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# EFFECT OF EXPERIMENTAL ECTOPARASITE CONTROL ON *BARTONELLA* INFECTIONS IN WILD RICHARDSON'S GROUND SQUIRRELS

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ABSTRACT: The purpose of this study was to investigate the role of ectoparasites in transmitting *Bartonella* infections in wild Richardson's ground squirrels (*Spermophilus richardsonii*). Richardson's ground squirrels were trapped, examined for fleas, and tested for *Bartonella* bacteremia once monthly, at six sites, from April to September 2004. After the initial trapping session in April, burrows at three sites were treated with deltamethrin insecticide. Richardson's ground squirrels trapped on treated sites were less likely to have fleas and had fewer fleas than squirrels on control sites in all months following treatment. We found no difference in the prevalence of *Bartonella* infections on control and treated sites in May, immediately following treatment; however, significantly fewer squirrels were infected with *Bartonella* on treated sites in June and July. We conclude that ectoparasites are a main route of transmission for *Bartonella* infections in Richardson's ground squirrels.

*Key words:* *Bartonella*, deltamethrin, ectoparasite, flea, insecticide, Richardson's ground squirrel, *Spermophilus richardsonii*.

## INTRODUCTION

The genus *Bartonella* is comprised of Gram-negative bacteria, many of which have been linked to a variety of emerging diseases in humans and animals (Breitschwerdt and Kordick, 2000). *Bartonella* species are generally considered to be vector-borne parasites. Known or suspected vectors include sand flies, lice, ticks, and fleas for *B. bacilliformis*, *B. quintana*, *B. vinsonii berkhoffii*, and *B. henselae*, respectively (Anderson and Neuman, 1997).

Several wild rodent-associated *Bartonella* species recently have been linked to human disease, including *B. elizabethae*, *B. grahamii*, *B. vinsonii arupensis*, and *B. washoensis* (Hofmeister et al., 1998; Ellis et al., 1999; Welch et al., 1999; Kosoy et al., 2003). The route of transmission for most rodent-associated *Bartonella* species has yet to be determined, yet this information is vital for understanding how these bacteria are maintained in wild rodents, and how and why they might emerge to infect humans.

*Bartonella* DNA has been detected in fleas (Stevenson et al., 2003), ticks (Chang et al., 2001), lice (Durden et al., 2004) and biting flies (Chung et al., 2004); however, the role of vectors in transmitting *Bartonella* infections among rodent hosts is still unclear. Bown et al. (2004) showed that a rodent flea, *Ctenophthalmus nobilis*, is a competent vector of *B. grahamii* and *B. taylorii* in rodents in a seminatural experimental trial, and Engbaek and Lawson (2004) suggested that ticks were likely important in the transmission of *Bartonella* in rodents in Denmark. Vertical transmission of *Bartonella* infections occurs in cotton rats (*Sigmodon hispidus*) and white-footed mice (*Peromyscus leucopus*) (Kosoy et al.,1998); however, Bown et al. (2004) found no evidence of vertical transmission in bank voles (*Clethrionomys glareolus*). These diverse findings suggest



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TABLE 1. Description and location of sites used for trapping Richardson's ground squirrels from April to September 2004.

| Site ID | Site Description | Latitude/Longitude | Group |
|---|---|---|---|
| A | Horse pasture (mildly to moderately grazed); rural | 52°1′N, 106°36′W | Control |
| B | Unused sheep pasture; within city | 52°8′N, 106°37′W | Treatment |
| C | Edge of crop field (mowed grass); within city | 52°8′N, 106°36′W | Control |
| D | Parking lot of conservation area (sage and grass); rural | 51°5′N, 106°42′W | Control |
| E | Empty lot (mowed grass); within city | 52°11′N, 106°40′W | Treatment |
| F | Alfalfa field; within city | 52°9′N, 106°37′W | Treatment |
| G | Alfalfa field; within city | 52°9′N, 106°37′W | Control |

that different vectors and routes of transmission occur in different settings, and that careful examination of specific *Bartonella*/rodent host systems is necessary to draw conclusions about the nature of transmission in that system.

