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Host specificity of microsporidia pathogenic to the gypsy moth, *Lymantria dispar* (L.): Field studies in Slovakia

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ABSTRACT

Several species of microsporidia are important chronic pathogens of *Lymantria dispar* in Europe but have never been recovered from North American gypsy moth populations. The major issue for their introduction into North American *L. dispar* populations is concern about their safety to native non-target insects. In this study, we evaluated the susceptibility of sympatric non-target Lepidoptera to two species of microsporidia, *Nosema lymantriae* and *Vairimorpha disparis*, isolated from European populations of *L. dispar* and applied in field plots in Slovakia. Application of ultra low volume sprays of the microsporidia maximized coverage of infective spores in a complex natural environment and, thus, exposure of non-target species to the pathogens. Of 653 non-target larvae collected from plots treated with *V. disparis* in 2002, 18 individual larvae representing nine species in four families were infected. These plots were monitored for two subsequent seasons and *V. disparis* was not recovered from non-target species. Of 2571 non-target larvae collected in *N. lymantriae*-treated sites, one larva was found to be infected. Both species of microsporidia, particularly *N. lymantriae*, appear to have a very narrow host range in the field, even when an inundative technique is used for their introduction. *V. disparis* infections in *L. dispar* exceeded 40% of recovered larvae in the treated study sites; infection rates were lower in sites sprayed with *N. lymantriae*. Several naturally-occurring pathogens were recorded from the non-target species. The most common pathogen, isolated from 21 species in eight families, was a microsporidium in the genus *Cystosporogenes*.

1. Introduction

Eradication of the introduced gypsy moth, *Lymantria dispar* (L.), from North America was deemed impossible by the mid-20th Century, resulting in extensive evaluation and introduction of natural enemies from Europe and Asia for biological control of this serious forest and urban pest (McManus and McIntre, 1981; Solter and Hajek, 2009). Of the numerous predators, parasites and pathogens studied, the *L. dispar* pathogens are, as a group, decidedly important biological control agents, particularly during outbreaks of the pest (Solter and Hajek, 2009).

Pathogens have been manipulated in a variety of ways for control of *L. dispar*, ranging from their development as microbial pesticides to introduction as classical biological control agents. Two pathogens have been developed and registered as pesticides for use against *L. dispar*, the bacterium *Bacillus thuringiensis kurstaki* as a broad-scale microbial pesticide (indeed, the first such use of *Btk*) (Dubois, 1981; Reardon et al., 1994), and the nuclear polyhedrosis virus, *Ld*MNPV, commonly present in European *L. dispar* populations and first reported from US *L. dispar* populations in 1907 (Hajek et al., 2005). The virus has been formulated as the microbial pesticide Gypchek® (Reardon et al., 1996) and is also a naturally occurring, density dependent pathogen that can cause precipitous decline of outbreak *L. dispar* populations (Elkinton and Liebhold, 1990). *Entomophaga maimaiga*, an entomophthoralean fungal pathogen originating in Japan (Nielsen et al., 2005), was introduced in 1910 or 1911 and was not recovered after its release (Speare and Colley, 1912). Epizootics were recorded for the first time in 1989 in the northeastern US, possibly from a later introduction (Wesloh, 1998), and caused a dramatic decline in *L. dispar* populations (Andreadis and Weseloh, 1990; Hajek et al., 1995). In some areas within the *L. dispar* range, this fungus appears to be responsible for a general decline of *L. dispar* outbreaks (Weseloh, 2003) and is acting in a density independent manner

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by causing populations to collapse prior to reaching outbreak densities.

A rich complex of entomopathogens, including *Ld*MNPV, occurs naturally in *L. dispar* populations in Europe. Particularly interesting in their ubiquity and diversity are the microsporidia (Fungi: Microsporidia); the first known, *Endoreticulatus* (*Pleistophora*) *schubergi*, was described by Zwölfer (1927). An additional six species of *L. dispar* microsporidia have been described (McManus and Solter, 2003; Vavra et al., 2006). Of these, five may be valid species, although three, *Nosema lymantriae*, *N. serbica*, and *N. portugal*, are very closely related (Maddox et al., 1999; Vavra et al., 2006). Two other species, *Thelohania disparis* (Timofejeva, 1956) and *T. similis*, the latter reported in a mixed infection with *N. lymantriae* (Weiser, 1957), actually represent one species, a dimorphic microsporidium, closely related to the monomorphic *L. dispar Nosema* group (Baker et al., 1994), that was recently re-described as *Vairimorpha disparis* (Vavra et al., 2006).

Microsporidia are typically chronic rather than acutely virulent parasites, nevertheless, the literature (summarized briefly by McManus and Solter, 2003) supports their importance as primary pathogens of *L. dispar*, and species in the *Nosema–Vairimorpha* clade have been implicated in declines of *L. dispar* populations in Eastern Europe (Sidor, 1979; Sidor and Jodal, 1983; Zelinskaya, 1980). Interestingly, microsporidia have never been recovered from *L. dispar* in surveys of pathogens in North American populations (Podgwaite, 1981; A. Hajek, personal communication).

Entomopathogenic microsporidia are relatively environmentally safe; they produce no toxins and are generally host specific at or below the family level. Rarely, species in multiple families within an insect order are ecologically susceptible (Solter and Becnel, 2003), and occasionally taxonomic orders or classes are crossed when the association between hosts is obligatory. Some species of hymenopteran parasitoids, for example, have been reported to acquire infections when they develop in an infected lepidopteran or dipteran host (Cossentine and Lewis, 1987; Schuld et al., 1999; Futerman et al., 2006), and two reports of microsporidia in ticks suggested that the infections were acquired from their mammalian hosts, an interaction above the taxonomic class level (Ribeiro and Guimães, 1998; Ribiero and Passos, 2006). The major issue regarding introduction of the *L. dispar* microsporidia into North American host populations, therefore, concerns their specificity to *L. dispar* and, thus, their safety to native non-target insects. Laboratory host specificity testing predicted a narrow host range for several *L. dispar* microsporidian isolates (Solter et al., 1997), as well as for other lepidopteran microsporidia (Solter and Maddox, 1998a; Solter et al., 2005). Field collections in Bulgaria found that Lepidoptera sympatric with *L. dispar* did not serve as reservoir hosts for three naturally occurring *L. dispar* microsporidian species, *V. disparis, N. lymantriae*, and *E. schubergi*, (Solter et al., 2000).

