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The effects of a myrmecochore-produced chemical on entomopathogenic fungal growth and seed-dispersing ant survival rates and foraging patterns

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Abstract

Myrmecochory, a type of ant-mediated seed dispersal, is a diffuse, widespread mutualism in which both partners are purported to benefit from the services or rewards of the other. However, ant benefits in this interaction are conflicted and understudied, especially in the context of microbial third parties. Here, we investigate the effect of a myrmecochore plant-produced antimicrobial chemical (sanguinarine) on the growth of a common entomopathogenic fungus (*Beauveria bassiana*). We then explore whether sanguinarine, through its effect on entomopathogen growth, might influence ant survival and foraging behavior. At high concentrations, sanguinarine increased the growth of *B. bassiana*, but fungal growth was not affected at concentrations of sanguinarine near natural levels produced in seeds. When ant colonies were exposed to *B. bassiana*, survival was not affected by a sanguinarine-supplemented diet. Furthermore, ant foraging patterns (preference for or avoidance of food items with sanguinarine) did not change when ants were exposed to the entomopathogen. Though sanguinarine promotes the growth of an entomopathogen at higher concentrations, which might pose an additional risk for ants in myrmecochory, we assert that social immune behavioral defenses (such as grooming or redispersal of seeds after elaiosome

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consumption) help ants mitigate this risk. By incorporating a microbial third party into this ant-plant interaction, we seek to more fully understand the risks and benefits provided to both partners in this mutualism. We encourage the investigation of third-party influences in reciprocal pairwise interactions to assist in the understanding of the evolution and persistence of mutualisms.

Keywords

Ant seed dispersal; plant antimicrobial chemicals; entomopathogenic fungi; mutualisms

Introduction

Though ants participate in numerous mutualisms with plant partners (see Beattie and Hughes 2002), myrmecochory, a pervasive form of ant seed dispersal, is the only one that actively and directly promotes the recruitment of the next plant generation. Myrmecochory exists on almost every continent and has evolved independently over one hundred times (Lengyel et al. 2010). Over 11,000 myrmecochorous plant species are dependent on ants for dispersal (Lengyel et al. 2010). Myrmecochorous interactions vary along elevational gradients (Del Toro and Ribbons 2019, Zelikova et al. 2008), with ant abundance and community composition (Del Toro and Ribbons 2019), with plant community composition (Del Toro and Ribbons 2019), in the presence of invasive ant species (Rodriguez-Cabal et al. 2011, Warren et al. 2015), and as climate warms (Stuble et al. 2014, Warren II et al. 2011). Although ant interactions with myrmecochorous plant seeds range from true myrmecochory to incomplete dispersal to parasitism (see Penn and Crist 2018 and references therein), here we constrain our definition of myrmecochory to the framework of the exchange of food rewards for dispersal services (as described in Warren II and Giladi 2014 or originally cast by Beattie 1985 or Handel and Beattie 1990). Upon myrmecochorous fruit dehiscence (release of seeds from the fruit), foraging ants are attracted to a fleshy seed-coat derived appendage, known as the elaiosome, which signals ants to bring diaspores (seed-elaiosome complex) back to the nest (Rico-Gray and Oliveira 2007). In the nest, elaiosomes are removed and preferentially fed to larvae (Fischer et al. 2005). Then, ants most often redisperse seeds outside of the nest into a midden pile (Beattie 1985). Thus, in this purported mutualism, plants gain a number of benefits, including but not limited to: 1) escape from distance- and density-dependent mortality agents, 2) protection from seed predators, such as rodents, and 3) a nutrient-rich germination site in or near ant nests (Beattie 1985). For their services, ants are rewarded with food in the form of the elaiosome (Beattie 1985).

Evidence for the quality of the elaiosome reward to the ant partners in the above-posed framework of myrmecochory, however, is either conflicted or lacks support. Compared to studies of the plant partners, there are relatively few studies that investigate benefits to the ant (Caut et al. 2013), and the lack of evidence of long-term benefits to the ant partners has led some to question the classification of this interaction as a mutualism (Warren II et al. 2019). Elaiosome diets may increase gyne (reproductive female) production (Fischer et al. 2005; Fokuhl et al. 2007; Morales and Heithaus 1998), or they may not (Bono and Heithaus 2002; Fokuhl et al. 2007). Elaiosomes increase larvae production (Bono and Heithaus 2002; Fischer et al. 2005; Fokuhl et al. 2007) perhaps due to essential nutrients in the elaiosome

(Gammans et al. 2005), but that benefit might only occur when insect prey are not abundant (Clark and King 2012). However, other studies show that elaiosome diets, even as a supplement to a standard diet, do not increase brood production (Caut et al. 2013), which suggests lack of nutritional supplementation in the elaiosome (Marussich 2006). Furthermore, some seeds have misleading chemical cues, which advertise greater nutritional benefits to ants than they actually provide (Turner and Frederickson 2013). Finally, myrmecochory is a facultative, not an obligate, mutualism from the ants' perspective (Ness et al. 2009). For example, myrmecochores in eastern North America are primarily dependent on one ant genus (*Aphaenogaster* spp.) for dispersal while the ants can functionally exist without the plant species (Ness et al. 2009).

