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Impact of tachinid parasitoids of gypsy moth (*Lymantria dispar*) after the natural spreading and introduction of fungal pathogen *Entomophaga maimaiga* in Serbia

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Abstract
In 2013, parasitoids of Tachinidae family of *Lymantria dispar* (L.) were studied in 12 forest stands in four regions in Serbia. Biological material for the investigations was collected in mixed oak and beech stands in which gypsy moth increased in number and where entomopathogenic fungus *Entomophaga maimaiga* Humber, Shimazu & Soper was established or introduced. A total of 1117 larvae and 814 pupae of *L. dispar* were collected and analyzed. Host mortality caused by tachinids in different localities varied between 0.7% and 37.1%. A total of 274 tachinid larvae and pupae were reared from gypsy moth larvae and pupae. Out of these, 266 died as pupae, resulting in an extremely high overall mortality of 97.1%. Only eight tachinid adult specimens of 3 species (*Compsilura concinnata*, *Exorista larvarum* and *Carcelia gnava*) emerged from tachinid pupae. *E. maimaiga* azygospores were observed on the surface of 69.5% but not in internal tissues of the dead pupae. The results of this study support the hypothesis of competition between natural enemies of the pest.

Keywords: *Entomophaga maimaiga*, impact, *Lymantria dispar*, mortality, Tachinidae.

1. Introduction

Gypsy moth, *Lymantria dispar* (Linnaeus, 1758) (Lepidoptera: Erebidae), is one of the major serious defoliators of broadleaved forests and orchards throughout Northern Hemisphere. It is characterised by high reproductive capacity, considerable ecological plasticity and polyphagia. Gypsy moth populations undergo periodic outbreaks to extremely high densities. Although it is found on four continents (North Africa, Asia, Europe, North America), the pest causes the greatest damage to forests of Balkan Peninsula, which have all the favourable environmental conditions for gypsy moth development, and it often increases its number. In the central part of Serbia there were five outbreaks of *L. dispar* during the period of seventy years, and the sixth outbreak started in the period 2009-2010 [1]. The outbreaks do not occur in regular time intervals. Generally, pest outbreaks in Serbia lasted for an average time period of 4.3 years (minimum two to three, and maximum five to six years). Also, certain phases of outbreak do not happen at the same time due to variety of biotic and abiotic influence on gypsy moth dispersal and occupation of new territories. Regular occurrence and characteristics of all outbreaks is their spatial expansion.

The population density of *L. dispar* is regulated by many biotic factors – parasitoids, predators and pathogens. Based on the literature data, a total of 102 species have been reported in Central Serbia as natural enemies of gypsy moth, i.e. 24 predators, 60 parasitic insects, 10 saprophagous insects and 8 pathogens [2-3]. Regarding the number of the species, the representatives of Diptera and Hymenoptera orders are the most frequent and most important ones [2].

Entomopathogenic fungus *Entomophaga maimaiga* Humber, Shimazu & Soper (Entomophthorales: Entomophtoraceae) was isolated and described as a natural enemy of the gypsy moth in Japan, where it causes the periodical epizootics. It is also spread in some parts of China and Russian Far East. Despite of the fact that it was introduced in North America in 1910-1911, its presence in natural populations of gypsy moth was determined only in 1989, when the pathogen caused pandemic in several countries of USA [4]. Today *E. maimaiga* is a very significant pathogen of gypsy moth there and in Canada.

In 1999, *E. maimaiga* was introduced into Bulgaria from the USA [5] and thereafter, in 2005, caused epizootics and mortality in four gypsy moth outbreak populations, located 30-70 km from the introduction sites [6]. From 2008 to 2011, six introductions of *E. maimaiga* were performed in outbreak populations of *L. dispar* in oak forests in different parts of the country [7]. This entomopathogenic species has slowly spread along Balkan Peninsula and Southeast Europe, and so far its presence has been reported also in European part of Turkey [8], Serbia [9-10], Greece, FYR Macedonia [11], Croatia [12], Hungary [13] and Slovakia [14]. Regarding the situation in Serbia, *E. maimaiga* was first established to spread naturally in two regions in central part of the country, Belgrade and Valjevo [9]. Then, the fungus was introduced into *L. dispar* population situated in mountain Avala in Belgrade region and was also introduced or found in many places of the country [15-16].

