Biological Control 63 (2012) 31–39

Contents lists available at SciVerse ScienceDirect

Biological Control

journal homepage: www.elsevier.com/locate/ybcon

Release, establishment, and initial spread of the fungal pathogen *Entomophaga maimaiga* in island populations of *Lymantria dispar*

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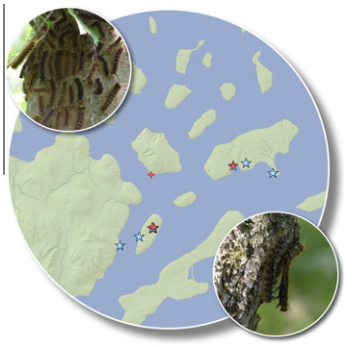
HIGHLIGHTS

- *Entomophaga maigaiga* established and spread at a mean rate of 0.8 km year^{-1}.
- Pathogen prevalence was highest on islands where pathogens had been released.
- Levels of larval parasitoids were significantly lower where pathogens were released.
- Understanding pathogen spatial and temporal dynamics optimizes their release.

GRAPHICAL ABSTRACT

Map: Recoveries of *Entomophaga maimaiga* in 2008 (red stars) and 2009 (blue stars) on the Apostle Islands National Lakeshore, Wisconsin, USA (Map Credit: Laura Blackburn). Top photo: *Lymantria dispar* larvae on Stockton Island, Wisconsin, USA, 2007 (Photo Credit: Patrick Tobin). Bottom photo: *L. dispar* larva killed by *E. maimaiga* at Rocky Arbor State Park, Wisconsin, USA, 2007 (Photo Credit: Patrick Tobin).



ARTICLE INFO

Article history:
Received 11 February 2012
Accepted 16 June 2012
Available online 23 June 2012

Keywords:
Biological invasions
Competition
Enemy release
Entomopathogen
Gypsy moth
Pathogen dispersal
Parasitoids
Agria housei

ABSTRACT

Biological invasions represent a major threat to the function and composition of ecosystems. Although the degree of invasion success of a non-native species and the consequent damage it causes can vary among and within invading species, the absence or presence of natural enemies associated with the invader can also play roles in the invasion dynamics. We used newly established, spatially isolated populations of the gypsy moth, *Lymantria dispar* (L.), on some of the islands within the archipelago of the Apostle Islands National Lakeshore in northwestern Wisconsin, USA, to study the establishment and initial spread of two releases of the entomophthoralean fungus *Entomophaga maimaiga*. We also explored patterns in rates of infection by the *L. dispar* nucleopolyhedrovirus, which was also released on three islands, and in the rates of parasitism by generalist parasitoids. The mean initial rate of spread by *E. maimaiga* following its successful establishment was 0.8 km year^{-1}, although it was detected as far as 6.1 km from a release site after one year. Infection rates by both entomopathogens were highest on those islands where they were released; however, rates of parasitism by larval parasitoids were highest where neither pathogen had been released, suggesting that pathogens outcompete larval parasitoids. Understanding the intricate relationship between an invading host species and their associated pathogens during the early stages of invasion could enhance the use of biological control in invasive species management.

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1049-9644/$ - see front matter Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.biocontrol.2012.06.004

1. Introduction

Biological invasions threaten native ecosystems and biodiversity (Liebhold et al., 1995b; Lockwood et al., 2007; Mack et al., 2000; Niemelä and Mattson, 1996), and a considerable amount of resources are allocated to their management (Holmes et al., 2009; Pimentel et al., 2000, 2005). The degree of invasion success of species can vary due to several factors, including climate suitability, resource availability, environmental and demographic stochasticity, founder population size, and Allee effects (Liebhold and Tobin, 2008; Lockwood et al., 2007; Taylor and Hastings, 2005; Tilman, 1997; Tobin et al., 2007b). In addition, the enemy release hypothesis suggests that the invasion success of a non-native species could be attributable to the lack of natural enemies in the area being invaded (Keane and Crawley, 2002). The ability of natural enemies to respond to biological invasions can thus have considerable influence on the dynamics of the invasion process (Fagan et al., 2002). We sought to investigate the establishment and spread dynamics of a fungal pathogen when experimentally released against island populations of its non-native host, *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae), within the Apostle Islands National Lakeshore in northwestern Wisconsin, USA (Fig. 1).

L. dispar is a univoltine forest defoliator that was introduced outside of Boston, Massachusetts in 1869 (Liebhold et al., 1989). Its current range in North America extends from Nova Scotia to Wisconsin, and Ontario to Virginia, but it continues to spread to the south and west (Tobin et al., 2007a). Larvae hatch from overwintering egg masses in spring, and adults emerge and mate in summer, after which females oviposit a single egg mass consisting of 200–400 eggs (Doane and McManus, 1981). Adult females in North America are not capable of flight (Keena et al., 2008). Forms of *L. dispar* dispersal include ballooning early instars and adult male flight, which generally occur over short distances, and the anthropogenic movement of life stages, which can occur over short and long distances (Liebhold et al., 1992; Tobin et al., 2007a). Because females lack flight, the detection of *L. dispar* egg masses is used as the most definitive evidence of established, reproducing populations in new areas. Egg masses were first detected on Basswood, Stockton, and Madeline Islands in August 2005, and these established populations were considerably ahead of the leading edge of the westward-spreading *L. dispar* population at the time (Tobin et al., 2010). Pheromone-baited traps (2–36 traps per year) used to detect incipient *L. dispar* populations had been deployed in the Apostle Islands National Lakeshore since 1992 (Tobin and Blackburn, 2007). The number of male moths per trap per year was 0 until 1997, but then averaged less than 1.5 moths per trap until populations sharply increased in 2004 to an average of ≈80 male moths/trap. In 2007, traps were deployed on every island (except for the small islands of Eagle, 0.11 km^2, and Gull, 0.006 km^2, which are also protected bird nesting sites), and male moth densities ranged from 10 moths/trap (on Sand Island) to >200 moths/trap on almost all other islands, including >600 moths/trap on Basswood and Stockton Islands (Supplementary data A).

