Nosema bombi (Microsporidia: Nosematidae) and Trypanosomatid Prevalence in Spring Bumble Bee Queens (Hymenoptera: Apidae: *Bombus*) in Kansas

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ABSTRACT: Several species of bumble bees are declining in the United States; these declining populations often show higher prevalence of *Nosema bombi*, a microsporidian pathogen. To date, surveys of bumble bee pathogens in the United States have only been conducted on workers and males, yet the health of a population is ultimately dependent on the success of colony-founding queens. We conducted a molecular-diagnostic survey of the prevalence of *N. bombi* and trypanosomatids, such as *Crithidia bombi*, in six species of spring queens (n = 142) collected in 2011 and 2013 at three sites in central Kansas. *Nosema bombi* was found in 27% of *Bombus pensylvanicus* and 13% of *B. auricomus* but was not found in the other species sampled. Trypanosomatids were only found in *B. pensylvanicus* (9%) during the May 2013 sampling period. The high prevalence of *N. bombi* in *B. pensylvanicus* is consistent with other surveys for this pathogen in other castes, but the high prevalence of *N. bombi* in *B. auricomus* is a novel finding. Although the conservation status of *B. auricomus* has not been thoroughly assessed, two recently published surveys showed that *B. auricomus* were less common in portions of the species' range. Based on those findings and an oft-cited link between *N. bombi* prevalence and bumble bee species' decline (e.g., *B. pensylvanicus*) in other studies, our findings suggest *B. auricomus* populations in Kansas may warrant further scrutiny.

KEY WORDS: *Bombus*, bumble bee, spring queen, *Nosema bombi*, *Crithidia bombi*, Kansas

Bumble bees are important native pollinators in temperate regions. Some species of *Bombus* Latreille (Hymenoptera: Apidae) are declining in North America (Colla and Packer, 2008; Cameron *et al.,* 2011). These declines are notably detectable not only on a range-wide scale (Colla *et al.,* 2012), but also at state (Grixti *et al.,* 2009) or province (Colla and Packer, 2008) levels. Although there are many factors that likely contribute to *Bombus* decline, such as habitat loss, habitat fragmentation, pesticide use and genetic homogeneity, pathogens have also been implicated (Colla *et al.,* 2006; Goulson *et al.,* 2008; Cameron *et al.,* 2011; Lozier *et al.,* 2011). Cameron *et al.* (2011) found that the prevalence of the microsporidian pathogen, *Nosema bombi* Fantham and Porter (Microsporidia: Nosematidae), was higher in the declining species *Bombus occidentalis* (Greene) and *B. pensylvanicus* De Geer than in the stable species *B. vosnesenskii* Radoszkowski, *B. bifarius* Cresson, *B. impatiens* Cresson and *B. bimaculatus* Cresson across their respective ranges. Interestingly, infections of the trypanosomatid pathogen *Crithidia bombi* Gorbunov (Kinetoplastida: Trypanosomatidae) are more common in stable *Bombus* species (Gillespie, 2010; Cordes *et al.,* 2012). Bumble bee colonies have an annual cycle in which all workers, males and old queens die at the end of the season. Only the newly emerged queens survive through the winter. Both *N. bombi* and *C. bombi* are incapable of surviving outside of a host for long, therefore, infected, overwintering queens must survive for the parasites to

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maintain populations in their hosts (Rutrecht and Brown, 2009; Tognazzo *et al.,* 2012).

Nosema bombi is an obligate, intracellular pathogen of multiple *Bombus* species worldwide (McIvor and Malone, 1995). *Nosema bombi* overwinters within host queens, being transmitted vertically from queen to workers most often (Otti and Schmid-Hempel, 2008). Experimental investigations to determine the impact of *Nosema* infections on host colony fitness have yielded contradictory conclusions. Infected queens have smaller colonies and produce fewer reproductives, potentially leading to population declines (Otti and Schmid-Hempel, 2008). Males from infected colonies produce very little viable sperm, and infected gynes exhibit distended abdomens and are unwilling to mate, effectively reducing colony fitness (Otti and Schmid-Hempel, 2007). Conversely, colonies with *Nosema* infections were equally as productive as *Nosema*-negative colonies in a New Zealand study (Fisher and Pomeroy, 1989).

