Comparison of Aerially-Applied Gypchek Strains Against Gypsy Moth (Lepidoptera: Lymantriidae) in the Presence of an *Entomophaga maimaiga* Epizootic[1]

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Abstract The standard strain (LDP-226) of Gypchek®, a nucleopolyhedrovirus product registered by the USDA Forest Service against the gypsy moth, *Lymantria dispar* (L.), was compared against a strain, LdMNPV-203NL (NL = nonliquefying), that was developed for production in cell culture. Both strains were applied by air to U.S. government property in Prince Georges Co., MD, in early May 2003 at the rate of 1×10^{12} occlusion bodies per ha. The two goals of the study were (1) to compare the first and second wave effects of the two strains against gypsy moth populations; and (2) to delineate the combined effects of the applied virus and the expected epizootic of the gypsy moth specialist fungal entomopathogen *Entomophaga maimaiga* Humber, Shimazu, and Soper. Heavy rainfall in May and June preceded a massive epizootic of *E. maimaiga*, whose effects did not mask the first wave of viral mortality. When the effect of application sequence was considered, it was concluded that the two strains were equivalent in their first-wave impacts. High fungal-induced mid and late-season gypsy moth larval mortality suppressed the second wave of virus at all evaluation sites. There were no obvious differences in the second waves engendered by the two LdNPV strains in the greatly reduced late-instar larval population.

Key Words *Lymantria dispar*, Gypchek strains, *Entomophaga maimaiga*, gypsy moth, entomopathogenic fungus, baculovirus, biological control

Gypchek® (USDA Forest Service, WA, DC), a product with the *Lymantria dispar* (L.) multienveloped nucleopolyhedrosis virus (LdMNPV) as the active ingredient, is registered by the USDA Forest Service with the U.S. Environmental Protection Agency as a general use bioinsecticide. Recent tests with Gypchek and a new commercially produced carrier (Webb et al. 1999a) demonstrated that one application at 10^{12} polyhedral occlusion bodies (OBs) per ha yielded results statistically equivalent

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to a double split-dose application of an earlier lignosulfonate-based tank mix formulation. Although the single application of the new formulation was statistically less efficacious than a double application of the new formulation, the single dose option has favorable from economic and programmatic aspects (Webb et al. 1999a) that make the single application attractive. Previously-published work evaluated the aerial application of Gypchek against high gypsy moth populations (Podgwaite et al. 1992a,b). These studies used foliage protection and egg mass reduction as the primary indicators of application success. A study of the aerial application of Gypchek against "low-density", but measurable (59-272 egg masses per ha) populations (Podgwaite et al. 1993), used egg mass reduction and counts of live larvae under burlap bands as measures of efficacy; as might be expected in such low populations, defoliation differences between sprayed and control plots were not significant, although larval and egg mass counts were significantly lower in the treated versus control plots.

Webb et al. (1999a,b), working in populations of various densities, used the virus-induced mortality calculated for resident larvae collected 6-11 days after treatment from treated or control plots as the primary indicator of treatment success. Measuring treatment effects soon after treatment avoided the confounding influence of late-season population collapse due to the fungal entomopathogen *Entomophaga maimaiga* Humber, Shimazu, and Soper. This entomophthoralean fungus was first detected in North America in 1989, and has because become a primary natural enemy of the gypsy moth in areas where it is established. The pathology and epizootiology of *E. maimaiga* has been reviewed by Hajek (1999) who found that the implications of *E. maimaiga* on NPV epizootics to be an open question, and that many years could pass before long-term empirical evidence to suggest trends is available.

The standard strain of Gypchek used in this study was LDP226. Gypchek, as currently produced in vivo, has some limitations. It is expensive to produce, contains extraneous material, and lacks potency at doses more affordable than those currently prescribed (Podgwaite et al., in press). A strain of the gypsy moth nucleopolyhedrovirus (LdMNPV), derived from a single genotype in the mixture of closely related genotypes that comprise the active ingredients in Gypchek, has been developed. The strain, designated LdMNPV-203NL (NL = nonliquefying), has been plaque purified, is stable in cell culture and, in laboratory bioassays, is two to three times as potent as Gypchek and kills larvae 2-3 days sooner than Gypchek. Laboratory cell culture of LdMNPV-203NL results in a clean product that has the potential for industrial-scale production at a reduced cost. Reduced cost would permit a higher dosage application that putatively would improve efficacy. However, LdMNPV-203NL does have one characteristic that may bear upon its further development; larvae killed by this strain do not "liquefy" after death in the manner that is associated with many baculoviruses including LdMNPV (Shapiro et al. 1987, Volkman and Keddie 1990). LdMNPV-203NL has a 3,798 base pair genomic deletion that includes the chitinase and Bro C genes, and most of the gp37 and Bro D genes (J. Slavicek et al., unpubl. data). Whatever the cause, lack of liquefaction has potentially negative implications for the horizontal transmission of LdMNPV-203NL in the year of treatment (i.e., liquefied larvae typically supply the secondary inoculum for subsequent waves of transmission in naturally-occurring epizootics) (Woods and Elkinton 1987). This issue has been explored in field work in 2003 and 2004 by D'Amico et al. (unpubl. data) testing the degree to which virus is transmitted from LdMNPV-203NL-killed larvae versus Gypchek-killed larvae. This work indicated that if LdMNPV-203NL were to be used for gypsy moth

suppression, the second wave of mortality that comes from healthy larvae feeding on inoculum from larvae killed by the spray may well be blunted. Although this could mean more defoliation and less kill than would be seen with standard Gypchek application, this issue remains unresolved. This implies that the late timing of a spray-induced epizootic, as opposed to a naturally-occurring one, makes horizontal transmission less important to total seasonal mortality. To test the hypothesis that an LdMNPV-203NL aerial application would affect a second wave of mortality, our second experiment, reported herein, involved monitoring a set of plots within Maryland gypsy moth cooperative suppression blocks that were treated aerially either with Gypchek or with LdMNPV-203NL.

