DISTRIBUTIONS OF *ENTOMOPHAGA MAIMAIGA* RESTING SPORES IN OAK FORESTS AND IMPLICATIONS FOR GYPSY MOTH POPULATIONS*

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Abstract. The gypsy moth (*Lymantria dispar*) is a major pest of Eastern hardwood forests, and studies of its population dynamics have important management applications. Previous studies have found that its population is regulated at low densities by the white-footed mouse, *Peromyscus leucopus*, and at high densities by the fungus *Entomophaga maimaiga*. To determine what role the fungus plays in regulating gypsy moth populations at low densities, this study examined the resting spore load in the soil of a forest where gypsy moth population density was extremely low (<0.3 egg masses/ha). Soil was sampled around the base of 112 trees and physical spore counts were performed. The counts were then used to estimate the current infection risk faced by gypsy moth larvae. Spore densities were found to be extremely low, with some trees having no spores present at all, and one or two trees having slightly higher densities. Risk calculations predict that approximately 11.08% of larvae will die from infection; however, only 1.85% of larvae will die in close proximity to another larva, providing the fungus with an opportunity for transmission. As a result of the low densities of fungal spores and the extremely low infection and transmission risks, it appears that the fungus does not play an active role in regulating moth populations at low densities. A certain threshold density of moths appears to be required in order for the fungus to operate. Furthermore, it appears that the mice are actually causing a local extinction of the fungus by keeping moth populations at such a low density that the fungus is unable to replenish itself in the soil. As a result, the next gypsy moth outbreak has the potential to cause massive damage. Potential management solutions include augmenting the spore load in the soil, introducing sterile egg masses, and supplemental watering.

INTRODUCTION

The gypsy moth (*Lymantria dispar*) was first introduced into the United States from Europe in the late 1860's. Since then, it has expanded its range throughout much of the Northeast and has become a major defoliator of hardwood forests (Campbell, 1974). Defoliation is harmful for trees because they have to expend more energy to produce a second growth of leaves, and this stress makes them more susceptible to other forest pests. Deciduous trees can survive one or two defoliations in a season, but not three, and coniferous trees die after a single defoliation (Leonard, 1981). Because the moth is such a severe threat to forest ecosystems, much research has been devoted to finding ways to control the pest and curb its spread.

The gypsy moth is an outbreak insect, meaning that it typically exists for many years at a low density, followed by an outbreak period, when the population density skyrockets (Elkinton & Liebhold, 1990). Many researchers have turned to the insect's natural enemies to understand factors regulating its population cycles. The moth's natural enemies include birds, small mammals, and various parasites and pathogens (Campbell, 1974). The white-footed deer mouse (*Peromyscus leucopus*) feeds on gypsy moth pupae and has been found to play a key role in regulating moth populations at low densities (Ostfeld et al., 1996; Jones et al., 1998). While mice are able to keep moth populations in check and prevent outbreaks, they are never able to completely extirpate moths from the forest. This is because of the distribution of mouse populations in the forest. Some areas are mouse "hot spots" with high mouse densities, and other areas are "cold spots" with no mice. Moth pupae are able to survive in areas that are mouse "cold spots," and this, in addition to the moth's limited dispersal abilities, allow the moth to persist in the forest

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even at high mouse densities (Goodwin et al., 2005). Egg masses are more likely to be laid in "cold spots" because adult female gypsy moths are flightless, and thus lay eggs wherever they survive pupation.

Another factor affecting moth populations is mortality caused by the entomopathogenic fungus *Entomophaga maimaiga*. The fungus is a natural enemy of the gypsy moth and infects populations in Asia. It was first reported to be infecting North American populations in 1989. *E. maimaiga* infects gypsy moth larvae, and has two spore forms, azygospores and conidia. Azygospores (resting spores) are produced inside cadavers that are left hanging on tree trunks, and when the cadaver falls from the tree trunk, the azygospores enter the soil. They remain dormant through the winter, germinating about the same time gypsy moth larvae start to hatch in the spring (Hajek, 1999). Azygospores are thick-walled and long-lived, and it is estimated they can survive as long as 10 years in the soil (Weseloh & Andreadis, 2002). By contrast, conidia spores are short-lived; they are produced externally on cadavers, spread by the wind, and do not overwinter (Hajek, 1999). Larvae pick up azygospores from walking around in the soil, and they are exposed to conidia spores any time they are downwind from a cadaver. When a larva picks up either type of spore, it attaches to their cuticle and burrows inside. Once inside the cuticle, the fungus replicates itself in the hemolymph, eventually killing the larva and leaving its reproductive spores (Hajek, 1999). An interesting thing to note is that larvae infected by resting spores produce only conidia, while larvae infected by conidia produce both types of spores (Hajek, 1997). The amount of time it takes for the fungus to kill a larva varies, but death generally occurs within several days of infection (Hajek, 1999).

