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Monitoring the establishment and prevalence of the fungal entomopathogen *Entomophaga maimaiga* in two *Lymantria dispar* L. populations in Bulgaria

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Abstract The establishment and prevalence of the entomopathogenic fungus *Entomophaga maimaiga*, (Zygomycetes, Entomophthorales), introduced into two gypsy moth populations in Bulgaria, was monitored during 2000–2004. In the Karlovo Region population, where *E. maimaiga* was introduced in 1999, the fungus was recovered every year and the prevalence of infection varied from 6.1% to 15.9%. A microsporidian infection caused by an *Endoreticulatus* sp. (Protista, Microsporidia) was also recorded every year and the prevalence varied from 2.1% and 5.0%. In the Svoge Region population, where *E. maimaiga* was introduced in late 2000 and first found in 2002, the prevalence of infection varied from 8.8% to 13.8%. Larval parasitism caused by *Cotesia melanoscela*, *Protapanteles liparidis* (Hymenoptera, Braconidae) and species of tachinids (Diptera, Tachinidae) was also recorded in the larvae of both populations. We provide a rationale as to why other countries should consider introducing *E. maimaiga* for biological control of *Lymantria dispar* populations.

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Introduction

The gypsy moth, *Lymantria dispar* (L.) is one of the most important pests of broadleaf forests, not only in Bulgaria, but also in much of Europe, Asia and North America. At present, the bacterial pathogen *Bacillus thuringiensis* subsp. *kurstaki* (*Btk*) is used to manage damaging larval populations, but its use is controversial because it is not highly specific and affects many other species within the order Lepidoptera (Miller 1990). Since the discovery of the entomopathogenic fungus *Entomophaga maimaiga* (Humber, Shimazu and Soper:Zygomycetes, Entomophtorales) in North America (Hajek et al. 1990; Andreadis and Weseloh 1990), there have been over 50 articles published on the biology, genetics, host specificity, and epizootiology of this organism. *Entomophaga maimaiga* has spread rapidly to encompass the host range of *L. dispar* in North America and has caused repeated epizootics among gypsy moth populations. Consequently, there has been keen interest in utilizing this fungus to manage *L. dispar* populations in North America and elsewhere.

Entomophaga maimaiga was originally described from a population of the Japanese gypsy moth (*L. dispar japonensis*) and was reported to periodically cause extensive epizootics in Japan (Koyama 1954). Soper et al. (1988) isolated and characterized *E. maimaiga* as a member of the *E. aulicae* species complex, which is restricted to the Lepidoptera (Humber 1984). Members of this complex are separable according to the size and nuclear numbers of their primary conidia, host specificity, enzyme polymorphism, and biogeographical factors. Soper et al. (1988) tested 14 isolates from within the *E. aulicae* complex and determined that only the Japanese isolate from *L. dispar* was pathogenic to gypsy moth larvae.

Entomophaga maimaiga was imported from Japan and introduced at several locations near Boston, Massachusetts during 1910–1911 (Speare and Colley 1912), however, despite numerous surveys, it was not recovered from gypsy moth populations until 1989 (Hajek et al. 1995a), at which time it caused a pandemic in the northeastern United States (Andreadis and Weseloh 1990; Hajek et al. 1990). A thorough review of what is known about the biology, pathology, and epizootiology of this fungus is provided in Hajek (1999).

Entomophaga maimaiga has two spore forms: conidia which are produced externally on cadavers, and azygospores (resting spores) produced from resting spores or conidia discharged from cadavers (Hajek 1999). Early instar cadavers produce mainly conidia whereas late instar larvae produce mainly resting spores which persist in the environment during adverse periods (Hajek and Shimazu 1996). Like most fungal entomopathogens, infection occurs through the host cuticle. The period of activity on *E. maimaiga* in the spring is closely aligned with the 6–8-week period when *L. dispar* larvae are present and active (Hajek 1999). Conidia are readily distributed by wind which facilitates the rapid spread of the fungus, and has been reported in the literature (Hajek et al. 1996a).