In 2003, approximately 2000 ha of U.S. Government land in Prince Georges Co., MD, were infested with gypsy moths, with a little over 1800 ha scheduled to be sprayed with Gypchek under a contract managed by the USDA Forest Service. This area included the Beltsville Agricultural Research Center (BARC) and Greenbelt National Park (GNP). It was decided to use this program to compare Strain LdMNPV-203NL against the standard Gypchek. The operational goal of the study was to compare Gypchek and Strain LdMNPV-203NL from the air. The test was to be considered successful if LdMNPV-203NL performed as well as Gypchek in the field.

Any effort to control gypsy moth populations in the eastern United States must take into account the impact of *E. maimaiga*, which was first recorded from North America in 1989 (Andreadis and Weseloh 1990, Hajek et al. 1990). This fungus over winters as resting spores (= azygospores). Under appropriate conditions in the spring, these resting spores germinate to produce a second type of spore (germ conidia) that can cause infections at any time from gypsy moth egg hatch until about 2 wks before pupation (Hajek et al. 1993, Hajek and Humber 1998). The fungus penetrates the larval cuticle and produces protoplasts and hyphal bodies that grow vegetatively in the larva, killing it in less than a week (Hajek 1999). Under appropriately moist weather conditions, hyphae grow through the outer wall of the insect, producing enormous numbers of conidia that are forcibly discharged and disseminated through the air, spreading the disease to surrounding gypsy moth populations. Infections arising from resting spores produce only conidia, never resting spores (Hajek 1997a). Hosts subsequently infected by these conidia can produce all conidia, all resting spores, or a mixture of conidia and resting spores, depending in part on host age (Hajek and Shimazu 1996, Hajek 1997b), as well as temperature, humidity, host molting status, fungal isolate, and dose (Hajek 1999). Resting spores must overwinter to be infective (Hajek and Humber 1998). Hajek (1997b) studied the coepizootics of *E. maimaiga* and LdMNPV in central New York State. Reviewing this work and that of other researchers, Hajek (1997b) suggested that the potential interference in NPV would be indirect; by decreasing host densities, thus preventing population collapse from NPV epizootics.

Experimental goals were (1) to compare the second wave levels of LdMNPV at sites treated with Gypchek versus those treated with Strain LdMNPV-203NL, and (2) to delineate the combined impacts of LdMNPV and *E. maimaiga* in the various plots.

Materials and Methods

Application parameters. The two products, the standard stain of Gypchek and the experimental strain LdMNPV, were sprayed, each at the rate of 1×10^{12} occlusion bodies per ha, by a rotary-winged aircraft equipped with a AG-NAV Differentially-

corrected Global Positioning System (Agnav Inc., Newmarket, Ontario, Canada) on 30 April 2003. The virus preparations were tank-mixed just before spray application. Strain LdMNPV-203NL was applied to 153 ha at BARC, whereas Gypchek was applied to 172 ha at BARC and GNP.

Plot establishment and characterization. Eighteen 1-ha evaluation sites were established (14 at BARC and 4 at GNP). Six sites were established in the area to be treated with the standard Gypchek, 6 sites were established in the area to be treated by the experimental strain LdMNPV-203NL, and 6 sites were established in nearby untreated woodland. Two of the control sites had to be dropped due to proximity to treatment areas and uncertainty of spray contamination, so subsequent evaluation was limited to 12 treated sites and 4 control sites. Because of differential treatment efficacy, presumably because of differences in spray coverage, the Gypchek sites were later arbitrarily split into 3 sites that received more-effective treatment (46.7-66.7% first wave mortality), designated Gypchek-A sites and 3 sites that received less-effective treatment (13.3-36.7% first-wave mortality), designated Gypchek-B sites. Egg mass numbers were estimated prior to eclosion from five 0.01-ha fixed-radius egg mass surveys per site as described by Liebhold et al. (1994).

Pretreatment assessment of naturally-occurring virus. Two days prior to virus application, 25 first-instar larvae were sampled from each plot and placed individually on artificial diet (Bell et al. 1981) in 30-ml plastic creamer cups (Solo Cup Co., Urbana, IL) with paper lids. All larvae were held on shelves in a wooden outdoor insectary (368 cm long, 215 cm wide, 92 cm deep, with hardware cloth across the front to allow natural conditions of light, temperature, and humidity but not rain). The number of larvae dead after 28 d was determined and used to calculate the percent preseason mortality for each plot. All of the larvae that died were examined in wet mounts under 400× using an Olympus BX40 (Olympus Optical Co., Tokyo, Japan) for the presence of viral OBs or spores of *E. maimaiga* (Hajek and Roberts 1992). If virus determinations could not be made with certainty using under 400×, smears of tissue samples were fixed over a flame, stained with dilute Giesma solution as per Glaser (1915), and then examined under oil emersion at 1000×. The presence of OBs confirmed the presence of LdNPV and characteristic conidia and/or azygospores (Hajek and Shimazu 1996) were considered positive for *E. maimaiga*, and the type of spore present in fungal-killed cadavers was recorded.

