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Virulence and fitness of the fungal pathogen *Entomophaga maimaiga* in its host *Lymantria dispar*, for pathogen and host strains originating from Asia, Europe, and North America

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Abstract

In this study, we tested (1) whether non-North American gypsy moth strains are susceptible to North American isolates of *Entomophaga maimaiga* and (2) the potential for erosion in the efficacy of *E. maimaiga* in controlling gypsy moth. We used bioassays to assess the variability in virulence (measured as time to death) as well as fitness of the pathogen (measured as spore production) in four gypsy strains challenged with six *E. maimaiga* isolates, using host and pathogen strains originating from Asia, Europe, and North America. We found that all *E. maimaiga* isolates tested were pathogenic to all strains of *Lymantria dispar*, regardless of the geographical origin of the fungal isolate, with at least 86% mortality for all combinations of fungal isolate and gypsy moth strain. We therefore conclude that Asian gypsy moths are susceptible to North American strains of *E. maimaiga*. No significant interactions between fungal isolates and gypsy moth strains with regard to time to death were found, indicating that each fungal isolate had the same overall effect on all the gypsy moth strains tested. However, fungal isolates differed significantly with regard to virulence, with a Russian isolate being the slowest to kill gypsy moth (5.1 ± 0.1 days) and a Japanese isolate being the overall fastest to kill its host (4.0 ± 0.1 days). Fungal isolates also differed in fitness, with variability in types of spores produced. These differences in virulence and fitness were, however, not correlated with geographical origin of the fungal isolate. Gypsy moth strains had no or only little effect on fungal virulence and fitness. Based on our studies with laboratory-reared gypsy moth strains, erosion of successful control of gypsy moth by *E. maimaiga* seems unlikely.

Keywords: *Lymantria dispar*; *Entomophaga maimaiga*; Bioassays; Co-evolution; Virulence; Fitness

1. Introduction

The potential for evolution of hosts or natural enemies that would lead to decreased pest control has received relatively little attention in the literature. To assess the possibility of erosion in the efficacy of a biological control agent, the fitnesses of both host and natural enemy need to be determined in relation to each other. Such a study was recently conducted to prove that control of the chestnut blight fungus, *Cryphonectria parasitica*, by its hypovirus is evolutionarily stable (Peever et al., 2000). On the other hand, there are also examples, albeit few, documenting the decreasing effectiveness of natural enemies in the field after applications of microbes (Briese, 1986; Holt and Hochberg, 1997). The classic example of decreased effectiveness of a microbe over time is the example of myxomatosis in rabbits, where the virulence of the myxoma virus released in Australia decreased through time and the resistance of rabbits increased, presumably due to strong natural selection (Fenner and Fantini, 1999). However, some

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surveys have suggested that control is greater for non-coevolved associations than co-evolved systems (Hokkanen and Pimentel, 1984, 1989), although this has been disputed (e.g., Goeden and Kok, 1986). Nevertheless, the efficacy of most biological control agents in populations of target pests has remained relatively stable despite widespread release over many years (Henter and Via, 1995; Holt and Hochberg, 1997). This stability of biological control agents contrasts remarkably with chemical pesticides, where numerous target pests have developed a resistance to a wide range of pesticides (Denholm et al., 1999; Georghiou, 1986). Holt and Hochberg (1997) summarized some of the hypotheses that have been proposed to explain why biological control agents can be effective yet are evolutionarily stable. These hypotheses include: (1) genetic constraints on selection arising from a negative genetic correlation among fitness components (high cost of resistance), (2) weak selection (e.g., spatial structuring can lead to situations where strong limitation is decoupled from strong selection), (3) temporally varying selection pressure, especially when control involves frequency-dependent selection (Red Queen dynamic), and (4) balanced co-evolution dynamics. The paucity of experimental data addressing these questions has precluded general predictions about the stability of biological control systems (Holt et al., 1999); this lack of data is particularly evident in biological control systems involving insect pathogens.

In insect pathology to date, strong examples of erosion in efficacy nearly exclusively involve viruses, bacteria or protozoa (Briese, 1986). However, for insect pathogenic fungi, clones of the pea aphid, *Acyrthosiphon pisum*, have shown variability in resistance to the entomophthoralean fungi *Pandora neoaphidis*, *P. kondoiensis*, and *Conidiobolus obscurus*, with some aphids being totally resistant to fungal infections (Ferrari et al., 2001; Hughes and Bryce, 1984; Hural, 1998; Milner, 1982; Papierok and Wilding, 1979). Such findings were used by Briese (1986) as a basis for the suggestion that there is a "trend in fungi towards development of non-susceptibility in hosts." This also agrees with the finding that longer-established populations of *Melolontha melolontha* in Switzerland were less susceptible to infection by the hyphomycete *Beauveria brongniartii* (lower infection rate and prolonged lethal time) than a more recently established host population in Italy (Keller et al., 1999). Interestingly, Boucias et al. (2000) demonstrated by AFLP analysis that haplotype populations of the *Nomuraea rileyi*, a fungus frequently causing epizootics in populations of the lepidopteran *Anticarsia gemmatalis*, were stable over their 5-year study period.