In this study, we evaluated the effects of microsporidia on non-target Lepidoptera when microsporidia were introduced via ultra low volume sprays into field plots consisting of natural oak stands in Central Slovakia. This method of introduction maximized the coverage of infective spores in a complex environment and, consequently, the exposure of sympatric non-target species to the pathogens. Our primary objectives were to determine, based on safety to non-target species, if microsporidia are appropriate organisms for introduction as classical biological control agents of *L. dispar* in North America and add to our basic understanding of microsporidian host range. The spray method of introduction into a natural environment allowed us to determine whether an inundative release of infective spores into a field population of *L. dispar* would pose a risk to non-target organisms. In addition, collections of non-target oak feeding lepidopteran species, some of concern as current and potentially invasive pests, provided an opportunity to survey for pathogens that could be of use in future control programs.

2. Methods

2.1. Production of microsporidia

New Jersey Standard *L. dispar* egg masses were obtained from the USDA APHIS rearing facility, Otis AFB, Massachusetts. The larvae were hatched and reared on high wheat germ diet (Bell et al., 1981) in 6 oz (177 ml) fluted plastic cups with DS-306 paper lids (Sweetheart®, Chicago, IL, USA) in growth chambers with conditions 24 ± 1 °C, 16:8 L:D, 60% RH.

Two species of microsporidia, *N. lymantriae* (INHS accession No. 1996-A; GenBank accession No. AF141129) isolated from a *L. dispar* population in Levishte, Bulgaria, and *V. disparis* (INHS accession No. 1995-D; GenBank accession No. DQ272237) isolated from *L. dispar* larvae in Rupite, Bulgaria, were chosen for these studies. Both species occur naturally in *L. dispar* populations in Slovakia and these particular isolates were evaluated in several previous and concurrent studies. To produce infective spores for field release, approximately 5×10^5 spores in 100 µl distilled water were spread on the surface of meridic diet in the rearing cups and 10 third-instar *L. dispar* larvae were added to each cup. The host larvae were dissected 20–23 days post inoculation and the silk glands and fat body tissues were excised. The infected tissues were homogenized in a 7-ml glass tissue grinder, filtered through fine mesh nylon material, and centrifuged in 15 ml tubes to pellet the spores. The spore pellets were re-suspended in sterile distilled water, the spores counted in a Petroff–Hausser hemocyte counter, and the inoculum stored at 5 °C for no more than 5 days post harvest.

2.2. Study sites

Two study sites in South Central Slovakia were selected based on the presence of native gypsy moth populations and a highly diverse complex of lepidopteran species. Locations of the sites were the following.

Krupina: 48°23′57.59″ N, 19°03′21.79″ E, elevation 480 m. This high-production forest stand is dominated by *Quercus petraea, Q. robur* and *Q. cerris* and the southwest oriented slopes are moderately steep. The area is at the northern distribution of gypsy moth habitat in Slovakia and has not experienced classical high-density outbreaks and defoliation.

Plaštovce: 48°09′40.47″N, 18°58′50.65″E, elevation 170 m. Slopes are predominantly southerly oriented. Relatively management-free oak stands are found at elevations slightly above old orchards and vineyards. The stands are composed of *Q. cerris* and *Q. pubescens*. Because of the somewhat hot and dry conditions, the trees rarely reach more than 10 or 15 m in height. Stands are interrupted by grassy meadows with high plant diversity. The site is considered to be a preferred habitat for gypsy moth populations and is highly susceptible to outbreaks. A broad spectrum of other oak foliage-feeding species including tortricids, geometrids and noctuids, are also commonly present.

The trees treated in both sites ranged from approximately 3–6 m in height, however lower branches of larger trees bordering the plots were also sprayed. Three 500 m^2 plots were positioned within each site with 25 or more meters separating the plots. In 2002, one plot in each site was treated with *V. disparis* and one plot (control) was unsprayed. The *V. disparis*-treated plots were monitored for persistence of the microsporidium for 2 years following treatment without further treatments. The control plots were maintained throughout the four seasons and a third plot in each site was treated with *N. lymantriae* in each 2003, 2004 and 2005.

Though both sites contained sparse natural *L. dispar* populations at the beginning of the project in May of 2001, populations increased steadily during the next 4 years. This population increase was not the focus of our study and, thus, was not quantitatively

determined; however, by 2005, outbreak conditions and defoliation occurred in the entire Plaštovce site area and the natural population density in the Krupina site was observably higher than in the previous seasons when few larvae were found in the area bordering the plots. The population increases we noted mirrored outbreaks (including widespread defoliation) in other areas of South Central Slovakia during the study period. Both test and control sites were augmented each season with 50 or 100 egg masses (25 or 50 egg masses per plot) produced at the USDA APHIS facility to ensure positive controls for the microsporidian treatments. *L. dispar* egg masses that were oviposited on brown paper were cut from the paper and attached with staples to *Quercus* sp. trees in both treated and untreated plots in late February or early March. Hatch matched that of the field populations in all study seasons.

2.3. Treatment with microsporidia

One billion infective spores of *V. disparis* or *N. lymantriae* were suspended in 2 l tap water for a final concentration of 500 spores/μl, and 0.5 ml Agrovital® (96% pinoline) adjuvant was added to each 2-l suspension to reduce droplet size and enhance adherence of the suspension to leaf surfaces (Prokop and Kejklicek, 2002). The spore concentration chosen was roughly based on laboratory bioassays in which 10^3 *V. disparis* spores/larva produced 87% infectivity in *L. dispar* and resulted in mortality during the late larval and pupal stages (Solter and Maddox, 1998a; unpublished data), and 200 *N. lymantriae* spores/larva infected 92% of third instar hosts (Goertz et al., 2004). The full 2-l container of suspension was sprayed with a hand-held ultra low volume sprayer (ULVA+, Micron Sprayers LTD, Bromyart Industrial Estate, UK) onto the upper and lower leaf surfaces of oaks in each treated plot. In 2001, collections were made in the four plots to record the composition of the lepidopteran populations feeding on oak foliage and determine whether microsporidia were present in the local *L. dispar* populations. *V. disparis* was sprayed in treatment plots in 2002, and these plots were monitored in 2003 and 2004 without further treatment. Although *N. lymantriae* was sprayed in a newly established plot in each site, due to lack of accessibility to areas with trees of appropriate sizes, the same plots were treated in 2003–2005. The spore concentration of *N. lymantriae* was quadrupled to 2×10^3 spores/μl in 2005 because infection rates were nearly zero in non-target species and low in the positive control (*L. dispar*) in 2003 and 2004; thus, we attempted to increase the potential for infections in non-target species to more clearly evaluate susceptibility of the non-target species to the microsporidium.