Treatment. Aircraft set up, atomizer calibration, spray droplet characterization, and weather data are detailed in Whiteman and Felton (2003). Treatment consisted of one application of 1×10^{12} OBs in 9.5 L of Carrier 038® formulation (Abbott Laboratories, N. Chicago, IL) per ha applied using a Bell UH-IH helicopter (Bell Helicopter Textron, Fort Worth, TX) equipped with boom carrying 8 AU 5,000 Micronair atomizers (Micronair, Newcastle under Lyme, UK) that were positioned 35° to the flight line with a variable restriction unit setting of 11. Before-treatment calibration of the aircraft delivery system and characterization of droplet deposit indicated that the required volume of spray and the desired droplet spectrum were achieved by spraying at an air speed of 137 km/h, with a boom pressure of 2.1 kg/cm^2, and using a lane separation of 46 m. The formulation handled and mixed well. The slurry for each treatment was prepared by adding the required amount of Gypchek or strain LdMNPV-203NL, formulated as a dry powder, to a measured volume of water and stirring to place the virus in suspension. The slurry was slowly added to a mix-tank in which the appropriate amount of Carrier 038 was circulating. Foliage expansion ranged from 30-70%, and insect development was determined to be 90% first and

10% second instar. Spray block parameters for each evaluation block, including egg mass density, time of spray, air temperature, relative humidity, wind speed, and wind direction, are given in Table 1.

Post-treatment effects of LdMNPV. Weekly live-larval collection 1. Four days after treatment, 30 larvae were sampled from each plot and placed individually in 30-ml diet cups half filled with gypsy moth diet. These larvae were used to measure first wave mortality. They were held in the outdoor field insectary for 30 days (or until death) and checked for mortality every 2-3 days. All cadavers were necropsied as above.

Weekly live larval collections 2-6. The viral epizootic in the plots was assessed by using the first week's mortality data from collection 1 and by making subsequent

Table 1. Spray block parameters. All sprays applied 30 April, 2003

Evaluation site	Egg mass per ha	Time of liftoff	Temp. °C	rh	Wind speed (meters/sec)	Wind direction
LdNPV-203NL Application						
S1	12,663	6:11	11	69	0	—
S2	26,673	6:11	11	69	0	—
S3	9,700	6:11	11	69	0	—
S4	2,375	6:11	11	69	0	—
S5	4,525	6:11	11	69	0	—
S6	5,825	6:11	11	69	0	—
Avg	10,293					
Gypchek Application						
G-A1	17,643	7:18	14	55	0.5 to 2	N-NW
G-A2	10,813	7:18	14	55	0.5 to 2	N-NW
G-A3	17,430	7:18	14	55	0.5 to 2	N-NW
Avg	15,295					
G-B1	18,508	8:20	16	50	1.5 to 3	N
G-B2	4,533	8:20	16	50	1.5 to 3	N
G-B3	7,220	7:18	14	55	0.5 to 2	N-NW
Avg	10,087					
Control Blocks						
C1	5,053					
C3	6,383					
C3	2,303					
C4	10,175					
Avg	5,979					

weekly collections from 13 of the 16 evaluation plots. These collections were made using methods similar to those of Woods and Elkinton (1987) and Webb et al. (1994, 1999c). We ceased to monitor 3 of the LdMNPV-203NL-treated plots either because we could find less than 5 larvae in a 30 min search after week 1or because they had insufficient foliage close to the ground to allow ready collection of larvae. Populations were sampled weekly (30 larvae per plot) for 6 wks, and each larva was placed in an individual diet cup. Larvae were held in the outdoor insectary and monitored for 1 wk until the next collection. Because larvae dying during the week in the cups should be representative of larvae dying that week in the plots, the 1-wk count from each weekly collection was used in calculating the course and size of the "second wave".

Dead larvae were labeled by date-of-examination and placed in a freezer to await necropsy. Tissue samples from all of the larvae that died were examined as previously described. Based on the number of larvae that died during the first week after each collection, season-long cumulative larval mortality (all sources) was calculated as per Wieber et al. (1995) and Webb et al. (1999c) by Eq. 1:

$$T_{mort} = 100 - [100 - (100 \times M_1) - (S_2 \times M_2) - \ldots - (S_n \times M_n)] \quad (1)$$

Where T_{mort} = % total season long mortality, M_x = proportion of mortality recorded for time period x, S_x = % survivorship at the start of time period x, and n = number of time periods.

Similarly, season-long cumulative larval mortality (NPV) was calculated by Eq. 2:

$$T_{NPV} = 100 - [100 - (100 \times NPV_1) - (S_2 \times NPV_2) - \ldots - (S_n \times NPV_n)] \quad (2)$$

Where T_{NPV} = % total season long mortality due to NPV, M_x = proportion of mortality due to NPV recorded for time period x, S_x = % survivorship at the start of time period x, and n = number of time periods. And, season-long cumulative larval mortality (*E. maimaiga* = Em) was calculated by Eq. 3:

$$T_{Em} = 100 - [100 - (100 \times Em_1) - (S_2 \times Em_2) - \ldots - (S_n \times Em_n)] \quad (3)$$

Where T_{Em} = % total season long mortality due to *E. maimaiga*, M_x = proportion of mortality due to *E. maimaiga* recorded for time period x, S_x = % survivorship at the start of time period x, and n = number of time periods. Cadavers with mixed infections were scored positive for both pathogens unless otherwise stated.

Weather data. Weather data for April, May and June were reported from BARC Weather Station Number 1 at the dairy complex off of Powder Mill Road, which was central to the various sites.

Statistical analysis. Percent mortality data, expressed as proportions, were arcsine-squareroot transformed prior to analysis of variance (ANOVA) using PROC GLM of the statistical software SAS ver 6.12 (SAS Institute 1989-1996). Means were separated using the least significant difference (LSD) procedure at a comparison-wise error rate of 0.05.

