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Identifying the Reservoir Hosts of the Lyme Disease Spirochete *Borrelia burgdorferi* in California: The Role of the Western Gray Squirrel (*Sciurus griseus*)

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Abstract

We investigated the role of the western gray squirrel (*Sciurus griseus*) as a reservoir host of the Lyme disease spirochete *Borrelia burgdorferi*. A survey of 222 western gray squirrels in California showed an overall prevalence of *B. burgdorferi* infection of 30%, although at a county level, prevalence of infection ranged from 0% to 50% by polymerase chain reaction. Laboratory trials with wild-caught western gray squirrels indicated that squirrels were competent reservoir hosts of the Lyme disease bacterium and infected up to 86% of feeding *Ixodes pacificus* larvae. Infections were long-lasting (up to 14 months), which demonstrated that western gray squirrels can maintain *B. burgdorferi* trans-seasonally. Non-native eastern gray squirrels (*Sciurus carolinensis*) and fox squirrels (*Sciurus niger*) were infrequently infected with *B. burgdorferi*.

INTRODUCTION

To predict and prevent human risk of exposure to vector-borne diseases, it is vital to identify the reservoir hosts of the pathogens. Lyme disease is the most commonly reported vector-borne disease in the United States and is caused by the spirochete *Borrelia burgdorferi* sensu stricto, a member of the bacterial complex of *Borrelia burgdorferi* sensu lato.[1] In the northeastern United States, the principal reservoir host of *B. burgdorferi* is the white-footed mouse (*Peromyscus leucopus*), but chipmunks (*Tamias striatus*), short-tailed and masked shrews (*Blarina brevicauda* and *Sorex cinereus*), and eastern gray squirrels (*Sciurus carolinensis*) also serve as infectious hosts.[2–5]

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Similarly, in California, *B. burgdorferi* has been isolated from several host species including western gray squirrels (*Sciurus griseus*), dusky-footed wood rats (*Neotoma fuscipes*), and California kangaroo rats (*Dipodomys californicus*).[6,7] However, prevalence of *B. burgdorferi* infection in wood rats and kangaroo rats typically has been low.[6–9] Conversely, a study of western gray squirrels in oak woodlands in Mendocino County, northwestern California, showed that squirrels are commonly infected with *B. burgdorferi* (80% by polymerase chain reaction [PCR]), and that larval *Ixodes pacificus* ticks readily acquire infections (47% of attached larvae were infected).6

We broaden the geographic survey of Lyme disease in Californian sciurids to sites throughout northern California and perform xenodiagnosis and transmission experiments using *I. pacificus* to determine whether western gray squirrels are competent reservoirs.

MATERIALS AND METHODS

Squirrel capture and sampling

Western gray squirrels were trapped using Tomahawk live traps (7″ × 7″ or 9″ × 9″; Tomahawk Live Trap Co., Tomahawk, WI). In 2006, 15–25 traps were placed in areas of likely squirrel habitat, i.e., in oak woodlands beneath trees with squirrel nests, and/or areas where squirrels were seen foraging. In 2007, traps were laid in transects of 5 traps, spaced 30 meters apart, in areas where squirrels had been seen. The number of transects varied depending on forest-patch size. Traps were baited with whole pecan nuts and peanut butter, wired open for a minimum of 2 weeks prior to trapping, and re-baited on 3–4 occasions during this period. During trapping sessions, traps were opened prior to sunset and left open for the following 2.5 days, and checked every 4–5 hours during daylight starting an hour after dawn. Traps were closed at night during cold weather and in the afternoons during hot weather.

Upon capture, squirrels were restrained using a canvas handling bag.[10] At first capture during each trapping session, animals were briefly anaesthetized with isoflurane (Isothesia; Abbott Laboratories, North Chicago, IL), and tissue samples were taken using a 2-mm ear punch to obtain ear punch biopsy (EPB) specimens of the pinna after the ears had been surface-sterilized sequentially with 10% povidone-iodine (Betadine solution; The Purdue Frederick Company, Norwalk, CT) and 70% ethanol. We marked squirrels with ear tags in 2006 and used passive integrated transponder tags (Biomark, Boise, ID) inserted subcutaneously in 2007 because ear tags were frequently removed by squirrels. Sex and body mass were recorded, and all ticks found were removed and stored in 95% ethanol. Squirrels were released at their sites of capture. Squirrels recaptured during the same trapping session were identified and released.

