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Inoculative Releases and Natural Spread of the Fungal Pathogen *Entomophaga maimaiga* (Entomophthorales: Entomophthoraceae) into U.S. Populations of Gypsy Moth, *Lymantria dispar* (Lepidoptera: Erebidae)

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Abstract

While emphasis with entomopathogens has often been on inundative releases, we describe here historic widespread inoculative releases of a fungal entomopathogen. Several U.S. states and municipalities conducted inoculative releases of the gypsy moth, *Lymantria dispar* (L.) (Lepidoptera: Erebidae), pathogen *Entomophaga maimaiga* Humber, Shimazu et Soper (Entomophthorales: Entomophthoraceae) after 1993, as gypsy moth populations spread into the Midwest and North Carolina. This Japanese pathogen first caused epizootics in northeastern North America in 1989 and methods for its inoculative release were tested and proven to be effective from 1991 to 1993. After 1993, spores in soil or in late instar cadavers were collected during or after epizootics and were released inoculatively into newly established populations of this spreading invasive; the goal was that spores would overwinter and germinate the next spring to infect larvae, thus speeding pathogen spread and hastening the development of epizootics in newly established populations. The fungus was released in gypsy moth populations that were separated from areas where the fungus was already established. In particular, extensive releases by natural resource managers in Wisconsin and Michigan aided the spread of *E. maimaiga* throughout these states. Where it has become established, this acute pathogen has become the dominant natural enemy and has exerted considerable influence in reducing gypsy moth damage. While this pathogen most likely would have invaded these new regions eventually, releases accelerated the spread of *E. maimaiga* and helped to reduce impacts of initial outbreaks, while further outbreaks were reduced by the pathogen's subsequent persistence and activity in those areas.

Key words: biological control, inoculative augmentation, epizootic, fungal entomopathogen

When natural enemies being released for biological control already occur in a region, they are either released inundatively or inoculatively. With inundative releases, the goal is that the released natural enemies will control the pest without needing to persist and reproduce. Alternatively, the goal of inoculative releases is that control will be due in part to the persistence and reproduction of the released natural enemies (Hajek and Eilenberg 2018). Inoculative augmentation of natural enemies is well known as a successful strategy for use against some greenhouse pests, where reproduction by released natural enemies occurs within a crop cycle, but the natural enemies cannot persist after crops are harvested (Heimpel and Mills 2017, Hajek and Eilenberg 2018). Inoculative augmentation is also the strategy used for biological control of weeds or plant pathogens, when released microbial natural enemies increase in response to pest populations and provide control.

Use of entomopathogens has usually emphasized inundative augmentation, especially for the protection of crops growing above ground. However, strategies for inoculative augmentation of entomopathogens have been developed (Shapiro-Ilan et al. 2018), especially for the control of pests in cryptic habitats or for pathogens that are difficult or impossible to produce in vitro. For example, inoculative augmentation has been developed using bacteria against scarab grubs in the soil (e.g., Jackson et al. 2018), nematodes against weevil larvae in soil (Shields and Testa 2017), and nematodes against woodwasp larvae within pines (Carnegie and Bashford 2012). As early as 1907, cadavers of the invasive browntail moth, *Euproctis chryssorhea* (L.) (Lepidoptera: Erebidae), that had been killed by the native *Entomophaga aulicae* (Reichardt) Humber (Entomophthorales: Entomophthoraceae) were released in a New England orchard (Hitchings 1908); in this case, *E. aulicae* could not be mass-produced but field-collected cadavers of *E. chryssorhea* containing spores were collected and released. While results from this release were not quantified, it was generally considered that *E. aulicae* caused extensive mortality of this pest. As seen in these examples, entomopathogens can respond to and control pest populations when inoculatively released in nature. Here, we report extensive releases of the gypsy moth fungal pathogen *Entomophaga maimaiga* based on collecting spore-filled soil or cadavers and releasing these inoculatively in the US to control invading populations of this non-native defoliator species.

The European gypsy moth, *Lymantria dispar dispar* (L.) (Lepidoptera: Erebidae), is one of the most serious forest pests in North America. It was accidentally introduced near Boston, MA in 1869 (Liebhold et al. 1989) and has been gradually expanding its North American range; it is currently established in 20 eastern and midwestern states (Fig. 1) (Tobin et al. 2012, Coleman et al. 2020). Through much of its native range, populations of this insect periodically erupt, causing widespread defoliation of host trees, which include hundreds of species but especially oaks and poplars (Johnson et al. 2005). Similarly, in many portions of its invaded North American range, oscillatory gypsy moth populations sometimes reach outbreak levels causing defoliation spanning millions of hectares. Historically, extensive applications of chemical and later microbial insecticides were aerially sprayed to suppress outbreaks, slow invasion spread, and eradicate isolated outlier populations (Liebhold and McManus 1999, Tobin et al. 2012).

The gypsy moth has been the focus of extensive efforts to control populations using classical biological control, with the first parasitoid introductions in 1906 (Fuester et al. 2014). Throughout the 1900s, the greatest emphasis for introductions was placed on parasitoids. However, of the 34 species released, only 12 became established, and the general impact of these parasitoids is not well understood. The *Lymantria dispar multicapsid nuclear polyhedrosis virus* (LdMNPV) (*Baculoviridae*: *Alphabaculovirus*) is conjectured to have been introduced as a contaminant with early parasitoid releases. In North America and elsewhere, epizootics caused by this virus are reported to cause crashes of outbreak gypsy moth populations (e.g., Kurenshchikova et al. 2020). The virus is strongly density-dependent (Dwyer and Elkinton 1993) and historically, viral epizootics have not prevented defoliation as populations increased (Woods et al. 1991), although this virus often has driven crashes of dense populations that end outbreaks.

