LYME DISEASE ECOLOGY IN SAN LUIS OBISPO COUNTY: THE ROLE OF THE WESTERN GRAY SQUIRREL

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ABSTRACT

Lyme disease ecology in San Luis Obispo County: The role of the western gray squirrel

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Despite the fact that eight cases of Lyme disease were diagnosed in San Luis Obispo County between 2005-2013, the identity of wildlife hosts serving as sources for tick infection in this region remained unidentified. The primary cause of Lyme disease in the U.S. is the spirochetal bacterium *Borrelia burgdorferi* sensu stricto, and this agent had not been previously isolated from the region. *Borrelia bissettii,* a related species that has not been implicated as a common causative agent of Lyme disease, was isolated in small rodents inhabiting coastal scrub and chaparral habitats in a previous San Luis Obispo County study. However, *B. burgdorferi* was not detected. In northwestern California, *B. burgdorferi* has been primarily associated with high populations of the tick vector *Ixodes pacificus* in dense woodlands or hardwood-conifer habitats, particularly in the western gray squirrel reservoir host, *Sciurus griseus*. My study investigated the role of *S. griseus* and other associated rodents as potential reservoirs for *B. burgdorferi* in central coastal California woodland habitats. Rodents were live-trapped at four sites in San Luis Obispo County in oak and mixed woodland. Rodent ear samples were tested for *B. burgdorferi* genospecies by bacterial culture and PCR. Ticks were collected from captured rodents and surrounding environments and tested by PCR for the presence of *Borrelia*. Of 119 captured rodents, seven were positive for *Borrelia* infection (5.9%) and of these, six were positive for *B. burgdorferi* (5.0%). There were multiple infected rodent species that included two western gray squirrels, three deer mice (*Peromyscus maniculatus),* and one brush mouse (*P. boylii*). *Borrelia* spp. were not detected by PCR from the 81 ticks recovered from the environment and rodents. Here, for the first time, we verify the presence of *B. burgdorferi* sensu stricto in San Luis Obispo county rodents. However, in contrast to previous Northern California studies, the western gray squirrel may not be the primary reservoir host for *B. burgdorferi* in this region. Multiple rodent species in oak woodlands may be involved in spirochete maintenance in San Luis Obispo County.

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Introduction

Lyme Disease History

Lyme disease is the most prevalent vector-borne disease in the U.S. and the most common tick-borne disease in North America (Stanek et al. 2012). There are approximately 30,000 new human cases diagnosed every year throughout the U.S. (**Figure 1**), but a recent report from the CDC estimates that the actual number of cases is approximately 300,000 per year (Kuehn 2013). Lyme disease can generate a variety of human clinical symptoms, including skin lesions, nerve inflammation, and recurrent arthritis (Stanek et al. 2012). While no fatalities have been attributed to Lyme disease, severe symptoms can be debilitating and reduce overall quality of life. Lyme disease affects companion animals such as dogs, with symptoms including intermittent lameness, migratory arthritis, neurological symptoms, and, rarely, fatal kidney failure (Bhide et al. 2004). Horses have also been diagnosed with Lyme disease, characterized by malaise, fever, swollen joints, and, rarely, cardiac issues (Gray et al. 2002). Lyme disease is an important public health concern that has become increasingly prevalent over the past few decades.

Lyme disease has probably been present in the U.S. for centuries, but the etiologic agent was not discovered until the late 1900's. At this time in Europe, bites from the castor bean tick (*Ixodes ricinus)* had been associated with erythema migrans, a "bullseye" rash at the bite site (Hellerstrom 1950). In 1975, a cluster of unusual childhood arthritis cases was investigated in Lyme, Connecticut (Steere et al. 1977). The Lyme investigation revealed that these arthritis cases had been associated with erythema migrans prior to arthritis onset, and the subsequent search for the vector of this disease implicated *Ixodes dammini* (subsequently renamed *Ixodes scapularis,* the eastern black-legged tick) (Steere et al. 1978; James & Oliver 1990). The infectious agent was identified as a spirochete bacterium in 1982 when it was isolated from *I. scapularis* and *I. ricinus* in the U.S. and Europe, respectively, and from patients in both regions. The causative agent was

named *Borrelia burgdorferi* after the discovering researcher (Burgdorfer et al. 1982). A cause of Lyme disease had been discovered, but further investigation revealed multiple Lyme disease-causing *Borrelia* species rather than a single species (Baranton et al. 1992).

Analysis of European and U.S. *B. burgdorferi* isolates revealed differences in clinical symptoms and antigenic characterization based on country of origin (Stanek et al. 1985). To better classify isolates, a serotyping system was developed using monoclonal antibodies to the outer surface protein (osp), ospA. Further characterization was performed using restriction endonuclease analysis, DNA-hybridization assays using whole *B. burgdorferi* DNA, and characterization of plasmid DNA (Stanek & Reiter 2011). These analyses delineated three distinct species: *B. burgdorferi* sensu stricto (hereafter referred to as *B. burgdorferi*), *B. garinii*, and group VS461 (subsequently classified as *B. afzelii*) (Baranton et al. 1992). *Borrelia burgdorferi* was isolated from the U.S., and *B. garinii* and *B. afzelii* were isolated from Europe. All three of these species were associated with clinical symptoms of Lyme borreliosis. Characterization of European and U.S. *B. burgdorferi* isolates differentiated three distinct *Borrelia* species that formed the initial Lyme *Borrelia* complex.

During the 1990's, the number of species in the Lyme *Borrelia* complex increased to ten. *Borrelia japonica* was discovered in Japan when it was isolated from *I. ovatus* ticks (Nakao et al. 1994). Using a characterization scheme based on restriction polymorphism (RFLP) analysis, three other species were differentiated: *B. andersonii* from the U.S. and *B. tanukii* and *B. turdi* from Japan (Marconi et al. 1995; Fukunaga et al. 1996). Two new species were characterized from *I. ricinus* ticks in Europe: *B. valaisiana* from Switzerland and *B. lusitaniae* from Portugal (Wang et al. 1997; Le Fleche et al. 1997). Lastly, *B. bissettii* was differentiated from *B. burgdorferi* in the U.S. using RFLP analysis and DNA sequencing of the 5S-23S region (Postic et al. 1998). This brought the total number of species in the *B. burgdorferi* sensu lato (s.l.) complex to ten. Of these species, only *B. burgdorferi*, *B. garinii*, and *B. afzelii* were implicated as routine human pathogens.

The Lyme *Borrelia* complex expanded further using new classification techniques and by testing previously unsurveyed ticks and rodents. *Borrelia sinica* was isolated from white-bellied rats and from *I. ovatus* ticks in Southern China (Masuzawa et al. 2001). Isolates associated with garden dormice were classified as the novel species *B. spielmanii* and were also recovered from patients in central Europe, implicating human pathogenicity in this species (Richter et al. 2004). Multilocus sequence analysis (MLSA) examines multiple gene loci simultaneously and allowed incorporation of more genetic information into species identification and classification. Seven new species were discovered using this technique. *Borrelia yangtze* was recovered from rodents and ticks in southwestern China (Chu et al. 2008), and *B. californiensis, B. americana, B. carolinensis,* and *B. kurtenbachii* were isolated from rodents and ticks in the U.S. (Margos et al. 2010; Postic et al. 2007; Rudenko et al. 2011; Rudenko et al. 2009). Some isolates associated with rodents that were originally classified as *B. garinii* in Europe were reclassified as the novel species B. *bavariensis* after MLSA analysis, and a novel isolate from *I. ricinus* in Finland was named *B. finlandensis* (Margos et al. 2009; Casjens et al. 2011). The latest novel species discovered was *B. chilensis* recovered from *I. stilesi* ticks in Chile, the first isolate found in South America (Ivanova et al. 2014). There are currently twenty species in the *B. burgdorferi* s. l. complex (**Figure 3**), representing a wide global distribution throughout a diverse group of tick vectors and vertebrate hosts.

Ecology of Lyme Disease

Lyme disease is a vector-borne zoonosis. A zoonosis is a disease caused by an infectious agent transmitted directly or indirectly (i.e, via an arthropod vector) from vertebrate reservoir hosts to humans (Gray et al. 2002). Lyme disease spirochete transmission occurs via a complex cycle that usually involves multiple hosts and vectors. For a tick to have vectorial competence, it must meet the following criteria: 1) feed on potentially infected hosts, 2) acquire the pathogen during a blood meal, 3) maintain infection through one or more life stages (transstadial passage),

and 4) re-transmit the pathogen to another host (Gray et al. 2002). The vectors of *B. burgdorferi* s.l. are ticks of the *Ixodes* genus, and the specific species responsible for *Borrelia* transmission vary based on geographic location and habitat.

Host species have variable impacts on *Borrelia* transmission depending on tick burden, tick life stages supported, and reservoir competence. A diverse group of avian and mammalian species serve as reservoir hosts for Lyme disease spirochetes. Laboratory experiments have been used to measure reservoir competence by experimentally exposing naïve ticks to infected hosts and measuring infection rate after successful feeding (Donahue et al. 1987). For a vertebrate to serve as a reservoir host in the Lyme disease transmission cycle, it must: 1) serve as a host for vector ticks, 2) acquire an infectious dose of the bacterium when fed upon by an infected tick, 3) support pathogen amplification and persistence, and 4) efficiently infect naïve ticks (Richter et al. 2000). Susceptible hosts, on the other hand, are hosts that exhibit disease symptoms and do not maintain long-term infections (Gray et al. 2002). Dogs, humans, and horses are examples of susceptible hosts for Lyme disease borreliae; these hosts are not considered important Lyme disease reservoirs because they do not commonly generate *Borrelia*-infected ticks (Gray et al. 2002; Bhide et al. 2004).

There are three major vectors for Lyme disease spirochetes: *I. scapularis, I. pacificus,* and *I. ricinus.* All three tick species are in the Ixodidae family ("hard ticks"), which includes ~850 described species (Guglielmone et al. 2010). The life cycle of an *Ixodes* tick includes four life stages: egg, larva, nymph, and adult (**Figure 4)**. As obligate parasites, hard ticks take a single bloodmeal from a vertebrate host during each life stage to obtain energy for molting, host-seeking, mating, and oviposition. Larvae and nymphs typically feed for three to six days, whereas adults can feed for as long as two weeks. Digestion of the bloodmeal starts within hours after drop-off from the host, and pathogens acquired from the host can cross the gut wall to infect other cells within the tick (Estrada-Peña & De La Fuente 2014). After molting to the next life stage, the

tick actively begins seeking a new host, known as "questing." When an infected tick feeds on a new host, pathogen transfer depends on attachment time and competence of the host for infection (Gray et al. 2002). Transovarial transmission (transmission from the female to her offspring) has not been demonstrated for *B. burgdorferi* s.l. with the primary vectors in the Eastern U.S. (*I. scapularis),* Western U.S. (*I. pacificus)* and Europe *(I. ricinus)* (Mannelli et al. 2012; Schoeler & Lane 1993; Richter et al. 2012). Consequently, emerging larvae are not expected to be infected with *B. burgdorferi* s.l. Only nymphs and adults serve as potential spirochete vectors to hosts, having acquired the pathogen during a previous life stage from feeding on an infected reservoir host. Maintenance of *B. burgdorferi* s.l. infection within an ecological community thus requires a sufficient density of both vector-competent ticks and reservoir hosts.

Disease ecology of *B. burgdorferi* shows significant variation based on geographic location. *Borrelia burgdorferi* is present in Europe and Eastern and Western U.S. For *I. ricinus,* the primary *B. burgdorferi* s.l. vector in Europe, 237 animals have been identified as hosts, including birds, rodents, and large ungulates such as deer (Gern 2008). In the Eastern U.S., the primary vector is *I. scapularis*, and reservoir hosts include 71 species of birds and a wide range of small to medium sized mammals (Brinkerhoff et al. 2011; Barbour & Fish 1993). In the Western U.S., *I. pacificus* is the primary reservoir, and 108 vertebrates from the Mammalia, Aves and Reptilia classes have been identified as hosts for *I. pacificus* in California (Castro & Wright 2007). Of these three geographical locations, Lyme disease transmission dynamics in the Eastern U.S. have been most clearly elucidated.

The most important reservoir host in the Eastern U.S. is the white-footed mouse (*Peromyscus leucopus).* This reservoir is abundant throughout the region, can harbor high numbers of immature *I. scapularis,* and has demonstrated a 92% vector infection rate to feeding *I. scapularis* larvae (LoGiudice et al. 2003). Two important aspects of reservoir hosts are average larval burden and reservoir competence level (for example, the percentage of naïve vector ticks

that become infected after feeding to repletion on a host). Using these criteria, eastern chipmunks (*Tamias striatus)*, shrews, and shrew-moles (*Sorex* and *Blarina* spp.) have demonstrated importance as reservoir hosts in a given community because of their combined competence levels (40-50%) and larval tick loads (450-1250 / ha^{-1}) (Ostfeld et al. 2006). Other reservoir hosts in the Eastern U.S., such as skunks, birds, raccoons, and opossums, are not considered as important for generation of *Borrelia*-infected *I. scapularis* nymphs because of low competence for these borreliae and low larval tick burdens (Ostfeld et al. 2006).

Reservoir incompetent hosts fail to infect naïve ticks, but play variable roles in pathogen maintenance within an ecological community. For example, white-tailed deer (*Odocoileus virginianus*) often serve as primary hosts for adult *Ixodes* and therefore increase tick vector populations, even though they do not generate infected ticks (Barbour & Fish, 1993). Birds have not demonstrated high levels of reservoir competence or substantial tick burdens, but are potential facilitators of *I. scapularis* dispersal in the Eastern U.S. (Brinkerhoff et al. 2011; Brinkerhoff et al. 2010). In the Western U.S., lizards such as the western fence lizard (*Sceloporus occidentalis*) may harbor high numbers of immature ticks, but possess a borreliacidal factor in the blood that prevents infection and can "cure" infected ticks (Lane & Quistad, 1998). Furthermore, certain hosts are not involved in *B. burgdorferi* transmission, but are involved in transmission cycles involving other *Borrelia* species that may co-occur in the same ecological community. For example, in Europe, rodent reservoirs are usually considered more important than avian hosts in generating *B. burgdorferi*-infected ticks (Mannelli et al. 2012). However, *B. bavariensis* and *B. garinii* are both exclusively associated with birds that are present in the same habitats (Margos et al. 2009). The diverse range of hosts for *Borrelia* infection and *Ixodes* infestation generate variable community-level dynamics that influence Lyme disease transmission.

Community assemblage is an important aspect to consider when analyzing Lyme disease risk (Ostfield & LoGiudice 2003). According to the "dilution effect" hypothesis, ecosystems low

in biodiversity and composed of highly competent reservoir hosts will result in increased human disease risk, measured by nymphal infection prevalence of the primary vector. As biodiversity increases and more hosts with low competence are introduced into the ecosystem, these low-competence hosts are predicted to serve as "dilution" hosts and decrease disease risk. Models simulating Lyme disease risk in different communities of animals in the Eastern U.S. supported these predictions (LoGiudice et al. 2003). However, ecosystems analyzed in Southern Canada did not exhibit the predicted negative correlation between rodent biodiversity (which included competent and incompetent hosts) and Lyme disease risk (Bouchard et al. 2013). A study in New York analyzed the impact of deer and predator (coyotes and red foxes) abundance on Lyme disease risk, measured by infected nymphal *I. scapularis* prevalence (Levi et al. 2012). Previously, decreased deer populations in Massachusetts were associated with decreased Lyme disease risk (Deblinger et al. 1993). In the New York study however, declines in red fox populations positively correlated with increased Lyme disease risk while deer abundance did not, suggesting that loss of predation as an ecosystem service was responsible for the increase (Levi et al. 2012). Lyme disease transmission cycles are complex, and many factors need to be taken into account to elucidate transmission dynamics for an ecosystem.

Lyme Disease in California

Most cases of Lyme disease in the U.S. are in the northeastern or midwestern states (**Figure 1**). However, California has approximately 100 cases per year throughout the state (California Department of Health, 2014), mostly in Northern California (**Figure 2**). Lyme disease ecology in California differs significantly compared to the Eastern U.S. The primary vector in the Western U.S. is the western black-legged tick, *I. pacificus,* and 108 vertebrate hosts have been demonstrated as hosts in California for at least one tick life stage (Castro & Wright 2007; Furman & Loomis 1984). This host range includes 48 bird, 52 mammal, and 8 reptile species. With

respect to *B. burgdorferi* s.l., over 50 mammalian and avian species were evaluated as potential reservoir hosts in Northern California (Lane et al. 2005). Western tree squirrels (*Sciurus griseus)* showed the highest rate of *B. burgdorferi* infection (80%) and possessed infected larval and nymphal *I. pacificus*. Infection rate of larval *I. pacificus* attached to *S. griseus* was 47%, and degree of engorgement was positively associated with acquisition of spirochetes (Lane et al. 2005). A later study focusing specifically on western gray squirrel yielded a 30% *B. burgdorferi* infection rate in squirrels from 15 California counties (Salkeld et al. 2008). *Sciurus griseus* was implicated as the primary reservoir of *B. burgdorferi* in Northern California.

