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# Emergent Fungal Entomopathogen Does Not Alter Density Dependence in a Viral Competitor

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# Emergent fungal entomopathogen does not alter density dependence in a viral competitor

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*Abstract*. Population cycles in forest Lepidoptera often result from recurring density-dependent epizootics of entomopathogens. While these systems are typically dominated by a single pathogen species, insects are often infected by multiple pathogens, yet little is known how pathogens interact to affect host dynamics. The apparent invasion of northeastern North America by the fungal entomopathogen *Entomophaga maimaiga* some time prior to 1989 provides a unique opportunity to evaluate such interactions. Prior to the arrival of *E. maimaga*, the oscillatory dynamics of host gypsy moth, *Lymantria dispar*, populations were apparently driven by epizootics of a nucleopolyhedrovirus. Subsequent to its emergence, *E. maimaiga* has caused extensive mortality in host populations, but little is known about how it has altered multigenerational dynamics of the gypsy moth and its virus. Here we compared demographic data collected in gypsy moth populations prior to vs. after *E. maimaiga*'s invasion. We found that the recently invading fungal pathogen virtually always causes greater levels of mortality in hosts than does the virus, but fungal mortality is largely density independent. Moreover, the presence of the fungus has apparently not altered the gypsy moth–virus density-dependent interactions that were shown to drive periodic oscillations in hosts before the arrival of the fungus.

*Key words: competition; density dependence;* Entomophaga maimaiga; *gypsy moth; host–pathogen dynamics; Lepidoptera;* Lymantria dispar; *nucleopolyhedrovirus; pathogens.*

## Introduction

Entomopathogens sometimes cause extensive mortality in insect populations and such epizootics can play a central role in the population dynamics of many insect species (Hajek and St. Leger 1994, Cory and Myers 2003). Host–pathogen dynamics can drive oscillatory host dynamics in some systems, while in other systems, host populations may be regulated at stable levels by pathogens (Anderson and May 1980, Hochberg 1989). Given the large impacts that pathogens sometimes exert on host dynamics, it is no surprise that they have often been used as biological control agents against pest species (Hajek and Delalibera 2009).

Most studies of population systems driven by host–pathogen dynamics have focused on the role of a single pathogen, although populations of individual insect species are often infected by multiple species of pathogens. Few studies have addressed interactions between multiple insect pathogens within one host, but evidence to date indicates that pathogens may act either in synergy or interference when they infect the same host individual (Malakar et al. 1999*b*, Ishii et al. 2002, Thomas et al. 2003, Hughes and Boomsma 2004). Virtually all studies of co-infection of insect hosts have been conducted in laboratory settings and have provided some evidence that infection by one pathogen may alter the host's physiology and make it more or less suitable for a second pathogen. Furthermore, co-infecting pathogens may require different amounts of time to complete a disease cycle; these differences, as well as differences in responses by pathogens to environmental conditions, ultimately determine which pathogen, if any, ultimately kills the host and reproduces.

While such information about interactions between pathogens at the physiological level is useful, it provides only a partial picture of possible interactions. In contrast, almost nothing is known about how multiple insect pathogens interact at the population level. Even when different pathogens do not simultaneously infect the same individual, they may still compete for hosts within a single population. Population-level pathogen interactions have been more widely studied in birds and mammals than insects. For example, Dobson and Hudson (1994) found that the differential effects of predators on disease-infected vs. healthy grouse populations, resulted in weaker oscillatory dynamics in geographical regions where predators are more abundant. In another system, Jolles et al. (2008) found that

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infection by gastrointestinal worms compromised immunity to bovine tuberculosis, leading to high rates of mortality in African buffalo co-infected with both organisms.

The pioneering work of Anderson and May (1980) captured the essence of pathogen epizootics as a mass-action process, in which transmission is dependent on both host and pathogen abundance. While the concept of mass action has been recognized as a simplification in many systems (e.g., D'Amico et al. 1996), density dependence of infection is generally recognized as a key ingredient to the role of pathogens in driving host population oscillations. Therefore, when two or more pathogens simultaneously exist in the same host population, they may each alter host abundance and thereby impact the density-dependent interactions between hosts and pathogens, thus affecting epizootics and oscillatory dynamics in host populations. Unfortunately, it is difficult to tease apart such numerical interactions experimentally because the presence of pathogens generally cannot be controlled in field populations. Consequently, we are not aware of any studies that have characterized entomopathogen interactions at the population level.

