, a second real-time PCR test targeting *flaB* was performed to confirm *Borrelia* DNA presence (Jenkins et al., 2012). cDNA samples from the ticks were also assayed using a real-time PCR targeting the citrate synthase (*gltA*) gene of *Rickettsia* spp. (Stenos et al., 2005). Briefly, the reactions were carried out on a C1000™ Thermal Cycler, CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, USA). The reaction mixture of a final volume of 20 µl consisted of Maxima Probe qPCR Master Mix (2X) (Invitrogen, Melbourne, Australia), 200 nM forward (5′-TCG CAA ATG TTC ACG GTA CTT T-3′) and reverse (5′-TCG TGC ATT TCT TTC CAT TGT G-3′) primers each, 200 nM probe (5′-FAM-TGC AAT AGC AAG AAC CGT AGG CTG GAT G-BHQ1–3′) (Biosearch Technologies Inc, Novato, CA), 2 µl of template cDNA and RNase-free water up to 20 µl. Cycling conditions were: 5 min 95 °C followed by 60 cycles of 95 °C for 20 s and 60 °C for 20 s. As a positive control, a synthetic plasmid containing the target sequence of the assay was used, spanning the nucleotides 1102–1231 of the *Rickettsia rickettsii* citrate synthase (*gltA*) gene (acc. no U59729), synthesized and cloned into a pUC57 vector (Genscript USA Inc, NJ). Samples that were real-time PCR-positive for *Rickettsia* spp. were further characterized to determine *Rickettsia* species by PCR amplification and sequencing of the 17-kDa gene, following (Carl et al., 1990). Nucleotide sequencing of the PCR products was performed by Macrogen Inc. (Amsterdam, The Netherlands). All sequences were confirmed by sequencing both strands. The obtained chromatograms were initially edited and analysed using BioEdit Software v7.0 (Tom Hall, Ibis Therapeutics, Carlsbad, CA), and the sequences were examined using Basic Local Alignment Tool (BLAST).

2.5. Growing season length (GSL)

The open portal seNorge (http://www.senorge.no/) delivered daily mean temperatures (DMT) at requested localities. The DMTs were determined in grids of 1 km x 1 km after interpolation of temperature data from the nearest meteorological stations. For both hourly and daily average temperature, the precision of the estimates at grid points varies between 0.8 °C and 2.4 °C (Lussana et al., 2018). GSL was defined according to the joint CCl/CLIVAR/JCOMM Expert Team (ET) on Climate Change Detection and Indice (ETCCDI) (http://etccdi.pacificclimate.org/list_27_indices.shtml) as the annual number of days between a first span of at least six days of DMT > 5 °C (i.e. GSL starting on the seventh day) and the first span (after July 1st) of six days of DMT < 5 °C (i.e. GSL ending on the day before the first day of the span of six days). For each of the intervals 1961–90 and 1991–2015, median GSL was calculated from DMT in 15 selected localities, all sampled for ticks by cloth-dragging.

Although Trøndelag County was not included in the study, PCR analyses, yearly DMT and GSL were calculated for two locations there for reference purposes. In central Dønna, *I. ricinus* larvae were collected from rodents and shrews in two separate seasons but only nymphs and adults (by cloth-dragging) in a third year; therefore, this site did not meet the strict criteria of a permanent tick population. To compare the GSL data in the study area with a location in southern Norway with a known tick population (Kjelland et al., 2010), the median GSL near Kristiansand (at Søgne, 58.10 °N, 7.81 °E), was calculated.

2.6. Statistical analysis

We used the Mann-Whitney test to calculate the differences in median GSL between localities of permanent populations of *I. ricinus* and those where *I. ricinus* had never been collected. The difference between the periods 1961–90 and 1991–2015 in both yearly mean temperature and median GSL was analysed with Wilcoxon matched-pairs signed rank test for the 15 localities. The ratio of ticks collected on dogs to all dogs in the municipality (with data from the Norwegian Kennel Club's register plus 20 % which is the kennel club's estimated percentage of unregistered dogs) was calculated as described earlier (Hvidsten et al., 2014). GraphPad Prism 7 software (San Diego, CA) was used for the calculations. Values of $p < 0.05$ (two-tailed) were considered statistically significant.

