Supplemental Material: Ogden et al. 2010: Active and Passive Surveillance, and Phylogenetic Analysis Elucidate the Process of Lyme Disease Risk Emergence in Canada

Materials and Methods

Data on ticks in passive surveillance:

PCR analysis of ticks: DNA was extracted from ticks using Qiagen® DNeasy® 96 Tissue kits (QIAGEN Inc., Mississauga, ON, Canada) optimized for recovery of low-copy number DNA from ticks, and extraction efficiency was assessed using primers specific for the tick 5.8S rRNA - 28S rRNA intergenic spacer (IGS). DNA was screened for evidence of *B. burgdorferi* infection using a multiplex real-time PCR targeting their 23S rRNA and *msp2* genes of *B. burgdorferi* and *A. phagocytophilum* as previously described (Courtney et al. 2004). Results for *A. phagocytophilum* are not presented here. *B. burgdorferi* infection was then confirmed in positive samples using primers and probe targeting the *ospA* gene (Ogden et al. 2006).

Active surveillance

Sites selection: All sites were selected on the following criteria:

- occurrence within the zone of high or moderate risk for *I. scapularis* occurrence in risk maps (Ogden et al. 2008);
- maple woodlands that, if mixed, comprised predominantly deciduous trees (determined from provincial government forest cover maps: http://www.mrnf.gouv.qc.ca/forets/connaissances/connaissances-inventaire-cartes-sief-peuplements.jsp);
- having minimal dimensions of continuous woodland of 0.5 km^2; iv) proximity of vehicular access.

Rodent capture and plasma and tick collection: At each field site three transects of 50 Sherman traps were placed and bated to trap a target minimum 15 rodents (with $\alpha = 0.05$, a sample of 15 rodents gives 80% power to identify an infested rodent if the true prevalence of infestation is 5%). Captured rodents were examined for the presence of ticks and a blood sample collected from the retro-orbital bleeding under light anaesthesia (or intra-cardiac puncture immediately post-mortem in some instances) as described elsewhere (Lindsay et al. 1999). Blood samples were collected only from *Peromyscus* spp. (white-footed mice and deer mice) in 2007, but also from *Tamias striatus* (eastern chipmunks) and *Tamiasciurus hudsonicus* (red squirrels) in 2008. The herbage of each site was 'flagged' to collect host-seeking ticks using a standard protocol of 3 person-hours of 'flagging' in the afternoon. This commonly-used technique comprised slowly dragging a 1m^2 flannel across the woodland floor or over herbage of the woodland understory to collect ticks that mistake the flannel for an animal host. All ticks collected from rodents and herbage were identified to species by standard keys (Clifford et al. 1961; Keirans et al. 1996), and analysed for the presence of *B. burgdorferi* infection by PCR as described above. The capture and sampling of rodents was conducted under

animal care licenses of the Université de Montréal and the Quebec Ministère des Ressources Naturelles et de la Faune.

Serological analysis of rodent plasma:
Blood from the rodents was collected into tubes containing EDTA, from which plasma was obtained and tested for the presence of a specific antibody response to *B. burgdorferi* infection using a two-tier IFA/ELISA and Western blot system similar to that previously described (Hofmeister et al. 1999).

For samples collected prior to May 2008 the first step was an immunofluorescence assay (IFA). For this test plasma samples were diluted to 1:64 in FTA hemagglutination buffer (Becton, Dickinson and Company, Cat. No. B11248) and added to wells on slides pre-coated with *B. burgdorferi* sensu stricto (Fuller Labs, CA. Cat. No. BB-12). Following a 30 minute incubation at 37° C, slides were washed in FTA buffer for 15 minutes then allowed to air dry. Fluorescein-conjugated goat-anti *Peromyscus* IgG H&L (KPL, Gaithersburg, MD., Cat. No. 022-33-06) diluted 1:20 in Evan's blue dye was added to each well and slides were incubated then washed as above. Once the slides were dry, they were mounted and observed for fluorescence by microscopy. Samples exhibiting fluorescence at the 1:64 screening dilution were confirmed by Western blot (Trevejo et al. 2001 and see below).

