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Quantifying horizontal transmission of *Nosema lymantriae*, a microsporidian pathogen of the gypsy moth, *Lymantria dispar* (Lep., Lymantriidae) in field cage studies

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ABSTRACT

Nosema lymantriae is a microsporidian pathogen of the gypsy moth, *Lymantria dispar* that has been documented to be at least partially responsible for the collapse of *L. dispar* outbreak populations in Europe. To quantify horizontal transmission of this pathogen under field conditions we performed caged-tree experiments that varied (1) the density of the pathogen through the introduction of laboratory-infected larvae, and (2) the total time that susceptible (test) larvae were exposed to these infected larvae. The time frame of the experiments extended from the early phase of colonization of the target tissues by the microsporidium to the onset of pathogen-induced mortality or pupation of test larvae. Upon termination of each experiment, the prevalence of infection in test larvae was evaluated. In the experiments performed over a range of pathogen densities, infection of test larvae increased with increasing density of inoculated larvae, from 14.2 ± 3.5% at density of 10 inoculated per 100 larvae to 36.7 ± 5.7% at 30 inoculated per 100 larvae. At higher densities, percent infection in test larvae appeared to level off (35.7 ± 5.5% at 50 inoculated per 100 larvae). When larval exposure to the pathogen was varied, transmission of *N. lymantriae* did not occur within the first 15 d post-inoculation (dpi) (11 d post-exposure of test larvae to inoculated larvae). We found the first infected test larvae in samples taken 20 dpi (16 d post-exposure). Transmission increased over time; in the cages sampled 25 dpi (21 d post-exposure), *Nosema* prevalence in test larvae ranged from 20.6% to 39.2%.

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1. Introduction

Microsporidia are frequently overlooked as natural enemies of the gypsy moth, *Lymantria dispar*, an important forest defoliator throughout the northern hemisphere, because they do not produce dramatic, visible epizootics like the gypsy moth nucleopolyhedrosis virus (*Ld*MNPV). A high prevalence of microsporidiosis has been reported in *L. dispar* populations from Sardinia, Poland, the Ukraine and the former Yugoslavia. (as reviewed in McManus and Solter, 2003). More typically, however, microsporidia occur at lower enzootic levels (Novotny, 1989; Hoch et al., 2001; Pilarska et al., 1998) and are detected in most populations in Europe. They must, therefore, have effective paths for disease transmission. A recent foreign exploration program was conducted by the US Forest Service to identify candidate microsporidia for introduction into North American *L. dispar* populations. More than 20 microsporidian isolates representing three genera were collected from *L. dispar* populations in several European countries (McManus and Solter, 2003).

One of the candidate species for introduction is *Nosema lymantriae* (Weiser, 1957), a microsporidium that is transmitted both horizontally and vertically among host individuals (Novotny and Weiser, 1993). Species of the genus *Nosema* isolated from the gypsy moth are characterized by a primary reproduction cycle in midgut cells with the production of primary, internally infective spores. A secondary cycle results in the maturation of single, binucleate environmental spores in "target tissues" such as silk glands, fat body tissues, and gonads (Maddox et al., 1999), as well as (in case of *N. lymantriae*) the Malpighian tubules (Pilarska et al., 2006). The infection of respective target tissues facilitates spore release either from living larvae through silk (Jeffords et al., 1987) and/or feces (Maddox et al., 1999; Goertz et al., 2007; Goertz and Hoch, 2008) and from cadavers, which are frequently laden with spores due to the infection of the fat body tissues. Effects of infection on host larvae (Goertz et al., 2004), competition with other species of microsporidia within hosts (Solter et al., 2002; Pilarska et al.,

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2006) and host specificity (Solter et al., 1997, 2000) have been well-studied for the *L. dispar* microsporidia. However, our understanding of horizontal transmission is still incomplete, particularly in natural populations. Recent studies that dealt with aspects of horizontal transmission of *L. dispar* microsporidia in some detail (Pilarska et al., 2006; Goertz et al., 2007; Goertz and Hoch, 2008) were carried out under standardized laboratory conditions using relatively small arenas (diet cups) and meridic diet. Quantitative laboratory data on spore production and spore release from infected host larvae are available for *N. lymantriae* (Goertz and Hoch, 2008), however, it is not clear how these findings relate to the situation in the field with variable food quality and a more complex and ecologically relevant habitat.