As has been the case in Europe, multiple overlapping transmission cycles of *B. burgdorferi* s.l. have been demonstrated in California. In Northern California, *B. burgdorferi* and *B. bissettii* are associated with different habitats, vectors, and hosts (Eisen et al. 2009). *Sciurus griseus* are associated with *B. burgdorferi* in oak woodland habitats, with *I. pacificus* as the primary vector. In chaparral habitats, dusky-footed woodrats (*Neotoma fuscipes)* and deer mice (*Peromyscus spp.*) were commonly infected with *B. bissettii*, with transmission perpetuated by *I. spinipalpis*. These California isolates were recovered primarily from *I. pacificus*, *I. spinipalpis* (including previously distinct *I. neotomae* [Norris et al. 1997]), and *N. fuscipes*. *Borrelia bissettii* has also been isolated from human samples, where it was implicated as the causative agent of Lyme disease-like symptoms in Mendocino County (Northern California) and Slovenia (Girard et al. 2011; Strle et al. 1997). *Ixodes spinipalpis* has been shown to indirectly contribute to human infection, since it has not been shown to bite humans, but can transfer infection between reservoir competent *N. fuscipes* (Brown et al. 2006).

San Luis Obispo County has had limited surveillance for *B. burgdorferi* s.l. infection. Only one of the squirrels sampled in the Salkeld et al. 2008 study was from San Luis Obispo County, and this squirrel tested negative for *B. burgdorferi* s.l. In 2002 and 2003, the prevalence of *B. burgdorferi* s.l. was examined in a variety of non-sciurid rodents across different habitats

(Vredevoe et al. 2004). Spirochetes identified as *B. bissettii* were isolated from 26.4% of *Peromyscus* mice and woodrats in seven distinct plant communities, primarily prickly pear and coastal scrub habitats. However, *B. burgdorferi* was not isolated from rodents or ticks during that study. Since 2005, eight cases of Lyme disease have been reported in San Luis Obispo County (California Derpartment of Health, 2014). To elucidate the transmission cycle of Lyme disease spirochetes in this region, further investigation was needed.

In light of previous findings in Northern California and San Luis Obispo County, I sought to expand the examination of rodents for *B. burgdorferi* s.l. in this region to include sciurid rodents. I focused my efforts on western gray squirrels in oak woodlands habitats at four distinct sites. Only one of the sites had been previously surveyed in the Vredevoe et al. (2004) study. My hypothesis was that the western gray squirrel is the primary reservoir of *B. burgdorferi* in San Luis Obispo County. This hypothesis led to two predictions: 1) Western gray squirrels will have higher rates of *B. burgdorferi* infection than all other rodents sampled, and 2) Western gray squirrels will have higher rates of *B. burgdorferi*-infected ticks recovered from them as compared to other captured rodents. I also speculated that western gray squirrels may have higher tick burdens of the primary vector, *I. pacificus*, as compared to other rodents in the same habitat.

Materials and Methods

Site Selection

Potential trapping locations in San Luis Obispo County were evaluated for the following criteria when considering potential study sites: 1) available public access, 2) suitable habitat for western gray squirrels, and 3) animal trapping and tick collection permissions. Public access to sites was an important criterion because the impact on community health would be more significant in areas frequently visited by the public. In previous Northern California studies, western gray squirrels showed strong associations with hardwood habitats containing oak species such as blue oak (*Quercus douglasii*), interior live oak (*Q. wislizenii*), scrub oak (*Q. dumosa*), and California black oak (*Q. kelloggii*) (Zeiner et al. 1990; Ingles 1950; Salkeld et al. 2008). Since these habitats occur in San Luis Obispo County and demonstrated strong association with *S. griseus*, they were the first priority for surveying, even though other habitats have been shown to support *S. griseus* populations, such as agricultural fields intermixed with sagebrush and juniper-sagebrush woodlands in northeastern California (Matson et al. 2010) and conifer-dominated forests in Baja Mexico (Escobar-Flores et al. 2011). Oak hardwood distribution in San Luis Obispo County was evaluated using GIS data to determine possible *S. griseus* habitats (**Figure 5**). This map was aligned with the previously described range of *S. griseus* (**Figure 6)** (Zeiner et al. 1990). Prospective sites were then evaluated for the presence of squirrels as part of a 2012 Cal Poly class project (Zoology 321, Mammalogy) (**Table 1**). The sites were scouted for the presence and / or evidence of gray squirrels, such as nests and hulled pinecones. To fulfill the public usage criterion, all trapping locations were within 500 yards of trails or roads accessible by the public. Lastly, trapping permits for specific sites needed to be available (Department of Fish and Wildlife), along with permission from California Polytechnic State University, City of San Luis Obispo, or San Luis Obispo County. Trapping permits and permissions were granted from the relevant authorities.

Four sites were selected for sampling. All of the sites were selected based on their fulfillment of the original criteria: western gray squirrel presence, public use, and access for study. The study sites included two California Polytechnic San Luis Obispo campus properties, Poly Canyon and a site adjacent to the Crop Sciences unit, as well as two off-campus properties, Reservoir Canyon City Park, and Santa Margarita Lake County Park. For all of the trapping sites, GPS coordinates (**Table 1**), map location (**Figure 7**), and habitat pictures (**Figure 8**) were obtained. Poly Canyon had a canyon topography with riparian, oak woodland, and coastal scrub habitats coinciding respectively with elevation increases. Reservoir Canyon had a hilly topography, characterized by riparian habitat at the lowest elevation, then oak woodland and coastal scrub with increasing elevation. Bordering the riparian area was working ranchland. Santa Margarita Lake had gradual hills and was populated by oak woodlands, with the lake bordering the trapping site. Crop Sciences had oak woodland bordered by riparian habitat on one side and agricultural fields on the other.

Animal Trapping

Trapping occurred throughout 2013 in Poly Canyon (January - May), Reservoir Canyon (July - August), Santa Margarita Lake (August - September), and Crop Sciences (October - November). Tomahawk live traps (15 x 15 x 50 cm; Tomahawk Live Trap, Tomahawk, WI) and Sherman live traps (8 x 8 - 10 x 23 - 30 cm; H.B. Sherman Traps Inc., Tallahassee, FL) were used to capture small rodents. Sherman traps were used to primarily capture small species, such as deer mice (*Peromyscus spp.*), California pocket mice (*Chaetodipus californicus*), and big-eared woodrats (*Neotoma macrotis* [previously classified as *N. fuscipes*] [Matocq 2002]). Tomahawk traps captured larger-bodied rodents including big-eared woodrats, California ground squirrels, and western gray squirrels. Twelve Sherman and twelve Tomahawk traps were deployed per plot

at Poly Canyon and Crop Sciences, while sets of twenty-four of each trap type were deployed per plot at Reservoir Canyon and Santa Margarita Lake.

Traps were deployed throughout each site in potential squirrel foraging locations, such as the base of trees harboring squirrel nests. Traps were not placed in exposed positions to prevent public interference with trapping. At each trap station, Tomahawk traps were deployed in pairs with one Tomahawk trap placed on a tree branch approximately 1.5m from the ground and another at the base of each tree. At locations where no appropriate branches were available, two tomahawk traps were deployed at the base of trees on opposite sides. At each Tomahawk deployment site, two Sherman traps were placed on the ground. Sherman traps were typically placed adjacent to cover frequented by rodents, such as rocky outcrops and woodrat nests.

Tomahawk traps were baited using peanut butter with one of five nut types: pecans, hazelnuts, pine nuts, walnuts, or almonds. Nut-embedded peanut butter was placed into a small paper cup in the back of the trap right behind the activation plate, to prevent access to the bait without activating the trap. A trail of nuts was then placed leading out of the trap. One of the five different nuts was used initially when trapping in an area, and nuts were rotated until one was used most successfully. Then this nut was used more frequently, which was usually pecans. Traps were covered with small branches and leaf litter to disguise the trap. Sherman traps were baited with rolled oats placed in the back of the trap with a trail leading out, and a piece of cotton was placed inside to minimize heat loss and mortality in captured animals.

Trapping plots were run for five days and five nights of trapping. After the plot was setup the first evening, Sherman traps were baited and opened to target nocturnal rodents, such as mice. In the morning, Sherman traps were checked and closed within an hour of sunrise. The Tomahawk traps were baited at this time. For the first two days of Tomahawk deployment, the traps were pre-baited to improve trapping success (Salkeld et al. 2008). Pre-baiting involved regularly baiting the traps with the door propped open and the activation mechanisms disabled.

On days three through five, Tomahawks were opened with the triggering mechanism activated within an hour after sunrise and then checked every four to six hours until closure at sunset, at which time the Sherman traps were baited and opened. Previously captured animals were identified by ear tag and immediately released. Previously uncaptured animals were promptly taken back to the laboratory and processed (see below). Trapping took place for five consecutive days at each location within a given study site.

Animal Processing

Animals were processed in the order that the traps were recovered. To remove captured mice and rats from Sherman traps, a plastic bag was securely attached to the end of the trap, the trap door was opened, and animals were shaken into the bag. Animals were anesthetized for all procedures using a cotton ball soaked in 1mL of the veterinary inhalant anesthetic isoflurane (Baxter, Deerfield, IL), which was deposited into the bag with the animal for initial sedation. The animal was observed until it lost consciousness, after which it was prepared for processing.

Larger rodents such as squirrels and woodrats captured in Tomahawk traps were sedated for processing without prior removal from the trap. To sedate these animals, the entire trap was placed into a custom-built Plexiglas chamber that completely enclosed the trap (**Figure 9**). Cotton balls soaked with 1mL of isoflurane were deposited into the front of the chamber, and the chamber was closed with a cap of Plexiglas and weather stripping. A blanket was placed over the chamber to reduce stress and decrease time to sedation. After the animal was unconscious, it was removed from the chamber and trap for processing.

The unconscious animal was transferred into a metal pan, and a nose cone containing an isoflurane-soaked cotton ball was applied to the animal's nose to maintain sedation during processing. The nose cone was placed 1 - 2cm from the animal's nose to ensure that adequate

oxygen mixed with the anesthetic to maintain light sedation. If the animal showed signs of regaining consciousness, the nose cone was moved closer to the animal, and if the signs continued, the animal was quickly transferred into the bag or Plexiglas box to provide additional anesthetic until it was unconscious again. The breathing rate and pulse rate (if visible) were monitored throughout procedures, and if breathing or pulse (if visible) ceased, the anesthetic was removed until the rate recovered.

During anesthetization, three animals were lost due to stress and / or overdosing. To prevent this from re-occurring, the following steps were taken. Stress was reduced during initial sedation by covering the anesthesia chamber with a blanket. This resulted in deeper anesthesia with less reawakening during the procedure. Additionally, the nose cone was applied immediately after removal from the trap to prevent reawakening and held 2 - 3cm from the animal's nose to ensure a proper mix of air and anesthetic. No animals were lost after these modifications were instituted.

Animal processing consisted of species and sex identification, body measurements, ear sampling, ear tagging, and tick surveys. First, the rodent was identified to species (Ingles, 1965), then sex and general body condition were recorded. Most species were clearly identified using general morphology, but *Peromyscus* species identification required mass and body size measurements. Length measurements were taken of the total body, tail, ear, and hind foot, and the animal was weighed using a digital scale. A sterile scalpel was then used to remove a small section of each ear for *Borrelia* testing. One ear sample was held at -20°C for testing by PCR, and the other ear sample was sterilized and placed in BSK-H culture for *Borrelia* screening. When possible, residual blood samples were collected from animal ears and held at -20°C for use in future studies.

Lastly, the animal was thoroughly inspected for attached ticks. Searches took place for approximately one minute and were always performed by the same individual. This amount of

time was enough to assume that most ticks had been removed. Body areas of frequent infestation such as the ears and neck were surveyed first before examining the rest of the body. Any discovered ticks were carefully removed using forceps, placed in microcentrifuge tubes, and held at -20°C. After recovery from anesthesia, animals were transported to and released at the original trap location.

Tick Flagging

Additional ticks were collected by flagging vegetation in areas where traps were deployed. Flagging was performed to capture additional life stages that would not be obtained through rodent trapping. Flagging took place at Poly Canyon from January - May 2013 and November 2013 - March 2014, at Reservoir Canyon from June - July 2013, at Santa Margarita Lake from August - September 2013, and at Crop Sciences from October - November 2013. White fleece flags ($1m^2$) attached to wooden or metal poles were swept over vegetation such as shrubs and grasses to target questing adult ticks. Since the primary host of adult *I. pacificus* in California is the Columbian black-tailed deer (*Odocoileus hemionus columbianus),* areas where deer trails were observed were specifically targeted (Furman & Loomis 1984). To target larval and nymphal *I. pacificus*, smaller flags (0.3m X 0.6m) were swept across tree trunks and leaf litter, because the target hosts for these tick stages, such as lizards and small rodents, reside in these microhabitats (Padgett & Bonilla 2011; Carroll 1996). Spatial targeting of flagging depended on the time of year flagging was performed. *Ixodes pacificus* have overlapping seasons for different life stages in California: adults from October to July and nymphs and larvae from February to October (Salkeld et al. 2014). The flags were examined for the presence of ticks at one-minute intervals during flagging; captured ticks were placed in vials. Ticks with low competence for *Borrelia* infection, such as *Dermacentor variabilis* and *D. occidentalis* were not

tested (Lane et al. 2010). Prior to processing, ticks were transferred into 1.5mL microcentrifuge tubes and stored at -20°C for identification and testing.

Ear Sample and Tick Processing

Of the two ear samples acquired from each animal, *Borrelia* detection was performed using BSK-H culture on one ear and DNA extraction followed by PCR on the other. The ear used for culture was placed into a HEPA-filtered laminar flow hood, then surface sterilized with Betadine (7.5% povidone-iodine, Stamford, CT) by immersion for 15 minutes. The Betadine was discarded, and the ear sample was soaked in 70% ethanol for 15 minutes. The ear sample was removed from the ethanol and air-dried for 2 - 3 minutes on sterile filter paper. Lastly, the excised ear sample was cut into small pieces (approximately $1mm^2$) with sterile scissors. The ear pieces were transferred with sterile forceps into snap-cap vials containing 4mL of BSK-H broth with rabbit sera and incubated at 35°C. Culture media used was 4mL of BSK-H medium complete (Sigma, St. Louis, MO) with added antibiotics for *Borrelia* spp. cultivation (Piesman & Sinsky 1988). To prevent contamination, BSK-H used in this study was prepared with 0.50g of rifampin, 0.0025g of fungizone, 0.20g of cycloheximide, 0.20g of phosphomycin, 0.24g of cysteine, and 0.50g of dithiothreitol (Fisher, Pittsburgh, PA) added per 1L. Cultures were checked weekly by dark field microscopy at 400X using an Olympus BX51-P microscope for evidence of spirochete growth, and positive cultures were re-passaged to increase concentration. Positive cultures from each animal were combined and flash frozen at -80°C as stocks using 700µL of culture media and 420µL 80% glycerol in a 1.5mL microcentrifuge tube for retesting or additional studies. Each positive culture was prepared for PCR by centrifugation at 12,000RPM for 15 minutes to concentrate the borreliae, then re-suspended in 200µL of BSK-H media and held at -20°C prior to PCR.

The second excised ear sample was tested by PCR for *Borrelia* infection after DNA extraction. DNA extractions were performed using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) following parameters for total DNA purification from animal tissues. Tissue lysis ATL buffer (180μL) and Proteinase K (20μL) were added to the ear sample and placed in a 56°C heat block overnight to fully degrade the ear tissue. Cell lysis AL buffer (200μL) was added, tubes were pulse-vortexed, then centrifuged to remove liquid from the tube inner lid. All centrifugation was performed at maximum speed (13,000RPM). Samples were placed in a 70°C heat block for 10 minutes. Next, 100% ethanol (200μL) was added, then pulse-vortexed and briefly centrifuged. The retentate, containing DNA and cellular debris, was transferred into filter tubes and centrifuged for 1 minute. Filtered liquid was discarded, then wash buffer AW1 (500μL) was added to the filter. After 1 minute of centrifuging, the filtered liquid was discarded, wash buffer AW2 (500μL) was added, and the sample was centrifuged for 3 minutes. The filter was placed in a sterile 1.5mL microcentrifuge tube, then 200μL of elution AE buffer was added and incubated for 1 minute. The sample was centrifuged again for 1 minute, and the eluent was added to the filter, then centrifuged for 1 minute. The filter was discarded and the sample was held at -20°C until PCR.