Because of the extensive extant database on the demonstrated efficacy of *E. maimaiga* against the gypsy moth and issues of environmental safety, a proposal was submitted to the Bulgarian Ministry of Environment and Water in March 1999 to introduce and establish this fungus as a permanent, classical biological control agent against the gypsy moth in Bulgarian forests. We present data on the results of our introductions, and on the prevalence of other mortality-causing organisms—a microsporidian pathogen and parasitoids—in the two gypsy moth populations during the period 2000–2004.

Materials and methods

In 1999, this fungus was first introduced into a medium density (660 egg masses per Ha) gypsy moth population in Central Bulgaria near the city of Karlovo (Pilarska et al. 2000). In the spring of 2000, collections (16) of larvae resting under burlap bands were made at approximate 10-day intervals in the Karlovo Forestry Region using the methodology described by Pilarska et al. (2000).

Larvae were reared in the laboratory at 20–25°C on oak foliage and observed daily. The day of larval death was recorded and the cadavers placed at 20°C in a humid chamber (sterile Petri dishes with wet filter paper) for 3–4 days. Subsequently, all cadavers were kept at room temperature for a week to allow formation of resting spores, and then stored at 4°C. Each cadaver was individually dissected and observed under light microscopy for the presence of conidia or resting spores. When microsporidian infections were observed, tissue smears were made, fixed with methanol, and stained with Giemsa (Vavra and Maddox 1976).

The second introduction of *E. maimaiga* was conducted in September 2000 in the village of Gabrovnica, in the Svoge Forestry Region (Sofia Forest Protection Station), 70 km north of Sofia. This site was chosen because it had been monitored for microsporidian infections for several years, and for the persistence of a low-density gypsy moth population at the location. . It was not possible to obtain an accurate estimate of the density of the gypsy moth population because very low numbers of gypsy moth egg masses were present.

Lymantria dispar cadavers containing *E. maimaiga* resting spores were obtained from M. L. McManus, US Department of Agriculture, Forest Service, and were collected in the summer of 2000 from a local population in Hamden, CT, USA. This was one of the locations where *E. maimaiga* was first reported in the US in 1989 (Hajek 1999).

Twenty dried cadavers were crushed and placed within 10 cm of the base of five overstory trees in the release sites; large leaves and stones were removed from around the base of the trees prior to the placement of the cadavers. After the cadavers were distributed, 4 l of water were sprinkled around the base of each tree to improve conditions for the germination of resting spores. The area at the base of the release trees was watered weekly thereafter until the observed gypsy moth larvae were in the fourth stage of development. Procedures for introduction were provided by A. Hajek (Personal communication).

Burlap bands were placed on ten overstory trees (including the release tree) nearest to the center of the release plot. The Burlap bands encircled the tree trunk and were attached 1.4 m above ground. Vertical cuts were made in both layers of each burlap band to facilitate collection of larvae. Collections (14) were initiated when gypsy moth larvae were observed to be in the late third and early fourth stage; larvae were processed as described previously.

Results and discussions

The survival and cause of mortality of all gypsy moth larvae collected in Karlovo and Svoge is presented in Tables 1 and 2. Conidia or resting spores of *E. maimaiga* were detected in larvae from the Karlovo Region every year after its introduction in 1999 (Table 1). The number of *L. dispar* larvae collected during the 5 years varied from 49 to 120 and reflected an overall decline in host density in the region. The prevalence of *E. maimaiga* fluctuated during this period from 6.1% to 15.9%, however the fungus persisted in the host population throughout the period of monitoring (Table 1).