Another pathogen, the fungal entomopathogen, *Entomophaga maimaiga* Humber, Shimazu et Soper (Entomophthorales: Entomophthoraceae), has long been observed causing epizootics in native gypsy moth populations in Japan (e.g., Jikumaru and Sano 2007). In 1910–1911, Speare and Colley (1912) attempted

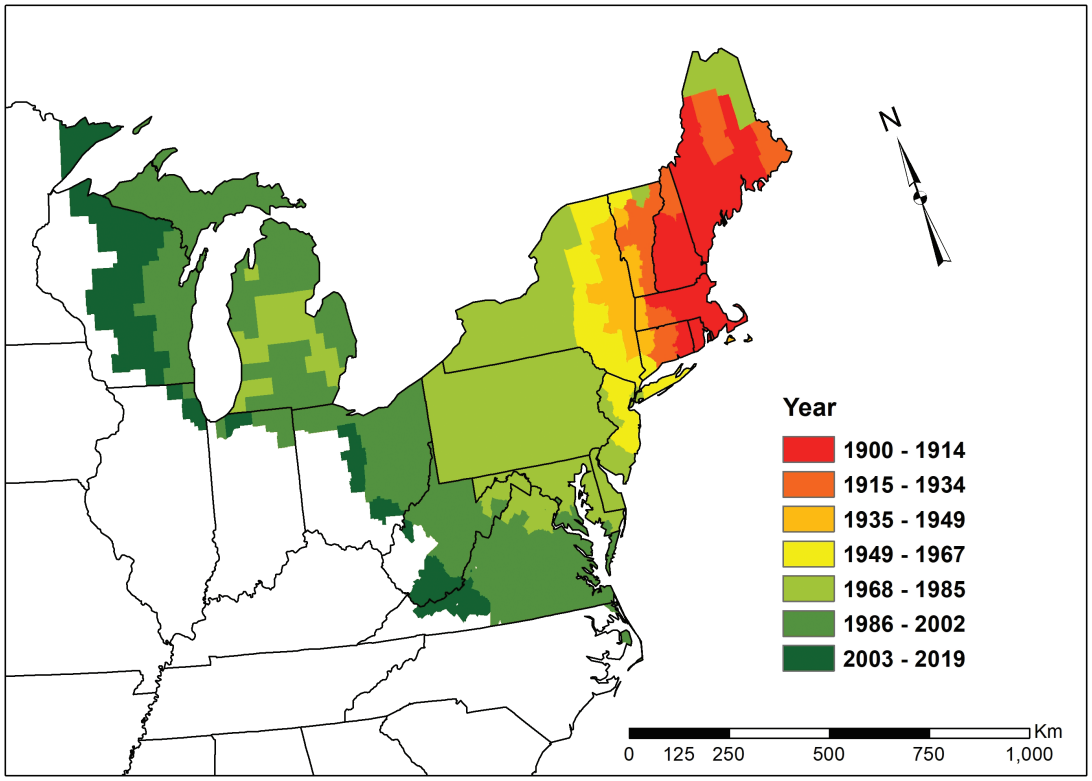

Fig. 1. Historical spread of the gypsy moth in the eastern US as documented by the year that each county was designated part of the US Dept. of Agriculture quarantine area.

to introduce this pathogen to North American gypsy moth populations but *E. maimaiga* was never considered to have established. However, in the exceptionally rainy spring of 1989, *E. maimaiga* epizootics were found causing the collapses of outbreak populations in seven northeastern states (Connecticut, Massachusetts, New Hampshire, New Jersey, New York, Pennsylvania, Vermont) (Hajek et al. 1990, Fig. 2). Analyses indicated that the origin of these populations most likely was an accidental introduction from Japan (Nielsen et al. 2005) to southern New England after 1971 (Weseloh 1998). During the three years following the discovery of *E. maimaiga* in the northeastern US (1989–1992), this fungus spread from the initial eight states (adding Rhode Island to the seven reporting in 1989) into five more states (Delaware, Maine, Maryland, Virginia, West Virginia); much of this spread likely occurred naturally (the asexual spores are actively ejected from cadavers and can become airborne), although purposeful introductions were also conducted (Hajek et al. 1996).

Subsequent studies of the epizootiology of *E. maimaiga* have shown that once *E. maimaiga* establishes in an area, a large fraction of hosts can become infected and this results in extensive mortality in gypsy moth populations (Hajek 1999). Although LdMNPV tends to cause extensive mortality only in high density gypsy moth populations, *E. maimaiga* can cause abundant mortality through a wide range of host densities. Currently, in most locations and in most years, *E. maimaiga* causes more gypsy moth mortality than LdMNPV (e.g., Hajek et al. 2015). Analyses of historical patterns of gypsy moth defoliation indicate that the emergence of *E. maimaiga* in North America has been associated with a decrease in the overall intensity of regional gypsy moth outbreaks (A.M. Liebhold et al., unpublished data).

During the last 25 yr, gypsy moth has expanded its range to the west (Illinois, Indiana, Michigan, Ohio, and Wisconsin) and south (North Carolina, Virginia and West Virginia) in the US (Fig. 1). Given the immense impacts of the gypsy moth and the negative impact of *E. maimaiga* on host gypsy moth populations, there has been sustained interest in promoting the spread of *E. maimaiga* into areas more recently invaded by the gypsy moth. Unfortunately, it has proven exceptionally difficult to produce large quantities of this fungus in vitro to apply as a biopesticide.