To test ticks for *Borrelia* infection, the same DNA extraction and PCR protocol used for ear samples was implemented. Ticks collected from rodents and ticks collected by flagging were examined using a dissection microscope to identify life stage, sex, and species. Identification to the species level was made using multiple keys (Clifford et al. 1961; Kleinjan & Lane 2008; Furman & Loomis 1984; Poucher et al. 1999), or by Lorenza Beati, Georgia Southern University U.S. National Tick Collection. Ticks removed from animals were occasionally damaged during removal, and in some cases, identification to only the genus level was possible. After identification, DNA was extracted from the ticks in a similar manner to the ear samples. Ticks

were first crushed using individual sterile pestles in 1.5mL microcentrifuge tubes prior to processing.

PCR Detection of Borrelia

Frozen ear samples, positive BSK cultures, and ticks were tested for *Borrelia* infection using a nested PCR approach. Nested PCR uses two rounds of PCR amplification to increase assay sensitivity. For the first round of PCR, extracted genomic DNA was used from ear samples and ticks as template. Positive BSK cultures did not require DNA extraction; the centrifuged borreliae culture pellet was used directly for colony PCR. The first round PCR primers targeted a ~250 base pair (bp) region in the 5S-23S intergenic spacer (IGS) of the *Borrelia burgdorferi* genome, then the second round PCR primers targeted a 200 bp region inside the first (**Figure 10**) (Postic et al. 1994; L. Eisen et al. 2004). The primers amplified a region frequently targeted in other studies. A library of these sequences were available for comparison in GenBank (Lane et al., 2005; Postic et al., 1994; Salkeld et al., 2008).

PCR was performed using reagents contained within PCR beads (GE Healthcare illustra PuReTaq Ready-to-Go PCR beads, Cat No. 46-001-011, Buckinghamshire, UK). PCR reactions were prepared with the following stoichiometry: 22μL molecular grade sterile water, 1μL 10mM forward primer, 1μL 10mM reverse primer, and 1μL of sample template. First round PCR used 1μL of DNA extraction or positive culture, and second round PCR used 1μL of first round PCR product. The reactions were aliquoted into tubes containing the PCR reagent bead to generate the PCR reaction mix. Each PCR run included negative (PCR water in lieu of template) and positive (B31 culture) controls. For the negative control in the first round PCR, 23μL of sterile water and 1μL each of the forward and reverse primers was added, and for the second round, 1μL of the first round negative control product was used as the PCR template. For the first round positive

control, the template was 1µL cell lysate from *B. burgdorferi* strain B31 (ATCC 35210), and for the second round, the template was 1µL of the first round positive control PCR product. Any runs that did not have appropriate results for controls were repeated before sample results were interpreted.

Samples were originally tested using the 5S-23S IGS primers. However, issues were encountered with negative control contamination and unclear DNA sequencing results. All rodent and tick samples were retested using two additional primer sets: ospC (Bunikis et al. 2004) and p66 (Clark et al. 2005). Each primer set utilized different running conditions (**Table 2**).

Gel Electrophoresis

PCR products from the second round of nested PCR were analyzed by gel electrophoresis. Gels composed of 1.5% agarose gel were prepared by adding 0.37g of agarose powder into 25 mL of TBE (Tris-Borate-EDTA buffer containing Tris Base 54g, Boric acid 27.5g, EDTA (0.5 M) 20mL, and sterile distilled water per 1L), then pulse-microwaved until agarose was completely dissolved. Ethidium bromide (2.5µL) was pipetted in and swirled to mix. The solution was promptly transferred into a gel box with a 10-well comb, then 1X TBE was added to the gel box to completely cover the hardened gel. Samples were prepared for loading by mixing 5µL of PCR product with 1µL of 6X Loading dye. Ladder was prepared using 1µL Thermo Scientific GeneRuler 100bp DNA ladder (Lithuania), 1uL 6X Loading dye, and 4uL of PCR-grade water. Samples were loaded into their lanes and run at 100V for 45 minutes. Gels were imaged using UV light housed inside a Gel Doc. Samples that exhibited appropriately-sized bands were noted as presumptively positive, and the remaining second round PCR product was stored at -20°C prior to DNA sequencing.

DNA Sequencing

DNA sequencing was performed on positive PCR samples to identify *Borrelia* species. Forward and reverse inner primers were used for DNA sequencing. PCR products were submitted for clean-up and sequencing (Molecular Cloning Lab, South San Francisco, CA). Sequencing data was cleaned up by trimming and aligning forward and reverse sequences using Sequencher® version 5.3 sequence analysis software (Gene Codes Corporation, Ann Arbor, MI USA, http://www.genecodes.com). MegaBLAST was performed on all sequenced samples to identify closely matching sequences in GenBank. Phylogenetic analysis was performed on sample sequences and representative reference samples obtained from GenBank. Reference samples were selected to obtain diverse *Borrelia* species and geographical representation. The primary pathogens of Lyme disease in Europe (*B. garinii* and *B. afzelii*) were used as outgroups for both p66 and ospC trees. jModelTest 2 was used to analyze best-fit models of nucleotide substitution using statistical selection (Darriba et al. 2012; Guindon & Gascuel 2003). Appropriate models and parameters were selected using Bayesian information criterion (BIC) scores. Using parameters obtained from jModelTest 2, phylogenetic trees were generated using *MEGA* version 6 (Tamura et al. 2013). The analysis was a Maximum Likelihood with bootstraps test (n = 1000 replicates) on the selected (ModelTest) model.

Statistical Analysis

All statistical analyses were performed using JMP statistical software, v. 11.1.1 (SAS Institute Inc., Cary, NC). For all analyses, α was set to 0.05.

Infection rate was analyzed for association with rodent sex, rodent species, site, infestation prevalence, and infestation intensity using an effect likelihood ratio test. The independent variable was infection rate, and the dependent variables were rodent sex, rodent

species, site, infestation prevalence, and infestation intensity. All dependent variables were fixed effects, and all combinations of dependent variables were analyzed for interaction effects.

Infestation intensity was analyzed for association with infection rate, rodent species, rodent sex, and site using Fit Least Squares. The independent variable was infestation intensity, and the dependent variables were rodent sex, rodent species, and site. All dependent variables were fixed effects, and all combinations of dependent variables were analyzed for interaction effects.

Infestation prevalence was analyzed for association with rodent sex, rodent species, site, and infection prevalence. The independent variable was infestation prevalence, and the dependent variables were rodent sex, rodent species, site, and infection intensity. All dependent variables were fixed effects, and all combinations of dependent variables were analyzed for interaction effects.

Rodent and tick richness, diversity and evenness at each successful trapping site were calculated using EcoComPaC (Drozd 2010). Shannon-Wiener diversity index was used for diversity calculations, and Pielou's evenness index was used for evenness calculations.

Results

Animal Trapping

Ten species of small animals were captured in San Luis Obispo County across three of four sites (Poly Canyon, Reservoir Canyon, and Santa Margarita Lake). The number of trap nights per site were as follows: 504 for Poly Canyon, 480 for Reservoir Canyon, 480 for Santa Margarita Lake, and 200 for Crop Sciences. Trapping at the Crop Sciences site was terminated after four weeks when no animals were captured at the site. It was subsequently discovered that rodent abatement was ongoing at this site, which likely impacted trapping success. This site was removed from statistical analyses.

Six animal species were captured in Tomahawk traps: western gray squirrel (*Sciurus griseus)*, California ground squirrel (*Otospermophilus beecheyi*), big-eared woodrat (*Neotoma macrotis*, formerly classified as a subspecies of *N. fuscipes*), striped skunk (*Mephitis mephitis*), American robin (*Turdus migatorius*), and western scrub jay (*Aphelocoma californica*). The skunk and birds were released without sampling as they were outside the handling and testing protocols of this study. Five animal species were captured in Sherman traps: deer mouse (*Peromyscus maniculatus*), California mouse (*Peromyscus californicus*), brush mouse (*Peromyscus boylii*), big eared-woodrat, and California pocket mouse (*Chaetodipus californicus).* A total of 119 rodents were trapped and processed from Poly Canyon, Reservoir Canyon and Santa Margarita Lake (**Table 3**).

Peromyscus identifications were made using standard taxonomic keys (Ingles, 1965; Reid, 2006). Primary characteristics used to differentiate species were body length measurements (total body, tail, ear, hind foot), weight and pelage. Three *Peromyscus* individuals were unidentifiable to the species level due to measurements that fell between acceptable ranges for *P. boylii* and *P. maniculatus*. Weight and pelage could not be used for successful identification;

these individuals were omitted for statistical measures of rodent diversity. Full measurements of all rodents and *Peromyscus* identification criteria are listed in **Appendix A**.

For the 116 identified rodents, site richness, diversity (Shannon-Wiener index), and evenness (Pielou's evenness index) were calculated for the three successful trapping sites (**Table 4)**. Richness was defined as total number of species captured at a given site. Diversity incorporated abundance of each species, so less abundant species did not increase diversity as much as more abundant species. Evenness measured how evenly species abundance was distributed, with a value of 1.0 representing uniform abundance between species; the value decreases as differences in species abundance increases. *Sciurus griseus* were captured only at Poly Canyon and Santa Margarita Lake, and *O. beecheyi* and *C. californicus* were solely captured at Santa Margarita Lake. All other species were collected across the three sites. Santa Margarita Lake had the highest infestation prevalence, rodent richness, and rodent diversity. Reservoir Canyon had the highest tick infestation, and Poly Canyon had the highest tick richness and diversity. However, statistical comparisons of these factors could not be made between sites because trapping effort and seasons were not equivalent.

Tick Collection

A total of 81 ticks (23 from flagging and 58 from rodents) were collected across 3 sites (Poly Canyon, Reservoir Canyon, and Santa Margarita Lake). Sites were analyzed for tick infestation prevalence and intensity, and tick richness, abundance, and diversity (**Table 4)**. No ticks were recovered from the Crop Sciences site, and this site was not included in statistical analyses. Five tick species were recovered: *Dermacentor variabilis, D. occidentalis, Ixodes pacificus, I. spinipalpis*, and *I. woodi*. All ticks captured by flagging were adults, whereas more nymphs and larvae were removed from small mammals. The ratio of ticks captured from rodents

was 0.03 : 1 : 0.58 for adults, nymphs, and larvae respectively, with nymphs being most commonly collected. Tick capture details are listed in **Appendix B**.

Flagging for adult ticks was performed from January 2013 to April 2014 and yielded *D. variabilis* and *I. pacificus* adult ticks. *Dermacentor variabilis* were frequently found at all four sites but were not tested because previous studies demonstrated they are inefficient vectors of *B. burgdorferi* (Piesman & Sinsky 1988). Flagging at Poly Canyon yielded 23 adult *I. pacificus*: 10 females and 6 males in 2013 and 4 females and 3 males in 2014. The seven ticks obtained in 2014 were located in pastures approximately ½ mile away from Poly Canyon. Capture dates at Poly Canyon were from January to April and October to November in 2013 and in March of 2014. Flagging attempts at Reservoir Canyon, Santa Margarita Lake, and Crop Sciences yielded no additional captures.

Flagging for larval and nymphal ticks using smaller flags took place from March to October 2013 at all four sites. No ticks were recovered using this method. Tree trunks and leaf litter were surveyed for larval and nymphal tick presence at all four sites, but no individuals were obtained.

There were 58 ticks recovered from rodents at Poly Canyon, Reservoir Canyon, and Santa Margarita Lake **(Table 5)**. Four tick species at various life stages were recovered: *I. pacificus, I. spinipalpis, I. woodi, and D. occidentalis* (**Table 6**). Most ticks were clearly identified to species level; however, seven larvae and one nymph could only be identified to genus level due to head and mouthpart damage. All unidentifiable ticks were belonged to either *Ixodes* or *Dermacentor* genera. Rodents yielding ticks included *S. griseus, O. beecheyi, N. macrotis, P. californicus, P. boylii,* and *P. maniculatus*. *Ixodes pacificus* nymphs were only recovered from *S. griseus*, while *I. pacificus* larvae were recovered from four species: *O. beecheyi, P. californicus, P. maniculatus,* and *P. boylii*.

Infestation prevalence and intensity were analyzed for associations with rodent species, rodent sex, infection prevalence, and site. Tick infestation prevalence was calculated as infested rodent abundance divided by total rodent abundance, and tick infestation intensity was calculated as tick abundance per infested individual. Infestation prevalence (DF = 7, χ^2 = 26.754, p = 0.0004) and infestation intensity (DF = 7, F = 2.672, p = 0.014) were significantly different between rodent species. Infestation prevalence did not show significant differences between rodent sex (DF = 1, χ^2 = 3.181, p = 0.204), infection prevalence (DF = 1, χ^2 = 0.207, p = 0.649) or sites (DF = 2, χ^2 = 4.812, p = 0.09). Infestation intensity did not show significant differences between rodent sex (DF = 1, F = 0.840, p = 0.138), infection prevalence (DF = 1, F = 0.108, p = 0.743), or site (DF = 2, F = 0.113, p = 0.89). No significant interactions between dependent variables were found for infestation prevalence or intensity.

Borrelia Culture and PCR (Animals)

Of the 119 captured rodents, 7 (5.9%) tested positive for *Borrelia* infection with 6 (5.0%) infected with *B. burgdorferi*, and one lost on follow up prior to identification (**Table 7)**. Of the seven infected individuals, five were captured at Poly Canyon, and the remaining two were from Reservoir Canyon. Three animals tested positive by BSK-H culture and four additional animals tested positive by PCR on ear DNA extractions. Of the seven *Borrelia* positives, only one rodent tested positive using both culture and DNA-extract PCR (concordance = 14.3%). Two samples tested positive by culture and culture PCR only, and four rodents tested positive by ear DNA extraction PCR only.

All three culture-positive animals were *S. griseus* trapped at Poly Canyon. Two of the positive cultures tested positive by PCR, one with both ospC and p66 primers, and the other with ospC primers only. The third *S. griseus* was culture positive, but bacterial growth was lost upon re-passage. PCR of the ear sample from this individual tested negative using both ospC and p66

primers. Because this sample could not be identified to species-level, this sample was omitted from statistical analysis for infection rates of *B. burgdorferi*.

Five animals had PCR-positive ear samples when tested at the ospC and / or p66 loci. One *S. griseus* and three *P. maniculatus* tested positive by ospC, and one *P. boylii* tested positive by p66. Of these animals, one *S. griseus,* one *P. boylii* and one *P. maniculatus* were from Poly Canyon and two *P. maniculatus* were from Reservoir Canyon.

Borrelia burgdorferi infection rate was analyzed for association with rodent sex, rodent species, site, infestation prevalence, and infestation intensity using an effect likelihood ratio test. There were no significant associations between infection rate and rodent species (DF = 7, χ^2 = 8.555, p = 0.286), rodent sex (DF = 1, χ^2 = 1.763, p = 0.414), infestation prevalence (DF = 1, χ^2 = 3.144, p = 0.0762), infestation intensity (DF = 1, χ^2 = 3.610, p = 0.0574), or site (DF = 2, χ^2 = 3.690, p = 0.158). No significant interactions between dependent variables were found.

Borrelia PCR (Ticks)

A total of 81 tick DNA extracts were tested by PCR using ospC and p66 primers. All tested negative by PCR with both sets of primers. This included ticks recovered from all sites by flagging and removal from rodents. Retesting of representative samples of species and sites were negative.

DNA Sequencing

Using DNA sequencing data, the overall *B. burgdorferi* infection rate for rodents was 6 of 119 (5.0%). All six samples formed well-supported clades with *B. burgdorferi* sensu stricto isolates. Phylogenetic trees were constructed for the p66 (**Figure 11)** and ospC (**Figure 12)** loci. One sample was analyzed using both loci, one sample using p66 only, and four samples using

ospC only. San Luis Obispo County samples were compared to sequences representing varied *Borrelia* species and geographic locations.

Base differences between rodent samples and their closest matches were analyzed (**Table 9)**. For p66, both samples grouped with *B. burgdorferi* strains from throughout the US: CHR45-88 (California), JD-1 (Colorado), B31 (New York), and FLCL-3 (Florida). For ospC, the five samples formed two distinct groups. One group contained one San Luis Obispo sample and three *B. burgdorferi:* CA6 and CHRW57 (California) and B31 (New York). The other group contained four San Luis Obispo samples and two *B. burgdorferi:* LAG24 and HUMB150 (California). All six samples were positively identified as *B. burgdorferi*.