Study sites

We trapped in woodland sites that were composed of oaks (black oak, *Quercus kelloggii*, or interior live oak *Quercus wislizenii*), Pacific madrone (*Arbutus menziesii*), and occasionally pines (*Pinus* spp.). The climate in northern California is predominantly Mediterranean with hot, dry summers and cool, moist winters.

We trapped at numerous sites but only captured multiple squirrels at China Camp State Park in Marin County (38.01°N, 122.46°W), Annadel State Park in Sonoma County (38.43°N, 122.62°W), and the Hopland Research and Extension Center (herein Hopland) in Mendocino County (39.00°, 124.15°W). We trapped at China Camp in April 2007, at Annadel State Park in June 2006 and March 2007, and repeatedly at Hopland from March 2006 until February 2008 as part of a long-term study. We report the results of PCR tests for only the first sampling of any individual squirrel.

Although we observed squirrels at additional sites, these animals were difficult to capture (0 squirrels captured in more than 400 trap nights), in part because wood rats and/or ground squirrels (*Spermophilus beecheyi*) removed bait from traps. Our success rate in capturing live squirrels appeared to be associated with longer pre-baits (two weeks), the presence of black oaks, and a lower density of competing rodents.

Additional EPB samples were collected from dead squirrels found on roads and from animals obtained by shooting. The EPB samples were obtained in the same way as described above. Ear samples, collected by Californian county vector-control agencies, were also provided by the California Department of Public Health as part of an investigation into West Nile virus.[11]

DNA extraction and pathogen detection in skin biopsy specimens and ticks

The EPB samples and tick specimens collected from infested animals were tested individually for *B. burgdorferi.* DNA was extracted from ticks and EPB samples using published methods.[6,12] DNA was extracted from each individual EPB sample using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) spin-column protocol for animal tissue with a few modifications. Squirrel EPB samples that had been stored in 95% ethanol were placed in a 1.5-mL tube and allowed to air-dry before adding ATL buffer. *Borrelia burgdorferi* infection was assessed with a nested PCR format that specifically targets the *rrf* (5S)–*rrl* (23S) ribosomal RNA intergenic spacer region. Cultured *B. burgdorferi* s.s. strain CA4 and UV-treated nuclease-free sterile water (Growcells, Irvine, CA) were used as positive and negative controls, respectively, during each PCR run. Using cultured, serially diluted strain CA4, we determined that the sensitivity of the nested PCR protocol was approximately 5 spirochetes/mL. To control for possible PCR inhibition, dilutions from a subset of samples that gave a negative result and their dilutions (10^{-1} to 10^{-10}) were tested by nested PCR. We found no evidence of PCR inhibition. Furthermore, each sample was tested twice to monitor the consistency of results.

Amplicons were purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and sequenced with internal PCR primers by the University of California DNA Sequencing Facility using a 96-capillary 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA). Forward and reverse sequences were assembled and manually edited using Sequencher version 4.6 (Gene Codes Corp., Ann Arbor, MI). All sequenced samples were aligned with representative *rrf-rrl* sequences available in GenBank for *B. burgdorferi* sensu stricto, *B. californiensis*, *Borrelia* genomospecies 1 and 2, *B. bissettii*, and unclassified borrelial strains found in North America using Clustal X multiple sequence alignment program (version 1.83.1).[13] The final alignment including representative genospecies sequences, along with tick- and squirrel-derived DNA sequences, was edited manually using Mesquite version 1.12[14] and used to generate a distance matrix and neighbor-joining phylogenetics tree using PAUP* 4.0 based on uncorrected *p* distance.[15]

Xenodiagnostic and transmission experiments

Wild-caught squirrels were brought into the laboratory for *B. burgdorferi* transmission experiments using immature *I. pacificus*. All squirrels were captured at Hopland and were transferred to the University of California, Berkeley, Office of Laboratory Animal Care. Squirrels were housed in galvanized steel cages, fitted with nest-boxes with aspen-chip bedding, and maintained on a 12:12 light cycle at 70–74°F. They were provided *ad libitum* with fresh tap water and food, consisting primarily of laboratory rodent chow and seed-based hamster diet supplemented with apple, Timothy hay-cubes, and nuts.

Questing adult *I. pacificus* ticks were collected from Marin County, California. Females were allowed to feed and mate on laboratory rabbits and the resultant F_1 larvae were used in

xenodiagnostic and transmission tests. Fifty larvae from each female tick were pooled and tested by PCR to ensure all broods were uninfected by *B. burgdorferi*.

