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Susceptibility of Larvae of Nun Moth, *Lymantria monacha* (Linnaeus, 1758) (Lepidoptera), to the Entomopathogenic Fungus *Entomophaga maimaiga* Humber, Shimazu and Soper (Entomophthorales) under Laboratory and Field Conditions

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Abstract: Susceptibility of *Lymantria monacha* larvae to *Entomophaga maimaiga* was investigated under laboratory and field conditions, using larvae of the natural host, *Lymantria dispar,* as positive controls. In laboratory bioassays, *L. monacha* and *L. dispar* were injected with protoplasts of two isolates of *E. maimaiga* and mortality was monitored for 20 days. While virtually all injected *L. dispar* died, with ST50s (median survival times for 50% of insects injected with the two isolates) of six – seven days, only 65.6-86.7% of the injected *L. monacha* died, with ST50s of 11-17 days. Both isolates produced conidia and resting spores more frequently within dead *L. dispar* than *L. monacha*. In more ecologically relevant host range assays, larvae of both species were exposed to germinating soil-borne *E. maimaiga* resting spores in the laboratory. More *L. dispar* than *L. monacha* larvae died after these exposures. However, while resting spores were formed within 100% of *L. dispar* larvae that died, significantly fewer (10%) dead *L. monacha* contained resting spores. When *L. monacha* larvae were collected during an *E. maimaiga* epizootic occurring in a sympatric *L. dispar* population, only 0.2 % of the *L. monacha* died and produced spores. These findings corroborate those of previous studies reporting a narrow host range for this fungal pathogen.

Key words: host specificity, physiological host range, ecological host range, *Lymantria monacha, Lymantria dispar*, *Entomophaga maimaiga*

Introduction

Entomophaga maimaiga Humber, Shimazu and Soper (Entomophthorales: Entomophthoraceae) is a fungal pathogen that was introduced to North America and was documented to be established in the invasive European gypsy moth, *Lymantria dispar* (Linnaeus, 1758), as of 1989 (Weseloh 1998, Hajek 1999). *Entomophaga maimaiga* appears to be providing control of the gypsy moth in some areas

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of north-eastern forests and is considered to be the most important natural enemy of this introduced, defoliating pest in the U.S. (Hajek 2007, Fuester *et al*. 2014).

During the period 1999-2001, *E. maimaiga* was introduced to three localities in Bulgaria through inoculum from the U.S. (Pilarska *et al.* 2000), and in 2005 strong epizootics caused by this fungus were reported in four *L. dispar* outbreak populations located 30-70 km from the introduction sites (Pilarska *et al.* 2006). From 2008 to 2011, six more introductions of *E. maimaiga* were performed in outbreak populations of *L. dispar* in Bulgaria where *E. maimaiga* did not yet occur and as a result, all outbreaks of the pest were suppressed (Georgiev *et al.* 2013; Mirchev *et al.* 2013). Today, this fungus is widespread in nearly all regions of Bulgaria in which *L. dispar* occurs (Georgiev *et al.* 2012a).

After the introductions of *E. maimaiga* in Bulgaria, the pathogen has quickly spread on the Balkan Peninsula and South-eastern Europe. In 2005 it was found in Georgia, where it was thought to have spread (Kereselidze *et al.* 2011). In 2011, *E. maimaiga* was found in the European part of Turkey (Georgiev *et al.* 2012b) and in Serbia (Tabaković-Tošić *et al.* 2012). In 2012, the fungus was introduced into a *L. dispar* population on Avala, a mountain overlooking Belgrade, and it was also introduced to or reported from numerous additional Serbian locations (Tabaković-Tošić 2014a, b). In 2012 *E. maimaiga* expanded its range into Greece and FYR Macedonia (Georgieva *et al.* 2013), and in 2013 it was found in Croatia (Hrašovec *et al.* 2013), Hungary (Csóka *et al.* 2014), Slovakia (Zúbrik *et al.* 2014) and Bosnia and Herzegovina (Milotic *et al.* 2015).

