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Nidicolous ticks of small mammals in *Anaplasma phagocytophilum*-enzootic sites in northern California

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Abstract

Ixodes spp. tick-borne zoonotic diseases are present across the Holarctic in humans, domestic animals, and wildlife. Small mammals are reservoirs for the rickettsial pathogen *Anaplasma phagocytophilum* and tick vectors may include catholic-feeding bridge vectors as well as host-specialist or nidicolous ticks. Far western North American communities in which *A. phagocytophilum* is maintained are complex ecologically, with multiple reservoir host and tick species, multiple strains of the bacterial pathogen *A. phagocytophilum* and differences in dynamics of hosts and vectors across heterogeneous landscapes. We evaluated sites in northern California in order to identify primarily nidicolous ticks and the hosts they infest. A total of 667 ticks was found in 11 study sites, including 288 on flags and 379 attached to small mammals. Larvae were over-represented among attached ticks and adults on flags. The most abundant species was *I. pacificus*. Two-hundred fourteen nidicolous ticks were found, most abundantly *I. angustus* and *I. spinipalpis*. All adult *I. ochotonae*, *I. auritulus*, *I. angustus*, *I. jellisoni*, and *I. woodi* were female, while the male:female ratio of *I. spinipalpis* was 1.2:1 and 1:1 for *I. pacificus*. The greatest number of ticks was obtained from *Tamias ochrogenys*, *Peromyscus* spp., and *Neotoma fuscipes*. Of 234 small mammal individuals that were infested with *Ixodes* spp., only 81 (34.6%) were infested with *I. pacificus*. The remaining infested small mammals hosted nidicolous tick species. Eight ticks were PCR-positive, including 6 *I. pacificus* (one adult, one larva, and 6 nymphs), and 2 adult *I. ochotonae* and high PCR prevalences of 18% and 9% were detected in woodrats and chipmunks, respectively. Nymphal *I. angustus* ticks were active year-long with a possible increase in August while larval activity was only observed in December and spring months and adults only during spring and fall. Overall, we show high tick species richness and year-round high levels of infestation on rodents by several different nidicolous ticks in areas where *A. phagocytophilum* is enzootic, including on reported reservoir species.

Keywords

Anaplasma phagocytophilum; Granulocytic anaplasmosis; *Ixodes angustus*; *Ixodes ochotonae*; *Ixodes pacificus*

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Introduction

Many tick-borne pathogens including *Borrelia hermsii*, *Rickettsia rickettsii*, and *Babesia microti* are zoonotic, with an enzootic cycle in wild small mammals as well as a zoonotic cycle in which a bridge vector transfers infection to humans and medically important domestic animals. This is true also for granulocytic anaplasmosis (GA) caused by *Anaplasma phagocytophilum*, which occurs across the Holarctic in humans, domestic animals, and wildlife (Madigan, 1993; Greig et al., 1996; Foley, 2000; Foley et al., 2001, 2004). Small mammals are reservoir hosts including white-footed mice (*Peromyscus leucopus*) in the eastern US (Telford et al., 1996) and woodrats (*Neotoma fuscipes*), squirrels (*Sciurus* spp.), and chipmunks (*Tamias* spp.) in the western US (Nicholson et al., 1999, Foley et al., 2002, Nieto and Foley, 2008, 2009).

