Retention and Transmission of *Entomophaga gryllii* in *Formica fusca* and *Camnula pellucida*

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My project experimentally determined the mechanisms by which *Formica fusca* ants carry the pathogen *Entomophaga grylli* to the clear-winged grasshopper (*Camnula pellucida pellucida*)**.** Prior studies have indicated that *E. grylli* is passively contracted by *C. pellucida*, yet there is little research on the role of ants as carriers of this fungal pathogen. We conducted four experiments to determine if ants (*Formica fusca*) can (1) Retain conidial spores (2) Transmit conidial spores to other ants (3) Carry conidia to grasshoppers (4) Contract resting spores from grasshopper cadavers and spread these spores within an ant farm. We found that condial spore presence in ants decreased over time, spores can be transferred to other ants and grasshoppers, and spores can be contracted from cadavers and distributed within an ant farm. The length of exposure time to infected ants did not affect the transfer of spores between ants and to grasshoppers. Our results indicate that ants may be a key carrier of *E. grylli* that has been overlooked in past research, and our findings can contribute to a predictive model for monitoring and anticipating *E. grylli* outbreaks.

Introduction

This research will expand current knowledge about disease ecology in social insects. Ants, specifically *Formica fusica*, are crucial to nutrient distribution in grasslands (Bestelmeyer and Wiens 2003). Ants are key drivers of nutrient distribution within the soil of prairie ecosystems, and this function is dependent upon the rate and range of ant foraging activity (Bestelmeyer and Weins 2003). This activity and behavior may also be important to driving the transmission of fungal spores between species in grassland ecosystems, which has epizootic implications.

Fungal-insect pathogen transmission is relevant to human interests, specifically agriculture, because fungi have been used as a form of biocontrol in controlling grasshopper and locust species (Baverstock et. al. 2010). Ants have potential to be used as a biocontrol for agricultural pests because they might be carriers of fungal pathogens that are deadly to grasshoppers, but this has not yet been experimentally verified (Bird et al 2004). This study is also important in understanding the influence of biotic and abiotic factors of *Entomophaga grylli* transmission between *Formica fusica* ants and *Camnula pellucida* grasshoppers, as current studies have only investigated E. grylli infection in grasshoppers without

considering vectoring or carrying (Carruthers et al. 1997). Increased understanding of fungal pathogen transmission between ants and grasshoppers has important implications for the understanding and application of disease ecology, to potentially allow ecologists to predict fungal disease outbreaks.

E. grylli is a fungi complex that infects locust and grasshopper species on a global scale (Carruthers et. al. 1997). *E. grylli* infection causes summit disease in grasshoppers, which climb to the top of grass stems and assume a distinctive pose shortly before death to facilitate the aerial transmission of conidial spores (Carruthers et al. 1997).

E. grylli Pathenotype 1 is the specific fungal variety that affects *C. pellucida* within the research site of this study. Pathenotype 1 produces two types of spores: resting spores with thick cell walls transmitted via soil and conidia transmitted aerially (Carruthers et. al. 1997, Hajek and Leger 1994). Once grasshoppers have contracted conidia, these mature into hyphal bodies that grow in the abdominal cavity of the insect. Passive transmission of *E. grylli* via condia or resting spores is thought to be the dominant mode of disease distribution (Figure 6). My study investigated the role ant scavengers have on disease transmission.

Ant are key scavangers of arthropod cadavers in grassland ecosystems (Bestelmeyer and Wiens 2003, Retina et al. 1991). Ants are known vectors and carriers of fungal pathogens and they may remove fungal spores by grooming (Baverstock et al. 2010, Bird et al. 2004, Gracia-Garza 1998). However, it is not clear what role ants play in the transmission of the fungal pathogen *Entomophaga grylli* and their contribution to the degradation process of *Camnula* cadavers (Retana et al 1991).