However, there have been considerable efforts to manually transplant *E. maimaiga* into areas newly invaded by the gypsy moth. Despite the impracticality of mass-producing *E. maimaiga,* it is relatively easy to field-collect large numbers of larval cadavers or soil containing the overwintering resting spores. In many states with newly invaded gypsy moth populations there were informal programs for the collection of cadavers or soil for the purpose of releasing this pathogen into newly invading gypsy moth populations. In this paper, our objective is to report on both the natural spread of *E. maimaiga* and purposeful spread due to inoculative releases into and within midwestern states (Illinois, Indiana, Michigan, and Wisconsin) as well as a southeastern state (North Carolina) (Fig. 2).

General Biology of *E. maimaiga* and Natural Spread

The gypsy moth stage infected by *E. maimaiga* is the larval stage. Larvae of this univoltine insect feed and develop over approximately 1.5–2. 5 mo in spring and early summer. After infected larvae die, *E. maimaiga* produces two types of spores that facilitate its dispersal: actively ejected airborne conidia often produced from the

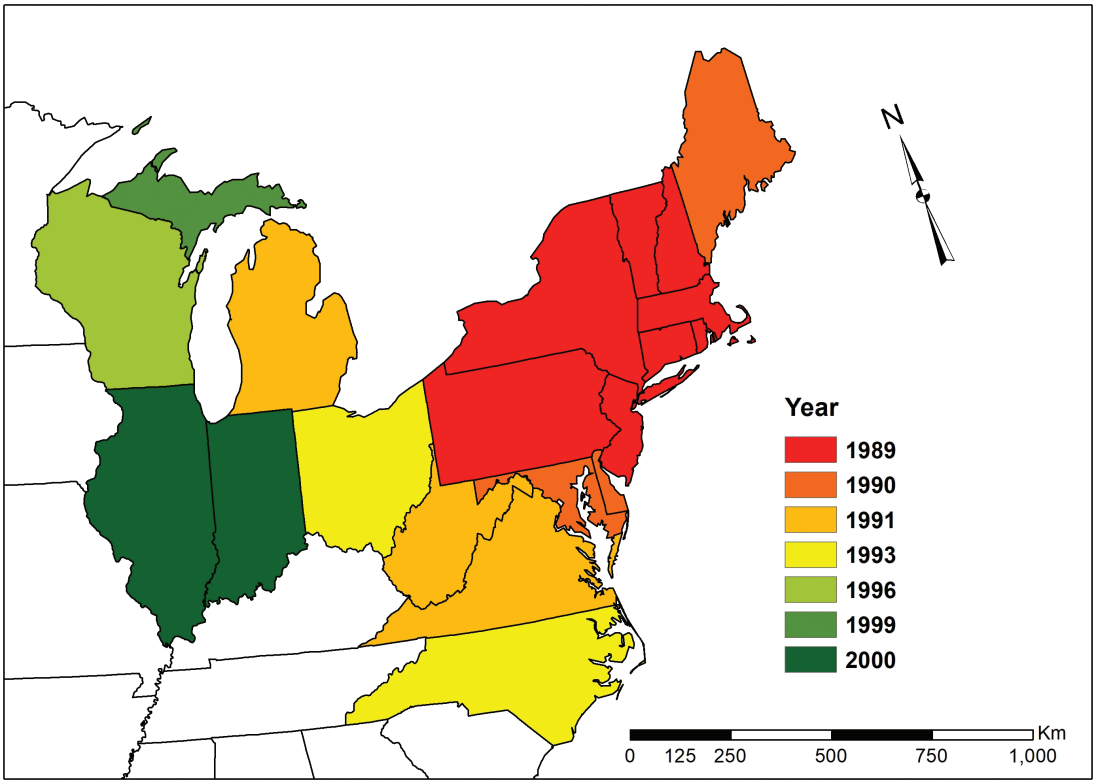

Fig. 2. Historical spread of *E. maimaiga* in the eastern US. Colors denote the year that *E. maimaiga* was discovered infecting *L. dispar* by state. Though there are no records of *E. maimaiga* from Rhode Island in 1989, it was surrounded by states where *E. maimaiga* was first seen so we assume that *E. maimaiga* occurred there in 1989 as well.