Classical biological control introductions for control of *L. dispar* in North America began in 1905 but the 12 parasitoid species and one predator that became established have not provided adequate control (Hajek, 2007). Another biological control option for the management of non-native insect pests is the use of entomopathogens, many of which are host-specific (Glare, 2009; Hajek et al., 2007; Hajek and Tobin, 2010). The microbial biopesticides *Bacillus thuringiensis kurstaki* (Reardon et al., 1994) and Gypchek®, the commercial formulation of the *L. dispar* nucleopolyhedrovirus (*Ld*NPV) (Reardon et al., 1996), are both registered for use in the eradication and suppression of *L. dispar* populations (Hajek and Tobin, 2009; Tobin and Blackburn, 2007).*B. kurstaki* can kill a diversity of lepidopteran species and hence is not specific to *L. dispar*, while Gypchek® is very specific to *L. dispar* and is consequently used in ecologically-sensitive habitats where there is a potential for non-target effects against other protected Lepidoptera. Another pathogen associated with *L. dispar* is *Entomophaga maimaiga* Humber, Shimazu and Soper (Entomophthoromycota: Entomophthorales), which was probably accidentally introduced into North America from Japan (Nielsen et al., 2005) at some time after 1971 (Weseloh, 1998).

E. maimaiga is not formulated or registered as a biopesticide, yet is very specific to *L. dispar* and can cause high levels of mortality

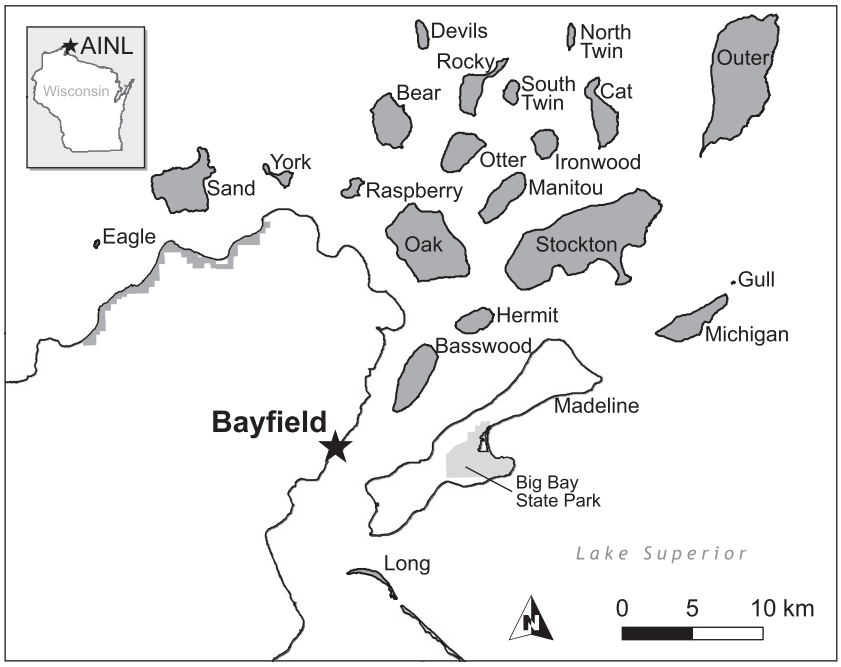

Fig. 1. Location of the Apostle Islands National Lakeshore (AINL, dark grey) and Big Bay State Park (light grey) along the Bayfield Peninsula.

(Hajek, 1999). Cadavers of late instar *L. dispar* killed by *E. maimaiga* are occasionally released by land managers to facilitate infection in newly-established *L. dispar* populations because there is a time lag between the arrival of *L. dispar* in new locations and arrival of associated pathogens (Hajek and Tobin, 2011). Past work has highlighted the effectiveness of introducing *E. maimaiga* through inoculative releases as a means to facilitate its establishment and spread (Hajek et al., 1996; Hajek and Roberts, 1991; Smitley et al., 1995). Also, a recent field study on the temporal lag between newly established populations of *L. dispar* and the arrival of entomopathogens demonstrated that although infection levels were low in newly-established host populations, host-specific pathogens moved into some host populations within 3 years, suggesting that spreading hosts were released completely from these natural enemies for a relatively short time (Hajek and Tobin, 2011). Because *L. dispar* was established in the Apostle Islands National Lakeshore ahead of its leading population front and these individual islands contained newly-established, spatially-isolated *L. dispar* populations that would not be impacted by anthropogenic disturbances (i.e., the development of lands for human use), we used this opportunity to study the establishment and initial spread of *E. maimaiga* from two point releases in the Apostle Islands National Lakeshore. Also, because *Ld*NPV had been applied against *L. dispar* on three islands in 2005, we also examined background populations of *Ld*NPV within these *L. dispar* populations, as well as larval parasitoids.