In a previous study, we found that 49% of Richardson's ground squirrels (*Spermophilus richardsonii*) (RGS), sampled in Saskatchewan, Canada, were infected with *Bartonella* (Jardine et al., 2005). Although *Bartonella* species found in Richardson's ground squirrels are not yet known to be zoonotic disease agents, *Bartonella washoensis*, isolated from a related host, *Spermophilus beecheyi*, has been associated with human disease (Kosoy et al., 2003). The route of transmission of *Bartonella* infections in RGS is not known. In this study, we investigated the role of ectoparasites in transmitting *Bartonella* infections among wild RGS by experimentally controlling ectoparasite numbers in burrows.

## MATERIALS AND METHODS

### Trapping and sample collection

Procedures for trapping and handling RGS were approved by the animal care committee at the University of Saskatchewan (University Committee on Animal Care and Supply Protocol #2020028).

Richardson's ground squirrels were trapped from approximately 1 hr after sunrise until 1 hr after midday at sites around Saskatoon, Saskatchewan, Canada, from April to September 2004. The study sites encompassed a variety of habitats (Table 1) and were chosen based on known presence of RGS.

Sites A through F were randomly assigned to treatment and control groups after the initial trapping session in April. Seven to ten grams of deltamethrin powder insecticide (Deltadust, Bayer CropScience, New Jersey, USA) was applied directly into burrows on three treatment areas using a DR 5 hand duster with burrow injection tube (Birchmeier Co. Ltd., Stetton, Switzerland) between 1 and 6 May, as weather permitted. We replaced control site D with control site G in June because we were unable to trap sufficient animals on site D at that time.

Richardson's ground squirrels were trapped at each site for 2 days each mo using unbaited burrow traps (Wobeser and Leighton, 1979), and were anesthetized prior to processing to facilitate sample collection. Anesthetic induction was performed in a small plexiglass chamber and animals were maintained on a mask, using isoflurane (Isoflo, Abbott Animal Health, Quebec, Canada) in all cases during processing.

Numbered metal ear tags (# 1005-1, National Band and Tag Co., Newport, Kentucky, USA) were placed in both ears for subsequent identification, and sex, age (adult or juvenile), and mass were recorded for each animal. Blood was collected from the medial saphenous vein.

Fleas were collected from the induction chamber and we brushed each RGS with a toothbrush for five minutes to collect additional fleas. We recorded presence or absence and number of fleas/squirrel to evaluate the effectiveness of our ectoparasite control treatment. Because fleas are relatively easy to see and collect, we used them as an index of ectoparasite numbers in general. Animals were released at the point of capture after recovery from anesthetic. We sampled individual squirrels only once per monthly trapping session; however, multiple samples were collected from the same individuals caught in different trapping sessions.

Blood and flea samples were stored in liquid nitrogen prior to transfer to a −70 C freezer in the laboratory. We only cultured blood sam-

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ples for *Bartonella* from RGS at sites where we collected five or more samples during a trapping session. Due to cost constraints we limited the number of samples submitted for culture. On some sites in May, June, and July we collected more blood samples than we could submit. In those cases we cultured roughly equal numbers of samples from different age and sex classes of RGS, from control and treatment sites.

### *Bartonella* isolation

Published procedures for isolating *Bartonella* from blood were used (Kosoy et al., 1997). Briefly, 0.15 ml of whole blood was plated on commercial sheep blood agar (BBC Columbia Agar) and on ATCC GC Agar media prepared at our institution. Plates were incubated at 37 C in an aerobic atmosphere with 7% carbon dioxide for up to 30 days. The plates were checked twice weekly for growth, and isolated colonies were subcultured to confirm purity. Bacterial colonies were tentatively identified as *Bartonella* based on morphology and standard biochemical tests. All morphologically distinct colony types from each sample were identified individually.

### *Bartonella* PCR

DNA was extracted using a standard phenol-chloroform extraction from bacterial colonies that were tentatively identified as *Bartonella*. The extracted DNA was stored in TE buffer at −70 C prior to PCR amplification.