2.4. Collection and evaluation of L. dispar and non-target larvae

Seven and 14 days post-spray, lepidopteran larvae feeding on oak foliage in the treated and untreated plots were collected from all trees and branches below 2 m height in each plot. Nylon beating sheets were held below oak branches and the branches were sharply tapped with a stick to dislodge feeding insects. For each plot, *L. dispar* larvae were collected and placed into one set of screened plastic containers and non-target Lepidoptera were placed into other containers.

Larvae were immediately returned to the laboratory, sorted by species and identified, then placed in separate containers with washed oak foliage cut from untreated areas. Larvae collected 7 days post-spray were held until death or for 5 days in the laboratory, at which time they were dissected and evaluated for infections. Because *L. dispar* larvae were held in large numbers in a small number of cages, transmission of the microsporidia was likely; therefore only larvae with mature spores in the tissues (generation time $\geqslant$7 d; Goertz and Hoch, 2007) were recorded as infected. Larvae collected from the field 14 days post-spray were sorted and dissected within 2 days of collection.

To determine susceptibility of the lepidopteran larvae to the *L. dispar* microsporidia, fat body tissues, Malpighian tubules, silk glands and midgut tissues, all of which support at least minimal pathogen reproduction in infected *L. dispar* larvae, were excised and examined under light microscopy at 400×. Infections observed in non-target species were confirmed to be the result of treatments based on morphological characteristics (e.g. spore size and shape, presence of *V. lymantriae* octospores) under 400× light microscopy. In addition, spores isolated from the non-target hosts were fed to young *L. dispar* larvae in the laboratory. Development of the microsporidia in the putative natural host (*L. dispar*) was then observed under light microscopy.

Spores of several microsporidian isolates that were recovered from non-target host species and were morphologically different from the *L. dispar* species (e.g. markedly smaller spores and spores contained within vesicles) were isolated and sequenced using standard PCR techniques. HotSHOT DNA preparation (Truett et al., 2000) was used to extract DNA from the spores. The procedures are briefly described as follows. The spores were centrifuged and re-suspended in Buffer A (25 mM NaOH and 0.2 mM EDTA), then transferred into 200 μl thin-wall PCR tubes and heated to 95 °C for 30 min in an iCycler (Biorad) thermocycler. After cooling to 4 °C, 20 μl Buffer B (40 mM Tris–HCl) was added to each reaction and then centrifuged at 13,780*g* for 10 min. The supernatants were stored at −20 °C before use. The PCR protocol used was described by Huang et al. (2007). The primer set 18f: 5′-CACCAGGTTG ATTCTGCC-3′/1492r: 5′-GGTTACCTTGTTACGACTT-3′ (Weiss and Vossbrinck, 1998) was used to amplify SSUrDNA (Huang et al., 2007). The PCR products were sequenced directly using the same primer set and compared with microsporidian SSUrDNA sequences in GenBank.

3. Results

3.1. Collection of non-target insects

Between 2001 and 2005, 6752 non-target lepidopteran larvae (99 species in 72 genera) were collected from the treated, monitored and control plots and identified to species by co-author J. Patocka, an expert on Central European lepidopteran larvae and pupae. An additional 204 larvae were identified to genus level (3 additional genera) and 15 to family (Table 1). In addition, seven species of phytophagous hymenoptera (498 individual larvae in two families) were collected and evaluated; three were identified to species and four to the genus level.

3.2. V. disparis and N. lymantriae in the L. dispar host

Microsporidia were not observed in the Krupina and Plaštovce *L. dispar* populations in 2001. Both species of microsporidia were recovered from the *L. dispar* host on day 7 and day 14 after treatment of the respective plots (Table 2). No *L. dispar* collected from the control sites were infected. Post-spray prevalence was generally higher for *V. disparis* than for *N. lymantriae*, with total prevalence per site for *V. disparis* at 55.7% and 41.7% for the Plaštovce and Krupina sites, respectively. No *L. dispar* larvae collected from the monitored sites in 2003 and 2004 were infected with *V. disparis*. Prevalence of *N. lymantriae* was 31.5% in Plaštovce and 40.0% in Krupina in 2005 when the higher spore concentration was used, but was lower in 2003 (6.7% and 21.2% for Plaštovce and Krupina, respectively) and 2004 (3.0% and 4.5% for Plaštovce and Krupina, respectively) when lower spore concentrations were sprayed. No *N. lymantriae* infections were recovered from control plots.

Table 1
Immature Lepidoptera collected May 12–20, 2001–2005, in South Central Slovakia.

