Reservoir Competence of the Redwood Chipmunk (*Tamias Ochrogenys*) for *Anaplasma Phagocytophilum*

Nathan C. Nieto and Janet E. Foley

Abstract

Granulocytic anaplasmosis (GA) is an emerging tick-transmitted disease that persists in rodent-*Ixodes ricinus*-complex tick cycles across the Holarctic. Although the putative reservoir for anaplasmosis in the western United States is the dusky-footed woodrat (*Neotoma fuscipes*), this rodent was not shown reservoir-competent because of failure of infection from woodrats to other animals via ticks. Redwood chipmunks are common in habitats where *Anaplasma phagocytophilum* is common, have high PCR- and seroprevalence, and are infested with a diversity of *Ixodes* spp. ticks. Experimental infection of seven wild-caught *A. phagocytophilum*-negative redwood chipmunks induced persistent periods of recurrent rickettsemia during the persistent phase of infection. Of three animals for which xenodiagnosis was attempted, all successfully infected pools of *I. pacificus* larvae during the primary rickettsemia. We show that chipmunks are reservoir-competent for GA and may be important for maintaining infection in nature.

Key Words: disease ecology—*Ixodes*—sciurid—tick-borne disease.

Introduction

Anaplasma phagocytophilum, which causes granulocytic anaplasmosis (GA), is an emerging pathogen across the Holarctic in humans, domestic animals, and wildlife (Madigan 1993; Greig et al. 1996; Foley 2000; Foley et al. 2001; Foley et al. 2004). It is an obligate intracellular tick-transmitted pathogen, vectored by ticks in the *Ixodes ricinus*-complex (Brown et al. 2005). Its ecology is known in many areas only in basic outline. *Ixodes ricinus* group ticks are three-host ticks, feeding on large mammals as adults and utilizing small mammals, reptiles, or birds in immature stages. Because there is no transovarial transmission of *A. phagocytophilum* infection must be acquired by the tick during larval or nymphal stages before it can be transferred to humans or domestic animals by adult ticks. Reported reservoirs are white-footed mice (*Peromyscus leucopus*) in the eastern United States (Telford et al. 1996), woodrats (*Neotoma fuscipes*) and squirrels (*Sciurus* spp.) in the western United States (Nicholson et al. 1999; Foley et al. 2002; Nieto and Foley 2008), and bank voles (*Myodes glareolus*) and wood mice (*Apodemus agrarius*) in the Europe and Asia (Blanco and Oteo 2002; Bown et al. 2003; Cao et al. 2006).

We have evidence that other sciurids in addition to western gray squirrels, including Douglas squirrels (*Tamiasciurus douglasii*), flying squirrels (*Glaucomys sabrinus*), and chip-

munks (*Tamias* spp.), may be infected with *A. phagocytophilum* and harbor ticks that could transmit the infection to other wildlife or humans as well (Foley et al. 2008a; Foley et al. 2007; Foley et al. 2008b). In a large survey of northern and central coastal California, the highest seroprevalence rates occurred in dusky-footed woodrats (50%), tree squirrels (71%), and some chipmunk species (up to 28%), and the highest PCR-prevalence was in tree squirrels (16%) and some chipmunk species (34%) (Foley et al. 2008). Five different species of ticks were found on redwood chipmunks (*T. ochrogenys*), representing the highest tick species richness found on any rodent species in the study. These findings are consistent with results from the eastern United States, where a PCR-positive eastern chipmunk (*T. striatus*) was reported from Minnesota (Walls et al. 1997). In addition to maintaining active infection, chipmunks in the eastern United States support tick numbers, with a strong correlation between chipmunk numbers and the density of nymphal deer ticks one year later (Ostfeld et al. 2006).

The goal of the present study was to evaluate the reservoir competence of the redwood chipmunk (*Tamias ochrogenys*) for *A. phagocytophilum* in north-coastal California by reporting infection and tick infestation in nature, chronic infection after experimental challenge, and infection to and transmission by *I. pacificus* ticks feeding on infected redwood chipmunks.