When gypsy moth populations start to increase prior to an outbreak, they eventually reach a certain threshold density above which predation by the white-footed mouse can no longer control their numbers. However, once they reach this density *E. maimaiga* is able to cause an epizootic or widespread infection event to prevent them from causing further damage (Hajek, 1999). Greater gypsy moth population densities ensure that conidia produced on cadavers will be able to reach and infect other gypsy moth larvae. Thus, it is the conidia spores that are responsible for the within-generation amplification of fungal infection leading to an epizootic. Because only larvae infected by conidia produce azygospores, the density of fungal spores in the soil can be used to indicate how prevalent the fungus is and how much amplification of infection is occurring.

We know that *P. leucopus* is effective at regulating gypsy moth populations at low densities, and that *E. maimaiga* can prevent outbreaks at higher densities. This study examined the possible regulatory role of *E. maimaiga* in low density gypsy moth populations. Soil was collected from a forest where the gypsy moth density has been low for several years and spore counts were performed to ascertain the prevalence of infection during those years.

METHODS AND MATERIALS

Site description

Three 2.25-ha grids on the Cary Institute of Ecosystem Studies (CIES) property in Millbrook, New York, USA were chosen to be sampled. The grids used in this study (Henry Experimental, Green Experimental, and Tea Experimental) along with three other grids (Henry Control, Green Control, and Tea Control) are used for several CIES long term paired grid studies, and are set up as rectangular arrays of 11x11 trap stations, with 15m between stations. All grids are located in mixed-hardwood forests dominated by oaks, and the four species of oak found on the grids are Red Oak (*Quercus rubra*), Chestnut Oak (*Q. prinus*), Black Oak (*Q. velutina*), and White Oak (*Q. alba*). Yearly egg mass surveys on all grids indicated that gypsy moth population density had been low for several years (<0.3 egg masses/ha). All trees on CIES grids are kept on a list with their species, DBH, and GPS location.

Soil sampling

Because the greatest density of resting spores is found at the base of gypsy moth host trees (Hajek et al., 1998), the decision was made to take samples from the base of oak trees on each of the plots. Trees for sampling were selected from the list of all trees on each grid using the following criteria: they must be oak (gypsy moth preferred host), and

their DBH must be within 1 standard deviation of the mean of each plot (because older trees would likely have more spores than younger trees). With these restrictions, 116 trees were designated for sampling. Trees were located using the grid system to gauge the approximate location of each tree. The identity of each tree was confirmed by comparing its DBH and species with the DBH and species for the tree at that location on the list. Four of the trees were found dead on the ground or missing altogether and these trees were not sampled, resulting in the collection of 112 samples rather than 116.

Approximately 100 ml of soil were collected using a soil corer from each of the four cardinal directions around the base of the tree and then pooled together into one sample, as in Weseloh & Andreadis (2002). The soil was collected within a 10 cm radius of the tree and no deeper than 3 cm from the surface, because this is where the highest concentration of spores is found (Hajek et al., 1998). Between trees, the corer was washed first with water to remove clumps of soil, then with ethanol to kill spores, then dried. Gloves used to handle the corer were also changed between samples, and the researchers avoided stepping within a 1ft radius of the tree to prevent contamination from shoes. Soil samples were stored in quart-sized freezer bags at 4°C in darkness until ready for use. This temperature is ideal for maintaining resting spore viability (Hajek et al., 2001), and spore viability was desired for future work.