The main tick vectors for GA are almost always reported to be catholic-feeding ticks in the *Ixodes ricinus* group including *I. scapularis* in the eastern US and *I. pacificus* in the western US (Foley et al., 2004). These three-host ticks feed primarily on large mammals as adults and utilize small mammals, reptiles, or birds in immature stages. Because transovarial transmission of *A. phagocytophilum* is not considered important (Munderloh and Kurtti, 1995), infection must be acquired by the tick during larval or nymphal stages before it can be transferred to humans or domestic animals by tick of subsequent stages. *I. pacificus* and *I. scapularis* are the most likely bridge vectors to humans, dogs, and horses in North America, but their importance as enzootic vectors may vary across regions. In several regions where the bridge vectors do not occur, studies have documented rodent-specialist ticks maintaining enzootic cycles of GA, including *I. spinipalpis* on the Mexican woodrat (*N. mexicana*) in Colorado and *I. trianguliceps* on small mammals in northwestern England (Zeidner et al., 2000; Bown et al., 2003). These ticks are nidicolous, i.e. living in small mammals. dens and burrows, where they gain access to their hosts. In far western North America, there are multiple reservoir host species, multiple strains of *A. phagocytophilum* (Foley et al., 2009b), and differences in dynamics of hosts and vectors across the diverse landscapes (Foley et al., 2009a). There also are numerous *Ixodes* spp. in the western US. In the present study, we evaluated multiple study sites in northern California and attempted to identify nidicolous ticks and the hosts they infest, any habitat and seasonal differences in their presence, and whether they were infected with *A. phagocytophilum*.

Materials and methods

Study sites, trapping, and sample collection

Small mammal trapping and tick collection were performed at 11 sites in northern California from February 2005 to July 2009 (Table 1). At each site, variable length transects were established by choosing among available deer trails or poorly used human trails or old roads. Flagging was performed over herbaceous and shrubby vegetation as well as duff and litter using a 1-m^2 white cotton flag. In order to obtain small mammals and their attached ticks, extra-large (4×4.5×15 in.) Sherman (HB Sherman, Tallahassee, FL) and Tomahawk (Tomahawk Live Trap, Tomahawk, WI) live traps were set overnight at locations of observed active rodent usage or dens and baited with peanut butter and oats. Rodents were anesthetized with approximately 20 mg/kg ketamine and 3 mg/kg xylazine delivered SC, examined visually for ectoparasites and body condition and given a permanent individually numbered metal ear tag. Ticks were removed with forceps and preserved in 70% ethanol. *Ixodes* spp. were identified to species using keys (Furman and Loomis, 1984; Webb et al., 1990). Larvae were viewed under a compound microscope in a depression slide as well as with a dissecting microscope before identification was confirmed. All work with small mammals was performed under the oversight of the UC Davis Attending Veterinarian and the Institutional Animal Care and Use Committee.

Nucleic acid extraction, real-time TaqMan polymerase chain reaction (PCR), and DNA sequencing

Ticks and small mammal blood samples were assessed for infection by polymerase chain reaction (PCR). DNA was extracted from ticks by surface cleaning with 70% ethanol, followed by grinding in a mortar and pestle, and then boiling in TE buffer for 10 min. Tick extracts were diluted 1:100 in water for PCR. DNA was extracted from mammalian blood using a kit (Qiagen Blood kit, Valencia, CA) following the manufacturer.s instructions. Real-time quantitative PCR was performed targeting the multiple-copy *msp2* gene of *A. phagocytophilum* as previously described (Drazenovich et al., 2006). Each 12-μl reaction contained 5 μl DNA, 1X TaqMan Universal Master Mix (Applied Biosystems), 2 nmol of each primer, and 400 pmol of probe. The thermocycling conditions consisted of 50°C for 2 min, 95°C for 10 min, and 40 cycles at 95°C for 15 s, followed by 60°C for 1 min. Samples were considered positive if they had a cycle threshold (CT) value <50 and characteristic amplification plots. For all reactions, 3 negative water controls were included during each run. In order to confirm that the animals were in fact infected with *A. phagocytophilum*, 2 rodent samples were randomly selected for DNA sequencing. A nested conventional PCR assay targeting the ribosomal RNA 23S-5S (*rrl–rrs*) intergenic spacer region was performed using newly designed external primers ITS2F, 5'-AGGATCTGACTCTAGTACGAG-3' and ITS2R, 5'-CTCCCATGTCTTAAGACAAAG-3', and internal primers ITS2iF, 5'-ATACCTCTGGTGTACCAGTTG-3' and ITS2iR, 5'-TTAACTTCCGGGTTCGGAATG-3' using the following thermocycler conditions: 94 °C for 2 min, followed by 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min, followed by a 5-min extension at 72°C. Amplified DNA was visualized on a 1% agarose gel stained with GelStar (Lonza, Rockland, ME) and then appropriately sized bands were excised and cleaned with a Qiagen gel extraction kit (Qiagen, Valencia, CA). Sequencing was performed in both forward and reverse directions on an ABI 3730 sequencer (Davis Sequencing, Davis, Ca). Sequences were aligned using the Clustal X program and evaluated for homology to previously reported sequencing by a BLAST search of the GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Data analysis