For tick infestation, squirrels were briefly anaesthetized with isoflurane followed by an intraperitoneal inoculation of a mixture of 0.15 mg/kg ketamine HCl (Ketaset; Fort Dodge Animal Health, Fort Dodge, IA) and 0.75 mg/kg medetomidine HCl (Domitor; Pfizer, Exton, PA). Supportive treatment, including 10 mL of normal saline solution and 0.5 mL of 50% dextrose solution (delivered subcutaneously) and an external heat source were also provided. Approximately 200 *I. pacificus* larvae were brushed onto each animal. After 1 hour, 3.75 mg/kg of atipamezole HCl (Antisedan; Pfizer) was administered to antagonize the medetomidine, and squirrels were returned to cages for recovery. Cages were suspended above water trays that were checked daily for replete ticks for up to 11 days. To determine the duration of infection, larvae were placed on squirrels at approximately two-month intervals. Molted nymphs were tested by PCR to determine the prevalence of *B. burgdorferi* infection in immature ticks.

We tested whether infected ticks were able to successfully infect a naive squirrel. Because of a combination of low numbers of uninfected animals in the wild and the difficulties associated with capturing and housing squirrels, we were able to conduct transmission experiments on only one squirrel. All EPB samples collected on three consecutive occasions over a six-month period from this squirrel were negative for *B. burgdorferi* and it also failed to infect any larvae during two xenodiagnoses (n = 82 larvae tested). To determine whether ticks are capable of infecting squirrels with *B. burgdorferi*, we placed on the uninfected individual nymphs that had been exposed to an infected squirrel in the laboratory as larvae. We used five nymphs from a cohort that had demonstrated an infection prevalence of 50% during xenodiagnoses, and after feeding confirmed by PCR that at least one nymph that fed on the uninfected squirrel contained *B. burgdorferi*. We collected EPB samples and exposed the squirrel to naive tick larvae one, two, and four months after exposure to the potentially infected nymphs.

Animal care and use

Animal handling and capture procedures were reviewed and approved by the Animal Care and Use Committee at the University of California at Berkeley, and complied with guidelines from the California Department of Fish and Game, and the National Institutes of Health.

Human Lyme disease incidence

We examined the relationship between *B. burgdorferi* infection prevalence in western gray squirrels and the Lyme disease incidence in humans using linear regression. Lyme disease incidence (reported cases per 100,000 person-years per county) was calculated for the 10-year period 1997–2006.[16] Western gray squirrel infection prevalence data were calculated for all California counties from which we tested a minimum of three squirrel samples.

RESULTS

Squirrel infection prevalence

We tested 222 western gray squirrels, 30% of which were positive for *B. burgdorferi* (Table 1). Prevalence of infection varied geographically, with a higher prevalence of infection in the northwestern counties of the state, predominantly Humboldt and Mendocino counties (Table 1 and Figure 1). On the basis of more extensive data from the Hopland area, prevalence of infection was similar between male (18 of 35 infected) and female squirrels (12 of 24; χ^2 = 0.02, degrees of freedom = 1, P = 0.88).

We sequenced *B. burgdorferi*-positive amplicons from a subset of western gray squirrels collected in Humboldt (n = 3), Lake (n = 1), Mendocino (n = 4), Placer (n = 1), Sonoma (n = 3), and Trinity (n = 4) counties. All amplicons were identified as *B. burgdorferi* sensu stricto by the neighbor-joining method with uncorrected (*p*) distances. No co-infections with other *Borrelia* genospecies were detected. The genetic distance between all sequenced samples derived from squirrels ranged from 0.000 to 0.018.

Although we tested EPB samples from other *Sciurus* species in California, few animals were positive for *B. burgdorferi*. One eastern gray squirrel (*S. carolinensis*) was infected in Santa Cruz County (n = 14), as was one fox squirrel (*Sciurus niger*) (n = 64) from Alameda County. We also found evidence of *B. burgdorferi* infection in 1 of 6 possums (*Didelphis virginiana*), but not in striped skunks (*Mephitis mephitis*) (n = 5).

There was a strong relationship between county-based western gray squirrel infection prevalence and the log-transformed incidence rates of humans cases (per 100,000 person years), ($F_{1,13} = 16.65$, $R^2 = 0.56$, $P = 0.0012$) (Figure 2).