Results and Discussion

Pretreatment assessment. Preseason egg mass counts were high at all evaluation sites, averaging 10,293, 15,295, 10,087, and 5,979 egg masses per ha, respectively, for the LdMNPV-203NL sites, Gypchek-A sites, Gypchek-B sites, and the un-

treated control sites. Six of the 350 larvae collected pretreatment from the sites died within 5 wks of collection. One of these 6 proved positive for NPV, one was parasitized, and 4 died of undetermined causes. The larva positive for NPV was from control site 2. These results indicate that there was little natural virus in the evaluation sites prior to treatment. There was no indication of early-season *E. maimaiga* activity at this time.

First wave mortality due to NPV, and application effects. First wave mortality was assessed on the basis of cumulative mortality due to NPV of collection 1 larvae followed for 5 wks. Per cent NPV-induced mortality averaged 62.2% in the LdMNPV-203NL-treated plots (range = 50%-73.3%) (Table 2). NPV-induced mortality in the Gypchek-treated plots varied from 13.3% to 66.7%. Examination of the application parameters in Table 1 in concert with the data in Table 2 indicates that the LdNPL-203NL was applied under excellent conditions as the first load of the morning. We concluded that the Gypchek sites received a variable application. We felt that G-A1, G-A2, and G-A3, which were applied during the second load of the morning, received an acceptable application, with NPV-induced mortality averaging 58.9% (range = 46.7%-66.7%). Two of the other 3 Gypchek-treated sites, G-B1 and G-B2, were applied later in load 3 under still favorable conditions, but with a little more heat, a little less humidity, and a bit more wind as recorded in Table 1. These plots were both small 1-ha plots that were treated in 1 (G-B2) or 3 (G-B1) passes of the spray ship. GB-1 was on the top of a hill and may have had an abnormal wind shear; an inversion layer cannot be ruled out. Site G-B3 was treated in load 2, but this site was on the extreme north edge of a block treated with the wind coming from the north, possibly resulting in a suboptimal spray deposition. Average NPV-induced mortality at these three Gypchek-B sites was 25.6% (range = 13.3%-36.7%), whereas average NPV-induced mortality at 4 untreated control sites was 6.7% (range = 0%-16.7%). Treatment effects for the four treatments (six LdMNPV-203NL-treated sites, three Gypchek-A sites, three Gypchek-B sites, and four untreated control sites) were significant

Table 2. Total LdNPV–induced mortality over a 5-wk period among larvae collected during the first wk after treatment. There were 30 larvae collected per site

LdNPV203NL sites		Gypchek A sites		Gypchek B sites		Control sites	
Evaluation site	% NPV	Evaluation site	% NPV	Evaluation site	% NPV	Evaluation site	% NPV
S1	50.0	G-A1	66.7	G-B1	26.7	C1	6.7
S2	73.3	G-A2	46.7	G-B2	36.7	C2	3.3
S3	73.3	G-A3	63.3	G-B3	13.3	C3	0.0
S4	50.0					C4	16.7
S5	60.0						
S6	66.7						
Avg	62.2 a		58.9 a		25.6 b		6.7 c

Means within a row followed by the same letter are not statistically different at the 0.05 level.

(F = 25.2; df = 3,11; P < 0.0001). The results from the LSD procedure indicated the following differences among the means: LdMNPV-203NL = Gypchek-A sites > Gypchek-B sites > controls. These differences indicate that LdMNPV applied under excellent conditions is statistically equivalent to Gypchek applied under similar conditions.

Post-treatment mortality followed for 5 weeks. The course of total mortality, for wk 1 through wk 5, is given in Fig. 1. Treatment effects (F = 61.3; df = 3,18; P < 0.0001), week (F = 167.3; df = 4,27; P < 0.0001), and treatment*week interaction (F = 4.8; df = 12,27; P < 0.0004) were all significant. Because the treatment*week interaction was significant, we analyzed each week separately. There were significant differences among treatment means on every week except week 5.

Weekly mortalities from the two pathogens are given for 5 wks after Gypchek/LdMNPV-203NL applications for the four treatment groups (Fig. 2). Cadavers with dual infections were counted for both pathogens. The *E. maimaiga* epizootic was similar to that recorded by our group during 1995 at Lexington, VA (Webb et al. 1999c) and consistent with the model of Malakar et al. (1999a). The fungus was essentially absent from all sites during the first 2 wks after treatment with Gypchek. *Entomophaga maimaiga* spores appeared in a few cadavers from all sites in the week 3 collections, reaching high levels at all sites in the week 4 to week 6 collections. NPV levels averaged 20-40% during the first 2 wks at the LdNPV-203NL and Gypchek-A sites, representing the first wave resulting from the virus application. NPV levels were considerably lower at the Gypchek-B sites and virtually absent from control sites at this time. NPV fell to low levels during weeks 3 and 4 at all treated sites, rising slightly during week 5 in what would have been a second wave of virus had not *E. maimaiga* severely reduced gypsy moth population levels by this time. The plot of the NPV epizootic is similar to the virus epizootics depicted by Dwyer and Elkinton (1993).

