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Original article

Infection of Siberian chipmunks (*Tamias sibiricus barberi*) with *Borrelia* sp. reveals a low reservoir competence under experimental conditions

Sarah Bonnet[a,*], Valérie Choumet[b,1], Sébastien Masseglia[c], Martine Cote[a], Elisabeth Ferquel[b,2], Thomas Lilin[d], Maud Marsot[c,e], Jean-Louis Chapuis[e], Gwenaël Vourc'h[c]

[a] *USC INRA Bartonella-tiques, UMR BIPAR ENVA-ANSES, 94706 Maisons-Alfort, France*
[b] *Institut Pasteur, CNR des Borrelia, 28 rue du Dr Roux, 75724 Paris cedex 15, France*
[c] *Unité Epidémiologie Animale (UR INRA 346), Centre de recherche INRA de Clermont-Ferrand/Theix, 63122 Saint Genès Champanelle, France*
[d] *Centre de recherches Biomédicales (CRBM), École Nationale Vétérinaire d'Alfort, 7 Avenue du Général de Gaulle, 94700 Maisons-Alfort, France*
[e] *Centre d'Ecologie et des Sciences de la Conservation (CESCO UMR7204), Sorbonne Universités, MNHN, CNRS, UPMC, CP51, 55 rue Buffon, 75005 Paris, France*

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ABSTRACT

Reservoir competence is a key parameter in understanding the role of host species in the epidemiology of multi-host—especially vector-borne—pathogens. With this aim in view, we studied the reservoir competence of the Siberian chipmunk (*Tamias sibiricus barberi*) recently introduced into Europe, for the multi-host tick-borne bacteria, *Borrelia burgdorferi* sl, the agent of Lyme borreliosis. *T. sibiricus* were experimentally exposed to bites from *Ixodes ricinus* ticks infected with *Borrelia burgdorferi* sensu stricto and *Borrelia afzelii*, with subsequent assessment of bacteremia and antibody responses. *Borrelia* was detected in chipmunk blood samples, ear biopsies and organ necropsies, and in nymphs used for xenodiagnosis (at one and six months after the initial chipmunk infection) via both serological and molecular methods. In total, eight out of twelve chipmunks showed evidence of infection by *Borrelia* sp., either by ELISA or PCR. Five chipmunks developed an immune response against the bacteria one month after infection. *Borrelia* infection in at least one organ was observed in seven animals at 14, 38, 93 or 178 days post-infection. Xenodiagnosis was positive for one chipmunk at 38 days, but no longer at 178 days post-infection. Four chipmunks remained uninfected, despite similar infection pressures to those observed in the field. Taken together, these results suggest that chipmunks can be infected through *Borrelia*-infected tick bites, and can transmit *Borrelia* to nymphs, but do not remain persistently infected.

Introduction

Ixodes ricinus is the most widespread and abundant tick species in Europe, and is the vector of several tick-borne diseases (TBD) of both medical and veterinary importance, including Lyme disease (Heyman et al., 2010). This disease is caused by bacteria belonging to the *Borrelia burgdorferi* sensu lato (sl) complex, and is the most prevalent vector-borne disease in Europe (Dantas-Torres et al., 2012). The major European genospecies pathogenic for humans are *B. afzelii*, which preferentially infects rodents; *B. burgdorferi* sensu strico (ss), found in birds and mammals; and *B. garinii* which preferentially infects birds and large mammals (Humair and Gern, 1998; Hanincova et al., 2003a,b). These bacteria are maintained in the natural environment in cycles of transmission between ticks and reservoir host vertebrates. Animals are only considered to be reservoirs if they can: (1) become infected following the bite of an infected tick, and then, (2) re-transmit pathogens to other ticks. Numerous vertebrate species have been identified as reservoir hosts for *Borrelia* sl (Gern et al., 1998), but not all hosts contribute equally to bacterial transmission dynamics. In addition to the importance of their density, tick load, and ability to infect ticks, their influence relies on the duration of host infection. Indeed, to be an effective reservoir, the host must be a preferential ectoparasite

* Corresponding author. Tel.: +33 0149774654; fax: +33 0149772828.
E-mail addresses: sbonnet@vet-alfort.fr (S. Bonnet), valerie.choumet@pasteur.fr (V. Choumet), sebastien.masseglia@clermont.inra.fr (S. Masseglia), mcote@vet-alfort.fr (M. Cote), elisabeth.ferquel@pasteur.fr (E. Ferquel), tlilin@vet-alfort.fr (T. Lilin), maud.marsot@anses.fr (M. Marsot), chapuis@mnhn.fr (J.-L. Chapuis), gvourch@clermont.inra.fr (G. Vourc'h).
[1] Current address: Unité Environnment Risques Infectieux, Institut Pasteur, 25 rue du Dr Roux, 75024 Paris cedex 15, France.
[2] Current address: Département de Virologie, Institut Pasteur, 25-28 rue du Dr Roux, 75024 Paris cedex 15, France.