Spore counts

Physical spore counts were performed on each of the samples using the simplified method outlined in Weseloh & Andreadis (2002). First, 100 ml of each sample were measured, weighed, and mixed with 100 ml of sterile water. This mixture was then poured through a set of sieves of decreasing size down to 60 μm using an additional 800 ml of water. This ensured that *E. maimaiga* spores were retained in the filtrate, because they have an average diameter of 32.1 μm (Soper et al., 1988). The filtrate was placed in a 1500 ml beaker and allowed to settle for 30 minutes. After this time about 800 ml of the water were decanted, leaving a 200 ml residue. The residue was then mixed thoroughly in a 250 ml beaker and allowed to settle for an additional 3 minutes. A 5 ml aliquot was then taken from the exact midpoint of the liquid. The aliquot was transferred to a small vial and shaken to mix. Then a 0.1 ml sample was taken from the aliquot, placed on a microscope slide with a cover glass, and examined under a microscope at 200x. The slide was moved in a raster pattern ensuring that all areas of the slide were seen and all resting spores counted. This was repeated with two more 0.1 ml samples from the aliquot, resulting in a total of 3 counts for each sample which were then averaged. For each sample, the number of spores per 0.1 ml was converted to number of spores per 1 g of soil using the weight in grams of the 100 ml subsample recorded earlier as a conversion factor.

Risk calculations

Calculations were performed to ascertain the risk of fungal mortality facing gypsy moth larvae on the CIES property. First, current egg mass density (no. egg masses/ha) in the forest was multiplied by gypsy moth average fecundity, percent hatch and percent incidental mortality found in Goodwin et al. (2005) to give an estimate of surviving gypsy moth larvae/ha. Next, this number was divided by oak tree density/ha to estimate the number of larvae per oak tree. The relative frequency distribution of soil spore densities obtained from spore counts was then multiplied by the oak tree density/ha to calculate the number of oak trees in each spore class. The number of oak trees/class was multiplied by the number of larvae/tree to yield the number of larvae being exposed to each class of spore density. An equation derived from biosassays performed in Weseloh & Andreadis (2002) was then used to calculate the expected larval mortality in each spore class. Larval mortality was summed across all spore classes to give the number of larvae expected to die from *E. maimaiga* per hectare which was then divided by total larvae per hectare to yield an estimate of percent larval mortality due to the fungus.

This number was compared to mortality data from the 2008 CIES Burlap Band survey (Goodwin et al., unpublished data). Burlap band surveys are carried out each summer on the CIES property. Trees are first banded and then later checked, and any arthropods that have crawled under the bands are recorded. If gypsy moth larvae are found, their

instar is recorded, and if they are dead, the cause of death is recorded as well. In this manner the percent of larvae dead from *E. maimaiga* during the current field season can be calculated, as well as the percent of trees where two larvae died.

RESULTS

Spore counts

Spore densities were found to be extremely low (Figure 1) considering that the average number of resting spores/g soil after an epizootic ranges from 1201-3895 (Hajek & Wheeler, 1994). Because the soil spore densities were found to be so low, it was hypothesized that *E. maimaiga* does not play a significant role in regulating gypsy moth populations at low densities.

Risk calculations

Risk calculations predict that 11.08% of caterpillars are likely to die from *E. maimaiga* infection based on the spore densities found. This number is comparable to the calculation from the 2008 burlap band survey, which found 15.48% of gypsy moth caterpillars dead from *E. maimaiga* infection. Only 0.14% of trees were found with more than one dead larva, indicating that only small amount of infection is due to conidia spores. This also means that less than 0.14% of trees will have potential "hot spots" of high spore density at their base (due to conidial infection being required for the production of azygospores). Because few trees are experiencing a replenishment of the spore load at their bases, it is appropriate that only 1.85% of trees sampled in this study were found to have even a moderately high spore count (>300 spores/g soil).

DISCUSSION

This study found extremely low spore densities at all three of the plots sampled on the Cary Institute property. These low densities are most likely a result of the last epizootic occurring in 1992, sixteen years prior to the study (C.G. Jones, personal communication). Most of the spores produced in the 1992 epizootic have probably either germinated or decomposed. Infection must have occurred frequently enough in the intervening years to keep a small number of spores in the soil but not enough to cause more epizootics, due to the scarcity of the host. Percent larval mortality estimated from spore counts (11.08%) and current field data (15.48%) reflect the fact that infection is rare, but does still occur. The spore distribution also indicates that infection is not homogenous throughout the forest, as a few trees have much higher spore counts than the others (Figure 1).