A unique opportunity to explore the potential for changes in the efficacy of a biological control interaction is provided by the *Lymantria dispar*/*Entomophaga maimaiga* system. Gypsy moth, *L. dispar*, is native in temperate Asia and across Europe. This pest was introduced to North America from Europe in 1868 or 1869 (Forbush and Fernald, 1896) and has become the most important defoliator of broad-leaved trees in eastern North America. In recent years, accidental introductions of the Asian gypsy moth into North America have also been documented (Wallner, 1996) but so far eradication programs, costing in excess of $30 million dollars, seem to have been successful in preventing establishment of this strain in North America. However, there is a reasonable fear that this new pest strain will eventually establish in North America. Gypsy moth is only occasionally a problem in the areas where it is native (Europe and temperate Asia) and damage from outbreaks in these areas never approaches the level of damage experienced in the US.

Entomophaga maimaiga is a major natural enemy in endemic Asian gypsy moth populations where spectacular epizootics have been reported (Koyama, 1954; Pemberton et al., 1993; Takamura and Sato, 1973a,b; Yanbe, 1976; Yurchenko et al., 2000; Shimazu, pers. comm.), yielding mortalities of up to 99% (Aoki, 1974). Curiously, there are very few records of entomophthoralean fungi attacking the gypsy moth populations in Europe, although Glowacka-Pilot (1982) described an epizootic in Poland. *E. maimaiga* was first seen in northeastern North America in 1989, when it caused widespread epizootics (Andreadis and Weseloh, 1990; Hajek et al., 1990) and from 1989 to 1992 it spread across the contiguous northeastern distribution of gypsy moth (Hajek et al., 1995b, 1996b, 1999). Epizootics caused by *E. maimaiga* in gypsy moth populations have been observed every year in the US since 1989 and *E. maimaiga* is probably the most effective mortality agent at present leading to suppression of outbreak gypsy moth populations.

The overall goal of this research was to investigate the evolutionary stability of biological control of gypsy moth with its fungal pathogen *E. maimaiga*. Specifically, we are interested in addressing two questions (1) Are non-North American gypsy moth strains susceptible to North American strains of *E. maimaiga*? and (2) What is the potential for erosion in the efficacy of *E. maimaiga* in controlling gypsy moth in North America?

We used bioassays to assess the variability in virulence (measured as time to death) as well as fitness of the pathogen (measured as fungal reproduction) in four gypsy strains challenged with six *E. maimaiga* isolates, using host and pathogen originating from Asia, Europe, and North America. Cross-inoculations with host and pathogen were accomplished by injecting fungal protoplasts into gypsy moth larvae or by subjecting larvae to conidial showers. This protocol allows comparison of variability in susceptibility to infection during the cuticular penetration process with effect of fungal infection at a later stage after penetration.

2. Materials and methods

2.1. Experimental insect

Four laboratory strains of *L. dispar* were tested in the present study (Table 1), including strains from (i) New Jersey, USA, (ii) Aichi Prefecture, Honshu, Japan, (iii) Primorski Krai, Far Eastern Russia, and (iv) Eastern Macedonia, Greece. The strain from Japan was of the *japonica* subspecies of *L. dispar* while the other strains were all of the *dispar* subspecies. Numbers of generations the colonies have been reared in the laboratory differed among strains, as did the ability of adult females to fly (Table 1), whereas the weights of early fourth-instar larvae were approximately the same for all four strains, ranging from 160 mg for the Greek strain to 180 mg for the Japanese strain (M.K., unpubl. data).

One hundred egg masses from each strain were crumbled and mixed together to provide a pool of eggs for use in these studies and were therefore subsequently randomized among treatments. Larvae that hatched from the pool of eggs were reared to either third or fourth instars, depending on the experiment, at 25 °C, 60% RH, and 16:8 L:D photoperiod in groups of 15 in 236.6-ml plastic cups closed with waxed lids containing approximately 80 ml of artificial diet. The high wheat germ artificial diet (Bell et al., 1981) was optimized for the individual strains using Wesson salt mix without iron and adding 0.27 g (Japanese and Russian strains) or 0.10 g (Greek and North American strains) amorphous $FePO_4$ per liter of diet. Voucher specimens for each strain were deposited at the Entomology Division, Yale Peabody Museum of Natural History, New Haven, CT.

Due to quarantine restrictions when working with non-native gypsy moth strains in the US and the availability of egg masses only once every 9 months, the numbers of larvae used in each bioassay and the number of bioassays had to be limited. Based on earlier experience demonstrating minimal variability in injection bioassays in our laboratory, only pseudo-replicates were carried out for this inoculation method.