No published experiments on microsporidia transmission in gypsy moth have been carried out under unconfined or caged field conditions. Related experiments, however, have been conducted using viruses infecting *L. dispar* and a related lymantriid, *Orgyia psuedotsugata*, to answer several basic questions of disease transmission (Dwyer, 1991; Dwyer and Elkinton, 1993; D'Amico et al., 1996) using a modified Anderson–May model. Such studies require (1) knowledge of the paths for release and acquisition of infective stages of the pathogen, (2) reliable estimates of pathogen density and host density, and (3) previous lab or field measurements of the time course of the disease. We designed our study of horizontal transmission of *N. lymantriae* in *L. dispar* larvae in semi-field conditions using two approaches: (1) we varied the density of the pathogen by varying the density of laboratory-infected larvae in the field cages, and (2) we varied the time that susceptible larvae were exposed to the laboratory-infected larvae in the cages.

2. Materials and methods

Study plots were established on young oak plantations near Nitra, in the Slovak Republic. The experimental trials were installed in the spring/early summer of 2004, 2005, and 2006 using two different plots similar in structure and in close proximity. *Quercus petraea* trees of similar size (approximately 2 m in height) and foliage quantity were selected as test trees each year, in oak plantations where *L. dispar* occurs naturally. Larvae were inoculated with the microsporidium in the laboratory and were confined with susceptible test larvae on the trees. The density of larvae placed in the cages was chosen to represent outbreak conditions (V. D'Amico, unpublished data). The time frame of the experiments was chosen to extend from the early phase of colonization of the host's target tissues by the microsporidium to the release of spores from living larvae and, finally, to the onset of first pathogen-induced mortality in laboratory-infected larvae, about 25 days. While in the field cages, *L. dispar* larvae developed from intermediate instars to mature larvae nearing pupation.

2.1. Insects and pathogens

Lymantria dispar larvae were hatched from egg masses provided by the USDA/APHIS Otis Method Development Center, Cape Cod, MA. Egg masses were from a colony that was constantly monitored for microsporidia and other pathogens, but none were found. They were reared on meridic wheat germ diet (Bell et al., 1981) in 250-ml plastic cups at 24 ± 1 °C/18 ± 1 °C, 16 h light/8 h dark unless otherwise indicated. The microsporidium chosen for the experiments was *N. lymantriae*, isolated from a *L. dispar* population near Levishte, Bulgaria (Isolate No. 1996-A, GenBank Accession No. AF141129). Spores for the experiments were propagated in *L. dispar* larvae following the methods of Hoch et al. (2000) and Solter et al. (2002). Mature spores were harvested from infected tissues 20 days post-inoculation (dpi), then cleaned by filtration through cellulose tissue and centrifugation. Spores were suspended in distilled water for no longer than two months and were then mixed 1:1 with glycerol and stored in liquid nitrogen (Maddox and Solter, 1996) until used in the experiments.

2.2. Experimental inoculations

Lymantria dispar larvae were inoculated on the first day of the third instar following the method of Bauer et al. (1998), as used in previous studies (Hoch et al., 2000; Solter et al., 2002). Microsporidian spore suspensions were removed from liquid nitrogen storage, thawed, counted in a Neubauer hemacytometer and adjusted to a concentration of 1×10^3 spores/μl with distilled water. Blocks of wheat germ diet cut to 4 mm^3 were placed individually into 24-well tissue culture plates, and 1 μl of spore suspension was applied to the surface of each diet block. *L. dispar* larvae were placed individually into each well. Only larvae that consumed the entire diet block within 16 h were used in the experiments.

Two or 3 dpi, inoculated larvae were marked by clipping the left first proleg with ocular scissors. The larvae were placed in a Petri dish on cellulose tissue for 30 min; after bleeding had stopped, they were transferred to 250-ml diet cups in which they were held until transfer to the study plot. A group of larvae was reared in the laboratory to show that this treatment did not lead to mortality and that the injury was detectable in later instars. According to Weseloh (1985), clipping up to four prolegs does not significantly reduce mobility of larvae in the field.