Discussion

This was the first study to isolate *B. burgdorferi*, the causative agent of Lyme disease in the U.S., from animal reservoirs in San Luis Obispo County. Previously performed surveys for animal and tick infection in San Luis Obispo County were limited. In statewide testing of western gray squirrels (*S. griseus)* from 2006-2008, the one squirrel captured from San Luis Obispo County tested negative for *Borrelia* infection (Salkeld et al. 2008). Statewide tick surveillance recovered 230 adult *I. pacificus* from San Luis Obispo County from 2000-2009, but all ticks tested negative for *B. burgdorferi* s.l. (Padgett et al., 2014). The most extensive reservoir host surveillance of San Luis Obispo County was performed from 2001-2003 (Vredevoe et al. 2004). This study discovered *B. bissettii* across a variety of habitats in seven rodent species, but no *B. burgdorferi* was isolated. My current study focused on *S. griseus* and oak woodlands specifically due to previous Northern California research, but *B. burgdorferi* infection was isolated from multiple rodent species.

My study isolated *B. burgdorferi* from three rodent hosts: *S. griseus*, deer mice (*P. maniculatus)* and brush mice (*P. boylii)*. It was hypothesized that *S. griseus* would fulfill primary reservoir criteria for *B. burgdorferi* in this region based on three previous Northern California studies (Lane et al. 2005; Salkeld et al. 2008; Nieto et al. 2010). Northern California is the region of California with the highest incidence of Lyme disease (California Department of Health, 2014) (**Figure 2).** To determine important reservoir hosts of *B. burgdorferi*, over 50 avian and mammalian species were surveyed, and the conclusion was that *S. griseus* was the primary reservoir of *B. burgdorferi* (Lane et al. 2005). This conclusion was based on the *S. griseus* infection rate (80%; n = 10) and the presence of attached, infected *I. pacificus* larvae (46.9%; n = 64) and nymphs (32.6%; n = 46) (Lane et al. 2005). A follow-up study focused on *S. griseus* spanning 15 California counties, primarily in Northern California, and demonstrated an overall

30% *B. burgdorferi* infection rate that ranged from 0-50% per county (Salkeld et al. 2008). Most recently, sciurid species were evaluated in Northern California, and *S. griseus* showed the highest number of infected animals (37.8%; n = 37) (Nieto et al. 2010). Any other surveyed reservoir hosts in California have not matched the infection rates obtained from these studies at this time. However, *S. griseus* is distributed throughout California (**Figure 6**), and Lyme disease cases are focused primarily in the northern part of the state (**Figure 2)**. This implies that the presence of *S. griseus* is not in itself enough to yield high Lyme disease risk. In San Luis Obispo County, I found evidence of infection in three rodent species: *S. griseus, P. maniculatus*, and *P. boylii*, suggesting that multiple rodent reservoirs could be involved in maintenance of *B. burgdorferi* in this region.

Infection rate was not significantly different between the three infected rodent species (p = 0.286). Overall, the *B. burgdorferi* infection rate was 12.5% for *S. griseus* (n = 16), 9.7% for *P. maniculatus* (n = 31), and 2.9% for *P. boylii* (n = 35). Previous studies of *B. burgdorferi* rodent infection rates support natural infection of all three species, satisfying one of the reservoir host criteria (Brown & Lane 1996; Lane et al. 2005; Swei et al. 2015). As mentioned previously, infection rate for *S. griseus* was highly variable, ranging from 0-80% based on location and individual studies (Lane et al. 2005; Salkeld et al. 2008; Nieto et al. 2010). Studies of the *Peromyscus* species have showed generally lower infection rates, and limited California studies have isolated *B. burgdorferi* from *P. maniculatus* and *P. boylii.* In Marin and Sonoma counties (Northern California), the *B. burgdorferi* infection rate of *P. maniculatus* (n = 1028) was 7.39% (Swei et al. 2015). Surveys of woodland, grassland, and chaparral in Mendocino County (Northern California) yielded infection rates of 1.6% for *P. boylii* (n = 123) and 0% for *P. maniculatus* (n = 31) (Eisen et al. 2009). An earlier study in Mendocino County found *B. burgdorferi* infection in *P. boylii* (n = 14) at 22.2% (Brown & Lane 1996). Previous studies

demonstrated generally higher infection rates for *S. griseus* as opposed to the *Peromyscus* species, but the rates between all three species were not significantly different for my study.

Analysis of rodent infection rates for associated factors yielded no significant associations. Infection rate was not significantly different between sites ($p = 0.158$), rodent species ($p = 0.286$), rodent sexes ($p = 0.414$), tick infestation intensities ($p = 0.057$), or tick infestation prevalences ($p = 0.076$). This is likely due to the low overall infection rate of 5.0% ($n = 119$), which is influenced by the relatively low capture rate compared to similar studies. Lyme *Borrelia* surveys in Northern California have been performed at a larger scale and have recovered more rodents, such as $n = 379$ for an oak woodland, grassland and chaparral survey in Mendocino County (Eisen et al., 2009) and $n = 1825$ for an oak woodland survey in Marin County (Swei et al. 2011).

While three rodent species demonstrated *B. burgdorferi* infection in the current study, limited data was available to determine their roles as reservoir hosts. As mentioned previously, reservoir hosts for tick-borne pathogens require being susceptible to infection from infected ticks, maintaining infectivity long enough to infect naïve ticks, and serving as hosts for multiple stages of susceptible ticks (Richter et al. 2000). Natural infection has been previously demonstrated in all three species, which fulfills the first criterion. To fulfill the second criterion, naturally or experimentally infected hosts need to be monitored for infection status over time by directly testing the animal or exposing the host to naïve, susceptible ticks. *Sciurus griseus* experimentally ($n = 1$) and naturally ($n = 3$) infected with *B. burgdorferi* were surveyed for minimal infection duration and exhibited 7 months and between 9 - 22 months respectively (Leonhard et al. 2010). Infection duration beyond twelve months is particularly interesting since this will allow infected reservoir hosts to infect immature ticks trans-seasonally (Leonhard et al. 2010). Experimentally infected *P. maniculatus* ($n = 4$) demonstrated 13 months as their minimal infection duration (Brown & Lane 1994). However, naturally infected *P. maniculatus* have not been surveyed for

minimal infection duration, and *P. boylii* has not been assessed for duration of infection by either infection route. Natural minimal infection duration was not measured for my study since all rodents captured were only tested for *Borrelia* infection once, and no experimental infection was performed. For the first two reservoir host criteria, *S. griseus* has previously demonstrated both, while *P. boylii* and *P. maniculatus* have not been measured for natural infection duration. The third criterion, multiple tick life stages feeding on the host species, requires tick infestation data to verify.

Ixodes ticks were recovered from all three prospective reservoir host species (**Table 6**). *Ixodes pacificus* larvae were the only *Ixodes* species and life stage recovered from *P. boylii* and *P. maniculatus*, while *S. griseus* showed greater diversity with *I. spinipalpis* nymphs and adults, *I. woodi* nymphs, and *I. pacificus* nymphs. Following the third criterion of reservoir host designation, the only tick species that showed multiple life stages on a host species was *I. spinipalpis* on *S. griseus,* with one nymph and one adult on one individual. Although *I. spinipalpis* is not considered to be important in human Lyme disease transmission because this tick species rarely bites humans, it can transmit borreliae between rodents, with *I. pacificus* serving as a bridge vector to humans (Furman & Loomis 1984; Brown et al. 2006; Foley et al. 2011). Evidence from the current and previous studies provided the most support for *S. griseus* as a reservoir host, but *P. boylii* and *P. maniculatus* could still serve important roles in *B. burgdorferi* transmission. Even though infected *Peromyscus* species did not demonstrate infestation with multiple tick life stages, both species were fed on by *I. pacificus.*

All infected rodent species were infested by *I. pacificus*, with *P. boylii* and *P. maniculatus* infested with larvae only and *S. griseus* infested with nymphs only. Nymphal *I. pacificus* are most likely to infect humans with *B. burgdorferi* and are the most important vector of Lyme disease in California (Clover & Lane 1995). The nymphal stage is important for human infection, because possesses higher infection rates than adult *I. pacificus* and can avoid

detection while feeding due to its small size (Padgett & Bonilla 2011). This makes larval *I. pacificus* hosts important. Since transovarial transmission (mother to offspring) is very inefficient in *I. pacificus*, most infected nymphs are generated by larval ticks feeding on infected hosts. For those hosts to become initially infected however, they must be fed on by an infected nymphal or adult tick. Larval and nymphal *I. pacificus* have previously been associated with *S. griseus*, *P. boylii*, and *P. maniculatus* in California (Holdenried et al. 1951; Castro & Wright 2007; Furman & Loomis 1984). The presence of at least one life stage of *I. pacificus* on each of our infected host species and previous studies confirming multiple *I. pacificus* life stages from each host species supports the importance of *S. griseus*, *P. boylii,* and *P. maniculatus* in *Borrelia* transmission.

Two additional *Ixodes* species were recovered from *S. griseus*, one of which is a novel association. Both *I. woodi* and *I. spinipalpis* were recovered from *S. griseus* only. This is particularly interesting because both *I. woodi* and *I. spinipalpis* tick species are typically associated with dusky-footed woodrats (*N. fuscipes*) and rodents present around woodrat nests such as *Peromyscus* species and chipmunks (*Tamias* spp.) (Foley et al. 2011; Casher et al. 2002; Furman & Loomis 1984). *Ixodes woodi* had not been previously isolated from *S. griseus*, even though it is not surprising because of its previous recovery in oak woodlands, a typical habitat for both *S. griseus* and *N. fuscipes* (Casher et al. 2002; Lars Eisen et al. 2004). The lack of recovery of *I. woodi* from other hosts is probably due to limited trapping during the questing season for *I. woodi* nymphs (November to May) (Casher et al. 2002).

Neither *I. woodi* nor *I. spinipalpis* tested positive for *Borrelia* infection. It is unknown whether *I. woodi* is competent for *Borrelia* infection. Previously *I. woodi* capture studies have only tested for *Anaplasma phagocytophilum* or have not tested for bacterial infection (Casher et al. 2002; Rejmanek et al. 2011; Foley et al. 2011; Foley & Piovia-Scott 2014; Lars Eisen et al. 2004). On the other hand, *I. spinipalpis* are competent for *Borrelia* infection and were considered

to be a primary vector of *B. burgdorferi* between rodents (Brown & Lane 1992). *Ixodes spinipalpis* still may play a role in *B. burgdorferi* transmission, but recent studies have demonstrated the importance of *I. spinipalpis* in *B. bissettii* transmission in Northern California, which could be a separate transmission cycle that is taking place in San Luis Obispo County (Eisen et al. 2009; Vredevoe et al. 2004).

Of the six PCR-positive rodents, all six formed well-supported clades with *B. burgdorferi* isolates (**Figures 11 and 12)**, which provided an overall *B. burgdorferi* infection rate of 5.0% (n = 119). None of the samples clustered with *B. bissettii* isolates. This may seem surprising, since all fourteen sequenced *Borrelia* samples from a previous San Luis Obispo County study were identified as *B. bissettii* (Vredevoe et al. 2004). However, separate cycles have been identified for these two *Borrelia* species based on habitat, rodent species, and vector tick (Brown et al. 2006; Eisen et al. 2009). In oak woodland habitats, strong associations were demonstrated between *B. burgdorferi, S. griseus*, and *I. pacificus* as the pathogenic agent, primary reservoir host, and primary vector respectively (Lane et al. 2005; Salkeld et al. 2008; Eisen et al. 2009). In chaparral habitats, *I. spinipalpis* has been presumptively identified as the primary vector of *B. bissettii*, with *N. fuscipes* and *Peromyscus* spp. as the primary reservoirs (Eisen et al., 2009). A similar transmission cycle involving *I. spinipalpis* was found in Colorado (Burkot et al. 2000). As previously mentioned, *I. spinipalpis* is not directly important for *Borrelia* spp. transmission to humans since this species rarely feeds on humans (Furman & Loomis 1984; Burkot et al. 2001). Based on the presence of distinct *Borrelia* species among habitats surveyed during the present and prior San Luis Obispo studies, *B. bissettii* is presumably maintained in a variety of rodents in coastal scrub and chaparral habitats, whereas *B. burgdorferi* is present in multiple rodent species in oak woodlands.

All six PCR-positive samples were identified as *B. burgdorferi* using phylogenetic trees of the p66 and ospC loci (**Figures 11** and **12**). For the p66 loci, the two San Luis Obispo isolates

differed by one bp with the grouped sequences (**Table 10**). The p66 gene codes for a 66-kDa integral membrane protein, and the inner primers targeted a 236 bp region of the 1794 bp gene (Bunikis et al. 1998). The two samples from the p66 locus aligned most closely with four identical sequences isolated from throughout the U.S. (FLCL3, JD1, B31, and CHR45-88). FLCL3 was isolated from *I. affinis*, a vector tick for *B. burgdorferi* and *B. bissettii* in the Southern U.S. (Maggi et al. 2010), while JD-1 was isolated from *I. scapularis* in Massachusetts (Piesman et al. 1987). B31 was used as the control strain and was originally isolated from *I. scapularis* in New York (Barbour et al. 1984). CHR45-88 was isolated from a human patient in Mendocino County (Girard et al. 2011). Interestingly, none of these isolates were recovered from rodents and had a wide geographic range. The p66 phylogenetic tree showed a well-supported clade containing two of our samples and four *B. burgdorferi* sequences, conclusively identifying our sequences as *B. burgdorferi*.

Five PCR-positive samples at the ospC locus formed two well-supported clades with *B. burgdorferi* sequences. Four samples formed a group with two Northern California *B. burgdorferi* isolates, LAG24 and HUMB150. LAG24 was isolated from an *I. pacificus* nymph in Mendocino County, and HUMB150 was isolated from *Tamias senex* (Allen's chipmunk) from an unknown location (Fedorova et al., unpublished). The other sample aligned with three *B. burgdorferi* isolates: CA6 isolated from *I. pacificus* in Sonoma County (Northern California), CHRW57 from an *I. pacificus* nymph from an unknown location, and the previously described B31. While most associated isolates were from California, it is interesting that there was no overlap in rodent hosts between the reference isolates and the San Luis Obispo isolates from this study. It was surprising that Sgriseus1 aligned with the first group, because it aligned with B31 at the p66 locus. However, other studies have demonstrated that strains will exhibit different association based on loci (Margos et al. 2008). The ospC gene is highly polymorphic with many parsimony informative sites and is genetically linked with 5S-23S genes in *B. burgdorferi* and *B. afzelii*

(Bunikis et al. 1998). The ospC gene also exhibits frequent recombination events, which decreases its reliability for identification (Anderson & Norris 2006). Two of the sequences (Pmaniculatus1 and Pmaniculatus2) were shorter than the other sequences (**Table 9**) due to poor 5' sequence quality but still aligned well to the first group of isolates. The ospC phylogeny demonstrated that the additional four sequences grouped into two well-supported *B. burgdorferi* clades and conclusively identified our sequences as *B. burgdorferi*.

Genetic information was successfully obtained from only two loci, p66 and ospC. The two usable loci for this study allowed us to compare information between loci for one isolate and provide confirmation of negative results. Additional loci would yield more resolution with respect to association with other *Borrelia* sequences. MLSA has become more frequently used to classify *B. burgdorferi* s.l. sequences because clearer identification and more in-depth analysis can be performed using sequences from functional and housekeeping genes (Margos et al. 2008; Hanincova et al. 2013; Coipan et al. 2013). Analysis of further loci may yield other similar isolates for comparison and confirmation of established associations.

None of the 81 ticks recovered from rodents or by flagging tested positive for *B. burgdorferi*. Of the 81 ticks tested, 40 were *I. pacificus*: 23 adults, 3 nymphs, and 14 larvae. Of these *I. pacificus*, only nymphs and larvae (n = 17) were collected from rodents. It is not surprising that the overall tick infection rates are low. State-wide surveillance of 47 counties from 2000-2012 yielded an overall infection rate of 0.3% from 2184 pools of adult *I. pacificus* (Padgett et al. 2014). In a study performed in Contra Costa County (Northern California) from 2006-2008, the *B. burgdorferi* infection rate was 0.1% (n = 814) (Padgett & Bonilla, 2011), and 17 human cases of Lyme disease in this county have been confirmed since 2005 (California Department of Health, 2014). This is a similar disease incidence to San Luis Obispo County with only eight confirmed cases since 2005 (California Department of Health, 2014).