Family	Genus and species	Plaštovce site					Krupina site				
		2001	2002	2003	2004	2005	2001	2002	2003	2004	2005
Lepidoptera											
Arctiidae	*Eilema complana*	–	2	9	5	2	1	10	–	–	–
	Euplagia quadripunctaria	–	2	–	2	1	–	–	–	11	–
Drepanidae	*Asphalia ruficollis*	2	9	10	85	25	–	–	–	–	–
	Cymatophorima diluta	1	2	8	2	13	–	–	1	–	6
	Polyploca ridens	4	9	109	47	28	1	-	4	1	0
Eriocraniidae	*Eriocrania subpurpurella*	1	–	–	–	–	–	–	–	–	–
Gelechiidae	*Anacampsis timidella*	–	–	–	1	–	–	–	–	–	–
	Psoricoptera gibbosella	5	3	3	–	2	6	11	15	3	1
Geometridae	*Agriopis aurantiaria*	4	6	96	46	81	20	48	88	157	91
	Agriopis bajaria	–	2	–	–	1	–	–	–	–	–
	Agriopis leucophaearia	134	88	431	75	249	3	7	35	7	3
	Agriopis marginaria	4	13	23	71	55	1	15	28	4	21
	Alsophila aescularia	6	22	17	20	62	2	4	18	8	8
	Alsophila aceraria	2	4	3	–	45	2	2	5	25	22
	Apocheima hispidaria	–	–	–	3	4	–	–	–	–	1
	Apocheima pilosaria	1	3	3	9	31	1	2	10	12	7
	Biston strataria	3	–	56	–	5	–	–	2	–	–
	Colotois pennaria	–	–	1	3	8	1	14	11	163	8
	Crocallis elinguaria	–	–	–	–	–	–	–	–	1	–
	Cyclophora ruficiliaria	–	–	3	–	–	–	–	–	–	–
	Ennomos quercinaria	–	1	1	–	–	–	3	2	–	1
	Epirrita dilutata	–	–	–	–	–	1	2	1	3	–
	Erannis defoliaria	4	-	7	1	19	1	1	7	18	8
	Eupithecia abbreviata	–	–	–	–	–	–	11	–	–	–
	Eupithecia dodoneata	3	7	–	–	–	–	–	–	–	–
	Eupithecia sp.	1	–	7	–	–	–	–	–	–	–
	Hypomecis roboraria	–	5	–	–	–	–	6	–	–	–
	Lycia hirtaria	–	1	2	–	–	–	3	15	6	–
	Operophtera brumata	3	17	8	–	132	15	17	44	182	166
	Geometridae g. sp.	–	–	–	–	–	2	–	–	–	–
Lasiocampidae	*Eriogaster rimicola*	–	–	1	–	–	–	–	–	–	–
	Poecilocampa populi	–	–	–	–	–	–	1	–	–	–
	Trichiura crataegi	–	–	–	–	–	–	–	–	–	1
Lycaenidae	*Neozephyrus quercus*	7	1	20	9	4	4	12	38	18	2
	Satyrium ilicis	1	–	–	–	–	3	2	4	11	–
Lymantriidae	*Lymantria dispar*[a]	153	401	412	200	400	150	404	570	500	400
	Ocneria rubea	1	–	8	–	–	–	–	1	–	1
	Parocneria detrita	–	–	2	–	–	–	–	–	–	–
	Orgyia antiqua	–	1	–	–	–	–	10	–	–	–
Noctuidae	*Acronicta* sp.	–	–	–	–	1	–	–	–	–	–
	Agrochola laevis	1	–	–	–	5	3	2	1	1	5
	Allophyes oxyacanthae	–	–	–	–	–	–	–	–	1	–
	Amphipyra pyramidoides	–	–	4	–	3	–	2	16	33	3
	Asteroscopus sphinx	–	–	–	–	–	–	–	2	1	–
	Catocala nymphagoga	6	7	84	49	24	–	–	8	–	–
	Catocala promissa	4	–	–	–	–	–	–	–	3	–
	Catocala sponsa	1	–	1	–	–	–	4	2	7	–
	Conistra vaccinii	11	7	1	2	1	19	67	19	25	1
	Cosmia pyralina	–	–	2	–	2	–	–	1	5	–
	Cosmia trapezina	–	1	4	1	3	1	3	36	30	13
	Dichonia Aprilina	1	1	1	1	1	–	–	–	–	–
	Dichonia convergens	–	–	–	–	–	3	10	–	8	5
	Diloba caeruleocephala	–	–	–	–	1	–	–	–	–	–
	Dryobotodes eremita	8	11	20	22	42	–	1	1	–	1
	Dicycla oo	5	1	6	5	11	–	–	2	–	–
	Eupsilia transversa	–	2	1	–	1	1	6	10	13	6
	Jodia croceago	1	2	1	–	1	5	11	12	2	–
	Lithophane ornithopus	1	1	4	3	3	–	3	3	3	2
	Lygephila sp.	1	–	–	–	–	–	–	–	–	–
	Minucia lunaris	–	–	–	2	–	–	–	–	–	–
	Nycteola revayana	–	–	1	–	–	–	3	4	–	–
	Orthosia cerasi	3	19	101	41	30	2	11	21	7	13
	Orthosia cruda	12	5	26	73	126	–	7	14	29	27
	Orthosia incerta	1	2	6	–	1	–	1	3	1	1
	Orthosia miniosa	5	11	12	6	29	30	20	1	7	22
	Orthosia munda	–	2	4	–	–	–	5	4	43	13
	Orthosia schmidtii	–	–	–	–	1	–	–	–	–	–
	Orthosia sp.	1	–	–	–	–	–	–	–	–	2
	Noctuidae g. sp.	–	–	–	–	–	–	10	–	–	–

Table 1 (*continued*)