The trypanosomatid pathogen *Crithidia bombi* is an obligate, intestinal pathogen of multiple *Bombus* species (Otterstatter and Thomson, 2006). *Crithidia bombi* can be horizontally transmitted as bees forage, however, successful overwintering requires residence in a *Bombus* queen host (Durrer and Schmid-Hempel, 1994). Although *C. bombi* infection can reduce the overall body mass of overwintering queens and infected queens show a reduction in colony founding capabilities, no effect was observed on their survival (Brown *et al.,* 2003). Colonies that were successfully founded by infected queens were shown to be 40% less fit, displaying smaller colony sizes and producing fewer reproductive males (Brown *et al.,* 2003). Workers infected with *C. bombi* exhibit increased handling time while foraging for nectar, visiting fewer flowers than their uninfected counterparts in timed studies (Otterstatter *et al.,* 2005). The virulence of *C. bombi* may also vary under differing environmental conditions. Under starvation conditions, *C. bombi*-infected *B. impatiens* suffered 50% mortality as compared to 0% in those under no food stress (Brown *et al.,* 2000).

Bombus in North America have co-evolved with these pathogens thus pathogens are unlikely to be the main factor for their decline. However, sub-lethal infections can add additional stress to colonies and exacerbate colony decline (Kissinger *et al.,* 2011). As described above, both pathogens show a potential for reducing queen health and colony fitness in laboratory studies. Queen health in social insects is central to population stability over time, yet most surveys of natural populations have investigated the occurrence of these pathogens in workers and males, rather than queens (e.g., Gillespie, 2010; Kissinger *et al.,* 2011; Cordes *et al.,* 2012). Rates of infection are substantial in the few studies that have documented infection prevalence in colony-founding queens. In Switzerland, 13.5% of *B. terrestris* and 5.6% of *B. lucorum* spring queens had *N. bombi* infections, and 47.5% of *B. terrestris* and 29.9% of *B. lucorum* spring queens were infected with *C. bombi* (Shykoff and Schmid-Hempel, 1991). In New Zealand, about 10% of *B. terrestris* queens collected in fall and spring were infected with *N. bombi* (Fisher and Pomeroy, 1989). No comparable survey has been conducted on spring queens in the United States.

We used a trapping design from a companion ecosystem services survey conducted in Kansas to collect foraging spring queens from several locations across multiple years. The objectives of this survey were to 1) sample queens for *N. bombi* and *C. bombi* pathogens, which have been shown to impact populations of native bumble bees and 2) generate baseline prevalence data for these pathogens in native bumble bee species found in Kansas. Determination of *N. bombi* and *C. bombi* prevalence in