Comparisons of rodent abundance and diversity between sites were complicated by uneven trapping effort and seasonal differences. Trapping efforts for Reservoir Canyon and Santa Margarita Lake were slightly less than Poly Canyon (480 vs. 504 trap nights respectively). These discrepancies would lead us to slightly overestimate abundances and possible diversity measures at Poly Canyon with respect to the other sites. Seasonality was another difference between sites, since no sites were trapped at the same time of year. Poly Canyon was trapped at peak season for *I. pacificus* nymphs and larvae (April-June), while the other successful sites (Reservoir Canyon and Santa Margarita Lake) were trapped from July to September, past peak activity but still within the nymphal and larval seasons (February-August). This could lead us to overestimate infestation prevalence and intensity at the Poly Canyon site. Additionally, all sites were flagged within the range of adult and nymphal tick seasons, so it was surprising that no adult ticks were recovered from sites other than Poly Canyon, and no nymphal or larval ticks were recovered from these flagging efforts. To standardize effort across sites and to target peak tick activity, it would be been ideal to apply even trapping and larval / nymphal flagging effort to all sites from April to June, during peak periods of subadult tick activity. This would have targeted all sites at previously observed peak activity for larval and nymphal *I. pacificus* in Northern California (Salkeld et al. 2014). However, this was not feasible with the amount of time, personnel, and traps available.

Limited flagging success was probably influenced by California's drought conditions. California in general and San Luis Obispo County specifically have experienced more dry days and lower mean precipitation than previous years (Mastrandrea et al. 2011). *Ixodes pacificus* is more active and abundant with moderate temperatures and humid conditions. Nymphal *I. pacificus* showed longer questing seasons and slower declines in abundance under cooler and moister conditions (Eisen et al. 2002). Nymphal intensity was shown to decline when maximum daily temperatures exceeded 22.7 - 25°C (73 - 77°F) and maximum daily relative humidity

decreased below 83 - 85% (Eisen et al. 2002; Eisen et al. 2003). In Napa County (Northern California), adult *I. pacificus* questing season starts with the first rain of the season and ends a few days after the last rain (Salkeld et al. 2014). There were very few days with precipitation in 2013 and 2014, and because of previously observed increases in tick activity during periods of higher relative humidity, flagging took place directly after days with precipitation. However, this did not increase flagging success.

Total rodent-recovered ticks are likely an underestimation of overall infestation intensity (Lane & Loye 1991). Tick inspection duration was constant between animals, and the same individual performed all inspections for consistency. However, factors influencing success of tick recovery, such as fur length and body size, varied among animals. *Sciurus griseus* and *O. beecheyi* were the largest animals captured, but their fur was significantly less dense and easier to find ticks on than other species, potentially offsetting the larger body size with regards to inspection time. The opposite was true for *Peromyscus spp. Neotoma macrotis* had dense fur and was larger than the *Peromyscus spp.*, so they were the most likely to have an underestimated tick load. Additionally, when *P. maniculatus* were assessed for the efficiency of field-sampling, seasonality was also a factor with spring and summer yielding 45% and 69% efficiency respectively (Lane & Loye 1991).

Conclusions

My study is the first to identify several *B. burgdorferi*-infected hosts in San Luis Obispo County. Rodent trapping across four public sites yielded three *B. burgdorferi*-infected rodent species: *S. griseus* (western gray squirrel), *P. maniculatus* (deer mouse), and *P. boylii* (brush mouse). I hypothesized that *S. griseus* would be the primary reservoir host based on previous studies in Northern California. However, I did not find strong evidence to support this hypothesis. My first prediction, that *S. griseus* would have higher infection rates than any other reservoir host,

was not supported at a statistically significant level ($p = 0.286$). My second prediction, that *S. griseus* would have higher infestation rates of *B. burgdorferi* s.l. infected ticks than other trapped rodents, was not supported, since no ticks tested positive for *Borrelia* infection. Tick infestation prevalence and intensity for *S. griseus* were not significantly different from other rodents either.

Furthermore, we did not find any *B. bissettii* infected individuals. This is probably due to the strong association of *B. bissettii* with *N. lepida* in chaparral and coastal scrub habitats (Vredevoe et al. 2004); this woodrat species was not present in the oak woodland habitats sampled in the current study. Additionally, transmission of *B. bissettii* by the *Neotoma*-associated tick species, *I. spinipalpis*, may be somewhat restricted to grasslands and chaparral (Eisen et al. 2009; Brown et al. 2006).

This study builds on previous work to provide evidence that *B. burgdorferi* is present in San Luis Obispo County in oak woodland communities, where it is associated with *S. griseus*, *P. boylii*, and *P. maniculatus*. I speculate that the primary vector is *I. pacificus* because of previous California research, but no infected *I. pacificus* were recovered, leading me to assume that overall disease risk to humans is probably low since other rodent-associated vector-competent ticks infrequently feed on humans (Furman & Loomis 1984; Burkot et al. 2001). Lyme disease risk is often measured by nymphal infection prevalence (LoGiudice et al. 2008; Leonhard et al. 2010), and my study recovered only three *I. pacificus* nymphs, none of which were infected. Since this is a very low recovery rate, further surveillance could confirm whether nymphal infection prevalence is indeed quite low in this area. Given the number of infected rodents, it appears likely that there are other competent *Ixodes* species transmitting *Borrelia* spp. between rodents (Clover & Lane 1995; Eisen et al. 2006). For the four sites surveyed using our study design, human Lyme disease risk appears to be low since no infected *I. pacificus* were recovered.

To better assess the transmission of *B. burgdorferi* in San Luis Obispo County, it would be useful to determine how long *P. maniculatus* and / or *P. boylii* retain infectivity. Experimentally infected *Sciurus griseus* exhibited 7 months minimal infected duration (Leonhard

et al. 2010), while experimentally infected *P. maniculatus* demonstrated 13 months as their minimal infected duration (Brown & Lane 1994). Naturally infected *P. maniculatus* and experimentally or naturally infected *P. boylii* have not been surveyed for *Borrelia* infection duration. To conduct this kind of study, trapping in oak woodlands with Sherman traps repeatedly over time would yield useful data. *Peromyscus* recapture rates were high when traps were redeployed in the previous exact location (data not shown), so testing for *Borrelia* infection in these species over multiple time points would presumably measure changes in infection rate. Infected individuals could be tracked over time to determine infection duration, and seasonal changes in infection rate could also be measured. Additionally, the same approach could be used on *S. griseus* to verify infection rates determined previously (Leonhard et al. 2010). However, difficulties recapturing *S. griseus* like those encountered in previous studies could complicate this endeavor (Carraway et al. 1994; Salkeld et al. 2008). If recapture rates were not high enough to yield useful data for any target species, trapped individuals could be maintained in the laboratory to measure change in infection status over time like in previous studies (Brown & Lane 1994). Taking these additional steps would help elucidate the role of *S. griseus, P. boylii,* and *P. maniculatus* as reservoirs for *B. burgdorferi* s.s. and s.l. in San Luis Obispo County.

Capturing additional ticks would also help elucidate *Borrelia* transmission in San Luis Obispo County. While California's drought contributed to low tick capture rates, additional techniques could be utilized to boost capture rates. Longer inspection times would possibly increase capture rate, but this approach would also increase the risk of accidental anesthetic overdose. Suspending caged rodents over water for days would ensure capture of all attached ticks, which was successfully utilized in previous studies (Lane & Loye 1991). Also, trapping and flagging could be concentrated during the peak seasons of *I. pacificus* to specifically target this species. Flagging for adults would be most useful if performed during their previously observed peak season in Northern California, November to January, while nymphal flagging and trapping animals would be more efficient if focused during nymphal and larval peak seasons of April to

June (Salkeld et al. 2014). Greater tick capture success would contribute to the body of knowledge on the transmission dynamics of Lyme disease in San Luis Obispo County.

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APPENDICES

Appendix A. Ear tag number, site, species, capture date, sex, weight, body measurements, culture results, and PCR results for all captures.

Peromyscus spp. identification criteria:

Peromyscus boylii - longer tail than body, ears smaller than feet (70-80%), hind foot 20-26mm, ear 15-20mm, tail 90-120 sometimes bicolored

Peromyscus maniculatus - tail less than 90% of total body (60-90mm), bicolored tail, hind foot 18-22mm

Peromyscus californicus - hind foot 25+mm, tail not bicolored

Site Abbreviations: Poly Canyon (PC), Reservoir Canyon (RC), Santa Margarita Lake (SML)

Ear Tag	Site	Animal	Date	Sex	Weight (g)	Ear length (mm)	Hind Foot length (mm)	Tail length (mm)	Total Body Length (mm)	Culture	PCR
301	PC	*S. griseus*	1/3/2013	F	> 610	N/A	N/A	N/A	N/A	-	-
302	PC	*S. griseus*	1/3/2013	M	> 610	N/A	N/A	N/A	N/A	-	-
303	PC	*P. boylii*	1/8/2013	M	32.0	20	24	120	N/A	-	-
304	PC	*P. boylii*	1/8/2013	M	31.53	20	25	125	N/A	-	+
324/ 325	PC	*S. griseus*	1/8/2013	M	> 610	N/A	N/A	N/A	N/A	-	-
305	PC	*S. griseus*	1/10/2013	F	> 610	N/A	N/A	N/A	N/A	+	+
306	PC	*P. maniculatus*	1/17/2013	F	16.78	15	20	90	170	-	-
307	PC	*N. macrotis*	1/17/2013	F	219	N/A	N/A	N/A	N/A	-	-
308	PC	*P. maniculatus*	1/24/2013	F	24.31	13	20	94	95	-	-
309	PC	*P. maniculatus*	1/24/2013	F	22.87	16	21	92	89	-	-
310	PC	*P. maniculatus*	1/31/2013	F	17.46	14	17	85	75	-	-
311	PC	*P. boylii*	1/31/2013	F	26.54	19	21	100	95	-	-
312	PC	*P. californicus*	2/19/2013	M	41.16	20	23	121	116	-	-
313	PC	*P. maniculatus*	2/19/2013	F	21.62	16	20	94	83	-	+
314	PC	*P. maniculatus*	2/19/2013	F	19.34	12	16	60	88	-	-
315	PC	*P. maniculatus*	2/19/2013	F	18.01	14	18	82	84	-	-
316	PC	*P. maniculatus*	2/21/2013	F	18.14	16	19	74	80	-	-
317	PC	*P. maniculatus*	2/21/2013	F	18.76	14	19	83	78	-	-
318	PC	*S. griseus*	3/5/2013	F	> 610	N/A	N/A	N/A	N/A	-	-
319	PC	*P. maniculatus*	3/7/2013	F	19.97	13	17	84	83	-	-
320	PC	*P. maniculatus*	3/7/2013	F	14.73	14	21	80	74	-	-
321	PC	*P. maniculatus*	3/7/2013	F	17.55	13	17	65	82	-	-
322	PC	*P. californicus*	3/7/2013	F	33.29	21	24	120	99	-	-
323	PC	*P. maniculatus*	3/7/2013	F	16.18	15	20	77	78	-	-
326	PC	*S. griseus*	4/10/2013	F	> 610	N/A	N/A	N/A	N/A	-	-
327	PC	*P. boylii*	4/15/2013	M	29.86	19	24	105	123	-	-

328	PC	*S. griseus*	4/15/2013	M	> 610	N/A	N/A	N/A	N/A	+	-
329	PC	*S. griseus*	4/22/2013	F	> 610	N/A	N/A	N/A	N/A	+	-
330	PC	*S. griseus*	4/24/2013	M	> 610	N/A	N/A	N/A	N/A	-	-
331	PC	*S. griseus*	5/8/2013	N/A	> 610	N/A	N/A	N/A	N/A	-	-
332	PC	*S. griseus*	5/15/2013	M	> 610	N/A	N/A	N/A	N/A	-	-
333	PC	*P. californicus*	5/22/2013	F	33.96	16	24	127	96	-	-
334	PC	*P. boylii*	5/22/2013	F	27.39	14	23	112	90	-	-
335	PC	*P. maniculatus*	5/22/2013	F	21.44	13	17	85	90	-	-
350	PC	*P. boylii*	5/22/2013	F	34.52	16	23	127	107	-	-
336	PC	*N. macrotis*	5/22/2013	F	N/A	N/A	N/A	N/A	N/A	-	-
12-S2	RC	*P. maniculatus*	7/9/2013	F	18.62	15	22	78	90	-	-
337	RC	*P. boylii*	7/9/2013	F	19.99	16	21	88	83	-	-
338	RC	*N. macrotis*	7/9/2013	F	147.84	N/A	N/A	N/A	N/A	-	-
339	RC	*P. boylii*	7/9/2013	F	17.37	12	23	89	81	-	-
12-S12	RC	*P. boylii*	7/9/2013	F	17.11	14	21	86	78	-	-
340	RC	*P. boylii*	7/9/2013	F	18.95	12	21	94	91	-	-
341	RC	*P. boylii*	7/9/2013	F	19.39	12	21	87	80	-	-
342	RC	*P. boylii*	7/9/2013	F	18.12	12	21	94	87	-	-
343	RC	*P. boylii*	7/9/2013	F	18.06	18	23	96	80	-	-
344	RC	*P. boylii*	7/9/2013	F	19.79	16	22	93	92	-	-
345	RC	*N. macrotis*	7/9/2013	F	157.84	N/A	N/A	N/A	N/A	-	-
346	RC	*P. boylii*	7/9/2013	F	15.59	12	22	93	76	-	-
347	RC	*N. macrotis*	7/9/2013	F	139.82	N/A	N/A	N/A	N/A	-	-
348	RC	*P. maniculatus*	7/10/2013	F	18.83	15	20	86	86	-	-
349	RC	*N. macrotis*	7/10/2013	N/A	268.42	N/A	N/A	N/A	N/A	-	-
351	RC	*P. maniculatus*	7/10/2013	F	21.45	16	16	88	82	-	-
352	RC	*N. macrotis*	7/10/2013	F	224.46	N/A	N/A	N/A	N/A	-	-
353	RC	*N. macrotis*	7/10/2013	F	227.76	N/A	N/A	N/A	N/A	-	-
354	RC	*N. macrotis*	7/11/2013	F	234.73	N/A	N/A	N/A	N/A	-	-
355	RC	*P. boylii*	7/16/2013	F	17.85	11	23	91	85	-	-
356	RC	*P. boylii*	7/16/2013	F	22.65	16	21	95	91	-	-
357	RC	*P. boylii*	7/16/2013	F	17.6	17	21	94	80	-	-
358	RC	*P. boylii*	7/16/2013	F	16.44	15	20	89	82	-	-
359	RC	*P. spp (boylii/ maniculatus)*	7/16/2013	F	16.89	17	20	89	87	-	-
360	RC	*P. maniculatus*	7/16/2013	F	19.22	17	19	88	86	-	-
361	RC	*P. boylii*	7/16/2013	F	17.78	16	21	90	83	-	-
362	RC	*P. boylii*	7/16/2013	F	20.36	12	22	94	89	-	-
363	RC	*P. boylii*	7/17/2013	F	18.37	16	21	88	87	-	-
364	RC	*N. macrotis*	7/17/2013	F	293.23	N/A	N/A	N/A	N/A	-	-
365	RC	*P. maniculatus*	7/17/2013	F	16.78	13	17	80	75	-	-
366	RC	*P. boylii*	7/17/2013	F	14.9	17	20	83	75	-	-

