Detection of *Borrelia burgdorferi*, *Ehrlichia chaffeensis*, and *Anaplasma phagocytophilum* in Ticks (Acari: Ixodidae) from a Coastal Region of California

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ABSTRACT A study was conducted in Santa Cruz County to estimate the prevalence and distribution of the agents of Lyme disease, human granulocytic (HGE), and human monocytic (HME) ehrlichiosis in 1,187 adult ixodid ticks collected from eight public-use recreation areas over a 2-yr period. *Borrelia burgdorferi*, the causative agent of Lyme disease, was detected by a polymerase chain reaction (PCR) in 44 of 776 (5.67%) *Ixodes pacificus* ticks and in 3 of 58 (5.17%) *Dermacentor variabilis* ticks. *Anaplasma phagocytophilum*, the causative agent of HGE, was detected by PCR in 48 (6.19%) *I. pacificus* ticks and 5 (8.62%) *D. variabilis* ticks. *Ehrlichia chaffeensis*, the causative agent of HME, was detected by nested PCR in just five (0.64%) *I. pacificus* ticks and four (6.9%) *D. variabilis* ticks. Interestingly, eight (1.03%) *I. pacificus* ticks were co-infected with *B. burgdorferi* and *A. phagocytophilum*, and just one (0.12%) tick was co-infected with *B. burgdorferi* and *E. chaffeensis*. Less than 1% of 353 *Dermacentor occidentalis* ticks showed evidence of infection with any of the agents tested. To our knowledge, this is the first reported identification of *A. phagocytophilum* and *E. chaffeensis* in *D. occidentalis* ticks from California. This study represents the first extensive survey of Lyme and the ehrlichial diseases across multiple areas of Santa Cruz County, and suggests that prevalence of *B. burgdorferi* in Santa Cruz County may be higher than other areas of the state.

KEY WORDS *Borrelia burgdorferi*, *Ehrlichia chaffeensis*, *Anaplasma phagocytophilum*, *Ixodes pacificus*, California

LYME BORRELIOSIS (LYME DISEASE) is a multiphased multisystem disease characterized by dermatological, musculoskeletal, and neurological manifestations. The etiological agent, *Borrelia burgdorferi* sensu lato, has been identified in a variety of tick species across the globe, and is the most common tick-borne zoonosis in humans worldwide (Steere 1989). In California, the western black-legged tick, *Ixodes pacificus* is the primary vector (Lane et al. 1991). Despite recent studies implicating the nymphal stages as the primary human vector (Clover and Lane 1995), more than 40% of reported cases with erythema migrans in California (1991–1999) were acquired during the winter and spring months when nymphal stages are not thought to be highly active (Monsen et al. 1999). Other tick species native to the state may become infected with the spirochete, but studies have shown that they may act as poor vectors for the disease (Johns et al. 1998). The Centers for Disease Control and Prevention (CDC) reported 1,619 cases in California from 1990 to 1999 and designated several northwestern counties of the state as endemic. Previous studies, often using microscopic identification rather than more specific sensitive molecular techniques, have estimated prevalence of 1–2% in adult ticks in selected areas of northern California, including Contra Costa (Kramer and Beesley 1993) and Mendocino (Lane and Loye 1989) counties. However, prevalence estimates for many parts of this large and ecologically diverse state remain limited.

Human ehrlichioses are recently described tick-borne diseases caused by rickettsiae of the genera *Ehrlichia* and *Anaplasma*, which are closely related to several veterinary pathogens. Pathogenesis of these diseases is poorly understood, and manifestations include a wide array of symptoms involving the hematopoietic, immune, and nervous systems. Human monocytic ehrlichiosis (HME) is caused by *Ehrlichia chaffeensis*, which infects mononuclear leukocytes. It was first recognized in Arkansas in 1986 (Maeda et al. 1987) and in California in 1994 (Vugia et al. 1996). Human granulocytic ehrlichiosis (HGE) is caused by *Anaplasma phagocytophilum* (Dumler et al. 2001), which infects polymorphonuclear leukocytes. It was first described in Minnesota and Wisconsin in 1994 (Bakken et al. 1994, Chen et al. 1994) and in California in 1995 (Gewirtz et al. 1996). Although most cases of

