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Bot Fly Infestation of Thirteen-Lined Ground Squirrels in Colorado Shortgrass Steppe

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ABSTRACT We studied prevalence of bot fly infestation of thirteen-lined ground squirrels (*Ictidomys tridecemlineatus)* trapped during 13 years of population monitoring in shrub and grassland habitats in northern Colorado. We also investigated effects of prescribed burning, a common habitat management practice in grasslands, on bot fly prevalence. Infested squirrels were rarely located on shrub sites and during spring (May–Jun) trapping. Across all summers, mean prevalence in grasslands was 7.9% (range: 2.1–23.8%), with years of highest prevalence corresponding to years when the fewest hosts were captured in spring. Infested squirrels had from one to seven warbles, with 46.7% having only one warble. Prevalence did not vary significantly with host sex, age, or body weight. Prevalence was significantly higher (31.6%) in burned sites one year after a prescribed fire and tended to remain higher on burned than on unburned sites. Our results indicate that fires may alter the grassland environment in ways that increase the susceptibility of squirrels to bot fly infestation or the ability of flies to infest hosts.

KEY WORDS bot fly, *Cuterebra*, *Ictidomys tridecemlineatus*, infestation prevalence, parasitism, prescribed fire, shortgrass steppe, thirteen-lined ground squirrel

Many parasites require healthy hosts to survive and successfully reproduce and may not pose an immediate risk unless infestation loads are high. For example, bot flies (Family Oestridae) spend their entire larval cycle inside mammalian hosts (Catts 1982) and benefit when their host is healthy (Slansky 2007). Bot flies of the genus *Cuterebra* (Cuterebrinae) are generally host-specific (Catts 1967, Sabrosky 1986) and typically infest small North American rodents, including mice, chipmunks and tree squirrels (e.g., Catts 1967, Sabrosky 1986). Although bot fly infestation is rarely fatal, it can cause some energy loss and malnutrition, and secondary infection at the site of larva emergence (Catts 1982, Munger and Karasov 1994, Cramer and Cameron 2006, Slansky 2007, Careau et al. 2010). High loads of bot flies also may interfere with a host's ability to forage, escape predators, and reproduce (Wecker 1962, Miller and Getz 1969, Smith 1978).

Host traits such as age and sex may affect the rate of bot fly infestation (Jacobson et al. 1961). Further, habitat structure may influence parasitism rates. For instance, Miller and Getz (1969) reported higher rates of bot fly parasitism in rodents in upland woodlands than in swamp areas, whereas Blair (1942) determined that bot fly parasitism was higher in shrub-dominated areas than in grassland vegetation. Prescribed burning, a common habitat management technique in grasslands that alters habitat structure (Converse et al. 2006, Augustine and Skagen 2014), can affect the abundance of rodent hosts and their parasites. For example, Boggs et al. (1991) reported that prevalence of bot flies in rodents was higher in unburned than burned areas of Oklahoma grasslands.

Between 1999–2011, we live-trapped thirteen-lined ground squirrels (*Ictidomys* [*Spermophilus*] *tridecemlineatus*) as part of long-term population studies in northern Colorado (Stapp et al. 2008) to determine prevalence of bot fly infestation. Previous studies have estimated prevalence of bot flies on small mammals, including the white-footed mouse (*Peromyscus leucopus*; Clark and Kaufman 1990, Barko 2003, Jaffe et al. 2005, Brown and Fuller 2006), Ord's kangaroo rat (*Dipodomys ordii*; Gummer et al. 1997), red-backed vole (*Myodes gapperi*; Lemaitre et al. 2009), eastern gray squirrel (*Sciurus carolinensis*; Jacobson et al. 1961), and eastern chipmunk (*Tamias striatus*; Bergstrom 1992, Jaffe et al. 2005, Careau et al. 2010). However, there are no published reports of bot fly parasitism of thirteen-lined ground squirrels, despite the fact that these ground squirrels are widespread and common in Great Plains grasslands (Streubel and Fitzgerald 1978). Catts (1982) reported that *Cuterebra* infest tree squirrels and other small rodents but not ground squirrels. None of 179 and 46 thirteen-lined ground squirrels trapped in Kansas and Alberta, Canada, respectively, had bot fly warbles (Clark and Kaufman 1990, Gummer et al. 1997). Moreover, a review of external parasites of thirteen-lined ground squirrels from Indiana (Whitaker 1972) did not mention any bot flies. Similarly, Wilson et al. (1997) failed to detect evidence of bot fly parasitism in 103 spotted ground squirrels (*Xerospermophilus spilosoma*), an ecologically similar species of small ground squirrel that cooccurs with the thirteen-lined ground squirrel across much of its southwestern range (Streubel and Fitzgerald 1978). Aside from two brief, anecdotal references (Lugger 1896, cited by Woods 1980, Sabrosky 1986), there are no published records to indicate which bot fly species might parasitize the thirteen-lined ground squirrel. Taxonomic keys for identifying bot flies based on morphology (Baird 1972, Sabrosky 1986), typically rely on traits of adult flies, which live less than 2