Family	Genus and species	Plaštovce site					Krupina site				
		2001	2002	2003	2004	2005	2001	2002	2003	2004	2005
	Nycteola revayana	–	–	1	–	–	–	3	4	–	–
Nolidae	*Bena bicolarana*	3	–	–	–	4	1	–	–	2	1
	Meganola strigula	2	–	–	–	–	–	–	–	–	–
	Pseudoips prasinana	–	–	2	–	1	–	2	–	–	–
Notodontidae	*Drymonia querna*	–	–	–	5	–	–	–	–	–	–
	Drymonia ruficornis	9	12	21	2	1	–	30	6	–	–
	Peridea anceps	–	1	5	–	–	–	–	–	–	–
	Spatalia argentina	–	–	1	–	–	–	1	–	–	–
Oecophoridae	*Carcina quercana*	–	–	–	–	–	–	1	–	–	–
	Diurnea lipsiella	4	1	–	–	3	1	–		–	–
Pyralidae	*Acrobasis consociella*	–	–	1	–	–	2	1	1	4	–
	Acrobasis sodalella	–	–	–	–	–	–	–	1	1	–
	Conobathra repandana	1	–	4	1	2	2	1	6	10	6
	Conobathra tumidana	2	–	1	2	6	–	–	2	–	2
	Phycita roborella	4	5	12	18	32	–	11	16	5	2
Tortricidae	*Aleimma loeflingiana*	1	3	1	–	1	14	3	6	65	49
	Archips crataegana	–	–	–	–	–	1	6	5	16	7
	Archips podana	1	–	–	–	–	–	–	5	–	–
	Archips rosana	1	–	1	–	1	–	–	–	–	1
	Archips xylosteana	2	1	0	–	1	3	3	14	6	5
	Choristoneura hebenstreitella	–	3	2	–	1	2	–	2	2	6
	Eudemis profundana	–	2	–	–	1	6	11	15	28	7
	Orthotaenia undulana	–	–	–	–	–	–	–	–	3	3
	Pandemis cerasana	–	3	–	–	1	1	2	1	2	4
	Pandemis corylana	–	–	–	–	–	2	7	5	–	5
	Pandemis heparana	–	–	–	–	–	–	–	–	–	1
	Ptycholoma lecheana	–	2	–	–	1	–	–	–	12	16
	Pommene sp.	4	2	–	–	–	–	–	–	–	–
	Tortricodes alternella	11	3	–	–	1	7	19	17	7	3
	Tortrix viridana	20	11	9	6	119	6	18	7	23	7
	Zeiraphera isertana	–	–	–	–	–	–	2	1	4	–
	Tortricidae g. sp.	–	–	–	–	–	1	2	–	–	–
Ypsolophidae	*Ypsolopha alpella*	9	–	1	5	24	10	–	4	2	4
	Ypsolopha lucella	13	–	–	–	1	–	–	–	–	1
	Ypsolopha parenthesella	–	–	–	–	–	–	1	–	–	–
	Ypsolopha sylvella	3	–	12	11	2	16	2	117	3	8
	Ypsolopha ustella	–	–	7	–	–	–	3	1	–	1
	Ypsolopha sp.	50	49	2	–	1	13	69	1	–	–
Zyganidae	*Rhagades pruni*	1	–	–	–	1	2	–	–	–	–
	Zygaena filipendulae	–	–	1	–	–	–	–	–	–	–
Hymenoptera											
Pamphiliidae	*Pamphilius silvaticus*	1	–	3	–	–	–	–	–	–	–
	Pamphilius sp.	–	–	–	–	–	–	1	–	–	–
Tenthredinidae	*Apethymus* sp.	9	11	7	4	1	5	31	13	44	13
	Emphytus cerris	7	4	14	–	6	1	–	1	–	–
	Mesoneura opaca	20	9	7	10	2	24	17	24	10	12
	Periclista sp.	20	9	12	5	2	22	43	27	23	22

N = Number collected in each site.
2003 and 2004: 3 plots per site (1500 m^2total area).
2002 and 2005: 2 plots per site (1000 m^2 total area).
[a] Augmented *L. dispar* population.

Table 2
Percent infections produced in augmented field populations (treated plots only) of *Lymantria dispar* using ultra low volume sprays of the microsporidia *Vairimorpha disparis* and *Nosema lymantriae*. No infected larvae were recovered from control plots (no spray).

Microsporidian species (year)	Site			
	Plaštovce		Krupina	
	7 dpi[a] (N)[b]	14 dpi (N)	7 dpi (N)	14 dpi (N)
Vairimorpha disparis (2002)	74.0 (100)	61.0 (100)	36.5 (104)	47.0 (100)
Nosema lymantriae (2003)	12.1 (33)	<8.0[c]	19.6 (107)	23.0 (100)
Nosema lymantriae (2004)	–[d]	3.0 (100)	5.0 (100)	4.0 (100)
Nosema lymantriae (2005)	42.0 (100)	21.0 (100)	51.0 (100)	29.0 (100)

[a] Days post inoculation (spray). Larvae collected at 7 dpi were held 5 additional days in the laboratory before examination.
[b] N = total number examined for infection.
[c] Approx 30% of larvae from this treatment were inadvertently collected from outside the plot-total larvae adjusted to approx. 57.
[d] Adverse weather conditions prevented a first week collection in the Plaštovce site.

3.3. L. dispar microsporidia infecting non-target hosts

V. disparis was confirmed to infect 18 individual non-target host insects, representing nine species in four families (Table 3); Lymantriidae (one species), Noctuidae (six species), Oecophoridae (one species) and Tortricidae (one species). In addition, two specimens of one geometrid species appear to have been infected, but infections were not confirmed in feeding bioassays. If all infections are considered to be *V. disparis*, the percentage infection in non-target species was 0.68% in the Krupina site and 7.94% in the Plaštovce site, 3.06% overall. *V. disparis* infections in one noctuid individual (*Cosmia trapezina*) and one tortricid individual (*Aleimma loeflingiana*) produced atypical spores, and infection resulted in acute death of all four infected *Dryobotodes eremita* (Noctuidae) larvae. Infection in one *D. eremita* larva appeared typical of infections observed in *L. dispar*, however, infections in three other *D. eremita* individuals were atypical, producing mortality despite low production of mature spores.

In the 2003 treatment, *N. lymantriae* infection was reported in one non-target insect, a *Conistra vaccinii* larva. No other *N. lymantriae* infections were recovered from non-target larvae, including during the 2005 season when spore concentration was quadrupled. No non-target lepidopteran larvae or oak-feeding hymenoptera recovered from control and monitored sites were infected with either species of *L. dispar* microsporidia.

Although differences in sites and treatments from year to year precluded statistical analysis, numbers of non-target lepidopteran larvae did not appear to be reduced in treated plots (Tables 4 and 5). Table 4 represents combined totals of non-target Lepidoptera for each year and site in treated (sprayed) vs. control (unsprayed) plots. Numbers of collected non-target individuals were higher in *V. disparis*-treated plots than in control plots in 2002 and, in *N. lymantriae*-treated plots, varied between control and treated plots and between sites annually. Table 5 reports family-specific data for treated vs. control plots in each site. To avoid inaccurate reporting for lepidopteran species that were infrequently recovered in the sites during the study period (Wagner et al., 1996), only species for which more than 20 individuals were reported from one site in at least one season were included. Collection numbers were higher in the control plots than in *N. lymantriae*-treated plots for geometrids and noctuids in 2003 and 2004 (primarily in the Krupina plot), but were not lower in the treated plots in 2005 when the spore concentration sprayed was 4× the concentration used in 2003 and 2004 and a higher percentage of infected *L. dispar* was recovered.

Table 4
Total number of non-target Lepidoptera collected from treated and untreated plots in Krupina and Plaštovce, Slovakia, 7 and 14 days post treatment with *Vairimorpha disparis* and *Nosema lymantriae*. Larvae collected from monitored plots are not included.