From May 2008, the first step was an enzyme-linked immunosorbent assay (ELISA) (Magnarelli et al. 2006). Plasma samples were tested at a 1:100 dilution using a modification of a commercially available EuroImmun Anti-Borrelia plus VlsE ELISA kit (EuroImmun, Lubeck, Germany, Cat. No. EI 2132-9601-2 G). In order to detect immunoglobulin from a variety of small mammal sources, the kit conjugate was substituted with commercially prepared (Calbiochem, EMD Biosciences, CA, Cat. No. 539322) affinity purified horseradish peroxidise-labelled Protein G (Akerstrom et al. 1985), diluted to 1:4000. All plates contained strongly positive, weakly positive and negative controls as well as the 20 RU/ml calibrator provided in the kit.

To determine consistent cut-off values for positive results between IFA and ELISA, samples from 50 positive and 50 negative *P. leucopus*, as determined by IFA and Western blot, were analyzed by ELISA. For each ELISA test for each sample a ratio of the test optical density for the sample to that of the test calibrator was calculated. Positive sera consistently provided an extinction ratio significantly greater than 2.0 while negative samples had ratios less than 0.7. Therefore, samples from rodents that gave a ratio greater than 2.0 were reported as reactive and considered positive for antibodies to *B. burgdorferi* and no further testing was performed. Samples with a ratio between 0.75 and 2.0 were considered borderline and were subsequently confirmed by Western blot (see below). Lastly, samples with a ratio less than 0.75 were reported as negative (Aguero-Rosenfeld et al. 1993).

Samples positive by IFA or borderline positive by ELISA were confirmed using a commercially prepared Western Blot strip (MarDx, Trinity Biotech, Inc.). The test kit was adapted to detect *B. burgdorferi* antibodies in small mammal samples by substituting

the kit conjugate with alkaline phosphatase-conjugated Protein G (Calbiochem, EMD Biosciences, CA, Cat. No. 539305) diluted to 1:2500. Samples were reported as positive if 5 or more of the significant bands specified by the manufacturer were present.

At a known Lyme disease endemic site at Lunenberg county in Nova Scotia, 137/602 (22.8%) of *Peromyscus* spp. plasma samples collected from 2004-2007, were positive using the IFA-Western blot testing procedure. Excepting samples collected in May 2004 (when prevalence was 5%), the prevalence of positive rodents captured in the Lunenberg county study was always greater than 30% (unpublished data).

Calculation of the 'Tick population index' for I. scapularis *population establishment:* The index was calculated for each site as follows: presence of larvae = 1 point; presence of 3 or more larvae = 1 additional point; presence of 10 or more larvae = 1 additional point; presence of nymphs = 1 point; presence of 3 or more nymphs = 1 additional point; presence of 10 or more nymphs = 1 additional point; presence of adult ticks = 1 point; presence of 3 or more adults = 1 additional point; presence of 10 or more adults = 1 additional point. Thus the index had a minimum value of 0 (no *I. scapularis* found at the site) and a maximum of 9 (when 10 or more ticks of each of the three instars were found at the site).

MLST analysis

Eight housekeeping genes (*clpA, clpX, nifS, pepX, pyrG, recG, rplB*, and *uvrA*) were amplified by nested PCR using Qiagen HotStarTaq mastermix (Qiagen, UK) for both amplification steps. For primer information and PCR conditions see Margos et al. 2008, 2009 and also the MLST website hosted at Imperial College London, UK (www.mlst.net). PCR products were sequenced in forward and reverse directions and trace files assessed for quality using Lasergene software Seqman (DNASTAR, Madison, USA). Sequences of individual housekeeping genes were compared to alleles already in the *Borrelia* database at borrelia.mlst.net where details of the sequences in this study can be found. Allele numbers and sequence types (ST) were assigned using the NRDB software available at the website.

Results

Passive surveillance:

Supplemental Material, Table 1. The number of *I. scapularis* ticks submitted and tested in passive surveillance in Quebec by year, and the number of ticks detected as infected with *B. burgdorferi*. The number of ticks used in cluster analysis was lower than these values because ticks from animals or humans that had travelled within Quebec within 2 weeks prior to the tick being detected were removed from analysis. Data from only 3 ticks collected in 2008 were available at the time of analysis.