A microsporidian infection caused by an *Endoreticulatus* sp. (Haplophasea, Encephalitozoonidae) was also recorded in *L. dispar* collected each year, however, the prevalence of the microsporidium was very low

Table 1 Survival and mortality of *L. dispar* larvae collected from Karlovo Region

Collection date	Number of larvae collected	Live larvae of *L. dispar*		Dead larvae infected with					
				E. maimaiga		*Endoreticulatus* sp.		Parasitoids	
		n	%	*n*	%	*n*	%	*n*	%
01.06.2000	42	31	73.8	3	7.1	4	9.5	4	9.5
10.06 2000	65	51	78.5	8	12.3	1	1.5	5	7.7
25.06.2000	13	9	69.2	1	7.7	1	7.7	2	15.4
Total 2000	120	91	75.8	12	10.0	6	5.0	11	9.2
30.05.2001	7	1	14.3	2	28.6	1	14.3	3	42.8
08.06.2001	8	5	62.5	0	0	0	0	3	37.5
15.06.2001	31	27	87.1	3	9.7	1	3.2	0	0
25.06.2001	36	28	77.8	8	22.2	0	0	0	0
Total 2001	82	61	74.4	13	15.9	2	2.4	6	7.3
29.05.2002	28	25	89.3	2	7.1	1	3.6	0	0
10.06.2002	21	14	66.7	3	14.3	0	0	4	19.0
20.06.2002	13	5	38.5	1	7.7	1	7.7	6	46.2
Total 2002	62	44	70.9	6	9.7	2	3.2	10	16.1
01.06.2003	25	21	84.0	3	12.0	1	4.0	0	0
15.06.2003	19	17	89.5	2	10.5	0	0	0	0
24.06.2003	5	5	100.0	0	0	0	0	0	0
Total 2003	49	42	85.7	5	10.2	1	2.1	0	0
08.06.2004	34	28	82.3	2	5.9	2	5.9	2	5.9
23.06.2004	18	16	88.9	2	11.1	0	0	0	0
29.06.2004	14	14	100.0	0	0	0	0	0	0
Total 2004	66	58	87.9	4	6.1	2	3.0	2	3.0
Total 2000–2004	379	297	78.3	40	10.6	13	3.4	29	7.7

n number

Table 2 Survival and mortality of *L. dispar* larvae collected from Svoge Region

Collection date	Number of larvae collected	Live larvae of *L. dispar*		Dead larvae infected with			
				E. maimaiga		Parasitoids	
		n	%	*n*	%	*n*	%
02.06.2001	8	5	62.5	0	0	3	37.5
10.06.2001	8	6	75.0	0	0	2	25.0
20.06.2001	7	5	71.4	0	0	2	28.6
29.06.2001	7	7	100.0	0	0	0	0
Total 2001	30	23	76.7	0	0	7	23.3
01.06.2002	6	3	50.0	0	0	3	50.0
08.06.2002	12	8	66.6	2	16.7	2	16.7
18.06.2002	4	1	25.0	1	25.0	2	50.0
01.07.2002	7	6	85.7	1	14.3	0	0
Total 2002	29	18	62.1	4	13.8	7	24.1
03.06.2003	15	13	86.7	0	0	2	13.3
11.06.2003	12	9	75.0	2	16.7	1	8.3
21.06.2003	5	2	40.0	1	20.0	2	40.0
Total 2003	34	28	82.4	3	8.8	3	8.8
7.06.2004	26	21	80.8	3	11.5	2	7.7
20.06.2004	15	14	93.3	1	6.7	0	0
05.07.2004	1	1	100.0	0	0	0	0
Total 2004	42	33	78.6	4	9.5	5	11.9
Total 2000–2004	135	102	75.6	11	8.1	22	16.3

n number

(2.1–5.0%) (Table 1). The morphological characteristics of this microsoporidium are very similar to the *Endoreticulatus* sp. found by Pilarska et al. (1998) in Asenovgrad. The prevalence of infection of *Endoreticulatus* sp. at this site varied from 2.0% to 11.5% over a period of 3 years (1995–1997).

It is possible that the microsporidium we found in Karlovo was *E. schubergi*, described by Zwölfer (1927) as *Pleistophora schubergi* and later renamed by Cali and Garhy (1991). Detailed studies of the host specificity, ultrastructure development, and rDNA sequence analyses are being conducted in order to identify this species.