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host, but should also remain infected and infectious, at least until a new tick bite, which is especially important during arthropod inactivity periods throughout colder months in temperate areas. However, *Borrelia* infection duration has only been studied in a few species. In particular, only two species of rodents (*Apodemus* mice and *Myodes* voles) are known to stay bacteriemic over winter, becoming a renewed source of infection for active ticks in spring (Humair et al., 1999). Introduced vertebrate species capable of acting as new hosts both for ticks and *Borrelia* sp. may modify the transmission dynamics of Lyme disease, as has been demonstrated for other infectious diseases (Begon, 2008).

The Siberian chipmunk (*Tamias sibiricus barberi*) has been found in pet shops across Europe since the late 1960s (Pisanu et al., 2013). Some chipmunks have been intentionally released into the wild, and have formed populations in several peri-urban woods and parks in France (Chapuis et al., 2011). There is abundant field evidence that *T. sibiricus* can be infected by *Borrelia* sp. including both *B. burgdorferi* ss and *B. afzelii* (Chu, 2006; Pisanu et al., 2010; Marsot et al., 2011, 2013; Jacquot et al., 2014a,b). In addition, Siberian chipmunks are suspected to greatly contribute to Lyme borreliosis risk, as they host higher numbers ticks and are more often infected by diverse *Borrelia* genospecies than the native rodent reservoir species (Pisanu et al., 2010; Marsot et al., 2011; Jacquot et al., 2014a). Indeed, *Borrelia* prevalence as high as 53% was reported in chipmunks captured in 2007 from the Sénart forest ($N = 91$), in the South of Paris (France) (Marsot et al., 2011). Recently, an epidemiological study conducted in the same area demonstrated that chipmunks contribute more to borreliosis risk than the two native rodent reservoir hosts, bank voles (*Myodes glareolus*) and wood mice (*Apodemus sylvaticus*), combined (Marsot et al., 2013). However studies performed in Siberian chipmunks collected from their original native Chinese habitats did not demonstrate increased levels of infection, or greater numbers of *B. burgdorferi* sl genospecies when compared to other indigenous rodents (Chu, 2006).

To date, no experimental data are available on the reservoir competence of Siberian chipmunks for *Borrelia* sp. Tick xenodiagnosis is the only way to conclusively demonstrate the infectivity of a vertebrate species. However, no studies under controlled laboratory conditions have ascertained the ability of Siberian chipmunks to transmit *Borrelia* species to *I. ricinus* ticks, or their predisposition toward maintaining *Borrelia* infection for several months. This is probably due to the fact that maintaining and manipulating chipmunks for several months under laboratory conditions represents a heavy laboratory workload, in addition, pathogen-free tick colonies are scarce in laboratory settings (Bonnet and Liu, 2012). Indeed, the only experimental study aiming to evaluate the reservoir competence of chipmunks for *Borrelia* sp. investigated the Eastern chipmunk (*Tamias striatus*) (McLean et al., 1993), and the Siberian chipmunk was only tested for *Mycobacterium leprae* reservoir competence (Lew et al., 1974).

In this study, our objective was to evaluate the potential of the Siberian chipmunk to exist as a reservoir host for both *B. burgdorferi* ss and *B. afzelii*. In particular, we wanted to determine if chipmunks infected via tick bites were able to maintain *Borrelia* sp. infection over several months, and then if they were capable of then re-transmitting the bacteria to ticks, under laboratory conditions.

Material and methods

Animals and ethics statement

In total, 13 Siberian chipmunks (7 females and 6 males) aged between 4 and 6 months, and weighing from 71 g to 109 g (mean of 86.3 g) (Table 1), were purchased from a local pet shop in Paris. They were maintained with a 12 h light/dark cycle, in the CRBM (*Centre de Recherche BioMédicale*) based at the Alfort Veterinary school (France) in adapted cages (42 cm × 25 cm × 55 cm) suspended over water during tick feeding periods to collect detached fed ticks. Chipmunks were individually housed and fed *ad libitum* with boiled corn, vegetables, walnuts, and hazelnuts, with free access to water, throughout the experiment. Prior to infection by ticks, chipmunks were acclimatized to laboratory conditions over two months. For all manipulations (ear biopsies, blood sampling, tick placement and recovery), chipmunks were anaesthetized with isoflurane (Abbott, France). The use of volatile anesthetics allowed anesthesia induction without manipulating the animals, because when cages were opened, chipmunks systematically retreated to their nest boxes. Therefore entire nest boxes were placed in the anesthesia induction chamber prefilled with a 3% isoflurane gas mix. After removing anesthetized chipmunks from their nests, anesthesia was maintained via a rat mask delivering a gas mix with between 1 and 1.5% isoflurane. Throughout all procedures, animals were placed on a heating blanket. After sampling (10–15 min), chipmunks were returned to their nest boxes for recovery. This procedure offered two advantages: it reduced the stress of the animals compared to injectable anesthesia, and avoided the risk of bites or contamination for laboratory personnel.

This study was carried out in strict accordance with good animal care practices as recommended by European guidelines. The protocol was approved by the ENVA (*Ecole Nationale Vétérinaire d'Alfort*) Ethics Committee for Animal Experiments (Permit Number: 10-0058).

Bacteria

B. burgdorferi ss (IPT209) was obtained from a female *T. sibiricus* trapped in 2008 from the Sénart forest (France). *B. afzelii* (IPT110) was obtained from a female *I. ricinus* collected in Alsace (France) in 2004. Both strains were isolated and cultured at the Pasteur Institute (Paris, France) as described in (Sinsky and Piesman, 1989) and (Postic et al., 1994).