367	RC	*P. maniculatus*	7/17/2013	F	23.96	18	21	76	88	-	-
368	RC	*N. macrotis*	7/17/2013	F	166.8	N/A	N/A	N/A	N/A	-	-
369	RC	*P. boylii*	7/17/2013	F	22.15	16	21	100	89	-	-
370	RC	*P. maniculatus*	7/17/2013	F	22.14	14	22	82	88	-	-
371	RC	*P. boylii*	7/17/2013	F	18.2	17	20	90	81	-	-
372	RC	*N. macrotis*	7/17/2013	F	128.77	N/A	N/A	N/A	N/A	-	-
373	RC	*P. boylii*	7/17/2013	F	19.69	14	22	92	91	-	-
374	RC	*N. macrotis*	7/18/2013	F	184.88	N/A	N/A	N/A	N/A	-	-
375	RC	*N. macrotis*	7/18/2013	F	213.36	N/A	N/A	N/A	N/A	-	-
376	RC	*N. macrotis*	7/18/2013	F	191.97	N/A	N/A	N/A	N/A	-	-
377	RC	*N. macrotis*	7/18/2013	M	255.92	N/A	N/A	N/A	N/A	-	-
378	RC	*P. californicus*	7/23/2013	F	33.91	21	24	121	105	-	-
379	RC	*P. boylii*	7/23/2013	F	17.29	13	20	92	85	-	-
380	RC	*P. boylii*	7/23/2013	F	17.93	18	20	93	79	-	-
14-S5	RC	*P. boylii*	7/23/2013	F	18.2	15	21	91	80	-	-
381	RC	*P. spp (boylii/ maniculatus)*	7/23/2013	F	18.91	13	20	90	91	-	-
382	RC	*P. californicus*	7/23/2013	F	37.91	21	25	124	105	-	-
383	RC	*P. boylii*	7/23/2013	F	14.8	17	21	82	71	-	-
384	RC	*P. boylii*	7/23/2013	F	19.21	17	20	87	89	-	+
385	RC	*P. boylii*	7/23/2013	F	18.45	12	19	93	88	-	-
386	RC	*P. californicus*	7/23/2013	F	40.23	20	25	134	117	-	-
387	RC	*P. boylii*	7/23/2013	F	16	13	20	87	78	-	-
388	RC	*P. maniculatus*	7/23/2013	F	20.41	14	18	89	89	-	+
14-S23	RC	*P. boylii*	7/23/2013	F	19.22	13	21	93	89	-	-
389	SML	*S. griseus*	8/7/2013	F	N/A	N/A	N/A	N/A	N/A	-	-
390	SML	*P. maniculatus*	8/7/2013	M	13.44	20	14	77	89	-	-
391	SML	*S. beecheyi*	8/8/2013	F	298.54	N/A	N/A	N/A	N/A	-	-
392	SML	*S. beecheyi*	8/8/2013	F	271.88	N/A	N/A	N/A	N/A	-	-
16-T4	SML	*S. griseus*	8/13/2013	M	> 610	N/A	N/A	N/A	N/A	-	-
16-T12	SML	*S. beecheyi*	8/13/2013	M	570.94	N/A	N/A	N/A	N/A	-	-
393	SML	*S. griseus*	8/14/2013	F	> 610	N/A	N/A	N/A	N/A	-	-
394	SML	*S. griseus*	8/14/2013	F	> 610	N/A	N/A	N/A	278	-	-
395	SML	*P. californicus*	8/14/2013	F	32.66	21	27	128	96	-	-
396	SML	*S. griseus*	8/14/2013	M	> 610	N/A	N/A	N/A	298	-	-
397	SML	*S. beecheyi*	8/14/2013	F	389.73	N/A	N/A	N/A	N/A	-	-
398	SML	*N. macrotis*	8/14/2013	F	204.27	N/A	N/A	N/A	N/A	-	-
399	SML	*P. maniculatus*	8/14/2013	F	N/A	N/A	N/A	N/A	N/A	-	-
17-S8	SML	*C. californicus*	8/14/2013	M	25.87	14	25	116	89	-	-
400	SML	*P. maniculatus*	8/14/2013	F	N/A	N/A	N/A	N/A	N/A	-	-
401	SML	*P. maniculatus*	8/21/2013	F	22.43	18	22	67	95	-	-
402	SML	*P. maniculatus*	8/21/2013	F	21.6	18	20	56	85	-	-

403	SML	*P. spp (boylii/ maniculatus)*	8/21/2013	F	16.55	11	21	93	90	-	-
404	SML	*P. maniculatus*	8/21/2013	F	20.64	18	21	78	90	-	-
405	SML	*N. macrotis*	8/21/2013	F	117.03	N/A	N/A	N/A	N/A	-	-
406	SML	*P. maniculatus*	8/21/2013	F	19.46	15	19	83	89	-	-
407	SML	*P. boylii*	8/21/2013	F	19.2	19	19	92	84	-	-
408	SML	*P. boylii*	8/21/2013	F	20.68	13	24	99	91	-	-
409	SML	*O. beecheyi*	8/22/2013	F	360.65	N/A	N/A	N/A	N/A	-	-
410	SML	*O. beecheyi*	8/22/2013	N/A	458.72	N/A	N/A	N/A	N/A	-	-
411	SML	*P. boylii*	8/22/2013	F	20.54	12	21	95	91	-	-
412	SML	*O. beecheyi*	8/22/2013	F	507.08	N/A	N/A	N/A	N/A	-	-
413	SML	*N. macrotis*	8/22/2013	N/A	164.84	N/A	N/A	N/A	N/A	-	-

Appendix B. Tick capture data by date, site, collection method, species, life stage, sex, and engorged status.

Date	Site	Collection Method	Ear Tag	Species	Life Stage	Sex	Engorged
1/23/2013	Poly Canyon	Flagging	N/A	*I. pacificus*	Adult	Female	N
				I. pacificus	Adult	Female	N
				I. pacificus	Adult	Female	N
				I. pacificus	Adult	Male	N
				I. pacificus	Adult	Male	N
3/5/2013	Poly Canyon	Flagging	N/A	*I. pacificus*	Adult	Female	N
				I. pacificus	Adult	Male	N
4/3/2013	Poly Canyon	Flagging	N/A	*I. pacificus*	Adult	Female	N
				I. pacificus	Adult	Male	N
				I. pacificus	Adult	Male	N
				I. pacificus	Adult	Female	N
4/8/2013	Poly Canyon	Flagging	N/A	*I. pacificus*	Adult	Female	N
				I. pacificus	Adult	Female	N
				I. pacificus	Adult	Female	N
10/30/2013	Poly Canyon	Flagging	N/A	*I. pacificus*	Adult	Male	N
11/4/2013	Poly Canyon	Flagging	N/A	*I. pacificus*	Adult	Female	N
3/5/2014	Poly Canyon	Flagging	N/A	*I. pacificus*	Adult	Female	N
				I. pacificus	Adult	Female	N
				I. pacificus	Adult	Female	N
				I. pacificus	Adult	Female	N
				I. pacificus	Adult	Male	N
				I. pacificus	Adult	Male	N
				I. pacificus	Adult	Male	N
1/3/2013	Poly Canyon	*S. griseus*	301	*I. spinipalpis*	Nymph	N/A	N
4/15/2013	Poly Canyon	*S. griseus*	328	*I. spinipalpis*	Adult	Female	N
4/22/2013	Poly Canyon	*S. griseus*	329	*I. woodi*	Nymph	N/A	Y
	Poly Canyon			*I. pacificus*	Nymph	N/A	N
	Poly Canyon			*I. spp*	Nymph	N/A	N
4/24/2013	Poly Canyon	*S. griseus*	330	*I. spinipalpis*	Nymph	N/A	N
5/8/2013	Poly Canyon	*S. griseus*	331	*I. spp*	Larvae	N/A	N
	Poly Canyon			*I. pacificus*	Nymph	N/A	N
	Poly Canyon			*D. occidentalis*	Nymph	N/A	Y

5/15/2013	Poly Canyon	*S. griseus*	332	*I. pacificus*	Nymph	N/A	N
5/22/2013	Poly Canyon	*P. californicus*	333	*I. pacificus*	Larvae	N/A	N
5/22/2013	Poly Canyon	*P. maniculatus*	335	*I. spp*	Larvae	N/A	N
	Poly Canyon			*I. pacificus*	Larvae	N/A	N
	Poly Canyon			*I. pacificus*	Larvae	N/A	N
5/22/2013	Poly Canyon	*P. californicus*	336	*D. spp.*	Larvae	N/A	N
	Poly Canyon			*D. spp.*	Larvae	N/A	N
7/9/2013	Reservoir Canyon	*P. boylii*	344	*D. spp.*	Larvae	N/A	
7/18/2013	Reservoir Canyon	*N . macrotis*	374	*D. occidentalis*	Nymph	N/A	N
7/18/2013	Reservoir Canyon	*N . macrotis*	375	*D. occidentalis*	Nymph	N/A	N
7/18/2013	Reservoir Canyon	*N . macrotis*	376	*D. occidentalis*	Nymph	N/A	N
7/23/2013	Reservoir Canyon	*P. californicus*	382	*I. pacificus*	Larvae	N/A	N
7/23/2013	Reservoir Canyon	*P. californicus*	386	*I. pacificus*	Larvae	N/A	Y
				I. spp	Larvae	N/A	N
				I. pacificus	Larvae	N/A	N
				I. pacificus	Larvae	N/A	Y
				D. occidentalis	Nymph	N/A	N
				D. occidentalis	Nymph	N/A	N
				D. occidentalis	Nymph	N/A	Y
				I. pacificus	Larvae	N/A	Y
				I. pacificus	Larvae	N/A	Y
				D. occidentalis	Nymph	N/A	N
				D. occidentalis	Nymph	N/A	Y
				D. occidentalis	Nymph	N/A	N
				D. occidentalis	Nymph	N/A	Y
				D. occidentalis	Nymph	N/A	Y
				D. occidentalis	Nymph	N/A	Y
				D. occidentalis	Nymph	N/A	Y
				I. pacificus	Larvae	N/A	N
8/7/2013	Santa Margarita Lake	*S. griseus*	389	*D. occidentalis*	Nymph	N/A	Y
8/14/2013	Santa Margarita Lake	*S. griseus*	394	*D. occidentalis*	Nymph	N/A	Y
8/14/2013	Santa Margarita	*P. californicus*	395	*I. pacificus*	Larvae	N/A	

	Lake						
8/14/2013	Santa Margarita Lake	*O. beecheyi*	397	*D. occidentalis*	Nymph	N/A	Y
				D. occidentalis	Nymph	N/A	Y
				D. occidentalis	Nymph	N/A	Y
				D. occidentalis	Nymph	N/A	Y
				D. occidentalis	Nymph	N/A	Y
				D. occidentalis	Nymph	N/A	Y
8/14/2013	Santa Margarita Lake	*N. macrotis*	398	*D. occidentalis*	Nymph	N/A	N
				D. occidentalis	Nymph	N/A	N
				D. occidentalis	Nymph	N/A	N
8/21/2013	Santa Margarita Lake	*N. macrotis*	405	*D. occidentalis*	Nymph	N/A	N
8/21/2013	Santa Margarita Lake	*P. boylii*	408	*I. pacificus*	Larvae	N/A	N
				D. spp.	Larvae	N/A	N
				D. occidentalis	Nymph	N/A	Y
8/22/2013	Santa Margarita Lake	*O. beecheyi*	410	*I. pacificus*	Larvae	N/A	N
8/22/2013	Santa Margarita Lake	*P. boylii*	411	*D. occidentalis*	Nymph	N/A	N
				I. pacificus	Larvae	N/A	N
8/22/2013	Santa Margarita Lake	*N. macrotis*	412	*D. occidentalis*	Nymph	N/A	N

.

Appendix C. Tables.

Site Name	Latitude	Longitude
Poly Canyon – Cerro Vista Entrance	**35°18'20.2"N**	**120°39'30.4"W**
Reservoir Canyon	**35°17'27.9"N**	**120°37'40.4"W**
Santa Margarita Lake	**35°19'37.4"N**	**120°29'30.9"W**
Crop Sciences	**35°18'05.3"N**	**120°40'16.3"W**
Davis Canyon Rd	35°13'32.49"N	120°45'19.12"W
Coon Creek Rd	35°14'27.85"N	120°46'47.21"W
Upper Arroyo Grande	35°12'36.8"N	120°22'04.5"W
Green Valley Rd	35°32'51.27"N	120°47'32.58"W
Noel Way	35°38'54.85"N	121°5'6.69"W
North Reservoir Canyon	35°16'10.8"N	120°35'20.4"W
Gates Ranch	35°31'37.4"N	120°45'33.8"W
Chorro Creek Ranch	35°19'28.4"N	120°44'14.4"W
Hi Mountain Rd and Big Baldy Way	35°12'24.8"N	120°24'36.2"W
Mt. Bishop - Highland Dr Trailhead	35°18'05.5"N	120°41'25.4"W
Irish Hills - Mariposa Trailhead	35°15'14.1"N	120°41'46.6"W
Los Osos Oaks State Reserve	35°18'11.2"N	120°48'58.1"W

Table 1. GPS coordinates for prospective trapping sites. Sites were scouted for the presence and / or evidence of western gray squirrels. Selected sites used in this study are in bold.

B. burgdorferi sensu lato target gene	Primer Type	Primer Sequence (5' - 3')	References
5S-23S IGS	Outer Forward	CTG-CGA-GTC-GCG-GGA-GA	(Postic et al. 1994)
	Outer Reverse	TCC-TAG-GCA-TTC-ACC-ATA	
	Inner Forward	TAT-TTT-TAT-CTT-CCA-TCT-CTA-TTT-TGC-C	(Lane et al. 2004)
	Inner Reverse	GAG-TAG-GTT-ATT-GCC-AGG-GTT-TTA-TT	
ospC	Outer Forward	ATG-AAA-AAG-AAT-ACA-TTA-AGT-GC	(Bunikis et al. 2004)
	Outer Reverse	ATT-AAT-CTT-ATA-ATA-TTG-ATT-TTA-ATT-AAG-G	
	Inner Forward	TAT-TAA-TGA-CTT-TAT-TTT-TAT-TTA-TAT-CT	
	Inner Reverse	TTG-ATT-TTA-ATT-AAG-GTT-TTT-TTG-G	
p66	Outer Forward	CGA-AGA-TAC-TAA-ATC-TGT	(Clark et al. 2005)
	Outer Reverse	GCT-GCT-TTT-GAG-ATG-TGT-CC	
	Inner Forward	TGC-AGA-AAC-ACC-TTT-TGA-AT	
	Inner Reverse	AAT-CAG-TTC-CCA-TTT-GCA	

(A)

	5S-23S	**ospC**	**p66**
Denaturation	60s@94°C	30s@94°C	60s@95°C
Annealing	60s@55°C	30s@52°C/50°C	30s@50°C
Elongation	60s@72°C	60s@74°C	45s@68°C
Cycle Number	40 - 1st Round 40 - 2nd Round	35 - 1st Round 40 - 2nd Round	40 - 1st Round 30 - 2nd Round

(B)

Table 2. PCR amplification conditions for *Borrelia burgdorferi* sensu lato primers. Inner and outer primer sequences for 5S-23S, ospC, and p66 (A) and PCR Amplification Conditions for 5S-23S IGS, ospC, and p66 primers (B).

Species	Poly Canyon	Reservoir Canyon	Santa Margarita Lake	Crop Sciences
Sciurus griseus	11	0	5	0
Otospermophilus beecheyi	0	0	7	0
Neotoma macrotis	2	14	3	0
Peromyscus californicus	3	3	1	0
Peromyscus maniculatus	14	10	7	0
Peromyscus boylii	6	26	3	0
Peromyscus spp.	0	2	1	0
Chaetodipus californicus	0	0	1	0
Total	36	55	28	0

Table 3. Captured rodent abundance by site. Abundance of captured rodents listed by species and site for trapping conducted from January 2013 to November 2013. Reservoir Canyon had the highest total abundance. No animals were captured from the Crop Sciences site.

Site	Poly Canyon	Reservoir Canyon	Santa Margarita Lake
Tick Infestation Prevalence	9/36	6/55	10/28
	25%	10.9%	35.7%
Mean Tick Infestation Intensity (SE)	1.778 (0.324)	3.667 (2.667)	2 (0.516)
Rodent Richness	5	4	7
Rodent Diversity	2.014	1.7	2.517
Rodent Evenness	0.867	0.85	0.897
Tick Richness	4	2	2
Tick Diversity	1.617	0.934	0.742
Tick Evenness	0.809	0.934	0.742

Table 4. Tick infestation prevalence and intensity and rodent and tick diversity by site. Tick infestation prevalence was calculated by infested individuals out of total captured individuals, and tick infestation intensity was calculated as mean tick load of infested individuals. Richness is total species per site, diversity measures species number and total abundance using the Shannon-Weiner diversity index, and evenness calculates how evenly species are distributed within sites using Pielou's evenness index. Santa Margarita Lake had the highest infestation prevalence, rodent richness, and rodent diversity. Reservoir Canyon had the highest tick infestation, and Poly Canyon had the highest tick richness and diversity. Statistical comparisons could not be made between sites because trapping effort and season were not equivalent.

Rodent Species	**Tick Prevalence**	**Mean tick load (SE)**	***I. pacificus* nymphal load (SE)**	***I. pacificus* larval load (SE)**
S. griseus	8/16 (50%)	1.5 (0.327)	1.0 (0)	0
O. beecheyi	3/7 (42.9%)	2.67 (1.667)	0	1.0
N. macrotis	6/19 (31.6%)	1.5 (0.342)	0	0
P. californicus	4/7 (57.1%)	5.0 (4.0)	0	2.25 (1.25)
P. maniculatus	1/31 (3.2%)	3.0	0	2
P. boylii	3/35 (8.6%)	2.0 (0.577)	0	1.0 (0)
P. spp	0/3 (0%)	0	0	0
C. californicus	0/1 (0%)	0	0	0

Table 5. Tick prevalence, aggregation, and load by rodent species. Tick infestation prevalence was calculated by infested individuals out of total captured individuals, and tick infestation intensity was calculated as mean tick load of infested individuals for all ticks and *I. pacificus* nymphs and larvae individually. *P. californicus* had the highest infestation prevalence and tick load.

Species	Total Rodents	*I. pacificus*	*I. spinipalpis*	*I. woodi*	*D. occidentalis*	*Ixodes* spp.	*Dermacentor* spp.
S. griseus	N = 16	3n	2n, 1a	1n	3n	1l, 1n	0
O. beecheyi	N = 7	1l	0	0	7n	0	0
N. macrotis	N = 19	0	0	0	7n	0	2l
P. californicus	N = 7	9l	0	0	10n	1l	0
P. maniculatus	N = 31	2l	0	0	0	1l	0
P. boylii	N = 35	2l	0	0	2n	0	2l
P. spp	N = 3	0	0	0	0	0	0
C. californicus	N = 1	0	0	0	0	0	0

Table 6. Number of ticks recovered from each rodent species. Larvae = l, nymphs = n, adults = a. All ticks recovered from rodents are presented as total tick number grouped by tick species and life stage. Captured rodents from all sites are grouped by species.