In the Karlovo Region, the average parasitism of *L. dispar* larvae during the same 5-year period varied from 0% to 16.1% (Table 1). Mortality was caused by *Cotesia melanoscela* (Ratz.) and *Protapanteles liparidis* (Bouché) (Hymenoptera, Braconidae), species that attack early instar larvae, and species of tachinids (Diptera, Tachinidae), that attack later instar larvae.

In Bulgaria, *Cotesia melanoscela* and *P. liparidis* are not considered very important in reducing the population density of *L. dispar* (Gantschew 1990; Georgiev et al. 1998). In some areas of Europe and Asia, *P. liparidis*, which has three generations/year, is one of the major parasitoids of *L. dispar* though its success is dependent on the availability of alternate hosts for overwintering. Species of tachinids and ichneumonids are also of little importance in regulating *L. dispar* populations in Bulgaria (Tsankov et al. 1998; Mirchev et al. 1999).

In the Svoge Forestry Region, *E. maimaiga* was not found in the first year of its introduction, though it was found in subsequent years in low numbers.Table 2). In 2002, a year after introduction, the fungus was detected in 13.8% of larvae collected. In the following two years, the prevalence declined slightly to 8.8% (2003) and 9.5% (2004). The parasitism of *L. dispar* larvae collected at this site was caused by the same species that we recovered from Karlovo, but the incidence was slightly higher (24.1% in 2002, 8.8% in 2003).

One of our concerns after introducing the fungus into the *L. dispar* population at the Karlovo Forestry site was that the *L. dispar* populations were at very low densities –which was not conducive to producing the inoculum (resting spores) required to maintain a minimum level of the fungus in the environment. In fact, Weseloh and Andreadis (1992) reported that the number of resting spores in a site was inversely related to the number of years since the site had last been defoliated by the gypsy moth. However, our results from both sites indicate that *E. maimaiga* has persisted despite the low density of its host, very unusual for most entomopathogens which are usually density dependent. The prevalence rates were quite low and similar to these reported by Smitley et al. (1995).

In several central and south European countries, *L. dispar* is an episodic pest that reaches outbreak levels every 8–10 years (Johnson et al. in press); gypsy moth outbreaks have occurred in many of these countries since 2003 and they currently exist in other countries such as Austria, Slovakia, Hungary, and Croatia. We suggest that other European countries should consider introducing *E. maimaiga* as a classical biological control agent against damaging *L. dispar* populations based on the scientific knowledge available about this entomopathogen and its characteristics: (1) *E. maimaiga* is reasonably host specific—several studies have documented that it has little or no impact on non-target organisms and specifically on forest Lepidoptera (Hajek et al. 1995b, 1996b, 2000a) (2) *E. maimaiga* is easily introduced into gypsy moth populations (Hajek and Roberts 1991) and spreads readily from its point of introduction (Hajek et al. 1996a; Reardon and Hajek 1998); (3) resting spores (azygospores) can persist in forest soils for 10–11 years, thus providing a source of inoculum over time (Hajek et al. 2000b; Weseloh and Andreadis 1997); (4) *E. maimaiga* has been shown to cause high larval mortality even at gypsy moth densities that are below those that normally cause defoliation, thus helping to maintain populations at an endemic phase. This is not typical of most entomopathogens, e.g., viruses and bacteria which are density dependent and exhibit a high prevalence of infection when the density of host populations are very high. Elkinton et al. (1991) concluded that *L. dispar* mortality caused by *E. maimaiga* is correlated with precipitation in May and June and not population density.

Populations of *L. dispar* in Bulgaria have increased since 2004 (Report of National Forestry Management), which should provide better conditions for the establishment and spread of the fungus. In fact, Forestry officials have reported that massive mortality of gypsy moth larvae has occurred at three sites in Bulgaria (located 10 and 70 km from where we conducted our introductions.) Preliminary dissection of larvae from the three sites indicates that the mortality was caused by *E. maimaiga*. Cadavers have been sent to A. Hajek for positive identification of the fungus. Additional information on the cause and scope of this epizootic will be reported at a later date. In the meantime, we will continue to monitor gypsy moth population at release sites in subsequent years in order to assess the overall success of our introduction.

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