Common defense mechanisms by which social insects defend against fungal pathogens can be defined by two categories: contact avoidance and restorative measures. Contact avoidance prevents transmission and hinders the infection rate of a colony, but it is rarely successful because it can be difficult for insects to anticipate or detect a fungal pathogen (Baverstock et al. 2010). Restorative measures are those taken by infected insects to either alleviate the effects of the pathogen and to prevent its spread to other insects. A common restorative measure is self-grooming and allo-grooming (Baverstock et. al. 2010, Okuno et al. 2012).

The impact of a pathogen on an infected insect population can be understood in two ways: pathogenicity and virulence. Pathogenicity is ability to cause disease (rate of infection) while virulence is the intensity of the disease (Carruthers et al. 1997). *E. grylli* has been noted as an especially virulent species of pathenogenic fungi (Carruthers et al. 2007), but the conidia are ephemeral and difficult to sustain outside of an infected insect body.

Objectives

The goal of this research is to address the following questions: (1) What is the retention rate of conidia spores by ants? (2) Can ants carry conidia spores to other ants? (3) Can ants contract resting spores from grasshopper cadavers and distribute them throughout a nest? (4) Can live grasshoppers contract resting spores from ants?

We expected that (1) spore retention rates by ants would decrease over time (2) uninfected ants would contract conidia from infected ants (3) resting spore count in ants would increase with the proportion of infected *Camnula* cadaver placed in ant farms (4) grasshoppers can contract resting spores from infected ants.

Methods

We collected specimens on the National Bison Range in northwest Montana between June 8 and August 4, 2012, as this is the highest period of ant activity during the year (Bestelmeyer and Wiens 2003). Previous studies on the National Bison Range determined that *Formica fusca* was the dominant species group (Chan 2010, not published). We collected ants manually and using a BioQuip Insect Aspirator, which minimized the risk of crushing ants or **interfering pheromone trails**. All ants were collected from the same nest and only worker ants were collected to prevent aggression by defender ants.

Prior to experimentation, the ants were fed a mixture of honey, crushed human multivitamin tablets, and water (Bird et al. 2004). Active spores of *E. grylli* were sustained by maintaining a population of infected *Camnula pellucida*. Infected *Camnula pellucida* cadavers were collected and frozen until needed for experiments.

All infected ants were exposed to 1×10^3 ul of conidia solution with a concentration of 120 conidia spores / m^3. Lachto fuschin stain was used to detect spore presence. We used a diluted solution of Virex to clean experimental equipment.

Ant – Conidia Retention

We placed infected ants into petri dishes with moistened paper towel and stained the ant for viable spores at 6, 12, 18, and 24 hours (Okuno et al. 2012), with 10 replicates per time interval. There were separate ants for each time period.

Ant – Ant Transmission

We placed an infect ant and uninfected ant, which was marked with nail polish, into a petri dish. We stained the ants for viable spores at 6, 12, 18, and 24 hours, with ten petri dishes per treatment (Okuno et al. 2012). There were separate ants for each time period.

Cadaver – Ant Transmission

The ants were kept in Tree Toys Ant Mine farms, which have a plaster of paris base and clear acrylic top. Only ants from the same nest were housed in the same ant farm. Temperature and humidity were regulated in the ant farms in order to maintain stable living conditions for the ants by moistening the plaster of Paris at least three times per day.

The ants were starved for 24 hours prior to beginning the experiment. The control group was fed uncontaminated *Camnula pellucida* cadavers. There was a control group fed uninfected cadavers, and there were three different treatment levels of *Camnula pellucida* cadavers infected with resting spores: Early instars (25%), late instars (50%), and Adults (100%). There were fifteen ants per ant farm and four ant farm replicates per treatment level, for a total of sixteen ant farms. The experiment ran for 7 days because prior experiments demonstrate that cadavers are consumed between 7 to 10 days (Sawyer et. al. 1997).