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weeks after they emerge from the soil, and thus are extremely difficult to observe and capture (Catts 1982).

The objectives of our study were to 1) determine prevalence of bot fly infestation in thirteen-lined ground squirrels in north-central Colorado, 2) compare prevalence of infestation of squirrels in burned and unburned grassland habitats to examine the effects of prescribed fires on infestation, and 3) identify the bot fly species by molecular genetics analysis of samples of late-instar larvae collected from ground squirrels.

STUDY AREA

Information on rates of bot fly infestation of thirteen-lined ground squirrels from 1999–2011 was collected during live-trapping studies conducted as part of the Shortgrass Steppe-Long Term Ecological Research (SGS-LTER) project. Our study site was the Central Plains Experimental Range (CPER; 519720E, 4517164N), a research station of the U.S. Department of Agriculture Agricultural Research Service (USDA-ARS) located approximately 14 km north of Nunn, Colorado, USA. The climate was semi-arid, with most (79%) of the 341 mm of mean annual precipitation falling as rain during the April–September growing season. Vegetation was characterized as shortgrass steppe, which was dominated by two perennial, warm-season shortgrass species (blue grama, [*Bouteloua gracilis*]; buffalograss, [*Buchloe dactyloides*]), although some areas also had woody shrubs, especially four-wing saltbush (*Atriplex canescens*).

METHODS

Field Methods

In Colorado, adult squirrels are typically active aboveground from April to August, with young squirrels entering hibernacula for the first time in September (Streubel and Fitzgerald 1978). We live-trapped squirrels during spring (May/Jun) and summer (Jul) each year on six, 3.14-ha sites (three grassland, three shrub) from 1999–2011. Trapping times corresponded to periods after adult emergence from hibernacula and mating (spring), and after emergence of juveniles from natal burrows (summer). In addition, we trapped squirrels in July on three additional grassland sites that were burned in the previous autumn to examine the effect of prescribed fire on squirrel populations. We studied bot fly infestation at one, two, three, and four years post-fire. Three sites were burned by USDA-ARS during autumn 2007 and we subsequently trapped squirrels on these same sites in July each year from 2008 to 2011. Other grassland sites that were burned in autumn 2008, 2009 or 2010 were trapped only once, in July 2011, representing different numbers of years post-fire.

Our trapping sites consisted of twelve 100-m linear transects arranged in a spoke-like fashion, with large, non-folding Sherman live-traps (Model LNAHD, 7.6 cm × 8.9 cm × 22.9 cm, H. B. Sherman Company, Tallahassee, FL, USA) every 20 m along each transect and two traps placed haphazardly at the center, for a total of 62 traps (a "trapping web" *sensu* Anderson et al. 1983). We baited traps with peanut butter and oats, and covered traps with polyvinyl chloride (PVC) pipe to shade traps from sunlight. We set traps at dawn and checked them during mid-morning for four consecutive days in each session (a total of 744 trap-days per habitat type per session); our trapping effort was similar across years. We collected information on sex, age, weight, and physical condition of squirrels, including presence of parasites. A combination of weight and pelage characteristics was used to determine age. We marked each individual with a colored Sharpie marker to distinguish recaptures from newly caught individuals, and released individuals unharmed at their capture locations and used data from squirrels only on their first date of capture. Prior to analysis, we combined juvenile and subadult squirrels into one age class (young-of-year; YOY). In some instances, we recorded evidence of multiple bot fly warbles on each host. Because we did not consistently record exact numbers, squirrels were categorized as having no flies, one bot fly, or more than one bot fly.