Uninfected, unmarked larvae to be used as susceptible (test) larvae in the transmission experiments were reared to the third stadium in 250-ml diet cups.

2.3. Transmission experiments

Wood-framed cages of dimensions 1 m wide × 1 m deep × 2 m high, with fine gauze, ca. 0.5 mm mesh, stretched across all sides were installed around 15 randomly selected trees. The ground inside the cages was cleared of vegetation and the trees were thoroughly searched for naturally-occurring insects, which were removed before introducing the test insects. In 2004, 200 field-collected *L. dispar* larvae from the site were examined microscopically for naturally-occurring microsporidian infections. No infections were observed. The only pathogen found in *L. dispar* larvae outside the cages during the research period was the virus *Ld*MNPV. Cages were disinfected after they were disassembled each year and different trees were used for each trial.

2.3.1. Transmission at different pathogen densities

Inoculated *L. dispar* larvae (late third instars; 4 dpi) and uninfected, susceptible larvae (day 1 or 2 post-molt to the third stadium) were placed in the cages May 21, 2004 and July 6, 2005. The later date was chosen in 2005 because there was an inadvertent pesticide spray application in the vicinity, which may have compromised the conduct of the study on an earlier date. Hereafter, the two groups will be referred to as inoculated larvae and test larvae, respectively. Care was taken to avoid all possible sources of contamination during handling of test larvae.

Numbers of inoculated larvae and test larvae per cage were 10:90, 20:80, 30:70, 40:60, 50:50; each density was represented by three replicates and the experiment was conducted twice, once each in 2004 and 2005. Larvae were removed after 21 d of exposure. Inoculated and test larvae were immediately separated according to the proleg markings. Very few larvae had pupated when the experiment was terminated; pupae were discarded because we were not able to distinguish between inoculated and test larvae. Inoculated larvae were transported to the laboratory in 250-ml cups and frozen for later examination for microsporidia infections under phase contrast microscopy (400×). Test larvae

were placed into 50-ml cups containing meridic diet, one larva/cup, upon recovery from the cages and were reared in the laboratory for another 11 d to allow acquired infections to progress before microscopic examination for microsporidia.

2.3.2. Transmission at different times of exposure

Marked inoculated larvae (late third instars; 4 dpi) and test larvae (day 2 post-molt to the third stadium) were placed into field cages on June 16, 2006 as described above. Numbers of inoculated and test larvae were 30:70 in all 15 cages based on the previous 2 years transmission results. Larvae were removed from the cages at three different points in time: five cages were emptied 15 dpi (11 d post-exposure), five cages at 20 dpi (16 d post-exposure), and five cages at 25 dpi (21 d post-exposure). Inoculated larvae and test larvae were separated immediately upon removal from the cage and treated as described above. Test larvae were reared individually on meridic diet for 12 d before dissection.

2.4. Diagnosis of infections

Mature, 25 d infections in inoculated larvae are evaluated on tissue smears of larval cross-sections that include silk gland, fat body, midgut and Malpighian tubule tissues (Solter et al., 1997). Test larvae were dissected and preparations containing individually excised silk glands, fat body and Malpighian tubules were examined. This more elaborate technique allowed diagnosis of very light and early stage infections in test larvae. The fresh smears were examined under phase contrast microscopy at 400×, which allowed detection of spores and immature stages of microsporidia (Solter and Maddox, 1998). Larvae were considered infected when environmental spores were observed in the tissues.

2.5. Data analysis

Statistical analysis was performed with SPSS 12.0.1 (SPSS Inc. 1989–2003). The percentage of larval recovery after exposure in the cages was compared by Spearman's *rho* correlation as well as cross-table χ^2 tests. Prevalence of infections was arcsin transformed before further analysis: A two-way ANOVA (using GLM procedure in SPSS) was performed to compare trials and different densities of inoculated larvae. Regression analysis was computed to explore the relationship between inoculum density and prevalence of infection in test larvae. Data lacking normal distribution were compared by Kruskal–Wallis H test followed-up by pair-wise Mann–Whitney U tests (controlled for type I errors by the Bonferroni method).