2.2. Fungal isolates

The six fungal isolates tested in laboratory bioassays are listed in Table 2. Isolates included two from the US, two from Japan, one from Far Eastern Russia, and one from China. All fungal isolates were isolated in vitro from hemolymph of infected gypsy moth larvae as described by Papierok and Hajek (1997). Infected larvae were obtained by exposing larvae (New Jersey strain) in the laboratory to soil containing germinating resting spores, as described by Hajek et al. (2000). All fungal isolates were grown in liquid cell culture media (Grace's Insect Tissue Culture Medium, Cellgro, Herndorn, VA supplemented with 5% fetal bovine serum, Gibco-BRL, Grand Island, NY). The isolates

Table 1
List of *L. dispar* strains included in this study

Country of origin	Nearest city and state/prefecture/region	Location[a]	Year of collection	Laboratory generation	Female flight
Japan	Nagoya, Aichi (Honshu)	35.15N 128.50E	1996	13	Yes
Russia	Mineralni, Primorski Krai	44.10N 133.15E	1993	16	Yes
Greece	Kavala, Macedonia	41.00N 24.25E	1997	10	No
USA	Blairstown, New Jersey	40.59N 74.58W	1967	55	No

[a] Approximate latitude and longitude of collection sites.

Table 2
List of *E. maimaiga* isolates included in this study

Cornell No.[a]	ARSEF No.	Country of origin	State/prefecture/province	Year of collection
03MA3-1-1	7123	USA	Massachusetts	2003
03PA1-1-5	7190	USA	Pennsylvania	2003
01JP4-11-1	7104	Japan	Iwate	2001
03JP5-1-2	7186	Japan	Hiroshima	2003
99RU1-1-1	7127	Russia	Primorski Krai	1999
02CN1-1-1	7139	China	Heilongjiang	2002

[a] In the Cornell identification number, the first digits give the year for collection of soil samples containing resting spores or collection of a cadaver in the field; the next two letters indicate either state (USA) or country of origin; the next digits indicate the site number, followed by the soil sample number/plot number or tree number from which soil was collected, and the last number indicates the isolate number in cases in which more than one isolate was isolated from a field.

are stored at Cornell University (Ithaca, NY, USA) and the Agricultural Research Service Collection of Entomopathogenic Fungal Cultures (ARSEF, Ithaca, NY, USA).

2.3. Injection bioassay procedure

The protocol for protoplast injection generally followed the techniques described by Soper et al. (1988). For all bioassays, the fungal concentration was adjusted to 1×10^5 cells/ml with Grace's Insect Tissue Culture Medium. Ten microliters of this suspension was injected intrahemocoelically into a third-instar larva at the base of a proleg using a manual microinjector fitted with a 3-ml syringe and a 23-gauge needle. For each bioassay, 30 randomly assigned larvae were injected for each combination of the four gypsy moth strains and six fungal isolates (in total, 24 treatments). Controls consisted of 30 larvae of each strain injected only with 10 μl Grace's Insect Culture Tissue Medium. After injection, larvae were incubated individually in 29-ml plastic cups containing artificial diet optimized for the individual strains, and incubated at 20 °C with a 16:8 h L:D photoperiod. All fungal injections were done on the same day but control larvae were injected the following day.

2.4. Showering bioassay procedure

These studies were conducted basically using the techniques developed by Vandenberg and Soper (1979), improved by Filotas et al. (2003) and adapted to the *L. dispar*/*E. maimaiga* system. Newly molted fourth-instar larvae of each of the four gypsy moth strains were exposed to conidial showers from sporulating cadavers produced by injection of fourth-instar larvae of the New Jersey strain following the procedure described in Section 2.3. Larvae used for conidial production were injected over five successive days in order to ensure that enough fresh sporulating cadavers were available for the showering experiments at the right time. Exposure of larvae that had molted to the fourth-instar within the previous 1–2 days took place in an enclosed 11.3 liter polyethylene storage box (Rubbermaid, Wooster, Ohio) in which a platform (195 mm) attached to a 0.5-rpm gear motor was mounted. Between 10 and 35 fresh sporulating cadavers were placed on 0.5 cm mesh nylon netting, covering 1.2 cm mesh hardware cloth, 10 cm above the rotating platform. Conidia were discharged onto 10 healthy larvae placed on the rotating platform individually within 29-ml cups closed with 0.5 cm mesh nylon netting to prevent escape of the larvae but still allow conidial inoculation. High humidity levels were maintained using moistened paper towels and pieces of 1.5% water-agar. Dose was quantified by counting the conidial density (per square millimeter) in three fields on the bottom of three exposed cups in each bioassay. Doses ranging between 15 and 130 conidia per mm^2 were attempted, by varying the exposure time. Based on experience from preliminary dose experiment with the New Jersey gypsy moth strain, we knew that this range would not affect either percent infection, time to death or the percentages of cadaver supporting conidia, resting spores or both spore types (C.N., unpubl. data). After showering, larvae were incubated individually in plastic cups (29 ml) containing artificial diet optimized for the individual strains, and incubated at 20 °C with a 16:8 h L:D photoperiod. Controls consisted of 10 non-exposed larvae of each gypsy moth strain. Each bioassay was repeated three times over three successive days for each combination of the four gypsy moth strains and six fungal isolates. However, the fungal isolate 99RU1-1-1 was never exposed to the Greek gypsy moth strain and only two replicates were performed for this isolate against the Russian gypsy moth strain due to poor sporulation by this isolate. Each day new sporulating cadavers were used in the bioassay.