2. Materials and methods

2.1. Study site

There are 22 Apostle Islands in Lake Superior off the Bayfield Peninsula in northwestern Wisconsin, USA, of which 21 are part of the Apostle Islands National Lakeshore, along with a portion of the shoreline on the northwestern part of the Bayfield Peninsula (Fig. 1). Within this archipelago, only Madeline Island is not part of the Apostle Islands National Lakeshore and contains both private- and state-owned lands. The forests of this region can be classified generally as hemlock-white pine-northern hardwoods, and the dominant *L. dispar* host tree species (Liebhold et al., 1995a) include northern red oak, *Quercus rubra* L., and quaking aspen, *Populus tremuloides* Michaux (Supplementary data B; Beals and Cottam, 1960). In 1970, the Apostle Islands National Lakeshore was established and currently 80% of the park falls within the federally-protected Gaylord Nelson Wilderness area. Prior to the 1940s, the Islands had an extensive history of agriculture, logging, fur-trading, and quarrying (Beals and Cottam, 1960; Holzhueter, 1986).

2.2. Pre-release monitoring

A map depicting all pre-release survey locations is presented in Fig. 2. On 17–18 July 2006, we sampled sites on Basswood (2 sites), Hermit (1 site), Manitou (1 site), Michigan (1 site), Oak (1 site), and Stockton (2 sites) Islands of the Apostle Islands National Lakeshore for *L. dispar* larval populations to ascertain rates of infection by pathogens and parasitization of larvae by insect parasitoids. We also sampled sites at Big Bay State Park (1 site) and on private lands (2 sites) on Madeline Island, and on property owned by the Wisconsin Department of Natural Resources on the mainland (1 site). Sampling efforts were conducted when late instars were present. At each site, larval collections generally occurred over 2–3 ha with the exception of most privately-owned lands, which were generally 0.5–1 ha in size, with 5–15 total worker-hours spent at each site. In this year and in subsequent years, we searched the sites randomly and collected larvae from the lower bole of the tree or from the lower branches. Also, in this year and subsequent years, we submerged the soles of our shoes in a 10% bleach solution between sites to limit anthropogenic movement of pathogens as a direct result of this study.

Gypchek® was aerially applied in the spring of 2006 to approximately 7.5 (of 7.8), 1.2 (of 58.9), and 0.6 (of 40.1) km^2 of Basswood, Madeline, and Stockton Islands, respectively (Roberts et al., 2011), after the detection of egg masses in 2005. Although these applications were conducted as part of the *L. dispar* Slow-the-Spread program (Tobin and Blackburn, 2007), and hence were not a component of the present study, we monitored subsequent levels of viral infection throughout our study.

Based on the preliminary surveys in 2006, we modified our pre-release sampling protocol in 2007 to incorporate a more comprehensive and systematic approach. We also targeted our sampling efforts, using phenological predictions from BioSIM (Régnière and Sharov, 1998) and real-time weather data from Bayfield and Ashland Counties, Wisconsin, to coincide with the period of 3rd to 5th *L. dispar* instars. We timed our sampling efforts within these instars because *E. maimaiga* activity is most likely to be detected during this period (Hajek, 1999). Sites on Basswood, Hermit, Madeline, Manitou, Oak, and Stockton Islands, and on the mainland were sampled for larvae 1 or 2 times from 13 June to 20 June, and 1 or 2 times from 21 June to 29. Larval collections generally occurred over 2–3 ha with 2–6 worker-hours spent at each site each time we sampled at the site. In addition, we supplemented field collections at low-density sites with caged, laboratory-reared larvae as a means to determine rates of *E. maimaiga* infection (Hajek and Tobin, 2011). *L. dispar* larvae were obtained from a laboratory colony maintained at the USDA Animal and Plant Health Inspection Service, Center for Plant Health Science and Technology, Buzzards Bay, Massachusetts. For deploying larvae in cages, 10 newly molted 4th instars (⩽24 h since molt) were placed in a cage made by folding aluminum window screening into a pocket (20.3 × 25.4 cm) and taping and stapling the sides to prevent escape. The leaf litter was brushed away at the north side of the base of a tree, and approximately 1 liter of water was poured over the soil. The cage was placed directly on top of the organic soil layer. A larger cage fabricated from hardware cloth (mesh size = 1.2 × 1.2 cm) was placed over the screen cage to protect caged larvae from predatory small mammals. Cages were left in the field for 4 days, and were deployed four times for a total of 16 days of exposure, from 13 June to 29 June. For each exposure interval, four cages were deployed at each of three sites: one site at the Bayfield Fish Hatchery (located on the mainland) and two sites at Big Bay State Park, Madeline Island. Each time cages were deployed in the field, an additional cage of 10 larvae was maintained in the laboratory as a control to verify the lack of infection during rearing and handling. Including the sites at which we deployed caged larvae, a total of 17 unique sites were sampled in 2007.

2.3. Collection and release of E. maimaiga

In 2007, an *L. dispar* outbreak and consequent *E. maimaiga* epizootic were occurring at Rocky Arbor State Park (43.64° N, 89.81° W) in central Wisconsin. In late June, we collected 250 cadavers of *L. dispar* killed by *E. maimaiga* and filled with resting spores from this park for point releases on two islands: Basswood (at 46.86° N, 90.74° W, 237.1 m above sea level) and Stockton (at 46.92° N, 90.61° W, 213.4 m above sea level) (Fig. 2). These release sites were chosen in part due to increasing *L. dispar* populations on both of these islands (Supplementary data A). Moreover, release sites were both <50 m from locations where egg masses were detected in 2005. At each release site, 125 cadavers were released around the base of one dominant *Q. rubra*. An additional 50 *E. maimaiga* resting spore-filled cadavers were collected from Rocky Arbor State