Here, we exploited a unique system to "experimentally" probe the interactions between two competing insect pathogens. North American populations of the invasive gypsy moth, *Lymantria dispar*, are currently associated with two host-specific pathogens that were also introduced. The *L. dispar* nucleopolyhedrovirus (LdNPV), present in virtually every gypsy moth population in the world, was apparently introduced to North America early with gypsy moth parasitoids introduced for biological control and historically has been known to play a key role in outbreak collapse (Dwyer and Elkinton 1993) prior to the arrival of a fungal pathogen also capable of causing epizootics. In contrast, the fungal pathogen, *Entomophaga maimaiga*, does not have a global distribution; this host-specific pathogenic fungus is native to Japan, northeastern China, and the Russian Far East (Nielsen et al. 2005), but was discovered in the northeastern USA in 1989 (Hajek et al. 1990). There, *Entomophaga maimaiga* increased and spread, and has now been recovered throughout the range of the gypsy moth in North America, often causing extensive mortality in gypsy moth populations (Hajek 1999).

Though both pathogens share an ability to persist for extended periods in soil, the epizootiology of these pathogens otherwise differs markedly. The first transmission of LdNPV each season occurs when occlusion bodies that are contaminating the surfaces of egg masses are consumed by hatching larvae, resulting in infection (Woods et al. 1991). Horizontal transmission occurs within a season via contamination of leaf surfaces by virus-killed cadavers and subsequent infection of healthy larvae via ingestion of contaminated foliage. Typical disease cycles span 10–20 days (Woods and Elkinton 1987), and density dependence in LdNPV arises from the mass-action phenomenon associated with horizontal transmission (Dwyer and Elkinton 1993). In contrast, *E. maimaiga* resting spores persist in soil (Hajek 1999), and each spring some fraction germinates and actively ejects germ conidia that infect larvae after landing on host cuticle. Horizontal transmission of *E. maimaiga* also occurs via infection of healthy larvae by windborne conidia actively ejected from cadavers (see Plate 1). The disease cycle of *E. maimaiga* spans only 4–7 days (Hajek 1999), and infection rates for *E. maimaiga* are closely associated with environmental moisture (Hajek 1999).

In this study, we made use of the fact that *E. maimaiga* was not present in gypsy moth populations prior to 1989 to quantify the density-dependent interactions between LdNPV and its host pre-*E. maimaiga*, from data collected in the 1980s. This relationship was then compared with that derived from similar data collected after the establishment of *E. maimaiga* to quantify the population-level interactions of LdNPV with its host pre- and post-*E. maimaiga* emergence. Our fortuitous collection of detailed survival data in gypsy moth populations prior to and after the establishment of *E. maimaiga* provides a unique opportunity for teasing out the interactions between these two entomopathogens, as well as providing information about gypsy moth–*E. maimaiga* density relations.

## Materials and Methods

### *Sampling*

Sampling of gypsy moth populations prior to the invasion of *E. maimaiga* was conducted from 1987 to 1989 in three 9-ha forested study sites located on Cape Cod, Massachusetts, USA (Barnstable County; Appendix A). Sampling after *E. maimaiga* invasion was carried out from 2007 to 2009 at 12 ~7-ha forested study sites located in central Pennsylvania, USA (Centre and Huntingdon counties). Both three-year intervals over which sampling was carried out corresponded with the rise of regional gypsy moth populations to outbreak levels followed by a synchronous population crash in the final year. Vegetation at each site was dominated by oaks, *Quercus* spp.

Gypsy moth population densities were estimated yearly by counting egg masses within 16 circular 0.01-ha plots at the Massachusetts study sites and 6 circular 0.01-ha plots at each site in Pennsylvania (Liebhold et al. 1994). Counts were made during winter months when trees were leafless, which facilitates counting. Density was expressed as egg masses per hectare.