2.5. Data recording

Mortality was checked daily for 9 days and time to death was recorded. All larvae dying during this period were examined daily for 3 days after death under a dissecting microscope and conidial production was recorded. After approximately 14 days, cadavers were dissected and smeared on glass microscope slides and examined under a compound microscope at 200× magnification to record the presence of resting spores and fungal hyphae. Each cadaver was scored as one of the following four categories: (i) production of conidia, (ii) production of resting spores, (iii) production of both resting spore and conidia, and (iv) no spore production.

2.6. Data analysis

The effects of isolate and gypsy moth strain on time to death and, for the showering experiment, effect of the logarithm of dose on time to death were analyzed by multiple regression (PROC GLM; SAS Institute, 1999). Survivors were omitted from the analysis and fungal isolate and gypsy moth strain were used as class variables. Injection bioassays and showering bioassays were analyzed separately, since different larval instars and inoculation procedures were used in the two assays. The full model, including all parameters and possible interactions, was reduced stepwise by excluding the least significant parameter for each step and always by excluding interactions before main factors (Jensen and Skovgaard, 1995).

The effect of the bioassay method, isolate, gypsy moth strain and log dose for the showering experiment and the percentage of gypsy moth larvae bearing conidia, resting spores, both resting spores and conidia or no spore

production after larval death was analyzed by logistic regression (Proc GENMOD; SAS Institute, 1999) with a binomial distribution and logit as link function (Jensen and Skovgaard, 1995). Bioassay method, isolate and gypsy moth strain were class variables. Overdispersion was taken into account (Collet, 1991). The full model was reduced stepwise by excluding the least significant parameter for each step (Jensen and Skovgaard, 1995).

3. Results

3.1. Virulence in injection bioassay

All isolates were pathogenic to the target, with percent mortality varying between 93.3% (28 out of 30 injected) and 100% for all combinations of fungal isolate and gypsy moth strain except for the Japanese gypsy moth strain injected with the Japanese isolate 01JP4-11-1 where only 26 out of the 30 injected larvae died over the 9 days following injection. None of the gypsy moth larvae injected with only Grace's Insect Tissue Culture Media (=controls) died in any of the bioassays. The days to death for all combinations of fungal isolates and gypsy moth strains are given in Table 3. Among the isolates tested, days to death ranged from 3.9 ± 0.1 (means $\pm$ SE) days for the Greek gypsy moth strain injected with the Japanese isolate 03JP5-1-2 to 5.3 ± 0.1 days for the US gypsy moth strain injected with the Russian isolate 99RU1-1-1. In most bioassays, all gypsy moth larvae died within a 2-day interval but occasionally they died over 3–4 days and, in some cases, all larvae died within 1 day. The interaction between gypsy moth strain and fungal isolate for time to death was not significant ($F_{15,\ 681} = 1.65$; $P = 0.0563$), indicating that the fungal isolates had the same overall effect on gypsy moth strain in terms of time to kill the host. The time to death was not significantly affected by gypsy moth strain ($F_{3,\ 696} = 2.02$; $P = 0.1099$) (Fig. 1). The isolate significantly affected the time to death ($F_{5,\ 699} = 143.64$; $P < 0.0001$) (Table 3), with the Russian isolate 99RU1-1-1 (5.1 ± 0.1 days) being the overall slowest isolate to kill gypsy moth and the Japanese isolate 03JP5-1-2 (4.0 ± 0.1 days) being the overall fastest to kill its host. However,

Table 3
Mortality and days to death for Russian, Greek, Japanese, and US laboratory strains of gypsy moth injected with protoplasts of fungal isolates from Russia, China, Japan, and the US

Isolate	GM strain	N	% mortality	Days to death	
Control	Russia	30	0.0	—	
	Greece	30	0.0	—	
	Japan	30	0.0	—	
	USA	30	0.0	—	
03MA3-1-1 (ARSEF 7123)	Russia	30	100	4.9 ± 0.1	c
	Greece	30	100	4.7 ± 0.1	
	Japan	30	100	4.9 ± 0.1	
	USA	30	100	5.0 ± 0.1	
03PA1-1-5 (ARSEF 7190)	Russia	30	96.7	4.5 ± 0.1	b
	Greece	30	93.3	4.5 ± 0.1	
	Japan	30	100	4.1 ± 0.1	
	USA	30	96.7	4.4 ± 0.1	
01JP4-11-1 (ARSEF 7104)	Russia	30	93.3	4.9 ± 0.1	d
	Greece	30	93.3	5.0 ± 0.1	
	Japan	30	86.7	5.1 ± 0.1	
	USA	30	90.0	5.0 ± 0.1	
03JP5-1-2 (ARSEF 7186)	Russia	30	96.7	4.0 ± 0.1	a
	Greece	30	100	3.9 ± 0.1	
	Japan	30	100	4.0 ± 0.0	
	USA	30	100	4.0 ± 0.0	
02CN1-1-1 (ARSEF 7139)	Russia	30	100	5.0 ± 0.1	d
	Greece	30	96.7	4.9 ± 0.1	
	Japan	30	100	5.1 ± 0.1	
	USA	30	96.7	5.0 ± 0.1	
99RU1-1-1 (ARSEF 7127)	Russia	30	100	5.0 ± 0.1	e
	Greece	30	100	5.1 ± 0.1	
	Japan	30	100	5.0 ± 0.1	
	USA	30	100	5.3 ± 0.1	

Different letters indicate statistical differences in days to death between fungal isolate at a 5% significance level.