Entomophaga maimaiga produces two types of spores, conidia and resting spores, on or in bodies of larvae that have died from infections. Conidia are formed externally on dead early season hosts and are actively ejected, thus spreading infection within the spring larval population. Thick-walled azygospores, or resting spores, are produced internally within dead, late instar larvae. Larval bodies containing resting spores are often located on tree trunks, rocks or vegetation above the soil but the bodies then fall to the soil and decompose, leaving resting spores at or near the soil surface (Hajek *et al.* 1998). Resting spores overwinter in the soil and germinate in the spring to produce germ conidia that infect the new generation of *L. dispar* larvae.

Entomophaga maimaiga only infects larval hosts. Based on previous laboratory bioassays, *E. maimaiga* appeared to be quite specific to hosts in the lepidopteran subfamily that includes the genus *Lymantria* Hübner, 1819, although it was shown to infect a number of other species at low levels (Hajek *et al.* 1995a). In field studies, only one individual of two species in the families Lasiocampidae and Erebidae were found to be infected with *E. maimaiga* (Hajek *et al.* 1996). Because high levels of *E. maimaiga* infection occur in *L. dispar* when late instar larvae spend time in or under leaf litter (Hajek 2001), non-target Lepidoptera specimens from leaf litter were evaluated. Only two non-target larvae of the families Noctuidae and Gelechiidae collected from leaf litter produced *E. maimaiga* spores (Hajek *et al.* 2000). Finally, studies conducted across five years of naturally occurring epizootics in *L. dispar* found relatively lower levels of infection in tussock moths of two genera, *Dasychira* and *Orgyia* (Erebidae: Lymantriinae; Hajek *et al.* 2004). Investigations in forest stands with *E. maimaiga* epizootics in Bulgaria found no alternative hosts among 1,499 non-target individuals belonging to 38 species from ten lepidopteran and one hymenopteran families (Georgieva *et al.* 2014).

The nun moth, *Lymantria monacha* (Linnaeus, 1758), is an outbreak pest in Eurasian conifers that poses an ever-present threat of being accidentally introduced to North America. It has high potential of being transported via commerce because, although eggs are typically oviposited in bark crevices, they are potentially oviposited in crevices of wood that is used for containers, pallets, and ship surfaces, etc. *Lymantria monacha* feeds primarily on *Picea*, *Pinus*, *Abies*, and *Larix* spp. but can also develop when feeding on leaves of deciduous trees and shrubs. European forest managers continue to seek natural control measures for *L. monacha*.

We present results from studies of the susceptibility of *L. monacha* larvae to *E. maimaiga*. We tested *L. monacha* larvae under laboratory conditions using protoplast injections and next via exposure to germinating resting spores. *Lymantria monacha* is not normally known in Bulgaria as a serious pest and has not caused heavy damage to forest stands in the recent past. However, in 2014 high densities of both *L. dispar* and *L. monacha* were observed in the Kirkovo State Forestry region and this change may have been due to a favourable combination of natural beech and oak forests intermixed with conifer plantations. We therefore used this opportunity to also investigate the potential for *L. monacha* to become infected with *E. maimaiga* under field conditions by collecting, rearing and diagnosing larvae during epizootics in several *L. dispar* populations.

Materials and Methods

Laboratory studies

Larval injection with protoplasts of *E. maimaiga*

Lymantria monacha larvae were reared on artificial diet at the USDA Forest Service Quarantine Facility in Ansonia, Connecticut (Keena *et al.* 2010) for ten days post hatch and then transferred to *Quercus velutina* foliage. *Lymantria dispar* larvae were reared on high wheat germ artificial diet (Bell *et al.* 1981). Bioassays were conducted using two isolates of *E. maimaiga* stored in the Agricultural Research Service Collection of Entomopathogenic Fungi (ARSEF) in Ithaca, NY. Isolates were thawed and propagated in 95% Grace's insect medium plus 5% fetal bovine serum (Life Technologies, Grand Island, New York). Early fourth instar larvae of *L. dispar* (New Jersey Standard Strain, 67th generation) and *L. monacha* (Czech Republic Strain, 26th generation) were individually injected with 10 µl of Grace's insect media containing 1 x 10^5 *E. maimaiga* protoplasts/ml (Hajek *et al.* 1995a). Syringes containing concentrations of protoplasts were prepared with separately quantified and adjusted suspensions of protoplasts and 30 larvae were injected per treatment using the same syringe. This bioassay included three replicates with 30 larvae each with *E. maimaiga* isolate ARSEF 6625 (from New York State), two replicates with 30 larvae each with *E. maimaiga* isolate ARSEF 7126 (from Massachusetts), and one replicate of 30 larvae as controls injected only with Grace's insect medium. Numbers of insects that could be included in the bioassays were limited due to availability of *L. monacha* of the suitable instar stage from the quarantine colony of this univoltine European insect. After injection, larvae were placed individually in 59.6 ml clear plastic cups containing artificial diet and held at 20 °C, and were checked daily for 20 days to record mortality. Dead larvae were held at 20 °C for one to three further days, were checked daily to record conidial production and were then moved to 5 ml microcentrifuge tubes and held at 4 °C until further diagnosis was possible. Both conidia and resting spores can be produced from the same dead insect. Unlike conidia that form on the ends of conidiophores that grow externally on cadavers and then degrade fairly quickly, resting spores are formed internally and are persistent. Bodies of dead larvae were dissected and examined microscopically at 200× to record the presence of resting spores.