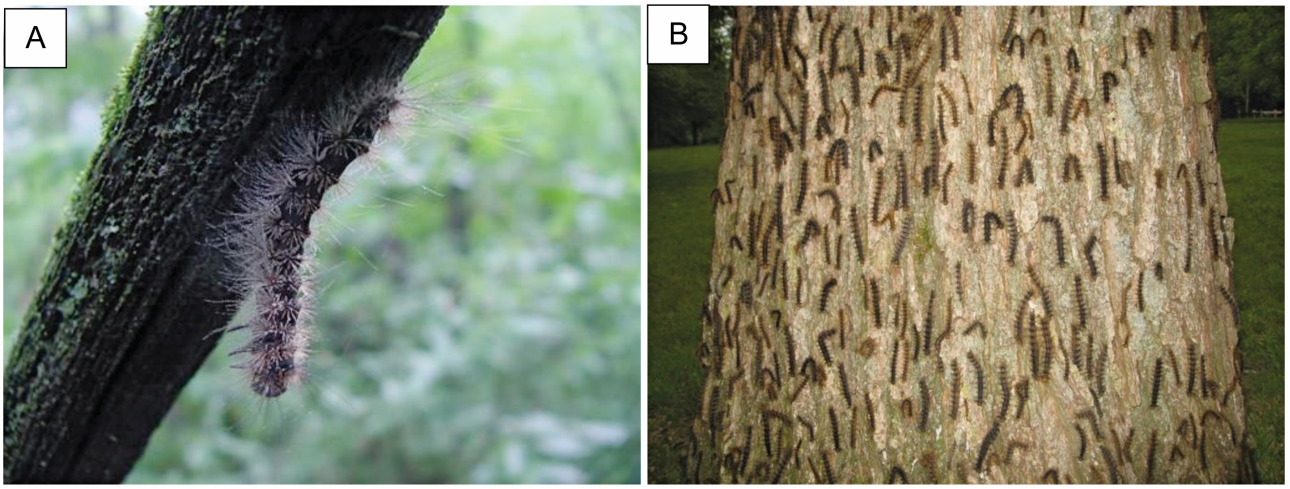

Fig. 3. A. *Entomophaga maimaiga* has grown out of this dead gypsy moth larva and has ejected conidia, which look like white sugar granules on the setae. Photo by Mark Guthmiller. B. *Entomophaga maimaiga* epizootic in an outbreak gypsy moth population. Late instars killed by *E. maimaiga* often are oriented vertically with head downward, although this is not always the positioning of late instars killed by *E. maimaiga*. Photo by Heather Faubert (Hajek et al. 2018).

bodies of early instars (Fig. 3A) and overwintering resting spores often produced within the bodies of later instars (see Blackburn and Hajek 2018). Few early instars are initially infected (with infections initiated by overwintering resting spores) and cadavers from these infected early-stage larvae are often inconspicuous, as these larvae typically die within tree canopies. Levels of infection, however, can build during the period of larval development due to secondary transmission from conidial infections of earlier instars, resulting in higher infection levels in later instars. Late instars killed by *E. maimaiga* typically do not exhibit external fungal growth but contain resting spores. Dried cadavers accumulate on lower tree trunks, sometimes in large numbers (Fig. 3B). These late instar cadavers can easily be collected and they provide a source of inoculum to introduce *E. maimaiga* into new areas. Eventually, dry cadavers fall from trees and decompose on the forest floor where resting spores are washed into surface layers of the soil (Hajek 1999).

Entomophaga maimaiga conidia were sampled from the air in high numbers near outbreak regions and their windborne dispersal is considered the principal means of natural spread by *E. maimaiga* (Hajek et al. 1999, Bittner et al. 2017, Elkinton et al. 2019). Based on fungal spread from 1989 to 1992, one model predicted movement of airborne conidia >120 km per season (Dwyer et al. 1998). However, most airborne spores travel shorter distances and only a small percentage travel > 10 km (Weseloh 2003, Bittner et al. 2017, Elkinton et al. 2019). The success of conidial production, airborne dispersal, and abundant infection is largely dependent on gypsy moth population density and weather. Spread of the fungus may also occur by purposefully or accidentally moved resting spore-laden soil.

Purposeful Introductions of *E. maimaiga*

As gypsy moth populations spread into and within midwestern states (Fig. 1), natural resource managers were often already aware of the dramatic 1989 epizootics that had decreased the widespread gypsy moth outbreaks in the Northeast. Little information was available to predict how quickly the fungus would naturally spread into areas that had been newly invaded. Therefore, inoculative releases of the fungus were conducted to facilitate its spread. Between 1990 and 2009, *E. maimaiga* was released in New York, Pennsylvania, Maryland, West Virginia, Virginia, North Carolina, Michigan, Ohio, Wisconsin, and Illinois. Methods for field collection and release were developed and improved throughout this period and interstate cooperation was extensive.

Soil containing resting spores was initially tested as a source of inoculum (Hajek and Roberts 1991) and then used to introduce the fungus around bases of trees in new areas (Smitley et al. 1995, Hajek et al. 1996). Late instar cadavers that contain resting spores are attached to tree trunks for only relatively short periods of time, usually in late June/early July. If field collection of *E. maimaiga* is conducted after this time, the only option is to collect soil around tree trunks where resting spores can be at high titers. Resting spores in soil can be sampled (Hajek et al. 2012) to verify that they are present and to quantify densities. When resting spore laden soil was introduced, densities of resting spores/g soil varied to some extent, but the volume of soil released could be adjusted to attain a target level of resting spores. For example, two early studies achieved successful establishment following release of $>5 \times 10^5$ *E. maimaiga* resting spores per site (Smitley et al. 1995, Hajek et al. 1996). Soil with resting spores can be collected and moved any time before the egg hatch the next spring and early projects frequently collected and released soil containing resting spores in April.

After successes with soil-borne resting spore releases in 1991–1993 (see below), more resource managers in midwestern states that were just becoming invaded by gypsy moth became interested in inoculative introduction of the fungus. However, problems had been encountered with moving resting spore laden soil. One issue encountered during some field-collection efforts occurred when wet soil was collected and stored in air-tight containers and bacteria killed the resting spores. To prevent this, collection of wet soil and long-term soil storage before release were avoided in later programs. Another issue was the possibility of accidentally moving unwanted organisms with soil collected from forests. State and federal regulations necessitate obtaining a permit before moving soil. Soil as an inoculant is bulky and heavy and thus expensive to ship. It is also unknown whether the density of spores in the soil is high or low, without quantification, a process that is not simple. Consequently, a new method was developed that involved collecting cadavers containing resting spores and moving these cadavers to release areas.