Ticks

All experiments were performed with *I. ricinus* pathogen-free laboratory colony ticks, reared at 22 °C with 95% relative humidity and with a 12 h light/dark cycle as previously described (Bonnet et al., 2007).

Infecting ticks via mice

Specific-pathogen-free mice C3H/HeN (5 to 7-week-old females) were purchased from Janvier (St. Berthevin, France). Mice were maintained under standard conditions in the Pasteur Institute animal facility. They were intradermally inoculated with 0.1 ml culture material (10^6 spirochetes) for each of the above-mentioned strains. To examine *Borrelia* infection in mice, weekly ear biopsies were performed, starting two weeks after inoculation, until bacterial DNA was detected.

To obtain infected nymphs, larvae fed on infected mice (C_3H/HeN, Janvier) until repletion. For each strain, two series of 50 larvae were fed per mouse, three mice were used for IPT 110 and five for IPT 209. Two non-infected mice were used to obtain control nymphs. Replete ticks were maintained at 20 °C and 95% relative humidity for two months after which they molted into potentially infected nymphs. Approximately 110 engorged *I. ricinus* larvae were obtained for each *Borrelia* strain and 200 from non-infected mice. Several of the resulting nymphs were tested for *Borrelia* sp. infection by nested PCR as described below, with 86% infection for *B. burgdorferi* ss IPT 110, and 30% for *B. afzelii* IPT 209.

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Table 1
Follow-up of the chipmunks used for experimental infection.

Chipmunk ID	Sex	Weight day 0 (g)	Weight day 31 (g)	Weight day 155 (g)	Infection	Death	Euthanasia
1	F	102	91	78	None		Day 178
2	F	71	–	–	*B. burgdorferi* ss	Day 14	
3	F	78	–	–	*B. burgdorferi* ss	Day 14	
4	F	82	76	–	*B. burgdorferi* ss	Day 93	
5	M	72	76	–	*B. burgdorferi* ss		Day 38
6	M	93	82	–	*B. burgdorferi* ss		Day 38
7	M	84	83	–	*B. burgdorferi* ss		Day 38
8	M	109	100	100	*B. burgdorferi* ss		Day 178
9	F	85	96	97	*B. burgdorferi* ss		Day 178
10	F	109	104	55	*B. burgdorferi* ss		Day 178
11	M	74	–	–	*B. afzelii*	Day 14	
12	M	93	90	–	*B. afzelii*		Day 38
13	F	70	84	–	*B. afzelii*		Day 38

Ticks feeding on chipmunks

The experimental design is presented in Fig. 1. Nymphs were placed into a capsule on the back of each chipmunk as previously described for mice (Reis et al., 2011a). Batches of 10 nymphs per chipmunk were used for each feeding experiment. Ticks were checked each day, and engorged nymphs were harvested from the capsule after seven days, or from the cage if chipmunks dislodged their capsules. At day 0, infected nymphs were used to infect the chipmunk. Three chipmunks were infected with nymphs fed on *B. afzelii*-infected mice, and nine chipmunks were infected with nymphs fed on *B. burgdorferi* ss-infected mice. One chipmunk was designated as a control by pairing with non-infected nymphs. Batches of non-infected nymphs were fed on *T. sibiricus* both at 31 and 155 days post-infection, to evaluate *Borrelia* sp. transmission from chipmunks to ticks (xenodiagnosis) 1 month after the infection and 5 month later, a longer duration period than the hibernation of chipmunks (Chapuis et al., 2011). Nymphs, rather than larvae, were used here for safety reasons. Larvae are more difficult to manipulate and to locate than nymphs in case of breakaway. In each case after feeding, engorged nymphs were maintained at 22 °C and 95% relative humidity to promote molting into adults, and *Borrelia* sp. detection was performed in adult ticks.

Borrelia detection in ticks, mice, and chipmunk ear biopsies

Ear tissue was punched out from each chipmunk at day 0, and at day 31 following exposure to infected tick bites. DNA extraction from ticks, mice or chipmunk biopsies was performed using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). Nested PCR was performed on the various samples to amplify the variable spacer region between two repeated genes encoding ribosomal 23S and 5S RNA as previously described (Cotte et al., 2014). The first set of primers used was Ins1 (5′-GAAAAGAGGAAACACCTGTT-3′) and S23R (5′-TCGGTAATCTTGGGATCAAT-3′), which amplified a 360-bp fragment. The second amplification was performed with RRC (5′-CTGCCGAGTTCGCGGGAGAG-3′) and RRB (5′-AAGCTCCTAGGCATTCACCATA-3′) primers resulting in a 250-bp fragment. PCR products from mice and chipmunks were sequenced using RRC and RRB primers.

