Mechanical Vectors Enhance Fungal Entomopathogen Reduction of the Grasshopper Pest *Camnula pellucida* (Orthoptera: Acrididae)

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ABSTRACT Mounting scientific evidence indicates that pathogens can regulate insect populations. However, limited dispersal and sensitivity to abiotic conditions often restricts pathogen regulation of host populations. While it is well established that arthropod biological vectors increase pathogen incidence in host populations, few studies have examined whether arthropod mechanical vectors (an organism that transmits pathogens but is not essential to the life cycle of the pathogen) influence host–pathogen dynamics. The importance of mechanical dispersal by ant scavengers, *Formica fusca* (L.), in a grasshopper–fungal entomopathogen system was investigated. We examined the ability of ants to mechanically disperse and transmit the pathogen, *Entomophaga grylli* (Fresenius) pathotype 1, to its host, the pest grasshopper *Camnula pellucida* (Scudder), in a series of laboratory experiments. Fungal spores were dispersed either externally on the ant's body surface or internally through fecal deposition. In addition, a third of all grasshoppers housed with fungal-inoculated ants became infected, indicating that ants can act as mechanical vectors of *E. grylli*. The effect of ant mechanical vectors on *E. grylli* incidence was also examined in a field experiment. Ant access to pathogen-exposed experimental grasshopper populations was restricted using organic ant repellent, thereby allowing us to directly compare mechanical and natural transmission. Ants increased grasshopper pathogen mortality by 58%, which led to greater pathogen reductions of grasshopper survival than natural transmission. Taken together, our results indicate that ants enhance *E. grylli* reduction of grasshopper pest numbers. Therefore, mechanical transmission of pathogens may be an important overlooking component of this grasshopper–fungal pathogen system.

KEY WORDS mechanical vector, transmission, entomopathogen, grasshopper, ant

Introduction

Although it is well documented that arthropod biological vectors are key components of many pathogen life cycles, the role arthropod mechanical vectors play in the dispersal and transmission of pathogens remains poorly understood (Foil and Gorham 2000). Unlike biological vectors, mechanical vectors disperse pathogens without affecting pathogen development or replication (Graczyk et al. 2001). This transportation can be either external, when the agent is located on the surface of an organism's body (Bird et al. 2004), or internal, when the agent passes through the digestive tract and is viable when excreted (Graczyk et al. 2001). These mechanical vectors may have important, but often overlooked implications, for humans (Sulaiman et al. 1988, Graczyk et al. 2005), livestock (Foil and Gorham 2000), wildlife (Asgari 1998, MacPhee and Greenwood 2013), and insect populations (Baverstock et al. 2010). For instance, outbreaks of human diarrheal diseases are closely linked to seasonal increases in filth flies that mechanically transmit the pathogens (Sulaiman et al. 1988; Graczyk et al. 2001, 2005). Unfortunately, most of these findings are correlative or based on observations. Little experimental work has examined the importance of mechanical transmission to overall disease dynamics in comparison to natural transmission.

In this study, we examine the importance of arthropod mechanical vectors in limiting host populations using a model system of a grasshopper–fungal entomopathogen and an ant mechanical vector. Entomopathogens are common pathogens of insects that are often dispersed through wind, water, or soil movement (Wilding 1970, Hajek and St. Leger 1994) and sometimes mechanically via arthropods (Evans 1989, Carruthers et al. 1997, Dromph 2003). Biocontrol studies suggest that arthropod mechanical vectors can increase entomopathogen mortality rates of pest insects (Bruck and Lewis 2002, Renker et al. 2005, Meyling et al. 2006, Meyling and Eilenberg 2007, Baverstock et al. 2010). However, it remains unclear if these mechanically driven increases in pathogen mortality can strengthen entomopathogen reduction of natural host populations.

The pest grasshopper, *Camnula pellucida* (Scudder) (Orthoptera: Acrididae), can be limited by the

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entomopathogen, *Entomophaga grylli* (Fresenius) pathotype 1 (Entomophthoramycota: Entomophthorales), whose dispersal is primarily through forcible ejection, as well as wind or water movement (MacLeod et al. 1966, Carruthers et al. 1988a). This fungal entomopathogen has evolved two modes of infection: conidia and resting spores (Supp Fig. 1 [online only]). Resting spores remain dormant in the soil, overwinter and germinate in the spring under suitable environmental conditions. Following germination, resting spores may infect a grasshopper by direct contact. Once inside the host hemocoel, the fungus multiplies rapidly (MacLeod et al. 1966). The infection leads to death within 7–10 d (Carruthers et al. 1997). Just before dying, the infected grasshopper climbs to the top of a plant where it dies grasping the foliage. This posture, known as summit disease, is characteristic of grasshopper mortality from *E. grylli* (Pickford and Riegert 1964, Hajek and St. Leger 1994). This infected cadaver can produce either conidia under cool, humid conditions, or resting spores under hot, dry conditions (Carruthers et al. 1997). Unlike resting spores, conidia can produce multiple disease cycles per season if moist conditions persist (MacLeod et al. 1966).

Consequently, epizootics vary spatially and temporally, given the limitations of both modes of infection (Carruthers et al. 1997). Conidia experience high mortality rates from heat, desiccation and ultraviolet exposure (Carruthers et al. 1988b). Unlike other fungal entomopathogens, *E. grylli* aerial conidia carry only a few meters in still air (Wilding 1970, Macleod and Müller-Kögler 1973). Likewise, resting spores may only be dispersed by initial forced projection and water movement in the soil (Carruthers et al. 1997). Given these points, we suspect that transmission may be enhanced via mechanical transport (Evans 1989, Foil and Gorham 2000) and a likely mode is scavenging by ants that are responsible for the rapid removal of grasshopper cadavers. For instance, ants removed 71% of grasshoppers cadavers placed on the ground ($n = 30$) and 31% of cadavers elevated in the canopy ($n = 30$) within 24 h at our site (E.J.K., unpublished data). Furthermore, ants have been documented to mechanically transmit entomopathogens to targeted insect pests (Gracia-Garza et al. 1998, Bird et al. 2004). Therefore, we conducted laboratory experiments to determine whether ants disperse and transmit *E. grylli* resting spores/conidia, and field experiments to determine whether ants strengthen the pathogen's negative impact on grasshopper populations.