Xenodiagnostic and transmission experiments

Exposure to *I. pacificus* larvae in the laboratory resulted in high numbers of successfully fed larvae, with a mean (SD) of 59.4 (64.00) larvae successfully feeding on individual squirrels, and a maximum of 241 larvae dropping off one squirrel. Most larvae fed for four or five days. When infectious, squirrels infected 10–75% of *I. pacificus* larvae, although the proportion of larvae infected appeared to decrease over time (Table 2). At Hopland, questing *I. pacificus* nymphs tend to peak in abundance during May and are virtually absent by late July.[17] In the laboratory, one squirrel collected from the wild in May was capable of infecting ticks the following February, nine months later. A second squirrel infected 13% of ticks approximately 14 months after its last exposure to infected nymphs in the wild (assuming exposure the previous June).

Infection status as measured by PCR testing of EPB samples varied across time in wild-caught squirrels kept in the laboratory (Table 2). Xenodiagnoses showed that squirrels were capable of infecting *I. pacificus* larvae even when EPB samples were PCR negative (Table 2).

The one naive squirrel had been exposed experimentally to one *B. burgdorferi* PCR-positive nymph. The EPB samples collected one, two, and four months after exposure were PCR positive, PCR negative, and PCR positive, respectively. Sequencing of the *B. burgdorferi* amplicon from the first month's positive EPB sample showed that it was identical to the sequence of the amplicon harvested from the positive nymph that had fed on the squirrel. Xenodiagnoses showed that this squirrel infected 0% (0 of 2), 86% (25 of 29), and 47% (14 of 30) nymphs one, two, and four months post-exposure, respectively.

DISCUSSION

Our discovery that *B. burgdorferi* is widespread in populations of western gray squirrels throughout northern California strengthens recent evidence that the western gray squirrel is likely an important reservoir host in woodlands of California.[6] Furthermore, the laboratory transmission trials demonstrated that the spirochete can be maintained in a squirrel-tick-squirrel transmission cycle. Because squirrels are capable of being infectious to ticks for over a year, they can maintain *B. burgdorferi* inter-annually. Consequently, western gray squirrels play an important role in the ecology of *B. burgdorferi* in the far-western United States.

The average prevalence of *B. burgdorferi* infection in squirrels was 30%, but ranged from 0% to nearly 50% in different counties, and was most common in the northwestern counties of

Humboldt and Mendocino.[18,19] In northern California, western gray squirrels are infected solely with *B. burgdorferi* sensu stricto, the only genospecies known to cause Lyme disease in the United States.

We also observed an apparent statewide association between squirrel infection prevalence and Lyme disease incidence, which suggests that squirrels are an important reservoir host responsible for maintaining this zoonotic disease regionally. Such a conclusion must be tempered by many potentially confounding factors. Human incidence was calculated from multiple years of data (1997–2006), whereas our squirrel research comprises two years of data. Furthermore, 59% of the samples tested were from road-killed animals for which data on habitat type, distance from urban centers, and tick abundance were unavailable. Both squirrel and human infection prevalence may be a function of the risk of exposure to ticks, and previous research has demonstrated that the likelihood of human cases is related to habitat type and tick abundance.[20]

The abundance and infection prevalence of both tick vectors and reservoir hosts are co-dependent, and therefore our observed relationship between human and squirrel infection prevalence is intriguing. In that regard, because some PCR-negative squirrels were capable of infecting ticks with *B. burgdorferi*, our reported prevalences of infection in squirrels must be considered conservative underestimates at best. We conclude that spirochete infection may be quite localized within an animal, and therefore it would be prudent to take multiple EPB samples from squirrels when conducting surveillance for *Borrelia* spp. It is unlikely that differences in PCR results are caused by our detection methods because we found no evidence of PCR inhibition. Previous work on deer mice also demonstrated that ear tissues are not the most reliable biopsy samples for diagnosing the presence of *B. burgdorferi*.[21] Similarly, *B. burgdorferi*-positive larvae have been observed feeding on PCR-negative squirrels.[6]