Two oligonucleotides (BhCs781.p and BhCs1137.n), specific for the citrate synthase (*glt*A) gene of *Bartonella*, were used as PCR primers, resulting in a 379-base-pair product (Norman et al., 1995). The PCR mixture consisted of 2 μl of sample DNA, 35.25 μl sterile ultrapure water, 5 μl 10× PCR buffer, 3 μl (25 mM) $MgCl_2$, 0.5 μl (25 mM) dNTPs, 2 μl (20 pmol) of each primer, and 0.25 μl (5 U/μl) Taq polymerase for a total volume of 50 μl. PCR amplifications were carried out in a PTC200 DNA-Engine (MJ Research, Watertown, Massachusetts, USA). The mixture was incubated at 94 C for 2 min, then amplified for 40 cycles at 94 C for 30 sec, 50 C for 60 sec, and 72 C for 60 sec, then held at 72 C for 5 min. To visualize the PCR amplicons, PCR products were separated by electrophoresis in 1.25% agarose gel with ethidium bromide staining, according to standard methods.

### Data analysis

Prior to treatment (April trapping session), we compared treatment and control groups for baseline differences in *Bartonella* prevalence, flea prevalence, and number of fleas/squirrel. In addition to treatment group, age, sex, and trapping month (May to August) were examined for associations with *Bartonella* prevalence, flea prevalence, and number of fleas/squirrel after treatment. We used multilevel mixed models with random intercepts to account for clustering of individual animals within site (for pre- and post-treatment analyses), and to account for repeated measures within individual animals over time (for post-treatment analysis) (MLwiN version 2.0, Centre for Multilevel Modelling, London, UK). A binomial distribution and logit link function were used to model *Bartonella* prevalence and flea prevalence. A Poisson distribution function was used to model flea numbers/squirrel. For each outcome, all factors associated with the outcome of interest at $P<0.25$ in univariate models were combined, and backwards elimination was used to identify a final multivariate model. In the final model, all factors where $P<0.05$ were considered to be significantly associated with outcome.

## RESULTS

### Prior to treatment

There was no significant difference in flea prevalence (Odds Ratio (OR),1.6; 95% Confidence Interval (CI), 0.1–41.5; $P=0.77$), number of fleas/squirrel (Incidence Ratio (IR), 1.0; 95% CI, 0.7–1.5; $P=0.96$), or *Bartonella* prevalence (OR, 2.2; 95% CI, 0.5–9.4; $P=0.29$) on three control (number of squirrels tested [$n$]=40) and three treated ($n=28$) sites prior to treatment in April (Figs. 1, 2; Table 2).

### Post-treatment

From May to September, we collected 140 and 107 samples for culture from squirrels on control and treatment sites, respectively, and we collected flea data from 190 and 159 squirrels on control and treatment sites, respectively. From April to July, we obtained sufficient samples from all sites; however, in August, we obtained samples from less than five squirrels on two treatment sites. Therefore, our estimate of *Bartonella* prevalence

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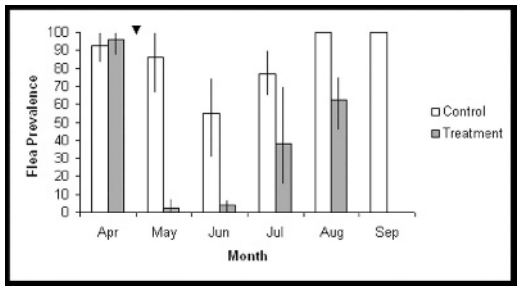



FIGURE 1. Mean prevalence of fleas on Richardson's ground squirrels (with maximum and minimum) on control and treatment sites from April to September 2004. Samples were collected from three control and three treatment sites from April to August and from two control sites in September. Sample sizes same as in Table 2. ▼ indicates timing of burrow treatment.

on treatment sites in August is based on one treatment site (Fig 2). We included flea data collected from these two treatment sites (Fig. 1 and Table 2). In September, we collected samples from only two control sites (Fig. 1) and did not include these data in the analysis.