Year	Number infected / total number of tested ticks (%)
1996	2/30 (6.7)
1997	7/35 (20)
1998	14/90(15.6)
1999	5/48 (10.4)
2000	14/97 (14.4)
2001	12/133 (9.0)
2002	24/160 (15.0)
2003	50/375 (13.3)
2004	36/384 (9.4)
2005	44/433 (10.2)
2006	62/663 (9.4)
2007	74/1034 (7.2)
2008	142/1709 (8.3)
Total	486/5191 (9.4)

Bi-variable logistic regression analysis of variables associated with infection prevalence in ticks submitted in passive surveillance:
In bi-variable logistic regression here were no significant differences amongst years in infection prevalence in ticks submitted from 1996 to 2004, and no significant differences amongst years for ticks submitted from 2004 to 2008 ($P > 0.1$ for both). However, the infection prevalence in ticks submitted after 2004 was significantly lower than that in ticks collected before 2004. There were significant differences in infection prevalence in ticks collected from different host species, and at different stages of engorgement (Supplemental Material, Table 2). There were only 11 immature ticks included in the analyses and there were no significant variations in prevalence amongst instars.

Supplemental Material, Table 2. Significant factors, in bi-variable logistic regression, associated with *B. burgdorferi* infection in ticks collected in passive surveillance

Explanatory variable	Odds Ratio (95% Confidence Interval)	Wald z	p-value
Ticks collected after 2003 versus those collected before 2004	0.65 (0.52 – 0.83)	-3.51	< 0.001
State of engorgement			
No engorgement	Reference		
Semi-engorged	1.85 (1.08 – 3.17)	2.24	0.025
Fully engorged	2.09 (1.16 – 3.75)	2.48	0.013
Host			
Dog	Reference		
Human	1.84 (1.07 – 3.16)	2.21	0.027
Cat	2.80 (1.61 – 4.87)	3.64	< 0.001

Active surveillance:

The 2159 rodents that were captured and examined for ticks comprised 1363 *Peromyscus* spp., 321 shrews (*Blarina brevicauda* and *Sorex* spp.), 170 voles (*Clethrionomys* and *Microtus* spp.), 168 eastern chipmunks (*Tamias striatus*), 95 jumping mice (*Zapus* and *Napaeozapus* spp.), 39 red squirrels (*Tamiasciurus hudsonicus*), 2 stoats (*Mustela erminea*) and 1 flying squirrel (*Glaucomys sabrinus*). Of the rodents tested serologically, 9/721 *Peromyscus leucopus*, 6/340 *P. maniculatus*,. 1/97 chipmunks, and 1/11 red squirrels were seropositive.

Supplemental Material, Table 3. The number of ticks of each instar found at field sites during active surveillance, during a single visit in 2007 or 2008, and after additional flagging in autumn, and then repeated visits during 2008.

	Larvae		Nymphs		Adults	Total
	Questing	Engorged	Questing	Engorged	Questing	
First visit	194	255	58	56	11	574
Autumn flagging	4		1		112	117
Repeat visits	57	426	78	117	23	701
Total	255	681	137	173	146	1392

Supplemental Material, Table 4. Details of active surveillance results. The numbers of rodents captured and *I. scapularis* ticks collected and tested for *B. burgdorferi* infection by site in active surveillance. Figures in bold indicate sites that had evidence of *B. burgdorferi* infection. QL = questing larvae, EL = engorged larvae, QN = questing nymphs, EN = engorged nymphs and QA = questing adults.