Borrelia detection in chipmunk necropsies

After euthanasia via intraperitoneal pentobarbital injection (150 mg/kg) under isoflurane anesthesia, or following natural death, necropsies were aseptically performed and various organs were harvested from each chipmunk: blood (heart puncture), heart, spleen, liver, kidney, lung, joint, bladder, skin (where the ticks fed), salivary glands, fat, brain and ear skin. Samples were frozen immediately at −80 °C in 100 μl of WFI (water for injection) until use. Each organ was then dilacerated using scissors, and portions distributed between two to five tubes according to total sample mass. Genomic DNA was isolated from each tube using the NucleoSpin Tissue Kit (Macherey-Nagel) according to supplier recommendations. DNA extracts from the same tissue were pooled before qPCR detection.

Borrelia sl were detected using a qPCR protocol adapted from (Gomez-Díaz et al., 2010) targeting a 198 bp fragment of the *Borrelia flaB* gene with the following primers: FlaB_F 5′-CAATATAACCAAATGCACATGTT-3′ and FlaB_R 5′-ACATTAGCWGMATAAATATTTACAG-3′. Reactions were performed in a 25 μl volume containing 2X iQ SYBR Green Supermix (Biorad), 1 μM of each primer, 2.5 μl of H_2O, and 5 μl of template DNA. qPCR were performed on a CFX96 Touch Real-Time PCR Detection System (Biorad). The amplification protocol was 95 °C for 3 min, 50 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 15 s, and extension at 72 °C for 30 s. Fluorescence was measured at the end of each extension step. After amplification, PCR products were denatured for 20 s at 95 °C and a melt curve was generated by heating from 60 to 90 °C with 0.5 °C/0.5 s increments and a plate read at each temperature. Negative controls were included for each PCR.

To identify *Borrelia* sl species, the variable 23S-5S spacer region of *Borrelia* sl species was amplified using B-5SBor and 23S-Bor primers (Alekseev et al., 2001), and then detected and identified by Reverse Line Blotting with specific probes targeting *B. burgdorferi* sl, *B. burgdorferi* ss and *B. afzelii* (Rijpkema et al., 1995). Controls were included for each PCR: a negative with ultra pure sterile water; and two positive with a 10 ng DNA mix extracted from *B. burgdorferi* ss (Johnson et al., 1984) and *B. afzelii* (Canica et al., 1993).

Detection of anti-Borrelia antibodies in chipmunk sera

After anesthesia, blood samples were collected from the suborbital sinus with capillary pipets at day 0 and 31. IPT 110 and 209 *Borrelia* strains were grown in BSK medium until a titer of 10^8 bacteria/ml was reached. After centrifugation, pellets were washed three times in PBS, then suspended in 100 ml of PBS and sonicated for 10 min at 4 °C. After sonication, protein extracts were centrifuged at 100,000 × *g* for 30 min. Supernatant protein concentrations were determined with a Nanodrop spectrophotometer.

For the first series of experiments, the sera of mice infected with the various *Borrelia* strains were tested for their reactivity to *Borrelia* protein extracts. Micro-titration plates were coated with 10 μg/ml of *Borrelia* proteins, then after washing, plates were saturated with 1% BSA in PBS (PBS/BSA) for 1 h at 37 °C and rinsed. Mice sera diluted in PBS/BSA were incubated for 1 h at 37 °C, then washed. Peroxidase-labelled anti-mouse antibodies (1/3000

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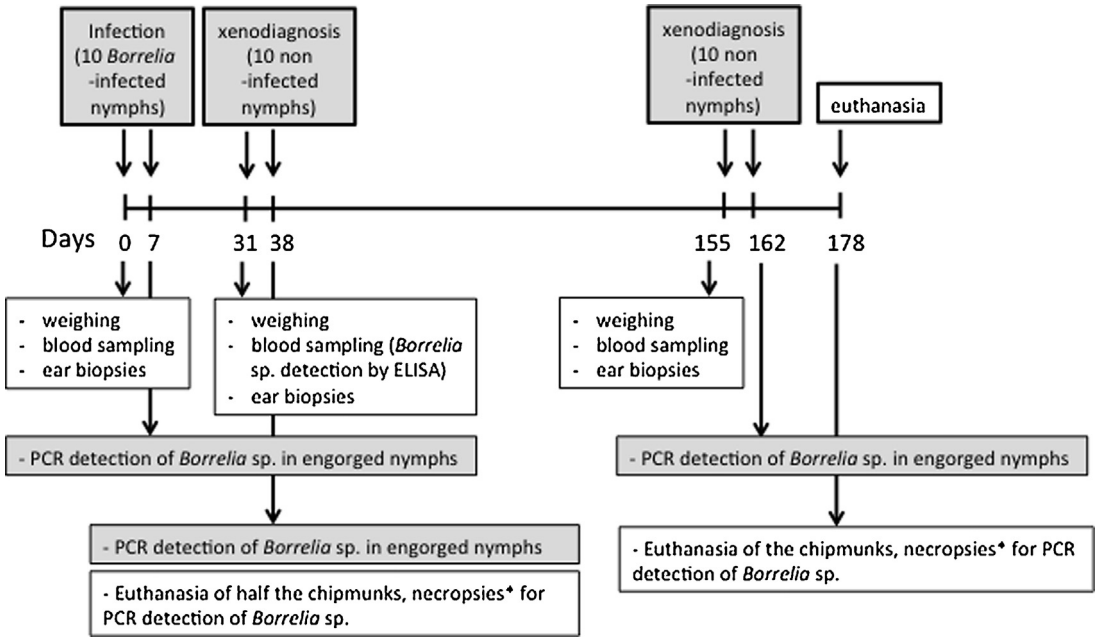