The roles of chemicals and microbes within myrmecochory have been largely overlooked in the context of benefits and costs to each partner. Plants produce secondary compounds, some of which have antimicrobial properties, as a defense against pathogenic plant microbes. For example, *Sanguinaria canadensis* (bloodroot), a common myrmecochore of forests in the eastern United States, contains an alkaloid called sanguinarine in all of its tissues, including its seeds (Campbell et al. 2007). Though antimicrobial secondary compounds are produced primarily to benefit the plant, ants likely encounter these antimicrobials throughout the dispersal process, especially during the removal and consumption of the elaiosome. If plant-based chemicals, such as sanguinarine, inhibit growth of entomopathogens (insect-specific pathogens), then ants exposed during seed-dispersal may experience additional byproduct benefits (sensu (Sachs et al. 2004)) beyond nutritional gain. Furthermore, ant colonies already suffering from high pathogen loads could augment this benefit by preferentially collecting food sources containing plant secondary compounds. However, the ability of these plant-produced chemicals (antimicrobial secondary compounds and others) to limit growth of entomopathogenic is unclear. Though most studies show inhibitory effects of plant chemicals on entomopathogens, some chemicals have the opposite effect (Cory and Ericsson 2009). For example, certain plant chemicals increase the toxicity of an entomopathogenic bacterium (Ludlum et al. 1991; Shikano et al. 2018), and methyl salicylate, a plant herbivore-induced volatile, increased growth (but not germination) of an entomopathogenic fungus that infects mites (Hountondji et al. 2006). If plant chemicals, such as sanguinarine, promote entomopathogenic growth, ant colonies might be increasing infection risk through dispersal actions, and might therefore preferentially avoid food sources with fungal growth-promoting chemicals.

Here, we investigate the effects of a known plant antimicrobial, sanguinarine, on a virulent seed-dispersing ant pathogen. By understanding the effects of this plant chemical on ant partners, we can begin to consider byproduct benefits or additional challenges for seed-dispersing ants in a myrmecochorous system, and we predict that these methods can be used to further explore the effects of plant chemicals on ant partners in myrmecochorous systems. To investigate the effects of sanguinarine on seed-dispersing ants in eastern North America, we first measure effects of sanguinarine on the growth of an entomopathogenic fungus. Then, we examine the effects of a plant-produced antimicrobial diet on ant survival when ant colonies are exposed to the entomopathogen. Finally, we document foraging preferences for food sources with and without plant-produced antimicrobials in ant colonies exposed to the entomopathogen.

Materials and Methods

Study System:

All ants used in our work are in the genus *Aphaenogaster*, the keystone seed-dispersing ant genus in eastern North America (Ness et al. 2009). We used both *A. picea* and *A. rudis* ants, which are species within the *Aphaenogaster rudis-fulva-texana* species complex (Umphrey 1996). Ant colonies were collected from the field using wooden nest boxes (Lubertazzi 2012) placed at our field sites (Blake Tract Nature Preserve and Falls Ridge Nature Preserve, Montgomery County, VA) in April of 2016 and 2017. Nest boxes were opened and ant colonies were placed into large plastic containers (23cm x 14cm x 8cm) with mesh-covered holes in the lid. We lined plastic containers with Insect-a-Slip (Bioquip) to prevent escape. Colonies were housed in environmental chambers located at Mountain Lake Biological Station (MLBS- University of Virginia, Giles County, VA) with the following settings: 14 hr 24°C:10 hr 13°C day/night cycle; ~60% relative humidity. These settings mimic the natural summer conditions at MLBS. We provided ant colonies with water *ad libitum* and fed colonies a modified Bhatkar-Whitcomb diet (prepared as in Dussutour and Simpson 2008 with 1L distilled water, 10g LB Agar Miller (Fisher), two raw eggs, 2g of Vanderzant vitamin mixture for insects (Sigma), and 170g honey). An aluminum foil-covered test tube with a water-soaked cotton ball in the base served as a makeshift nesting place for the colony in the plastic container. Test tubes were cleaned and replaced approximately every other week or when dirty. Feeding, water provisioning, and cleaning were modified when colonies were being used in an experiment.