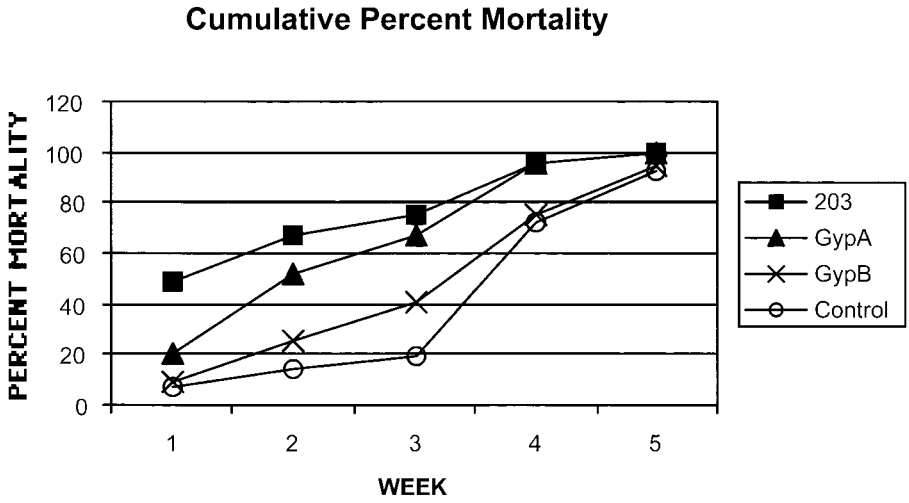

Fig. 1. Average cumulative percent mortality (all sources) calculated weekly for four treatment classes for 5 weeks posttreatment. Mortality recorded for the first week after collection only.

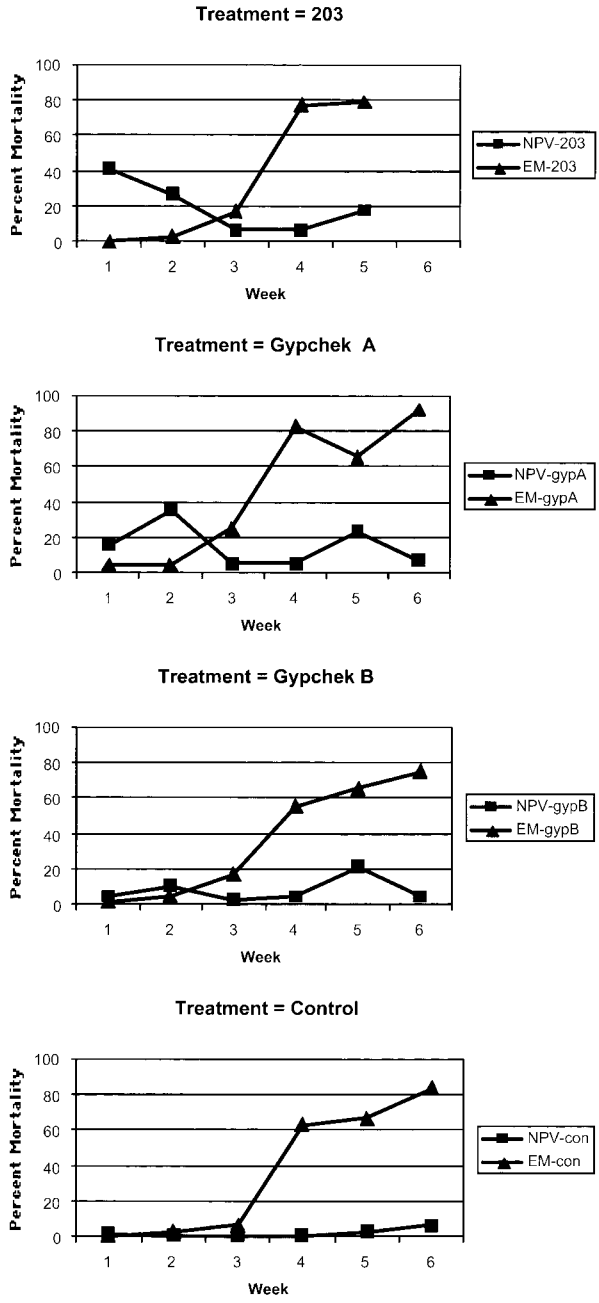

Fig. 2. Mortality from NPV and *Entomophaga maimaiga* calculated weekly for four treatment classes for 6 weeks posttreatment. Mortality recorded for the first week after collection only.

However, the timing is different, because our first wave began with the virus application whereas their first wave resulted from larvae chewing their way out of NPV-contaminated egg masses. Also, our second wave was attenuated by the *E. maimaiga* epizootic, which played no roll in the models of Dwyer and Elkinton (1993). Though the data are limited in the numbers of larvae collected (30 larvae/plot/week), the results indicated that post treatment viral dynamics were similar in both Gypchek and LdNPV-203NL plots (note data from week 5 where a second wave of mortality clearly is seen for both treatments). This indicates that "within generation" disease dynamics resulting from Gypchek and LDNPV-203NL treatments would be similar.

The total 5-wk cumulative mortality, and cumulative mortality due to nucleopolyhedrosis virus (NPV), *E. maimaiga* (Em), double infections (NPV + Em), and other mortality sources (other), based on the first week mortality from five weekly collections for the four treatment classes, are given in Table 3. By 5 wks after treatment, cumulative mortality was high in all plots, including the controls. However, a statistically significant treatment effect ($F = 12.5$; df = 3,9; $P < 0.0015$) was found for total mortality. Total mortality recorded and the statistical separation were 99.6% at 203 sites = 99.2% at Gypchek-A sites >94.8% at Gypchek-B sites = 92.4% at control sites. Whereas the total mortality was similar for all treatments, the relative contribution of NPV and *E. maimaiga* to total mortality varied among the treatments. Treatment effects were significant for both NPV ($F = 32.4$; df = 3,9; $P < 0.0001$) and *E. maimaiga* ($F = 11.5$; df = 3,9; $P = 0.0020$). Total mortality due to NPV (first and second wave combined) and the statistical separation were 57.8% at LdNPV-203NL sites, = 43.8% at Gypchek-A sites, >20.5% at Gypchek-B sites, and >3.7% at control sites. Total mortality due to *E. maimaiga* and the statistical separation were 28.7% at LdNPV-203NL sites, = 44.7% at Gypchek-A sites, <64.2% % at Gypchek-B sites, and = 78.2% at control sites. A few cadavers in all treatment categories had double infections (Table 3), but the treatment effect for double infections was not significant. Likewise, a few cadavers (designated as "other") in all treatment categories were negative for both pathogens (Table 3), but the treatment effect for "other" was also not significant at $\alpha = 0.05$.