3. Results

3.1. Ticks collected by cloth-dragging

During the summer seasons from 2010 to 2018, cloth-dragging was performed on 167 occasions at 109 different locations in 31 municipalities in the counties of Troms (n = 5), Nordland (n = 23) and Trøndelag (n = 3) (Fig. 1). In 2017, 56 of these locations were examined; in 2018, the two northernmost locations of *I. ricinus* determined by drag sampling were re-examined: i) at Nordøyvågen (66.2204 °N, 12.5913 °E), on the northern promontory of the island of Dønna, all three tick stages were found in two successive years (2017–18) in addition to the earlier findings (Table 1), and ii) at Dønnes Farm (66.2045 °N, 12.6001 °E), 1.6 km further south, all postembryonic stages had already been collected in 2016 and 2017. In total, we found one or more ticks in 28 (26 %) of the 109 examined locations, whereas larvae were collected in eight (7 %) of them (Fig. 2.B). Six locations (6 %) were found to fulfil the criteria proposed at the Consensus Conference on Lyme disease (Anonymous, 1991) for permanent *I. ricinus* populations. Temperature, time and examined area for the cloth-dragging procedures are given in Table 2.

3.2. Ticks from pets

In 2013, 13 adult ticks were collected at four of six pet clinics in Narvik city and Troms County, of which only three ticks were accepted for further analysis: ten specimens of *I. ricinus* were excluded because they were collected from dogs that had been outside the study area (the northern Norway region) in the last 10 days, or because of decay (Table 3). In 2015, 40 ticks were found on pets in Lofoten and Vesterålen: one dog owner found four females on his dog, another found one nymph, all *I. ricinus*. The other 35 findings were single *I. ricinus* females *per* dog. From the northernmost location of Nordland County, Andenes, and from the city Narvik, we did not receive any ticks. Two female *I. trianguliceps* were found on dogs and were not included in the study because this tick is not involved in the transmission of *B. burgdorferi* s.l. The highest frequency of 'ticks on dogs to all dogs in the municipality' was found in the cities of Svolvær (2.7 %, 12/440) and Leknes (1.1 %, 6/530), whereas in Sortland (Vesterålen), the figure was 0.6 % (4/686). The collected nymphs and adult ticks from pets on Dønnes Farm are shown in Table 3.

Fig. 1. Map of Norway and adjacent countries Blue dots: No *I. ricinus* ticks collected by cloth-dragging. **Red dots**: Locations where *I. ricinus* ticks were collected by cloth-dragging. C.; County. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Borrelia infections and coinfections in *Ixodes ricinus* collected by cloth-dragging.

Location Latitude, longitude	Season	Larvae collected	N/M/F[1]	*Borrelia*-positive (%)	*B. afzelii*	*B. garinii*	*B. valaisiana*	*Borrelia* spp.[2]/ *Borrelia* coinfections
Nordøyvågen	2014-15	No	1/0/0	0				
66.22035 °N, 12.5862 °E	2016	Yes	4/0/0	0				
	2017	Yes	2/1/1	NA[3]				
	2018	Yes	45/1/2	NA				
Dønnes Farm	2015-16	Yes	66/6/5	1 (1 %)	0	1	0	0/0
66.20131 °N, 12.5982 °E	2017	Yes	9/0/3	NA				
	2018	Yes	23/0/0	NA				
Alstahaug (except Tjøtta and Rosøya)	2011-12	No	5/1/1	0				
65.9 °N, 12.5 °E	2014, 2016							
Tjøtta	2011-12,	Yes	91/13/4	16 (15 %)	5	4	3	
65.8414 °N,12.4917 °E	2014, 2016							
	2017	Yes	30/1/0	NA				
Rosøya	2012, 2014	Yes	96/9/9	2 (2 %)	0	2	0	0/0
65.8429 °N, 12.3490 °E								
Årsetfjorden (Trøndelag County)	2012	No	27/2/1	8 (27 %)	7	0	0	0/1[4]
65.0492 °N, 11.8735 °E								
Rørvik (Trøndelag County)	2012	Yes	16/1/3	3 (15 %)	1	1	1	0/0
64.8610 °N, 11.0987 °E								

[1] N, nymphs; M, males; F, females.
[2] *Borrelia* genospecies.
[3] Not analysed.
[4] *B. afzelii/B. garinii.*