E. maimaiga is a host specific pathogen of gypsy moth in its natural range. In USA, the fungus causes low mortality in only 3 closely related species of Erebidae and single representative species from 2 other lepidopteran families [17]. In Bulgaria, a total of 1499 larvae of 38 non-target phyllophagous insect species from 10 lepidopteran and 1 hymenopteran families were studied but *E. maimaiga* was not found in any of them [18]. The high level of host specificity of *E. maimaiga* limits the negative impact on non-target species but in biological control programs it is also important to evaluate impact on other biological agents of the target host. This is especially true for the parasitoids in the family Tachinidae, which are strongly associated with late instar gypsy moth larvae where *E. maimaiga* also develops but insufficient information in entomological literature occurs. Currently there is only one more precise study on potential interactions between *E. maimaiga* and tachinid parasitoids during development in the gypsy moth larvae [19].

This paper reports results from the study on impact of tachinids of gypsy moth after natural spreading and introduction of *E. maimaiga* in Serbia and mortality of the parasitoids in pupal stage.

2. Material and Methods

The studies were conducted during the growing season in 2013, in some forest areas of Central Serbia (Kruševac, Donji Milanovac, Krupanj and Leskovac regions) in which *L. dispar* increases in number and *E. maimaiga* was found in the host populations.

Gypsy moth larvae were collected in oak or mixed forest stands dominated by different oak species, *Quercus cerris* L., *Q. petraea* (Matt.) Liebl., *Q. frainetto* Ten., and beech, *Fagus sylvatica* L. (Table -1). Each study plot was 100×100 m (1 ha) in size and included a minimum of 25 oak trees: five groups of five trees each, one chosen as the centre of the plot, and four additional trees located approximately 50 m from the centre tree at approximately 90º. During the spring and early summer, gypsy moth larvae were collected two to three times in each of the study sites. Larvae and pupae were collected manually from foliage in the lower parts of tree crowns and tree branches.

Table 1. Main characteristics of study localities.

Locality	Region	Altitude (m a.s.l.)	Geographic coordinates		Tree species
			N	E	
Srndaljska reka-1	Kruševac	493	43° 27' 18.73"	21° 26' 47.64"	*Quercus cerris, Quercus frainetto, Quercus petraea, Carpinus betulus*
Srndaljska reka-2	Kruševac	762	43° 26' 05.24"	21° 27' 42.25"	*Fagus sylvatica*
Srndaljska reka-3	Kruševac	500	43° 27' 08.71"	21° 29' 18.86"	*Quercus cerris, Quercus frainetto, Quercus petraea, Carpinus betulus*
Srndaljska reka-4	Kruševac	488	43° 27' 41.91"	21° 28' 38.07"	*Quercus cerris, Quercus frainetto, Quercus petraea, Carpinus betulus*
Monte Miroč	Donji Milanovac	365	44° 27' 28.10"	22° 12' 39.30"	*Quercus cerris, Quercus frainetto, Quercus petraea*
East Boranja-1	Krupanj	365	44° 22' 16.94"	19° 20' 31.77"	*Fagus sylvatica*
East Boranja-2	Krupanj	423	44° 22' 41.28"	19° 21' 40.19"	*Fagus sylvatica*
Village Miroševce-1	Leskovac	334	42° 52' 14.77"	21° 49' 30.44"	*Fagus sylvatica*
Village Miroševce-2	Leskovac	292	42° 51' 22.21"	21° 50' 06.19"	*Fagus sylvatica*
Monte Vučje-1	Leskovac	546	42° 50' 26.63"	21° 53' 19.99"	*Fagus sylvatica*
Monte Vučje-2	Leskovac	935	42° 50' 47.80"	21° 56' 05.97"	*Fagus sylvatica*
Monte Vučje-3	Leskovac	618	42° 51' 27.40"	21° 56' 01.10"	*Fagus sylvatica*