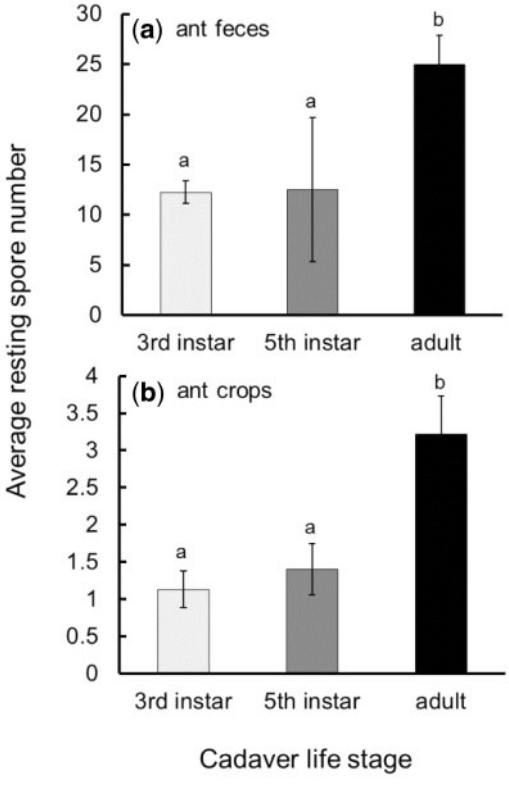

Fig. 1. Average number of resting spores (±SE) recovered from (a) ant feces and (b) ant crops of ants ($n = 240$) fed *E. grylli*-killed grasshoppers. Columns bearing the same letter were not significantly different within treatments (Tukey HSD test, $P > 0.05$).

First, we tested the hypothesis that ants can mechanically disperse and transmit *E. grylli*. In laboratory experiments, we measured ant external transport (on body surface) of conidia, ant internal (fecal deposition) dispersal of resting spores, and grasshopper infection rates when exposed to *E. grylli*-inoculated ants. *E. grylli* conidia are coated with a sticky residue that allows it to adhere to any object it contacts (Macleod and Müller-Kögler 1973), while resting spores have thick cell walls (Carruthers et al. 1997) that should retain viability after passing through an ant's digestive tract. If ants can mechanically vector *E. grylli*, we would expect to find viable spores on their bodies, as well as their feces, and healthy grasshoppers to become infected when exposed to ants carrying the pathogen. Second, we examined the contribution of ant-aided transmission to overall pathogen reduction of grasshopper populations by comparing mechanical transmission (ant aided) to natural transmission (pathogen ejection, wind, and water) in a field experiment. Ant presence in replicated, enclosed field populations of the host grasshopper was reduced with the use of an organic ant repellent. We also manipulated pathogen exposure (resting spores, conidia, and absent) given that ant dispersal of *E. grylli* may vary by pathogen spore type (Supp Fig. 1 [online only]). The combined effects of ant presence and pathogen exposure on pathogen mortality and grasshopper survival rates were measured. If mechanical vectors are an important component of the *E. grylli* life cycle, we would expect that grasshoppers experience lower pathogen mortality rates and exhibit increased survival when ant presence is restricted.

Materials and Methods

Study Site and Organisms. We conducted our field study at the National Bison Range, MT (47°21.040′ N, 114°10.190′ W), at an elevation of 832 m.

This site is primarily Palouse prairie dominated by C_3 grasses. *Poa pratensis* (L.) and *Elymus smithii* (Rydb.) A. Love were the dominant grasses. Dominant forbs include *Aster falcatus* (Lindl.) G. L. Nesom, *Achillea millefolium* (L.), and *Erigeron* (L.) spp. The graminivorous clear-winged grasshopper *C. pellucida* is common at the site and is univoltine, overwintering as eggs that hatch in late May through early June (Pfadt 1994). This grasshopper is often considered a pest species and undergoes fluctuating population sizes capable of very high densities (Pfadt 1994). *E. grylli* pathotype 1 (Entomophthoramycota: Entomophthorales), unofficially known as *Entomophaga macleodii* (Casique-Valdez 2012), is one member of a complex of obligate grasshopper fungal entomopathogens (Goettel et al. 1995, Carruthers et al. 1997). *E. grylli* pathotypes 1 and 2 are both endemic to North America, but have different life histories. Pathotype 2 only infects individuals of the subfamily Melanopline and produces resting spores that have one disease cycle per season. In contrast, pathotype 1 infects certain members of the subfamily Oedipodinae (Goettel et al. 1995, Carruthers et al. 1997) and can produce both resting spores and conidia, resulting in multiple disease cycles in a single season (Supp Fig. 1 [online only]). Periodically, this pathogen is known to dramatically decrease *C. pellucida* populations and is common at this site (Kistner and Belovsky 2013, 2014). *Formica fusca* (L.) (Hymenoptera: Formicidae) ants are the most abundant scavenger of grasshopper cadavers at this site (Belovsky et al. 1990). *F. fusca* are common in grasslands across Europe and North America (Tuzzolino and Brown 2010). These ants are active throughout the day and can remove as much as 59–100% of dead arthropod material from the ground within a 24-h time span (Retana et al. 1991, Bestelmeyer and Wiens 2003). While *F. fusca* ants mainly forage on the ground, workers were also observed removing grasshopper cadavers from the lower canopy (≈0.3 m in height) at our site (E.J.K., personal observation).