Data were maintained in Excel (Microsoft, Redmond, WA) and analyzed with the statistical package "R" (R-Development Core Team, http://www.r-project.org). The cut-off for statistical significance was P=0.05. A chi-square contingency test was performed in order to evaluate whether the stage distribution of ticks varied between flagged ticks and those removed from small mammals.

Results

A total of 667 ticks was evaluated across 11 study sites, including 288 on flags and 379 attached to small mammals. Of the flagged ticks, 13.1% were larvae, 26.1% nymphs, and 62.5% adults, while the stage distribution among attached ticks was 46.8% larvae, 33.7% nymphs, and 19.5% adults, i.e. significantly more larvae among attached ticks (P=2.2×10^{-16}). The most abundant species was *I. pacificus* (67.7% of all ticks collected) found in all study sites except Henry Cowell State Park and the nearby Fall Creek unit. Altogether, 214 nidicolous ticks were found: *I. angustus* and *I. spinipalpis* were abundant, while only one *I. auritulus* and one *I. soricis* were observed. Because tick collection effort was not equal across locations, statistical comparisons were not performed, but sites from which the greatest numbers of ticks were collected included Hendy Woods State Park, Samuel P. Taylor State Park, and Humboldt Redwoods State Park. Interestingly, all adults of the following species were female, including *I. ochotonae, I. auritulus, I. angustus, I.*

jellisoni, and *I. woodi*. The male:female ratio of *I. spinipalpis* was 1.2:1 and 1:1 for *I. pacificus*.

A greater proportion of ticks 273 (44.4%) was obtained by flagging than from any individual host species (Table 2). Of the *I. pacificus*, 197 (44.0% of total *I. pacificus*) were attached to rodents, while 251 (56.0%) were obtained from flagging. Of the other species of ticks, 24 were detected on flags, including 3 *I. angustus*, one *I. auritulus*, one *I. sculptus*, and 19 *I. spinipalpis*, even though these are species which rarely quest openly. Finally, of 234 small mammal individuals that were infested with *Ixodes* spp., only 81 (34.6%) were infested with *I. pacificus*. The remaining infested small mammals hosted nidicolous tick species.

A total of 628 ticks (those with usable DNA) were tested for *A. phagocytophilum* by PCR, of which 8 were positive, including 6 *I. pacificus* (one adult, one larva, and 6 nymphs) and 2 adult *I. ochotonae*. All except one of the *I. pacificus* were from flags, while the remaining individual, the larva, was removed from a *T. ochrogenys* from Hendy Woods. The 2 *I. ochotonae* were removed from *P. maniculatus* also from Hendy Woods. The mean CT among PCR-positive ticks was 36.1 (range 33.7–39.0). Small mammals hosting ticks were also evaluated for infection by PCR (Table 3). PCR-positive individuals were identified among 18.0% of woodrats and 8.9% of chipmunks with an overall PCR prevalence in study animals of 4.7%. The mean CT for PCR-positive woodrats was 33.3 (range 32.0–36.0) and for chipmunks 36.8 (31.0–39.0). Two samples from woodrats were evaluated by DNA sequencing to confirm infection with *A. phagocytophilum*. Good-quality DNA sequences were obtained and compared to a database, which revealed 99% identity over 300 nucleotides with both the HZ and USG3 strains of *A. phagocytophilum* (GenBank accession numbers AF416766.1 and CP000235.1). Aside from these 2 accessions, identity to other species including *A. marginale*, *Ehrlichia chaffeensis*, and *E. ruminantium* did not exceed 86%, confirming the identity of the target as *A. phagocytophilum*. In order to evaluate the phenology of ticks, we evaluated numbers of *I. pacificus* and *I. angustus* ticks collected in each month (i.e. focusing on tick species with enough individuals to allow for this analysis). Flagging efforts for *I. pacificus* were not standardized precluding statistical analysis, but strong peaks for adults and nymphs were observed from December through April and March to July, respectively (data not shown). Thirty-two larvae were removed from flags in May. Because numbers of rodents caught varied over each sampling time, we presented the number of ticks observed divided by the number of rodents evaluated (Fig. 1). Patterns for rodent-feeding *I. pacificus* were related to those from flagged ticks, with a nymphal peak March through May and a smaller secondary peak in September and October. Very few adults were observed, in spring and November, while larvae were the most abundant ticks, occurring also in spring and October. These results were distinctly different from those of *I. angustus* (Fig. 2). In that species, nymphal ticks were active year-long with a possible increase in August. In contrast, larval activity was only observed in December and spring months, and the December results are strongly influenced by the small number of rodents (n=3) evaluated in that time period. Adult activity also was seasonal during spring and fall, but not in summer months.