Because of its antimicrobial compounds (primarily sanguinarine) and its abundance in eastern North America, we used *S. canadensis* for our experiments. *Sanguinaria canadensis* fruits were collected from field sites in late May and early June of 2016 and 2017 when the fruits were close to natural dehiscence or were beginning to open. Fruits were frozen at -20°C upon return to the lab and remained frozen until used in experiments. In 2016, we supplemented collected *S. canadensis* fruits with fruits ordered from Amanda's Garden (Springwater, NY). Seeds were shipped on ice and immediately moved to a -20°C freezer upon arrival.

To challenge ants with infection, we used *Beauveria bassiana*, a common, generalist entomopathogen that is known to infect ants (Meyling and Eilenberg 2007; Zimmermann 2007). *Beauveria bassiana* spores attach to the cuticle of insects, penetrate the cuticle, kill the host, and sporulate from the cadaver (Zimmermann 2007); thus, cause of death can be identified. For our experiment investigating the effects of sanguinarine on fungal growth, we used *B. bassiana* strain Bb11-98 that was isolated from a click beetle and has been shown to infect numerous insects (Ownley et al. 2004). The isolate was a generous gift from B.H. Ownley, University of Tennessee, Knoxville. For all experiments involving ants, we used *Beauveria bassiana* strain GWA (BotaniGard).

Measuring Effects of Sanguinarine on *B. bassiana*:

To analyze the impact of sanguinarine on *B. bassiana* growth, we conducted growth assays using Bioscreen C (Oy Growth Curves Ab Ltd, Helsinki, Finland), an instrument that

measures fungal growth as a function of increased turbidity of the medium. A stock solution of 0.8mM sanguinarine was made by dissolving 1.5mg of Sanguinarine Chloride (Tocris Bioscience) in 5mL of sterile deionized water. Potato Dextrose Agar (PDA) was prepared at 0.125% strength for semi-solid media, which promoted germination of fungal spores and subsequent hyphal growth (Medina et al. 2012). *Beauveria bassiana* spores were harvested from stock plates and placed in a sterile 0.05% Tween 20 solution at a concentration of approximately 10^4 *B. bassiana* spores/mL; based on results of preliminary tests, spore germination was not inhibited at this concentration and hyphal growth was supported. Wells of a 100-well microtitre plate were filled with a mixture of the following: 98µL of Potato Dextrose Broth, 50µL of semi-solid PDA, and 20µL of the *B. bassiana* spore solution. Then the stock solution of sanguinarine was added to the wells at the following amounts: 0µL, 0.5µL, 1µL, 2µL, 4µL, and 8µL. This exposes *B. bassiana* to sanguinarine at 0mM, 0.0023mM, 0.0047mM, 0.0093, 0.019mM, and 0.037mM respectively. For reference, the approximate concentration of sanguinarine in *S. canadensis* diaspores is 0.0037mm (Campbell et al. 2007). To ensure all wells contained the same amount of liquid, sterile water was added to each well to achieve a total volume of 176µL. Ten replicates of each sanguinarine concentration were used. Plates were incubated at 25 °C, and optical density was measured at the wide band setting (450-580nm) typically used for turbidity measurements. Optical density of each well was measured every 15 mins for 22 hours for days 1, 2, 3, 4, and 6 (day 5 was skipped due to limited lab access). Measurements began approximately the same time every day, and at those times, hyphal growth was confirmed using a light microscope.

Monitoring Exposed Ant Survival on an Elaiosome-based Diet:

In summer 2016, we conducted a laboratory-based experiment to investigate the effects of an elaiosome-based diet on ants exposed to *B. bassiana*. We selected 63 captive ant colonies and pruned the colonies down to 20 workers (which is near the minimum worker number in *Aphaenogaster* colonies (Clark and King 2012)) for use in the experiment. The queen was retained in the experimental colonies to ensure normal colony behavior. Each colony was randomly placed into one of three *B. bassiana* exposure regimes: no exposure, low exposure, and high exposure. Ten workers from each colony were randomly selected for exposure. First, those ants were placed in Petri dish in a refrigerator (4°C) for one hour to slow movement. Then for the given exposure regime, each ant received a 0.3µL droplet of the following solutions on their thorax: a sterile 0.05% Triton-X solution (no exposure), a 1.88×10^6 *B. bassiana* spores/mL suspension in 0.05% Triton-X (low exposure), or a 2.44×10^8 *B. bassiana* spores/mL suspension in 0.05% Triton-X (high exposure). The exposed ants were returned to their colonies. Each colony was fed twice per week on one of three diets: 1- the modified Bhatkar-Whitcomb diet (Dussutour and Simpson 2008), 2- the modified Bhatkar-Whitcomb diet with a sanguinarine addition, or 3- *S. canadensis* diaspores. Diet 2 had a sanguinarine addition at approximately 1.2µg of sanguinarine per gram of the modified Bhatkar-Whitcomb diet, which approximately the concentration of sanguinarine that occurs naturally in *S. canadensis* diaspores (Campbell et al. 2007).