To analyse survival, median survival times (ST_{50}s) for larvae of the two species exposed to the different isolates were estimated using Kaplan-Meier analyses. Likelihood ratio tests were used to determine significant effects (JMP Version 10; SAS Institute 2012). Percentages of larvae dying and producing different types of spores or no spores were compared using Fisher's exact probability tests with the Bonferroni correction for conducting multiple tests.

Infection experiments with *E. maimaiga* resting spores

The susceptibility of *L. monacha* larvae to *E. maimaiga* was further evaluated at Eberswalde University of Sustainable Development, Germany by conducting infection experiments with the natural host, *L. dispar*, and with *L. monacha*. Egg masses of *L. dispar* were provided by the USDA, APHIS, CPHST at Otis Air Force Base, Buzzards Bay, Massachusetts, U.S. Eggs were hatched and larvae were reared on high wheat germ diet (Bell *et al.* 1981) in 250 ml plastic cups until the fourth instar, at 20 °C, 16 h light: 8 h dark. Early instar *L. monacha* larvae were collected on 15 May, 2013 and on 5 June, 2013, from *Pinus nigra* in forests in Southern Brandenburg, Germany. From these collections a laboratory colony was established, providing *Larix decidua* foliage as food.

Bioassays using early fourth instar *L. dispar* and *L. monacha* larvae were conducted in May and June 2013 following the protocols of Hajek, Wheeler (2004) and Pilarska *et al.* (2013). For the bioassays, sterile soil was mixed well with *E. maimaiga* resting spores from crushed dead larvae collected from tree boles in summer 2012 near Veliko Tarnovo, Northern Bulgaria. The larval cadavers containing the resting spores had been maintained in cotton bags 1-3 cm under the surface of the soil (Hajek *et al.* 2001) in a forested area in Bulgaria over the winter of 2012-2013.

Approximately 20-30 g of the resting spore-inoculated soil were placed in plastic containers (11 cm x 4.5 cm) with ventilated tops and were slightly moistened with distilled water. Ten *L. dispar* or *L. monacha* larvae were added to each of the containers, where they were maintained at 15 °C for three days without food, for a total of ten containers containing *L. dispar* and 37 containers containing *L. monacha*. At 4 d post inoculation, the larvae of both hosts were transferred from containers of soil to food sources (i.e. wheat germ diet for *L. dispar* and *L. decidua*, foliage for *L. monacha*). Larvae were then monitored daily for mortality for ten days. Dead larvae were removed from the treatment cups and placed in humid chambers at 20 °C, where they were monitored daily for seven days in order to detect formation of conidia. The samples were then stored at 4 °C until

dissection. Each dead larva was dissected individually and inspected under 200× to detect conidia or resting spores of *E. maimaiga*.

A total of 370 *L. monacha* and 98 *L. dispar* larvae were exposed to inoculated soil at the end of May. At the same time 100 *L. monacha* larvae used as controls were exposed to sterilised soil moistened with distilled water. In mid-June the experiment was repeated, with 110 *L. monacha* larvae and 50 *L. dispar* larvae.