Site	Year	Rodents Tested	Rodents Captured	QL	EL	QN	EN	QA
1	2007	0/5	5	0	0	0	0	0/2
	2008	0/3	3	0	0	0/5	0/2	0
2	2007	0/28	34	0	0/4	0	0	0
3	2007	0/9	12	0	0	0	0	0/4
4	2007	0/15	31	0	0	0	0	0
	2008	-	-	0	-	0	-	0/1 [f]
5	2007	0/11	13	0	0	0	0	0/1
6	**2007**	**0/4**	**14**	**0**	**0/3**	**0**	**0/1**	**1/11**
	2008	**3/40**	**57**	**0**	**1/42**	**0/3**	**1/8**	**1/8**
7	2007	0/10	15	0	0	0	0	0
8	**2007**	**2/15**	**23**	**1**	**0**	**0/13**	**2/12**	**1/15**
	2008	**1/33**	**57**	**1**	**0/34**	**1/23**	**4/23**	**0/4**
9	**2007**	**0/12**	**12**	**1**	**0/2**	**0**	**0/6**	**0/5**
	2008	**0/36**	**43**	**0**	**0/34**	**0/2**	**0/4**	**1/6**
10	2007	0/6	8	2	0	0	0	0/2
11	2007	0/4	15	0	0	0/1	0/1	0/4
12	2007	0	0	0	0	0	0	0/1
13	2007	0/15	28	0	0	0	0	0
	2008	-	-	0	-	0	-	0/2 [f]
14	2007	0/7	18	0	0	0	0	0
	2008	-	-	0	-	0	-	0/5 [f]
15	2007	0/14	34	0	0	0	0	0
16	**2007**	**0/15**	**27**	**0**	**0/3**	**0**	**0/1**	**0/2**
	2008	**1/35**	**65**	**15**	**0/35**	**0/7**	**0/8**	**0**
17	**2007**	**0/15**	**25**	**0**	**0**	**0**	**0/4**	**1/3**
	2008	**2/22**	**38**	**40**	**0/188**	**0/28**	**0/13**	**1/6**
18	2007	0/14	27	0	0/1	0	0	0
19	**2007**	**1/6**	**9**	**4**	**0/1**	**0/2**	**0/2**	**4/12**
	2008	**1/19**	**61**	**1**	**10/63**	**0/7**	**5/31**	**0/1**
20	2007	0/15	18	0	0	0	0	0
21	2007	0/18	21	0	0	0	0	0
22	2007	0/2	8	0	0	0	0	0
	2008	**-**	**-**	**0**	**-**	**0**	**-**	**2/5** [f]
23	**2007**	**0/9**	**33**	**0**	**0/1**	**0**	**0/3**	**0/8**
	2008	**0/22**	**66**	**0**	**0/23**	**0**	**3/15**	**0**
24	2007	0/15	27	0	0	0	0	0
25	2007	0/10	18	0	0/17	0	0/2	0
26	2007	0/15	24	0	0	0	0	0/1
27	2007	0/19	24	0	0	0	0	0
28	2007	0/14	48	0	0	0	0	0
29	2007	0/19	33	0	0	0	0	0
30	2007	0/7	10	0	0	0	0	0
31	2007	0/11	27	0	0/6	0	0	0/1
	2008	0/27	37	0	0/8	0/3	0/8	0/3
32	2007	0/15	35	0	0	0	0	0
33	2007	0/19	33	0	0	0	0	0
34	**2007**	**0/8**	**12**	**0**	**0/19**	**0**	**0**	**4/17**