#### Flea prevalence

Age was not associated with flea prevalence ($P=0.82$). Male RGS were more likely to have fleas than female RGS in a univariate model ($P=0.04$) and sex was included as a factor in model development; however, after adjusting for month and treatment group, there was no association between sex and flea prevalence ($P=0.32$). Treatment group and month (May, June, July, August) were associated with flea prevalence in separate univariate models ($P<0.001$) and together in the final model ($P<0.001$). The odds of finding fleas on animals from control sites were on average 62 times (95% CI, 9–149) greater than on animals from treatment sites, after accounting for month of data collection.

The prevalence of fleas varied over the summer on both control and treatment sites (Fig. 1) and we were interested in investigating possible interactions between session and treatment group. Because 100% of animals on control sites had fleas in August, we either had to omit August data or group it with data from July to investigate interactions between treatment group and month. We compared models that excluded August data with models that grouped August data with July and found no significant differences in the associations found in the two models.

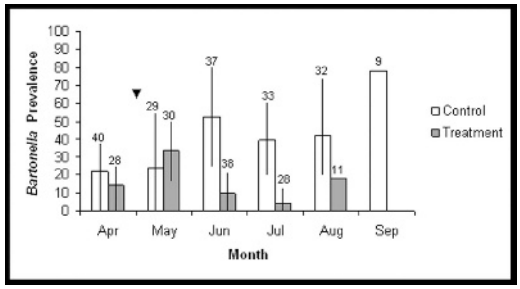



FIGURE 2. Mean prevalence of *Bartonella* infections in Richardson's ground squirrels (with maximum and minimum) on control and treatment sites from April to September 2004. Samples were collected from three control and three treatment sites from April to July, from three control sites and one treatment site in August, and from one control site in September. Sample sizes indicated by numbers above error bar. ▼ indicates timing of burrow treatment.

TABLE 2. Median number of fleas, with maximum and minimum in parentheses and sample size below, collected from Richardson's ground squirrels trapped on control and treatment areas from April to September 2004. Samples were collected from three control and three treatment sites from April to August and from two control sites in September. Burrows were treated in May, prior to May trapping session.

| Month | Control | Treatment |
|---|---|---|
| April | 6.5 (4–8) | 7 (6–7.5) |
| | 40 | 28 |
| May | 3 (3–6.5) | 0 (0) |
| | 39 | 32 |
| June | 1 (0–1) | 0 (0) |
| | 68 | 73 |
| July | 2 (1–4.5) | 0 (0–2) |
| | 38 | 36 |
| August | 5 (1–8) | 3 (0–8.5) |
| | 33 | 18 |
| September | 22.5 (11–34) | — |
| | 12 | — |

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There was a significant difference in the effect of treatment at different times during the summer ($P=0.009$). In May, animals on control sites were 575 times (95% CI, 29–11374, $P<0.001$) more likely to have fleas than animals on treatment sites. In June, animals on control sites were 71 times (95% CI, 9–545, $P<0.001$) more likely to have fleas than animals on treatment sites, and by July/August animals on control sites were only 11 times more likely to have fleas than animals on treatment sites (95% CI, 2–59, $P=0.006$). Although the treatment effect decreased over the summer, animals on control sites were significantly more likely to have fleas than animals on treated sites in May, June, and July/August.

#### Number of fleas/squirrel

Group, month, age, and sex all met the criteria for inclusion in further model building based on univariate models examining associations between these factors and number of fleas/squirrel ($P<0.001$, $<0.001$, 0.002, and 0.09, respectively). Age was not associated with number of fleas/squirrel after adjusting for month, treatment group, and sex ($P=0.2$) and sex was not significantly associated with number of fleas/squirrel after adjusting for treatment group and month ($P=0.06$). Treatment group and month were associated with number of fleas/squirrel in univariate models ($P<0.001$ for both factors) and both factors remained significant after adjusting for each other ($P<0.001$ for both factors). Squirrels from control sites had on average 11 times (95% CI, 5.7–20) more fleas/squirrel than squirrels from treated sites after accounting for the month of data collection.