3. Results

3.1. Transmission at different pathogen densities

After 21 d in the field, an average of 79% of the 1050 test larvae were collected in 2004 and 69% in 2005. Of the 450 exposed inoculated larvae, 45% were recovered in 2004 and 64% in 2005, respectively. These recovery rates differed between years (trials) ($\chi^2 = 23.043$, $P < 0.001$ for test larvae, $\chi^2 = 37.144$, $P < 0.001$ for inoculated larvae). While we recovered significantly more test larvae than inoculated larvae in 2004 ($\chi^2 = 163.050$, $P < 0.001$) the rate of recovery did not differ between the two groups in 2005 ($\chi^2 = 2.380$, $P > 0.05$). Moreover, recovery of test and inoculated larvae were positively correlated in 2005 (Spearman's $\rho = 0.719$, $P = 0.003$). In 2004, the correlation was not significant ($\rho = 0.473$, $P = 0.075$). In both years, the percentage of recovery did not vary with density of inoculated larvae. Of the inoculated larvae, 99.5% and 98.6% developed infections in 2004 and 2005, respectively.

The number of inoculated larvae per cage affected prevalence of infections in test larvae. This prevalence was variable (Fig. 1) but there were no differences between the trials and there was no significant interaction between trials and density of inoculated larvae (two-way ANOVA see Table 1). Thus, results from both trials were combined for further analysis.

Prevalence of infections in test larvae increased with increasing density of inoculated larvae (Fig. 1) from 14.2 ± 3.5% at density of 10 inoculated larvae to 36.7 ± 5.7% at a density of 30 inoculated larvae. At higher densities, percent infection in test larvae appeared to level off. The prevalence was 35.7 ± 5.5% at 50 inoculated larvae. Regression analysis to explore the dependence of transmission of infections on density of inoculated larvae is shown in Fig. 1. Our data could be explained with a logarithmic function, with levels of infection increasing at a decreasing rate as the density of inoculated larvae increased.

3.2. Transmission at different times of exposure

Recovery rates in 2006 were generally higher than in the previous years; we recovered 75% of inoculated and 79% of test larvae. There was not a significant correlation between recovery of inoculated larvae and test larvae (Spearman's $\rho = 0.04$, $P = 0.0886$). Recovery of inoculated larvae decreased over time; it was significantly lower 20 dpi and 25 dpi than on the first collection date ($P < 0.05$; Mann–Whitney tests following up significant Kruskal–Wallis H test, $\chi^2 = 8.77$, $P = 0.012$). No such relationship existed for test larvae. However, overall recovery rates of inoculated and test larvae were not significantly different ($\chi^2 = 2.691$, $P > 0.05$).

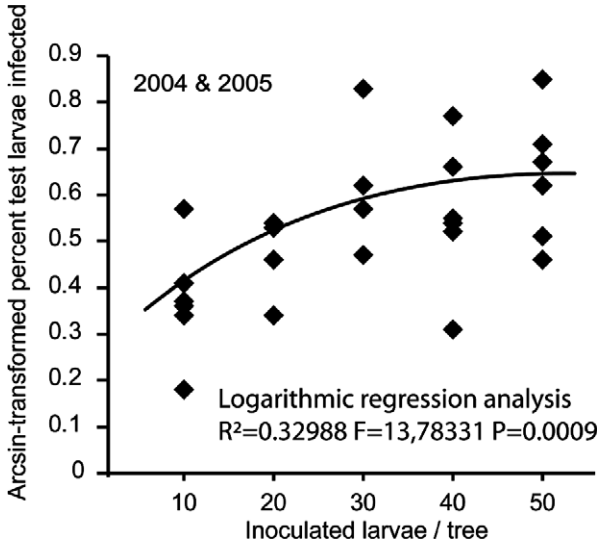

Fig. 1. Arcsin-transformed percentage of test larvae infected by *Nosema lymantriae* increases at a decreasing rate with an increase in the number of inoculated larvae on each tree. Inoculated larvae were infected in the laboratory prior to the start of the experiment.