Discussion

In this study, we show high tick species richness and year-round high levels of infestation on rodents by several different nidicolous ticks in areas where *A. phagocytophilum* is enzootic, including on reported reservoir species. *I. pacificus*, which is likely both an enzootic and bridge vector for *A. phagocytophilum*, was a common member of the tick fauna in most sites we evaluated and could be obtained both by flagging and by rodent trapping. Nidicolous tick species also found on small mammals in the areas we studied included *I. angustus*, *I.*

ochotonae, *I. spinipalpis*, *I. jellisoni*, *I. sculptus*, and *I. woodi* and in fact, the majority of infested small mammals hosted ticks that were not *I. pacificus*.

In our data, *I. angustus* was a very abundant tick, particularly on chipmunks, woodrats, and deer mice. This species feeds on a variety of small mammals and occasionally on humans, and ranges in California throughout northern coastal mountains and the Sierra Nevada Mountain range including to relatively high elevations (Furman and Loomis, 1984). Interestingly, only female ticks appear to be recoverable from hosts, although males have been found in small mammal nests (Easton and Goulding, 1974). *I. angustus* may be naturally infected with *B. burgdorferi* (Banerjee et al., 1994) and is a competent vector for *B. burgdorferi* sensu stricto (Peavey et al., 2000). As in prior reports, *I. angustus* could be found throughout the year (Furman and Loomis, 1984) in contrast to the strong seasonality observed for both questing and host-attached *I. pacificus*. This implies that pathogen transmission could be continual in contrast to the more seasonal questing activity and resultant probable pulses of pathogen transmission that occur with *I. pacificus* (Eisen et al., 2002).

The next most commonly encountered ticks in the present study were *I. spinipalpis* (n=39) and *I. ochotonae* (n=32). *I. spinipalpis* (which now includes *I. neotomae*; Norris et al., 1997) is a common nidicolous tick of numerous host species including principally rodents, but also lagomorphs and rarely birds and humans (Furman and Loomis, 1984). It occurs across western North America often in chaparral and oak habitats and becomes more nidicolous in more mesic environments (Norris et al., 1997). As for *I. angustus*, nymphs can be collected year-round (Furman and Loomis, 1984), although sample size in the present study was insufficient to observe this. In our study, *I. spinipalpis* was found mostly on woodrats with fewer on deer mice and chipmunks. Prior research indicated that *I. spinipalpis* was an important vector of *Borrelia* sp. (likely *B. bissettii*) among woodrats in California (Brown and Lane, 1992, Brown et al., 2006). *I. ochotonae* is a relatively infrequently encountered tick, but with a broad geographical distribution across most of western North American (Furman and Loomis, 1984). Reported hosts include woodrats, chipmunks, pikas (*Ochotona princeps*), and grey foxes (*Urocyon cinereoargenteus*), and the specimens obtained in this study were removed from deer mice, woodrats, and chipmunks. *I. ochotonae* adults bear a strong resemblance to *I. angustus*, but were distinguished in this data set on the basis of having 2/2 denticles at the base of the hypostome. Two *I. ochotonae* were PCR-positive for *A. phagocytophilum*, but not only were the individual deer mice from which they were obtained not PCR-positive – no deer mice in this study were PCR-positive –, suggesting that these ticks were infected during earlier stages while feeding on other host species. Although this tick was uncommon in this and other studies in western North America, the PCR-positive ticks suggest that further study of this tick is warranted.