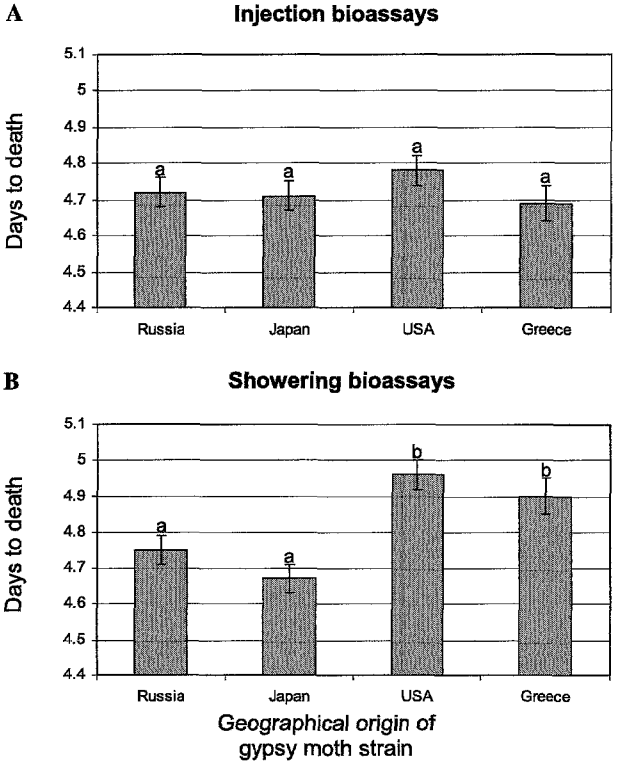

Fig. 1. Days to death (means $\pm$ SE) as a function of gypsy moth strain after inoculation with *E. maimaiga*. Data for six fungal isolates are combined in the figure. Different letters indicate statistical differences in days to death among gypsy moth strains at a 5% significance level. (A) Injection bioassays. (B) Conidial showering bioassays.

the time it took an isolate to kill a host was not consistent among isolates from the same continent (Table 3).

3.2. Virulence in showering bioassay

All isolates tested were pathogenic to the target, giving rise to 100% mortality in all bioassays and none of the control larvae died in any of the showering bioassays. The dose varied between 9 and 163 conidia per mm^2 among bioassays but did not affect the time to death ($F_{1,\ 660} = 1.68$; $P = 0.1948$). Therefore, the three replicates were combined in all additional analyses. The days to death for all combinations of fungal isolates and gypsy moth strains are given in Table 4. Among the isolates tested, days to death ranged from 4.0 ± 0.1 days for the Russian gypsy moth strain subjected to conidial showers of the Japanese isolate 03JP5-1-2 to 5.8 ± 0.1 days for the American gypsy moth strain subjected to conidial showers of the Russian isolate 99RU1-1-1. As for the injection bioassays, most larvae died within a 2-day interval, but occasionally over 3–4 days and in some cases all larvae died within 1 day. The interaction between gypsy moth and fungal isolate for time to death was not significant ($F_{14,\ 646} = 0.58$; $P = 0.8794$). Isolate significantly affected the time to death ($F_{5,\ 661} = 80.47$; $P < 0.0001$) (Table 4), with the Russian isolate 99RU1-1-1 being the overall slowest isolate to kill gypsy moth (5.6 ± 0.1 days) and the Japanese isolate 03JP5-1-2 being the overall fastest to kill its host (4.1 ± 0.1 days), just as in the injection bioassays. No consistency among isolates from the same continent in their time to kill their host. However, in contrast to the injection bioassay, gypsy moth strain significantly affected the time to death ($F_{3,\ 661} = 11.24$; $P < 0.0001$). Pairwise comparisons using least squares means to test effects of gypsy moth strain ($\Pr > |t|$ for H_0: LSMean(strain i) = LSMean(strain j)) showed that the Greek and American gypsy moth

Table 5
Results from least squares means test the effect of gypsy moth strain on mean survival time ($\Pr > |t|$ for H_0: LSMean(strain i) = LSMean(strain j)) after conidial showers with *E. maimaiga*

Origin of gypsy moth strain	Japan	Russia	USA
Greece	0.0001	0.0142	0.3402
Japan	—	0.1314	0.0001
Russia		—	0.0003

Table 4
Mortality and days to death for Russian, Greek, Japanese, and US laboratory strains of gypsy moth subjected to conidial shower of fungal isolates from Russia, China, Japan, and the US