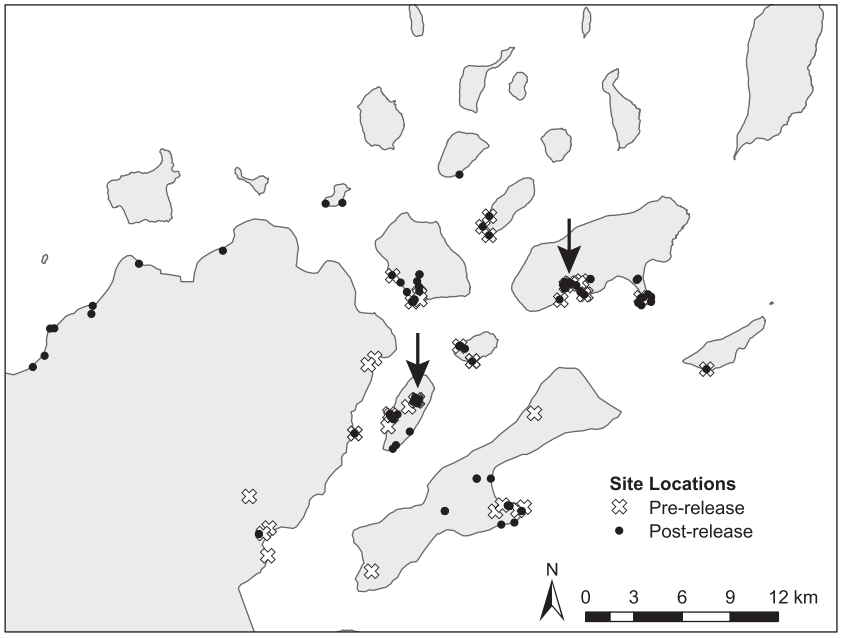

Fig. 2. Locations of gypsy moth monitoring sites before and after release of *E. maimaiga*. The arrows indicate *E. maimaiga* 2007 release locations.

Park to estimate that a total of 5.4×10^7 (SE = 1.25×10^7) resting spores were released at each site. Point releases of *E. maimaiga* resting spores on Basswood and Stockton Islands were made on 27 June and 28 June, 2007, respectively and were therefore intended to cause infections beginning in the next year.

2.4. Post-release monitoring

A map depicting all post-release survey locations is presented in Fig. 2. In both 2008 and 2009, we also timed our sampling efforts to coincide with the period of 3rd to 5th *L. dispar* instars using our 2007 method. In 2008, we sampled for live larvae and cadavers on Basswood, Hermit, Madeline, Oak, and Stockton Islands, and at the Bayfield Fish Hatchery 1 or 2 times from 9 July to 15 July, and 1 time from 16 July to 20 July. Collections generally occurred over 2–3 ha, with 2–12 total worker-hours spent each time we sampled each site. In addition to collecting all larvae and cadavers we detected during undirected searching, we implemented standardized sampling transects at the *E. maimaiga* release points on Basswood and Stockton Islands. At these sites, we collected 50 larvae from within a 10 m radius of each release point. On Basswood, we also collected up to 25 larvae along north, south, east, and west transects from the release point at approximately 25, 50, 100, and 200 m from the release point, for a total of ≈100 larvae collected along each transect. On Stockton, due to the location of the Lake Superior shoreline relative to the release site, we collected up to 25 larvae along transects emanating at 0, 60, 240, and 300°, at approximately 25, 50, 100, and 200 m from the release point, also for a total of ≈100 larvae collected along each transect. We also sampled for larvae and cadavers at more distant sites on Basswood that were ≈2100, 3200, and 1900 m from the release point, located at 194.5, 208.2, and 242.3°, respectively. On Stockton, we also sampled at ≈322, 1570, 2130, and 966 m from the release point, at 71.9°, 76.8°, 101.0°, and 200.5°, respectively. A total of 47 unique sites were sampled in 2008.

In 2009, we sampled for live larvae and cadavers on Basswood, Hermit, Madeline, Manitou, Oak, Otter, Raspberry and Stockton Islands 1 or 2 times during the week of 29 June to 4 July, and 1 or 2 times during the week of 5 July to 11 July. Larval collections generally occurred over 2–3 ha with 2–6 worker-hours total spent at each site during each sampling interval. On the mainland, we sampled at the Bayfield Fish Hatchery (1 time each week), Roy's Point Marina (2 times each week), and at several locations on Apostle Islands National Lakeshore along the northwestern shore of the Bayfield Peninsula (1 time over both weeks). Larval collections at these sites generally occurred over 0.5–3 ha with 2–6 total worker-hours spent at each site during each sampling interval. Collections from the 2008 sampling transects emanating from release points were modified in 2009: up to 120 larvae were collected from within 10 m of each release point, and up to 120 more larvae were collected along each transect vector but over an area that was ≈75–100 m from the release point. Approximately the same distant locations from release points on Basswood and Stockton Islands that had been sampled in 2008 were also sampled in 2009 (Fig. 2). A total of 48 unique sites were sampled in 2009.