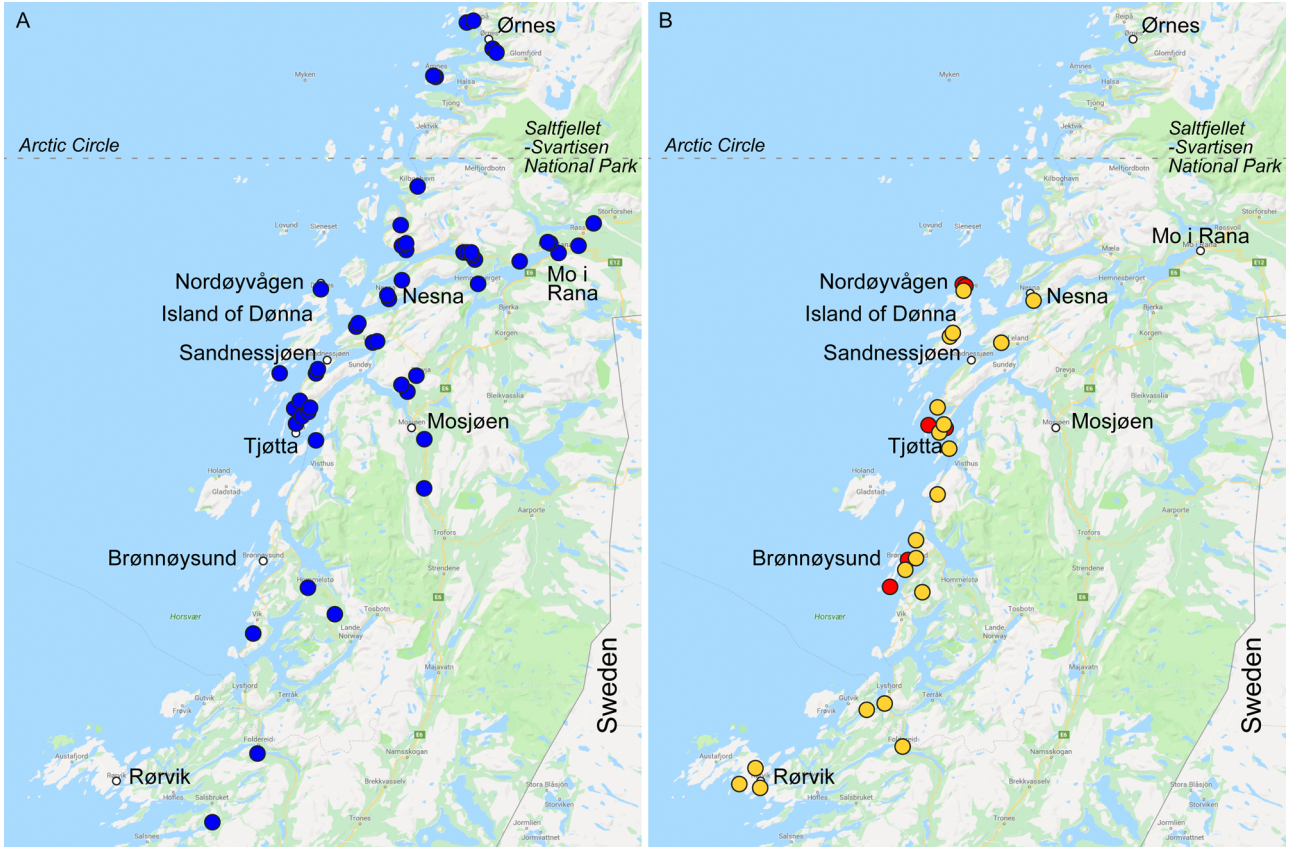

Fig. 2. Maps of the southern study area (and northern part of Trøndelag County) Focus on the northern distribution limit of *I. ricinus* on the coast of Nordland County where cloth-dragging was performed. Left (A). **Blue dots**: *I. ricinus* ticks not collected by cloth-dragging. Right (B). **Red dots**: Positive according to criteria of the Consensus Conference on Lyme disease (Anonymous, 1991) of permanent *I. ricinus* populations (all stages were collected during ≥ 2 consecutive years). **Orange dots**: ≥ 1 *I. ricinus* tick(s) of 1–3 stage(s) were reported but without satisfying the criteria of the Consensus Conference on Lyme disease. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Comparison of cloth-dragging procedures for tick sampling on sites with and without ticks.