Ant colonies were maintained for four weeks (28 days). We checked for dead ants in each colony daily. All dead ants were removed and surface-sterilized (Lacey and Brooks 1997) to

determine if deaths were from a presumed *B. bassiana* infection. Surface-sterilized dead ants were placed on PDA plates and were left at room temperature for 14 days. After 14 days, PDA plates were checked to determine if *B. bassiana* was the presumed cause of death. Carcasses with a white, powdery fungal growth coming out of the carcass were presumed to have died from a *B. bassiana* infection. We also counted the eggs present in the nest at the end of the 28-day period to use as a measure of reproductive health of the colony.

Investigating Foraging Preferences in Exposed Ant Colonies:

In summer 2017, we conducted preference experiments with captive ant colonies to determine if colonies exposed to *B. bassiana* would selectively feed on or avoid diaspores with sanguinarine. We pruned 56 colonies to include 60 workers and the queen. Then those colonies were split into 8 different treatments based off of three binomial factors (explained below): Brood Presence, Exposure to *B. bassiana* and Metapleural Gland Blockage. For Brood Presence treatments: colonies were either allowed to retain 100 larvae (B+) or all larvae were removed (B-). For Exposure to *B. bassiana* treatments: colonies were either exposed to *B. bassiana* inoculum or to a control solution on the carcasses of dead ants. This replicates one likely mode of transmission of *B. bassiana* in the natural environments of *Aphaenogaster* ants. For this exposure method, we removed ants from an unrelated colony, froze them at -20°C and beheaded the dead ants. This ensured observers did not confuse dead ants used for pathogen exposure with ants from the experimental colonies, in case any ants in the experimental colonies died during the course of the experiment. Beheaded ants were then either surface sterilized (Lacey and Brooks 1997) and sprayed with 2.44×10^8 *B. bassiana* spores/mL suspension (P+) in a 0.05% Triton-X solution or were surface sterilized then coated in a sterile 0.05% Triton-X solution (P-). Four *Bb*-coated or control ant carcasses were placed in front of the test tube nest three days prior to preference trials. For Metapleural Gland Treatments: the metapleural glands (MG) of workers were blocked (see below) or remained accessible to the ants. Ants secrete antimicrobial chemicals from metapleural glands, as a primary defense against pathogens (Yek and Mueller 2011). Ants actively apply metapleural gland secretions to exposed nestmates, brood, and nest material (Fernandez-Marin et al. 2006; Tranter et al. 2013) and brood survival decreases when ants do not have access to metapleural gland secretions when challenged with an entomopathogen (Tranter et al. 2013). For treatments that did not have access to their metapleural glands (MG-), we randomly selected 30 of the 60 workers and using a single bristle paintbrush, applied a drop of dental resin to the posterior end of the ant's thorax at the opening of the metapleural gland. Then, we dried the dental resin by holding the ant under a UV light for 10-15 seconds. For treatments that had open access to their metapleural glands (MG+), an equivalent amount of dental resin was applied to the top of the ant's thorax and was dried in the same manner. Prior to experimental colony set up, we experimented with the duration of the hold of the dental resin. Dental resin stayed in place for at least 5-6 days after application before needing reapplication; thus, one application was sufficient for the duration of experimentation.

All original colonies were starved for three days prior to experimental colony set up to ensure adequate hunger levels upon food presentation. Then all experimental treatments were established and given three more days to acclimate prior to the preference trials. After