To measure generational pathogen impact, gypsy moth larvae were collected every 4–7 d during the last four weeks of larval development. At the Pennsylvania sites, sampling generally began between 5–9 June and ended 22–26 June; at the Massachusetts sites, sampling began 11–17 June and ended 2–9 July (Appendix B). These sampling periods were targeted to sample late fourth to sixth instars. At Pennsylvania sites, an attempt

was made to collect at least 50 larvae per sample, although occasionally populations were too sparse, but at least 18 larvae were taken per collection. At Massachusetts sites, 50 or more larvae were collected for each weekly sample. Larvae were placed individually into 29-mL cups containing artificial diet (Bell et al. 1981). Cups were maintained at 18–22°C, and the status of each larva was checked daily until the next collection date, or for 30 d after the last collection. Gypsy moths that died as larvae or did not emerge as adults were checked daily for 3 d after death to detect conidial production by *E. maimaiga*. Cadavers were then frozen for subsequent microscopic examination for the presence of *E. maimaiga* resting spores and LdNPV occlusion bodies (Appendix C).

For each pathogen, season-long mortality was calculated by aggregating mortality from weekly or semiweekly collections. The fraction dying from each collection was first censored to only include hosts dying during the interval between collection dates. The majority of cadavers of pathogen-killed larvae contained either *E. maimaiga* spores (i.e., conidia and/or resting spores) or LdNPV occlusion bodies. For those cadavers with dual infections (both pathogens were able to reproduce), half were allocated as killed by *E. maimaiga* and half were designated as killed by LdNPV. Marginal mortality rates for each agent were calculated under the assumption of proportional hazards (Elkinton et al. 1992) and cumulative mortality (across the late larval period for a given year at a given site) was calculated for both LdNPV and for *E. maimaiga* as one minus the product of weekly or semiweekly proportions surviving (1 − marginal mortality rates; Varley et al. 1973). Simple least-squares linear regression of season-long mortality on $\log_{10}$(egg mass density) was used to test for the presence of density dependence in mortality caused by LdNPV, as well as that caused by *E. maimaiga*. A general linear model was used to test how the relationship of season-long LdNPV mortality to $\log_{10}$(egg mass density) varied pre- vs. post-1989 (the point at which *E. maimaiga* appeared in North American gypsy moth populations). The analysis was performed using the GLM procedure in the SAS language (SAS Institute 2004); code is provided in the Supplement.

## Results

A total of 12 580 larvae were collected and reared from the Cape Cod sites from 1987–1989; of those, 1061 died from LdNPV infections. A total of 5607 gypsy moth larvae were collected from the central Pennsylvania plots from 2007–2009; a total of 1946 were infected only by *E. maimaiga*, 422 were only by LdNPV, and 125 were infected by both pathogens. Though *E. maimaiga* consistently caused high levels (range = 61–99%) of season-long larval mortality during 2007–2009, levels almost always higher from those caused by LdNPV, mortality from *E. maimaiga* was not density dependent