Weather conditions at BARC, Spring 2003. Spring 2003 was cooler than normal at BARC. Average temperature (departure from normal) was 11.2°C (–0.6°C) for

Table 3. Total five-week cumulative mortality, and cumulative mortality due to nucleopolyhedrosis virus (NPV), *E. maimaiga* (Em), double infections (NPV + Em), and other mortality scores (other), based on mortality from five weekly collections, for four treatment classes

Treatment	Total dead	NPV	Em	NPV + Em	Other
203NL*	99.6 a	57.8 a	28.7 a	2.2	10.9
Gypchek A	99.2 a	43.8 a	44.7 a	6.7	4.0
Gypchek B	94.8 b	20.5 b	64.2 b	4.9	5.2
Control sites	92.4 b	3.7 c	78.2 b	1.6	9.0

Thirty larvae collected per site per week. Mortality recorded for the first week after collection only. Means within a column followed by the same letter are not statistically different at the 0.05 level.

* Mortality followed in only 3 of the 6 plots treated with 203 (sites S-1, S-2, S-3).

April, 15.6°C (–1.6°C) for May, and 21.1°C (–1.1°C) for June. Rainfall was fairly low for April, but above normal for May and June. Average rainfall (departure from normal) was 6.4 cm (–2.1 cm) for April, 17.5 cm (+5.9 cm) for May, and 18.5 cm (+9.4 cm) for June. The first significant period of rain that would activate the resting spores was probably a 3.6-cm event occurring 6-11 May. However, it was the 13.6-cm of rain that occurred between 16-30 May (on 12 out of 15 days) that probably accounted for the rapid increase in fungal kill noted during this period (Figs. 1, 2). Cooler than normal temperatures would have delayed larval development, giving more time for the viral and fungal epizootics to develop, whereas the higher than normal rainfalls for May and June would have promoted the fungal epizootic (Malakar et al. 1999a and numerous citations therein). However, cool weather would retard the germination of the fungal resting spores and slow the development of both the fungus and the NPV in the insect. Although *E. maimaiga* develops much faster than NPV in gypsy moth larvae, it has been established that NPV and *E. maimaiga* can coinfect if the virus gets a head start (Malakar et al. 1999b). The relatively dry April would have given the virus such a start.

Spore types found in cadavers. Although the first wave of NPV (collections 1 and 2) arose from an application rather than naturally through contaminated egg masses, most of the early mortality due to NPV occurred in instars 1 and 2 (Table 4). This reflected the fact that the application was made to first-instar caterpillars soon after egg hatch. Few cadavers contained *E. maimaiga* spores during the early collections, so that the fungus had little apparent impact on the first wave of NPV. This is in accordance with the predictions of Malakar et al. (1999a). However, the severe population reduction caused by the *E. maimaiga* epizootic later in the season should have negatively impacted the second wave of NPV (Anderson and May 1981, Onstad 1993). Moreover, 40 of the 65 cadavers positive for NPV in the second wave (collections 4-6) were coinfected with *E. maimaiga*. (Collection 6 was taken on June 12 and has not been previously mentioned because only a few of the sites, 4 treated and all 4 control sites, still had collectable populations). Because NPV-infected cadavers coinfected with *E. maimaiga* contain fewer OBs than cadavers containing NPV alone (Malakar et al. 1999b), this putatively would represent an additional negative impact of the fungus on the viral second wave. By the same reasoning, one would suppose that the early-season gypsy moth population reduction would have a negative impact on the *E. maimaiga* epizootic. However, this is not apparent in the late-season fungal epizootic recorded in Fig. 2. Moreover, of the 617 cadavers from collections 4-6 positive for *E. maimaiga*, only 40 were coinfected with NPV, indicating little interference of the developing fungal epizootic by the viral second wave.

The pattern of spores in the cadavers infested with *E. maimaiga* was instructive (Table 4). If just the pattern of spore production is considered, this *E. maimaiga* epizootic was more similar to that recorded during 1996, a relatively wet year at Lexington, VA, than for 1995, a relatively dry year (Webb et al. 1999c). In 1995, numerous cadavers containing azygospores were recorded mid-to-late season, whereas in 1996, no azygospores were found until late in the season. In the present study, of the 363 cadavers from collections 1-4 positive for *E. maimaiga*, 362 contained only conidia, and 1 contained a combination of conidia and resting spores. Moreover, 172 of 204 cadavers in collection 5 that were positive for *E. maimaiga* contained only conidia. Only in Collection 6 did resting spores predominate. As seen in Fig. 1, cumulative larval mortality by the end of collection 4 ranged from 72% (control sites) to 95.5% (Gypchek A sites), whereas cumulative larval mortality by the

Table 4. Number of gypsy moth larvae dying by instar, disease agent (NPV = nucleopolyhedrosis virus, Em = *E. maimaiga,* and NPV + Em = co-infection), and *E. maimaiga* spore type (c = conidia, r = resting spore)

	Gypsy moth larval instar						*E. maimaiga* spore type in cadavers			
Collection agent	1	2	3	4	5	6	c	c, r	r	Total
1 (May 8) NPV	34	39	1							74
Em	2	4	3				9			9
NPV + Em		1					1			1
2 (May 15) NPV	3	65	19	2						89
Em		7	9	2			18			18
NPV + Em		2					2			2
3 (May 22) NPV		2	4	4						10
Em		1	24	30	2		57			57
NPV + Em			2	2			4			4
4 (May 29) NPV			5							5
Em		1	44	169	45	2	260	1		261
NPV + Em			4	7			11			11
5 (June 5) NPV		1	3	11	2					17
Em			2	57	129	16	172	27	5	204
NPV + Em				15	9		23		1	24
5 (June 12) NPV				1	2					3
Em				2	84	26	11	59	42	112
NPV + Em					4	1	2	1	2	5