We calculated two metrics of bot fly infestation. First, prevalence was the proportion of individual hosts that were infested with bot flies, and was calculated for different sex, age, and weight classes. Because of the low numbers of host captures on any given site (typically <20) and the relative rarity of infested squirrels (typically <10%), we pooled captures across sites in a given season and year to estimate prevalence. For the same reasons, we combined data across sites and years to analyze prevalence of infestation by sex, age, and body weight. Second, for hosts that had at least one bot fly, we calculated the proportion of infested hosts that had one fly versus more than one fly as a rough measure of intensity. Here, intensity was estimated by dividing the number of hosts that had multiple fly larvae by the total number of infested hosts (e.g., squirrels with at least one warble).

Laboratory Methods

We collected late-instar larvae during July 2011 and stored individuals in separate vials in 95% ethanol. Unfortunately larvae were degraded somewhat between the time of collection and laboratory analysis, which precluded characterization of fine morphological features. Because no infested squirrels or warbles were observed from 2012–2014, we were unable to collect additional samples.

We dissected three larvae to obtain tissues from interior body and cuticle for DNA extraction. We extracted DNA using a DNeasy Blood and Tissue kit (QIAGEN®, Valencia, CA, USA) following the manufacturer's recommendations, with the adjustment of incubating samples overnight and eluting with 100 µL instead of 200 µL. We eluted three samples a second time and measured DNA concentration (ng/µL) using a NanoDrop spectrophotometer. We chose interior tissue samples for further analysis because they had the highest DNA concentration of all purified samples.

We used polymerase chain reaction (PCR) to amplify 657 base-pair (bp) regions of the cytochrome oxidase subunit I (*COI)* gene. The *COI* gene is a region of mitochondrial DNA that often is used for species identification (Hebert et al. 2003). The total reaction volume was 50 µL, including 1 µL of forward primer, 1 µL of reverse primer, 3 µL of DNA sample, and 45 µL of Platinum Taq Master Mix (Invitrogen™ Life Technologies). Samples were run in a BioRad t-100 Thermocycler at 94° C for 3 min, followed by 35 cycles of 94° C for 30 s, 49° C for 40 s, and 72° C for 1 min, with a final extension of 72° C for 5 min. We used universal primers (LC01490f: GGTCAACAAATCATAAAGATATTGG) and (HC02198r: TAAACTTCAGGGTGACCAAAA AATCA; Folmer et al. 1994) to create sequences.

We compared sequences from our samples to unpublished sequences generated using the same primers for seven other bot fly species from the subfamily Cuterebrinae (*Cuterebra fontinella, C. bajensis, C. lepusculi*, *C. atrox, C. austeni, C. tenebrosa, C. emasculator*; B. Wiegmann, North Carolina State University, unpublished data). We visualized PCR products on a 2% agarose gel using gel electrophoresis and sequenced them by SEQUETECH (Mountain View, CA, USA). Using bidirectional sequences, we assembled and aligned consensus sequences using CodonCode Aligner software (CodonCode Corporation, Centerville, MD, USA) and aligned using clustW in MEGA 5.1, respectively (Tamura et al. 2011). We conducted phylogenetic analysis with maximum-likelihood, maximum-parsimony, and neighbor-joining models using MEGA 5.1.

RESULTS

Between 1999 and 2011, the earliest date that bot fly warbles were reported on squirrels was 16 May, but only six of 105 (5.7%) instances of bot fly infestation were recorded in spring. Combining across all years, prevalence of bot flies was significantly lower (Fisher's exact test, $P < 0.001$) in May and June (1.1% of 571 hosts) than in July (5.8% of 1,055 hosts). In addition, prevalence was significantly higher (Fisher's exact test, $P < 0.001$) for squirrels from grassland sites (5.3% of 1,215 hosts) than from shrub sites (0.7% of 411 hosts). Therefore, we restricted our analysis to data from grassland sites trapped in July.