Ant - Grasshopper Transmission Experiment

We exposed grasshoppers to infected ants for time intervals of either 6, 12, 18 or 24 hours. We then transferred the grasshoppers to individual mason jars with screen lids and grass. After four days, we stained the grasshopper abdomen contents to measure their hyphael body count.

Results

Ant – Conidia Retention

A Pearson Chi – Square analysis indicates that conidia presence decreased over time and varied across treatment groups ($p < 0.001$), which confirmed our prediction that predicted that conidia spore presence would decrease over time

(Figure 1). Standard error was 3.520, which may have been due a wide range of affectivity in allogrooming behavior.

Ant – Ant Transmission

A multivariate analysis of variance (MANOVA) showed that there was a statistically significant difference in spore retention between the infected and uninfected ants (Figure 2). Infected ants showed a statistically significant decrease over time ($F_{3, 36}$ = 92.450, $p < 0.001$), while the uninfected ants showed a low and constant spore retention level ($F_{3, 36}$ = 1.180, p = 0.332).

Cadaver – Ant Transmission

We ran two one-way analysis of variance (ANOVA) tests to determine the resting spore count for the ants and frass by treatment level. Ant spore count was significantly different by treatment level ($F_{2, 177}$ = 8.752 $p < 0.001$) (Figure 3). Spore count in frass by treatment level was not significantly different ($F2, 9$ = 2.622, p = 0.127).

A Post-Hoc Tukey test determined that while there was no significant difference in spore count between the ants fed early instars (25%) and late instars (50%), ants fed adult cadavers (100%) had a significantly different spore count from ants fed late instars and early instars (Table 2).

Ant – Grasshopper Transmission

There was no significant difference between infected grasshoppers by level of exposure time (R squared = 0.026, p = 0.729) (Figure 4). A Kruskal Wallis test confirmed the lack of significant difference between different exposure times (Table 3).

Experiment Comparison

A Chi square analysis of average spore count on ants was significantly different between experiment types: solitary ants, ants paired with other ants, and ants paired with grasshoppers (Value = 34.242, p < 0.001). Ants paired with grasshoppers showed the greatest decline in spore count across time (Table 4, Figure 5).

Discussion

Our experiments confirmed our prediction that time affects spore persistence in ants, but time did not significantly influence transmission between ants and grasshoppers. We found that ants can contract and maintain resting spores within ant farms, and that ants can transfer conidia to other ants and to grasshoppers. Although the number of spores transferred between ants and to grasshoppers was numerically low in the ant-grasshopper experiment, the findings reflect the ephemerality of conidia within a field environment. Even if a grasshopper contracts a single conidial spore, the likelihood of infection onset is quite high due to *E. grylli*'s virulence (Fisher et al 2012).

Casual observation noted that ants displayed allogrooming and social grooming during the experiments, and this removal could have influenced the measured spore count across all experiments. The high standard of error in several of the experiments (See figures 3 and 5) may have been due to varying grooming behavior of individual ants.

While ants that were paired with grasshoppers in mason jars had the greatest decline in spore count, this trend may be due to the difference between the petri dish and mason jar environments. The decrease in spores might have been due to complexity of their laboratory environment, which caused increased spore dispersal

due to more frequent contact with organic material. Humidity and temperature variance might have also contributed to this trend.

Ants that consumed adult grasshopper cadavers had higher counts of resting spores in the ant farm experiment, which may have been caused by the larger body mass of adult cadavers, which held more resting spores. We did not control for variation in body mass between early instars, late instars, and adult cadavers, which may have affected the consumption and transmission of spores by ants.

These experiments confirmed our hypotheses and provide promising evidence that ants can vector *E. grylli* and redistribute resting spores with their mounds. These results, in congruence with further research, might provide a predictive model for anticipating *E. grylli outbreaks*, which would be valuable for biocontrol initiatives.