Year and treatment	Plaštovce treated	Plaštovce control	Krupina treated	Krupina control
2002-*Vairimorpha disparis*	214	159	439	239
2003-*Nosema lymantriae*	329	509	306	291
2004-*Nosema lymantriae*	264[a]	274[a]	303	527
2005-*Nosema lymantriae*	706	554	663	387
Total *Nosema lymantriae*	1299	1337	1272	1205

[a] No 7-day collection was made in Plaštovce in 2004 due to weather conditions.

3.4. Other pathogens recovered from non-target Lepidoptera

A number of pathogens, including microsporidia, fungi and nematodes, were recovered from non-target species (Table 6). No viruses visible under light microscopy (nuclear polyhedrosis virus, granulosis virus, cytoplasmic polyhedrosis virus and entomopox virus) were observed. A small microsporidium, enclosed in membrane-bound vesicles that typically contained 16 or more spores, was recovered from a number of the non-target species in the families Geometridae, Lasiocampidae, Lycaenidae, Noctuidae, Tortricidae and Ypsolophidae. Six samples isolated from *Operophtera brumata*, *Acrobasis sodalella, Alsophila aescularia*, *Erannis defoliaria*, *Phycita roborella* and *Ypsolopha sylvella* were sequenced. The sequences were identical to each other, and were 99% similar to *Cystosporogenes operophterae* (GenBank accession No. AJ302320), a microsporidium described from *O. brumata* (Canning, 1960; Can-

Table 3
Vairimorpha disparis infections in non-target lepidopteran larvae exposed to spores in treatment plots in 2002. *V. disparis* was not recovered from control plots or from the treated plots 1 and 2 years post-spray.

Species	Plaštovce site				Krupina site			
	7 days[a] (N)	14 days (N)	1 yr[b] (N)	2 yr[b] (N)	7 days (N)	14 days (N)	1 yr[b] (N)	2 yr[b] (N)
Geometridae								
Hypomecis roboraria	–	2 (5)[c,d]	–	–	–	0 (2)	–	–
Lymantriidae								
Lymantria dispar	74 (100)	61 (100)	0 (155)	0 (50)	38 (104)	47 (100)	0 (165)	0 (150)
Orgyia antiqua	–	1 (1)	–	–	–	–	–	–
Noctuidae								
Cosmia trapezina	–	1[d] (1)	0 (1)	–	–	0 (1)	0 (20)	0 (17)
Dryobotodes eremita	4[e] (5)	1 (2)	0 (8)	0 (3)	–	0 (1)	0 (1)	–
Orthosia cerasi	3 (7)	–	0 (19)	0 (4)	1 (4)	0 (3)	0 (10)	0 (2)
Orthosia cruda	1 (3)	–	0 (5)	0 (22)	0 (5)	0 (1)	0 (4)	0 (12)
Orthosia miniosa	2 (2)	0 (1)	0 (2)	0 (1)	1 (7)	0 (1)	–	0 (7)
Orthosia munda	1 (2)	–	0 (1)	–	0 (5)	–	0 (2)	0 (22)
Oecophoridae								
Diurnea lipsiella	–	1 (3)	0 (1)		–	–	0 (1)	–
Tortricidae								
Aleimma loeflingiana	0 (2)	–	–	–	1[d] (2)	–	0 (3)	0 (35)

N = total number of larvae collected.
[a] Time post ULV spray.
[b] Total collected for season (2 collections) in each site.
[c] *Vairimorpha disparis* infection not confirmed in feedbacks to *Lymantria dispar* larvae.
[d] Atypical spores produced.
[e] All four individuals died; three infections atypical with few spores; environmental spores and immature octospores produced in one larva.

Table 5
Numbers of larvae per lepidopteran family collected in treated, monitored, and untreated (control) plots. Species for which >20 individuals were collected from one or more plots in a single season are included.

Non-target species	2002 Plastovce		2002 Krupina		2003 Plastovce			2003 Krupina			2004 Plastovce			2004 Krupina			2005 Plastovce		2005 Krupina	
	V[a]	C[a]	V	C	N[a]	C	M[a]	N	C	M	N	C	M	N	C	M	N	C	N	C
Drepanidae	7	2	1	0	50	32	27	0	2	2	54	49	30	0	0	1	28	24	6	0
Geometridae	81	70	64	58	152	328	163	52	78	109	37[b]	18[b]	20[b]	87	232	195	246	220	187	142
Lycaenidae	1	0	4	8	5	9	6	12	7	19	6	2	1	8	10	14	0	0	0	0
Noctuidae	25	30	89	38	59	73	36	39	27	64	94	63	37	35	67	85	132	125	65	31
Notodontidae	5	7	28	2	6	8	6	0	1	5	0	0	0	0	0	0	0	0	0	0
Pyralidae	4	1	4	7	5	5	6	4	3	12	6	9	4	8	1	6	23	9	2	0
Tortricidae	10	6	23	25	3	1	5	20	13	20	2	2	2	43	32	53	89	32	43	29
Ypsolophidae	32	17	44	25	9	3	0	51	25	41	5	6	0	2	0	1	14	12	9	3

[a] V = treated with *Vairimorpha disparis* in 2002; M = monitored *V. disparis* plots in 2003 and 2004; C = control plot; N = Treated with *Nosema lymantriae* in 2003–2005.
[b] Collections extensive in all plots for two *Agriopus* species; only 25 of each counted and dissected, therefore not included in data set.

Table 6
Pathogens isolated from oak-feeding Lepidoptera in South Central Slovakia, 2001–2005.