			Ear Sample PCR		**BSK Culture PCR**	
Species	**Site**	**Ear Tag**	**ospC**	**p66**	**ospC**	**p66**
S. griseus	Poly Canyon	305	+	-	+	+
S. griseus	Poly Canyon	329	-	-	+	-
S. griseus	Poly Canyon	328	-	-	*	*
P. boylii	Poly Canyon	304	-	+	-	-
P. maniculatus	Poly Canyon	313	+	-	-	-
P. maniculatus	Reservoir Canyon	384	+	-	-	-
P. maniculatus	Reservoir Canyon	388	+	-	-	-

Table 7. *Borrelia burgdorferi* positive rodents by PCR and culture. Positive designation was assigned by phylogenetic analysis of p66 and / or ospC loci. DNA sequences from GenBank were used to positively identify samples as *B. burgdorferi*. Six samples were positive for *B. burgdorferi*.

* = BSK-H culture showed positive growth for *Borrelia* in the initial culture, but was lost upon re-passaging and not tested by PCR.

Site *Species*	Poly Canyon	Reservoir Canyon	Santa Margarita Lake	Total Infected per species
S. griseus	**2/11*** **18.2%**	N/A	0/5 0%	**2/16** **12.5%**
O. beecheyi	N/A	N/A	0/7 0%	0/7 0%
N. macrotis	0/2 0%	0/14 0%	0/3 0%	0/19 0%
P. californicus	0/3 0%	0/3 0%	0/1 0%	0/7 0%
P. maniculatus	**1/14** **7.1%**	**2/10** **20%**	0/7 0%	**3/31** **9.7%**
P. boylii	**1/6** **16.6%**	0/26 0%	0/3 0%	**1/35** **2.9%**
P. spp	N/A	0/2 0%	0/1 0%	0/3 0%
C. californicus	N/A	N/A	0/1 0%	0/1 0%
Total Infected per site	**4/36** **11.1%**	**2/55** **3.6%**	0/28 0%	

Table 8. *Borrelia burgdorferi* infected rodents by species and site. Positive rodents tested positive for p66 and / or ospC PCR and DNA sequenced from PCR products formed well-supported clades with *B. burgdorferi* isolates. *Sciurus griseus* exhibited the highest infection rate for rodents, while Poly Canyon showed the highest infection rate by site.
* 3rd *S. griseus* was culture positive for *Borrelia*, but was not identified to species level.

Rodent Sample	**Ear Tag**	**Loci**	**Amplicon Length (BP)**	**Closest Matches**	**BP Differences (from Closest Match)**
Pboylii1-PC	304	p66	236	CHR45-88, JD1, B31, FLCL3	1
Sgriseus1-PC	305	p66			1
Sgriseus1-PC	305	ospC	560	LAG24, HUMB150	4
Sgriseus2-PC	328	ospC			Exact Match
Pmaniculatus1-RC	313	ospC			66
Pmaniculatus2-RC	384	ospC			22
Pmaniculatus3-PC	388	ospC		CHRW-57, CA6, B31	2

Table 9. DNA sequence analysis of *B. burgdorferi* PCR-positive rodents. DNA sequences from p66 and ospC loci were compared with similar sequences from Gen Bank and MiST 2.2. Number of base differences between the isolate and closest matches are listed. All the closest matches of recovered isolates are strains of *B. burgdorferi*.

Appendix D. Figures.

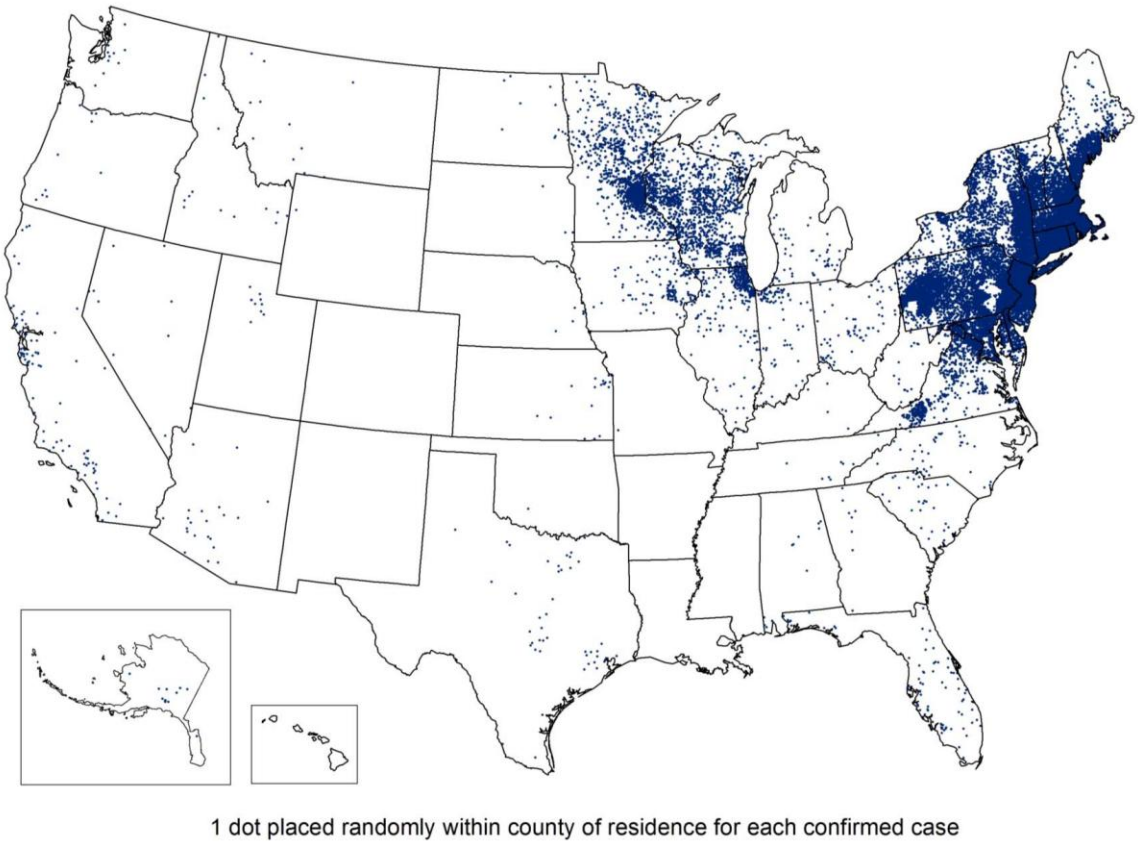


Figure 1. Reported cases of Lyme disease in the U.S., 2013. Each dot represents a confirmed case of Lyme disease in the county of residence of the infected patient. Most cases are located in the midwestern and northeastern U.S. (Compiled by the CDC, Retrieved from http://www.cdc.gov/lyme/stats/maps/map2013.html)

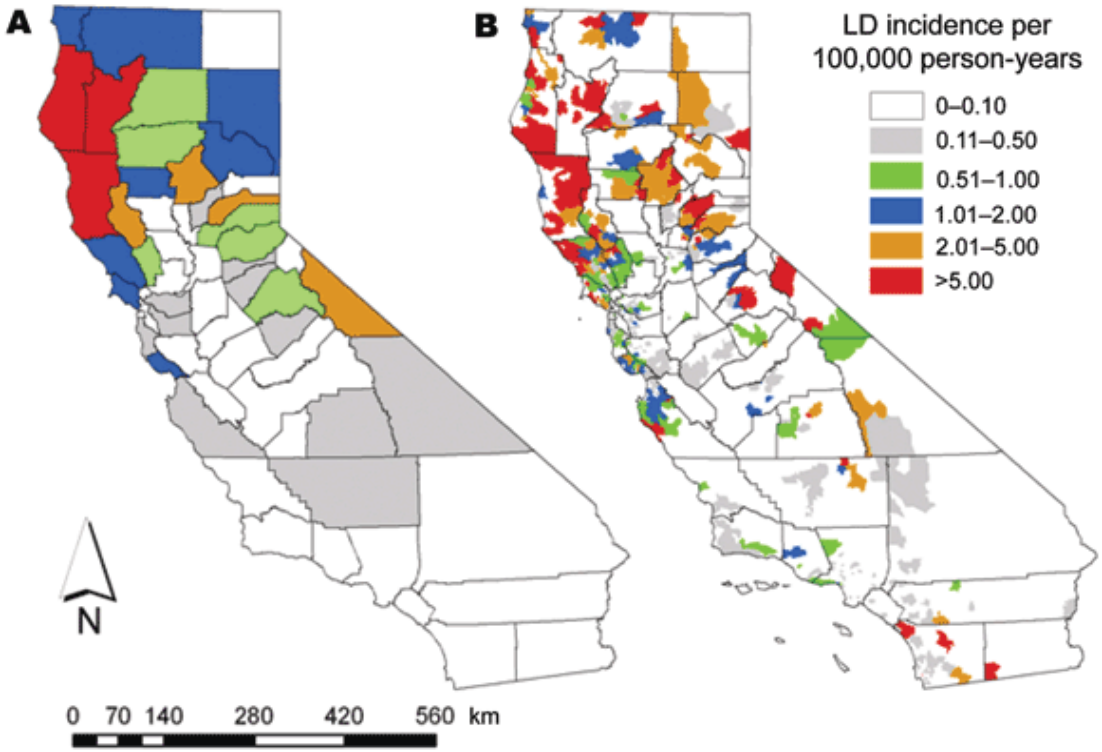


Figure 2. Lyme disease cases by county in California. Incidence of Lyme disease per 100,000 person-years in 2009 by county (A) and geographic distribution within the county (B). Lyme disease incidence rates are higher in Northern California than in Central and Southern California. (Compiled by the CDC, retrieved from http://lymeadvocate.com/lyme-disease-map-california/)

	1	2	3	4	5
1984	*Borrelia burgdorferi*				
1992		*B. garinii*	*B. afzelii*		
1994				*B. japonica*	
1995					*B. andersonii*
	6	**7**	**8**	**9**	**10**
1996	*B. turdi*	*B. tanukii*			
1997			*B. lusitaniae*	*B. valaisiana*	
1998					*B. bissettii*
	11	**12**	**13**	**14**	**15**
2001	*B. sinica*				
2006		*B. spielmanii*			
2007			*B. californiensis*		
2008				*B. yangtze*	
2009					*B. carolinensis*
	16	**17**	**18**	**19**	**20**
2009	*B. americana*	*B. bavariensis*			
2010			*B. kurtenbachii*		
2011				*B. finlandensis*	
2014					*B. chilensis*

North America
North America and Europe
Europe
Eurasia
Asia
South America

Figure 3. *Borrelia* species discovery by date and country. Twenty species are currently in the *Borrelia burgdorferi* sensu lato complex. Location of discovery is indicated by color. Expanded from Figure 1 of Stanek & Reiter 2011.

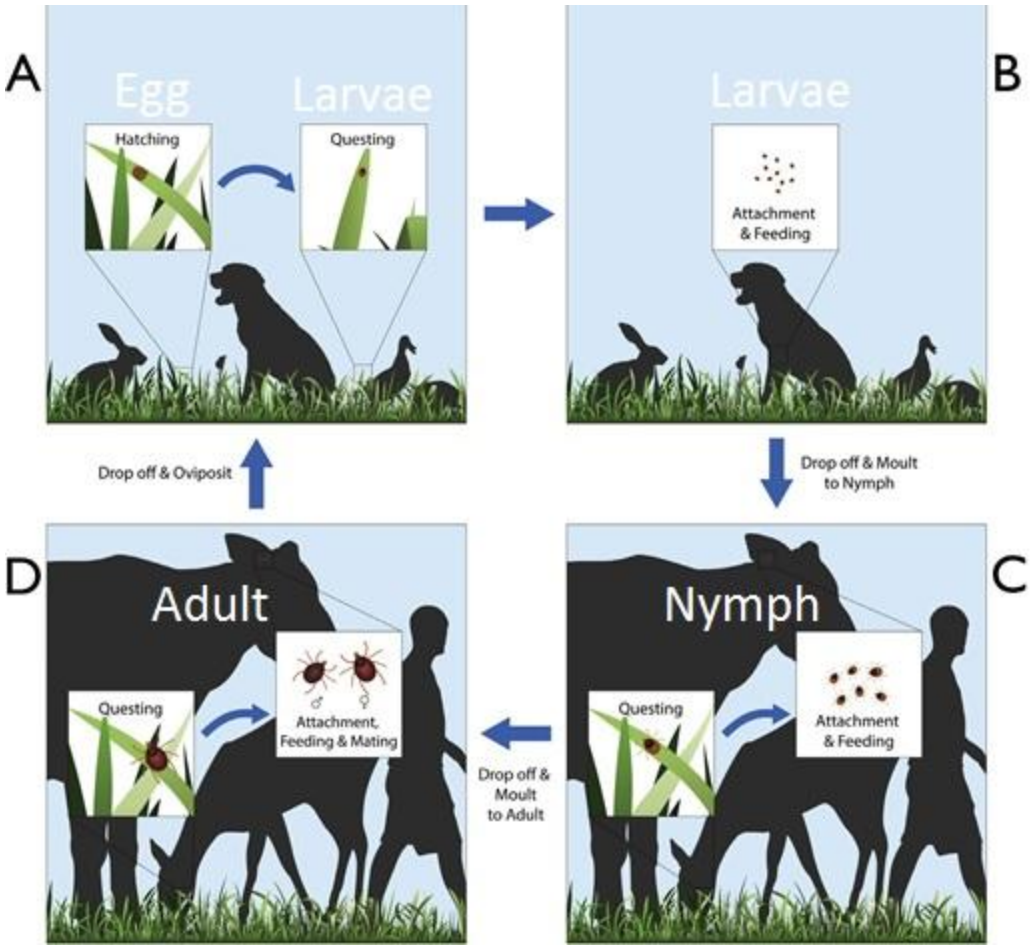


Figure 4. Typical *Ixodes* tick life cycle. Eggs mature into larvae and quest for their first vertebrate host (A). Larval *Ixodes* feeds, drops off the host, and molts into a nymph (B). Questing nymphal *Ixodes* attaches to the second vertebrate host, feeds, and drops off to molt to an adult (C). Questing male and female *Ixodes* adult attach to the third vertebrate host, feed, and mate (D). After females drop off the host, eggs are oviposited, and the life cycle begins again. Modified Figure 1 from Estrada-Peña & De La Fuente 2014.