The number of fleas/squirrel varied over the summer on both control and treatment sites (Table 2) and we found significant interactions between month and treatment group ($P<0.001$). In May, animals on control sites had 162 times (95% CI, 12.6–2,079.5; $P<0.001$) more fleas than animals on treatment sites. In June, animals on control sites had 41 times more fleas than animals on treatment sites (95% CI, 9.7–174.5; $P<0.001$) and by July, animals on control sites had 3.5 times (95% CI, 1.3–9.2; $P=0.01$) more fleas than animals on treatment sites. In August, animals on control sites had only 3.1 times (95% CI, 1.2–8.2; $P=0.02$) more fleas than animals on treatment sites. Although the treatment effect decreased over the summer, animals on control sites had significantly more fleas than animals on treated areas in May, June, July, and August.

#### *Bartonella* prevalence

Month, age, and sex did not meet criteria for inclusion in further model building based on univariate models examining associations between these factors and *Bartonella* prevalence ($P=0.5$, 0.5, and 0.3, respectively). However, treatment group was significantly associated with *Bartonella* prevalence ($P=0.03$), with animals on control sites being on average four times (95% CI, 1.1–14.6) more likely to be infected with *Bartonella* than animals on treatment sites.

*Bartonella* prevalence varied over the summer on both control and treatment sites (Fig. 2) and, although month was not by itself significantly associated with *Bartonella* prevalence, we found potentially important interactions between treatment group and month ($P=0.03$).

In May, there was no significant difference in the prevalence of *Bartonella* infections in animals on control and treatment sites (OR=0.7; 95% CI, 0.1–4; $P=0.7$). In June, animals on control sites were 11 times (95% CI, 2–69; $P=0.008$) more likely to have *Bartonella* infections than animals on treatment sites, and in July, animals on control sites were 22 times (95% CI, 1.3–358; $P=0.03$) more likely to have *Bartonella* infections than animals on treatment sites. In August, we found no significant difference in the prevalence of *Bartonella* infections on treatment and control sites (OR=4; 95% CI, 0.4–46; $P=0.2$).

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### Incidence

Blood samples from seven (27%) of 26 animals trapped on control sites, and two (6%) of 36 animals trapped on treated sites that were culture negative one month, became culture positive (confirmed by PCR) the following month.

## DISCUSSION

Significantly fewer squirrels were infested with fleas and there were fewer fleas/squirrel on areas treated with deltamethrin insecticide compared with control sites for all time periods sampled after treatment. The treatment effect decreased over time, with noticeable increases in flea prevalence on treated sites evident by July, two months after treatment. Mian et al. (2004) found a similar decrease in treatment effect, reporting no difference in the prevalence of fleas on California ground squirrels (*Spermophilus beecheyi*) 56 days after treatment with deltamethrin. Seery et al. (2003), however, found no fleas on black-tailed prairie dogs *(Cynomys ludovicianus)* trapped on a portion of a colony 84 days after treating all burrows in that colony with deltamethrin.

We suspect that the decreasing treatment effect seen in our study was a result of choosing to treat multiple smaller sites which were surrounded by untreated ground squirrel burrows. Although RGS are relatively sedentary, with the majority of female animals moving less than 50 m from their burrows (Michener and Michener, 1977), animals on the periphery of treated areas could move to untreated areas, bringing fleas back to colonize their burrows as the insecticidal activity of deltamethrin decreased with time. Despite the decreasing treatment effect seen over the summer, we obtained sufficient ectoparasite control to allow us to investigate the role of ectoparasites in the transmission and maintenance of *Bartonella* infections.

We found no difference in the prevalence of *Bartonella* infections between control and treatment sites in May despite seeing decreases in flea prevalence and intensity of flea infestations on treatment sites that month. Experimentally infected cotton rats can remain bacteremic for up to 15 wk (Kosoy et al., 1999) and long term bacteremia has also been shown to occur in rodents in the wild (Kosoy et al., 2004a). Although our sample size was small, we found that fewer *Bartonella*-negative animals became *Bartonella* positive the following month on treated areas (6%) compared to control areas (27%) and we suspect that our findings in May are a result of chronic, rather than newly acquired, infections.