Collected larvae were transported to the laboratory of Institute of Forestry (Belgrade) and reared in 15×10×8 cm plastic boxes, ten larvae per box. The larvae were grown in a climate chamber under the laboratory conditions. During the experiment, temperature and light conditions were constant (temperature 21 °C, light regime - 8 h dark, 16 h light). The larvae were fed on daily basis on fresh oak leaves, brought from the sample plots.

Larvae that were parasitized with braconid (Hymenoptera) puparia and those from which tachinid larvae emerged were separated and held individually in Petri dishes for observation and microscopic examination. Individual gypsy moth pupae were placed in 50 cm^3 plastic containers, one pupa per container, and mortality caused by parasitoids was recorded. Tachinid pupae were maintained in sterile Petri dishes containing sand moistened with sterile water. Relative humidity of 75-80% was maintained in order to prevent drying of pupae.

Microscopic analyses of dead tachinid pupae were conducted two or more months after pupation using the protocol described in another publication [28].

3. Results

A total of 1117 larvae and 814 pupae of *L. dispar* were collected and analyzed in 2013 (Table -2). Host mortality due to tachinid parasitism in different localities varied from 0.7% to 37.1%; the overall average parasitism by tachinids was 14.2%.

Mortality occurred in late larval stage (≥ IV instar) but much more in the pupal stage of gypsy moth. Mortality due to the hymenopteran species *Protapanteles liparidis* (Bouché, 1834) and *Cotesia melanoscela* (Ratzeburg, 1844) (Hymenoptera: Braconidae) was recorded in earlier stage larvae (≤ III instar) (unpublished data). *E. maimaiga* was not observed in early instar larvae from which hymenopteran parasitoids emerged. No pathogens (*Lymantria dispar* multicapsid nuclear polyhedrosis virus - LdMNPV or microsporidia) were observed in the parasitized larvae.

A total of 274 tachinid larvae were reared from gypsy moth larvae and pupae. Of these, 266 died as pupae, resulting in an extremely high overall mortality of 97.1% (Table -2). No *E. maimaiga* azygospores were observed in internal tissues of dead pupae, but were detected on the surface of 69.5% of the dead pupae. No other pathogens were established in the tissues of the dead parasitoids.

Table 2: Impact of tachinids on gypsy moth and mortality of parasitoids in pupal stage in 2013.

Locality	*Lymantria dispar* L.					Tachinid pupae		
	Larvae		Pupae		Mortality caused by tachinids (%)	N	Mortality (%)	With *E. maimaiga* azygospores (%)
	Collection dates	N	Collection dates	N				
Srndaljska reka-1	11 May- 1 June	60	18 June	75	15.6	21	100.0	72.2
Srndaljska reka-2		71		42	11.5	13	92.3	36.6
Srndaljska reka-3		34		51	10.6	9	100.0	77.8
Srndaljska reka-4		53		30	4.8	4	100.0	50.0
Monte Miroč	9 May	342	13 June	209	19.1	105	98.1	83.3
East Boranja-1	5 May 5 June	176	11 June	55	5.6	15	80.0	33.3
East Boranja-2		93		46	0.7	1	0.0	0
Miroševce-1	16 May 20 June	74	3 July	50	8.1	10	90.0	70.0
Miroševce-2		56		73	10.1	13	100.0	69.2
Monte Vučje-1	11 May	60	3 July	64	37.1	46	93.5	62.8
Monte Vučje-2		41		37	17.9	14	100.0	64.3
Monte Vučje-3		57		82	16.5	23	100.0	52.2
Total		**1117**		**814**	**14.2**	**274**	**97.1**	**69.5**

Only eight tachinid adult specimens emerged from the pupae and three species were identified: *Compsilura concinnata* (Meigen, 1824) – 37.5%, *Exorista larvarum* (Linnaeus, 1758) – 37.5%, and *Carcelia gnava* (Meigen, 1824) – 25.0% (Table -3). The high tachinid mortality in the pupal stage did not allow identification of predominate part of specimens or evaluation of the role of different species in the regulation of gypsy moth density.