Fig. 1. Experimental framework of chipmunk infection with *Borrelia* sp. via *I. ricinus* nymphs and transmission from chipmunks to *I. ricinus* nymphs. *I. ricinus* nymphs were first infected with *B. afzelii* or *B. burgdorferi* ss on infected mice. Three chipmunks were then infected with *B. afzelii*-infected nymphs, nine with B. *burgdorferi* ss-infected nymphs and one non-infected chipmunk was used as a negative control. Ten ticks were used per chipmunk. Bacterial presence was assessed in all samples (ticks, blood, biopsies, necropsies) by PCR, except at day 31 when antibodies against *Borrelia* sp. were detected by ELISA in serum samples. In the case of accidental death, ELISA serum analysis and necropsy analysis were performed immediately, the day of death. *Necropsies included heart, spleen, liver, kidney, lung, joint, bladder, skin, salivary glands, fat and brain.

in PBS/BSA) were then incubated for 1 h at 37 °C. After a final washing, plates were incubated with 0.2% orthophenylendiamine containing 0.03% H_2O_2 in 0.01 M sodium phosphate buffer, pH 7.3. The reaction was stopped by the addition of 3 M HCl, and absorbance measured at 490 nm.

The dilution of infected mice sera resulting in 50% maximal ELISA response was utilized for competition experiments with chipmunk sera serial dilutions.

Results

Chipmunk follow-up

Three chipmunks died before the end of the protocol, two at day 14, and one at day 93, the remainders were either euthanized at day 38 ($N = 5$) or day 178 ($N = 4$) (Table 1). At their death, chipmunk mean weight was 75 ± 14 g, with no significant difference compared to day 0 (Student's t test for paired samples between days 0 and 31: $t = 0.557$, $df = 9$, P value = 0.30; and between days 0 and 155: $t = 0.27$, $df = 3$, P value = 0.40). Mean necropsy weights were as follows (mean, SD): heart blood samples (272 ± 210 mg), heart (210 ± 39 mg), spleen (78 ± 51 mg), liver (257 ± 97 mg), kidney (141 ± 41 mg), lung (73 ± 25 mg), joint (406 ± 88 mg), bladder (60 ± 24 mg), skin (111 ± 48 mg), salivary glands (88 ± 25 mg), fat (62 ± 45 mg), brain (370 ± 167 mg), and skin ear (44 ± 19 mg). In spite of the unexplained deaths, no chipmunks developed signs of illness throughout the experiment.

Prior to the start of the experiment, no bacteria were detected in the ear tissue collected from chipmunks at day 0. Similarly, no anti-*Borrelia* antibodies were detected by ELISA at day 0. The negative control (chipmunk N°1) remained infection free throughout the experiment (Table 2).

Chipmunk infection by ticks

Eight out of 12 chipmunks showed evidence of *Borrelia* sp. infection after exposure to ticks fed on infected mice, either by ELISA, or PCR from several tissue samples (Table 2). No *Borrelia* sp. or antibodies against the bacteria could be detected in four chipmunks (N° 2, 9, 11, and 13), although for two chipmunks (N° 2 and 9), *B. burgdorferi* ss-positive ticks were collected after the feeding period. For the two *B. afzelii*-infected chipmunks (N° 11 and 13) for which no bacteria or antibodies could be detected, none of the 8/10 nor 9/10 ticks used for infection at day 0 tested positive. In this case, it could be supposed that the absence of chipmunk infection was due to non-infected ticks, as only 30% of ticks carried infection after feeding on infected mice.

ELISA to detect anti-Borrelia antibodies in chipmunks

Anti-mouse antibodies did not cross-react with chipmunk antibodies (data not shown). In order to test the reactivity of chipmunk sera to *B. afzelii* and *B. burgdorferi* ss strains at D31, we designed a competition assay in which we used sera of mice infected by *B. afzelii* or *B. burgdorferi* ss, known to be positive by direct ELISA. One serum was chosen for each strain and was diluted in order to obtain half of the maximal ELISA reaction, and mixed with serial dilutions of chipmunk sera. As shown in Fig. 2, four out of seven sera from chipmunks exposed to ticks fed on *B. burgdorferi* ss-infected mice, and one of two sera from chipmunks exposed to ticks fed on *B. afzelii*-infected mice, reduced the reaction of mice sera to *Borrelia* protein-coated plates. Five chipmunks (N° 4, 7, 8, 10, and 12) out of the ten tested (including the negative control), revealed a positive ELISA immune reaction at day 31 post-inoculation by ticks (Table 2).

PCR detection of Borrelia in chipmunk organs and ticks

Bacteria were detected in the tissues of seven chipmunks: in the heart from number 12 infected with *B. afzelii*; and then in the ear from number 8; salivary glands from number 5; brain from number 6; kidney, lung and brown fat from number 7; heart, spleen and bladder from number 4; and skin from number 3; for chipmunks

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Table 2
PCR and ELISA results from 13 chipmunks experimentally infected by the bite of *I. ricinus* nymphs infected by *B. burgdorferi* ss or *B. afzelii*, and PCR detection of the bacteria in ticks used for xenodiagnoses.