2.5. Detection of pathogens and parasitoids from samples

Each field-collected larva (2006–2009) and each larva recovered from deployed cages (in 2007) was individually placed in a 29 ml clear plastic cup containing high wheat germ artificial diet (Bell et al., 1981), reared at room temperature (23 ± 1 °C), and monitored daily for death for up to 30 days. Larvae that died were checked daily for 3 days after death to detect conidial production by *E. maimaiga*. Seven-ten days after death, cadavers were stored at 4 °C for subsequent individual dissection and microscopic observation at 200–400× to diagnose cause of death. Field-collected cadavers were stored individually at 4 °C, and subsequently diagnosed microscopically to determine a cause of death. Dissected cadavers were diagnosed as death due to *E. maimaiga* if they contained characteristically shaped resting spores and death due to *Ld*NPV if, under phase contrast, shining occlusion bodies dissolved with 1 M KOH (Lacey and Brooks, 1997). Parasitoids emerging from larvae were identified based on adult flies and wasps enclosing or based on morphology of the puparia (Simons et al., 1974).

2.6. Data analyses

We compared prevalence of *E. maimaiga* or *Ld*NPV in larvae or cadavers, and rates of larval parasitism between release and

non-release areas to determine if pathogen infection rates differed based upon where *E. maimaiga* or *Ld*NPV had been released (i.e., *Ld*NPV in the form of Gypchek® and *E. maimaiga* in the form of point releases of resting spores). We also determined if rates of infection or parasitization were a function of the number of larvae collected (i.e., sampling effort). The number of larvae collected (independent variable), and the number of larvae infected by *E. maimaiga* or *Ld*NPV or parasitized (dependent variables) were transformed using $\log_{10} + 1$ to correct for normality, and subjected to a general linear model for analysis. Using samples collected following the release of *E. maimaiga* resting spores, we also quantified the distance between point releases and subsequent detections of larvae infected with *E. maimaiga* in 2008 and 2009 to estimate the initial spread of this pathogen using least squares regression, analogous to methods used to estimate *L. dispar* spread rates (Liebhold et al., 1992; Tobin et al., 2007a). All analyses were conducted in R (R Development Core Team, 2011).

3. Results

3.1. Pre-release monitoring

We collected a total of 113 and 900 *L. dispar* larvae (living larvae plus cadavers) in 2006 and 2007, respectively, and recovered a total of 575 larvae from deployed cages on Big Bay State Park and mainland sites in 2007. We did not detect *E. maimaiga* in any of these samples (Table 1). Larvae and cadavers that were collected documented a 62.8% infection rate due to *Ld*NPV in 2006, which decreased to 8% in 2007 (Table 1); all of these larvae were collected from either Basswood or Stockton Islands and within the areas that were treated with Gypchek® in 2006. We also detected larval parasitoids in both years from samples collected from Basswood, Manitou, Oak, and Stockton Islands (Table 1). Twenty-three larvae were parasitized by *Compsilura concinnata* (Meigen) (Diptera: Tachinidae), while one larva was parasitized by *Cotesia melanoscela* (Ratzeburg) (Hymenoptera: Braconidae).

3.2. Post-release monitoring

We collected a total of 2,034 live larvae and cadavers in 2008 (Table 1), of which 904 and 886 were collected specifically from the *E. maimaiga* release sites and radiating transects on Basswood and Stockton Islands, respectively. A total of 209 additional larvae were collected from distant sites on Basswood (77) and Stockton (132) Islands, while the remaining 35 larvae were collected from Oak (30) and Hermit (5) Islands. We recovered *E. maimaiga* from a total of 22 larvae, including from one and eight larvae collected from within 10 m of the Basswood and Stockton release sites, respectively (Fig. 3). Some additional larvae collected from transect samples 100 m away on Stockton, and 200 m away on Basswood, also tested positive for *E. maimaiga*. The most distant detection of *E. maimaiga* was recovered from one of the 30 larvae collected on Oak Island, ≈6.2 and 9.5 km from the respective release sites on Basswood and Stockton Islands. Rates of infection by *Ld*NPV in 2008 and 2009 were 52.5% and 39.8%, respectively (Table 1).

In 2009, we collected a total of 4,630 live larvae and cadavers (Table 1), of which 896 and 900 were from the *E. maimaiga* release sites and radiating transects from the release sites on Basswood and Stockton Islands, respectively. The remaining larvae were collected from more distant sites on Basswood (540) and Stockton (899), Oak (780), Big Bay State Park on Madeline (189), Hermit (114), Manitou (5), Otter (1), and Raspberry (1) Islands, and mainland sites (305). We detected *E. maimaiga* infections in a total of 38 larvae, of which 24 were from sites located within 100 m of 2007 resting spore release sites (Fig. 3). In addition, we detected *E. maimaiga* from distant locations on Basswood and Stockton Islands. These included a site on Stockton Island located 4.7 km from the release location on that island, and a site on the mainland located 4.4 km from the release location on Basswood (Fig. 3).

A total of 117 and 999 larvae collected in 2008 and 2009 were parasitized, respectively (Table 1). Unlike our pre-release samples in which *C. concinnata* accounted for ≈96% of parasitized larvae, the most common parasitoid in our post-release samples was *C. melanoscela* (903 parasitized larvae across both years), while *C. concinnata* was observed from a total of 113 larvae across both post-release years. Forty-five larvae were parasitized by a tachinid in the Exoristini, with identification based on puparia, and the only adult eclosing from these puparia was *Parasetigena silvestris* (Fallén) (Diptera: Tachinidae). Because *P. silvestris* is considered the most commonly occurring gypsy moth parasitoid in this tribe in North America (e.g., Fuester, 1992; Nealis et al., 1999), we hypothesize that these uneclosed puparia were *P. silvestris* in both years. In 2008, *Agria housei* Shewell (Diptera: Sarcophagidae) parasitized one larva on Oak Island, and on Stockton Island 10 puparia identical to the *A. housei* puparium emerged from gypsy moth larvae. In 8 of these instances, the host larva was also infected with *Ld*NPV. One larva parasitized by *A. housei* was also infected with *Ld*NPV and parasitized by *C. concinnata*.