Laboratory Experiments. Worker ants were collected from identified *F. fusca* ant mounds at the National Bison Range (D.C., unpublished data) in July 2012. In July, *E. grylli* incidence at this site was low with <2% of sampled *C. pellucida* grasshoppers testing positive for infection (E.J.K., unpublished data). Therefore, ants used in study were unlikely to have contacted *E. grylli* before experimentation. We collected ants at the base of an ant mound using a handheld insect aspirator attached to a collecting vial (BioQuip, Rancho Dominquez, CA). Ants were then stored in a cooler and transported to the laboratory. Ants were cooled in a refrigerator for 5 min and then examined under a dissecting microscope before assignment to treatments to ensure all experimental individuals were uninjured worker ants (Wallis 1962).

Internal Pathogen Retention and Dispersal. Twenty worker ants were placed in 0.3- × 0.2-m ant farms made of a plaster of Paris base and clear acrylic top (Interplay UK, Buckinghamshire, UK). Only ants from the same mound were housed together to avoid aggressive behavior between different colony members (Tuzzolino and Brown 2010). Plastic cap covers over the acrylic top allowed for easy access. Experimental ants were allowed to acclimate to their new environment for 48 h and were fed honey mixed with multivitamins (Bird et al. 2004). Ants were randomly assigned to grasshopper cadaver treatments with four replicates per treatment level, for a total of 16 ant farms. *E. grylli*-killed grasshoppers were collected from Moiese, MT, in July 2012 and examined under a dissecting microscope to determine whether the cadavers contained resting spores (Carruthers et al. 1997). Ants were fed either a single: *E. grylli*-killed third instar, *E. grylli*-killed fifth instar, or *E. grylli*-killed adult. All cadavers were female grasshoppers to minimize size differences. Control ant farms (fed *E. grylli*-free fifth instars) were kept in a separate room from treatment ant farms. After 2 wk, experimental ants were frozen, dissected, stained with lacto fuchsin (AEML, Inc., Pompano Beach, FL), and examined under a compound microscope to count the number of spores inside their crops. In addition, ant feces were collected from ant farms, stained, and examined for spores under a compound microscope.

External Pathogen Transport and Transmission. *E. grylli* conidia were obtained from *E. grylli*-killed grasshoppers collected from Moiese, MT, in July 2012. Cadavers were stored in individual petri dishes and soaked with deionized water until conidiophores developed (Macleod and Müller-Kögler 1973). Sporulating cadavers were then placed in a desiccator jar for 12 h. Newly formed conidia were collected in deionized water from the bottom of the desiccator jar (Carruthers et al. 1988b). Individual ants were inoculated with *E. grylli* conidia (≈100 conidia in 0.1 ml of water) with a sterile loop on the surface of the dorsal abdomen. Inoculated ants were then placed in glass containers (99 mm in diameter by 177 mm in depth) with a moistened paper towel before treatment assignment. *E. grylli* conidia were easily distinguished from other particles by their unique pear shape (Macleod and Müller-Kögler 1973). We tested for the effect of self and allo-grooming on conidia dispersal by housing an inoculated ant in the glass container alone or with a second *E. grylli*-free ant marked with nail polish (Wallis 1962, Baverstock et al. 2010). In addition, we placed a single *E. grylli* conidia-inoculated ant with a single *E. grylli*-free third instar grasshopper to estimate mechanical transmission rates. Inoculated ants were stained with lacto fuchsin and examined under a compound microscope for conidia at 0, 6, 12, 18, and 24 h with 10 replicates per time interval. Grasshoppers were transferred to a sterile glass container after 6, 12, 18, and 24 h of exposure to inoculated ants, where they were fed a diet of grass for 7 d. After 1 wk, grasshoppers were dissected and examined for the presence of *E. grylli* infection.

Field Experiment. Grasshopper populations were established in aluminum screen cages placed over natural vegetation (basal area: 0.5 m^{-2}; height: 1 m). Cages were originally installed in August 2011. Each cage was buried in the ground and secured with stakes (Belovsky and Slade 1995). Cages were spaced about 1.5 m apart

with attempts to place cages over areas of similar vegetation. Prior to stocking cages with grasshoppers, all cages were treated with diluted VIREX II 256 Germicidal Cleaner (Diversey Inc., Surtevant, WI), and PT Infuse Systemic Fungicide (Bonide Products Inc., Oriskany, NY) to control for level of pathogen exposure.

Two treatments were used: ant presence (two levels: restricted and unrestricted) and pathogen exposure (three levels: resting spores, conidia, and absent). We used a 2×3 factorial design with six replicates of each treatment for a total of 36 cages. Experimental grasshoppers were reared from eggs laid by captive individuals collected from Moiese, MT, in the previous year to insure that they were not exposed to the fungal pathogen before stocking into experimental cages (Kistner and Belovsky 2014). Ten laboratory-reared second to third instar grasshoppers were stocked in each cage. Fungal pathogen exposure in field cages was conducted in a manner that mimics *E. grylli* life history (Carruthers et al. 1997). Resting spore exposure was instigated by placing fifth instar cadavers containing *E. grylli* resting spores (one per cage) on the ground a year prior to stocking. Trace amounts of water (100 ml per cage) was applied on the ground of resting spores-exposed cages once a week in June 2012, a month prior to stocking, to help facilitate resting spore germination. For conidia exposure, sporulating fifth instar cadavers (one per cage) were tied on a wire 0.6 m above the ground, which mimics the effects of *E. grylli* summit disease (Kistner and Belovsky 2014).