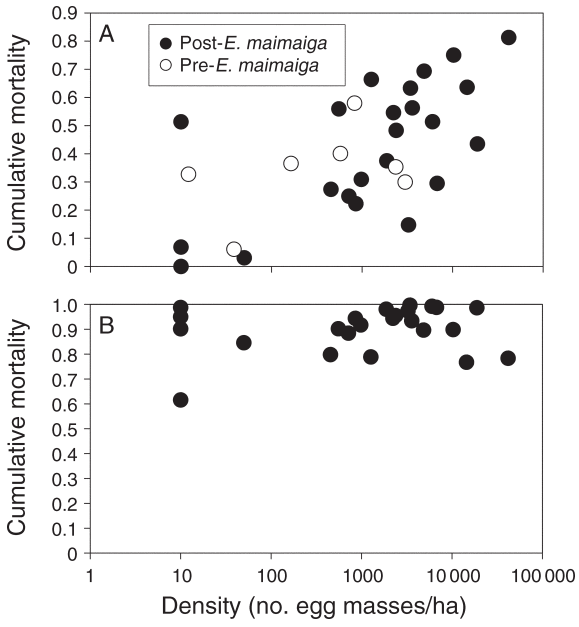


FIG. 1. Patterns of density dependence of pathogen mortality pre-*Entomophaga maimaiga* (Massachusetts [USA] sites, 1985–1987) and post-*E. maimaiga* establishment (Pennsylvania [USA] plots, 2007–2009). Each dot corresponds to season-long cumulative mortality (summarizing mortality among weekly or biweekly [every two weeks] samples of late-instar larvae) plotted vs. gypsy moth (*Lymantria dispar*) egg mass density (note the log scale) at an individual site in a given year. (A) Density dependence in season-long mortality caused by *L. dispar* nucleopolyhedrovirus (LdNPV). (B) Density dependence in season-long mortality caused by *E. maimaiga*.

(Fig. 1B); regression of season-long *E. maimaiga* mortality on $\log_{10}$(gypsy moth egg mass density) was not significant ($F_{1,21} = 0.87$, $P = 0.362$). In contrast, season-long LdNPV mortality was highly variable (range = 0–77%) and strongly density dependent (Fig. 1a); effect of $\log_{10}$(egg mass density) on mortality from LdNPV was highly significant ($F_{1,23} = 22.5$, $P = 0.0001$). The pattern of LdNPV density dependence did not differ between data collected pre-*Entomophaga* and post-*Entomophaga*; there were no significant differences in the intercept ($F_{1,21} = 0.75$, $P = 0.396$) or slope ($F_{1,21} = 1.35$, $P = 0.258$).

## Discussion

The sudden emergence of *E. maimaiga* in North American gypsy moth populations in 1989 offered a unique opportunity to observe the population-level interactions between LdNPV and host populations, with and without a competing pathogen. These two pathogens now co-occur in most North American gypsy moth populations, sharing similar niches. At the host level, they can co-occur and reproduce within the same host, but infection order is crucial for their reproduction. Because *E. maimaiga* kills larvae faster, LdNPV is only able to reproduce if it has infected long before *E. maimaiga* (Malakar et al.1999*b*). Our collection of relatively few

PLATE 1. Fourth-instar gypsy moth larva killed by *Entomophaga maimaiga*. *E. maimaiga* conidia have already been ejected from the cadaver, and some conidia landed on dark larval setae, which now appear white due to a coating of conidia. Larval prolegs are gripping a red oak twig, while the anterior portion of the body is bent downward. Photo credit: A. E. Hajek.

larvae that died in which both pathogens were able to reproduce is consistent with the finding of Malakar et al. (1999*b*) of the temporal precedence of infection.

Malakar et al. (1999*a*) found that within a single host generation, the presence of *E. maimaiga* inoculum does not substantially reduce generation-level mortality caused by LdNPV. Indeed, results reported here from naturally occurring populations, namely that density dependence in LdNPV mortality is unaltered by the presence of *E. maimaiga*, support the conclusion of lack of pathogen interference. The lack of an impact of *E. maimaiga* on LdNPV density dependence is surprising given that the short disease cycle of *E. maimaiga* allows this fungal pathogen to "beat" LdNPV when hosts are simultaneously infected. Additionally, there is good evidence from human disease systems that the presence of one fatal pathogen can interfere with the dynamics of a co-occurring disease via "ecological interference" in which susceptibles are removed by a competing pathogen, thereby altering the conditions for mass action (Rohani et al. 1998). Malakar et al. (1999*a*) suggested that one reason for the lack of a direct effect of *E. maimaiga* on LdNPV seasonal mortality may be that *E. maimaiga* mortality is typically highest in late instars, and by that time, the course of any LdNPV epizootic may have already been determined. They argued that the primary potential for an impact of *E. maimaiga* may be in depressing the late-season LdNPV infection and consequent environmental contamination with virus particles that transmit LdNPV to the next generation. Given such a decrease in vertical transmission of LdNPV, one could anticipate that this would result in overall lower levels of mortality from LdNPV in subsequent generations than would result if *E. maimaiga* was absent.