Materials and Methods

XL (4 × 4.5 × 15 in.) Sherman (HB Sherman, Tallahassee, FL) and 6 × 6 × 19 in. wire mesh Tomahawk (Tomahawk Live Trap, Tomahawk, WI) live traps were set overnight and checked every few hours throughout the day in Hendy Woods State Park, central Mendocino County, California (39.1284 N, -123.7121 W). Traps were set at locations of observed active rodent usage and baited with peanut butter and oats. Rodents were anesthetized with 20 mg/kg ketamine and 3 mg/kg xylazine delivered SC, examined visually for ectoparasites, bled by retro-orbital abrasion, and the blood anticoagulated with EDTA. In most cases, animals were recovered from anesthesia and transported to the live animal facilities at UC Davis. All blood was kept cool or frozen at -20 to -80°C until plasma could be separated by centrifugation. Once in the laboratory chipmunks were held above water for 10 days to retrieve replete ticks. Ticks were collected from the water and then preserved in 70% ethanol. *Ixodes* spp. ticks were identified to species using keys (Furman and Loomis 1984; Webb et al. 1990). Larvae were viewed under a compound microscope with a depression slide as well as a dissecting microscope before identification was confirmed. All chipmunks were screened for current infection and exposure to *A. phagocytophilum* using PCR and with serology. Only *A. phagocytophilum* free animals with PCR-negative ticks were included in the experiment.

Seven non-infected and unexposed chipmunks (*T. ochrogenys*) individuals were experimentally infected with a well-characterized MRK strain of *A. phagocytophilum*. Each chipmunk received 1.5 ml of purified horse neutrophils infected with *A. phagocytophilum* via intra-peritoneal (IP) injection. After inoculation, each animal was monitored daily for responsiveness, bled weekly via retro-orbital abrasion, and weighed.

Questing adult *I. pacificus* were collected by flagging in western Yolo County, California, an area where extensive work has not identified *A. phagocytophilum* infection (Foley and Nieto, unpub. data). Larval *I. pacificus* were obtained by feeding and mating adult *I. pacificus* on laboratory rabbits and the progeny used for transmission experiments. Three experimentally infected PCR-positive chipmunks were chosen for tick transmission experiments. Each chipmunk was anesthetized with a combination of ketamine (20-40 mg/kg), xylazine (4 mg/kg), and infested with ~200 larval *I. pacificus*. Animals were then held over water as previously de-

scribed for ~10 days or until no replete ticks were recovered. Engorged larval ticks were disinfected with 10% bleach and rinsed with 70% ethanol and sterile saline, then housed in scintillation vials at 24°C, 85% relative humidity, 12 hrs light:12 hrs dark through the molt. Following larval molt and hardening, nymphs were fed on naïve C3H/HeJ mice. Three *A. phagocytophilum* naïve C3H/HeJ mice were each infested with 15 nymphal ticks that fed on infected chipmunks. Infected nymphal ticks were separated as to which infected chipmunk they fed on. Mice were individually housed and bled after six and nine days. Blood was analyzed for infection and exposure to *A. phagocytophilum* using PCR and with serology.

Plasma anti-*A. phagocytophilum* IgG was detected by an indirect immunofluorescent antibody (IFA) assay (Dumler et al. 1995), using *A. phagocytophilum*-infected HL-60 cells as substrate and fluorescein isothiocyanate-labeled goat anti-rat heavy and light chain IgG (Kirkegaard & Perry, Gaithersburg, MD). Samples were tested starting at dilutions of 1:25 and positive and negative control sera were included on each run. Samples were considered positive if strong fluorescence was detected at dilutions of at least 80, consistent with previously published cutoff values (Dumler et al. 1995).

DNA was extracted from 200 µl of whole blood using a kit (DNeasy Tissue kit, Qiagen, Valencia, CA) according to manufacturer's instructions. Real-time PCR was performed using primers and probes as described previously (Drazenovich et al. 2006) in a combined thermocycler/fluorometer (ABI Prism 7700, Applied Biosystems, Foster City, CA). Results were considered positive if the cycle threshold C_T was <40 and there was a characteristic amplification plot. For interpretation, C_T values were rescored. A C_T of 30 was scored as 10, 31 was scored as 9, and so forth, with C_T = 40 scored as 0.