	I. ricinus collected, median (5–95 p.[1])	*I. ricinus* not collected, median (5–95 p.)
Time (hr) (2010–16)	1.00 (0.41–3.00)	0.93 (0.25–2.50)
Area (m^2) (2017–18)	210 (105–591)	315 (158–679)
Temperature at 1 m (°C) (2010–16)	19.0 (10.9–26.6)	20.1 (11.6–24.5)
Ground temperature (°C) (2017–18)	14.0 (7.3–16.5)	18.7 (9.0–25.2)

[1] p., percentile.

3.3. Ticks from small mammals

In the autumns of 2011 and 2013, 23 specimens of *M. glareolus* and 42 of *S. araneus* were caught on the island of Dønna. At central Dønna, six *I. ricinus* larvae were found altogether, and on the island's northern coast, at Nordøyvågen, one *I. ricinus* larva was found in 2011 (Table 4). At Utskarpen on the mainland, 45 km east-northeast of Nordøyvågen, on three sampling sites within an area of 10 km^2, none of the 159 ticks on 17 *M. glareolus* and 31 *S. araneus* were *I. ricinus,* all were *I. trianguliceps*. These ticks were not analysed by PCR.

3.4. Analysis of ticks for Borrelia spp. and Rickettsia spp.

A total of 545 nymphs and adult ticks were collected in 2011–16 of which 27 ticks were not accepted because of damage or decay (n = 21) or likely origin outside the study area (n = 6); 518 ticks and 20 tubes of larvae were analysed by the *Borrelia* spp. and *Rickettsia* spp. PCR. The *Borrelia* spp. prevalence in different areas is presented in Tables 1 and 3. Sequencing failed to characterize 14 *Borrelia*-positive samples to the genospecies level, and the *flaB* real-time PCR only detected *Borrelia* in three out of 14 positive samples. *Rickettsia* spp. analysis was positive in five (1.0 %) ticks out of 518 specimens (nymphs, n = 1; females, n = 4). Three (2.0 %) ticks out of 147 ticks collected from pets or by cloth-dragging on the island of Dønna were positive, but this was not significantly different from the rest of the material. Our analysis of the amplified 17-kDa sequences revealed that all five *Rickettsia*-positive samples had a 100 % signature match of *Rickettsia helvetica*, with *R. helvetica* sequences deposited in GenBank (acc.no LC379447).

None of the 20 tubes containing larvae were positive for either *B. burgdorferi* s.l. or *Rickettsia* spp. The ticks collected in 2017–18 were not subjected to PCR analysis.

3.5. Growing season length

In the climatologic standard reference period 1961–91, on sites harbouring permanent populations of *I. ricinus*, median GSL varied between 163 and 166.5 days (n = 4); but on sites where *I. ricinus* was never collected by cloth-dragging or on small mammals, median GSL was 158.5 days or shorter (n = 9). In the 25-year span 1991–2015, the corresponding figures were 173–184 days and 160 days or shorter, respectively (Fig. 3; Table 5). In both periods, the median GSL was calculated and found significantly higher at the sites of a permanent *I. ricinus* population compared with the sites where ticks were never collected even after cloth-dragging twice or more in separate seasons (n = 5) (Table 5). For all selected sites, the increase of median GSL from 1961–90 to 1991–2015 was 9.5 days (n = 15; $p < 0.0003$; rank sums 17, –3; Spearman rank statistics 0.9425), and yearly mean temperature (4.3 °C) increased for all 15 locations; the increase was 1.1 °C, ($p < 0.0001$; Spearman rank statistics 0.9357). At Søgne (near Kristiansand in southern Norway), GSL was 198.5 days in 1961–90 and 221 days in 1991–2015, and the yearly mean temperature (6.7 °C) increased by 1.1 °C from the first to the second period.

4. Discussion

The collecting of all post-embryonic life stages over two successive seasons showed that *I. ricinus* completes a full life cycle at Nordøyvågen on the island of Dønna at the northern latitude of 66.2204 °N, 35 km south of the Arctic Circle. To our knowledge, this is the northernmost documented location of a permanent *I. ricinus* population. In 26 % of the examined areas from the present, new distribution limit, *I. ricinus* were collected by cloth-dragging, and six areas were found to harbour tick populations completing a full life-cycle, as defined by the presence of all three living stages in two consecutive seasons or more. At Nordøyvågen, larvae were found attached to small mammals at a location where they could also be collected by cloth-dragging, which is a strong indication of a permanent tick population, and contrasts with the great difficulty of determining the geographical origin of larvae and other stages attached to humans, pets, birds and large mammals.