35	2007	0/23	26	0	0	0	0	0
36	2007	0/17	41	0	0	0	0	0
37	2007	0/10	26	0	0	0	0	0
38	**2007**	**0/24**	**30**	**0**	**0**	**0**	**1/1**	**0**
	2008	0/10	20	0	0	0	0	0
39	2007	0/19	35	0	0/7	0	0	0
	2008	0/12	18	0	0	0	0/2	0/1
40	2007	0/16	28	0	0	0	0	0
41	2007	0/12	29	0	0/3	0	0	0
	2008	0/13	19	0	0	0	0/1	0
42	2007	0/5	15	0	0	0	0	0
43	2007	0/12	41	0	0	0	0	0
44	**2007**	**0/16**	**23**	**0**	**0/5**	**0**	**0**	**0**
	2008	**2/10**	**20**	**0**	**0/1**	**0**	**0/2**	**0**
45	2007	0/15	16	6	0/14	0	0	0/1
46	2007	0/16	16	0	0/1	0	0	0
47	2008	0/4	5	0	0	0/3	0	0
48	2008	0/11	14	0	0	0	0	0
49	2008	0/15	19	0	0	0	0	0
50	2008	0/14	28	0	0	0	0	0
51	2008	0/9	18	0	0/3	0	0	0
52	2008	0/2	8	0	0	0	0/1	0
53	2008	0/12	15	0	0/6	0/1	0/2	0
54	2008	0/9	17	15	0/9	0/6	0/2	0
55	2008	0/4	12	15	0	0	0/2	0
56	2008	0/9	29	1	0	0/1	0/1	0
57	2008	0/15	30	0	0	0	0	0
58	2008	0/10	25	0	0	0	0	0
59	2008	0/2	14	1	0/10	0/3	0	0
60	2008	0/10	29	0	0	0	0	0
61	2008	0/18	32	0	0	0	0	0
62	2008	0/9	33	10	0/13	0/7	0/2	0
63	2008	0/17	38	0	0	0	0	0
64	**2008**	**1/16**	**32**	**0**	**0**	**0**	**0/1**	**0**
65	2008	0/16	30	0	0	0	0	0
66	2008	0/23	28	0	0	0	0	0
67	2008	0/18	23	0	0	0/1	0	0
68	2008	0/11	16	0	0	0	0	0
69	**2008**	**3/13**	**36**	**137**	**11/97***	**0/17**	**1/8**	**3/12**
70	2008	0/19	38	0	0/10	0/1	0/1	0/2
71	2008	0/15	39	5	0/20	0/3	0/3	0
Totals		17/1169	2159	255	22/681	1/137	17/173	19/146

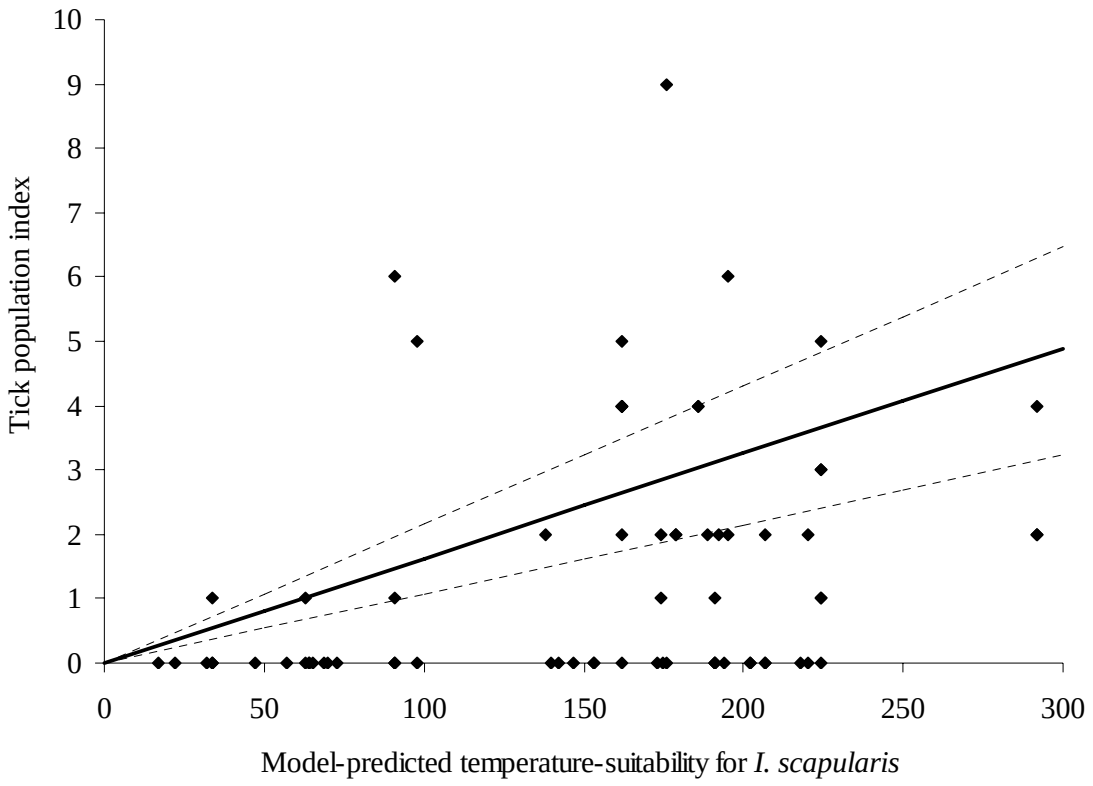
* these positive engorged larvae comprised a pool of ticks. Superscript ‘f’ indicates ticks that were collected by flagging the herbage at sites re-visited in October 2008.