Ants move freely in and out of these enclosures via small cracks in the ground at the base of each cage. These ants often scavenge grasshopper cadavers within experimental cages (Belovsky et al. 1990). To prevent ants from entering cages while not disturbing natural ant densities at the study site, we applied an organic repellent (Orange Guard Inc., Carmel Valley, CA) around each cage of the "ants restricted" treatments. This repellent contains the citrus-based terpene, d-limonene, a proven plant-based repellent to ants (Vogt et al. 2002). A month prior to the start of the field experiment, we assessed the efficacy of the organic ant repellent. In five randomly chosen experimental cages, we estimated ant abundance with and without ant repellent. All counts were conducted during the afternoon when ant activity was high. For 5-min intervals, ants passing by a landmark point inside the cage bottom were counted. After the initial ant survey was conducted, organic ant repellent was applied to the five cages. Twenty-four h after applying the repellent, we repeated the ant count survey. A paired t-test indicated that organic repellent application reduced ant abundance within cages by 67% ($t = 4.22$; $df = 4$; $P < 0.01$). Throughout the course of the field experiment, ant repellent was applied to cages every day and after any rainfall event.

The experiment ran from July to August 2012 (42 d total) until senescence and cold nights began to kill grasshoppers. Laboratory-reared grasshoppers were stocked in July when ants are most active (Bestelmeyer and Wiens 2003, Tuzzolino and Brown 2010). Individual grasshoppers were counted every 2 d. Any grasshopper cadavers found clinging to the vegetation or cage screening in the characteristic summit disease position (Carruthers et al. 1997) were categorized as pathogen-induced mortality and left in the cages to maintain natural disease transmission (Kistner and Belovsky 2013, 2014). All other cadavers found within cages were frozen, later thawed, dissected and samples of internal tissues stained with lacto fuchsin. Stained samples were then examined under a compound microscope to determine whether they died of *E. grylli* infection (Macleod and Muller-Kogler 1973, Sánchez Peña 2005). The majority of cadavers removed from cages (97%) were not infected with *E. grylli*, indicating that this cadaver removal did not affect overall transmission rates. Only individuals whose cause of death could be determined (11% of all experimental individuals were never recovered) were included in the mortality analysis. Mortality due to *E. grylli* infection was the sum of elevated cadavers plus the number of other cadavers found to be infected, and is presented as a proportion relative to all accounted for deaths.

Statistical Analysis. Generalized linear models (GLM) with quasi-Poisson distributions and log link functions were conducted to 1) determine differences in average resting spore number in ant crops and feces across ant farm treatments, and 2) the effect of time and treatment on average number of conidia present on conidia-inoculated ants across pairing treatments (Wilson and Grenfell 1997). Significant differences between treatment means were identified with Tukey honestly significant difference (HSD) tests (Torsten et al. 2008). A Kruskal–Wallis test was performed to test the effect of time on pathogen incidence of grasshoppers housed with conidia-inoculated ants. For the field experiment, analyses were conducted on the first 42 d. We used GLM with a binomial distribution and a logit link function to assess whether the proportion of pathogen mortality varied with ant presence and fungal pathogen exposure. Differences in grasshopper survivorship were analyzed using parametric survival models with a Weibull distribution and the following predictive factors: ant presence and pathogen exposure (Therneau 2013). To directly compare differences in grasshopper survival of ant restricted and ant unrestricted treatments across pathogen exposure treatments, we used Kaplan-Meier survivorship analysis and the Breslow–-Gehan test. We choose to use the Breslow–Gehan test because it gives more weight to early deaths which coincide with the timing of the majority of *E. grylli* mortality; 78% of fungal deaths occurred in the first 2 wk (Crawley 2013). All analyses were conducted in R 3.0.1 (R Development Core Team 2013).

Results

Ant Retention and Deposition of Resting Spores. *E. grylli* resting spores were only recovered from the crops and feces of ants fed *E. grylli*-killed grasshoppers, indicating that the sampled ants were not acting as a reservoir for the pathogen before the start of the experiment (Fig. 1). Ants fed *E. grylli*-killed adults yielded two times more resting spores in their

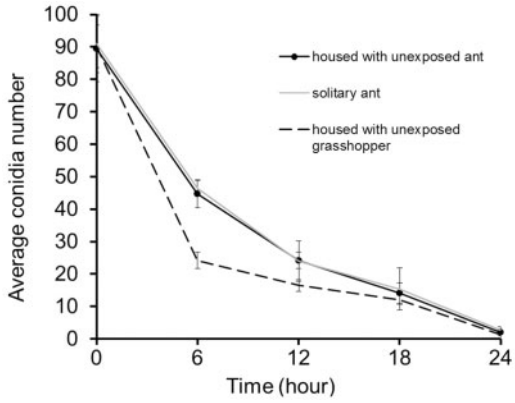

Fig. 2. Average number of conidia (±SE) recovered from inoculated solitary ants, inoculated ants housed with *E. grylli*-free ants and inoculated ants housed with *E. grylli*-free grasshoppers over time.

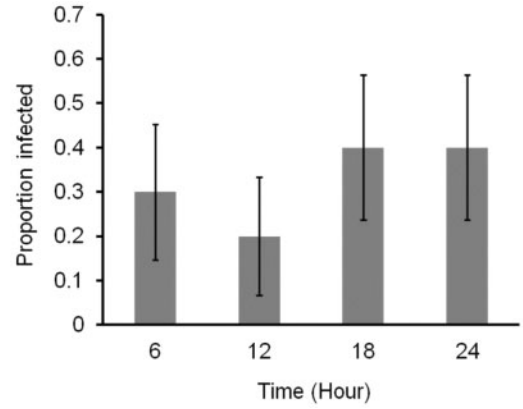

Fig. 3. Proportion of grasshoppers that became infected when exposed to *E. grylli*-inoculated ants (±SE) under laboratory conditions. Length of exposure time had no effect on ant to grasshopper transmission success.

feces than the third and fifth instar fed ants ($\chi^2 = 24.15$; df = 2,9; $P < 0.0001$; Fig. 1a). The mean number of resting spores in the adult treatment was significantly different from both third and fifth instars treatments ($P = 0.005$; $P = 0.002$; respectively), while third and fifth instars treatments did not differ ($P = 0.96$). The same trend was seen in the ant crop analysis with ants fed *E. grylli*-killed adults yielding the highest resting spore numbers ($\chi^2 = 75.63$; df = 2,177; $P < 0.0001$; Fig. 1b). Pair-wise comparisons revealed significant differences between the mean number of resting spores in adult treatments compared with both third and fifth instars treatments ($P = 0.03$ and $P = 0.04$; respectively). There was no difference in the mean number of resting spores recovered from ants in the third and fifth instars treatments ($P = 0.92$). No conidia were recovered from ants housed in experimental ant farms, which suggest that only resting spores may pass through an ant's digestive tract intact.