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HME have been reported from the southern United States, and most cases of HGE from the midwest and northeast, at least three cases of HGE and two cases of HME have been identified in California (California Department of Health Services 1999). Two of the HGE cases occurred in Santa Cruz County (Barlough et al. 1997). Most cases of ehrlichiosis in horses occur during the winter and early spring months (Vredevoe et al. 1999), correlating to the period of peak activity for adult *I. pacificus* ticks, which is implicated as the primary vector for these diseases in California (Barlough et al. 1997, Kramer et al. 1999). Infection has also been demonstrated in the American dog tick, *Dermacentor variabilis,* but not in the Pacific coast tick, *Dermacentor occidentalis* (Barlough et al. 1997, Kramer et al. 1999). The natural reservoirs of HME in California are not known, but for HGE dusky-footed wood rats are implicated (Nicholson et al. 1999). Prevalence estimates of *Ehrlichia* and *Anaplasma* spp. in California tick populations are limited.

Many public-use recreation areas of Santa Cruz County lie along the damp coastal ranges and feature large deer populations, long grasses, and lush shrubbery. These moist habitats are ideal for ixodid ticks, and potentially represent locations of high risk of human exposure to tick bites. Because of the temperate climate, eight public-use recreation areas in particular experience heavy usage even during the cooler winter months, when adult ticks are likely to be questing. Given these conditions, it is probable that the prevalence of *B. burgdorferi* is higher in ticks collected from these niches in comparison to other previously surveyed areas of the state. Furthermore, it is likely that ticks questing in these habitats are also harboring *E. chaffeensis* and *A. phagocytophilum,* as preliminary studies using smaller sample sizes have shown (Barlough et al. 1997, Kramer et al. 1999). Because these two agents were identified in more than one tick species, it is necessary to examine all three commonly found tick species in Santa Cruz County.

Materials and Methods

Tick Collection. A total of 1,187 adult *I. pacificus, D. occidentalis,* and *D. variabilis* ticks were collected from eight public-use recreation areas in Santa Cruz County, CA, between January and May in 2000 and 2001. Collection sites (Big Basin State Park, Castle Rock State Park, DeLaveaga City Park, Forest of Nisene Marks State Park, Henry Cowell State Park, New Brighton State Beach, Sunset State Beach, and Wilder Ranch State Park) were chosen in part because of their year-round public use and high recreational traffic. Additional factors included the identification of potential tick habitats within these locations, characterized by the presence of lush vegetation, such as long grasses and shrubbery, along the edges of well-defined hiking and game trails. A flannel flag was used to collect questing adult ticks from such areas. Ticks were stored individually in 70% ethanol until processing, and species, sex, location, and collection date of each was recorded.

DNA Extraction. Each tick DNA extraction was performed individually using the DNeasy tissue kit (Qiagen, Valencia, CA) following the manufacturer's recommended protocol, with minor modifications as follows: before extraction, ticks were removed from 70% ethanol, individually placed into 1.5-ml microtubes, and allowed to dry. Microtubes were immersed in liquid nitrogen for 15 min before maceration in 180 μl of Buffer ATL (Qiagen) using sterile disposable microtube pestles (VWR, San Francisco, CA). After addition of 20 μl of proteinase K (Qiagen), samples were incubated at 55°C on a heat block for 3 h with occasional vortexing, and DNA was extracted according to the manufacturer's protocol. The DNA was eluted once using 200 μl of Buffer AE (Qiagen) and the eluate reapplied to the purification column to maximize DNA concentration. All eluates were stored in 96-well format deep well blocks (Wheaton, Millville, NJ) at −20°C before polymerase chain reaction (PCR) analysis.

Bacterial Cultures and DNA Controls. *Borrelia burgdorferi* B31 cultures (ATCC 35210) were maintained in BSKII medium supplemented with rabbit serum at 33°C and passaged once per week (Barbour 1984). Cells were enumerated by dark-field microscopy in a Petroff-Hausser counting chamber. Total genomic DNA was extracted from these cultures using the DNeasy tissue kit (Qiagen) following the manufacturer's recommended protocol for bacterial cultures. These extracts served as positive controls for PCR. For *A. phagocytophilum* and *E. chaffeensis,* previously isolated genomic DNA was obtained to serve as positive controls (CDC, Atlanta, GA).