Supplemental Material, Table 5. Full results of analysis of MLST sequence types (ST) including the allelic profile for each of the eight housekeeping genes used in MLST analysis (Margos et al. 2008), IGS type and *ospC* major group (MG), as well as the corresponding RST type for each sample analyzed. The region of the USA where each ST has been found is indicated in the last column: NE = northeast, MW = mid-west. ST 238 is novel to this study.

Tick	ST	*clpA*	*clpX*	*nifS*	*pepX*	*pyrG*	*recG*	*rplB*	*uvrA*	IGS type	*ospC* MG	RST	US region
QC07-785	1	1	1	1	1	1	1	1	1	1	A	1	NE
QC07-908	1	1	1	1	1	1	1	1	1	1	A	1	NE
QC07-161-5	1	1	1	1	1	1	1	1	1	1	A	1	NE
QC07-83	1	1	1	1	1	1	1	1	1	1	A	1	NE
QC07-493	3	4	1	1	1	1	6	1	7	2	K	2	NE
QC07-765	3	4	1	1	1	1	6	1	7	2	K	2	NE
QC07-84	3	4	1	1	1	1	6	1	7	2	K	2	NE
QC07-951	3	4	1	1	1	1	6	1	7	2	K	2	NE
QC07-1054	3	4	1	1	1	1	6	1	7	2	K	2	NE
QC07-565b	4	8	1	1	1	4	6	1	7	2	H	2	NE
QC07-755	7	6	1	5	1	1	7	1	8	3	B	1	NE
QC07-399	8	5	5	4	5	5	5	1	6	4	F	3	NE
QC07-603	8	5	5	4	5	5	5	1	6	4	F	3	NE
QC07-815	8	5	5	4	5	5	5	1	6	4	F	3	NE
QC07-819	8	5	5	4	5	5	5	1	6	4	F	3	NE
QC07-723	11	5	7	5	1	6	1	4	9	5	C	3	NE
QC07-776	12	3	3	2	4	3	4	4	4	6	M	3	MW
QC07-1008	14	9	1	1	7	1	6	1	10	6	G	3	NE
QC07-181-1	14	9	1	1	7	1	6	1	10	6	G	3	NE
QC07-181-4	14	9	1	1	7	1	6	1	10	6	G	3	NE
QC07-182-4	14	9	1	1	7	1	6	1	10	6	G	3	NE
QC07-182-6	14	9	1	1	7	1	6	1	10	6	A	3	NE
QC07-650	16	2	2	1	2	2	2	2	2	7	I	3	NE
QC07-484	29	18	12	1	11	2	15	1	2	2	L	2	MW

QC07-344	38	21	1	15	8	1	18	4	7	5	D	3	NE
QC07-362	34	8	1	1	7	1	6	1	10	5	J	3	NE
QC07-402	238	10	5	75	6	1	15	1	6	4	N	3	-
QC07-595	36	10	5	4	6	1	15	1	6	4	N	3	NE
QC07-851	59	6	1	5	1	1	7	1	19	3	B	1	NE
QC07-1048	59	6	1	5	1	1	7	1	19	3	B	1	NE
QC07-175-3	59	6	1	5	1	1	7	1	19	3	B	1	NE

Supplemental Material, Figure 1. The relationship between model-predicted climate (temperature) suitability for *I. scapularis* and the tick population index calculated for each study site from the number of tick instars and number of ticks found at each site (see supplementary methods above). The values on the x-axis are an index of suitability for tick population establishment (i.e. model-generated tick abundance) predicted by an *I. scapularis* population model (Ogden et al. 2005) for the temperature conditions (mean annual cumulative degree-days > 0°C) at each site. The solid line shows the statistical relationship (+/- SE indicated by the dashed lines) adjusted for clustering of positive sites and inter-year differences. Temperature conditions were ascribed to each site as described in Ogden et al. (2008).



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