Chipmunk ID.	Infection		Blood (ELISA)			Biopsies/necropsies[a]						Infected ticks/ engorged ticks[b]	
	Borrelia sp.	Infected ticks/engorged ticks[b]	D0	D14	D31	DO	D14	D31	D38	D93	D178	D 38	D162
1	–	0/7	–	ND	–	–	ND	–	–	ND	–[a]	0/0	0/6
2	*Bb*	2/4	–	–	ND	–	–[a]	NA	NA	NA	NA	NA	NA
3	*Bb*	1+/9	–	–	ND	–	Skin +[a]	NA	NA	NA	NA	NA	NA
4	*Bb*	0/1	–	ND	+	–	ND	–	–	Heart, spleen, bladder +[a]	NA	0/2	NA
5	*Bb*	0/2	–	ND	–	–	ND	–	Salivary glands +[a]	NA	NA	0/7	NA
6	*Bb*	0/3	–	ND	–	–	ND	–	Brain +[a]	NA	NA	0/4	NA
7	*Bb*	2/10	–	ND	+	–	ND	–	Kidney, lung, fat +[a]	NA	NA	0/1	NA
8	*Bb*	1/4	–	ND	+	–	ND	–	–	ND	Ear +[a]	3/4	0/8
9	*Bb*	1/5	–	ND	–	–	ND	–	–	ND	–[a]	0/1	0/7
10	*Bb*	1/1	–	ND	+	–	ND	–	–	ND	–[a]	0/4	0/0
11	*Ba*	0/8	–	–	ND	–	–[a]	NA	NA	NA	NA	ND	NA
12	*Ba*	1/2	–	ND	+	–	ND	–	Heart +[a]	NA	NA	0/6	NA
13	*Ba*	0/9	–	ND	–	–	ND	–	–[a]	NA	NA	0/2	NA

Bb: *B. burgdorferi* ss, *Ba: B. afzelii.* ND: not determined. D: days post tick infestation, +: positive result, –: negative result, NA: not appropriate.
Ear biopsies were performed for all chipmunks at days 0 and 31.

[a] Necropsies analyzed by PCR in order to detect *Borrelia* sp. were performed at days 14, 38, 93 or 178, and included heart, spleen, liver, kidney, lung, joint, bladder, skin (where the ticks fed), salivary glands, fat, brain, and ear, and only positive PCR or RLBH PCR results are listed in the table. At day 0 only ear biopsies were performed.

[b] Ten nymphs were used both for to infect chipmunks, and for xenodiagnoses. Values correspond to the number of positive ticks detected by nested PCR for *Borrelia* sp./the number of engorged ticks that were recovered from the chipmunk cages after seven days of feeding.

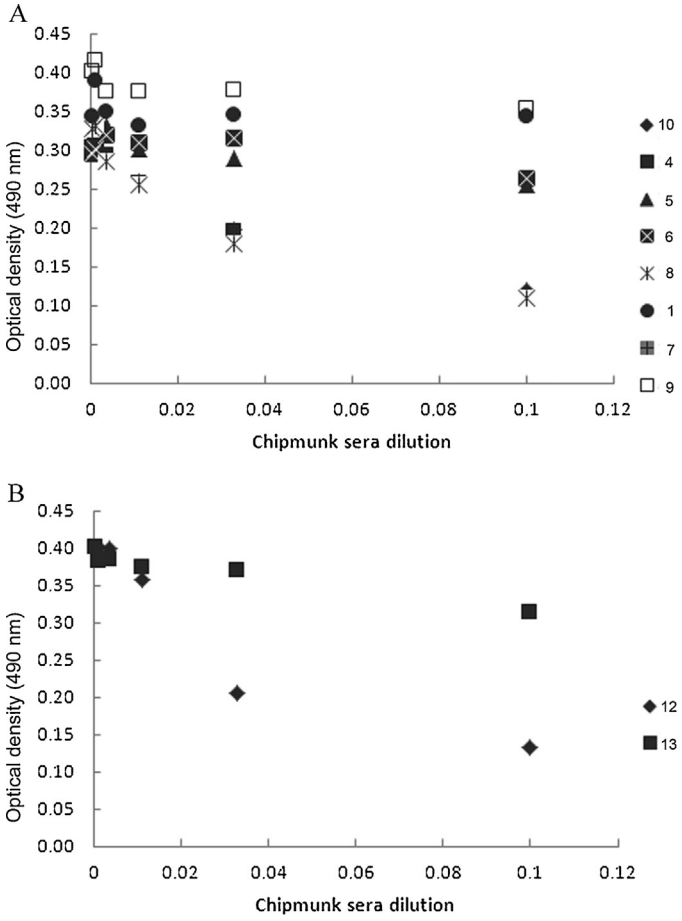

Fig. 2. Detection of anti-*Borrelia* antibodies in chipmunk sera using a competitive ELISA.

infected with *B. burgdorferi* ss (Table 2). For three *B. burgdorferi* ss-infected chipmunks (N° 4, 5, and 6), no positive ticks could be recovered after engorgement at day 0, despite positive bacterial detection in necropsies. However, of the ticks initially used to transmit infection to each of these chipmunks, only 2/10, 3/10, and 1/10 ticks, were able to be tested respectively. In two chipmunks, bacteria were detected in tissues despite a negative ELISA result (N° 5 and 6). This result could be explained by a possible weak immune reaction to inoculated bacteria, thus no competition with mice anti-*Borrelia* antibodies. One chipmunk (N° 10) generated a positive ELISA result at day 31, but no bacteria could be recovered from tissues analyzed at day 178. One chipmunk (N° 3) which died at D14, had a negative ELISA result, but simultaneously had a positive skin sample result. It is likely that fourteen days is too soon to detect any anti-*Borrelia* antibodies.