Numerous investigations have demonstrated that other squirrel species in Europe and the United States can harbor high tick loads and are competent reservoir hosts that have the ability to infect both tick larvae and nymphs. Eurasian red squirrels (*Sciurus vulgaris*) in Switzerland infect 15–19% of immature ticks, and are the source of a large proportion of infected questing ticks.[22,23] In the United Kingdom, up to 26% of non-native eastern gray squirrels are infected with *B. burgdorferi* s.l. and infect up to 75% of feeding ticks.[24] Eastern gray squirrels in the northeastern United States show a prevalence of infection as high as 100%, infect 15–32% of their feeding larvae, and have mean larval loads that range from 5 to 142 per squirrel.[2,3,5] Compared with white-footed mice, shrews, and chipmunks, eastern gray squirrels infect lower proportions of larvae with *B. burgdorferi*. Consequently, eastern gray squirrels have recently been considered to act as dilution hosts, that is, they reduce overall disease risk to humans because despite harboring high tick loads they are less competent reservoir hosts.[2,5,25] However, because they harbor such high numbers of larvae, eastern gray squirrels nonetheless contribute to the local abundance of infected ticks in an area and are therefore probably more important in the ecology and epidemiology of Lyme disease in the northeastern United States than previously appreciated.

In New England, a suite of reservoir hosts are involved in maintaining *B. burgdorferi*.[2,5] In northern California, it appears that western gray squirrels are a primary reservoir in dense oak woodlands. Other small mammals, such as dusky-footed wood rats and *Peromyscus* mice, have lower tick loads and low to moderate prevalence of *B. burgdorferi* s.l. infection.[8,9] Further research into the relative importance of different host species in *B. burgdorferi* dynamics will determine whether additional mammals or birds are important reservoirs, as well as the importance of habitat in determining regional disease prevalence. Nonetheless, western gray squirrels may act as good sentinel species to determine whether *B. burgdorferi* is present in certain areas, although they can be difficult to capture alive. With regard to habitat, western

gray squirrels are normally associated with oak-woodlands.[26] In contrast, non-native eastern gray and fox squirrels mainly occupy suburban areas, where they are infrequently infected with *B. burgdorferi* because of a lack of exposure to tick vectors. Therefore, human risk of exposure to *B. burgdorferi* is restricted largely to activities that take place in oak woodlands,[27] and as such, personal-protective measures or squirrel-targeted tick-control initiatives are most apt to reduce Lyme disease risk in California.[6]

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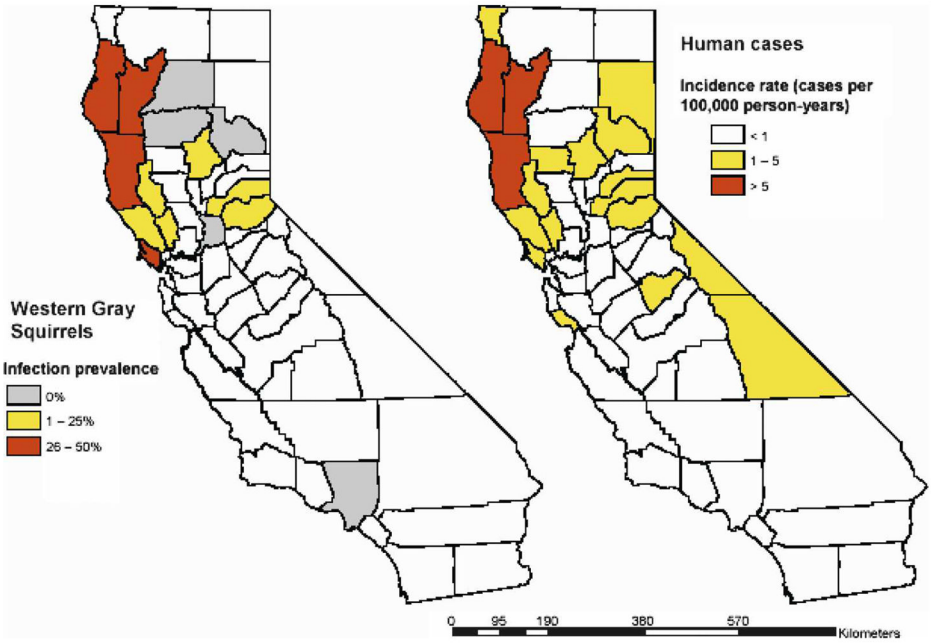

Figure 1.

Left, classification of Californian counties by *Borrelia burgdorferi* infection prevalence in western gray squirrels (*Sciurus griseus*). Gray = 0%; yellow = 1–25%; red = 26–50%; white = sample sizes < 3. Right, classification of Californian counties by Lyme disease incidence per 100,000 person-years, based on data for 1997–2006 from the California Department of Public Health. White = < 1; yellow = 1–5; red = > 5. This figure appears in color at www.ajtmh.org.