Ant Dispersal of Conidia and Ant to Grasshopper Transmission. Conidia-inoculated ants ($n = 30$) retained 92 ± 4% of applied conidia when examined immediately after inoculation ($t = 0$), indicating that conidia readily adhere to the body surface of ants (Fig. 2). Conidia persistence on inoculated ants varied between treatments ($\chi^2 = 522$; df = 2,147; $P < 0.001$), across time ($\chi^2 = 40073$; df = 4,143; $P < 0.001$), and the time × treatment interaction was significant ($\chi^2 = 837$; df = 8,135; $P < 0.001$). The majority of applied conidia were dislodged within 6 h of application and this effect varied across treatments (Fig. 2) Inoculated ants paired with grasshoppers lost 73% of their conidia after 6 h compared with the 48% loss experienced by ants housed alone ($P = 0.038$) or with a second ant ($P = 0.032$). While there was no difference in conidia retention between solitary inoculated ants and paired inoculated ants ($P = 0.98$), trace amounts of conidia (2 ± 0.04 conidia) were found on 43% of all unexposed ants. A third of all experimental grasshoppers (0.33 ± 0.05) housed with conidia-inoculated ants became infected with *E. grylli* (Fig. 3). Length of exposure time to conidia-inoculated ants had no effect on the proportion of grasshoppers that became infected (Kruskal–Wallis = 1.22; df = 3; $P = 0.75$).

Effects of Ant Treatments on Experimental Cages. In experimental field cages, grasshopper mortality from *E. grylli* peaked in late July and was no longer detected by mid-August. Only one grasshopper (<1%) died from the pathogen in cages where the pathogen was absent. Grasshoppers in cages treated with ant repellent (ants restricted) exhibited a 58% reduction in pathogen mortality than those in cages where ants were not restricted ($F = 5.12$; df = 1,20; $P = 0.03$; Fig. 4). Only 20% of grasshoppers in pathogen-exposed cages treated with ant repellent died of disease (24 deaths), while 32% of grasshoppers in pathogen-exposed cages with unrestricted ant access died of disease (38 deaths). The type of pathogen exposure (resting spores vs. conidia) had no effect on overall pathogen mortality rates ($F = 3.83$; df = 1,20; $P = 0.65$) and the ant presence × pathogen exposure interaction term was not significant ($F = 0.95$, df = 1,20; $P = 0.34$).

Over the entire 42 d of the experiment, pathogen-exposed grasshoppers exhibited shorter survival times compared with pathogen absent grasshoppers ($\chi^2 = 29.99$; df = 2,341; $P < 0.001$; Fig. 4) Conidia and resting spore exposure decreased grasshopper survival time by 54% and 47%, respectively. Ants restriction, through the use of a citrus-based ant repellent, increased overall grasshopper survival time by 32% ($\chi^2 = 5.94$; df = 1,340; $P < 0.05$). The ant presence × pathogen exposure interaction was not significant ($\chi^2 = 0.21$; df = 2,338; $P = 0.90$). While ant restriction increased grasshopper survival in conidia-exposed treatments, this trend was not significant (Breslow–Gehan = 0.67; df = 1; $P = 0.41$; Fig. 5a). Survival of grasshoppers exposed to resting spores increased when ants were restricted (Breslow–Gehan = 6.38; df = 1; $P = 0.01$; Fig. 5b). Survival of pathogen-free

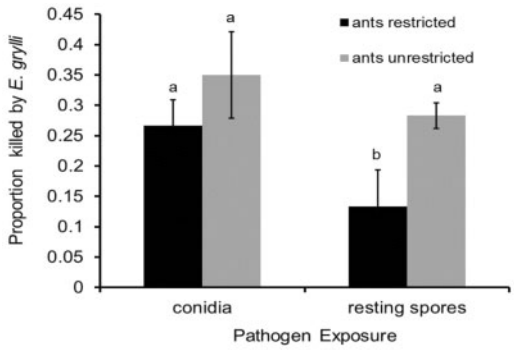

Fig. 4. Proportion of grasshoppers exposed to *E. grylli* that died from the pathogen (±SE) for ant presence and pathogen exposure treatment combinations. Columns bearing the same letter were not significantly different within treatments (Tukey HSD test, $P > 0.05$).

grasshoppers was not affected by ant presence (Breslow–Gehan = 0.20; df = 1; $P = 0.66$; Fig. 5c).

Discussion

Experimental results from this study support the view that arthropod mechanical vectors can increase fungal entomopathogen incidence in grasshopper host populations and may enhance pathogen reduction of insect pest populations. We found that ants can mechanically transmit fungal entomopathogens and that ant-aided transmission resulted in greater reductions of grasshopper numbers than natural transmission (wind, soil, and water) alone. These results reinforce the findings of past biocontrol studies (Evans 1989, Gracia-Garza et al. 1998, Bruck and Lewis 2002, Dromph 2003, Bird et al. 2004, Meyling and Eilenberg 2007), and suggest that ant mechanical vectors may play a larger role in host–entomopathogen dynamics than previously suggested (Evans 1989).