Future Research

Because the experimental design created ideal conditions for ant, grasshopper and conidia survivorship, our laboratory findings are unlikely to be replicated in a field environment. Future studies should then measure the effect of abiotic factors, such as temperature and moisture, on spore transmission and retention. An increased number of replicates and longer time periods would also allow us to better determine the success rate of spore transmission, especially in the ant farm experiment. Due to the noted variation in ant behavior, future research should then investigate the specific behavioral effects of spore presence and transmission in ants by measuring the frequency of allogrooming and social grooming.

Acknowledgements

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Tables and Figures

Table 1. Chi square analysis of conidia counts on ants over time by treatment. Treatment type had a statistically significant impact on conidia presence ($p < 0.001$, Value = 34.242). Ants paired with grasshoppers had significantly fewer conidia.

Pearson Chi-Square

Time (Hour)	Solitary	Paired with Ant	Paired with Grasshopper	Total
6	561	583	304	1448
12	367	301	207	875
18	164	193	152	509
24	39	35	8	82
Total	1131	1112	671	2914

Table 2. Tukey test of 1-way analysis of variance (ANOVA) comparing the resting spore count on ants by treatment type for the ant farm experiment. There was a significant difference between ants that ate adult cadavers and those that ate early or late instar cadavers ($F_{2,177} = 8.752$, $p < 0.001$).

Tukey Test

Treatment	Treatment	Difference	P Value	95% Confidence Interval	
				Lower	Upper
Early	Late	-0.267	0.875	-1.537	1.003
Early	Adult	-2.083	0	-3.353	-0.813
Late	Adult	-1.817	0.002	-3.087	-0.547

Table 3. Pairwise comparison of resting spore count (s/m^3) in grasshoppers by exposure time (hour). There was no significant difference in resting spore count between different exposure times (R squared = 0.026, $p = 0.729$).

Pairwise Comparison - Resting Spore Count by Treatment

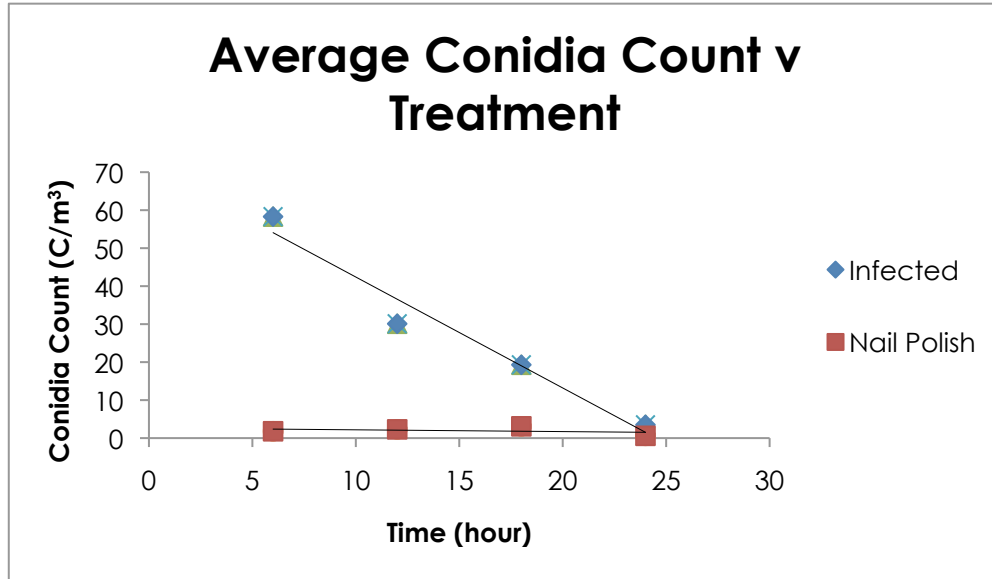| Exposure Time	Exposure Time	Statistic	p-Value
6	12	-0.712	0.958
6	18	0.646	0.968
6	24	0.646	0.968
12	18	1.345	0.777
12	24	1.345	0.777
18	24	0	1

Table 4. Pearson Chi-Square analysis of spore counts versus exposure time by experiment. There was a significant difference in spore counts between experiments, and there was a significant decrease in spore count over time (Value = 34.242, $p < 0.001$).