acclimation, experimental ant colonies were subject to food preference trials. Plastic seed grids (4cm x 5cm) were sterilized with ethanol, and 20 spots in the grids were filled with alternating food choices: *S. canadensis* diaspores or cricket halves. Small crickets were frozen at -20°C . Limbs were removed from dead crickets after freezing and bodies were cut in half to ensure that the cricket pieces were approximately the same size as the diaspores. Crickets were chosen as *Aphaenogaster* ants often consume dead insect prey in natural environments (Lubertazzi 2012). We observed the experimental colonies for 60 mins after food placement, recording which food items were taken into the test tube nest or moved elsewhere in the nest environment. We also scored ant behavior based on interactions with the food types. A score of 1 indicated antennation of the food type. A score of 2 indicated that the food was moved somewhere on the seed grid but was not removed from the seed grid. A score of 3 indicated removal from the seed grid to somewhere else in the container, and a score of 4 indicated that the food item was moved into the ant nest. After the initial 60-minute observation, the colonies were left for 48 hours. After 24 hours and 48 hours, food choices taken into the nest and removed from the grid were counted again. Care was taken to minimally disturb colonies during 24 and 48 hours checks. We removed lids from plastic containers to count food items, and only if necessary, removed aluminum foil from test tubes to count any changes in number of dispersed food items taken into the test tube nest.

Statistical Analyses

To determine effects of sanguinarine on the growth of *B. bassiana*, we ran a beta regression (package “glmmTMB” version 1.0.2.1 (Magnusson et al. 2020)) on the final optical density readings from each day with day, treatment and their interaction as response variables. We included replicate (n=10) as a random variable. We conducted hypothesis testing on the models using Wald Chi-Squared tests (package “car” version 3.0-6 (Fox et al. 2019)) to determine significant factors. Post-hoc pairwise comparisons were made using estimated least squares means (package “emmeans” version 1.4.4 (Lenth et al. 2020)) to determine differences between pairs of treatments and between days.

For survival analysis of ant colonies (n=63) on different diets, we used mixed effects Cox Proportional Hazard Regression models (package “coxme” version 2.2-16 (Therneau 2020)) with diet and exposure level as explanatory variables, and colony, ant species ID and collection location as random effects. We again ran Wald Chi-Squared tests to determine significant factors for hypothesis testing and ran post-hoc pairwise comparisons testing using estimated least squares means for differences within significant variables. Final egg count data was normally distributed, so we used an ANOVA (package “car”) to test effects of diet and exposure level on final egg counts.

Ant colony (n=56) dispersal of food types back to the ant nest and to other locations in the nest environment were analyzed using generalized linear models with a quasibinomial distribution (package “stats”). Brood presence, metapleural gland access, pathogen exposure and their interactions were included as response variables at each time point (60 mins, 24 hours, or 48 hours). We conducted hypothesis testing using Wald Chi-Squared tests. Ant behavior scores by food type were analyzed via a contingency table with a Chi-square test for independence. To test the different treatments (brood presence, MG access, pathogen

exposure) on behavior types, we ran a generalized linear model using the Poisson distribution. The interaction between food choice and behavior type as well as brood presence, metapleural gland access and pathogen exposure were included as response variables. Wald Chi-Squared tests and post-hoc pairwise comparisons were used to determine significant predictors. All analyses were done in R (version 3.6.0). An alpha value of 0.05 was used to determine significance for all statistical testing.

Results

Measuring Effects of Sanguinarine on *B. bassiana*:

The effect of sanguinarine on *B. bassiana* growth was concentration dependent with higher concentrations of sanguinarine promoting *B. bassiana* growth. We found a significant interaction of the effects of day and treatment on *B. bassiana* growth ($X^2=139.82$, $df=20$, $p<2.2\times10^{-16}$). Until Day 6, there were no significant differences between pairs of treatments on the same day (based on pairwise comparisons of means; all $ps>0.05$). On Day 6, the control and the two treatments with the lowest concentrations of sanguinarine (0.0023mM and 0.0046mM), all had significantly less growth than the three treatments with the highest concentrations of sanguinarine (0.0093mM, 0.019mM, and 0.037mM) (based on pairwise comparisons of means; all $ps<0.0001$; Fig 1). On average, the lower concentrations of sanguinarine exhibited half the growth of the higher concentrations of sanguinarine.

Significant growth of *B. bassiana* for many treatments occurred between Days 1 and 2 and between Days 4 and 6. Generally, in lower sanguinarine concentration treatments, fungal growth as measured by an increase in turbidity was greater on Day 2 than on Day 1, and in the higher sanguinarine concentration treatments, turbidity increased from Day 4 to Day 6. Pairwise comparisons of means indicated that growth in the same treatments on consecutive reading days significantly increased for the following treatments: 0.0023mM (Days 1-2, $p=0.027$; Online Resource 1), 0.0046mM (Days 1-2, $p=0.048$; Online Resource 1), 0.019mM (Days 1-2, $p=0.031$; Online Resource 1), 0.0046mM (Days 4-6, $p<0.0001$; Fig 2), 0.0093mM (Days 4-6, $p<0.0001$; Fig 2), 0.019mM (Days 4-6, $p<0.0001$; Fig 2) and 0.037mM (Day 4-6, $p<0.0001$; Fig 2).