On average, 91.9 (SD = 28.6) squirrels were captured annually between 1999 and 2011. Squirrel numbers peaked in 2006 (135), then declined through 2011 (Fig. 1a). Mean prevalence of infestation was 7.9% of squirrels captured in a given summer (SD = 5.9%, $n = 13$ summers); prevalence was highest in 2008 (Fig. 1b), with little variation in other years. Prevalence varied little (7–16%) by weight class, although because of the large number of YOY squirrels captured, most (79%) of the warbles were detected on YOY squirrels (typically ≤100 g; Fig. 2). Prevalence was similar ($\chi^2_3 = 0.15$, $P = 0.99$) among ages and sexes (adult females, 0.083, $n = 97$; adult males, 0.070, $n = 71$; YOY females, 0.074, $n = 296$; YOY males, 0.071, $n = 350$). Also, intensity of infestation was similar ($\chi^2_3 = 1.98$, $P = 0.58$) among different sex and age classes, with most (53.3% of 60 hosts) infested hosts having more than one warble, and adult females tending to have multiple warbles more often (75%) than other sex-age groups. For hosts where the actual number of warbles was reliably recorded, the number of larvae ranged from one to seven (all ages and sexes of hosts combined).

On average, bot fly prevalence was higher ($t_3 = 3.92$, $P = 0.03$) for squirrels from the three sites that were burned in 2007 than for squirrels from the three unburned grassland sites sampled during the same years (Fig. 3), although confidence intervals overlapped considerably in any given year. To determine if prevalence differed based on the number of years since burning, we combined data from the three sites that were burned in 2007 and sampled annually from 2008–2011, with those that were sampled only in 2011 but represented different numbers of years post-fire. Our approach was justified based on the large amount of overlap in confidence intervals, for each time interval, between these two groups. Combining results from sites based on the number of years post-fire, prevalence of bot flies was significantly higher (31.6%) on sites one year after being burned (based on the lack of overlap in 90% confidence intervals; Fig. 4). Prevalence returned to approximately the same rate (11–13%) by the second year post-fire, which was similar to that on unburned grassland sites over the same time period (15.0%; Fig. 4).

Consensus trees generated from *COI* sequences (GenBank Accession Numbers BankIt1727142 Cuterebra KJ922157, BankIt1727498 Cuterebra KJ922158, BankIt1727500 Cuterebra KJ922159) from our unidentified larvae and those from seven other North American Cuterebrinae species indicated that our three samples were, on average, 0.2% different from each other and 4.7% different from *C. fontinella*, a species typically associated with white-footed mice (genus *Peromyscus*; Catts 1982).

DISCUSSION

Our study represents the first description of infestation of thirteen-lined ground squirrels with bot flies. Prevalence was significantly higher in summer than late spring, and higher in grasslands than in shrub-dominated vegetation. These results differ from those of Clark and Kaufman (1990), who determined that, in tallgrass prairie, bot fly parasitism of rodents was higher in shrub areas than grassland vegetation. In our study, prevalence did not vary significantly by host sex, age or body weight, and without individually marking squirrels, it is not possible to determine if bot fly infestation affected the timing of when squirrels enter hibernation or overwinter survival.

Across 13 years of summer sampling in grassland habitats, prevalence ranged from 2.1% to 23.8%, with an average of 7.9%. Prevalence was unusually high in 2008 (and to a lesser extent, 2001), although the reason for this spike is not clear. This year was the third of three consecutive years with low spring (Apr–Jun) precipitation and represented a period during which squirrel population numbers at the site had been declining since 2006 (Fig. 1a). Few squirrels were captured during spring trapping sessions associated with relatively high summer prevalence (7 in May 2008 and 19 in 2001, compared to an average of 32.5 ± 8.2 squirrels in other years), and it is possible that the number of hosts available in spring for recently emerged adult flies may influence prevalence of infestation in summer. Summer trapping success continued to be low from 2012–2014 (*n* = 9, 2 and 0 squirrels, respectively; P. Stapp, unpublished data), and none of these squirrels had warbles. It is not clear if the bot flies could have switched to a different, more abundant rodent host, although, to date, we have no records of bot flies on other small rodents captured at CPER since 1994 (P. Stapp, unpublished data). Nocturnal rodents are uncommon in upland grassland areas where squirrels were abundant, but Ord's kangaroo rats, northern grasshopper mice (*Onychomys leucogaster*),