Pearson Chi-Square – Spore Count (Ant) v Exposure Time by Experiment

| Time (Hour)	Solitary	Paired with Ant	Paired with Grasshopper	Total
6	561	583	304	1448
12	367	301	207	875
18	164	193	152	509
24	39	35	8	82
Total	1131	1112	671	2914
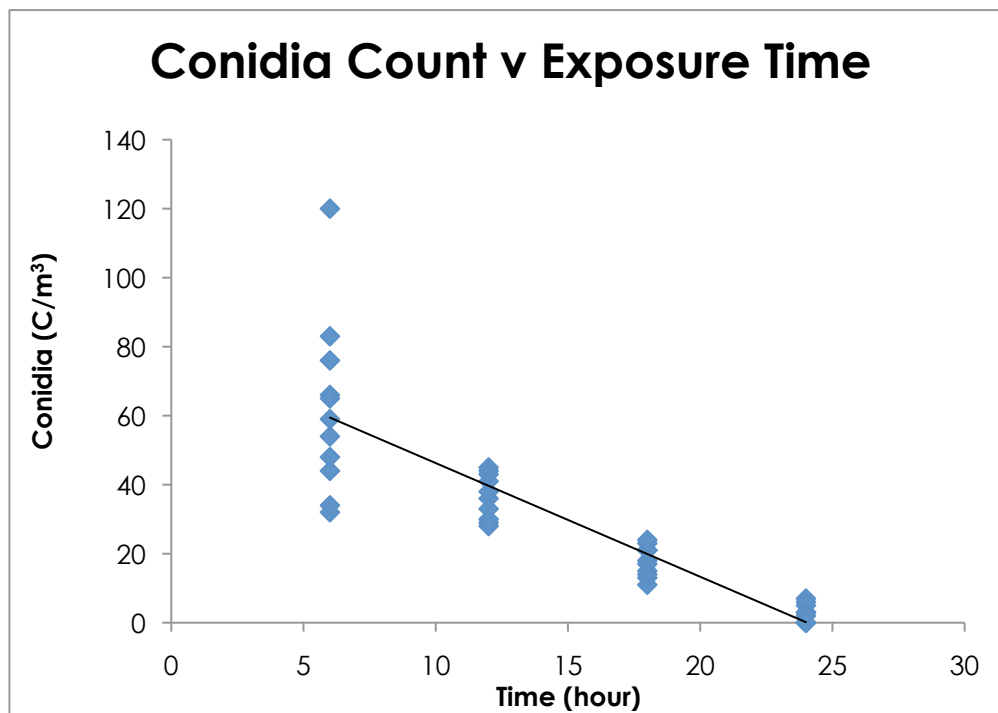
Figure 1. We tested conidia count (C/m^3) by exposure time (hour) in solitary ants. Exposure time has a statistically significant effect on conidia counts ($p < 0.001$).