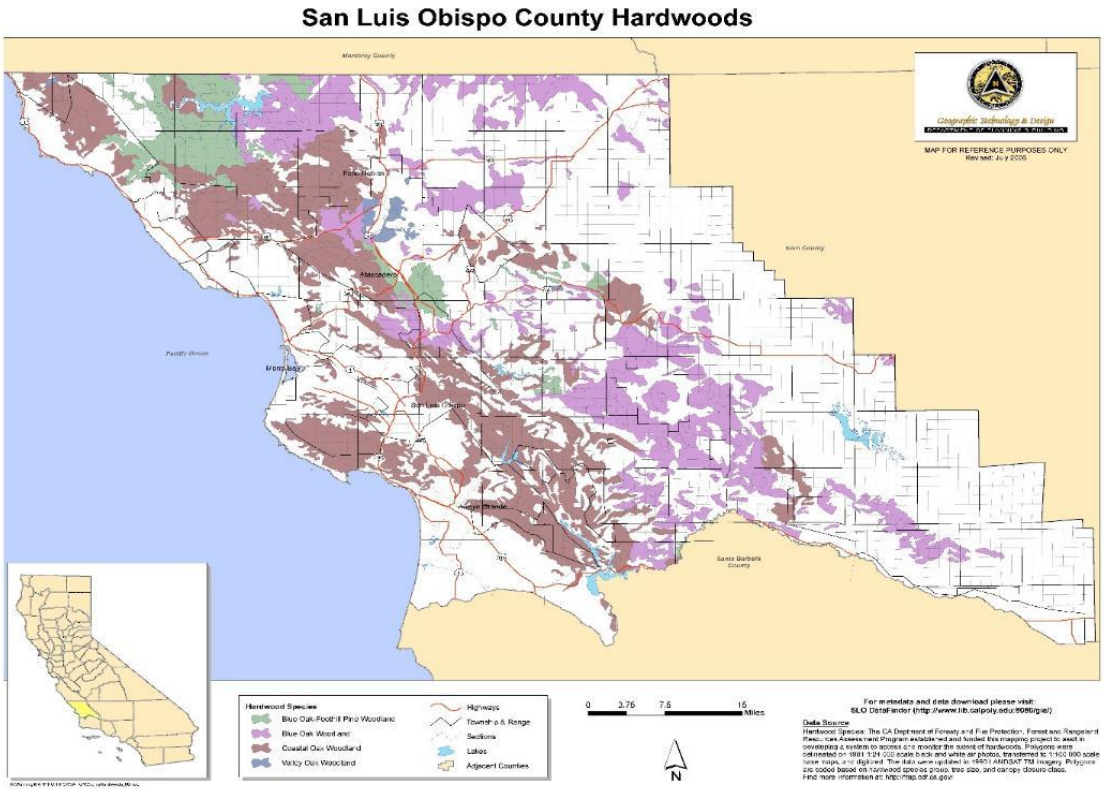


Figure 5. Hardwood distribution in San Luis Obispo County. Oak distribution in San Luis Obispo County was generated using GIS data collected by the CA Department of Forestry and Fire Protection. (Compiled by the San Luis Obispo County Department of Planning and Building, Retrieved from frap.cdf.ca.gov/webdata/maps/san_luis_obispo/hdwd_map.40.pdf)

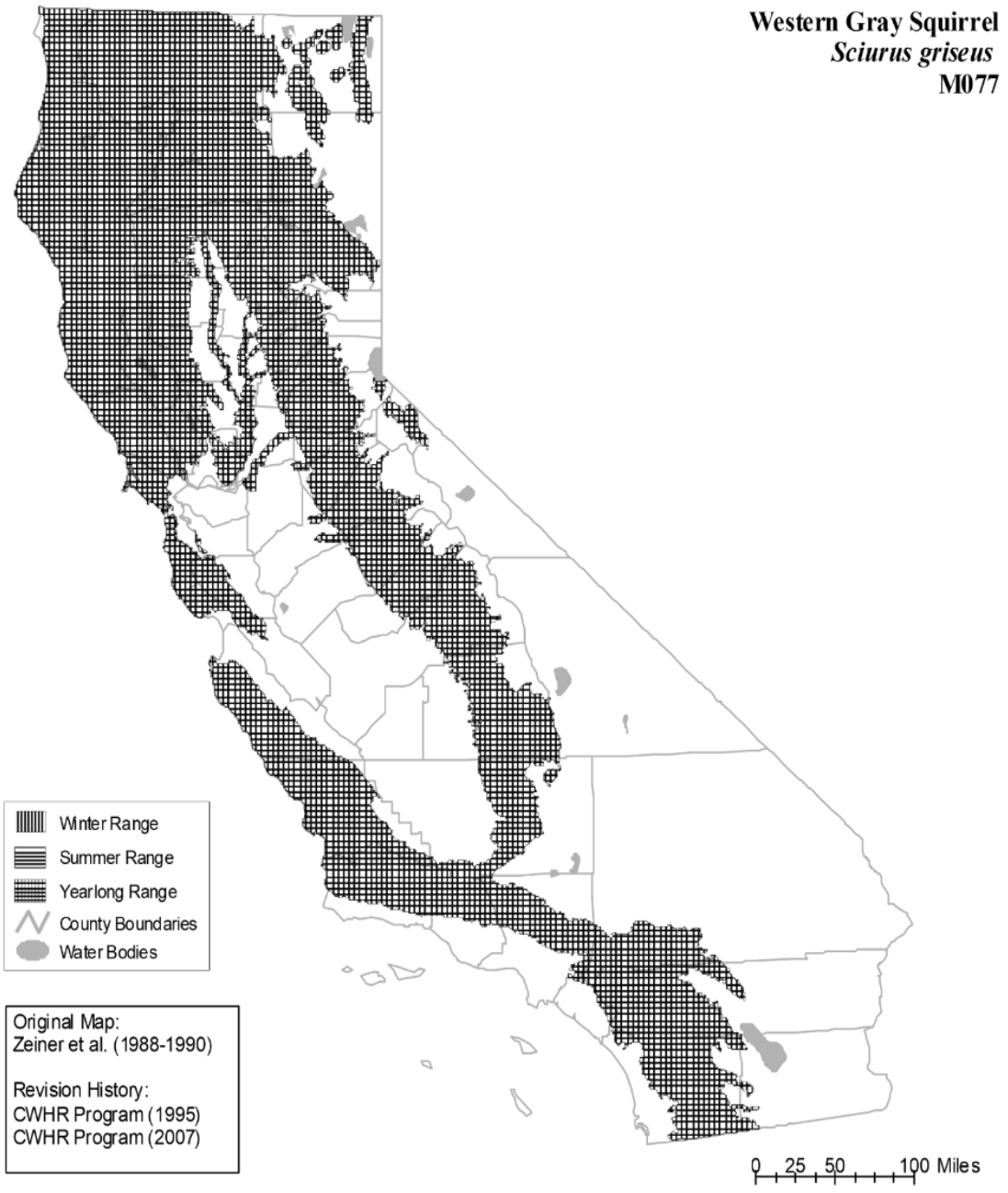


Figure 6. Distribution of western gray squirrel (*Sciurus griseus*) in California. Range maps were generated by the California Wildlife Habitat Relationships system from the California Department of Fish and Game (Zeiner et al. 1990). *Sciurus griseus* are located throughout San Luis Obispo County. (Retrieved from http://www.dfg.ca.gov/biogeodata/cwhr/)

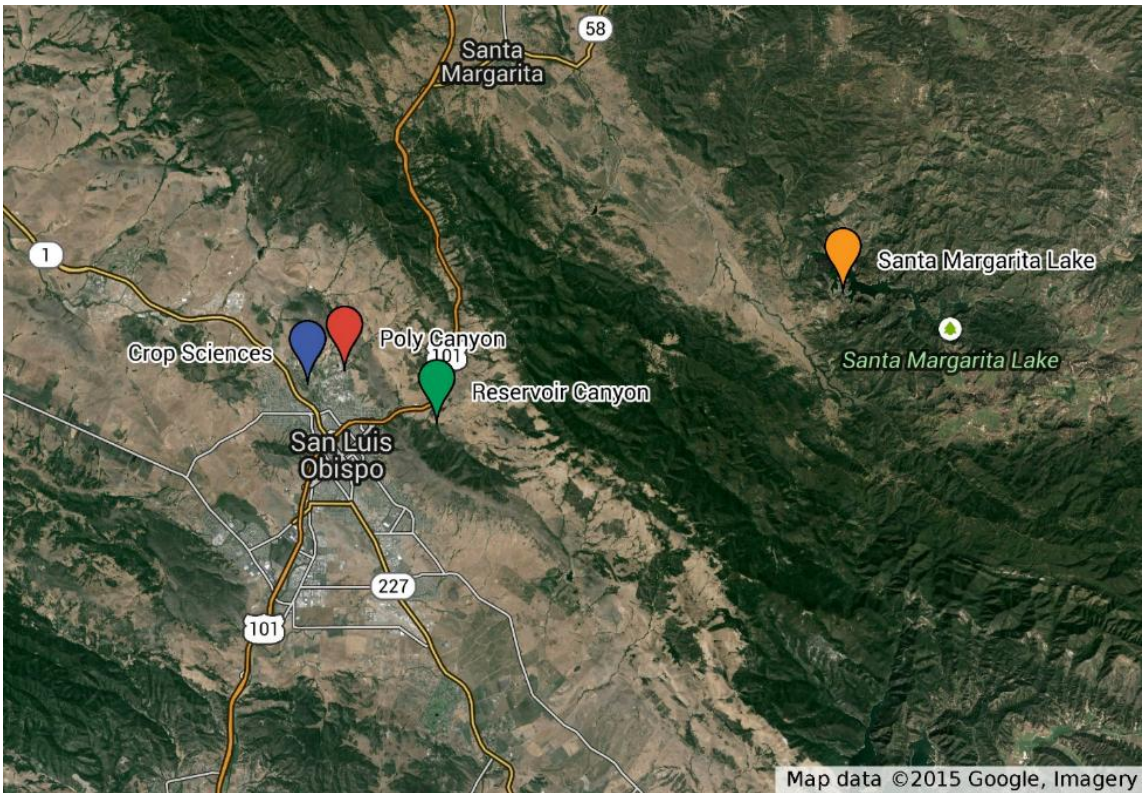


Figure 7. Trapping location map. Trapping sites are indicated: Poly Canyon (red), Reservoir Canyon (green), Santa Margarita Lake (orange), and Crop Sciences (blue). Map generated using Google Maps (Google, TerraMetrics).

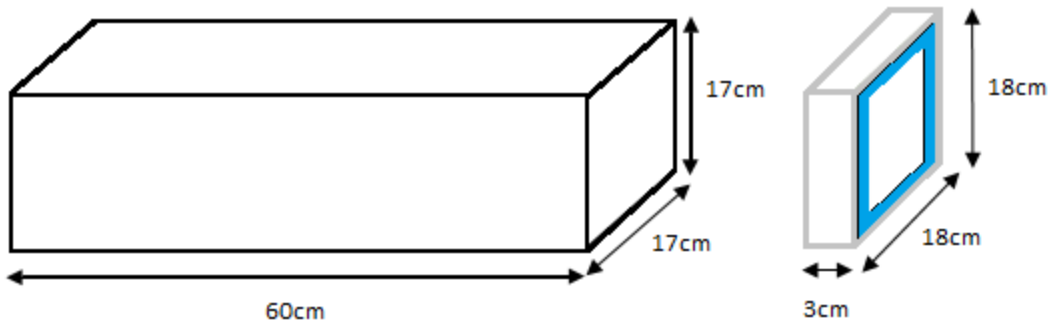
(A)

(B)

(C)

(D)

Figure 8. Trapping sites. Pictures of representative habitats at the trapping sites: Santa Margarita Lake (A), Reservoir Canyon (B), Poly Canyon (C), and Crop Sciences (D).



(A)

(B)

Figure 9. Plexiglas anesthesia chamber. Diagram of anesthesia chamber and lid (A) and photograph of chamber setup (B). Chamber and lid were constructed with Plexiglas, 2" corner mold, caulking and weather stripping (inner edge of lid - blue).

Figure 10. Nested primer design for 5S-23S intergenic spacer region. Outer primers (red) produce amplicons approximately 250bp, and inner primers (blue) produce amplicons approximately 200bp. The region targeted is the intergenic spacer region between 5S and 23S ribosomal subunit genes.

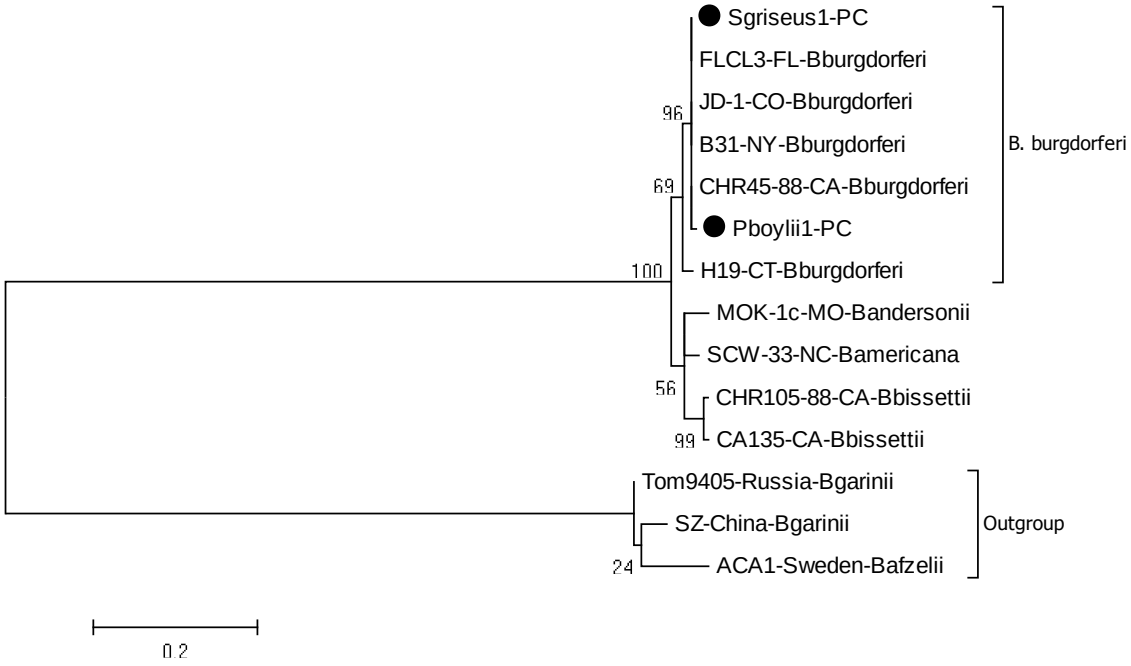


Figure 11. Molecular phylogeny of the *B. burgdorferi* s.l. p66 locus. MEGA 6 was used to generate phylogenies utilizing the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model (Hasegawa et al. 1985; Tamura et al. 2013) with model parameters from ModelTest. The percentage of trees (1000 bootstrap replicates) in which the associated taxa clustered together is shown next to the branches, with branch lengths measured in the number of substitutions per site. Sequences obtained from this study are labeled with black dots, species name, number, and site, while sequences obtained from GenBank are labeled with strain, location, and species. Both isolates are contained within a well-supported *B. burgdorferi* clade.

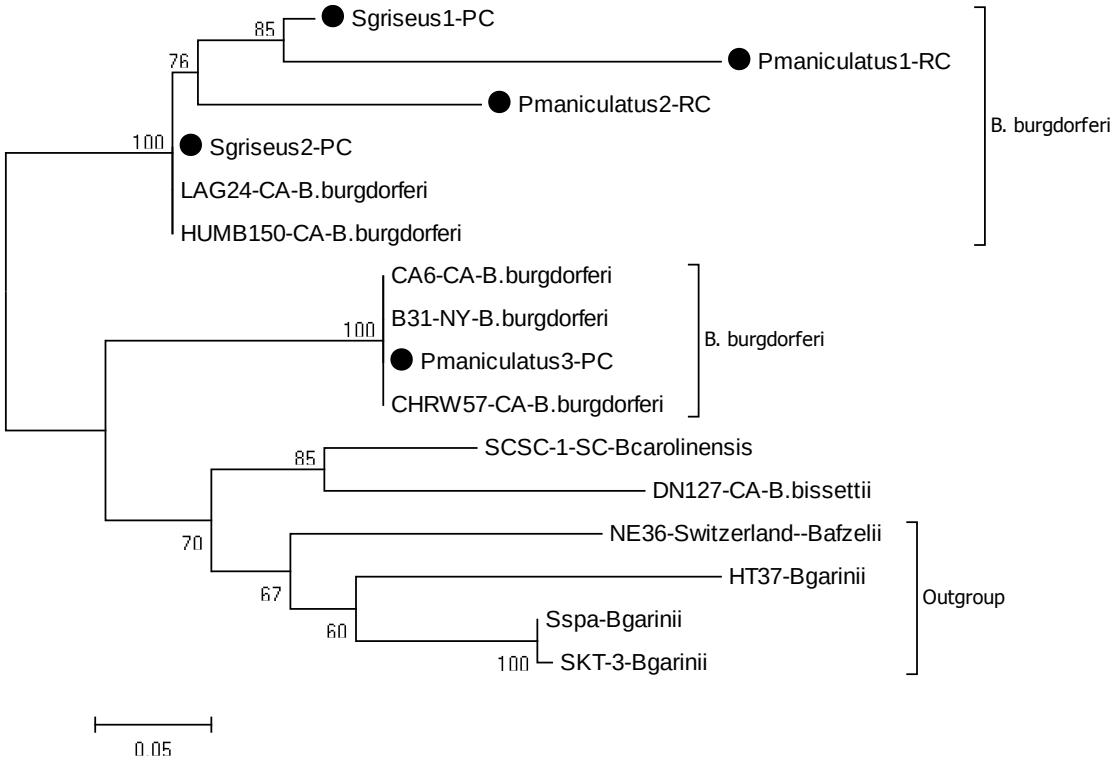


Figure 12. Molecular phylogeny of the *B. burgdorferi* s.l. ospC locus. MEGA 6 was used to generate phylogenies utilizing the Maximum Likelihood method based on the Kimura 2-parameter model with discrete gamma distribution (Kimura 1980; Tamura et al. 2013) with model parameter values from ModelTest. The percentage of trees (out of 1000 bootstrap replicates) in which the associated taxa clustered together is shown next to the branches, with branch lengths measured in the number of substitutions per site. Sequences obtained from this study are labeled with black dots, species name, number, and site, while sequences obtained from GenBank are labeled with strain, location, and species. All five isolates are contained within well-supported *B. burgdorferi* clades.