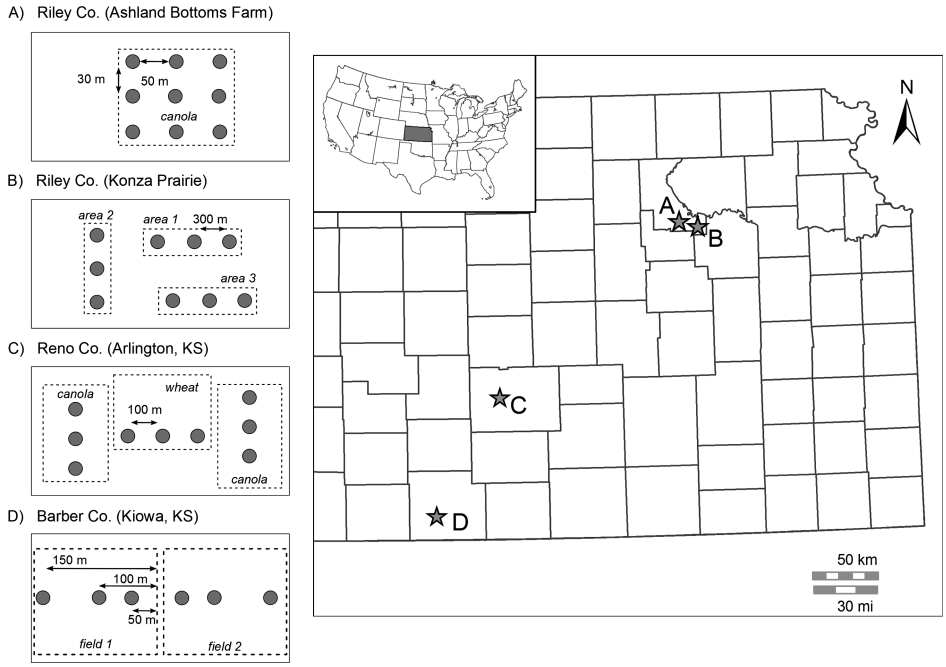

Fig. 1. Map of locations surveyed during springs of 2011 to 2013 and their corresponding blue-vane-trap arrangements (A–D). A) a production canola field at Ashland Bottoms Research Farm in Riley County, B) transects at Konza Prairie Biological Station in Riley County, C) production wheat and canola fields near Arlington, Kansas in Reno County, and D) paired fields (canola with wheat or pasture/unmanaged prairie) near Kiowa, KS in Barber County. Locations were spaced along a longitudinal gradient and are denoted with stars; traps within a location are denoted with grey circles (A–D). Inset in upper left depicts the location of Kansas within the continental United States in grey.

spring queens may help inform studies on the population decline or stability of captured species in the surveyed area.

Materials and Methods

Passive surveys were conducted in multiple field sites located in three counties (Barber, Reno and Riley) along a longitudinal gradient in Kansas from 2011 to 2013 (Fig. 1). Samples for this survey were part of a broader ecosystem services study which used production fields planted to winter canola (*Brassica napus*) or winter wheat (*Triticum aestivum*) in addition to uncultivated grass pasture and tallgrass prairie landscapes consisting of mixed plant species. Unscented, translucent blue-vane traps (Spring Star^{TM} LLC, Woodinville, WA, USA) were deployed following the Stephen and Rao (2007) protocol. At each study location, blue-vane traps were suspended above the plant canopy from a 1 m tall metal fence post.

Different trapping arrays were employed at each site, as shown in Fig. 1. Two sampling locations were used in Riley County: Ashland Bottoms Agricultural Research Farm operated by Kansas State University (Fig. 1A) and Konza Prairie Biological Station (Fig. 1B), a native tallgrass prairie preserve. Both locations are near Manhattan, Kansas. At Ashland Bottoms Agricultural Research Farm, traps (n = 12 traps) were deployed 30 m apart from one another in three parallel transects

50 m apart within a 0.3 ha cultivated winter canola field (Riley variety). Samples were collected after 2–4 days from mid-April to mid-May in 2011. Three transects of three traps each were deployed at the Konza Prairie Biological Station (Fig. 1B). Traps were approximately 300 m apart from each other within each area sampled (n = 9 total traps) and were deployed once in mid-May for a 4-day period in 2011. Three production fields were surveyed (2 canola fields adjacent to 1 wheat field) in Reno County (Fig. 1C). Within each field, traps were spaced 100 m apart from each other along a single transect (n = 18 traps) and were deployed for a 4-day period once during mid-May in 2011. A total of 13 canola, 8 wheat and 2 pasture fields were surveyed in pairs (e.g., canola:wheat, canola:pasture) in Barber County. Three traps along a single transect were deployed per field (n = 30 traps in 2011, n = 30 traps in 2012 and n = 18 traps in 2013) per collection date (Fig. 1D). Traps were located 50, 150 and 300 m from the interfacing edges of each field in the sampled pair. These traps were collected 1 to 5 days after deployment from mid-April to mid-May across years (2011–2013). Specimens collected in traps from each location were emptied into1gl zip-lock bags, transported to the lab at Kansas State University on ice and stored at −20°C. Specimens were then shipped to the University of Arkansas on dry ice, identified to species (Mitchell, 1962) and stored at −20°C until dissection and DNA extraction.