Polymerase Chain Reaction. All 50-μl PCR reactions contained 10 pmoles of each primer (Invitrogen, Carlsbad, CA), 200 μM of each dNTP (Amersham Biosciences, Piscataway, NJ), 5 μl of 10X PCR buffer (100 mM Tris-HCl (pH 8.6), 500 mM KCl, 15 mM $MgCl_2$, 1% Triton X-100, Amersham), 5 μl of template, nuclease-free water (Invitrogen) and 2.5 U of *Taq* cloned hot start DNA polymerase (Amersham). For the amplification of *B. burgdorferi* DNA, primers specific for the flagellin gene, FL6 F (5′TTCAGGGTCTCAAGCGTCTTGGACT) and FL7R (5′GCATTTTCAATTTTAGCAAGTGATG), were used to produce a 276-bp fragment (Picken 1992). Cycling conditions were 5 min at 95°C, then 30 cycles (1 min at 94°C, 1 min at 55°C, and 2 min at 72°C) followed by 7 min at 72°C. Amplification of *A. phagocytophilum* DNA used primers specific for the p44 gene, MSP3 F (5′CCAGCGTTTAGCAAGATAAGAG) and MSP3R (5′GCCCAGTAACAACATCATAAGC), to produce a 334-bp fragment (Zeidner et al. 2000). Cycling conditions were 5 min at 95°C, then 40 cycles (1 min at 94°C, 1 min at 55°C, and 2 min at 72°C) followed by 7 min at 72°C. *Ehrlichia chaffeensis* DNA was amplified using primers specific for the VLPT gene, FB3AF (5′AAGACTGAAACGTTATAGAG) and FB5AR (5′GTGACATCTTAGTTTAATAGAAC), which after a nested PCR produces four amplicons, a 396-bp (three tandem repeats), a 486-bp (four tandem repeats), a 576-bp (five tandem repeats), and a 666-bp

Table 1. Number of adult ticks, by species, collected from public recreation areas in Santa Cruz County, 2000–2001

Location	No. of *I. pacificus* (%)[a]	No. of *D. occidentalis* (%)[a]	No. of *D. variabilis* (%)[a]	Total (%)[b]
Big Basin SP	113 (75.84%)	33 (22.15%)	3 (2.01%)	149 (12.55%)
Castle Rock SP	264 (70.40%)	104 (27.73%)	7 (1.87%)	375 (31.59%)
DeLaveaga CP	91 (75.21%)	30 (24.79%)	0	121 (10.19%)
Forest of Nisene Marks SP	28 (73.68%)	4 (10.53%)	6 (15.79%)	38 (3.20%)
Henry Cowell SP	133 (54.73%)	101 (41.56%)	9 (3.71%)	243 (20.47%)
New Brighton SB	85 (80.19%)	21 (19.81%)	0	106 (8.93%)
Sunset SB	6 (28.57%)	15 (71.43%)	0	21 (1.77%)
Wilder Ranch SP	56 (41.79%)	45 (33.58%)	33 (24.63%)	134 (11.30%)
All Locations	776 (65.37%)[b]	353 (29.74%)[b]	58 (4.89%)[b]	1187 (100%)

SP, State Park; CP, City Park; SB, State Beach.
[a] Percent of ticks collected within each location.
[b] Percent of all ticks collected.

(six tandem repeats) (Sumner et al. 1999). Nested PCR was performed using 0.5 μl of primary PCR product as template and 10 pmoles of each nested primer, FB3F (5′GCCTAATTCAGATAAACTAAC) and FB5CR (5′GTTGATCATGTACCTGTGTG). Cycling conditions were 5 min at 95°C, then 30 cycles (1 min at 94°C, 1 min at 55°C, and 45 s at 72°C) followed by 7 min at 72°C. For the nested reaction, annealing temperature was adjusted to 60°C.