In general, nidicolous ticks are relatively host-specialist, non-‘questing’ ticks, relying on intimate associations with hosts in their nest sites to ensure a stable microenvironment and access to feeding resources, although this varies somewhat for some species such as *I. spinipalpis* in different environments (Furman and Loomis, 1984). This is a very different strategy than a catholic-feeding, questing tick such as *I. pacificus* which remains protected in leaf litter and duff, but seasonally emerges to seek mammalian, reptilian, or avian hosts. Because nidicolous ticks either are attached to mammalian hosts during feeding or spend non-feeding periods in host nest sites, standard methods of flagging litter and vegetation may overlook the majority of these ticks in an ecosystem. In the present study, most nidicolous ticks were removed directly from small mammals. However, we did obtain a low number of nidicolous ticks through flagging, notably *I. spinipalpis*, which reinforces the importance of individually examining all flagged ticks. Similarly, work in Colorado demonstrated *I. spinipalpis* questing more than 20 feet from a nearby woodrat house and no

significant association of this tick on sentinel mice near or more distant from woodrat houses (Burkot et al., 2001). Eisen et al. (2006) also recovered host-seeking *I. spinipalpis, I. angustus*, and *I. auritulus* in dense woodlands in Mendocino County, California. We found *I. angustus* abundantly on *Peromyscus* spp. and to a lesser degree on voles (*Microtus californicus*), woodrats, house mice (*Mus musculus*), and rats (*Rattus* spp.). Less common ticks were *I. pacificus* on *Peromyscus* spp. and *I. spinipalpis* on woodrats and *Peromyscus* spp. One study compared ticks in small mammal and reptile communities from the Willamette Valley of Oregon and a coastal site with Sitka spruce (*Picea sitchensis*) and western hemlock (*Tsuga heterophylla*) (Easton and Goulding, 1974). In the Valley, abundant ticks were *I. pacificus* on deer mice, shrews, and alligator lizards (*Elgaria multicarinatus*). *I. angustus* was abundant at both sites: on the coast, notably on chipmunks, deer mice, flying squirrels (*Glaucomys sabrinus*), and shrews and in the Valley on shrews, woodrats, and deer mice. When rodent burrows were excavated, *I. angustus* was disproportionately found in voles. nests compared with nests of deer mice, woodrats, and shrews.

In the present study, pathogen testing revealed *A. phagocytophilum* DNA in *I. pacificus* and *I. ochotonae*. Overall, this is quite a low prevalence (1.3%) of positive ticks, but it is noteworthy that ticks in the present study were tested individually as opposed to some studies where ticks were pooled. Additionally, *A. phagocytophilum* loads in ticks tend to be relatively low, potentially reducing sensitivity of the assay (Foley and Nieto, 2007). Several PCR-positive ticks were adult, and it is not possible to determine what host species infected these ticks because they could have acquired infection while feeding earlier as larvae or nymphs. The finding of PCR-positive larvae could be consistent with transovarial transmission, although this is reported not to occur for *A. phagocytophilum* (Munderloh and Kurtti, 1995). It is more likely that the host on which these ticks fed was infected, but at very low levels that were below the detection ability of the PCR assay. In support of this, previous work has documented that tick-based xenodiagnosis can be more sensitive than direct testing of the rodent host (Levin and Ross, 2004).

Ecological complexity may increase persistence of some diseases in nature, while often also reducing the prevalence of certain pathogens, such as *B. burgdorferi*, in some ecosystems by a dilution effect. Very simple tick–*A. phagocytophilum* systems have been described comprising *I. trianguliceps*–small mammal hosts in England and *I. spinipalpis*–Mexican woodrats in Colorado (Zeidner et al., 2000). At the other extreme, California systems comprise at least 3 reservoir-competent hosts, potentially zooprophylactic lizards, possibly dozens of other tick host species of varying or unknown reservoir competence, multiple strains of the pathogen, and up to 10 different *Ixodes* species, each with different host preferences. Important future research will be to further evaluate the vector competence of nidicolous ticks for *A. phagocytophilum* including through experimental infection studies using natural small mammal hosts.