Borrelia sp. transmission from chipmunks to ticks (xenodiagnosis)

The ability of chipmunks to transmit *Borrelia* to ticks was determined by xenodiagnosis. Only one chipmunk (N°12) that was positive via ELISA at day 31, led to infected xenodiagnosis ticks at day 38, with three out of the four recovered ticks found to be positive. However, after confirming continual chipmunk infection via ear necropsy at day 178, no xenodiagnosis ticks were found to be infected at day 178, even though eight out of ten ticks were able to be tested (Table 2).

None of the other chipmunks, even those positive via ELISA and/or necropsy analysis, were able to infect ticks during xenodiagnosis experiments. However, it must be stressed that the number of ticks collected for these experiments was low because the majority of ticks were eaten by the chipmunks (between one to seven ticks harvested per chipmunk, from the 10 ticks initially deposited, with a mean of 3.4) (Table 2).

Borrelia persistence in chipmunks

At 38 days post-infection, some bacteria could be detected in four of the five euthanized chipmunks (taking into account that

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the fifth, N° 13, was possibly not infected by ticks at day 0, see above). At 178 days post-infection, only one chipmunk (N° 8) of the three euthanized (and which were bitten by infected ticks at day 0) presented a positive ear biopsy suggesting that infection prevalence is greatly reduced at six months as compared to one month following infection.

Despite a positive ear necropsy, chipmunk N° 8, which was able to infect ticks at day 38, was no longer able to infect ticks at day 178, six months after the initial infection. This result suggests that this chipmunk lost its infectious capacity after six months, revealing short infection duration in these animals.

Discussion

Overall disease incidence can be thought of as a function of the host community's composition (Keesing et al., 2006). To understand this relationship and predict Lyme disease risk, we must be able to measure the contributions of different species to overall rates of transmission, including new invasive species like the Siberian chipmunk *Tamias sibiricus barberi*. To achieve such a goal, both epidemiological and experimental studies verifying if vertebrate potential hosts are able to infect tick vectors, are required. Here, we evaluated the reservoir competence of *T. sibiricus* for two Lyme disease agents and showed evidence that *T. sibiricus* is a competent reservoir host for both *B. burgdorferi* ss and *B. afzelii*, but can only maintain *Borrelia* for a short time period.

Only 59 of the 130 ticks used to infect chipmunks could be harvested and tested for *Borrelia* sp. infection. Chipmunks often ate their ticks, and thus we were not able to recover all engorged nymphs from chipmunks, and to test the real number of ticks used to infect them; as evidenced by bacterial-positive chipmunks but without evidence of infection in the ticks used for the initial infection. Indeed, of the total number of nymphs recovered from chipmunk only 20% for *B. burgdorferi* ss and 5% for *B. afzelii* were found to be infected, despite an estimated infection prevalence of 86% and 30% respectively, after feeding on *Borrelia*-infected mice. To avoid this problem, chipmunks could have been infected via subcutaneous inoculation. However, it is well known that tick saliva is implicated in both pathogen transmission and dissemination to vertebrate hosts (Wikel, 1999), and our aim was to mimic biological reality as much as possible.

However, and in total, 8/12 chipmunk (67%), displayed evidence of *Borrelia* infection, one via positive ELISA and the others harboring bacteria in at least one biopsy or necropsy sample. Marsot et al. (2011) found that 35% of chipmunks collected from the Sénart forest (years 2006–2008, $N = 335$) were infected by *Borrelia*. In spite of being exposed to positive ticks at day 0, two chipmunks showed no signs of *B. burgdorferi* ss infection, and one chipmunk found to be ELISA positive showed no sign of biopsy infection, and a negative xenodiagnosis test. The moderate transmission of *Borrelia* to chipmunk via ticks in experimental conditions could be explained by low numbers of infected nymphs used to infect *T. sibiricus* as mentioned above. Indeed, infection was only detected in one or two nymphs used at day 0 per chipmunk. In the wild, *T. sibiricus* is often concurrently infested by several nymphs, over the whole period of the nymph activity with a range in average 1.7–5.2 nymphs per adult, depending on the age, sex and collection year (Pisanu et al., 2010). However, the percentage of *B. burgdorferi* ss-infected nymphs collected from the location where chipmunks were captured to isolate the *B. burgdorferi* ss strain used in this study, was shown to range between 3 and 11% according to the study and the year (Marsot et al., 2011; Reis et al., 2011b). Lastly, we were also able to quantify the number of *Borrelia* in the salivary glands of adult *Ixodes ricinus* infected on *Borrelia*-infected mice by qPCR, and found more than 10^3 spirochetes/gland (data not shown). We can therefore state that our experimental conditions mimicked natural field infectious pressures.