Before evaluating tick DNA extracts from field samples, *I. pacificus* tick DNA testing negative by PCR for *B. burgdorferi*, *A. phagocytophilum*, and *E. chaffeensis* was used in separate analyses to dilute DNA isolated from *B. burgdorferi*, *A. phagocytophilum*, and *E. chaffeensis* to determine the limit of detection (LOD) for each PCR assay. All primer pairs were reacted with homologous DNA templates and a panel of heterologous DNA control templates for each PCR assay to confirm species specificity. The specificity panel included DNA extracts from *B. burgdorferi*, *A. phagocytophilum*, *E. chaffeensis*, *B. coriaceae*, *Escherichia coli* and human genomic DNA (Clontech, Palo Alto, CA). All positive and negative control reactions were carried out at a different location from actual test reactions. All amplifications were performed in 96-well plate formats using MJ Research DNA Engine Tetrad cyclers (MJ Research, Inc., Waltham, MA). With the exception of the nested PCR for *E. chaffeensis*, all of the initial amplifications testing for the presence of a single pathogen were performed at the same time, to minimize possible amplicon contamination.

Gel Electrophoresis. PCR products were diluted 10-fold in Blue Juice (Invitrogen) and subjected to electrophoresis in Vistra-Green (Amersham) stained 2% agarose gels. Images were captured using the Typhoon (Amersham) scanner. All presumptive positive reactions were confirmed with a second round of PCR and gel electrophoresis.

Results

During the months of January to May in 2000 and 2001, a total of 1187 ticks were collected from eight public recreational areas in Santa Cruz County (Table 1). These included 776 *I. pacificus* adults (370 females, 406 males), 353 *D. occidentalis* adults (162 females, 191 males), and 58 *D. variabilis* adults (24 females, 34 males). *Ixodes pacificus* and *D. occidentalis* ticks were collected from all eight sites, and *D. variabilis* were collected from five of the eight sites.

Each tick was subjected to individual PCR analysis for *B. burgdorferi*, *E. chaffeensis*, and *A. phagocytophilum.* Concentrations of extracted tick DNA averaged 21.96 ng/μl (range = 113.35, SD = 13.61) for all species. Positives were successfully identified throughout the entire range of DNA extraction concentrations. The *B. burgdorferi* assay was determined to have a LOD of 37 fg DNA, corresponding to ≈25 spirochetes (based on an approximate DNA content of 1500 kilobases); the *A. phagocytophilum* assay LOD was 135 fg of DNA, corresponding to ≈90 bacteria (based on an approximate DNA content of 1500 kilobases); the *E. chaffeensis* assay LOD was 350 fg of DNA, corresponding to ≈292 bacteria (based on an approximate DNA content of 1200 kilobases). No amplification product was detected from the negative controls. *Borrelia burgdorferi* was detected in 44 of 776 (5.67%) *I. pacificus*, 3 of 58 (5.17%) *D. variabilis*, and 3 of 353 (0.85%) *D. occidentalis* ticks (Table 2). *Anaplasma phagocytophilum* was detected in 48 of 776 (6.19%) *I. pacificus*, 5 of 58 (8.62%) *D. variabilis*, and 4 of 353 (1.53%) *D. occidentalis* ticks. *Ehrlichia chaffeensis* was detected in 5 of 776 (0.64%) *I. pacificus*, 4 of 58 (6.90%) *D. variabilis*, and 1 of 353 (0.28%) *D. occidentalis* ticks. According to a chi-square test of independence, male and female ticks did not differ significantly in rates of infection for *B. burgdorferi* ($\chi^2(1) = 2.08$, $P = 0.15$), *E. chaffeensis* ($\chi^2(1) = 1.31$, $P = 0.25$), or *A. phagocytophilum* ($\chi^2(1) = 1.41$, $P = 0.24$). Furthermore, a separate chi-square test of independence showed that a positive result was not dependent on the year or the sex of the tick collected ($\chi^2(1) = 0.03$, $P = 0.87$).

Co-infection with *B. burgdorferi* and *E. chaffeensis* was detected in *I. pacificus* (one of 776, 0.12%), but not in *D. variabilis* and *D. occidentalis* ticks. Co-infection with *B. burgdorferi* and *A. phagocytophilum* was detected in *I. pacificus* (8 of 776, 1.03%), but not in *D. variabilis* and *D. occidentalis* ticks. Finally, co-infection with *E. chaffeensis* and *A. phagocytophilum* was not detected in any tick species.