Forty-five km east-northeast of the new limit Nordøyvågen, at Utskarpen in Rana, *I. trianguliceps* was the only tick species found on small mammals (Table 4), although female *I. ricinus* has often been collected on pets at this location (data not shown). In Rana, we examined 13 different locations by cloth-dragging, many of them several times but always with negative results (Fig. 2.A). North of

Table 3
Ticks collected from dogs and cats in 2013–16.

County/ Region	Veterinary practice location	Latitude (°N)	Longitude (°E)	Summer season	Ticks, n received/ accepted	Ticks, n dog/ cat	*Borrelia-positive, n*	Stage[1] of *I. ricinus*, n
Cities at *or* north of 68.44°N								
Troms	Tromsø	69.66	18.94	2013	5/0			
Troms	Finnsnes	69.23	17.98	2013	4/1	1/0	0	F:1
Troms	Harstad	68.78	16.51	2013	6/2	No data		F:2
Cities in Vesterålen								
Nordland	Sortland	68.69	15.40	2015	8/7	4/3		F:7
Nordland	Bø	68.69	14.69	2015	3/3	1/2		F:3
Nordland	Stokmarknes	68.57	14.91	2015	2/1	1/0		F:1
Cities in Lofoten								
Nordland	Svolvær	68.23	14.56	2015	15/15	12/3		F:15
Nordland	Leknes	68.15	13.61	2015	20/15	6/9	1[2]	N:1; F:14
Other sources								
Nordland	Dønnes Farm	66.19	12.59	2014–16	No data/65		2[3]	N:14;M:10;F:39;ND:2
Northern Norway[4]	Project			2015–16	No data/48		1[3]	N:2; M:1; F:44

[1] N, nymphs; M, males; F, females.
[2] *Borrelia afzelii.*
[3] *B. afzelii* and *Borrelia valaisiana.*
[4] Ticks were sent to the project from the region's three counties. Only ticks from the study area north of Dønna (66.22 °N) were included.

Table 4
Ticks collected from captured *Myodes glareolus* and *Sorex araneus*.

Year–month	Municipality	Location	Latitude (°N)	Longitude (°E)	*Myodes glareolus*, n	Ticks, n, L[1] (*I. ricinus*)/N[2]	*Sorex araneus*, n	Ticks, n, L[1] (*I. ricinus*)/N[2]
2013–09	Tromsø	Kaldfjord	69.6900	18.7412	5	0/0	6	0/0
2011–09	Rana	Utskarpen[3]	66.30	13.57	17	53/0	31	106/1
2011–09	Dønna	Nordøyvågen	66.2184	12.5927	8[4]	10(1)/0	14	49/3
2013–09	Dønna	Nordøyvågen	66.2184	12.5927	1	0/0	16	3/1
2011–08	Dønna	Central Dønna	66.1007	12.5169	4	2(1)/1	7	1(1)/7
2013–09	Dønna	Central Dønna	66.1007	12.5169	10	7(3)/1	5	2(1)/0

[1]L, larvae: The sum of *Ixodes trianguliceps* and *I. ricinus* (the number of *I. ricinus* larvae in parentheses).
[2]N, nymphs of *I. trianguliceps.*
[3]Three locations within an area of 10 km$^{2.}$
[4]In addition, one specimen of *Mus musculus* (house mouse) was collected but had no ticks.

Nordøyvågen, we never collected any ticks in 56 areas surveyed by cloth-dragging (Fig. 1) or by examination of trapped small mammals. The archipelago of Lofoten, located above the Arctic Circle, is affected by the Gulf Stream and has a climate of mild winters and cool summers, heavy autumn rain in the western areas and strong wind from the sea (http://www.yr.no/). Despite this, drag sampling of five locations in three municipalities yielded negative results. Moreover, pet owners collected only 41 ticks in this archipelago, mainly in the surroundings of Leknes and Svolvær. This is comparable with the finding in Bodø in 2010 (Hvidsten et al., 2014). The number of collected ticks in Vesterålen (Table 3) seems to be lower than that indicated by a questionnaire completed by veterinarians, where, at least in some municipalities, ticks were seen weekly or monthly (Jore et al., 2011). At the same latitude, during the summer seasons 2009, 2011 and 2013, two pet clinics in Harstad only provided 14 female *I. ricinus* to the study (Jenkins et al., 2012; Hvidsten et al., 2014). In the northerly part of the study area, the number of pet-collected ticks in 2013 was negligible (Table 3), thereby confirming earlier findings (Jenkins et al., 2012). In summary, the results of ticks collected from pets and by cloth-dragging do not suggest the existence of sustainable *I. ricinus* populations north of Nordøyvågen. This is in agreement with a recently published article that reported no ticks detected by flagging in nine locations northeast of Dønna (Soleng et al., 2018).