Figure 2. MANOVA testing conidia count (C/m^3) by exposure time (hour) and treatment (infected and nail polish). Time had a statistically significant impact on infected ants $F_{3, 36}$ = 92.450, p < 0.001), while the uninfected ants did not ($F_{3, 36}$ = 1.180, p = 0.332). Error Bars are SE.
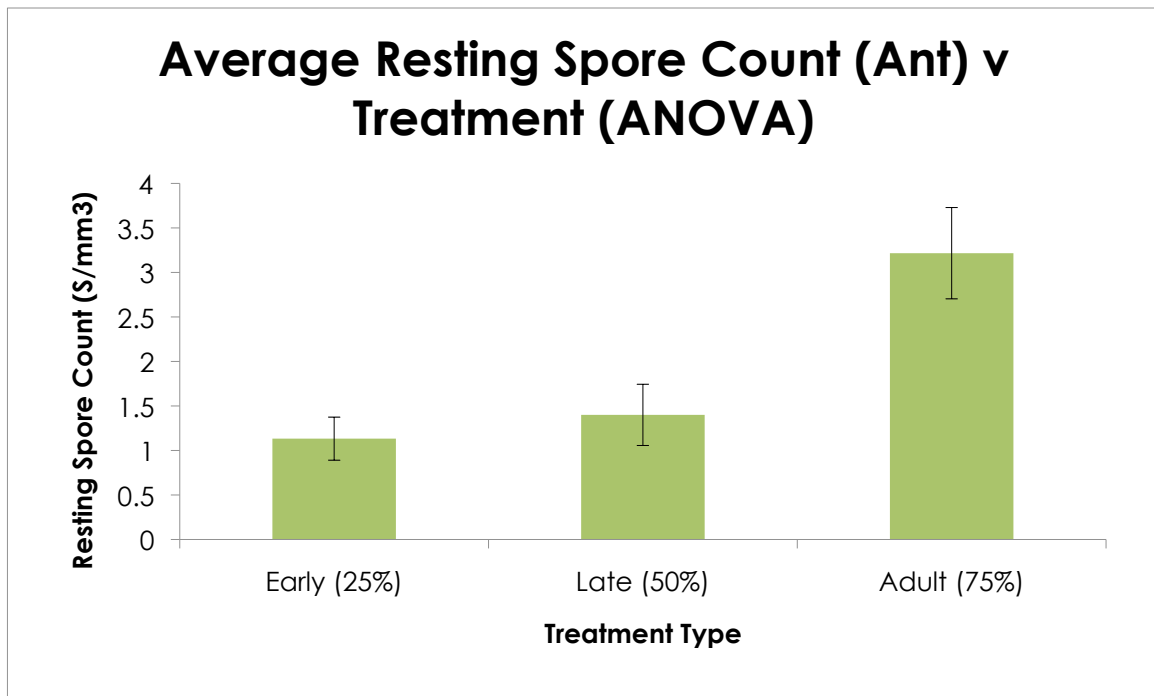