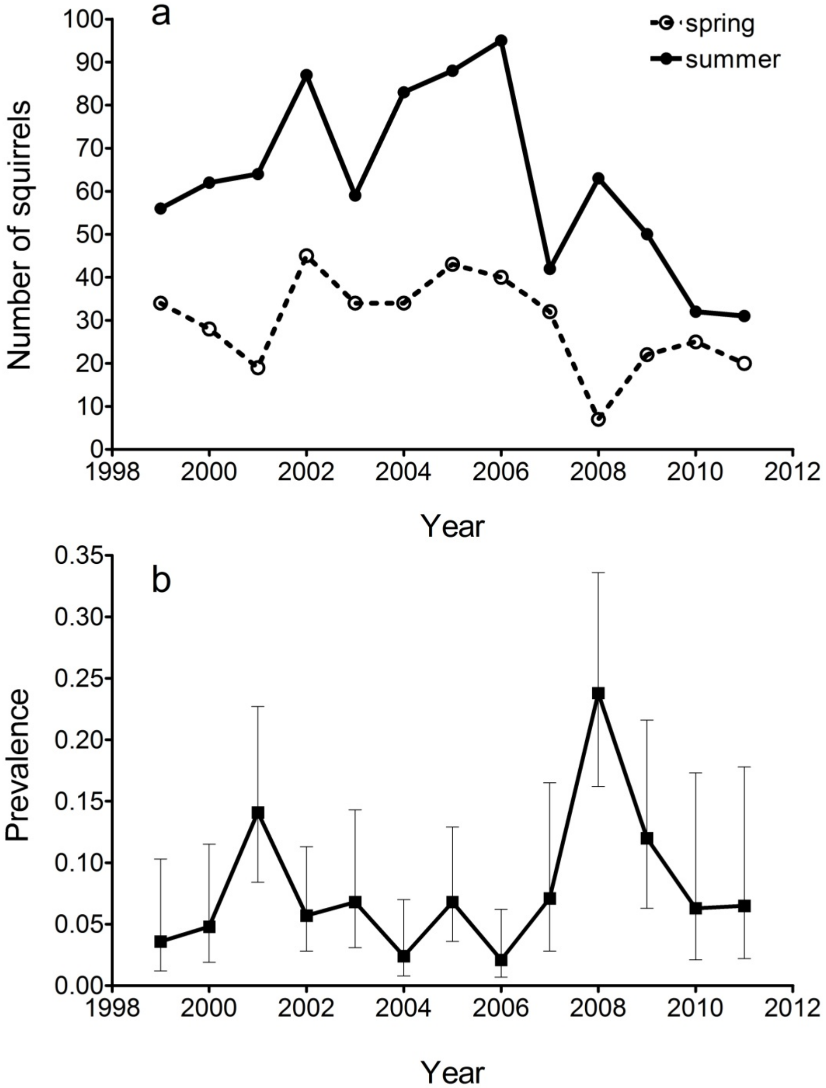

Figure 1. a) Number of thirteen-lined ground squirrels trapped in north-central Colorado during spring and summer from 1990–2011, ranging from 51 to 135 squirrels per year and b) prevalence of bot-fly infestation in thirteen-lined ground squirrels in long-term grassland trapping sites in north-central Colorado during summer from 1999–2011. Error bars are 90% confidence intervals calculated using the Wilson score approximation, as described by Zelmer (2013). Number of hosts in a given year ranged from 31 to 101, with 59 hosts in 2008, the year with the highest prevalence.