Table 2. Prevalence and distribution of *Borrelia burgdorferi*, *Anaplasma phagocytophilum*, and *Ehrlichia chaffeensis* in adult *Ixodes pacificus*, *Dermacentor occidentalis*, and *Dermacentor variabilis* ticks

Location Pathogen(s)	No. of positive *I. pacificus* (%)[a]	No. of positive *D. occidentalis* (%)[a]	No. of positive *D. variabilis* (%)[a]	Total (%)[b]
All Locations				
B. burgdorferi	44 (5.67%)	3 (0.85%)	3 (5.17%)	50 (4.21%)
A. phagocytophilum	48 (6.19%)	4 (1.13%)	5 (8.62%)	57 (4.80%)
E. chaffeensis	5 (0.64%)	1 (0.28%)	4 (6.90%)	10 (0.84%)
B. burgdorferi + *A. phag.*	8 (1.03%)	0	0	8 (0.67%)
B. burgdorferi + *E. chaffeensis*	1 (0.13%)	0	0	1 (0.08%)
E. chaffeensis + *A. phag.*	0	0	0	0
Big Basin SP				
B. burgdorferi	7 (6.19%)[c]	0	0	7 (4.70%)
A. phagocytophilum	5 (4.42%)[c]	2 (6.06%)	0	7 (4.70%)
E. chaffeensis	0	0	0	0
Castle Rock SP				
B. burgdorferi	13 (4.92%)[d,e]	1 (0.96%)	1 (14.29%)	15 (4.00%)
A. phagocytophilum	13 (4.92%)[d]	1 (0.96%)	0	14 (3.73%)
E. chaffeensis	2 (0.76%)[e]	0	1 (14.29%)	3 (0.80%)
DeLaveaga CP				
B. burgdorferi	4 (4.40%)[f]	0	0	4 (3.31%)
A. phagocytophilum	5 (5.49%)[f]	0	0	5 (4.13%)
E. chaffeensis	0	0	0	0
Forest of Nisene Marks SP				
B. burgdorferi	5 (17.86%)[g]	0	0	5 (13.16%)
A. phagocytophilum	3 (10.71%)[g]	0	0	3 (7.89%)
E. chaffeensis	0	0	1 (16.67%)	1 (2.63%)
Henry Cowell SP				
B. burgdorferi	7 (5.26%)	1 (0.99%)	0	8 (3.29%)
A. phagocytophilum	9 (6.77%)	1 (0.99%)	0	10 (4.12%)
E. chaffeensis	3 (2.26%)	1 (0.99%)	1 (11.11%)	5 (2.06%)
New Brighton SB				
B. burgdorferi	5 (5.88%)[h]	0	0	5 (4.72%)
A. phagocytophilum	10 (11.76%)[h]	0	0	10 (9.43%)
E. chaffeensis	0	0	0	0
Sunset SB				
B. burgdorferi	0	0	0	0
A. phagocytophilum	1 (16.67%)	0	0	1 (4.76%)
E. chaffeensis	0	0	0	0
Wilder Ranch SP				
B. burgdorferi	3 (5.36%)	1 (2.22%)	2 (6.06%)	6 (4.48%)
A. phagocytophilum	2 (3.57%)	0	5 (15.15%)	7 (5.22%)
E. chaffeensis	0	0	1 (3.03%)	1 (0.75%)

SP, State Park; CP, City Park; SB, State Beach.
[a] Percent of this tick species positive for this agent at this location(s).
[b] Percent of all tick species positive for this agent at this location(s).
[c] Two *I. pacificus* ticks with *B. burgdorferi* + *A. phagocytophilum.*
[d,e] Three *I. pacificus* ticks with *B. burgdorferi* + *A. phagocytophilum* and one with *B. burgdorferi* + *E. chaffeensis.*
[f,g,h] One *I. pacificus* tick with *B. burgdorferi* + *A. phagocytophilum.*

Discussion

This study represents the first widespread effort to determine prevalence estimates of the etiologic agents of tick-borne diseases among the adult tick populations in Santa Cruz County. We found evidence that the agents of Lyme disease, HME, and HGE are present in actively questing adult ticks in Santa Cruz County, and that these vectors may be infected with more than one of these pathogens.