Figure 3. 1-way analysis of variance (ANOVA) testing the resting spore count in ants by treatment level. Treatment type had a statistically significant impact on resting spore count in ants ($F_{2,177}$ = 8.752, p < 0.001). There was a significant difference between ants fed adult cadavers and early instars (p <0.001) and late instars (p = 0.002).
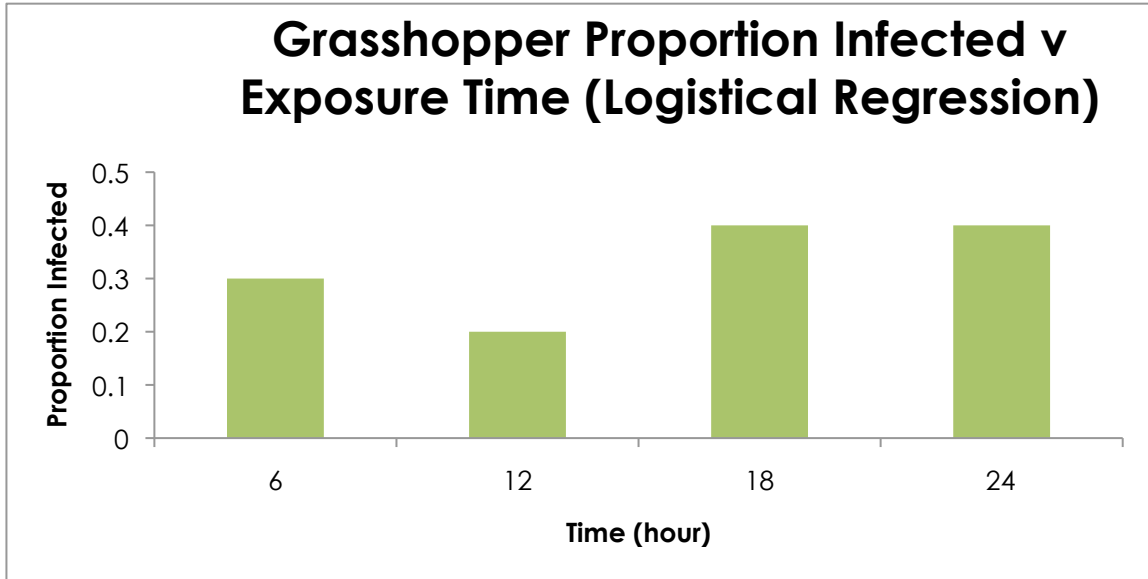
Figure 4. Logistical regression measuring proportion of grasshoppers infected with resting spores by exposure time to infected ants.