The non-homogeneous build-up of spores may result from the peculiar life cycle of *E. maimaiga*. When a larva is infected by azygospores, only conidia are produced, but when a larva is infected by conidia, both types of spores are produced (Hajek & Wheeler, 1994). Thus, in order to return spores to the soil, a larva must be infected by conidia spores, which must come from another larva that has been killed by the fungus (Hajek, 1999). It is likely that areas of higher spore counts are areas where more than one larva has died on the same tree, assuming that at least one of them infected the others. Burlap band data indicate that only 0.14% of trees contained more than one larva that died from the fungus, while only 1.85% of trees had high spore counts. These numbers are both extremely small and this provides further support for the idea that they are related. Future research questions could address whether two larvae dying of *E. maimaiga* on the same tree causes an increase in spore load at its base.

The low spore densities and low fungal mortality signify low levels of infection. High levels of infection, such as those found during an epizootic, would result in large numbers of spores being returned to the soil. (More amplification means more conidia are produced, more larvae are infected by conidia, and as a result more resting spores are produced). Low spore densities and thus low infection rates indicate that *E. maimaiga* is not having much impact on gypsy moth populations on the CIES property, particularly when considering not all of the spores counted are active in any given year. Only a subset of spores germinates each year, and there is no way to tell if the spores are active from physical spore counts (Hajek, 1999). Because the counts were low to begin with, it can be

assumed that the number of spores becoming active and germinating each year is even lower. Thus it appears (at least in the case of the CIES oak forests) that *E. maimaiga* does not play a significant role in regulating low density gypsy moth populations. A certain threshold density of gypsy moth is required in order for the fungus to be effective.

White-footed mice are effective at keeping gypsy moth populations below this density with steady predation pressure. When mice populations crash, this pressure is removed and gypsy moth populations begin to increase rapidly (Ostfeld et al. 1996). It is at this point that a fungal epizootic can prevent a gypsy moth outbreak. However, if mice keep gypsy moth populations at low densities for long periods of time, the fungus is not able to replenish itself in the soil. Low densities of gypsy moth decrease the probability of one infected larva dying in close proximity to another. This in turn decreases the probability of conidial transmission, which is the only type of infection that results in production of azygospores. Although long-lived in comparison to conidia, these spores are not infinitely persistent; eventually all will either germinate or decompose. If mice continue to keep moth populations at low densities, the fungus will eventually go extinct from the soil. This is an interesting case of a generalist predator (the white-footed mouse) driving a specialist pathogen (*E. maimaiga*) to extinction.

If the fungus does go extinct from the soil, the next time the mouse population crashes, the gypsy moth population will skyrocket and cause massive defoliation. These results have important management implications for areas where gypsy moth populations have been kept at low densities in recent years. A simple solution at first appears to be inoculative augmentation (adding to the presence of a pathogen already present in an area). This would involve developing a way to produce spores *in vitro* and then scattering them in the forest. However, entomophthoralean species (such as *E. maimaiga)* are more difficult to grow than hyphomycetes (Hajek, 1999). Kogan & Hajek (2000) were able to produce spores *in vitro*; however, their method is not feasible for the mass production of spores, and a more suitable method has yet to be developed. Mass production of the spores would be required in order to institute spore augmentation as a management strategy.

While spores cannot currently be produced in the lab and added to the soil, several studies have found other ways of increasing *E. maimaiga* infection. Other methods of inoculative augmentation include adding soil with high *E. maimaiga* titers, adding infected larvae, or adding cadavers containing resting spores. Hajek & Webb (1999) added soil with relatively high titers of resting spores to the bases of trees on experimental plots. While augmented plots experienced higher infection than control plots, the differences were not always significant. They suggest that this is due to the fact that they added only 1×10^6 spores per plot, which is about the amount of spores produced from one cadaver (Hajek & Humber, 1997). They are confident that using a greater number of spores would produce significant results.