3.3. Patterns of infection and parasitization

During the two years following point releases of *E. maimaiga* resting spores in 2007, the overall mean percent infection by *E. maimaiga* was very low (0.7%) but highest at sites where we released this pathogen (0.8%) relative to all other sites (0.2%). Larvae infected with *E. maimaiga* and cadavers of larvae killed by *E. maimaiga* were recovered at both release sites in both years following *E. maimaiga* release, suggesting successful establishment (Fig. 3). Across both post-release years (2008–2009), the number of larvae infected with *E. maimaiga* significantly increased with increasing sample size (e.g., numbers of larvae and cadavers collected, $F_{1,169} = 13.9$, $P < 0.01$). There was also a significant interaction effect between sample size and whether *E. maimaiga* was released or not at a site ($F_{1,169} = 4.6$, $P < 0.03$), but the main effect of site was not significant ($F_{1,169} = 0.3$, $P = 0.58$). This suggests a common intercept but different slopes when considering the relationship between the number of *L. dispar* killed by *E. maimaiga* relative to sample size for the release versus non-release sites; in other words, at low host densities where it was difficult to find larvae and cadavers for collection, infection by *E. maimaiga* did not differ between sites whether the fungus has been released or not (Fig. 4A).

At sites where Gypchek® was applied, the *Ld*NPV infection rate was 47.8% (SE = 2.0) compared to 15.1% (SE = 3.8) at sites where Gypchek® was not applied. The main effects of site (e.g., where

Table 1
Yearly summary of the number of infected or parasitized *L. dispar* larvae and cadavers prior to and following two point releases of *E. maimaiga* on Basswood and Stockton Islands.

Year	Number of larvae and cadavers collected	Number of deployed larvae[a]	Number infected with *E. maimaiga*	Number infected with *Ld*NPV	Number parasitized
Pre-release					
2006	113	0	0	71	5
2007	900	575	0	72	19
Post-release					
2008	2.034	0	22	1.067	117
2009	4.630	0	38	1.843	999

[a] Deployed larvae can only be used to detect infection by *E. maimaiga*.

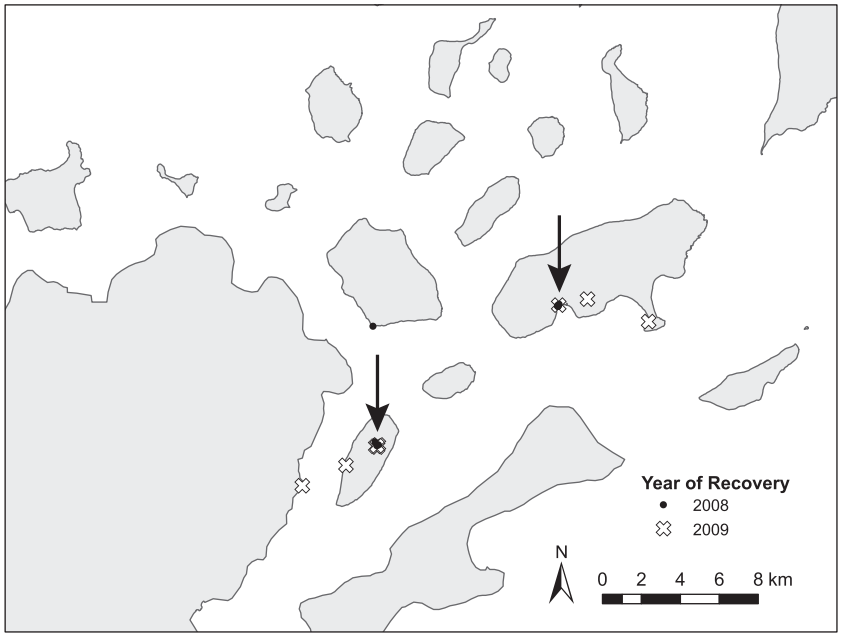

Fig. 3. Recovery of *E. maimaiga* in 2008 and 2009 relative to release sites in 2007 (denoted by arrows).

Gypchek® was applied or not; $F_{1,169} = 40.8$, $P < 0.01$), sample size ($F_{1,169} = 205.5$, $P < 0.01$), and their interaction ($F_{1,169} = 9.4$, $P < 0.01$) were all significant. In this case, and unlike our observations with *E. maimaiga* infection patterns, rates of virus infection differed significantly across the range of sample sizes, including when host densities were low (Fig. 4B). Overall, higher rates of infection by *Ld*NPV in 2008 and 2009 were observed from areas where Gypchek® was applied in 2006 relative to other areas (Fig. 4B).

In contrast, rates of larval parasitism were significantly negatively associated with the rate of infection by pathogens. The main effect of whether pathogens were released or not ($F_{1,169} = 58.6$, $P < 0.01$), sample size ($F_{1,169} = 182.5$, $P < 0.01$), and their interaction ($F_{1,169} = 4.6$, $P < 0.03$) were all significant (Fig. 4C). The overall mean percent parasitism at sites without release of either pathogen was 36.8% (SE = 5.0) compared to 9.1% (SE = 1.0) at sites receiving one or both pathogen releases.