Results

A total of seven chipmunks (1 female, 6 males) was captured and experimentally infected in the laboratory. No animals captured for experimental infection had any evidence of *A. phagocytophilum* infection prior to inoculation. Two larval *I. pacificus* were collected replete from a single chipmunk and tested negative by PCR for *A. phagocytophilum*. All experimentally infected animals became PCR-positive and seroconverted. The first day of active infection varied between 19 days post-infection (PI) and 42 days PI (mean = 30.0, SD = 10.36, Table 1). Infection of the first two animals, designated C1-1 and C2-1, was terminated at 6 wks PI due to

TABLE 1. PCR RESULTS FROM FIVE CHIPMUNKS EXPERIMENTALLY INFECTED WITH *A. PHAGOCYTOPHILUM*-MRK

Chipmunk #	Lowest C_T value	Total duration of infection (days)	First day of detectable infection (days PI)	Successful tick transmission
C1-1	33	21	19	NA
C2-1	31	21	19	NA
C1	37	22	29	NA
C2	38	21	37	Yes
C3	37	35	22	NA
C4	36	14	42	Yes
C5	33	36	42	Yes
Mean (SD)	34.8 (2.91)	NA	30.0 (10.36)	

poor body condition: both were PCR-positive at the time of euthanasia. The total duration of infection in the remaining five animals, (C1-C5), varied from four to seven weeks (Fig. 1). The longest consecutive period of positive PCR test results was for animal C1 which remained positive for 4 weeks, but with a high C_T and was euthanized due to poor body condition (Fig. 1). Four additional chipmunks were PCR-positive for three consecutive weeks: of these, two (C1-1 and C2-1) were euthanized, while two (C3 and C5) continued repeated pulses of positive and negative test results until euthanasia. The remaining chipmunks, C2 and C4, showed brief periods of test positivity interspersed with negative test results (Fig. 1). All chipmunks infected with *A. phagocytophilum*-MRK seroconverted after 9–19 days and remained seropositive for the duration of the experiment, often prior to being detectably PCR-positive. The magnitude of rickettsemia varied from a C_T of 31 to 39 (scores 1 to 9) in PCR-positive animals.

Ixodes pacificus larvae were fed on three of the experimentally infected chipmunks at 30 days PI, at a time when all three were PCR-negative. Two hundred xenodiagnostic larvae were fed on each chipmunk. Fifteen molted nymphs from each infected chipmunk were then fed on naïve C3H/HeJ mice. All three mice became PCR-positive by day 8 and seroconverted by day 13 PI.

Discussion

The redwood chipmunk is reservoir-competent for *A. phagocytophilum* and may be an important component of the ecology of this pathogen in the western United States. We have shown high rates of infection with, and exposure to, this pathogen in redwood chipmunks from numerous sites (Foley et al. 2008b), a high density and diversity of potential vector ticks infesting individuals of this species, and easily-induced chronic infection after experimental inoculation with successful transmission to ticks.

The rates of infection and exposure to *A. phagocytophilum* in chipmunks are as high or higher than woodrats in many locations. Our previous work showed a seroprevalence of 33% and PCR-prevalence of 7% for redwood chipmunks, compared to sero- and PCR-prevalence of 50% and 4%, respectively, in woodrats, even though the woodrat sample was heavily biased by inclusion of a large number of animals from a highly enzootic northern site (Foley et al. 2008). In fact, only the western gray squirrel had rates higher than those for chipmunks.