Host family	Genus and species	Plaštovce site	Krupina site
		No. infected & pathogen group[a] (N)[b]	No. infected & pathogen group (N)
Drepanidae	*Asphalia ruficollis*	4 – fungal pathogen (131)	–[c]
Gelechiidae	*Psoricoptera gibbosella*	0 (13)	1 – *Cystosporogenes* sp.[d] (36)
Geometridae	*Agriopis aurantiaria*	0 (233)	1 – fungal pathogen (404)
	Agriopis leucophaearia[a]	10 – *Cystosporogenes* sp. 2 – fungal pathogen 1 – possible GV (977)	2 – *Cystosporogenes* sp. (55)
	Agriopis marginaria	12 – *Cystosporogenes* sp. (166)	0 (69)
	Alsophila aescularia	4 – *Cystosporogenes* sp. (127)	1 – *Cystosporogenes operophterae*[d] (40)
	Alsophila aceraria	1 – fungal pathogen (54)	1 – *Cystosporogenes* sp. (56)
	Colotois pennaria	1 – *Cystosporogenes* sp. (11)	11 – *Cystosporogenes* sp. 1 – fungal pathogen (197)
	Epirrita dilutata	–	1 – *Cystosporogenes* sp. (7)
	Erannis defoliaria	0 (31)	1 – *Cystosporogenes operophterae*[d] (35)
	Eupithecia abbreviata	–	2 – Unknown microsporidium (11)
	Operophtera brumata	1 – *Nosema*-type 1 – *Cystosporogenes* sp. 1 – *fungal pathogen* 1 – nematode sp. (160)	46 – *Cystosporogenes* sp. 23 – *Cystosporogenes operophterae*[d] 2 – *Endoreticulatus*-type? (424)
Lasiocampidae	*Poecilocampa populi*	–	1 – *Cystosporogenes* sp. (1)
Lycaenidae	*Neozephyrus quercus*	0 (41)	1 – *Cystosporogenes* sp. (74)
Noctuidae	*Amphipyra pyramidoides*	0 (7)	1 – *Cystosporogenes* sp. (54)
	Conistra vaccinii	0 (22)	3 – *Cystosporogenes* sp.[d] 1 – *Orthosomella operothophterae*[d] (131)
	Dryobotodes eremita	1 – fungal pathogen (103)	0 (4)
	Orthosia cruda	1 – *Cystosporogenes* sp. (242)	0 (77)
Pyralidae	*Acrobasis sodalella*	–	1 – *Cystosporogenes operophterae*[d] (2)
	Phycita roborella	0 (71)	1 – Unknown microsporidium 1 – *Cystosporogenes operophterae*[d] (34)
Tortricidae	*Aleimma loeflingiana*	0 (6)	2 – fungal pathogen 1 – *Cystosporogenes* sp. (137)
	Archips crataegana	–	1 – Unknown micro sp. 3 – *Cystosporogenes*-type (35)
	Archips xylosteana	0 (4)	1 – *Endoreticulatus*-type (31)
	Eudemis profundana	0 (3)	1 – *Cystosporogenes* sp. (67)
	Tortrix viridana	1 – *Cystosporogenes* sp. 1 – nematode 1 – possible NPV 1 – fungal pathogen (165)	0 (61)
Ypsolophidae	*Ypsolopha sylvella*	0 (28)	2 – *Cystosporogenes operophterae*[d] (146)

[a] *Vairimorpha disparis* infections in 2002 excluded. See Table 3.
[b] N = total number collected in site over five seasons (2001–2005); two 500 m^2 plots/site in 2001, 2004, 2005; three plots/site in 2002 and 2003.
[c] No specimens collected.
[d] SSU-rDNA sequenced.

ning et al., 1985; Canning and Curry, 2004). The sequences were also 99% similar to *C. legeri* isolated from a laboratory colony of *Lobesia botrana* in Germany (GenBank accession No. AY233131). The new isolate was deposited into GenBank, accession No. GU299511. A microsporidian isolate from *Conistra vaccinii* collected in the Krupina site shared 99% identity with *Orthosomella operophterae* (GenBank accession No. AJ302316), and the sequence was deposited into GenBank, accession No. GY299512. Slight sequence errors combined with morphological variability in one *O. brumata* sample suggested a mixed infection of two microsporidian species. Sequence errors also occurred in the sample from *A. sodalella.* The spores in the *A. sodalella* sample appeared to be a small *Nosema*-like species rather than *Cystosporogenes*; it is possible that this isolate also represented a mixed infection. *Cystosporogenes* sp. was also sequenced from a *Psoricoptera gibbosella* larva from our collection (M. Hylis, personal communication). No pathogens other than the released microsporidia were observed in recovered *L. dispar* larvae.

4. Discussion

Laboratory and field studies (Solter et al., 1997; Solter et al., 2000) suggested that *Nosema* and *Vairimorpha* microsporidia isolated from *L. dispar* have a narrow host range and should be safe for use as classical biological control agents against this pest in North America. Nevertheless, questions remained about their infectivity to non-target species, particularly during the period of establishment when infective spores are introduced into the environment. Because *L. dispar* and the microsporidian pathogens of interest are native to Slovakia, and because the temperate oak woodlands in Slovakia host many congeners of North American lepidopteran species, it was possible to address these questions using a 'worst case scenario' method of directly spraying suspensions of infective spores onto oak foliage where both early instar *L. dispar* and non-target lepidopteran species were feeding. The primary effort was focused on providing information and data needed to pursue permission from federal and state regulatory agencies in the US to release *V. disparis, N. lymantriae* and possibly *Nosema portugal* into US *L. dispar* populations, with the goal of enhancing biological control of the pest. We also provide a five-season collection record of oak-feeding Lepidoptera in two sites in Slovakia (Table 1) and report observations and prevalence of naturally-occurring pathogens in the larvae (Table 6). This collection included species that are potential forest pests if introduced into temperate North American forests, including *Archips crataegana*, *Tortrix viridana, A. loeflingiana*, *E. defoliaria*, and *O. brumata* (Tables 1 and 6). *O. brumata* has already been introduced (Elkinton et al., 2009).

Species in three families of Lepidoptera, Geometridae, Noctuidae, and Tortricidae, dominated the collections during the first 2 weeks of May in Slovakia (Table 1). Other species that occurred in high densities represented the families Drepanidae (*Polyploca ridens*), Lymantriidae (*L. dispar*), and Ypsolophidae (several species). Peak populations for the high-density species differed across years, but the total non-target lepidopteran population increased yearly from 2002 to 2005; collection totals in 2005 were double those in 2002 using a consistent sampling time of approximately 2 h per site and two collection teams per collection event. This total population increase coincided with an observed increase in the natural *L. dispar* population density.