Our results suggest that ants are capable of transmitting *E. grylli* to grasshopper hosts through external transport of conidia and fecal deposition of resting spores. Ants fed *E. grylli*-killed adult grasshoppers retained and excreted more resting spores because larger adult bodies provide more nutrients for pathogen reproduction than smaller juvenile cadavers. Resting spores remained intact inside ant crops and ant feces, whereas conidia did not. This is unsurprising given that resting spores have thick cell walls and can remain in the soil for several years, whereas conidia are highly sensitive to abiotic stressors (Carruthers et al. 1997). Although, conidia readily adhere to the bodies of ants, conidia retention time is short with only 2% of total applied conidia remaining on the ants after 24 h. We suspect that the conidia died, fell off, or were forcibly removed through self-grooming (Wallis 1962) or allogrooming (Baverstock et al. 2010). However, conidia-free ants did pick up conidia off the ground or from contact with the conidia-inoculated ants (Bird et al. 2004). It is plausible that conidia more readily adheres

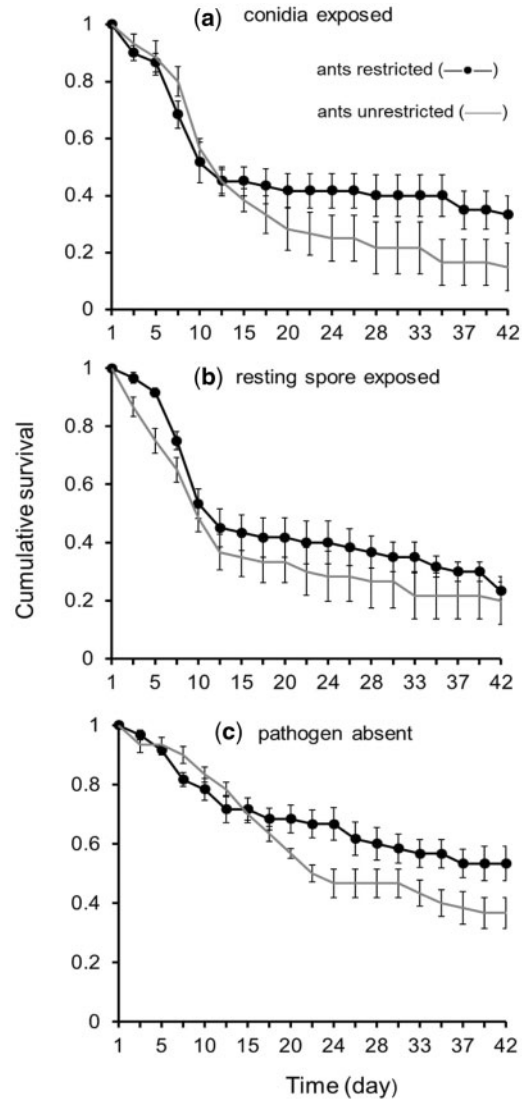

Fig. 5. Grasshopper survival (±SE) over time according to ant presence and pathogen exposure treatment combinations. (a) conidia exposed. (b) resting spore exposed. (c) pathogen absent.

to grasshopper hosts than non-host organisms by recognizing chemical cues on the cuticle surface, given that a third of all *E. grylli*-free grasshoppers housed with inoculated ants became infected (Hajek and St. Leger 1994). Combined, these laboratory experiments indicate that ants can mechanically transmit *E. grylli* to grasshoppers by physically dispersing conidia, or through internal transportation of resting spores via fecal deposition.

Our field experiment results are in agreement with our laboratory findings, with the effect of ant-aided transmission on grasshopper pathogen mortality and survivorship varying by spore type (conidia vs. resting spore). Clearly, ants can affect grasshopper pathogen

mortality rates through the mechanical vectoring of this virulent entomopathogen and these increases in mortality rates are comparable to similar biocontrol studies (Gracia-Garza et al. 1998, Bird et al. 2004).

Survival time of pathogen-exposed grasshoppers is reduced more when ant presence is unrestricted (mechanical transmission) compared with when ant presence is restricted (natural transmission). However, ant-aided increases in pathogen mortality rates do not always lead to greater reductions in overall grasshopper survival. For instance, ants did not significantly decrease grasshopper survival time in conidia-exposed treatments (Fig. 5a). Furthermore, while ants did decrease grasshopper survival time in resting spore-exposed treatments, the effect was not dramatic and resulted in only a 10% additional decrease in overall survival time (Fig. 5b). This outcome makes intuitive sense when considering the life history of both the fungal entomopathogen and mechanical vector. *E. grylli* conidia are short-lived and sensitive to changing abiotic conditions (Carruthers et al. 1988b). *F. fusca* ants forage more frequently on the ground than in the canopy (E.J.K., personal observation), where they would likely encounter *E. grylli*-killed grasshoppers (Sawyer et al. 1997). Yet, even a small decrease in grasshopper survival may help reduce damage to rangeland forage or crops by reducing grasshopper consumption rates within a growing season and by decreasing the number of breeding adults (Belovsky and Joern 1995, Branson et al. 2006). For example, ant restriction does increase the final number of surviving grasshoppers by 17% in the conidia-exposed treatments. Ants did not significantly decrease survival time of pathogen-free grasshopper (Fig. 5c) and this outcome supports past work indicating that ant predation has little effect on overall grasshopper numbers at this site (Belovsky et al. 1990). Taken together, our findings suggest that ant-aided transmission of *E. grylli* may enhance fungal entomopathogen reduction of grasshopper populations if favorable abiotic conditions for *E. grylli* development coincide with high ant abundance and activity.