Table 1
Results of two-way ANOVA with number of inoculated larvae and trial (year) as factors

Factor	df	*F*	*P*	r^2
Number of inoculated larvae	4	4.000	0.015	0.444
Trial	1	0.551	0.466	0.027
Interaction number * trial	4	0.639	0.641	0.113

The dependent variable, percent of test larvae that became infected, was arcsin transformed prior to analysis.

The percentage of infections in inoculated larvae was lower in the experiment carried out in 2006 than in the previous years; overall, 88% of inoculated larvae were diagnosed with infections. The infected test larvae showed mature, heavy infections in silk glands, fat body and Malpighian tubules.

Transmission of *N. lymantria* infections did not occur until 20 dpi (16 d post-exposure) (Fig. 2). Prevalence of infection in test larvae was >10% in two cages, while in two cages it was 1.7%, and in one cage no test larvae were infected. Transmission increased over time; in the cages sampled 25 dpi (21 d post-exposure), *Nosema* prevalence in test larvae ranged from 20.6% to 39.2%. This was within the range of cages with the same density (30 inoculated and 70 test larvae) in the previous 2 years.

4. Discussion

Nosema lymantriae was efficiently transmitted among the *L. dispar* larvae on small oak trees during the period of the experimental trials. Transmission occurred at the lowest pathogen density; 14% of the test larvae developed infections in cages with 10 inoculated larvae per tree. The maximum prevalence of acquired microsporidiosis in test larvae was achieved on a tree on with the highest pathogen density (50 inoculated larvae). The process of transmission began slowly; the first infected test larvae were found after 16 days of exposure to inoculated larvae (20 days after inoculated larvae had been fed the microsporidium). This important delay is partially due to the reproductive cycle of *N. lymantriae*. The first environmental spores, the mature, infective stages that can survive outside host tissues and are thus the incoculum for horizontal transmission, are produced 6–8 days after inoculation (Solter et al., 2002; Goertz et al., 2004). Apparently, additional time is required before environmental spores are released in the feces of the larvae. A latent period of ca. 11 days is necessary before *L. dispar* larvae inoculated with *N. lymantriae* become infectious (Goertz et al., 2004); after 12 dpi, spores can be detected regularly in feces (Goertz and Hoch, 2008). A similar latent period of 11–15 days was observed for *Nosema fumiferanae* in *Choristoneura occidentalis* (Campbell et al., 2007). Both of these studies were conducted in the laboratory. In the field situation, >16 d was required for the first susceptible *L. dispar* larvae to acquire infections, possibly due to the more complex environment and, consequently, a reduced probability for a susceptible larva to encounter sufficient spores to initiate infection.

It is not surprising that the levels of infection among test larvae increased with an increase in the density of inoculated larvae in these pathogen density experiments. A non-linear trend in the increase of infections over pathogen density has been noted in other

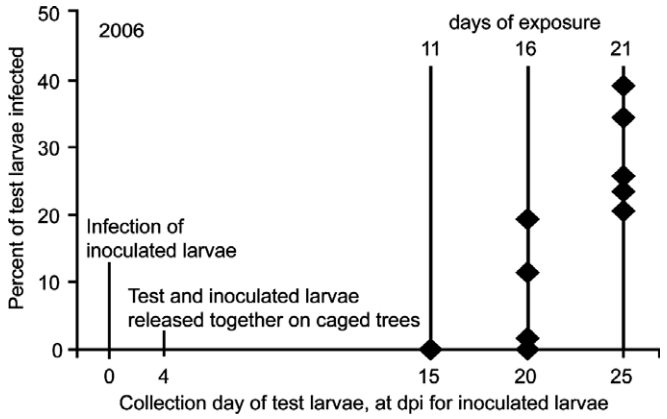

Fig. 2. Percentage of test larvae infected after increasing number of days caged with inoculated larvae. Cages destructively sampled 11, 16, and 21 days after deployment. Larvae inoculated in the laboratory produced no infections in test larvae until 20 days post-inoculation (=16 days of exposure).