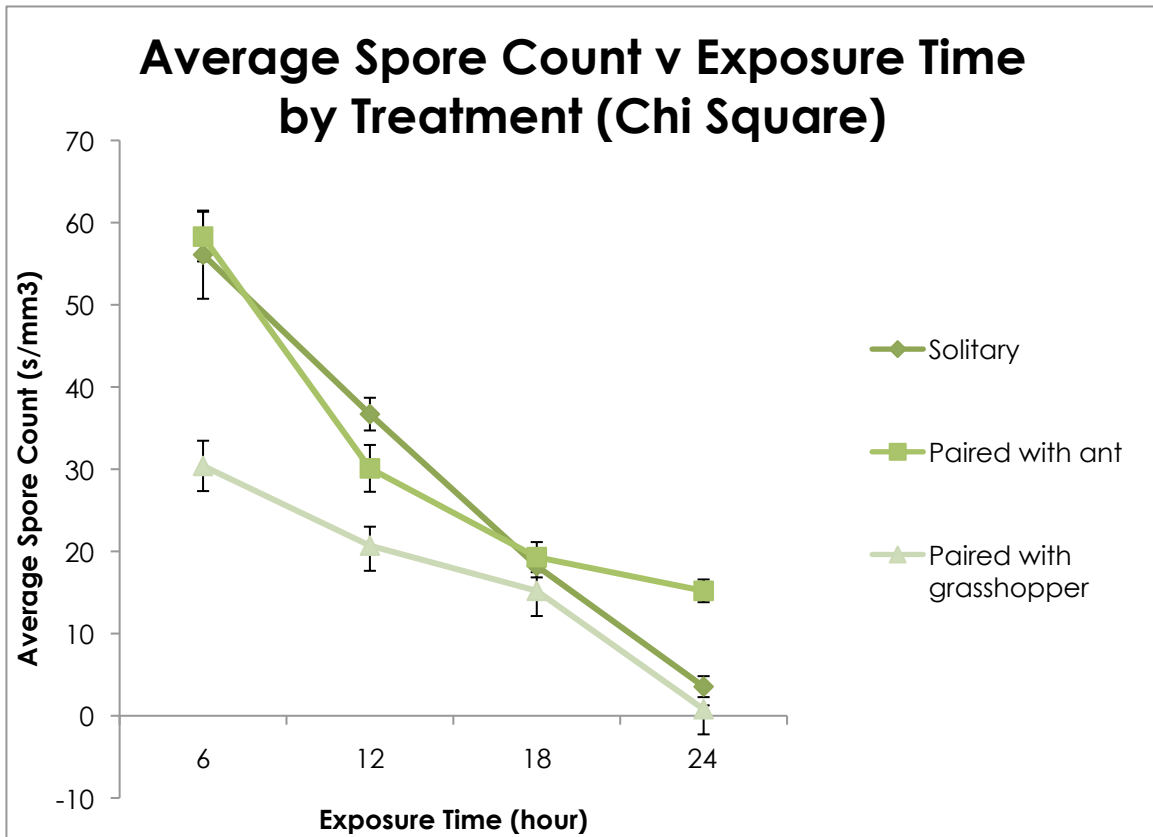
Figure 5. Chi square analysis of average spore count on ants by experiment. There was a significant difference between average spore count and experiment type (Value = 34.242, $p < 0.001$), and ants paired with grasshoppers showed the greatest decline in spore count across time. Error bars are SE.
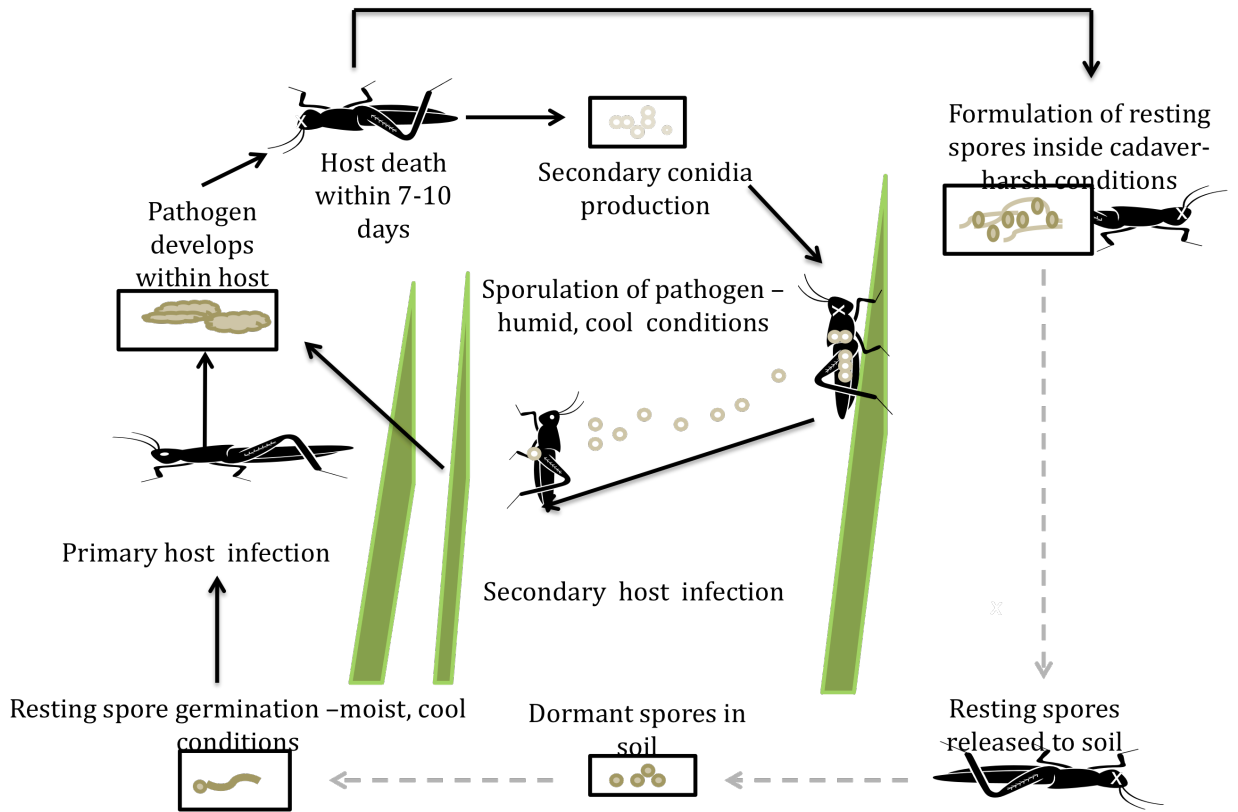
Figure 6. Diagram of *E. grylli* life cycle Pathenotype 1. Used with permission from Erica Kistner.