Larvae are from 6 weekly collections from untreated plots and plots treated with aerial applications of NPV. Data are from larvae dying during the first week after collection only.

end of collection 5 ranged from 92.4% (control sites) to 99.6% (strain 203 sites). Although *E. maimaiga* killed a high proportion of the gypsy moth population, the titer of resting spores produced to carry on future epizootics, whereas still substantial, will be considerably reduced over that expected during drier years. This is because most *E. maimaiga*-induced mortality occurs late in the season, resulting in late-instar cadavers filled with resting spores.

Comparison of Gypchek and strain LdMNPV-203NL. Gypchek and strain LdNPV-203NL gave equivalent control when both were applied under appropriate conditions (Gypchek-A sites). The level of control recorded in this study was similar to that in our previous reports. Webb et al. (1999a) found that a single application of Gypchek applied experimentally in Virginia under excellent conditions resulted in a 68% first wave infection (compared with 80-86% when applied twice with a split dose). Webb et al. (1999b) found that a single application of Gypchek applied experimentally

in West Virginia under excellent conditions resulted in a 67% first wave infection, whereas providing only a 50% first wave when applied under marginal conditions in Maryland. In Wisconsin studies, a single application of Gypchek resulted in first wave mortality ranging from 67% for the first load out at 6:05 AM down to 24% for the last load out at 11:15 AM (Webb et al. 2004b). A decrease in NPV efficacy was correlated with increasing temperature and wind speed and decreasing relative humidity. Thus, the first wave seen for LdMNPV-203NL in the present study is consistent with our previous work with Gypchek and we may conclude that the two strains give equivalent first wave efficacy.

The lower first-wave NPV-induced mortality seen in the Gypchek-B sites was fortuitous in that we were able to follow second wave development, and interactions with the developing fungal epizootic, at sites receiving favorable treatment and at sites receiving less favorable treatment. As seen in Fig. 2 and Table 3, the *E. maimaiga* epizootic largely compensated for the less efficacious virus application.

Control implications. By the end of the week after collection 6, larvae at all sites were below detectable or collectable levels, and first pupation had yet to occur. Heavy rains continued throughout June, further aiding the fungus. Any surviving larvae would have been subject to additional mortality. No defoliation was noted at any site. A postseason egg mass survey was attempted. No obviously new egg masses were seen, but some of the egg masses high in the trees had not sufficiently weathered to rule out being new, and we abandoned the survey attempt. The official USDA Forest Service survey found no new egg masses on BARC or in GNP. Clearly, outstanding control occurred. During this rainy spring, outstanding control would have occurred due to the fungus whether the virus was sprayed or not. However, during years with normal rainfall, such as occurred in Lexington, VA in 1995 (Webb et al.1999c), *E. maimaiga*-induced mortality will be considerably less, and will occur late in the season after much gypsy moth larval feeding. In an analysis of gypsy moth mortality along Skyline Drive in Virginia during 2000, a year of normal rainfall, Webb et al. (2004a) calculated that close to 22% of the larval population survived to adulthood at favorable locations, with moderate to heavy defoliation occurring in spots. Larval mortality, due largely to a late-season *E. maimaiga* epizootic, was calculated at about 29%. During such years, the level of early season suppression due to the applied virus reported herein, combined with a considerable late-season *E. maimaiga* epizootic expected even in years of normal rainfall, would likely be sufficient to prevent serious defoliation even at large gypsy moth population levels. Thus, the applied Gypchek + natural *E. maimaiga* system, which essentially kills only gypsy moths, can be considered an outstanding example of applied biological control in areas where the fungus is established.

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References Cited

Anderson, R. M. and R. M. May. 1981. The population dynamics of microparasites and their invertebrate hosts. Philos. Trans. R. Soc. Lond. B Biol. Sci. 291: 451-524.

Andreadis, T. G. and R. M. Weseloh. 1990. Discovery of *Entomophaga maimaiga* in North American gypsy moth, *Lymantria dispar.* Proc. Natl. Acad. Sci. USA 87: 2461-2465.

Bell, R. A., C. D. Owens, M. Shapiro and J. G. R. Tardif. 1981. Development of mass rearing technology, Pp. 599-633. *In* C. C. Doane and M. L. McManus [eds.], The gypsy moth: research toward integrated pest management. U.S.D.A. Tech. Bull. 1584.

Dwyer, G. and J. S. Elkinton. 1993. Using simple models to predict virus epizootics in gypsy moth populations. J. Anim. Ecol. 62: 1-11.

Glaser, R. W. 1915. Wilt of gipsy moth caterpillars. J. Agric. Res. 4: 101-128.

Hajek, A. E. 1997a. *Entomophaga maimaiga* reproductive output is determined by the spore type initiating the infection. Mycol. Res. 101: 971-974.

1997b. Fungal and viral epizootics in gypsy moth (Lepidoptera: Lymantriidae) populations in central New York. Biol. Control 10: 58-68.

1999. Pathology and epizootiology of *Entomophaga maimaiga* infections in forest Lepidoptera. Microbiol. Mol. Biol. Rev. 1999: 814-835.

Hajek, A. E. and R. A. Humber. 1998. Formation and germination of *Entomophaga maimaiga* azygospores. Can. J. Bot. 75: 1739-1745.