For the same reason, xenodiagnosis results should be interpreted with caution as for chipmunks designated "non-infectious", only 27 out 80 and 15 out of 30 of the original ticks that were introduced onto the animals, could be analyzed, at day 38 and 162, respectively, and all were found to be non-infected. However, chipmunk N° 8 demonstrated an ability to retransmit *B. burgdorferi* ss to three out of four ticks after one month of infection, but not after six months of infection. This suggests that infection duration may be shorter in chipmunks, leading to weaker reservoir competence for *T. sibiricus*. Marsot et al. (2011) found higher *B. burgdorferi* ss infection prevalence in adult chipmunks compared with juveniles. However, this result could either be due to the fact that chipmunks remain infected throughout their lifetime, or rather that juveniles are less frequently exposed to ticks due to their lower activity (Boyer et al., 2010; Marsot et al., 2011). In addition, our results align with infection data gathered from chipmunks recaptured after hibernation, which revealed that only 3/10 infected chipmunks captured before hibernation, remained infected, and that only one was infected with the same *Borrelia* species, revealing new infections for the other two ones (M. Marsot, personal communication). More recently, Marsot et al. (2013) have also demonstrated that the dominant contribution of Siberian chipmunk's to *Borrelia* prevalence was not due to its ability to re-infect ticks, but rather its high larval burden and infection prevalence. In addition, in the native Chinese habitat of Siberian chipmunk (*T. sibiricus senescens*) (Obolenskaya et al., 2009), studies have demonstrated that *Myodes rufocanus* and *Rattus confucianus* probably serve as the major reservoir hosts for *B. burgdorferi* in northeastern forests and Zhejiang province forests respectively (Chu, 2006). Finally, it should be mentioned that to be a suitable bacterial reservoir host, chipmunks have to maintain infection during tick non-activity period that corresponds to their hibernation. Although the duration of our experiment corresponds to this period, our laboratory conditions have not correctly simulated chipmunk hibernation (with no decrease in temperature and no change in dark/light cycle), potentially leading to differences compared to that which is observed to the wild.

Small mammals, including rodents, have been known to act as reservoir hosts for both *B. burgdorferi* ss and *B. afzelii* for several years (Gern and Humair, 2002; Franke et al., 2013). Subsequently, the frequent observations of *B. burgdorferi* ss and *B. afzelii* in the skin of gray and red squirrels (*Sciurus carolinensis*, and *S. vulgaris*), combined with the high prevalence of these two *Borrelia* species in ticks collected from the squirrels, suggested that *B. burgdorferi* ss and *B. afzelii* were transmitted from squirrels to feeding ticks (Craine et al., 1997; Humair and Gern, 1998; Pisanu et al., 2014). Tick xenodiagnosis has been successfully performed for three species including *Sciurus carolinensis* (Craine et al., 1997), *Apodemus* mice (Huegli et al., 2002) as well as *Myodes* voles (Humair et al., 1999). The only experimental study aiming to evaluate the reservoir competence of chipmunks for *Borrelia* sp. under laboratory conditions was performed in 1993 and utilized the Eastern chipmunk (*Tamias striatus*) and a *B. burgdorferi* isolate from the USA (McLean et al., 1993). In this study, chipmunks were infected via subcutaneous inoculation with approximately 10^5 bacteria. *B. burgdorferi* was isolated from the ear of all tested animals ($N = 11$), at one week and two months after inoculation, and from internal organs from five of eight tested chipmunks 133 days after inoculation. Interestingly, three of the chipmunks seem to clear their infection within four months, similar to what we observed for all animals five months after infection. They reported that over 50 laboratory-reared larval *Ixodes scapularis* ticks became infected with bacteria after feeding on two infected chipmunks, four months after the initial inoculation, whereas in our study, only one chipmunk was able to infect

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ticks one month after infection. It is possible that the differences between both studies are linked to the route of infection (injection *vs* ticks) as well as the inoculating bacterial dose. On the other hand, the high prevalence of *Borrelia* sp. infection observed in wild *T. sibiricus* (Marsot et al., 2011), could be due to repeated tick bites producing recurrent infections, rather than high host competence resulting in persistent chipmunk infections.

Conclusion

In conclusion, we demonstrated that *I. ricinus* infected by *B. burgdorferi* ss or *B. afzelii* are able to infect *T. sibiricus* via infectious bites, and that one of the *B. burgdorferi* ss-infected chipmunks was able to re-transmit the bacteria to naïve ticks. However, although we were unable to quantify infectivity with our data (rate of *Borrelia* transmission from chipmunk to ticks), we did indicate impaired *Borrelia* persistence in *T. sibiricus*. Additional experiments are required to confirm this putative xenodiagnoses result, as well as whether *T. sibiricus* are able to maintain *Borrelia* infection for long periods. One of the main difficulties faced during this experiment was that chipmunks dislodged their capsules and ate escaping ticks, whether they were engorged or not. Indeed, repeated experiments with a more effective recovery system for engorged ticks would confirm results obtained.

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