Adult *I. ricinus* (almost exclusively females) are regularly collected on pets north of the present, new distribution limit (Hvidsten et al., 2014). During the project period, several ticks and reports of tick findings (i.e. passive surveillance) were received in Nordland and Troms counties but fewer in Finnmark County. Migratory birds are the likely origin of ticks detected randomly beyond the distribution limit. In Canada, thousands of *I. scapularis,* collected north of the suggested distribution limit, were thought to have been transported from endemic areas (Ogden et al., 2006). On the coast of southern Norway, Hasle et al. (2009) reported that 7 % of migratory birds transported ticks, among which about 75 % of the ticks were nymphs. The Consensus Conference on Lyme disease (Anonymous, 1991) claimed that in areas beyond the distribution limits of *I. scapularis* and *I. pacificus*, ticks are found sporadically, usually involving a single stage. Likewise, north of 66.22 °N, we received mostly engorged, (adult) female ticks collected from pets (Table 3). This implies that younger life stages are extremely rare in this region and suggests that sustainable populations are not present.

It is well known that the factors determining successful establishment of *I. ricinus* populations include the presence of suitable vertebrate hosts, especially for adult females, vegetation that provides a humid microhabitat enabling the survival of the off-host stages, temperatures that permit tick activity, and, probably more importantly, the development of fed ticks to the next stage before the onset of unfavourable conditions. The effect of temperature on tick activity is unlikely to be the main determinant of tick population establishment, since even in Tromsø, more than 450 km northeast of the newly established northern limit for *I. ricinus* populations, daily temperatures from June to September regularly exceed the tick activity temperature threshold, which for significant levels of activity has been found to lie within the range 7–10 °C (MacLeod, 1936; Gray, 1984; Perret et al., 2000; Randolph, 2004). Little is known about the precise limits of the accumulated temperatures required for successful development, but some insight may be obtained by examining climate in relation to the occurrence of permanent *I. ricinus* populations at the limit of their northern geographical distribution. Jaenson and Lindgren (2011) suggested that tick occurrence in Sweden can be correlated with the growing season. Although not necessarily reflecting the precise impact of temperature on tick development, growing season length serves as a useful index to assess the likelihood of tick establishment in a particular region, given that the other conditions are satisfied, and goes some way in explaining the distribution of established tick populations in Norway. Thus, it can be seen in Table 5, in the period 1991–2015, that the locations where permanent tick populations were observed have a median GSL of 173–184 days. Where no ticks have been detected, the values are significantly lower and are 160 days or less.

There is no clear-cut GSL value defining the sites harbouring *I. ricinus* and those which do not. Other factors may be operating at individual sites. Årsetfjorden in Trøndelag had, for instance, a density of 27 nymphs and three adults *per* 30 min of dragging (data not shown) despite a relative low GSL (159 days [Fig. 3]), although with a high density of roe deer (more than 200 roe deer killed by hunting yearly in the last years). In contrast, at Nesna, the GSL is above the supposed development threshold but without evidence of a permanent tick population, which may be due to the very low numbers of roe deer (https://www.hjorteviltregisteret.no/).

In the 15 selected locations in the study area, the median GSL increased from that of the reference period 1961–90 to the figure for the subsequent 25 years by 9.5 days. We observed that yearly mean DMT increased by more than one degree Celcius, hence showing that climate change has been taking place in this Arctic region. Likewise, the temperature increase in southern Norway was same as those in the north, and the increase in Trøndelag was one degree Celsius (data not shown). Overall, by correlating our findings at localities of permanent *I. ricinus* populations with the median GSL at 15 sites in Troms and Nordland, we confirm the relationship of tick presence to GSL, as suggested by Jaenson and Lindgren (2011).