Field studies

In May and June 2014 an epizootic in *L. dispar* caused by *E. maimaiga* was observed in the region of Kirkovo State Forestry, Rhodope Mountains, Bulgaria, in mixed hardwood/conifer forest stands formed by European beech (*Fagus sylvatica*), sessile oak (*Quercus petraea*), Turkey oak (*Quercus cerris*), Italian oak (*Quercus frainetto*) and Scots pine (*Pinus sylvestris*) (Table 1). *Entomophaga maimaiga* had been introduced to seven sites in this area by releasing bodies of dead, field-collected *L. dispar* larvae containing resting spores on 25 November 2013 and 19 March 2014, with the aim to suppress the outbreak of *L. dispar* in this region and to avoid the use of insecticides. We hypothesised that these introductions were the catalyst for the observed strong epizootic (Georgiev *et al.* 2014).

A total of 718 *L. dispar* larvae were collected from 7 May – 20 June 2014 using double layered burlap bands on *Fagus sylvatica*, over a total of four collections at the seven introduction sites. The larvae were transported to the Forest Research Institute (FRI), Bulgarian Academy of Sciences and reared on leaves of *Quercus robur* at room temperature (18-22 °C). Mortality was recorded daily and dead larvae were evaluated for the presence of resting spores under light microscopy at 100× and 400×. Overall 703 *L. dispar* larvae were evaluated microscopically.

Numerous *L. monacha* larvae were also found in the burlap bands (Fig. 1), although *L. monacha* had previously persisted for years at very low population densities in Bulgaria. This situation offered a unique opportunity to investigate whether *L. monacha* larvae are susceptible to *E. maimaiga* under field conditions. Each time that *L. dispar* larvae were collected, all *L. monacha* larvae that were found were also collected for a total of 1061 *L. monacha* larvae (Table 1). *Lymantria monacha* larvae were transported to the laboratory of FRI and reared on leaves of *Fagus sylvatica* or *Quercus robur* at room temperature to determine time to death and whether *E. maimaiga* spores were present in bodies of dead larvae after death.

Results

Laboratory studies

Protoplast injection bioassays

Lymantria monacha larvae began dying at 4-5 d after injections with both *E. maimaiga* iso-

Table 1. Field sites and numbers of *L. monacha* larvae collected in 2014

Locality (Forestry stand number)	Geographical coordinates	Altitude, m	Tree species	*L. monacha* larvae collected			
				7-8 May	26-27 May	9-10 June	18-19 June
Chakalarovo (209 d)	41° 16' 28.1''N 25° 17' 34.5''E	663	*Fagus sylvatica* *Quercus petraea*	-	6	5	-
Chakalarovo (217 b)	41° 16' 24.0''N 25° 18' 24.9''E	559	*Fagus sylvatica* *Quercus petraea*	-	369	304	22
Dolno Kapinovo (201 a)	41° 16' 04.1''N 25° 16' 36.1''E	618	*Fagus sylvatica* *Quercus petraea* *Pinus sylvestris*	6	36	55	74
Strizhba (392 a)	41° 18' 11.3''N 25° 25' 44.8''E	690	*Fagus sylvatica* *Quercus petraea*	-	2	6	2
Strizhba (387 g)	41° 17' 26.7''N 25° 25' 28.8''E	701	*Fagus sylvatica* *Pinus sylvestris*	1	22	52	16
Strizhba (452 v)	41° 17' 42.4''N 25° 26' 31.6''E	591	*Fagus sylvatica* *Quercus petraea*	-	34	41	-
Tihomir (442 e)	41° 18' 43.4''N 25° 28' 18.2''E	538	*Quercus frainetto* *Fagus sylvatica* *Quercus cerris*	-	4	4	-
Total				**7**	**473**	**467**	**114**