When considering the initial rate of spread of *E. maimaiga* from release sites, all but one recovery of *E. maimaiga* occurred within 200 m 1 year following its release; the exception was the recovery of one larva from Oak Island, ≈6.1 km from the nearest release location (Figs. 3 and 5). Two years after release of *E. maimaiga*, several recoveries were made >1.5 km from release locations, including one on the mainland. Using the distance of individual *E. maimaiga* recoveries relative to the years following its release, we estimated a maximum rate of spread of 6.1 km year^{-1}, a mean rate of spread of 1.3 km year^{-1}, and a median rate of spread of 0.2 km year^{-1}.

4. Discussion

Although relatively few resting spores of *E. maimaiga* were released (e.g., 125 cadavers at each site, ≈5.4×10^7 resting spores per release site), this fungal pathogen was still able to kill and reproduce in hosts at the site of release as well as in host populations farther away, both 1 and 2 years after release. Most interesting is the detection of *E. maimaiga* on Oak Island (1 year after release) and on the mainland (2 years after release, Fig. 3), suggesting initial spread. Establishment of *E. maimaiga* after the release of similarly small amounts of inoculum has been seen in other studies (Hajek et al., 1996; Smitley et al., 1995), but the initial spread from the point releases in this study (up to 6.1 km in a year, Fig. 5) is further than recorded spread from previous studies, in which spread was limited to ⩽1 km after 2 years. We also note that in July 2006, five point releases of cadavers of larvae killed by *E. maimaiga* (two on the mainland southwest of Bayfield including one at the Bayfield Fish Hatchery, one on the mainland north of Bayfield, and two at Big Bay State Park on Madeline Island) were made by the Wisconsin Department of Natural Resources (Hajek and Tobin, 2011). Although details of these releases are lacking (e.g., number of cadavers released, where cadavers were collected), the approximate location of these releases is known. All recoveries of *E. maimaiga* from our 2008–2009 sampling efforts were closer to our release sites than the 2006 release sites used by the Wisconsin Department of Natural Resources; thus, the estimates of the initial rate of *E. maimaiga* spread can be considered to be conservative. However, it is also important to note that we never recovered, in any year, *E. maimaiga* from the Bayfield Fish Hatchery or Big Bay State Park even though we sampled these 2006 release sites each year and deployed caged larvae there in 2007.

Infection by *Ld*NPV remained high 3 years following its aerial application in 2006 (Table 1). We assume that the viral infections documented in 2007–2009 were descendants from the 2006 *Ld*NPV releases, although background levels of *Ld*NPV are thought to be present in nearly every *L. dispar* population (Elkinton and Liebhold, 1990). It is well known that *Ld*NPV is density dependent (Dwyer, 1994; Dwyer and Elkinton, 1995), which is consistent with our observations of increasing levels of infection (Table 1) with increasing host populations.

The higher rates of infection by both *E. maimaiga* and *Ld*NPV on islands where each was released relative to those areas where they were not released (Fig. 4) is intuitive. More interesting, however, is the decline in the rate of larval parasitism at sites where either pathogen was released (9.4%) compared to those sites where neither pathogen was released (36.8%) (Fig. 4). This suggests that pathogens tend to outcompete larval parasitoids, or infect larvae before parasitoids and render subsequent success of parasitization less likely. This observation is also consistent with our previous work in central Wisconsin in which the highest rates of larval parasitism were consistently observed in areas with the lowest

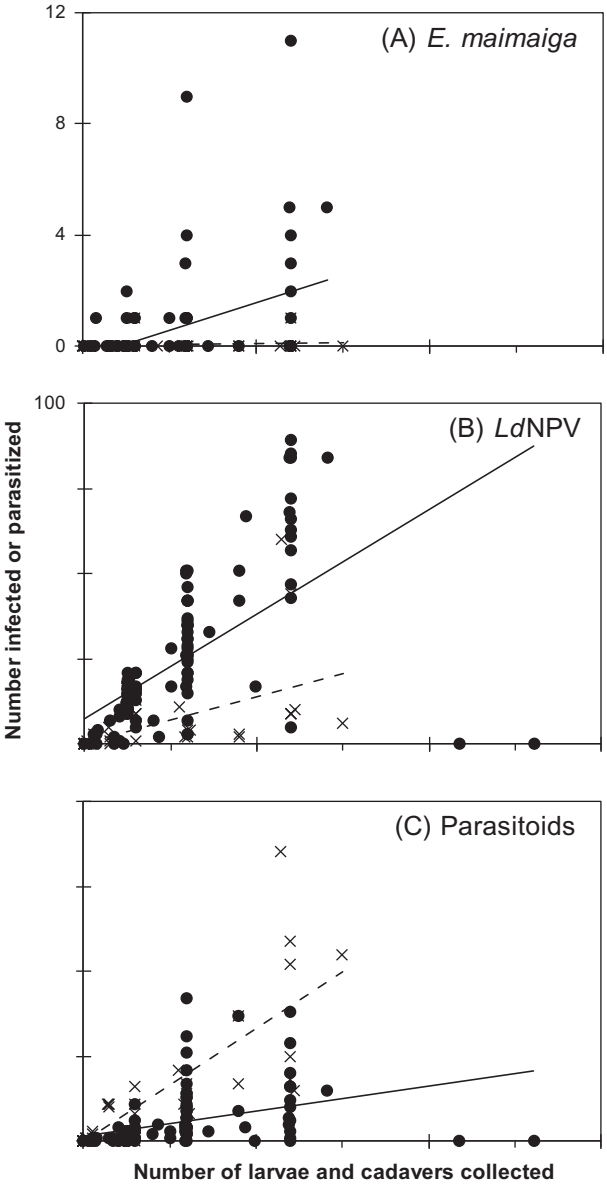

Fig. 4. Relationship between the number of larvae and cadavers infected (A and B) or parasitized (C) relative to the number of larvae and cadavers collected at each sampling site (cf. Fig. 2). Solid circles represent islands receiving point releases of *E. maimaiga* (Basswood and Stockton) (A), *Ld*NPV applied as Gypchek® (Basswood, Madeline, and Stockton) (B) or sites with one or both pathogens released (C), while crosses represent sites where neither pathogen was released. The solid and dashed lines are the regression fits for the data represented by solid circles or crosses, respectively. In the case of pathogens, higher rates of infection were observed at pathogen release sites when controlling for sampling effort, while the inverse was observed for larval parasitoids.

prevalence, and usually absence, of entomopathogens (Hajek and Tobin, 2011).