DNA was extracted from the entire abdominal contents of each specimen using a salting-out extraction protocol with in-house reagents (Sambrook and Russell, 2001). PCR reactions were conducted separately using the primers NbombiSSUJf1 (CCATGCATGTTTTTGAAGATTATTAT) plus NbombiSSUJr1 (CATATAT-TTTTAAAATATGAAACAATAA), which yields a 323 bp product only if *N. bombi* is present (Klee *et al.,* 2006), and SEF (CTTTTGGTCGGTGGAGTGAT) plus SER (GGACGTAATCGGCACAGTTT), which yields a 417 bp product if any organism in the parasitic order Trypanosomatida, including *Crithidia* spp., is present (Meeus *et al.,* 2010). Both primers target portions of the 18S small subunit rRNA region of the nuclear genomes of each taxon. Each PCR reaction contained 1–4 µl of DNA extraction, 2.5 µl of 10× reaction buffer (ThermoPol, New England BioLabs, Ipswich, MA), 2.0 µl of each nucleotide (dATP, dTTP, dCTP and dGTP at 10 mM), 0.25–0.5 µl of each primer, (at 20 mM), 1 unit of *Taq* polymerase (New England BioLabs, Ipswich, MA) and DNase/RNase-free, distilled water to bring the reaction to a total volume of 25 µl. The thermal cycling profile consisted of an initial denaturation step of 94°C for 2 min, followed by 40 cycles of 94°C for 45 s, annealing at 50°C (NbombiSSUJf1/NbombiSSUJr1) or 57°C (SEF/SER) for 45 s and 72°C for 45 s, plus a final extension step of 72°C for 5 min (C1000 Touch Thermal Cycler, Bio-Rad, Hercules, CA). A positive control extracted from specimens with verified infections and a negative control of DNase/RNase-free, distilled water was included with each reaction batch. PCR products were subjected to electrophoresis in 2% agarose gels and then visualized under UV light (BioDocit Imaging System, UVP, LLC, Upland, CA). The presence or absence of diagnostic bands was recorded.

Generalized linear models (GLM) were used to assess the factors that contributed to the prevalence of *N. bombi* and trypanosomatids. Initially, a full model was conducted using each factor (site, month, species and year) independently and as second-degree interactions, with *N. bombi* or trypanosomatid occurrence as the response variable. For each pathogen, the untested pathogen was included as an independent factor in the model as well. Errors were tested under a quasipoisson distribution to account for overdispersion in the data. Factors that were significant

Table 1. *Nosema bombi* and trypanosomatid prevalence in spring bumble bee queens by month at sampled locations in Kansas in 2011 and 2013.

	2011						2013					
	April			May			April			May		
Species	*n*	NB	Tryp.	*n*	NB	Tryp.	*n*	NB	Tryp.	*n*	NB	Tryp.
B. auricomus	28	6	0	35	2	0	0	0	0	0	0	0
B. bimaculatus	0	0	0	12	0	0	0	0	0	0	0	0
B. fraternus	0	0	0	1	0	0	0	0	0	0	0	0
B. griseocollis	3	0	0	21	0	0	0	0	0	0	0	0
B. impatiens	2	0	0	6	0	0	0	0	0	0	0	0
B. pensylvanicus	8	6	0	2	0	0	0	0	0	24	3	3

n = number of specimens sampled, NB = number of specimens positive for *N. bombi*, Tryp. = number of specimens positive for trypanosomatids.

via *F* tests in the full model were retained for the final, simplified model. The final models were assessed for significance by comparison with a null model. All statistics were conducted in R (R Core Team, 2012) with $\alpha = 0.05$.

Results

A total of 142 queens were collected from the sites described above, with 118 captured in 2011 and 24 captured in 2013 (Table 1). One *B. auricomus* and two *B. pensylvanicus* queens were captured in 2012, but these were not analyzed for pathogen presence. *Bombus auricomus* (44%, $n = 63$), *B. pensylvanicus* (24%, $n = 34$) and *B. griseocollis* (17%, $n = 24$) were most commonly collected. Overall, 12% of *Bombus* queens ($n = 17$) were positive for *N. bombi,* and 2% ($n = 3$) were positive for trypanosomatids. *Nosema bombi* was found in 13% of *B. auricomus* ($n = 8$) and 27% of *B. pensylvanicus* ($n = 9$), but was not observed in any other species at these sites. Trypanosomatids were found only in *B. pensylvanicus* (9%, $n = 3$). Both *B. auricomus* and B. *pensylvanicus* showed a lower prevalence of *N. bombi* in May (6% and 11.5%, respectively) than in April (21.4% and 75%, respectively) even though more specimens were collected in May. Trypanosomatids only occurred in samples of *B. pensylvanicus* collected in May 2013.