Significantly fewer squirrels were infected with *Bartonella* on treated sites compared to control sites in June and July, indicating that ectoparasites are important for transmitting *Bartonella* infections in RGS populations. We were unable to detect any difference in the prevalence of *Bartonella* infections on control and treatment sites in August despite finding significantly fewer squirrels infested with fleas and significantly fewer fleas/squirrel on treated areas in July and August. This suggests the possibility that other routes of transmission might be important for maintaining *Bartonella* infections in RGS. Alternatively (and we think more likely based on our results in June and July) it is possible that our inability to detect a difference in the prevalence of *Bartonella* infections on control and treatment sites in August might have been a consequence of the decreasing effect of treatment on ectoparasite prevalence and numbers in the late summer (Fig. 2, Table 2) combined with small sample sizes in August.

Trapping sufficient animals on certain sites became more challenging as the summer progressed. Not only were there fewer animals to trap as animals entered hibernation, but we found that the animals that were present became more difficult to trap. We were able to collect sufficient samples from control sites in August because we pursued animals at the edge of our defined areas. We could not do this

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on treatment sites because of the risk of trapping animals that lived beyond the treated area. As a result, we trapped fewer animals on treatment sites compared with control sites. Nonetheless, the treatment effect was large and was evident despite small sample sizes.

We measured the prevalence and intensity of flea infestations as an index of ectoparasite numbers in general. Deltamethrin is a nonspecific pesticide, killing fleas, ticks, mites, and lice. Although we found that the prevalence and intensity of flea infestations and the prevalence of *Bartonella* infections decreased in areas treated with deltamethrin, we cannot conclude that fleas are the main vector of *Bartonella* in RGS; further studies would be necessary to determine which specific ectoparasites are important vectors of *Bartonella* among RGS.

Hilton and Mahrt (1971) found few RGS with ticks (1 of 63 animals) or parasitic mites (7 of 63) in a study in Alberta; however, they found that lice (51 of 63) and fleas (22 of 63) occurred commonly. Assuming similar relative occurrence of ectoparasites in our study populations, lice and/or fleas, rather than ticks and/or mites, are more likely to be important vectors for maintaining the high prevalence of *Bartonella* infections found in RGS populations.

*Bartonella* DNA has been detected in fleas and lice taken from rodents (Stevenson et al., 2003; Durden et al., 2004), but this is insufficient evidence to conclude that they are vectors. To date, only fleas have been definitively identified as vectors of a rodent-associated *Bartonella* species (Bown et al., 2004). Transmission trials will be required to determine the role of different ectoparasites as vectors of *Bartonella* infections in RGS.

Concurrent studies have shown that RGS in our area are infected with only four closely related genotypes of *Bartonella* and in fact, the majority of animals (87%), are infected with a single genotype (Jardine et al., unpubl. data). More detailed studies will be required to tease apart the roles that different vectors might have in transmitting different genotypes of *Bartonella* among RGS. We feel that we have started this process by showing that ectoparasites have an important role to play in transmitting *Bartonella* infections, in general, among RGS.

In this 7-mo study, we were unable to detect any association between *Bartonella* prevalence, flea prevalence, and age. In a concurrent 3-yr study, which included some of the same RGS populations used here, we found that juvenile RGS were more likely to have *Bartonella* infections than adult RGS, whereas adult animals were more likely to have fleas than juvenile animals (Jardine et al., 2006). We suspect that our inability to detect associations between *Bartonella* prevalence, flea prevalence, and age in this study are due to the small number of samples collected. We refer readers who are interested in the demographic features of *Bartonella* infections in rodents to larger, long term studies (Fichet-Calvet et al., 2000; Kosoy et al., 2004b; Jardine et al., 2006).

Although we found a significant decrease in the prevalence of *Bartonella* infections on insecticide-treated areas, treatment never completely eliminated *Bartonella* infections. It seems likely that ectoparasites remained sufficiently abundant on treated sites to allow for some transmission of *Bartonella* infections between ground squirrels. It is also possible that other routes of transmission, including vertical transmission, which has been found to occur in other North American rodents (Kosoy et al., 1998), played some role in *Bartonella* transmission on our study sites. However, the results of this study show that ectoparasites are a main route of transmission for *Bartonella* infections in RGS populations.

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