By this time, resource managers were also aware that the fungus would eventually naturally spread into their states. However,

natural spread by *E. maimaiga* would potentially be slowed because new gypsy moth populations in some states were not contiguous with northeastern populations. For example, the majority of gypsy moth populations in Michigan were isolated from northeastern populations by Lake Erie. Wisconsin and Illinois populations were isolated from the contiguous gypsy moth populations by Lake Michigan. Therefore geography, in combination with the relatively low-density populations of the invading host, would potentially significantly slow the establishment of the fungus. Land managers believed that field releases could function to establish *E. maimaiga* in areas sooner than this fungus would naturally spread and this would increase background levels of mortality in newly invaded gypsy moth populations. The ultimate goal, therefore, was to accelerate the occurrence and efficacy of epizootics caused by *E. maimaiga*. Here, we describe some of these release programs, that progressed from releases of resting spore laden soil to cadavers.

Mid-Atlantic State Releases 1991–1992. Soil containing *E. maimaiga* resting spores was collected in Massachusetts or New York for redistribution in Maryland, Pennsylvania, Virginia, and West Virginia (Hajek et al. 1996). In 1991, 6×10^5 resting spores in soil were placed at the bases of oaks in early spring in each of 34 plots along the leading edge of gypsy moth spread, where *E. maimaiga* had not previously been found (Hajek et al. 1996, see Figs. 1 and 2). From larvae sampled in the same year, *E. maimaiga* infections were detected in >80% of plots, with infection at >40% in six plots. Low levels of infection were also found in control plots and this was assumed to have resulted from airborne dispersal of infective spores. In 1992, similar releases were made at seven additional plots. In 1992, in 31 of the plots where releases were made in 1991 (24) or 1992 (7), epizootics (>70% infection) occurred (see Hajek et al. 1996).

In 1991, *E. maimaiga* infections occurred up to 350 m from 1991 release sites (Hajek et al. 1996, see Fig. 7) and by 1992, infections were abundant at 1000 m from the 1991 release sites. In plots where releases were made in 1992, egg mass densities decreased to lower levels in the next generation than in controls. In these experiments, infection levels were greater where the resting spore laden soil was watered each week and infection levels were positively associated with May rainfall each year (Hajek et al. 1996). Based on the results from releases, a survey was conducted in 1992 across these four states and *E. maimaiga* was found to have spread throughout the gypsy moth distribution in the northeast, including areas as far south as gypsy moth was known to occur and not only where *E. maimaiga* had been released. While it appeared that natural dispersal of the fungus eventually allowed it to catch up with the expanding range boundary of the gypsy moth, we assume that the introductions made in 1991 and 1992 accelerated infection levels and reduction of host populations.

Michigan, 1991–1996. Gypsy moth was first found in Michigan in the early 1950s near Midland in the lower peninsula where populations were geographically discontinuous with populations in the northeastern US (Hanna 2017). In the early-mid 1990s, gypsy moth populations were increasing, and the invaded area was expanding. In June 1991, gypsy moth larvae were collected from 50 survey sites in 10 counties to document that *E. maimaiga* could not be detected, although levels of LdMNPV infection ranged from 16 to 69%. Along the leading edge of gypsy moth spread in the lower peninsula of Michigan, three sites were chosen for release studies (Smitley et al. 1995). In May of 1991 or 1992, one of three procedures was conducted at each of three study sites: 1) soil containing *E. maimaiga* resting spores, collected in Massachusetts in April, was placed at the bases of 4 oaks/plot ($2.6–3.3 \times 10^6$ resting spores/plot), 2) 15 laboratory-infected, living larvae were released on boles of 4 oaks/plot, or 3) no inoculation took place. Little infection was found in 1991 at any study sites but by 1992, *E. maimaiga* was found in both types of release plots (9–12% infection at one study site) and very low levels of infection were also found in control sites. By 1993, epizootics (92–96% infection) occurred at all three treatments at one site, with infection levels ranging from 20 to 43% in both treated and control plots at the other two sites. No significant difference in infection level was detected due to releasing infected larvae versus resting spore laden soil. After 1993, subsequent egg mass counts were lower than in adjacent forests. Also, *E. maimaiga* was detected up to 425 m from additional single tree release sites.

Releases in Michigan continued in 1993 and 1994 (N. Siegert unpubl. data), with a mix of either resting spores in soil or cadavers (Supp Table 1 [online only]) (Fig. 4A), and by 1996, *E. maimaiga* was found throughout much of the lower peninsula (Mott 1998) (Fig. 4B). In total, *E. maimaiga* was released 48 times in a total of 20 counties and was recovered 267 times in 36 counties, meaning that *E. maimaiga* had spread on its own into 16 counties where it was not released. Thus, *E. maimaiga* was successfully introduced even when high levels of LdMNPV infection were already present and gypsy moth populations were subsequently reduced to levels lower than in stands where naturally occurring LdMNPV acted alone.