Another method of increasing infection does not add spores directly, but rather takes advantage of the density-dependence of the fungus. Gillock & Hain (2004) found that adding sterilized egg masses to trees in the beginning of a season can greatly increase the soil spore load by the end of the season. The egg masses temporarily increase the gypsy moth population size enough to cause small-scale epizootics, but because they are sterile, there is no danger of them causing an outbreak in future years. However, sterilized egg masses are more difficult to obtain than cadavers. While the former requires extensive permits, the latter requires only obtaining cadavers—although collecting cadavers introduces problems of its own. These problems include a need to collect them almost immediately after death and then store them until areas needing augmentation are identified by counting egg masses in the fall (Hajek, 1999).

A third study utilizes the observation that more infection occurs in years with a wet spring, so the fungus appears to prefer more humid environments. Webb et al. (2004) provided supplemental watering around the bases of trees as a method to increase fungal infection, but because their plots were outdoors and the study was conducted during a year with a large amount of rainfall the supplemental watering did not have much of an effect. However they believe that supplemental watering during dry years could prove to be an inexpensive and successful management strategy to increase *E. maimaiga* infection.

Future studies could retest these techniques using different parameters or test new techniques for increasing fungal spore load. In addition, studies could further examine whether the presence of more than one larva dead from *E. maimaiga* on one tree is a predictable indicator for high spore load. Sites that have ongoing surveys such as the burlap band survey on the CIES property would be ideal assuming that past surveyed trees could be located. This study would be a simple matter of finding the trees that had more than one dead larva in the previous field season, sampling soil from the base, and counting the spores. Because physical spore count techniques are not able to distinguish conclusively between *E. maimaiga* spores and other entomophthorlean spores of similar size, future studies could also retest the samples from this study to confirm that the spores counted in the study were actually those of *E. maimaiga*. Other techniques that would confirm presence of *E. maimaiga* spores in particular include an ELISA test developed by Hajek et al. (1991) and real-time PCR (Castrillo et al., 2007).

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APPENDIX

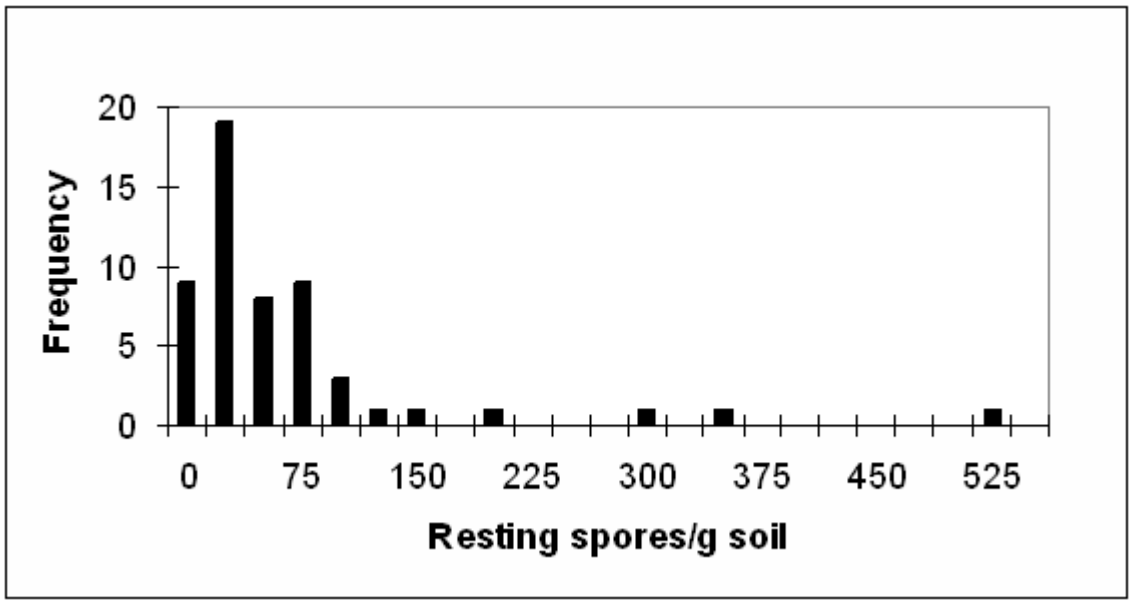

FIGURE 1. Frequency diagram showing number of resting spores/g soil found in spore counts.