Monitoring Exposed Ant Survival on an Elaiosome-based Diet:

Of 1206 ants, 217 died from a presumed *B. bassiana* infection with most deaths occurring in the high exposure treatments. The interaction between diet and exposure level was tested, but was not significant ($X^2=2.94$, $df=4$, $p=0.57$) and hence, was not included in the final model. Survival differed by treatment ($X^2=290.75$, $df=7$, $p<0.0001$; Fig 3), and exposure level was the only significant predictor ($X^2=196.53$, $df=2$, $p<2\times10^{-16}$). Ants exposed to a high dosage of *B. bassiana* had approximately a 25-40% reduction in survival rate compared to ants exposed to a low dosage of *B. bassiana* or a control solution. Post-hoc testing revealed that high exposure level survival rates were significantly lower than low exposure levels and control treatments ($p<0.0001$ in both instances), but that low exposure levels and control treatments did not differ significantly from one another ($p=0.091$). Diet had no significant impact on survival ($X^2=1.43$, $df=2$, $p=0.49$). Egg production did not significantly differ by diet ($F=1092$, $df=2$, $p=0.53$) or by exposure level ($F=214$, $df=2$, $p=0.88$).

Investigating Foraging Preferences in Exposed Ant Colonies:

We found no evidence of foraging preferences in ant colonies. Neither brood presence, nor metapleural gland access, nor pathogen exposure predicted the type of food (cricket or diaspore) taken into the ant nest at any time point (all $p>0.05$, Fig 4). Likewise, no significant interactions were found between explanatory variables (all $p>0.05$). Furthermore, no explanatory variables or combination predicted the food items moved off of the grid but elsewhere in the nest environment at any time point (all $p>0.05$, Online Resource 2).

There was no difference in ant dispersal behavior by food type ($X^2=2.94$, $df=3$, $p=0.40$; Table 1). We also found no effects of treatment on behavior by food type ($X^2=2.99$, $df=3$, $p=0.394$). However, absence of brood decreased foraging behavior overall (based on pairwise comparisons of means; all $p<0.05$) and decreased metapleural gland access increased foraging behavior overall (based on pairwise comparisons of means; all $p<0.05$).

Discussion

Here, we document that an antimicrobial chemical, sanguinarine, found in seeds of a myrmecochorous plant promotes the growth of a common ant pathogen, *Beauveria bassiana*, at certain concentrations. While this might present challenges to seed-dispersing ants that bring seeds with this chemical into the nest, ant survival and foraging patterns when exposed to *B. bassiana* were not affected by the presence of this chemical. This work begins the incorporation of plant chemicals' effects on ant microbial mortality agents in myrmecochory. Particularly, chemicals and microbial pathogens have important implications for the costs incurred and benefits offered to ant partners in myrmecochorous systems, which ultimately impacts the persistence of these mutualistic interactions.

We found that at concentrations of sanguinarine higher than those found naturally in *S. canadensis* seeds growth of *B. bassiana* was promoted. However, at concentrations close to the natural concentration in seeds, growth was not affected. More specifically, we found that concentrations of sanguinarine over 0.0093mM had a greater effect on growth of the entomopathogen at Day 6 of the experiment. The approximate concentration of sanguinarine in *S. canadensis* diaspores is 0.0037mM (Campbell et al. 2007). Though this concentration is for the seed-elaiosome complex (diaspore) rather than just the elaiosome, we expect that the ants are being exposed to sanguinarine at a relatively lower concentration, which may not be enough to promote the growth of *B. bassiana* in the nest. However, sanguinarine concentration might not be static in plant tissues. In the opium poppy (*Papaver somniferum*), cell cultures increase the production of sanguinarine when challenged with a common plant pathogen (*Botrytis* sp.) (Facchini et al. 1996; Holkova et al. 2010). Thus, if challenged with a pathogen, plant tissues might respond with increasing production of antimicrobials. If this occurs in *S. canadensis* tissues, ants might indeed be exposed to sanguinarine at a higher concentration during the dispersal process, which might result in increased growth of their own entomopathogens. If the natural concentration of sanguinarine in *S. canadensis* seeds increases by a factor of 2.5, concentrations would reach the point at which we saw increased growth in *B. bassiana* in our study. Thus, fluctuations in sanguinarine concentrations in seeds could have important implications for ant dispersers.