No interaction terms were significant in either full GLM model. The overall simplest model for *N. bombi* prevalence included host species ($F = 4.85$; d.f. = 5, 136; $P < 0.001$) and month ($F = 8.76$; d.f. = 2, 134; $P < 0.001$) as explanatory variables and was significantly better than the null model ($F = 46.13$; d.f. = 7, 134; $P < 0.001$). The overall model for trypanosomatid prevalence included host species ($F = 9.92$; d.f. = 5, 136; $P < 0.001$), month ($F = 4.65$; d.f. = 2, 134; $P = 0.01$) and *N. bombi* prevalence ($F = 5.73$; d.f. = 1, 133; $P = 0.02$) as explanatory variables and was significantly better than the null model ($F = 11.20$; d.f. = 8, 133; $P < 0.001$). Tracheal mites (*Locustacarus buchneri* Stammer) were observed in two *B. bimaculatus* specimens during pre-extraction dissections. Since these were not targeted for identification in all specimens, tracheal mite prevalence was not analyzed in this study.

Discussion

Bombus auricomus was the most common species (44%) collected during springs 2011 and 2013 in Kansas. Out of the 63 *B. auricomus* specimens collected, 13% were

infected with *N. bombi*. Prevalence was higher in April than in May, although more specimens of *B. auricomus* were collected in May. This may be an artifact of sampling, rather than an indication of a natural disease cycle. *Bombus pensylvanicus*, the second most commonly collected species, accounted for only 24% of the specimens collected but accounted for 53% of all *N. bombi* positives. Therefore, *B. pensylvanicus* showed disproportionately higher *N. bombi* prevalence across these sites than *B. auricomus*. Higher prevalence of *N. bombi* in *B. pensylvanicus* than in other species in the eastern United States has been previously reported in the literature (e.g., Cameron *et al.,* 2011; Kissinger *et al.,* 2011; Cordes *et al.,* 2012), but few studies have recovered *N. bombi* in *B. auricomus*. In the eastern United States, Cordes *et al.* (2012) found *N. bombi* in 15.2% of *B. pensylvanicus* and 1.59% of *B. auricomus* specimens, with an overall prevalence rate of 2.1% across all species. Similarly, Kissinger *et al.* (2011) found a relatively high (3.57%) prevalence of *N. bombi* in *B. pensylvanicus* in Illinois, although the prevalence of *N. bombi* was very low (<1%) overall. *Nosema bombi* was not detected in *B. auricomus* in the Illinois survey (Kissinger *et al.,* 2011). In Massachusetts, Gillespie (2010) found *N. bombi* in 12% of all worker and male specimens surveyed, but prevalence rates were much higher (42%) among *B. pensylvanicus* alone. Based on previous studies, our finding of higher prevalence of *N. bombi* in *B. pensylvanicus* spring queens in Kansas was expected, but a high prevalence of *N. bombi* in *B. auricomus* queens was not.

Other studies have indicated that *B. auricomus* may be susceptible to decline. A survey of species in eastern North America rated *B. auricomus* as "vulnerable" based on a decrease in its detectable range in contemporary times as compared to records prior to 1990 (Colla *et al.,* 2012). *Bombus auricomus* was not recovered from Blackland Prairie sites in Arkansas in recent surveys (Warriner, 2011), although in a similar survey in Illinois, *B. auricomus* was found in all expected locations (Grixti *et al.,* 2009). If infection with *N. bombi* is indeed linked with species decline as other studies have suggested (e.g., Cameron *et al.,* 2011), this may be another indication that populations of *B. auricomus* are currently susceptible to decline, at least in some regions of the United States.