We noted that prevalence of naturally-occurring disease in non-target insects was highest in 2004 for four lepidopteran species, *Agriopis leucophaearia*, *Agriopis marginaria*, *Colotois pennaria*, and *O. brumata*, that were collected in large numbers (more than 200 individuals) and with more than five individuals infected with naturally-occurring pathogens from 2001–2005 (Table 6). Overall, 11.5% of these species were infected by various pathogens. By far the most frequently recovered pathogen was *Cystosporogenes* sp., accounting for 132 of the 159 infections we identified (Table 6). Sequencing showed that all tested isolates, including those from *O. brumata*, were 99% similar to *C. operophterae* and *C. legeri*, suggesting that this microsporidium or species complex has an unusually broad host range. Although not all samples were sequenced, the morphotype was observed in 21 species in eight families (Table 6).

It is acknowledged that behind the leading edge of invasion in North America, *L. dispar* is irretrievably established, and occasional outbreaks in large forested areas will need to be addressed using biological and chemical pesticides to prevent or limit defoliation and associated human (nuisance, recreational) and ecological impacts (Sharov et al., 2002; Tobin et al., 2004). Both *Ld*MNPV and *Entomophaga maimaiga* are established in North American *L. dispar* populations, but the microsporidia, which commonly occur in European *L. dispar* populations, have never been recovered from populations in the US or Canada. The literature suggests that microsporidia are persistent in *L. dispar* populations (Pilarska et al., 1998), are host density-dependent, often occur in populations in which *Ld*MNPV is present (Zelinskaya, 1980, 1981), and possibly synergize the effect of *Ld*MNPV (Bauer et al., 1998). Therefore, introducing an additional host specific pathogen to the natural enemy complex of *L. dispar* in North America, and potentially augmenting naïve populations in Europe, could improve the natural control of *L. dispar* populations and further reduce the frequency, duration and severity of outbreaks.

Host specificity testing is critical to introduction of classical biological control agents (Hokkanen et al., 2003). The field studies described herein, in concert with studies of natural field populations (Solter et al., 2000), provided evidence that *V. disparis*, *N. lymantriae* and, by extrapolation, the more host specific *N. portugal* (Solter et al., 1997), would not endanger non-target species by host-switching if introduced. Many non-target species that did not occur in high densities were collected in insufficient numbers to evaluate susceptibility to the *L. dispar* microsporidia; however these species were recovered in roughly equivalent numbers from treated and untreated plots, suggesting that no acute mortality occurred due to spraying of the microsporidia.

Combined data for all species (Table 4) suggest that spraying microsporidia did not produce overall deleterious effects on non-target populations, and data for common species combined at the family level (Table 5) were sufficiently variable among sites to suggest no impact on common species.

As predicted from earlier laboratory studies (Solter et al., 1997), *V. disparis* was less host specific than *N. lymantriae*. In general, *Vairimorpha* species appear to be relatively more virulent; they attack the fat body tissues of the host, reproducing quickly and filling the target cells with spores (Solter and Maddox, 1998b; Vavra et al., 2006). Nevertheless, similar to findings in laboratory studies (Solter et al., 1997), several of the non-target infections produced in this field study were atypical, producing abnormal spores and/or low numbers of mature spores, or resulting in acute death of the host before optimal reproduction of the pathogen. Atypical infections in non-target hosts, as well as many infections that appeared similar to those in the natural host, were noted to be most likely "dead end" infections and result in inability of the pathogen to be transmitted to conspecific individuals (Solter et al. 1997; Solter and Maddox, 1998a; Solter et al. 2005).

Having observed a number of non-target infections in 2002 and being limited in accessible areas to establish plots, we chose to monitor the two plots where *V. disparis* was sprayed rather than treat the plots again. We recovered no infected non-target lepidopteran larvae in 2003 and 2004. Additionally, a comparison of total numbers of individuals collected and of numbers of individuals per family in the spray sites during the treatment year and the two

years of monitoring showed no overall impact of *V. disparis* on non-target species (Tables 4 and 5).

N. lymantriae was recovered from one individual non-target larva in 2003 but no infections were recovered from non-target species in 2004 and 2005, despite application in 2005 at a concentration 4× the concentration used for the previous two applications. While *V. disparis* causes larval or pupal mortality at dosages over 100 spores and when orally inoculated at any larval stage (Goertz and Hoch, 2008), *N. lymantriae*-infected larvae may survive if dosages are low or larvae are late stage when infection is acquired. This species may be transovarially transmitted to the next *L. dispar* generation by infected adults (Goertz and Hoch, 2008).

In 2003, collections of Geometridae were slightly lower for *N. lymantriae*-treated plots, In 2004, collections for both Geometridae and Noctuidae were lower in the Krupina sites but not in Plaštovce. In 2005, however, collections were not lower in treated plots for either family in either site. As mentioned previously, in 2005 the plots had been treated for three consecutive years and the higher dosage was used for the final treatment.

The results of these studies, particularly when considered with those from our previous laboratory and field research, suggest that *V. disparis* and *N. lymantriae* are quite specific to *L. dispar* and are not a risk to non-target lepidopteran populations. The results of introducing these microsporidia, particularly *V. disparis*, by spraying infective spores, however, indicate that methods other than spraying should be used for inoculative introduction. Possible alternative techniques include introducing contaminated egg masses (Jeffords et al., 1989) or releasing laboratory-reared infected *L. dispar* larvae into field populations. Transmission and persistence in the target host may be the most problematic issue for successful establishment of these microsporidia in North American *L. dispar* populations. In Slovakia, we failed to find infected *L. dispar* larvae during two seasons (2003 and 2004) of intensive monitoring following introduction of *V. disparis* in 2002. The *N. lymantriae* plots were not monitored in the years post-spray because the same plots were treated each year, however, Jeffords et al. (1989) recovered the closely related *N. portugal* from *L. dispar* larvae the year following release in a trial introduction in Maryland. *N. lymantriae*, like *N. portugal*, is transovarially as well as orally transmitted and may have more opportunity to persist.

Trial releases (<10 acre plots) of *V. disparis, N. lymantriae*, and *N. portugal* have been approved by the US Environmental Protection Agency and the US Department of Agriculture, Animal and Plant Health Inspection Service, Plant Protection and Quarantine. The method of release will not include spraying of infective spores. Once release and monitoring are completed and evaluated, it is hoped that the microsporidia will establish and persist in *L. dispar* populations to provide an additional natural control for this serious pest.

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