Wisconsin 1997–2009. After *E. maimaiga* had been found to have naturally spread to the lower Door Peninsula of Wisconsin in 1996, releases elsewhere in Wisconsin were approved by federal and state agencies. The gypsy moth population in Wisconsin was not contiguous with that in Michigan or the eastern states and Wisconsin was concerned that natural enemies would lag significantly behind invading gypsy moth because there was no natural corridor for dispersal. To improve environmental safety, instead of moving soil containing resting spores, field-collected cadavers filled with resting spores were used as the source of inoculum (Fig. 3B). This change required additional planning and communication; epizootics can be very localized and late instar cadavers can be easily collected for only limited periods of time after the insects die. To use this type of inoculum, land managers and researchers in areas where *E. maimaiga* was well established would be contacted to identify locations of epizootics. During the first and second weeks of July, cadavers were collected from Michigan, western Maryland, and adjoining areas of Pennsylvania and West Virginia, and Massachusetts. Collected cadavers were dried in the laboratory if they were damp and then ground in a blender to a powder. The powder was mixed with sterile potting soil so that the equivalent of 10 dry cadavers in 335 ml of soil would be inoculated around the base of each of 10 trees per release site. With the estimate of 1 million (1×10^6) resting spores per cadaver, the base of each tree was then inoculated with 10 million (1×10^7) resting spores. Introductions were made from August through the fall so that the resting spores subsequently received natural overwintering conditions. Populations selected for introduction were typically at low densities.

Between 1997 and 2009, as gypsy moth populations spread through Wisconsin, *E. maimaiga* was released in 106 locations in 32 counties (A. Diss-Torrance unpubl. data) (Fig. 5A). These releases were operational and resources were not available to regularly sample larvae or cadavers at all sites to quantify infection levels. In total, *E. maimaiga*-infected larvae were recovered in 135 locations in 34 counties (Fig. 5B) but in seven of those counties, *E. maimaiga* had not been released and we assume had moved into those counties on its own. For counties in the center of the state where *E. maimaiga* had not been released but was recovered, high levels of infection were found in dense gypsy moth populations.

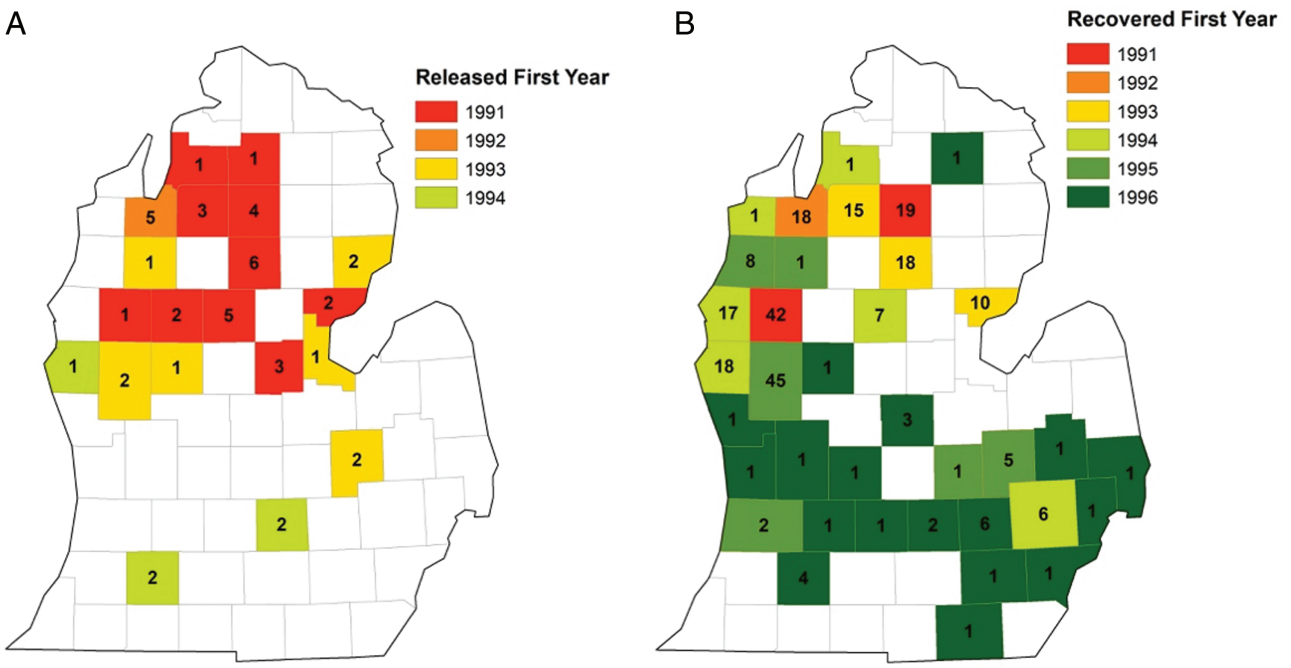

Fig. 4. Maps of *E. maimaiga* releases and recoveries in Michigan from 1991 to 1996. A. Releases. B. Recoveries. Colors of counties on the map indicate only the first year that *E. maimaiga* was released or recovered in each county while the numbers within the counties indicate the numbers of releases or recoveries in that county in subsequent years.