Evidence of *B. burgdorferi* was found in 5.67% of 776 adult *I. pacificus* ticks collected in public parklands in Santa Cruz County, supporting the concept that *I. pacificus* is a vector of Lyme borreliosis to humans in California. This finding is notably higher than previously reported prevalence estimates of 1–2% in adult *I. pacificus* collected elsewhere in the state, including Contra Costa (Kramer and Beesley 1993) and Mendocino (Lane and Loye 1989) counties. However, these findings do coincide more closely with other reports suggesting a prevalence of at least 4–5% in Sonoma County (Lane and Lavoie 1988) and Del Norte and Lake counties (Bissett and Hill 1987). Many areas of Santa Cruz County may provide a more suitable habitat for *I. pacificus* ticks and *B. burgdorferi* reservoirs than previously surveyed localities. *Ixodes pacificus* ticks are more likely to quest in locations that are humid and well shaded (Lane et al. 1995), and the damp, foggy coastal mountains and foothills of Santa Cruz County provide such a habitat. The protected parklands surveyed in our study may also provide optimal habitats for tick hosts, such as deer and rodents. However, our sampling transects were placed in areas of likely tick habitat, and areas of lower prevalence may have been overlooked.

In *D. variabilis* and *D. occidentalis* ticks, the prevalence of *B. burgdorferi* was 5.17% and 0.85%, respectively. However, because the number of *D. variabilis* ticks collected represented <5% of the total number of ticks (Table 1) compared with *I. pacificus* or *D. occidentalis* species, our prevalence estimate for this species should be considered with caution. A lower abundance of *D. variabilis* than other species in Santa Cruz County is consistent with previous studies (Furman and Loomis 1984). Although some *D. variabilis* ticks may contain *B. burgdorferi,* they probably act as poor vectors for this pathogen and may not sustain infection because of antimicrobial proteins in their hemolymph (Johns et al. 1998). Because *D. occidentalis* ticks demonstrated such a low prevalence, 0.85%, despite greater numbers collected, similar mechanisms may be active.

Anaplasma phagocytophilum was found in 6.19% of adult *I. pacificus,* 8.62% of *D. variabilis* ticks, and 1.03% of *D. occidentalis* ticks. To our knowledge, this is the first identification of *A. phagocytophilum* in *D. occidentalis* ticks from California. These findings support the concept that *I. pacificus* ticks appear to be the primary vectors for this agent in California (Barlough et al. 1997, Kramer et al. 1999). However, the competency of *I. pacificus* and *Dermacentor* sp. to act as vectors for *A. phagocytophilum* has yet to be demonstrated. The similar rate of infection with *A. phagocytophilum* observed in *I. pacificus* and *D. variabilis* may indicate that the immature stages of both these ticks feed on the same infected hosts. Conclusions regarding actual prevalence in *D. variabilis* ticks should be guarded because of the smaller sample numbers tested.

Ehrlichia chaffeensis occurred in 10 of 1187 ticks (*I. pacificus* 5 of 776, 0.64%; *D. variabilis* 4 of 58, 6.90%; and *D. occidentalis* 1 of 353, 0.28%). To our knowledge, this is the first identification of *E. chaffeensis* in *D. occidentalis* ticks from California. These data confirm the previous findings of infection with *E. chaffeensis* in *I. pacificus* and *D. variabilis* ticks from California, and are consistent in portraying a higher rate of infection in *D. variabilis* than *I. pacificus* ticks (Kramer et al. 1999). Caution should be taken in assigning a prevalence estimate for this potential vector because few *D. variabilis* ticks were tested. Vector competency studies will be required to determine if any of these tick species are capable of transmitting *E. chaffeensis.*

We found the prevalence of *B. burgdorferi* may be higher in Santa Cruz County than in other areas of the state, where prevalence of 1–2% has been reported (Lane and Loye 1989, Kramer and Beesley 1993). We confirmed the presence of *A. phagocytophilum* and *E. chaffeensis* in questing adult ticks from California. For the first time, *A. phagocytophilum* and *E. chaffeensis* were found in *D. occidentalis* ticks. Several ticks in this study supported more than one etiological agent. Studies using a greater number of samples, locations, and habitats for adult ticks and nymphs will be needed to confirm the levels of infection and the availability of competent reservoirs for each of these pathogens. Furthermore, vector competency studies should be completed for both *A. phagocytophilum* and *E. chaffeensis* to determine which tick species are capable vectors for these pathogens. Our results indicate that *B. burgdorferi, A. phagocytophilum,* and *E. chaffeensis* are present in three species of actively questing adult ticks in Santa Cruz County, and represent a potential health risk in public recreation areas.

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