While we might expect that an antimicrobial chemical would inhibit the growth of all fungi, our results show the opposite. Insect-plant-fungal interactions are complicated. Most studies conducted on insect-plant-entomopathogenic fungal interactions have been done in a herbivory context (Shikano 2017). Some plant chemicals increased the toxicity of an entomopathogenic bacterium (Ludlum et al. 1991; Shikano et al. 2018). However, investigations into the effects of plant compounds on cuticle-entering entomopathogens (like *B. bassiana*) are few (Shikano 2017). A plant volatile increased sporulation in an entomopathogenic fungus that infects mites (Hountondji et al. 2006). Likewise, a combination of plant volatiles induced by herbivory promotes performance (including germination, sporulation, pathogenicity and virulence) of another entomopathogenic fungus (Lin et al. 2016; Lin et al. 2017). We found that another plant-produced chemical, sanguinarine, promotes entomopathogenic fungal growth. At a certain concentration sanguinarine appears to signal increased *B. bassiana* growth. Overall, our work suggests that the effects of plant chemicals on the growth of entomopathogens or pests in general should be examined in other plant-insect relationships, rather than just herbivory.

Ant behaviors (both foraging and social immune defenses) likely help to counteract any risk associated with growth-promoting compounds. This idea is supported by the results of our survival analysis, as exposed ant colonies did not suffer decreases in survival rates or egg production when maintained on a sanguinarine-enhanced diet. One such behavior may be redispersal. Redispersal, or the removal of the seeds from the nest after elaiosome removal and consumption, has been primarily investigated in a plant-centric view (Canner et al. 2012; Connell et al. 2016), but we suggest that this practice might also benefit the ant colonies. *Aphaenogaster* ants redisperse most of the seeds that they take into the nest after seven days (Canner et al. 2012). In laboratory and field experiments, we have observed that seeds often do not remain in the nest longer than 48 hours. Though sanguinarine is low in concentration in seeds, timing is also important. A stockpile of seeds left for days could act as a Petri dish of growth for *B. bassiana*, as even the concentrations of sanguinarine slightly higher than natural concentrations had higher growth of *B. bassiana* after Day 6. Thus, quick removal of the elaiosome for consumption and quick redispersal of the seed from the nest might reduce the risk of pathogen growth on seeds in the ant nest. Other ant species, notably leaf cutter ants, manage waste piles either inside or outside of the nest, depending on what environmental conditions are least favorable for pathogen growth (Farji-Brener et al. 2016). Further work is needed to understand the risk of seed waste in *Aphaenogaster* nests but removal of seeds after the consumption of the elaiosome could help mitigate pathogen growth in food waste.

Furthermore, ants, like other social insects, exhibit a variety of behaviors that contribute to overall nest sanitation (Cremer et al. 2007; Meunier 2015; Vander Meer 2012; Yek and Mueller 2011), which must not be disregarded in our consideration of entomopathogen presence in the nest. Ant behavioral defenses include the cleaning of nest material through active grooming of the metapleural glands, which allows ants to lower entomopathogen loads (Tranter et al. 2013). It is likely that seeds come into contact with antimicrobial secretions from the metapleural and perhaps other glands during the elaiosome removal and consumption process, and also passively as a byproduct of being in the nest environment. Thus, even if plant chemicals promote the growth of a ubiquitous entomopathogen at certain

concentrations, ants are not defenseless against infection. We saw that the addition of sanguinarine to the ant diet (either supplemental or through elaiosomes) did not alter the survival of the colony overall, regardless of the level of exposure. We assert that this can be attributed to the robust social immunity exhibited by *Aphaenogaster* ants.

Application of chemical secretions is not the only aspect of ant social immunity. Ant behavior, beyond grooming, also changes upon detection of a pathogen (Bos et al. 2012). For example, infected *Camponotus aethiops* ants spend more time outside of the nest and less time with brood than their non-infected nestmates (Bos et al. 2012), and *Lasius niger* ants change social interactions after pathogen exposure (Stroeymeyt et al. 2018). Research such as this suggests that ants are aware of their immunocompromised states and alter behaviors accordingly. Thus, ants might be able to detect the presence of an entomopathogen and alter their foraging behaviors. We found no preference for or avoidance of sanguinarine-containing diaspores in ant colonies whether or not they had brood present, access to their metapleural gland, or exposure to *B. bassiana*. Though overall documented behaviors decreased when brood were absent and when the metapleural gland was accessible (which can both be attributed to the energetic needs of the colony), preference did not change. Thus, other aspects of social immunity may eliminate the need for this kind of food choice, or behaviors might be specific to entomopathogen presence on seeds rather than the presence of a potential entomopathogen growth promoting compound. Furthermore, perhaps the antimicrobial chemicals from the seed signal nothing for ant foraging. This lack of preference also indicates that the plants are not sending misleading signals, as far as antimicrobial action. Some myrmecochores cue higher nutritional value than what is actually available to ants in the elaiosome (Turner and Frederickson 2013), but, in this experiment, ants did not select or avoid food with sanguinarine when exposed to a pathogen. We also acknowledge that the nutritional content of elaiosomes is not identical to the nutritional content of crickets, and future studies might help elucidate the distinction between dispersal trade-offs between antimicrobial chemical signaling and nutritional reward (as started by Turner and Frederickson 2013 and Miller et al. 2020).