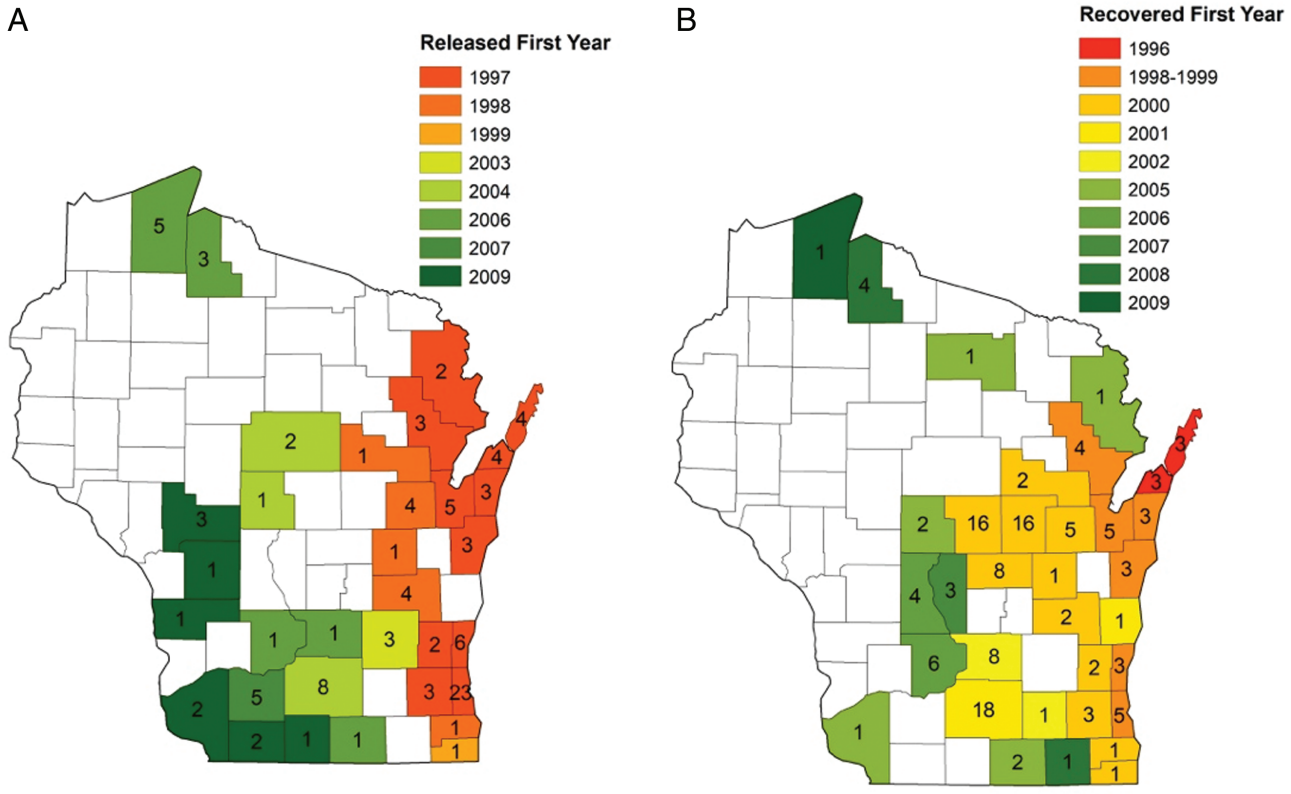

Fig. 5. Maps of *E. maimaiga* releases and recoveries in Wisconsin from 1996 to 2009. A. Releases. B. Recoveries. Colors of counties on the map indicate only the first year that *E. maimaiga* was released or recovered in each county while the numbers within the counties indicate the numbers of releases and recoveries in that county in subsequent years.

Cooperation Among States

Releases and recoveries were made by different agencies and organizations. For example, in Michigan, releases were carried out by personnel from Michigan State University in cooperation with the Michigan Department of Natural Resources and the Michigan Department of Agriculture, as well as county gypsy moth program coordinators. In many states, these activities were often not separately funded programs but instead were often initiated by land managers based on communications with others (which was usually necessary to obtain inoculum). Many of their efforts were motivated by community foresters who wanted to use all control options available toward an IPM approach. Once methods had been developed, it was common that states would assist others with more recent gypsy moth invasions. For example, Illinois and Minnesota both received assistance from Wisconsin.

Gypsy moth is more recently established in Minnesota. While *E. maimaiga* has been released in five counties (2010 and 2019) (Supp Table 2 [online only]) low gypsy moth densities (which make sampling of larval populations difficult) and limited resources for field sampling may have contributed to the lack of recoveries of infected larvae in Minnesota. As the gypsy moth range continues to spread, isolated populations are occasionally found in Kentucky, Iowa, and Tennessee but these are either eradicated or have gone extinct with no intervention. To our knowledge, no *E. maimaiga* releases have been made in these states.

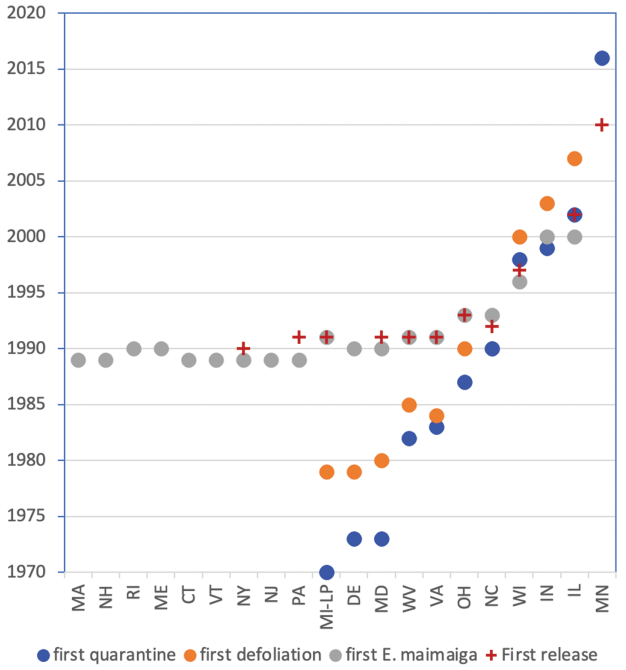

Fig. 6. Timing of gypsy moth invasion (indicated by year of first quarantine), first gypsy moth defoliation, first *E. maimaiga* release, and first *E. maimaiga* recoveries by state. States are shown in order of their initial year of first quarantine.