Tick diversity was high on redwood chipmunks. Previously, we reported also that this species had the highest mean sub-adult tick burden (1.01 ticks/rodent), and third highest adult mean tick burden (0.08 ticks/rodent) compared with other sciurids (Nieto and Foley 2008). Transmission of anaplasmosis using *I. pacificus*, the established vector in California (Richter et al. 1996), was documented in the present study, but there may be other sylvatic or enzootic vectors that acquire infection from chipmunks as well. *Ixodes angustus*, a nidicolous tick that feeds on a variety of rodents and occasionally humans (Furman and Loomis 1984), was found commonly on redwood chipmunks (Foley et al. 2008). This species may be naturally infected with *Borrelia burgdorferi* (Banerjee et al. 1994) and is a competent vector for *B. burgdorferi* sensu stricto (Peavey et al. 2000). It is possible that this tick could transmit infection among rodents and large mammals if it were vector-competent for *A. phagocytophilum*. *Ixodes spinipalpis* also was found on redwood chipmunks but also occurs on woodrats, deer mice, and squirrels (Furman and Loomis 1984; Foley et al. 2008). This tick is reported to function as a primary vector for *A. phagocytophilum* in Mexican woodrats (*N. mexicanus*) in Colorado, where *I. pacificus* is absent, but whether it vectors this pathogen among chipmunks in California is not known (Zeidner et al. 2000).

In contrast to woodrats, which were not documented reservoir-competent because they failed to transmit infection after xenodiagnostic *I. pacificus* tick feeding (Foley et al. 2002),

FIG. 1. Dynamics of *A. phagocytophilum*-MRK infection in seven experimentally inoculated redwood chipmunks (*T. ochrogenys*) based on PCR results.

chipmunks were easily infected with *A. phagocytophilum* experimentally, supported and infected larval ticks, and facilitated transmission of the pathogen through those ticks to naïve mice. Infection with equine-origin MRK-*A. phagocytophilum* strain, which has been used as a model of infection in horses and mice, induced PCR-positive test results, although with relatively low rickettsemia, in 100% of the inoculated chipmunks. Although the TaqMan C_Ts generally suggested low rickettsemia (and at the time of tick feeding, PCR-test results were negative), infection was nevertheless sufficient to infect all pools of ticks fed on these infected chipmunks. These results also suggest that xenodiagnosis in this system was more sensitive than direct hemodiagnosis by PCR, as has been reported previously (Massung et al. 2004). Interestingly, chipmunk infection was of chronic duration, but intermittent, at least within the detectable sensitivity of the PCR. The significance of this waxing and waning of infection is not known, but could relate to cyclical immunity and emergence of novel antigens as has been shown for *A. phagocytophilum* and *A. marginale* infection in other laboratory animal models (Barbet et al. 2001; Barbet et al. 2006).

The ecology of GA in chipmunks is not completely known. These rodents are abundant in and around redwood and Douglas fir forests throughout coastal California from north of Marin County to southern Humboldt County. Closely related species, including *T. sonomae* and *T. senex*, are found just south and north to northeast of *T. ochrogenys*, and these species harbor anaplasmosis as well (Foley et al. 2008a; Foley et al. 2008b). To some extent, chipmunks co-occupy habitats of both woodrats and tree squirrels. They have been trapped frequently on forest floor and at 1 m heights in trees in second- and old-growth redwood and Douglas fir forests, as well as in campgrounds (Nieto and Foley, unpub. data). Thus, the range of redwood chipmunks at both coarse and fine scales considerably overlaps regions of high risk for *I. pacificus* infestation, human and animal GA, and borreliosis (Madigan 1993; Foley et al. 2001; Eisen et al. 2003; VBDS 2006). Chipmunk populations are characterized by high density and relatively low turnover, compared to other rodents such as deer mice (Brand 1974; Carey 1991). It is intriguing to speculate that chipmunks have important roles on the ground maintaining sylvatic granulocytic anaplasmosis, while also potentially bridging infection via ticks to arboreal sciurids as well.

In summary, we show that chipmunks are reservoir-competent for GA and may be important for maintaining infection in nature and thus contributing to risk of infection in humans and domestic animals. These data provide an important starting point for future ecological work to clarify the spatial extent of infection in different chipmunk species, as well as the extent to which chipmunk interactions with other small mammals and diverse tick species could facilitate transmission of infection.