Crithidia bombi was uncommon in our samples of spring queens and only found in *B. pensylvanicus*. Previous studies have recovered a higher prevalence of *C. bombi* in stable species than in declining ones. In the eastern United States, Cordes *et al.* (2012) found *C. bombi* in 2.7% of all specimens surveyed, with higher prevalence in the stable species *B. impatiens* (3.2%) than in the declining species *B. pensylvanicus* (0.2%). In Illinois, Kissinger *et al.* (2011) found an annual prevalence rate of *C. bombi* ranging 1.0 to 5.7% (all host species combined), with the highest rates in *B. impatiens* (14.1%) and none recovered from *B. pensylvanicus*. In Massachusetts, Gillespie (2010) found *C. bombi* in 24.0 to 57.0% of all specimens surveyed. Prevalence rates were much higher (>60.0%,) in *B. impatiens*, and as in Illinois, none were recovered in *B. pensylvanicus* specimens (Gillespie, 2010). Unlike previous studies, trypanosomatids were only detected in *B. pensylvanicus* queens from our Kansas specimens. This may be due to the seasonal cycle of *C. bombi* in natural populations, which shows an increase over a season (Schmid-Hempel, 2001; Gillespie, 2010). In Switzerland, *C. bombi* infection rates were lower in spring queens (5.3–7.8%) as compared to newly emerged, fall queens (50.0%) or all castes pooled (18.1%) (Tognazzo *et al.,* 2012). If *C. bombi* phenology follows the same pattern in Kansas, lower prevalence of *Crithidia* would be expected in Kansas spring

queens as well. All trypanosomatids observed in *Bombus* hosts have been assumed to be *Crithidia bombi*, yet *C. expoeki* Schmid-Hempel and Tognazzo, a cryptic species of *Crithidia,* has recently been described in *Bombus* specimens from Switzerland and Alaska based on molecular evidence (Schmid-Hempel and Tognazzo, 2010). The PCR detection technique we used here cannot distinguish among trypanosomatid species, although the mitochondrial region amplified by SEF/SER is conserved within Trypanosomatida and should recover any trypanosomatids present (Meeus *et al.,* 2010).

The difference in sampling abundance between years is notable. After collecting 118 queens of six species in the first year of the study, only three *Bombus* queens were collected in 2012. Although 24 queens were captured in 2013, species richness declined, and only *B. pensylvanicus* was collected. This drop in abundance and richness may be indicative of the impact of passive trapping on local populations caused by our removal of colony-founding queens during the first year of the study. However, our increased sampling intensity in 2011 may account for the higher richness and abundance observed in that year. On the other hand, year-to-year variability in local bee abundance is commonly cited as a confounding factor in detecting bee declines (Williams *et al.,* 2001; Lebuhn *et al.,* 2013). Even studies with non-destructive sampling protocols have noted the high year-to-year variation in both the abundance of spring *Bombus* queens and the timing of their active periods (Cane and Payne, 1993). Our sampling dates were consistent over the three-year study, without regard for environmental variables that may have advanced or delayed the timing of spring queen flights. Removing bees from the system or missing spring activity periods may have led to an underestimation of pathogen presence.

This study is the first to examine the prevalence of two common *Bombus* pathogens in colony-founding, spring queens in the United States. In addition to finding *N. bombi* at higher levels in *B. pensylvanicus* queens, we also show that *B. auricomus* queens are commonly infected with *N. bombi* in Kansas. Trypanosomatids, presumably *Crithidia* spp., were only recovered from *B. pensylvanicus* queens in this survey. Although declining species in the United States exhibit higher *N. bombi* prevalence rates (Cameron *et al.,* 2011), the link between *N. bombi* prevalence rates and species' decline is still unclear. In surveys of *B. occidentalis* workers in Alaska, Koch and Strange (2012) found that *N. bombi* infections were more common in *B. occidentalis* than in other Alaskan species. Although *B. occidentalis* is thought to be in decline in the contiguous United States (Cameron *et al.,* 2011; Lozier *et al.,* 2011), this species was the most abundant bumble bee species in their Alaskan survey (Koch and Strange, 2012). Because bumble bee populations ultimately depend upon successful colony founding by spring queens, the higher prevalence of these pathogens in *B. pensylvanicus* and *B. auricomus* spring queens is an indication that Kansas populations of these species warrant greater scrutiny. The interaction between disease and population health is clearly complex and deserving of further attention.

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