Isolate	GM strain	*N*	Range of doses (conidia/mm^2)	% mortality	Days to death	
Control	Russia	30	—	0.0	—	
	Greece	30	—	0.0	—	
	Japan	30	—	0.0	—	
	USA	30	—	0.0	—	
03MA3-1-1 (ARSEF 7123)	Russia	30	[40–125]	100	4.4 ± 0.1	b
	Greece	30	[56–93]	100	4.7 ± 0.1	
	Japan	30	[36–53]	100	4.5 ± 0.1	
	USA	30	[81–104]	100	4.8 ± 0.1	
03PA1-1-5 (ARSEF 7190)	Russia	30	[51–69]	100	4.8 ± 0.1	c
	Greece	30	[92–115]	100	4.8 ± 0.2	
	Japan	30	[33–61]	100	4.6 ± 0.1	
	USA	30	[61–87]	100	4.8 ± 0.1	
01JP4-11-1 (ARSEF 7104)	Russia	30	[19–34]	100	4.8 ± 0.1	d
	Greece	30	[18–36]	100	5.0 ± 0.1	
	Japan	30	[16–38]	100	4.7 ± 0.1	
	USA	30	[15–32]	100	5.1 ± 0.1	
03JP5-1-2 (ARSEF 7186)	Russia	30	[48–130]	100	4.0 ± 0.1	a
	Greece	30	[54–162]	100	4.2 ± 0.1	
	Japan	30	[13–55]	100	4.0 ± 0.1	
	USA	30	[45–97]	100	4.4 ± 0.1	
02CN1-1-1 (ARSEF 7139)	Russia	30	[42–56]	100	4.9 ± 0.1	d
	Greece	30	[35–63]	100	5.1 ± 0.1	
	Japan	30	[16–40]	100	4.8 ± 0.1	
	USA	30	[35–64]	100	5.0 ± 0.1	
99RU1-1-1 (ARSEF 7127)	Russia	20	[9–44]	100	5.6 ± 0.2	e
	Greece	—	—	—	—	
	Japan	30	[17–28]	100	5.4 ± 0.1	
	USA	30	[17–40]	100	5.8 ± 0.1	

Different letters indicate statistical differences in days to death between fungal isolate at a 5% significance level.

strains (European biotype) took longer to die compared to the Japanese and Russian gypsy moth strains (Asian biotype) but with no differences within each gypsy moth biotype (Table 5; Fig. 1).

3.3. Fitness of *E. maimaiga*

Initially, the dose used in the showering experiment was tested for significant effects in fungal reproduction resulting from infection (conidia, resting spores or both), or the number of larvae dying with no spore production. The tests showed that dose did not affect any of the categories ($P \geqslant 0.0556$) and neither did inoculation method ($P \geqslant 0.3444$). Therefore, but also due to biological rationale, data for the two inoculation methods were combined in all further analyses. Numbers of cadavers with the different symptoms of infection (conidia, resting spores or both) as well as the number of larvae dying with no spore production varied significantly among isolates for all response categories ($P < 0.0001$). These differences were, however, not correlated with geographical origin of the fungal isolate. Thus, larvae subjected to the isolate from Pennsylvania supported mostly conidial cadavers whereas larvae subjected to the fungal isolate from Massachusetts supported mostly cadavers with both spore types. The most notable differences among isolates concerning production of conidia, resting spores or both spore types, were between the Japanese isolate 03JP5-1-2 and the Russian isolate 99RU1-1-1 (Table 6). The only sign of infection observed for 03JP5-1-2 was production of conidia whereas all but one cadaver infected with the Russian isolate produced resting spores (either as the only spore form or in combination with conidia).

The effect of host strain on fungal reproduction was less clear. Gypsy moth strain did not affect the percentage of cadavers supporting conidial infection ($P = 0.1853$). However, χ^2 tests showed an effect of gypsy moth strain on percentage of cadavers supporting resting spore production ($P = 0.0119$), production of a combination of conidia and resting spores ($P = 0.0098$), and no spore production after larval death ($P = 0.0242$). Nevertheless, if the

Table 6
Percentage of larvae supporting production of conidia, resting spores (RS), both conidia and resting spores or no spores for Russian, Greek, Japanese, and US laboratory strains of gypsy moth challenged with fungal isolates from Russia, China, Japan, and the US (data from injection bioassays and showering bioassays combined)