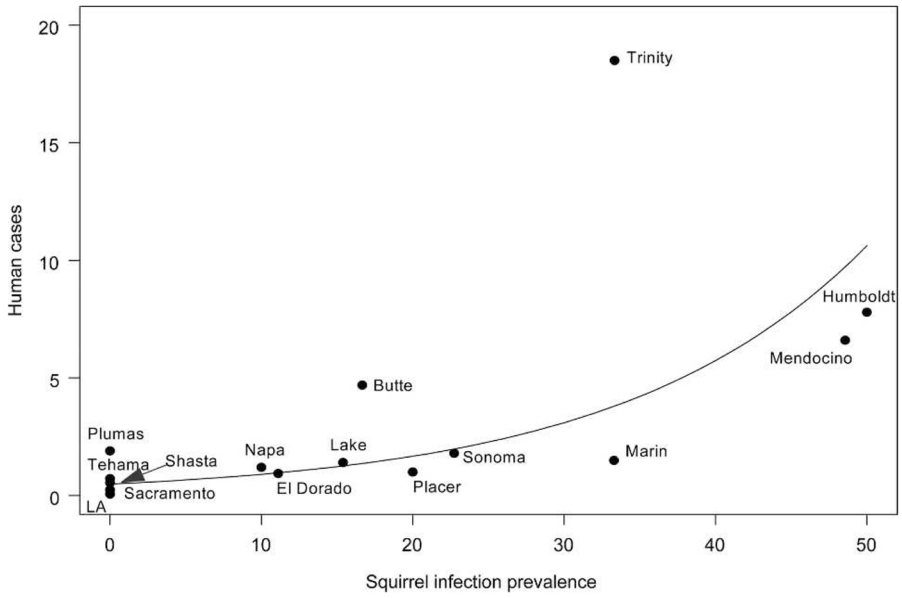

Figure 2.

Relationship between western gray squirrel (*Sciurus griseus*) infection prevalence and human Lyme disease incidence (cases per 100,000 person-years) in California. Sample sizes varied (from 3 to 71) and can be found in Table 1.

Table 1

Prevalence of *Borrelia burgdorferi* infection in western gray squirrels (*Sciurus griseus*) in California counties

County	Live captures* (no. infected)	Road-kill† samples (no. infected)	Total (no. infected)	Prevalence (% infected)
Butte	1 (0)	5 (1)	6 (1)	16.7
El Dorado		9 (1)	9 (1)	11.1
Humboldt		28 (14)	28 (14)	50.0
Lake		13 (2)	13 (2)	15.4
Los Angeles		4 (0)	4 (0)	0
Marin	3 (1)	3 (1)	6 (2)	33.3
Mendocino	65 (32)	7 (3)	72 (35)	48.6
Napa		20 (2)	20 (2)	10.0
Placer		5 (1)	5 (1)	20.0
Plumas		3 (0)	3 (0)	0
Sacramento		5 (0)	5 (0)	0
Shasta		4 (0)	4 (0)	0
Sonoma	21 (5)	1 (0)	22 (5)	22.7
Tehama		4 (0)	4 (0)	0
Trinity		9 (3)	9 (3)	33.3
Total	90 (38)	132‡ (28)	222‡ (66)	29.7

* All live captures were located in rural woodland habitats.

† Includes samples taken from both suburban and rural areas; therefore, they may not be directly comparable with live captures.

‡ Including single road–killed squirrels from Colusa, Fresno, San Mateo, San Luis Obispo, Santa Clara, and Yuba counties. Two squirrels each were collected in Siskiyou, Tuolumne, and Yolo counties. All of these samples were negative for *B. burgdorferi* (n = 12).

Table 2

Temporal variation in *Borrelia burgdorferi* infection status of captive, wild-caught squirrels (*Sciurus griseus*) shown by xenodiagnoses (PCR-positive nymphs) and concurrent ear-punch biopsy specimens*

Squirrel ID	Sep 2006	Dec 2006	Feb 2007	Apr 2007	Aug 2007	Dec 2007
1	**50% (5/10)**†	**10% (3/30)**	17% (3/18)	0% (0/7)	no ticks	
2	30% (9/30)	12% (4/34)	44% (4/9)	**0% (0/4)**	0% (0/12)	
3		**23% (7/31)**	**PCR only**‡	**PCR only**	**13% (4/30)**	
4					**PCR only**	**22% (4/18)**
5					**75% (3/4)**	

*PCR = polymerase chain reaction. PCR-positive infection is shown in **bold**.

†Prevalence of infection in molted nymphs (no. positive/no. tested).

‡No ticks fed successfully.