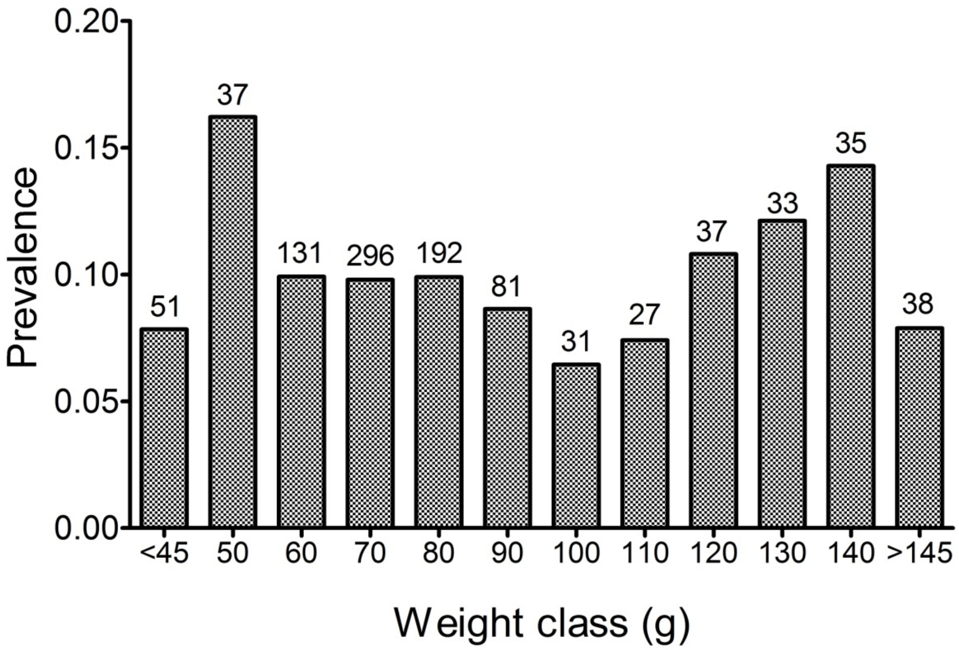

Figure 2. Prevalence of bot fly infestation in thirteen-lined ground squirrels in long-term grassland trapping sites in north-central Colorado in summer from 1999–2011. Values on the x-axis are the midpoints of the 10-g weight classes, combining both sexes. Number of hosts examined in each weight class is shown above bars. Confidence intervals were omitted for clarity and because of the similarity in prevalence values across weight classes.

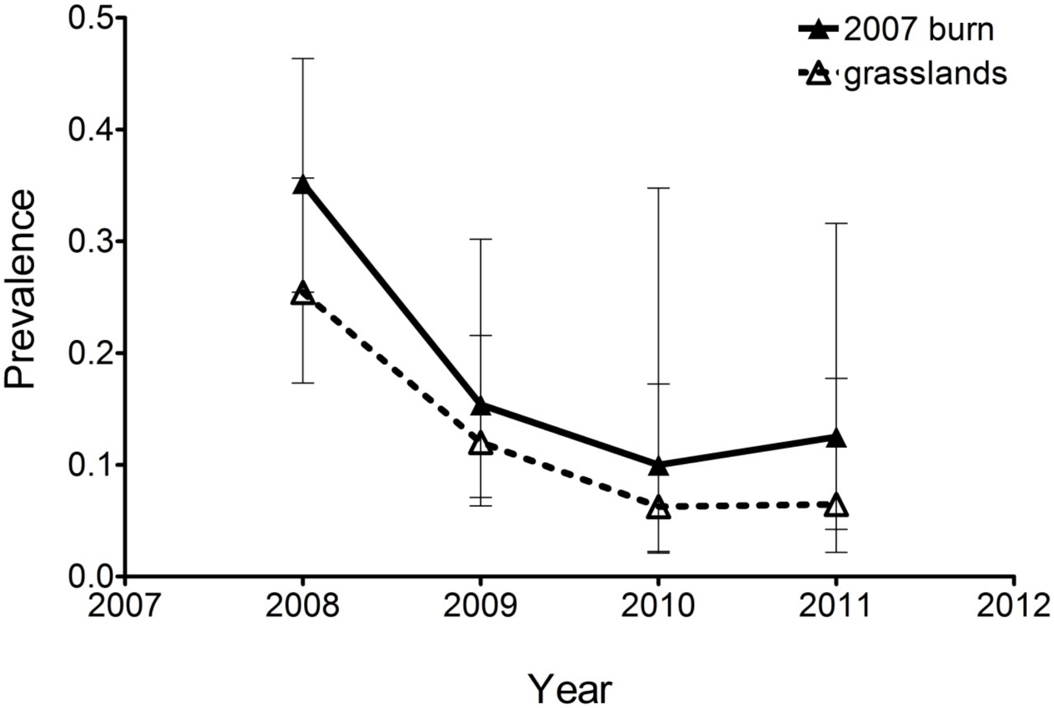

Figure 3. Prevalence of bot fly infestation of thirteen-lined ground squirrels from 2008–2011 in three grassland trapping sites that were burned in autumn 2007, compared to three long-term grassland sites that were never burned. Error bars are 90% confidence intervals calculated using the Wilson score approximation, as described by Zelmer (2013).