lates. However, the observed mortality was far from synchronous and differences in speed of kill were significantly different for the fungal isolates (chi-square = 13.77, df = 1, P = 0.0002; Fig. 2; ARSEF 6625 ST_{50} = 17 d, 95% CI = 16-20; ARSEF 7126 ST_{50} = 11 d, 95% CI = 9-13). At 20 d post inoculation 65.6% (ARSEF 7216) and 86.7% (ARSEF 6625) of *L. monacha* larvae had died. In contrast, time to death for inoculated *L. dispar* larvae did not differ by isolate (chi-square = 0.339, df = 1, P = 0.5601; Fig. 2; ARSEF 6625 ST_{50} = 6 d, 95% CI = 6-9; ARSEF 7126 ST_{50} = 7 d, 95% CI = 6-8; P = 0.5561) and nearly all *L. dispar* larvae that had been injected with protoplasts died within 20 d (percent mortality: ARSEF 6625, 97.8%; ARSEF 7126, 98.3%). For both fungal isolates, the survival curves for *L. dispar* differed significantly from *L. monacha* (ARSEF 6625: chi-square = 31.85, 1 df, P < 0.0001; ARSEF 7126: chi-square = 7.565, 1 df, P = 0.006). Significantly fewer controls died than for either fungal isolate (Fig. 2).

No conidia or resting spores were produced in circa 20% of *L. monacha* cadavers for each fungal isolate (Fig. 3; P < 0.01). Both fungal isolates produced both resting spores and conidia in individual hosts more frequently in *L. dispar* than in *L. monacha*. Conidia of ARSEF 6625 were produced on more *L. monacha* than *L. dispar* cadavers. ARSEF 7126 differed from ARSEF 6625 in that resting spores were always produced along with conidia from the same individual, regardless of the host species.

Fig. 1. Larvae of *Lymantria monacha* on a burlap band

Laboratory infection experiments with *E. maimaiga* resting spores

More *L. dispar* larvae exposed to resting spore-inoculated soil died (15.3%) compared with mortality of 8.1% for *L. monacha* larvae (chi-square = 4.62; df = 1; P = 0.031). *Entomophaga maimaiga* resting spores were produced within significantly fewer dead *L. monacha* (10.0%) compared with *L. dispar* (100.0%; Fisher's exact test P < 0.0001). In the three *L. monacha* larvae that died and in which spores were formed, only resting spores were found and many were atypically shaped. No infections were detected in any of the *L. dispar* and *L. monacha* larvae from the second trial that was conducted in mid-June.

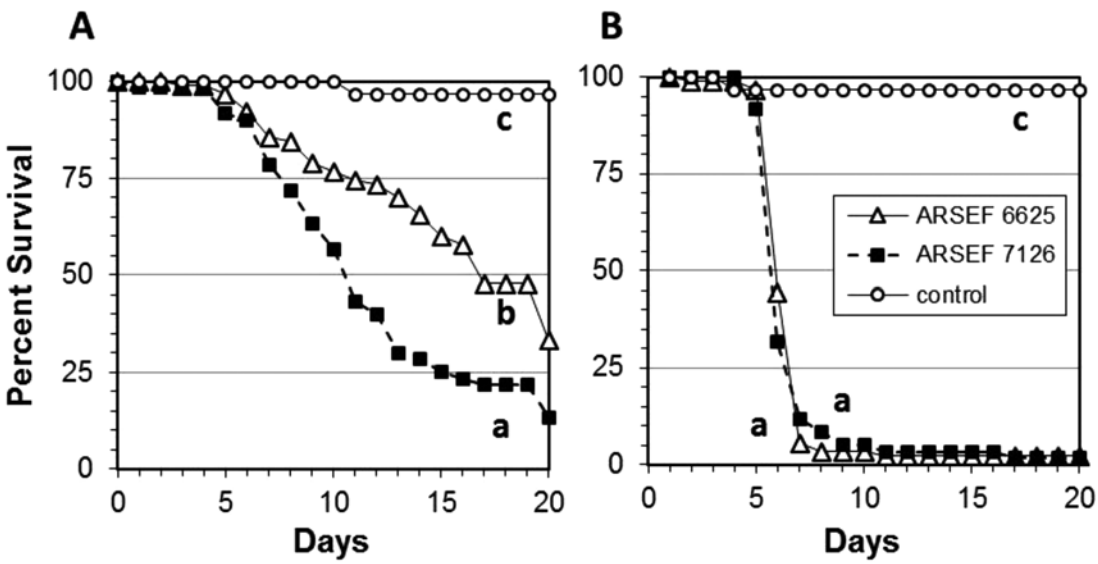

Fig. 2. Percent survival over the 20 d experiment for (A) *L. monacha* and (B) *L. dispar* larvae injected with protoplasts of *E. maimaiga* isolate ARSEF 6625 or ARSEF 7126. Control larvae were injected with Grace's insect medium. Within each species, different letters indicate statistical significance based on likelihood ratios of survival times in the three treatment groups, with Bonferroni corrections

Field studies

The mortality of *L. dispar* at field sites was extremely high, averaging 97.9% for the second-third larval instars and 100.0% for the fourth-sixth larval instars. Conidia were observed on bodies of dead early instar larvae while later instar larvae produced either both conidia and resting spores, or resting spores only.