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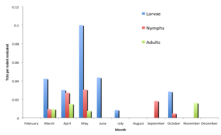

Fig. 1.
Monthly number of *I. pacificus* collected per rodents trapped in 11 sites in northern California.

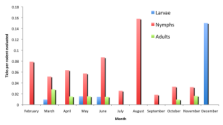

Fig. 2.
Monthly number of *I. angustus* collected per rodents trapped in 11 sites in northern California.

Table 1

Study sites evaluated for granulocytic anaplasmosis in nidicolous ticks and wild rodents across northern and central coastal California.

Study Site	Region	Latitude and longitude (decimal degrees)	Elevation (m)	Distance to coast (km)
Big Basin State Park	Central coast range	37°10.621; 122°12.328	368	21
Hendy Woods State Park	Northern coast range	39°04.25; 123°28.238	120	18
Henry Cowell/Fall Creek State Park	Central coast range	37°04.01; 122°06.40	150	10
Humboldt Redwoods State Park	Northern coast range	40°17.770; 123°59.178	610	26
Hoopa Valley Indian Reservation	Northern coast range	41°10.333; 123°56.520	109–1200	26
King Range National Conservation Area	Northern coast range	40°08.059; 124°07.404	61–610	3.9
Quail Ridge/Cold Canyon Preserves	Northern interior coast range	38°82.06; 122°23.47	300	72
Samuel P. Taylor State Park	Northern coast range	38°01.232; 122°40.774	134	11
Soquel Demonstration Forest	Central coast range	37°06.30; 121°83.19	450–600	8
Sutter Buttes State Park	Central Valley butte	39°12.805; 121°48.167	247	151
Yosemite National Park	Central Sierra Nevada	37°42–54; 119.15–51	1200–3000	217

Table 2

Number of *Ixodes* spp. obtained from flagging vegetation and trapping 14 species groups of rodents in 11 sites in northern California. In addition, one *I. soricis* was found on a *Sorex townsendi*.

	Number of host individuals captured	***I. angustus***	***I. auritulus***	***I. jellisoni***	***I. ochotonae***	***I. pacificus***	***I. sculptus***	***I. Spinipalpis***	***I. woodi***
Flag	N/A	3	1	0	0	249	1	19	0
Sciurus carolinensis	20	2	0	0	0	2	0	0	0
Sciurus griseus	31	0	0	0	0	4	0	3	0
Tamiasciurus douglasii	5	1	0	0	0	0	0	0	0
Spermophilus beecheyi	36	0	0	0	2	2	1	0	0
Spermophilus beldingi	151	0	0	0	0	0	1	0	0
Glaucomys sabrinus	13	4	0	0	0	0	0	0	0
Neotoma fuscipes	185	13	0	0	11	12	0	10	3
Tamias ochrogenys	206	35	0	0	7	128	0	4	0
Tamias merriami	9	0	0	0	1	0	0	0	0
Tamias sonomae	5	2	0	0	0	1	0	0	0
Peromyscus spp.	1134	40	0	3	11	34	0	3	0
Myodes californicus	18	0	0	0	0	1	0	0	0
Microtus californicus	19	0	0	0	0	1	0	0	0

Table 3

Prevalence of *Anaplasma phagocytophilum* infection, based on PCR test results of blood samples in small mammals from 11 sites in northern California.

Host	PCR-negative	PCR-positive	Total	Prevalence (%)
Ground squirrels	5	0	5	0
Voles	2	0	2	0
Woodrats	30	4	42	11.8
Flying squirrels	1	0	1	0
Deer mice	89	0	89	0
Tree squirrels	8	0	8	0
Shrews	5	0	5	0
Chipmunks	61	6	81	8.9
Grand total	201	10	233	4.7