Acknowledgements

We thank Elizabeth Holmes, Nat Lim, and Edwin Saada for laboratory assistance and Karyn Tschida and staff at the UCD Center for Laboratory Animal Science for assistance with animals. Personnel at Hendy Woods and the California State Parks, in particular Pat Freeling and Rene Pasquinelli, provided invaluable access and logistical support. Robert

Lane and Douglas Kelt provided insightful ideas in experimental design. Financial support was provided by the UC Davis Center for Vectorborne Diseases and the National Institutes of Allergy and Infectious Disease Evolution of Infectious Disease program.

Disclosure Statement

No competing interests exist.

References

- Barbet, A. F., J. Yi, A. Lundgren, B. R. McEwen, et al. Antigenic variation of *Anaplasma marginale*: major surface protein 2 diversity during cyclic transmission between ticks and cattle. *Infection and Immunity* 2001; 69: 3057–3066.
- Barbet, A. F., A. Lundgren, R. Alleman, S. Stuenkel, et al. Structure of the expression site reveals extensive global diversity in MSP2/P44 variants of *Anaplasma phagocytophilum*. *Infection and Immunity* 2006; 74: 6429–6437.
- Blanco, J. R., and J. A. Oteo. Human granulocytic ehrlichiosis in Europe. *Clinical Microbiology and Infection* 2002; 8: 763–762.
- Bown, K., M. Begon, M. Bennett, Z. Woldehiwet, et al. Seasonal dynamics of *Anaplasma phagocytophilum* in a rodent-tick (*Ixodes trianguliceps*) system, United Kingdom. *Emerging Infectious Diseases* 2003; 9: 63–70.
- Brand, L. Tree nests of California chipmunks (*Eutamias*). *American Midland Naturalist* 1974; 91: 469–491.
- Brown, R. N., R. Lane, and D. T. Dennis. 2005. Geographic distributions of tick-borne diseases and their vectors, pp. 363–391. In J. L. Goodman, D. T. Dennis and D. E. Sonenshine [eds.], *Tick-Borne Diseases of Humans*. ASM Press, Washington DC.
- Cao, W., Z. Lin, J. He, J. Foley, B. Jiang, et al. Natural infection of *Anaplasma phagocytophilum* in ticks and rodents from a forest area of Jilin Province, China. *American Journal of Tropical Medicine and Hygiene* 2006; 75: 664–668.
- Carey, A. 1991. The biology of arboreal rodents in Douglas-fir forests, pp. 46. United States Dept. of Agriculture Forest Service Pacific Northwest Research Station.
- Drazenovich, N. L., R. N. Brown, and J. E. Foley. Use of real-time quantitative PCR targeting the msp2 protein gene to identify cryptic *Anaplasma phagocytophilum* infections in wildlife and domestic animals. *Vector Borne and Zoonotic Disease* 2006; 6: 83–90.
- Dumler, S. J., K. M. Asanovich, J. S. Bakken, P. Richter, et al. Serologic cross-reactions among *Ehrlichia equi*, *Ehrlichia phagocytophilum*, and human granulocytic ehrlichiosis. *Journal of Clinical Microbiology* 1995; 33: 1098–1103.
- Eisen, R., L. Eisen, M. B. Castro, and R. S. Lane. Environmentally related variability in risk of exposure to Lyme disease spirochetes in northern California: effect of climatic conditions and habitat type. *Environmental Entomology* 2003; 32: 1010–1018.
- Foley, J. Human ehrlichiosis: a review of clinical disease and epidemiology for the physician. *Infectious Disease in Clinical Practice* 2000; 9: 93–98.
- Foley, J., S. Clueit, and R. N. Brown. Differential exposure to *Anaplasma phagocytophilum* in rodent species in northern California. *Vector Borne and Zoonotic Disease* 2008a; 8: 49–55.
- Foley, J., N. Nieto, J. Adjemian, H. Dabritz, et al. *Anaplasma phagocytophilum* infection in small mammal hosts of *Ixodes* spp. ticks in the western United States *Emerging Infectious Diseases* 2008b; 14:1147–1150.
- Foley, J., N. Nieto, S. Clueit, P. Foley, et al. Survey for zoonotic rickettsial pathogens in northern flying squirrels, *Glaucomys*