The *Borrelia* prevalence in the ticks collected by cloth-dragging on Dønna, Tjøtta and Rosøya is lower than that in Brønnøysund and Trøndelag (Table 1). In an earlier study, the *Borrelia* prevalence in nymphs and adult ticks collected from pets in Brønnøysund was 29 % (54/188), whereas further north, the prevalence was only 4 % (12/305) (Hvidsten et al., 2014). The non-permissive climate in the surroundings of the present distribution limit may play a role in the transmission dynamics of *Borrelia*. However, Lyme borreliosis incidence in Brønnøysund, located in the southern part of the study area, is the highest in northern Norway, which may be attributed to the high tick abundance and relatively high prevalence of *Borrelia* species in the ticks (Jenkins et al., 2012; Soleng and Kjelland, 2013; Hvidsten et al., 2015); but in general, the overall incidence of Lyme borreliosis in northern

Fig. 3. Growing season length (GSL) in 1961–1990 and 1991–2015 Growing season length (days) in two periods, 1961–1990 and 1991–2015, at 15 selected locations in the study area of northern Norway and two locations in the northern part of Trøndelag County. **Blue dots**: Areas where no *I. ricinus* ticks were collected by cloth-dragging. **Red dots:** Permanent *I. ricinus* populations according to the criteria of the Consensus Conference on Lyme disease (Anonymous, 1991). **Orange dots:** ≥1 *I. ricinus* tick(s) of 1–3 stage(s) were collected but without satisfying the criteria of the Consensus Conference on Lyme disease. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Norway is very low (http://www.msis.no) with low seroprevalence (Hvidsten et al., 2017), which is most likely due to the scarcity of *I. ricinus,* as shown in the present study. The prevalence of *R. helvetica* infection in the ticks was also low, but was a little higher than that in the ticks collected in southern Norway (Quarsten et al., 2015).

Previous records of *I. ricinus* north of the present, new distribution limit have been mainly based only on indirect reports or the finding of nymphs or adults, either on vegetation or on pets or domestic animals. However, since migratory birds may deposit engorged larvae or nymphs in areas where temperatures permit development to the next stage, but not completion of the life cycle, such records do not constitute evidence for established tick populations. Reliable larval presence (together with the other life cycle stages) as an indication of life cycle completion is the only acceptable criterion for determination of the *I. ricinus'* northern

Table 5
Median growing season length in northern Norway (and Søgne).

		Growing season length (days)			
		1961–1990		1991–2015	
Location	*I. ricinus* presence	Median	5–95 percentile	Median	5–95 percentile
Bodø	No	158.5	122–182	160	144–205
Ørnes	No	154	116–178	157	138–193
Mo i Rana	No	139	111–174	147	124–175
Utskarpen	No	158.5	128–187	159	142–187
Mosjøen	No	154	123–183	160	141–200
Median (n = 5)		154		159	
Nordøyvågen	Permanent	166.5	136–199	184	159–239
Tjøtta	Permanent	163	134–193	174	157–225
Rosøya	Permanent	164.5	134–193	179	157–227
Brønnøysund	Permanent	163.5	138–196	173	157–247
Median (n = 4)		164		176.5	
Difference between the groups		10.0[1]		17.5[1]	
Søgne (lat. 58.10°N)	No data[2]	198.5	175–238	221	178–309

[1] $p = 0.0079$ (ties among values are taken into account. Sum of ranks 30, 15).
[2] See Kjelland et al. (2010).

geographical distribution limit, which is an important part of the monitoring of tick-borne pathogens in a climate change scenario.

5. Conclusion

All the postembryonic tick stages (larva, nymph, female, male) have been demonstrated by cloth-dragging to occur in successive years at Nordøyvågen (latitude of 66.22 °N), on the northern promontory of the island of Dønna. This constitutes evidence of a permanent *I. ricinus* population, and to our knowledge, this may be the northernmost sustainable *I. ricinus* population reported so far. Analysis of climate data showed that the established tick populations occur where the growing season is 170–180 days or longer.

Ethics

The method of capturing rodents and shrews was approved by the Norwegian Environment Agency (reference numbers 2011/10865 and 2013/5002) in accordance with the regulations on the capture and collection of game for scientific or other special purposes.

Declaration of Competing Interest

The authors declare no conflict of interest.

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