When rearing *L. monacha* in the laboratory after collection from the sites with *E. maimaiga* epizootics, 86.5 % of the larvae died over a period of three weeks and > 60% mortality occurred three – seven days after collection. Microscopic analysis showed that only two dead larvae (0.2%) contained resting spores that looked like *E. maimaiga*: one larva from Tihomir (site 442 e) collected on 26 May, and one larva from Chakalarovo (site 217 b) collected on 9 June. Both larvae died eight – nine days after collection. Unlike the infections caused by *E. maimaiga* in *L. dispar* under laboratory conditions, the numbers of resting spores in the bodies of the two *L. monacha* larvae were very low, two – three resting spores per microscopic field, while microscopic fields were filled with spores in typical *L. dispar* infections collected at that time. No dead *L. monacha* larvae were observed on trees or burlap bands in the study sites.

The high mortality of *L. dispar* larvae and lack of *L. monacha* mortality in the field may partially

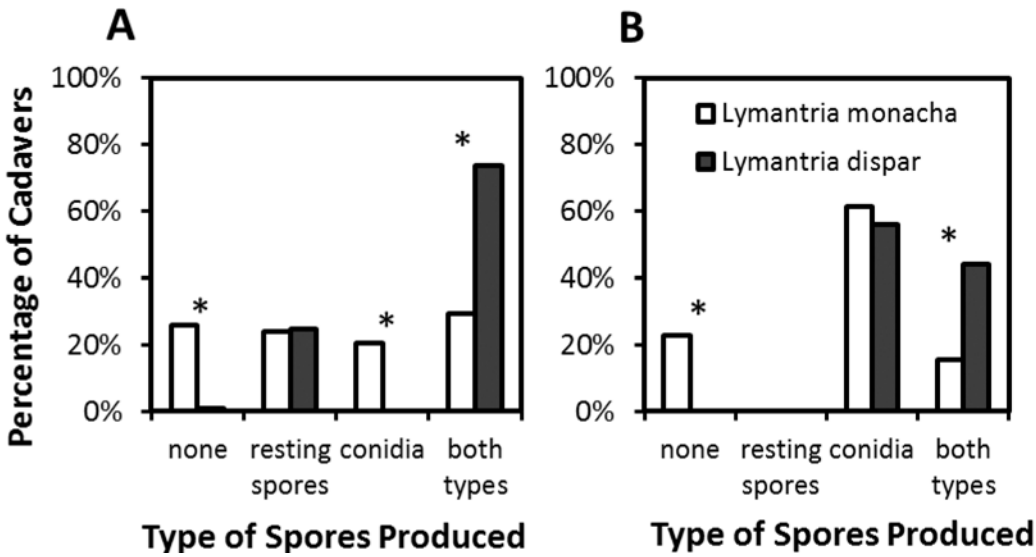

Fig. 3. Percentages of cadavers of *L. monacha* and *L. dispar* larvae that had been injected with protoplasts of either ARSEF 6625 (A) or ARSEF 7126 (B) that produced either conidia only, resting spores only, both spore types (in the same cadaver) or no spores. Asterisks indicate significant differences between the two host species using Fisher's exact probability test with Bonferroni correction