- sabrinus*, in northern California. *Journal of Wildlife Diseases* 2007; 43: 684–689.
- Foley, J. E., P. Foley, and J. E. Madigan. The distribution of granulocytic ehrlichia seroreactive dogs in California. *American Journal of Veterinary Research* 2001; 62: 1599–1605.
- Foley, J. E., V. L. Kramer, and D. Weber. Experimental ehrlichiosis in dusky footed woodrats (*Neotoma fuscipes*). *Journal of Wildlife Diseases* 2002; 38: 194–198.
- Foley, J. E., P. Foley, R. N. Brown, R. S. Lane, et al. Ecology of granulocytic ehrlichiosis and Lyme disease in the western United States. *Journal of Vector Ecology* 2004; 29: 41–50.
- Furman, D. P., and E. C. Loomis. 1984. The ticks of California (Acari: Ixodida). University of California Press, Berkeley, CA.
- Greig, B., K. M. Asanovich, P. J. Armstrong, and J. S. Dumler. Geographic, clinical, serologic, and molecular evidence of granulocytic ehrlichiosis, a likely zoonotic disease, in Minnesota and Wisconsin dogs. *Journal of Clinical Microbiology* 1996; 34: 44–48.
- Madigan, J. Equine ehrlichiosis. *Veterinary Clinics of North America: Equine Practice* 1993; 9: 423–428.
- Massung, R. F., R. A. Priestley, and M. L. Levin. Transmission route efficacy and kinetics of *Anaplasma phagocytophilum* infection in white-footed mouse, *Peromyscus leucopus*. *Vector Borne and Zoonotic Disease* 2004; 4: 310–318.
- Nicholson, W. L., M. B. Castro, V. L. Kramer, J. W. Sumner, et al. Dusky-footed wood rats (*Neotoma fuscipes*) as reservoirs of granulocytic Ehrlichiae (Rickettsiales: Ehrlichieae) in northern California. *Journal of Clinical Microbiology* 1999; 37: 3323–3327.
- Nieto, N. C., and J. Foley. Evaluation of squirrels as ecologically significant hosts for *Anaplasma phagocytophilum* in California. *Journal of Medical Entomology* 2008; 45: 763–769.
- Ostfeld, R. S., C. D. Canham, K. Oggenfuss, R. J. Winchcombe, et al. Climate, deer, rodents, and acorns as determinants of variation in Lyme-disease risk. *PLoS Biol* 2006; 4: e145.
- Richter, P. J., R. B. Kimsey, J. E. Madigan, J. E. Barlough, et al. *Ixodes pacificus* (Acari: Ixodidae) as a vector of *Ehrlichia equi* (Rickettsiales: Ehrlichieae). *Journal of Medical Entomology* 1996; 33: 1–5.
- Telford, S. R., J. E. Dawson, P. Katavolos, C. K. Warner, et al. Perpetuation of the agent of human granulocytic ehrlichiosis in a deer tick-rodent cycle. *Proceedings of National Academy of Sciences USA* 1996; 93: 6209–6214.
- VBDS. 2006. Vector-borne diseases in California. 2004 Annual Report, pp. 58. Infectious Diseases Branch, California Department of Health Services, Sacramento CA.
- Walls, J., B. Greig, D. Neitzel, and J. Dumler. Natural infection of small mammal species in Minnesota with the agent of human granulocytic ehrlichiosis. *Journal of Clinical Microbiology* 1997; 35: 853–855.
- Zeidner, N. S., T. R. Burkot, R. Massung, W. L. Nicholson, et al. Transmission of the agent of human granulocytic ehrlichiosis by *Ixodes spinipalpis* ticks: evidence of an enzootic cycle of dual infection with *Borrelia burgdorferi* in Northern Colorado. *Journal of Infectious Diseases* 2000; 182: 616–619.

Address correspondence to:

Nathan Nieto
Department of Medicine and Epidemiology
University of California
School of Veterinary Medicine
2108 Tupper Hall
Davis, CA 95616-8737

E-mail: ncniето@ucdavis.edu