Discussion

Since its introduction in 1869, the gypsy moth has expanded its North American range relatively slowly; despite being present in the continent for over 150 yr, it currently is established in about 1/3 of its suitable habitat (Morin et al. 2005). This slow spread can be attributed in part to the life history of this insect (females are flightless) but also to management efforts that have successfully constrained spread (Liebhold et al. 2021). Once gypsy moth becomes established in a state, there typically is a delay of 5–10 yr before populations grow to defoliating levels (Sharov et al. 1996). When *E. maimaiga* suddenly emerged in N. America in 1989 it was quickly found within the next few years in all states that had already been previously invaded by the gypsy moth (i.e., quarantined before 1989) (Fig. 6). The only exception was Ohio, which was first quarantined in 1987 but where *E. maimaiga* was first found in 1993.

For states that were invaded by gypsy moth after 1993, there has been an approximately parallel timing of gypsy moth establishment, first defoliation and establishment of *E. maimaiga* (Fig. 6). In all cases, *E. maimaiga* has been first detected after a state has been quarantined but before it first experienced defoliation. Hajek and Tobin (2011) found that in individual stands, *E. maimaiga* could be detected within a few years of initial gypsy moth establishment. In some states, *E. maimaiga* was discovered before the releases were first made (Fig. 6).

Release studies at various locations have frequently detected *E. maimaiga* infection of gypsy moth larvae at control sites where no fungus had been released in addition to at release sites. In these studies, *E. maimaiga* appears to have spread on its own, and the infection levels at control sites were always lower than at release sites. It is consistent that *E. maimaiga* first spread naturally into Wisconsin, Illinois, and Indiana (Supp Table 2 [online only]) from neighboring states. Given this natural spread, one could ask whether the redistribution efforts were necessary after all. In fact, analysis from one study conducted in central Wisconsin between 2005 and 2007, suggested that infection levels were not related to the time since releases or distance from release sites (Hajek and Tobin 2011). However, the establishment and spread of *E. maimaiga* from releases in isolated gypsy moth populations in Michigan (Smitley et al. 1995) and the Apostle Islands in Wisconsin (Tobin and Hajek 2012), demonstrated that these releases likely accelerated the widespread establishment of *E. maimaiga* by adding inoculum to individual sites from which the fungus spread and accelerated its impacts on host populations.

Releases of *E. maimaiga* in Bulgaria provide another example, demonstrating the utility of this practice. Gypsy moth is native across Eurasia. Its frequency of outbreaking varies across Eurasia but high density, defoliating populations historically have occurred frequently throughout the Balkan Peninsula (McManus and Csóka 2007), which includes Bulgaria. *Entomophaga maimaiga* was successfully introduced for neoclassical biological control of native gypsy moth populations at 3 Bulgarian sites in 1999 and 2000. To accelerate spread and impact, from 2000 to 2014, inoculum collected from epizootics in Bulgaria and the US was released at 17 additional forested locations (Pilarska et al. 2016). Larval mortality between 80.4–100% was subsequently documented from most release sites. Releases in Bulgaria were considered so beneficial that almost no insecticides were used for gypsy moth control over this period.

The story of gypsy moth establishment and spread and resulting control efforts does not stop at the northern US border. *Entomophaga maimaiga* was first found in southeastern Ontario, next to Lake Ontario, in 1990 (Welton 1991). It had naturally spread there, as would be consistent with the detection of infected gypsy moth larvae in northeastern New York and northern Vermont in 1990 (Elkinton et al. 1991). A successful release of *E. maimaiga* was made in 1992 near Niagara Falls (Belme 1995) and releases in the mid-1990s were made on the southern shore of Lake Huron. There were unsubstantiated reports of *E. maimaiga* infections in gypsy moth populations along the

northern shores of Lake Huron, west to Lake Superior before 1994. The presence of *E. maimaiga* there and at other sites in Ontario was confirmed in 1994–1996 (Nealis et al. 1999). Further to the west in Ontario, *E. maimaiga*-infected gypsy moth larvae were first recovered in 2002 in Sault Ste. Marie, during the first year of moderate defoliation by gypsy moth (Villedieu and van Frankenhuyzen 2004).

Confirming the establishment of *E. maimaiga* is not always straightforward. Most confirmations of the presence of *E. maimaiga* made by natural resource managers have been based on the presence of late instar cadavers on tree trunks. While there is a characteristic positioning, cadavers of gypsy moth larvae killed by *E. maimaiga* can also be quite variable in positioning and appearance, especially soon after death when cadavers are internally liquid and can be confused with larvae killed by LdMNPV (Hajek 1999). While cadavers of gypsy moth larvae killed by LdMNPV are pungent and break easily, there is still a possibility for inaccuracy as *E. maimaiga* and LdMNPV can co-infect the same individual larvae. Absolute confirmation of *E. maimaiga* infection requires microscopic observations of the contents of cadavers, but this is often not possible during field surveys (Hajek and Roberts 1992).

In summary, the releases we have described are significantly different from inoculative use in greenhouses, the most commonly used type of inoculative augmentation against insect pests. Instead of releasing natural enemies that will not persist, releases were made to speed establishment in spreading populations of an invasive species and to accelerate the development of epizootics, with the goal of long-term persistence. In particular, releases and recoveries we have documented are unique because this was largely a community-driven activity by land managers and university students, and personnel that was based on communication and cooperation, rather than the release of natural enemies purchased from companies. Overall, the result was faster establishment and spread of *E. maimaiga* due to releases, and many participants reported results of high levels of infection and decreased defoliation.

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