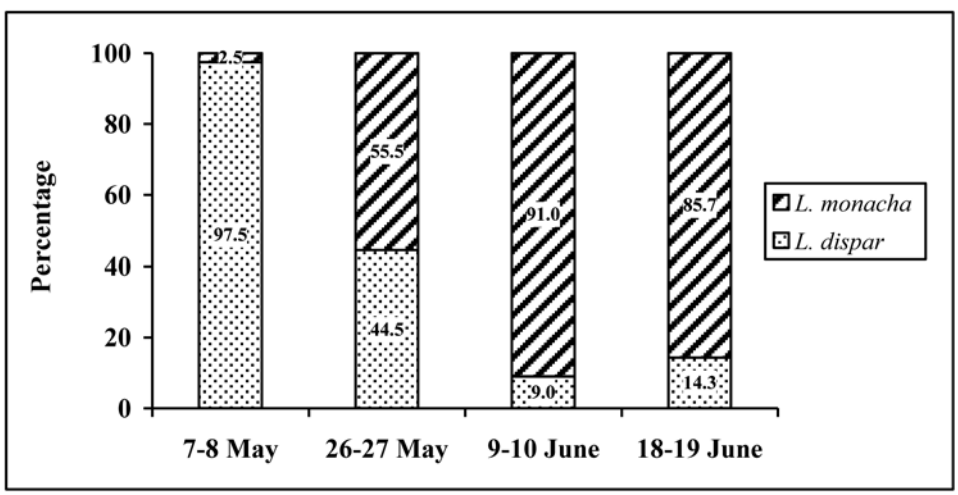

Fig. 4. Percent *L. dispar* and *L. monacha* in the lymantriid larval complex (N=1779) at seven sites in Bulgaria during May and June 2014

explain the numbers and ratio of the two lymantriid species at study sites through the season in which the proportion of *L. dispar* decreased from 97.5% to 9.0% (Fig. 4).

Discussion

Studies on the physiological host range of *E. maimaiga* (Soper *et al.* 1988, Hajek *et al.* 1995, 2000) and ecological host range (Hajek *et al.* 1996, 2000, 2004) have reported that the only group of lepidopteran species with relatively higher susceptibility is the subfamily Lymantriinae, within the family Erebidae. This study presents results from physiological and ecological host range studies on susceptibility of the European pine-defoliating *L. monacha* to *E. maimaiga*. Although *L. monacha* is more closely related to *L. dispar* than any other lymantriines previously evaluated (i.e. *Orgyia* and *Dasychira* species), overall this potential host was not highly susceptible to *E. maimaiga*. Levels of *L. monacha* dying and producing spores were higher in injection bioassays than in field studies. This is typical of studies testing physiological host range in the laboratory and usually involving optimal pathogen challenges as in this study. However, in laboratory bioassays, fewer *L. monacha* died and they died slowlier than *L. dispar* and the fungus produced spores in fewer *L. monacha* than *L. dispar* cadavers. We also conducted intermediate experiments, exposing *L. monacha* larvae to resting spores mixed in soil that resulted in levels of mortality and sporulation that were lower than for *L. dispar* larvae. During field studies, although many *L. dispar* died and produced spores, we found no *L. monacha* cadavers in the field. These results suggest that *L. monacha* is not normally susceptible to *E. maimaiga* even when occurring at the same site as an epizootic in an *L. dispar* population. In addition to native resistance of *L. monacha*, the difference in susceptibility may be behavioural; high levels of infection among *L. dispar* larvae during epizootics are thought to be due, at least in part, to the late instar *L. dispar* larval behaviour of resting in the leaf litter each day (Hajek 2001), where high densities of inoculum can occur (Hajek *et al.* 1998). We are not aware that *L. monacha* exhibits this behaviour, but finding older instar larvae in the burlap bands might be an indication for diurnal vertical migrations for feeding and rest.

Solter *et al.* (1997) suggest that there are three categories for responses of organisms being challenged with a pathogen: heavy (optimal) infections, atypical infections, or the host is refractory. In the physiological host range studies, fewer *L. monacha* than *L. dispar* died when injected with protoplasts and it took longer for *L. monacha* to die. In these bioassays, approximately 20% of *L. monacha* cadavers produced no spores, while in the resting spore exposures this percentage decreased to 10.0% and the infections could be classified as atypical. In these cases, no subsequent horizontal transmission would be expected. Thus, *L. monacha* is not as susceptible to *E. maimaiga* as *L. dispar*. However, when directly exposed to high doses *E. maimaiga* can make spores in *L. monacha* although it seems that sporulation will not occur in at least some of the infected individuals.