Our results should, however, be approached with some caution because we could not completely exclude all ants from our experimental cages. In addition, ants at our site are fairly dense with 7.8 ($\pm$0.4) mounds per 1,000 m^{-2} (D.C., unpublished data), each containing about 1,000 workers (Tuzzolino and Brown 2010). These high ant densities make contact with *E. grylli*-killed grasshoppers far more likely than in areas where ants are less abundant. Finally, the viability of resting spores excreted by experimental ants or conidia applied topically to ants was never confirmed. Future studies should consider examining ant mechanical transmission across an ant density gradient, directly measuring ant to grasshopper contact rates and testing the viability of the pathogens transported by these mechanical vectors. Moreover, ant, grasshopper, and entomopathogen abundance vary temporally, which necessitates multi-year experiments to confirm the importance of mechanical transmission in this system over time.

Despite these limitations, this study suggests that ants can mechanically transmit *E. grylli* and may be able to vector other entomopathogens including biocontrol agents to target pest species (Gracia-Garza et al. 1998, Bird et al. 2004). Ant-aided transmission of biocontrol agents would be most advantageous when ants are both abundant and active. For example, peak ant activity (June–July) coincides with high grasshopper densities and *E. grylli* emergence in our system (Kistner and Belovsky 2013). However, concern over grasshopper damage often occurs in rangelands (Branson et al. 2006), where cattle grazing may decrease ant abundance and diversity (Tuzzolino and Brown 2010, Schmidt et al. 2012). In addition, insecticides commonly used for grasshopper control could negatively affect sensitive fungal entomopathogens and ant vectors (Branson 2006, St. Leger and Hejek 1994). Thus, insecticide application and cattle grazing should be altered in conjunction with the use of fungal entomopathogens and ant vectors for grasshopper control.

Our results indicate that both natural and ant-aided transmission of *E. grylli* reduced grasshopper survival time. However, mechanical transmission resulted in greater reductions of grasshoppers than natural transmission alone. While arthropod mechanical vectors have been shown to be important in some disease outbreaks (Sulaiman et al. 1988, Asgari et al. 1998, Foil and Gorham 2000, Graczyk et al. 2005, MacPhee and Greenwood 2013), our study provides empirical evidence that mechanical transmission may be an important overlooked component in entomopathogen systems like *E. grylli* pathotype 1 (Carruthers et al. 1997). As such, arthropod mechanical vectors may need to be considered when examining the effects of pathogens on host population dynamics (Graczyk et al. 2005). Nevertheless, our results are tentative given that our experiment was temporally and spatially limited. Further research is needed to clarify the effect of arthropod mechanical vectors on grasshopper population dynamics.

Supplementary Data

Supplementary data are available at *Environmental Entomology* online.

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References Cited

Asgari, S., J. R. Hardy, R. G. Sinclair, and B. D. Cooke. 1998. Field evidence for mechanical transmission of rabbit haemorrhagic disease virus (RHDV) by flies (Diptera: Calliphoridae) among wild rabbits in Australia. Virus Res. 54: 123–132.

Baverstock, J., H. E. Roy, and J. K. Pell. 2010. Entomopathogenic fungi and insect behavior: from unsuspecting hosts to targeted vectors. Biocontrol 55: 89–102.

Belovsky, G. E., and A. Joern. 1995. The dominance of different regulating mechanisms for rangeland grasshoppers, pp 359–386. *In* N. Cappuccino and P. Price (eds.), Population dynamics: new approaches and synthesis, Academic Press, New York, NY.

Belovsky, G. E., and J. B. Slade. 1995. Dynamics of two Montana grasshopper populations: relationships among weather, food abundance and intraspecific competition. Oecologia 101: 383–396.

Belovsky, G. E., J. B. Slade, and B. A. Stockhoff. 1990. Susceptibility to predation for different grasshoppers: an experimental study. Ecology 71: 624–634.

Bestelmeyer, B. T., and J. A. Wiens. 2003. Scavenging ant foraging behavior and variation in the scale of nutrient redistribution among semi-arid grasslands. J. Arid Environ. 53: 373–386.

Bird, A. E., H. Hesketh, J. V. Cross, and M. Copland. 2004. The common black ant, *Lasius niger* (Hymenoptera: Formicidae), as a vector of the entomopathogen *Lecanicillium longisporum* to rosy apple aphid, *Dysaphis plantaginea* (Homoptera: Aphididae). Biocontrol Sci. Technol. 14: 757-767.

Branson, D. H., A. Joern, and G. A. Sword. 2006. Sustainable management of insect herbivores in grassland ecosystems: new perspectives in grasshopper control. Bioscience 56: 743–755.

Bruck, D. J., and L. C. Lewis. 2002. *Carpophilus freemani* (Coleoptera: Nitidulidae) as a vector of *Beauveria bassiana*. J. Invertebr. Pathol. 80: 188–190.

Carruthers, R. I., Z. Feng, M. E. Ramos, and R. S. Soper. 1988a. The effect of solar radiation on the survival of *Entomophaga grylli* (Entomophthorales: Entomophthoraceae) conidia. J. Invertebr. Pathol. 52: 154–162.

Carruthers, R. I., T. S. Larkin, and R. S. Soper. 1988b. Simulation of insect disease dynamics: an application of SERB to a rangeland ecosystem. Simulation 51: 101–109.

Carruthers, R. I., M. E. Ramos, T. S. Larkin, D. L. Hostetter, and R. S. Soper. 1997. The *Entomophaga grylli* (Fresenius) Batko species complex: its biology, ecology, and use for biological control of pest grasshoppers. Mem. Entomol. Soc. Can. 129: 329–353.

Casique-Valdez, R., S. Sanchez-Peña, R. Ivonne Torres-Acosta, and M. J. Bidochka. 2012. A PCR-based method to identify Entomophaga spp. infections in North American grasshoppers. J. Invertebr. Pathol. 109: 169–171.

Crawley, M. J. 2013. The R book, 2nd ed. John Wiley & Sons Ltd, West Sussex, United Kingdom.

Dromph, K. M. 2003. Collembolans as vectors of entomopathogenic fungi. Pedobiologia 47: 245–256.