Isolate	GM strain	*N*	% conidia		% RS		% conidia and RS		% no spore production	
Control	Russia	60	—		—		—		—	
	Greece	60	—		—		—		—	
	Japan	60	—		—		—		—	
	USA	60	—		—		—		—	
03MA3-1-1 (ARSEF 7123)	Russia	60	28.3	b	8.3	b	63.3	ab	0.0	a
	Greece	60	28.3		5.0		65.0		1.7	
	Japan	60	20.0		1.7		78.3		0.0	
	USA	60	41.7		0.0		56.7		1.7	
03PA1-1-5 (ARSEF 7190)	Russia	60	96.7	c	0.0	a	0.0	a	1.7	b
	Greece	60	65.0		0.0		0.0		31.7	
	Japan	60	80.0		0.0		0.0		20.0	
	USA	60	71.7		0.0		0.0		26.7	
01JP4-11-1 (ARSEF 7104)	Russia	60	93.3	d	0.0	a	0.0	a	3.3	a
	Greece	60	78.3		0.0		0.0		1.7	
	Japan	60	90.0		0.0		0.0		3.3	
	USA	60	88.3		0.0		0.0		6.7	
03JP5-1-2 (ARSEF 7186)	Russia	60	98.3	d	0.0	a	0.0	a	0.0	a
	Greece	60	98.3		0.0		0.0		1.7	
	Japan	60	100		0.0		0.0		0.0	
	USA	60	100		0.0		0.0		0.0	
02CN1-1-1 (ARSEF 7139)	Russia	60	8.3	a	11.7	b	78.3	bc	1.7	a
	Greece	60	0.0		6.7		90.0		1.7	
	Japan	60	6.7		0.0		93.3		0.0	
	USA	60	1.7		25.0		70.0		1.7	
99RU1-1-1 (ARSEF 7127)	Russia	50	0.0	a	60.0	c	38.0	ab	2.0	a
	Greece	30	0.0		20.0		76.7		3.3	
	Japan	60	0.0		36.7		63.3		0.0	
	USA	60	1.7		45.0		51.7		1.7	

In each column different letters indicate statistical differences of least squares means (pairwise χ^2 test) in the percentage of larvae supporting the respective category, at 5% significance level.

two response categories supporting resting spores are combined (resting spores alone or in combination with conidia) no effect of gypsy strain was seen ($P = 0.1143$). A remarkable result was that between 20.0 and 31.7% of the Japanese, Greek, and Russian gypsy moth larvae dying after inoculations with the North American isolate 03PA1-1-5 failed to produce spores (Table 6). In contrast, this phenomenon was only observed in 1.7% of the cases when the Russian gypsy moth was inoculated with this specific fungal isolate. Upon dissection of the cadavers of larvae challenged with the Pennsylvanian isolate, only a few single-walled hyphal bodies were found within some of the cadavers whereas other cadavers had no obvious fungal structures present.

4. Discussion

All six *E. maimaiga* isolates tested in this study were pathogenic to the four strains of *L. dispar* tested, regardless of the geographical origin of the isolate as well as the inoculation method. The percentage of mortality varied between 87 and 100% for all combinations of fungal isolate, gypsy moth strain, and inoculation method, with time from inoculation to death varying between 3.9 days to 5.8 days. The time to death determined in this study is in the range of earlier studies evaluating the lethal time for the New Jersey gypsy moth strain infected by Japanese and North American *E. maimaiga* isolates (e.g., Hajek et al., 1993, 1995a,c; Shimazu and Soper, 1986; Soper et al., 1988).

Gypsy moth strain did not affect the time to death in any of the injection bioassays; however, the gypsy moth strain did affect the time to death when larvae were subjected to conidial showers. The Greek and North American gypsy moths (European biotype) took longer to die than the Japanese and Russian strains (Asian biotype), suggesting a greater physical barrier in the cuticle of European gypsy moths compared with Asian gypsy moths. These two biotypes of the gypsy moth differ in several important ways that could have contributed to the development of differences in response to *E. maimaiga*. Females of the European biotype of gypsy moth introduced into Massachusetts in 1868 or 1869 do not fly (Forbush and Fernald, 1896), whereas Asian females are reported as flying up to 100 km (Rozkhov and Vasilyeva, 1982). Female flight allows individuals to move away from densely populated areas annually which could reduce the chances of exposure to the fungus in the subsequent generation and reduce selection pressures. In addition, females of the Asian strains used tend to go through 6 instars (100% of Japanese and 50% of Russian) while those from the two European strains predominantly go through only 5 instars (Greek 93% and North American 100%) (M. K., unpubl. data). Thus, the fourth instar is not the same phenological stage for the two biotypes and so could differ in ways that affect susceptibility to the fungus. In contrast to our findings, Thomas (pers. com.) has found that both Japanese and North American isolates of *E. maimaiga* were more virulent in terms of time to death toward North American gypsy moth compared with Japanese gypsy moth. At present, we do not have any explanations for these differences in findings among studies. Both studies, however, struggle from the fact that the experiments were done with gypsy moths reared in the laboratory for 10–55 generations rather than naturally occurring populations and during these years of rearing, the strains have not been under selective pressure for development of resistance toward *E. maimaiga*. In addition, each gypsy moth strain only represents a sample of the genotypes present in the field. Unfortunately, an experiment with wild populations would be nearly impossible to perform due to quarantine restrictions for gypsy moth. Nevertheless, our study does answer one of the main questions posed in this paper: non-North American gypsy moth larvae are susceptible to North American strains of *E. maimaiga* under laboratory conditions. We therefore expect that the populations of *E. maimaiga* already present in North America can be expected to help control the Asian gypsy moth if it became established in North America.