In the field studies, only 0.2% of all dead larvae contained resting spores and resting spore loads were low. The cause of the high mortality of *L. monacha* larvae that were field-collected and reared in the laboratory is unknown; in field studies, mass mortality of *L. monacha* larvae occurred approximately three – seven days after larvae were collected and transported to the lab. Microscopic analysis did not indicate presence of other pathogens (e.g. virus, other fungi or microsporidia). Higher mortality in treated compared with control larvae and lack of spore production from cadavers was previously reported for seven out of 78 non-target lepidopteran species challenged with conidia of *E. maimaiga* (Hajek *et al.* 1995a); these instances would be atypical infections as occurred in some *L. monacha* during injection with protoplasts and exposure to germinating resting spores.

In the present field study, no cadavers of *L. monacha* were observed in the field. High mortality of *L. monacha* larvae collected in the field and reared in the lab might be connected with disturbance during transportation or poor nutrition during laboratory rearing. It is known that *L. monacha* larvae reared on beech (*Fagus*) experience severe digestive issues and mortality climbs quickly especially in late instars (Keena 2003). Mortality of nun moth larvae caused by feeding on beech could also occur in the field since no defoliation in beech stands was observed during the field studies. Besides the direct impact of feeding on a suboptimal host plant on *L. monacha* larval development and survival, poor growth of host larvae could have impacted *E. maimaiga* infection. Studies with *L. dispar* have shown that *E. maimaiga* does not sporulate as much or cause mortality as frequently when host larvae were feeding on tree species on which larvae do not develop well (Hajek *et al.* 1995b).

Although *L. dispar* and *L. monacha* are congeneric, results from the protoplast injection bioassays demonstrated that under optimal conditions in the laboratory, when protoplasts of *E. maimaiga* are directly injected into healthy larvae, this fungus is not

as successful in killing *L. monacha* and producing spores as it is for *L. dispar*. The lower levels of sporulation from *L. monacha* were atypical and mortality of *L. monacha* larvae reached only 65.6% (ARSEF 7216) and 86.7% (ARSEF 6625) by 20 d after injection. Perhaps *L. monacha* is able to mount some immune response to *E. maimaiga* that does not occur in *L. dispar*. A main host defence, the cuticular barrier, is overcome when protoplasts are injected. According to Butt *et al.* (1996), *E. maimaiga* protoplasts lack cell walls and have fewer sugars in small amounts on their membranous surfaces, which therefore evoke minimal cellular immune responses by *L. dispar*.

In this study, different isolates of *E. maimaiga* produced different ratios of the two spore types. Variability in the production of conidia and resting spores by different *E. maimaiga* isolates has been documented previously (Hajek, Plymale 2010). However, we also observed that the types of spores produced by a cadaver differed by host species.

No infections occurred in *L. dispar* and *L. monacha* larvae exposed to soil containing resting spores of *E. maimaiga* in mid-June, confirming that resting spores generally germinate earlier in spring (Hajek, Humber 1997). Percentages of mortality and sporulation differed for *L. dispar* and *L. monacha* during resting spore exposure studies but *L. dispar* did not die at high percentages as might be expected. We hypothesise that the densities of resting spores that were germinating in the soil samples at the time of the study were not high enough to yield high levels of infection in this primary host.

In Bulgaria *L. monacha* was found previously on *Pinus*, *Picea*, *Fagus* spp. and other coniferous and deciduous tree species in many regions of the country (Buresch 1915, 1934, Drenovsky 1923, Tschorbadjieff 1925, Buresch, Tuleschkow 1930, Drenski 1940). Unlike Central Europe, the species is not normally known as a serious pest in Bulgaria and has not caused heavy damage to forest stands in recent records. It is difficult to explain the high density population of *L. monacha* observed in the present studies in the region of Kirkovo State Forestry (up to 200 larvae collected per tree). However, it is possible that this is a result of a favourable combination of natural beech and oak forests intermixed with many plantations of *Pinus sylvestris* L., *P. nigra* Arn., *Pseudotsuga menziesii* (Mirb.) Franco, *Picea abies* (L.) Karst. and *Larix decidua* Mill.

Our results clearly shows that *L. monacha* larvae are less susceptible to *E. maimaiga* than *L. dispar* under optimal laboratory conditions, where in around 20% of *L. monacha* larvae that died spores were not produced in cadavers. In contrast, *L. monacha* was rarely infected in the field. These studies corroborate the high host specificity of this fungal pathogen and low risk to entomofauna in forest ecosystems.

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