Although numerous larval parasitoids are associated with *L. dispar* (Hajek, 2007), to the best of our knowledge this is the first published recovery of *A. housei* from *L. dispar*. Results from general collections and experimentation in the 1900s with sarcophagids and *L. dispar* in New England (Patterson, 1911) and collections from weakened lepidopteran larvae from Isle Royale, Michigan (Shewell, 1971) suggested that these flies principally act as scavengers of weakened larvae or pupae (Patterson, 1911). In agreement, in our study, *A. housei* was predominantly recovered from weakened and dying larvae infected with *Ld*NPV, either alone or in combination with *C. concinnata*.

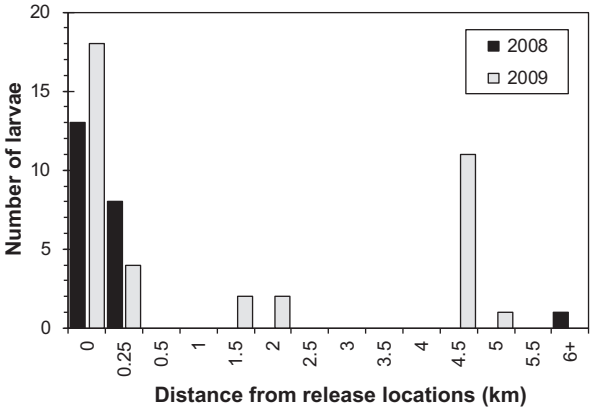

Fig. 5. Histogram of the number of larvae infected with *E. maimaiga* based upon the distance from release locations in 2008 and 2009.

Understanding the relationship between *L. dispar* and associated pathogens, specifically the lag in space and time following biological control agent releases, could facilitate the deployment of *L. dispar* pathogens against establishing host populations. In this study, we focused on the establishment and initial spread of a released entomopathogen in spatially isolated host populations that formed ahead of the advancing contiguous population front of this invasive host. We also focused our efforts on quantifying *E. maimaiga* spread from point releases prior to the natural arrival of entomopathogens from advancing populations. Our past field work in Wisconsin suggested that *E. maimaiga* invades newly-established *L. dispar* populations prior to *Ld*NPV (Hajek and Tobin, 2011). Moreover, in some cases *E. maimaiga* can follow the moving *L. dispar* population front at the same speed as the front of its host but generally lags behind by 3 years (Hajek and Tobin, 2011).

When new invaders first become established, there is often a need to determine the extent of the potential damage they may cause, and to develop effective management strategies against those invaders that pose significant threats. One management tactic is the release of non-native natural enemies against a non-native pest species, even though finding effective agents and the required non-target testing make it difficult to gain effective control very quickly using this strategy (Hoddle, 2004). In the *L. dispar* system, we studied two non-native entomopathogens attacking a non-native pest that were both introduced accidentally to North America. Future ecological studies that provide more information about the potential for each of these pathogens to cause epizootics separately as well as the effect of their interactions on the development of epizootics could enhance their use as biological control agents of *L. dispar*. Furthermore, because *L. dispar* is a non-native insect pest for which the knowledge base is comprehensive (e.g., Doane and McManus, 1981; Elkinton and Liebhold, 1990; Sharov and Liebhold, 1998) and for which there is information on the pathology and spread of its associated entomopathogens (e.g., Dwyer and Elkinton, 1995, 1988; Hajek et al., 1996, 1995; Hajek and Tobin, 2011), this system could serve as a conceptual framework from which other classical biological control programs against new forest invaders can be explored. This is especially important because for many non-native species limited information on their interactions with natural enemies exist, both in their area of endemism and their area of introduction.

Acknowledgments

We sincerely thank Joshua Hannam, Ruth Plymale, Keith Ciccaglione, and Stefan Long (Cornell University); Ken Raffa, Mario Contarini and Kyle Buechel (University of Wisconsin); and Drew

Cantis (USDA Forest Service) for valuable field and laboratory assistance. Norm Woodley of the USDA Systematic Entomology Laboratory kindly identified parasitic flies. Gypsy moth neonates were provided by John Tanner (USDA APHIS). We also thank Andrea Diss-Torrance and William McNee (Wisconsin Department of Natural Resources), Chris Lettau (Wisconsin Department of Trade and Consumer Protection), Richard Reardon and Laura Blackburn (USDA Forest Service), and Roger Fuester (USDA Agricultural Research Service) for technical assistance. We thank Julie Van Stappen (National Parks Service, Apostle Islands National Lakeshore) for all her help in facilitating this study. We are very grateful to Tim Olson (Bayfield, WI) and Jim Hughs (Ashland, WI) for providing excellent water taxi service on the often unpredictable waves of Lake Superior. This study was funded by the USDA Forest Service, Northern Research Station (05-CA11242343-044).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocontrol.2012.06.004.

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