This does not mean that introductions of Asian gypsy moth strains into North America should not still be a concern, since the dynamics and behavior of the Asian and North American populations are extremely different. In addition to the ability of Asian females to fly, the gypsy moth strains from Asia also possess other traits that make them more threatening to North American forests than the established strain, including a broader host range (Baranchikov, 1988), shortened egg chill requirements (Keena, 1996), and female attractancy to lights that results in egg deposition on vehicles or cargo (Wallner et al., 1995). These attributes could influence the effect of *E. maimaiga* on gypsy moth populations since it is a well-known fact that environmental factors (Blanford et al., 2003; Hajek et al., 1995c) and behavioral factors (Hajek et al., 1996a) affect the impact and dynamics of fungal infections.

Naturally, the most important factor for success of a pathogen used as a biological control agent is the ability to infect the host. For classical and inoculation biological control, however, the ability to multiply and reproduce in the host populations as well as the ability to survive in the environment at times when the host is not active are probably much more essential for success than the time to kill the host. In this study, we measured the fitness of *E. maimaiga* isolates using production of spores from cadavers of dead gypsy moth larvae (e.g., conidia, resting spores, both spore types, and no fungal reproduction). Fungal reproduction varied significantly among isolates. To our surprise, we found that in many cases gypsy moth larvae challenged with the Pennsylva-

nian isolate 03PA1-1-5 died without any spore production, a phenomenon we have observed for several other Pennsylvanian isolates (C. N. and A.E.H., unpubl. data). We do not believe that this lack of spore production can be attributed to handling of the insects since no control mortality occurred in any of the bioassays and also since we found fungal hyphal bodies internally in some cadavers upon dissection. The lack of production of any kind of spores impedes contribution of these fungal genotypes to conidial cycling in the population and/or to the resting spore load in the soil. This may explain why some gypsy moth populations in Pennsylvania in recent years have rebounded, causing considerable defoliation (Butalla, 2001). Potentially, this phenomenon could be due to a negative trade off between virulence and spore production (i.e., the fungus would be less able to produce spores when larvae die quickly), although gypsy moth subjected to isolate 03PA1-1-5 had intermediate lethal times. We hypothesize that other explanations than selection are possible, including presence of mycoviruses, which are known to affect virulence of *Metarhizium anisopliae* (Frazzon et al., 2000) and have been reported from *Entomophaga aulicae* (Nolan et al., 1988). The effect of gypsy moth strain on fungal reproduction was less clear in this study and no clear trends in fungal production from specific gypsy moth strains emerged, although the low numbers of larvae in some symptom categories could have weakened our ability to find differences during statistical testing.

Unfortunately, since our data were obtained from gypsy moth strains reared in the laboratory for up to 55 generations before use in the bioassay, this study does not allow us to test whether there was greater virulence in non-coevolved associations than co-evolved systems, as proposed by Hokkanen and Pimentel (1984, 1989). However, no significant effects in time to death were obtained for interactions between gypsy moth strain and fungal isolate, even though both a gypsy moth strain from Greece, a geographical area where *E. maimaiga* has never has been documented, and two gypsy moth strains from Asia where gypsy moth/*E. maimaiga* associations supposedly have co-existed for centuries, were included in the study.

We found the same trends in variability in virulence as well as fitness of the pathogen in both injection bioassays and showering bioassays, so even though only pseudo-replicates were carried out in our injection bioassay we feel confident that this does not affect our main conclusions. Thus, in none of our bioassays significant interactions between fungal isolate and gypsy moth strain were found with regard to mortality or time to death, indicating that each fungal isolate had the same overall effect on all the gypsy moth strains used. Fungal isolates differed significantly with regard to both virulence and fitness, whereas gypsy moth strain seemed to have little effect on fungal virulence and fitness.

In studies of virulence reported in the literature on other insect pathogens infecting North American gypsy moth, variability in virulence among strains of *Bacillus thuringiensis* (Rossiter et al., 1990), *Nosema* sp. (Goertz et al., 2004), and NPV (Shapiro et al., 1984) have been documented. The studies of *B. thuringiensis* furthermore demonstrated variability in susceptibility among natural gypsy moth populations from Pennsylvania (Rossiter et al., 1990), suggesting the potential for development of resistance. However, in our study the only significant variation among the gypsy moth strains tested was found in the conidial shower experiment, where prolonged time to death was found for the North American and European gypsy moth compared to the two Asian strains. We hypothesize that these differences are due to differences in fungal ability to penetrate gypsy moth larval cuticle, rather than evolution of resistance to the fungus, since we did not see differences in the injection bioassay.

In conclusion, we did not find any evidence for frequency-dependent selection (Red Queen dynamic) since no significant interactions between gypsy moth strain and fungal isolate were found. Therefore, erosion of successful control of gypsy moth by *E. maimaiga* seems unlikely based on the apparent equilibrium between gypsy moth and *E. maimaiga* found in this study. Nevertheless, to fully understand the potential for changes in control of gypsy moth by *E. maimaiga* in North America, studies including natural, established gypsy moth populations are needed.

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