Results presented here provide clear evidence that *E. maimaiga* has neither taken on the density-dependent role previously played by LdNPV, nor has it substantially altered the density-dependent regulation of host gypsy moth populations by LdNPV. The density-dependent interaction of LdNPV with gypsy moth populations has previously been identified as the dominant process responsible for quasi-periodic oscillations in gypsy moth populations (Dwyer et al. 2004, Bjørnstad et al. 2010), although the data on which this was based were largely collected before the establishment of *E. maimaiga*. Given that generation-long mortality caused by *E. maimaiga* is consistently high, typically exceeding that of LdNPV, it is remarkable that this mortality has apparently not altered the numerical interaction between LdNPV and host populations.

Our finding of density independence in mortality caused by *E. maimaiga* is consistent with other field

studies; Weseloh and Andreadis (1992*a*, *b*) also did not find any consistent pattern of density dependence. This is surprising given that *E. maimaiga* typically completes several disease cycles within a single host generation and this, coupled with a mass-action process for disease transmission, holds potential for generating density-dependent behavior. Because initial infections each year originate from resting spores that persist and accumulate in the soil over many generations, disease levels in any year may be influenced by host densities over many years and this may prevent the occurrence of direct density dependence. It is also possible that the highly mobile nature of conidia prevents fungal pathogen populations from tracking local host populations both within and among host generations. Perhaps as a result, variation in mortality caused by *E. maimaiga* is more strongly influenced by environmental conditions (i.e., moisture; Hajek 1999) than by host density.

The unaltered density-dependent behavior of LdNPV following the invasion of *E. maimaiga* would suggest that this virus may continue to play a dominant role as a driver of oscillations in gypsy moth populations. Indeed, Allstadt et al. (2013), analyzing historical records of defoliation, found that the period of oscillations in gypsy moth populations has not clearly changed following the appearance of *E. maimaiga* in 1989. They noted, however, that the amplitude of oscillations may have diminished since 1989, although such a change was not unprecedented in historical records dating back to 1924. Thus, at present, it is not clear if the recent decrease in outbreak amplitude can definitively be attributed to the activity of *E. maimaiga*. We note here that the mortality caused by *E. maimaga* is consistently high, and this could logically result in a diminution of the amplitude of cycles driven by the interaction of LdNPV with gypsy moth populations.

The little that is known about population-level interactions between competing pathogens indicates that such relationships are complex and difficult to predict from laboratory studies alone. There are examples from the animal disease literature illustrating synergy, antagonistic, and neutral population-level interactions among competing pathogens (Rohani et al. 2003, Jolles et al. 2006, 2008). Results from the present study indicate an apparently neutral impact of *E. maimaiga* on the density dependence of LdNPV in North American gypsy moth populations. Laboratory studies (Malakar et al. 1999*b*) indicate that *E. maimaiga* has relatively little impact on LdNPV transmission, although this has not been confirmed in the field. It is also remains to be determined if *E. maimaiga* alters host demographics (e.g., age structure) that might influence longer term LdNPV–host interactions. Nevertheless, the lack of a more pronounced impact of *E. maimaga* on LdNPV dynamics is a remarkable result given the obvious superior competitive characteristics of this fungal pathogen. Moreover, *E. maimaiga* may have modified the dynamics of host populations despite its neutral impact on the primary driver of host gypsy moth oscillations. The impact of *E. maimaiga* reported here is slightly atypical compared to other emergent fungal pathogens that have been found to exhibit strong density dependence and some of which have driven host populations to extinction (Fisher et al. 2012).

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## Supplemental Material

### Appendix A

A description of the study sites (*Ecological Archives* E094-110-A1).

### Appendix B

Numbers of gypsy moth larvae collected (*Ecological Archives* E094-110-A2).

### Appendix C

Microscopic identification of LdNPV and *Entomophaga maimaiga* in larval cadavers (*Ecological Archives* E094-110-A3).

### Supplement

SAS code for general linear model analysis (*Ecological Archives* E094-110-S1).