Evans, H. 1989. Mycopathogens of insects of epigeal and aerial habitats, pp. 205–238. *In* N. Wilding, N. M. Collins, P. Hammond and J. F. Webber (eds.), Insect-Fungus Interactions. Academic Press, London, United Kingdom.

Foil, L. D., and J. R. Gorham. 2000. Mechanical transmission of disease agents by arthropods, pp. 461–514. *In* B. F. Edridge and J. D. Edman (eds.), Medical Entomology. Springer Publishing, New York, NY.

Goettel, M. S., D. L. Johnson, and G. D. Inglis. 1995. The role of fungi in the control of grasshoppers. Can. J. Bot. 73: 71–75.

Gracia-Garza, J., D. Fravel, B. Bailey, and P. Hebbar. 1998. Dispersal of formulations of *Fusarium oxysporum f. sp. erythroxyli* and *F. oxysporum f. sp. melonis* by ants. Phytopathology 88: 185–189.

Graczyk, T. K., R. Knight, R. H. Gilman, and M. R. Cranfield. 2001. The role of non-biting flies in the epidemiology of human infectious diseases. Microb. Infect. 3: 231–235.

Graczyk, T. K., R. Knight, and L. Tamang. 2005. Mechanical transmission of human protozoan parasites by insects. Clin. Microbiol. Rev. 18: 128–132.

Hajek, A., and R. St. Leger. 1994. Interactions between fungal pathogens and insect hosts. Annu. Rev. Entomol. 39: 293–322.

Kistner, E. J., and G. Belovsky. 2013. Susceptibility to disease across developmental stages: Examining the effects of an entomopathogen on a grasshopper (Orthoptera: Acrididae) pest. J. Orthoptera Res. 22: 73–77.

Kistner, E. J. and G. E. Belovsky. 2014. Host dynamics determine responses to disease: Additive versus compensatory mortality in a grasshopper-pathogen system. Ecology 95: 2579–2588.

MacLeod, D. M., and E. Müller-Kögler. 1973. Entomogenous fungi: Entomophthora species with pear-shaped to almost spherical conidia (Entomophthorales: Entomophthoraceae). Mycologia 63: 823–893.

MacLeod, D., J. Cameron, and R. Soper. 1966. The influence of environmental conditions on epizootics caused by entomogenous fungi. Rev. Roum. de Biol. 11: 125–131.

MacPhee, R. D., and A. D. Greenwood. 2013. Infectious Disease, Endangerment, and Extinction. Int. J. Evol. Biol. 2013**:** 1–9.

Meyling, N. V., and J. Eilenberg. 2007. Ecology of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* in temperate agroecosystems: Potential for conservation biological control. Biol. Control 43: 145–155.

Meyling, N. V., J. K. Pell, and J. Eilenberg. 2006. Dispersal of *Beauveria bassiana* by the activity of nettle insects. J. Invertebr. Pathol. 93: 121–126.

Pfadt, R. E. 1994. Field guide to common western grasshoppers. Wyoming Agricultural Experiment Station, MN.

Pickford, R., and P. Riegert. 1964. The fungous disease caused by *Entomophthora grylli* Fres., and its effects on grasshopper populations in Saskatchewan in 1963. Can. Enotomol. 96: 1158–1166.

R Development Core Team. 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Renker, C., P. Otto, K. Schneider, B. Zimdars, M. Maraun, and F. Buscot. 2005. Oribatid mites as potential vectors for soil microfungi: study of mite-associated fungal species. Microb. Ecol. 50: 518–528.

Sánchez Peña, S. R. 2005. In vitro production of hyphae of the grasshopper pathogen *Entomophaga grylli* (Zygomycota: Entomophthorales): Potential for production of conidia. Fla. Entomol. 88: 332–334.

Sawyer, A., M. Ramos, T. Poprawski, R. Soper, and R. Carruthers. 1997. Seasonal patterns of cadaver persistence and sporulation by the fungal pathogen *Entomophaga grylli* (Fresenius) Batko (Entomophthorales: Entomophthoraceae) infecting *Camnula pellucida* (Scudder)(Orthoptera: Acrididae). Mem. Entomol. Soc. Can. 129: 355–374.

Schmidt, A. C., L. H. Fraser, C. N. Carlyle, and E. R. Bassett. 2012. Does cattle grazing affect ant abundance and diversity in temperate grasslands? Rangeland Ecol. Manage. 65: 292–298.

Sulaiman, S., A. R. Sohadi, H. Yunus, and R. Iberahim. 1988. The role of some cyclorrhaphan flies as carriers of human helminths in Malaysia. Med. Vet. Entomol. 2: 1–6.

Therneau, T. 2013. A package for survival analysis in S. R package version 2.37-4. (http://CRAN.R-project.org/package=survival).

Torsten, H., B. Frank, and W. Peter. 2008. Simultaneous Inference in General Parametric Models. Biom. J. 50: 346–363.

Tuzzolino, K. E., and W. D. Brown. 2010. Effects of nest size and dispersion on brood production in a North American population of wood ant *Formica fusca* (Hymenoptera: Formicidae). Entomol. Sci. 13: 162–165.

Vogt, J. T., T. G. Shelton, M. E. Merchant, S. A. Russell, M. J. Tanley, and A. G. Appel. 2002. Efficacy of three citrus oil formulations against *Solenopsis invicta buren* (Hymenoptera: Formicidae), the red imported fire ant. J. Agric. Urban Entomol. 19: 159–171.

Wallis, D. 1962. Behaviour patterns of the ant, *Formica fusca*. Anim. Behav. 10: 105–111.

Wilding, N. 1970. Entomophthora conidia in the air-spora. J. Gen. Microbiol. 62: 149–157.

Wilson, K., and B. Grenfell. 1997. Generalized linear modelling for parasitologists. Parasitol. Today 13: 33–38.

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