Table 3. Parasitoid adults reared from *Lymantria dispar*.

Parasitoid species	Locality	Parasitoid number	Date of emergence
Compsilura conciunata (Meigen, 1824)	East Boranja-1	2♂, 1♀	4-6 July 2013
	East Boranja-2	1♂	6 July 2013
Exorista larvarum (Linnaeus, 1758)	Miroševce-1	1♂	19 July 2013
	Srndaljska reka-2	1♂	3 July 2013
Carcelia gnava (Meigen, 1824)	Monte Miroč	1♂, 1♀	8-10 July 2013

4. Discussion

Family Tachinidae (Diptera) occupies an important place among the parasitoids, not only by the richness of species and wide geographical distribution but also because they parasitize a large number of economically important insect pests. For Serbia, Hubenov [3] reported 288 tachinid species from which 21 (*Exorista larvarum*, *E. segregata*, *E. sorbillans*, *Parasetigena* silvestris, *Phorocera assimilis*, *Blondelia inclusa*, *B. nigripes*, *Compsilura concinnata*, *Drino inconspicua*, *Carcelia gnava*, *C. lucorum*, *Senometopia* *confudens*, *S. excisa*, *S. separata*, *S. susurrans*, *Zenillia libatrix*, *Pales pavida*, *Blepharipa pratensis*, *B. schineri*, *Baumhaueria goniaeformis*, *Tachina fera*) are connected with *L. dispar* [1, 3, 9].

Previous studies in Serbia showed that some tachinids are important in gypsy moth population dynamics with relatively high host mortality: *C. conncinata*, 60.0% [20]; *E. larvarum*, most frequent species [21]; *B. pratensis*, 4.7-92.5% [22]; and *P. silvestris*, 3.0-61.0% [20]. The four species are one of the most effective gypsy moth tachinid parasitoids in Europe, and were introduced into North America. These are polyphagous on a variety of phytophagous host species [23-24]. They are typically multivoltine with two or more generations per year, the first parasitizing gypsy moth. In Serbia, the degree of parasitism of gypsy moth caused by polyphagous tachinid species is the largest in the progressive stage of gradation, as well as the stage of latency.

The lack of azygospores inside dead tachinid pupae is an indication of the absence of a direct effect of fungal pathogen on parasitoids. Tachinids evidently do not become infected by *E. maimaiga* while parasitizing the infected host but the presence of azygospores on the surface of 69.5% of tachinid puparia is an evidence of development of entomophthorous fungus in parasitized gypsy moth larvae.

In this study in Serbia, higher mortality of tachinid pupae (97.1%) was established during their development in gypsy moth larvae infected with *E. maimaiga*, in comparison to a similar study in Bulgaria (86.5%). Georgiev *et al.* [19] hypothesized that, during fungal development, nutritional resources available to the parasitoids decrease, limiting available energy for successful pupation and eclosion.

The causes of tachinid mortality during their development in gypsy moth larvae which have been parasitized by *E. maimaiga* are not clarified but it is likely a result of the competition between the fungus and tachinids. *E. maimaiga* has become the primary biological control agent in gypsy moth populations in USA [25], Bulgaria [7, 19] and Serbia [15] but its impact on other natural enemies of gypsy moth is still not well known. Additional studies are needed to clarify the specific causes of mortality of tachinid parasitoids in order to obtain an objective assessment of the impact of *E. maimaiga* on gypsy moth and role of the pathogen in programs for biological control of the pest.

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