Overall, this work demonstrates that there are no byproduct benefits to ants in the form of antimicrobials in plant tissue, at least in the *S. canadensis* system. Instead, our work suggests that, during their myrmecochorous interactions, ants may experience additional challenges resulting from the influences of a microbial third party, such as *B. bassiana*. However, there is much work left to be done to fully understand the effects of plant chemicals on seed-dispersing ants and their entomopathogens. Other myrmecochores have antimicrobial or antibiotic properties (such as *Asarum canadense* (Cavallito and Bailey 1946)) and *B. bassiana* is not the only entomopathogen to challenge *Aphaenogaster* ants. We suggest future work should incorporate larger sample sizes or maintain focus on specific aspects of ant behavior or factors that alter their susceptibility, which will allow us to make more firm conclusions about diaspore-related myrmecochore chemicals and seed-dispersing ant survival. Finally, by incorporating microbes into what is traditionally framed as a simple pairwise ant-plant interaction, we can begin to understand mutualisms in a community context (Palmer et al. 2015), which will aid in the understanding of the evolution and persistence of mutualistic interactions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig 1.

On Day 6, the last day of growth monitoring of *Beauveria bassiana*, treatments with higher concentrations of the plant-produced chemical sanguinarine had greater growth than those with lower sanguinarine concentrations. Higher optical density measures indicate increased growth of *B. bassiana*. Treatments in gray (0.0093mM, 0.019mM, 0.037mM) showed significantly more growth than treatments in white (0.0023mM, 0.0046mM, Control) (Pairwise comparisons: all $ps < 0.0001$). For reference, the approximate concentration of sanguinarine in *S. canadensis* diaspores is 0.0037mm (Campbell et al. 2007)

Fig 2.

Growth comparisons of *Beauveria bassiana* from Day 4 to Day 6 at different concentrations of sanguinarine. Treatments in gray (0.0046mM, 0.0093mM, 0.019mM, 0.037mM) showed a significant increase in growth from Day 4 to Day 6 (Pairwise comparisons: 0.0046mM ($p < 0.0001$), 0.0093mM ($p < 0.0001$), 0.019mM ($p < 0.0001$) and 0.03705mM ($p < 0.0001$). The approximate concentration of sanguinarine in *S. canadensis* diaspores is 0.0037mm (Campbell et al. 2007)

Fig 3.

Ant survival by diet and exposure regime. Panel a. represents ant survival on the diaspore diet. Panel b. represents ant survival on the modified Bhatkar-Whitcomb diet, and panel c. represents ant survival on the modified Bhatkar-Whitcomb diet with the sanguinarine addition. Shape corresponds to the exposure regimes: squares are the no exposure regime, triangles are the low exposure regime, and circles are high exposure regime. Survival differed by treatment ($X^2=290.75$, $df=7$, $p<0.0001$). Exposure level was the only significant predictor ($X^2=196.53$, $df=2$, $p<2 \times 10^{-16}$). High exposure level treatments differed significantly from low and no exposure levels ($p<0.0001$ in both instances). Low and no exposure level treatments did not differ significantly from one another ($p=0.091$).

Fig 4.

Brood presence (B+/B-), access to metapleural glands (MG+/MG-), and exposure to a pathogen (P+/P-) had no effect on movement of food items into ant nests (all p s > 0.05). These independent variables did not affect ant preference for movement of crickets (top row) or diaspores (bottom row) taken into the ant nest

Table 1

Counts of behavior scores by food choice. Type 1 behavior indicates an instance of antennation of a food choice. Type 2 behavior indicates that a food item was moved but not off of the seed grid. Type 3 indicates removal of a food item from the seed grid. Type 4 indicates movement of a food item into the ant nest. There was no difference between behavior scores by food type ($\chi^2=2.94$, $df=3$, $p=0.40$)

Food Choice	Behavior Type 1	Behavior Type 2	Behavior Type 3	Behavior Type 4
Cricket	214	21	17	22
Diaspore	197	11	11	17