systems (D'Amico et al., 1996; Knell et al., 1998), although calculating transmission coefficients remains the best method of detecting true non-linearity. However, determining transmission coefficients for these data remains problematic. The Anderson–May model variants that are appropriate for simple and straightforward calculations of disease transmission in experiments such as these are based on several important assumptions. Random encounters between pathogen and susceptible insects are more likely in this system than those studied by Dwyer (1991) or D'Amico et al. (1996) because the microsporidian species we tested is not released solely in a clumped distribution upon the death of the host as are nucleopolyhedrosis viruses. Other assumptions, such as constant density of pathogen during a round of infection, are certainly violated by the gradual release of spores from the host. Release of environmental spores in feces is the most important transmission pathway for *N. lymantriae* while infected larvae are still alive. Goertz and Hoch (2008) showed in laboratory studies that more than 80% of infected larvae released spores with feces continuously from the end of the latent period until death. Thus a continuous time model may be more appropriate for the gypsy moth–microsporidia system than a discrete model. We feel that further elucidation of this system is necessary before valid attempts to model transmission can be made.

Other avenues of spore release may be of significant importance. Jeffords et al. (1987) reported for *Nosema portugal*, another pathogen of *L. dispar* that is closely related to *N. lymantriae*, that larval silk frequently contained environmental spores. They assumed that this was an important route for horizontal transmission, aided by the fact that *L. dispar* larvae tend to follow silk trails during their diurnal movements (Leonard, 1967; McManus and Smith, 1972). For *N. lymantriae* infections, however, spore release with silk rarely occur (Goertz and Hoch, 2008). Be it via feces or silk, spore release from living larvae contributes to a continuous build-up of pathogen density in the environment. This is reflected in the increase in the prevalence of microsporidiosis in test larvae from 15 to 25 dpi of inoculated larvae.

At the dosage of 1×10^3 spores used for these experiments, *N. lymantriae* is lethal for a high percentage (>90%) of hosts in the larval stage (Goertz and Hoch, 2008). Release of spores from cadavers can therefore be a second important source for transmission. *N. lymantriae* proliferates in the fat body tissues as well as in the silk glands of the host, and high numbers of spores are produced in infected larvae. When infected orally with *N. lymantriae* at the same dosage and larval instar as in our present study, *L. dispar* cadavers contained approximately 5×10^9 spores at the time of death (25 dpi) (Goertz and Hoch, 2008). The negative correlation between time of collection and recovery of inoculated larvae, as well as the lower recovery rate of inoculated larvae than test larvae in our experiment with different exposure times, indicate that some mortality due to microsporidiosis occurred and was probably an additional source of infection for test larvae in the cages. Laboratory studies demonstrated that test larvae can acquire infections when confined with *Nosema*-infected cadavers (Goertz and Hoch, 2008). Release of inoculum after host death is the primary transmission pathway for *Ld*MNPV, however, *Nosema*-killed larvae are not liquefied like those that succumbed to the virus. We must, therefore, assume that the spread of the pathogen takes place in a different manner; spore release may be considerably slower due to slower decomposition of the microsporidia-infected cadavers.

The *N. lymantriae* infections acquired by the test larvae in the field cages were typically very light; often only a few spores were detected upon microscopic examination. This may be an indication of ingestion of a very low spore dosage or infection acquired at a late stage of larval development. We know that the infective dose of *N. lymantriae* is low. Dosages of 100 spores administered to third

instars lead to 92–99% infection in the laboratory (Goertz et al., 2004). Light infections in test larvae, together with the fact that the larval population was already close to pupation at that time, suggests that it is unlikely, despite high infectivity, that a second cycle of horizontal transmission could take place in the field. Lightly infected larvae, however, may develop into infected adults and vertically transmit the infection to their offspring. Vertical transmission has been reported for a different isolate of *N. lymantriae* (Novotny and Weiser, 1993) and for *N. portugal* (Maddox et al., 1999). Transovum or transovarially infected progeny could be a major source of infection of the next generation of *L. dispar* populations. Microsporidia like *N. lymantriae* are able to survive freezing and thawing to a certain extent (Maddox and Solter, 1996). With sufficient protection against UV radiation, environmental spores may be able to persist in the field and function as inoculum for the next larval generation. It was demonstrated that debris mats consisting of silk, frass, cadavers, and old pupal exuviae are an important source of inoculum of gypsy moth nucleopolyhedrosis virus for infection of the following generation (Doane, 1970). Such structures may function in a similar manner for the trans-generational transmission of microsporidia.

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