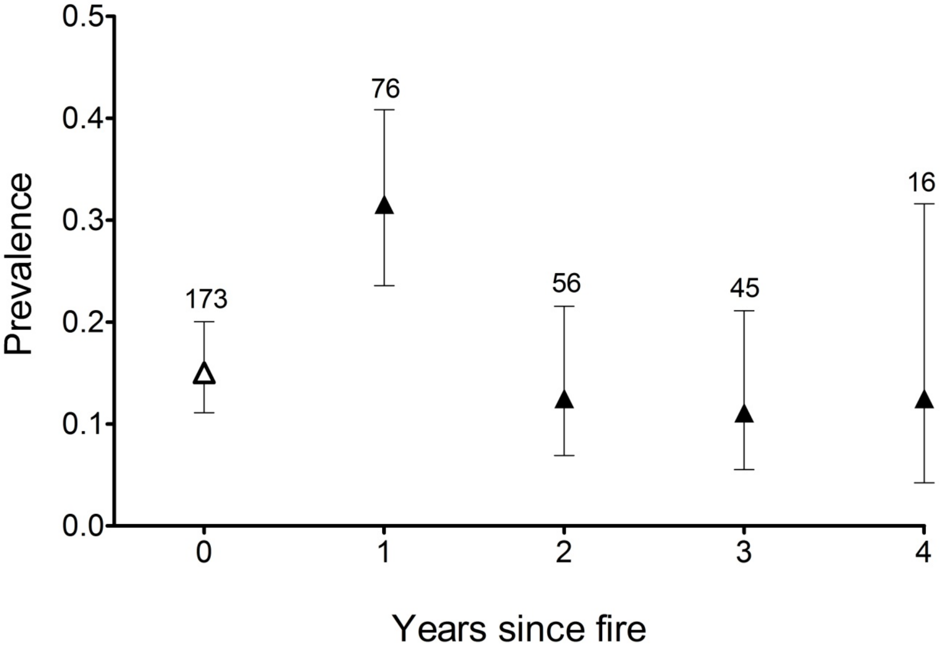

Figure 4. Prevalence of bot fly infestation in thirteen-lined ground squirrels in north-central Colorado on trapping sites in July as a function of the number of years since autumn prescribed burns. Six sites were trapped one, two, and three years post-fire, and three sites were sampled four years post-fire, from 2008–2011. Data from unburned sites (0 years post-fire; open triangle) were from three grassland sites trapped in July from 2008–2011. Number of hosts examined is shown above points. Error bars are 90% confidence intervals calculated using the Wilson score approximation, as described by Zelmer (2013).

deer mice (*P. maniculatus*), and western harvest mice (*Reithrodontomys megalotis*) inhabit shrub-dominated areas (in approximate order of relative abundance), and black-tailed prairie dogs (*Cynomys ludovicianus*) and northern pocket gophers (*Thomomys talpoides*) are common on our study area (Stapp et al. 2008). Nocturnal rodents were trapped twice per year on the same sites where we trapped squirrels and individuals were inspected for injuries and presence of parasites. We have not systematically surveyed prairie dogs or pocket gophers for bot flies.

Prescribed burns affected prevalence of bot fly infestation of ground squirrels, with prevalence significantly higher the first year after an autumn fire, and tending to remain higher on burned sites compared to unburned ones trapped at the same time. These results differ from those of Boggs et al. (2007), who detected lower levels of bot fly infestation of small mammals in burned tallgrass prairie in Oklahoma and argued that fire might have killed eggs and larvae belowground, or that the removal of litter by burning made the microclimate unsuitable for developing larvae. Our results indicate that, in shortgrass steppe, where there is no significant litter layer (Lauenroth and Milchunas 1992), fires may cause environmental changes that increase the susceptibility of squirrels to bot fly infestation. Burning of grasses may alter vegetation near burrows in ways that make it easier for squirrels to come in contact with bot fly eggs. In addition, fires may modify the environment in ways that increase survival of bot flies, such as warming the soil. Warmer soil temperatures during the time larvae pupate may increase the number of bot flies that emerge in spring. Also, autumn fires may alter the suitability of habitat for oviposition by adult flies.

MANAGEMENT IMPLICATIONS

Information on seasonal and inter-annual variation in the frequency of bot fly parasitism across habitat types, and especially, how it is influenced by land-management practices such as prescribed fire, contributes to our knowledge of the ecology of this regionally important species. Future work should attempt to determine the fate of the bot fly populations that parasitize thirteen-lined ground squirrels, especially given the current low squirrel densities, and to identify other possible hosts. The degree to which bot fly infestation, versus other ecological factors, contributed to the recent decline in ground squirrel numbers remains unclear.

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