Hajek, A. E., R. A. Humber, J. S. Elkinton, B. May, S. R. A. Walsh and J. C. Silver. 1990. Allozyme and restriction fragment length polymorphism analysis confirm *Entomophaga maimaiga* responsible for 1989 epizootics in North American gypsy moth populations. Proc. Natl. Acad. Sci. USA 87: 6979-6982.

Hajek, A. E., T. S. Larkin, R. I. Carruthers and R. S. Soper. 1993. Modeling the dynamics of *Entomophaga maimaiga* (Zygomycetes: Entomophthorales) epizootics in gypsy moth (Lepidoptera: Lymantriidae) populations. Environ. Entomol. 22: 1172-1187.

Hajek, A. E. and D. W. Roberts. 1992. Field diagnosis of gypsy moth (Lepidoptera: Lymantriidae) larval mortality caused by *Entomophaga maimaiga* and the gypsy moth nuclear polyhedrosis virus. Environ. Entomol. 21: 706-713.

Hajek, A. E. and M. Shumazu. 1996. Types of spores produced by *Entomophaga maimaiga* infecting the gypsy moth, *Lymantria dispar.* Can. J. Bot. 74: 708-715.

Liebhold, A., K. Thorpe, J. Ghent and D. B. Lyons. 1994. Gypsy moth egg mass sampling for decision-making: a users' guide. USDA Forest Service, Forest Health Protection, Northeastern Area, Southern Region, NA-TP-04-94.

Malakar, R., J. S. Elkinton, S. D. Carroll and V. D'Amico. 1999a. Interactions between two gypsy moth (Lepidoptera: Lymantriidae) pathogens: nucleopolyhedrosis virus and *Entomophaga maimaiga* (Zygomycetes: Entomophthorales): Field studies and a simulation model. Biol. Control 16: 189-198.

Malakar, R., J. S. Elkinton, A. E. Hajek and J. P. Burand. 1999b. Within-host interactions of *Lymantria dispar* (Lepidoptera: Lymantriidae) nucleopolyhedrosis virus and *Entomophaga maimaiga* (Zygomycetes: Entomophthorales). J. Invertebr. Pathol. 73: 91-100.

Onstad, D. W. 1993. Thresholds and density dependence: the roles of pathogen and insect densities in disease dynamics. Biol. Control 3: 353-356.

Podgwaite, J. D., R. C. Reardon, G. S. Walton, L. Venables and D. M. Kolodny Hirsch. 1992a. Effects of aerially applied Gypchek on gypsy moth (Lepidoptera: Lymantriidae) populations in Maryland woodlots. J. Econ. Entomol. 85: 1136-1139.

Podgwaite, J. D., R. C. Reardon, G. S. Walton and J. Witcosky. 1992b. Efficacy of aerially applied Gypchek against gypsy moth (Lepidoptera: Lymantriidae) in the Appalachian highlands. J. Entomol. Sci. 27: 337-344.

Podgwaite, J. D., N. R. Dubois, R. C. Reardon and J. Witcosky. 1993. Retarding outbreak of low-density gypsy moth (Lepidoptera: Lymantriidae) populations with aerial applications of Gypchek and *Bacillus thuringiensis.* J. Econ. Entomol. 86: 730-734.

Podgwaite, J., R. Webb, J. Slavicek, K. Thorpe, V. D'Amico, B. Onken, R. Reardon, R. Fuester, J. Swearingen, R. Peiffer and M. Valenti. Improving Gypchek: Field evaluations of LdNPV-203. Proc. 2003 Ann. Gypsy Moth Rev., Grand Rapids, MI, Nov. 3-6, 2003. In Press.

SAS Institute. 1989-1996. SAS version 6.12. SAS Institute Inc., Cary, NC, USA.

Shapiro, M., H. F. Preisler and J. L. Robertson. 1987. Enhancement of baculovirus activity on gypsy moth (Lepidoptera: Lymantriidae) by chitinase. J. Econ. Entomol. 80: 1113-1116.

Volkman, L. E. and B. A. Keddie. 1990. Nuclear polyhedrosis pathogenesis. Semin. Virol. 1: 249-256.

Webb, R. E., M. W. Bair, G. B. White and K. W. Thorpe. 2004a. Expression of *Entomophaga maimaiga* at several gypsy moth (Lepidoptera: Lymantriidae) population densities and the effect of supplemental watering. J. Entomol. Sci. 39: 223-234.

Webb, R. E., M. Shapiro, J. D. Podgwaite, R. L. Ridgway, L. Venables, G. B. White, R. J. Argauer, D. L. Cohen, J. Witcosky, K. M. Kester and K. W. Thorpe. 1994. The effect of optical brighteners on the efficacy of gypsy moth (Lepidoptera: Lymantriidae) nuclear polyhedrosis virus in forest plots with high or low levels of natural virus. J. Econ. Entomol. 87: 134-143.

Webb, R. E., K. W. Thorpe, J. D. Podgwaite, R. C. Reardon, G. B. White and S. E. Talley. 1999a. Field evaluation of Gypchek (a nuclear polyhedrosis virus product) against the gypsy moth (Lepidoptera: Lymantriidae). J. Entomol. Sci. 34: 72-83.

1999b. Efficacy of Gypchek against the gypsy moth (Lepidoptera: Lymantriidae), and residual effects in the year following treatment. J. Entomol. Sci. 34: 404-414.

Webb, R. E., G. B. White, K. W. Thorpe and S. E. Talley. 1999c. Quantitative analysis of a pathogen-induced premature collapse of a "leading edge" gypsy moth (Lepidoptera: Lymantriidae) population near Lexington, Virginia. J. Entomol. Sci. 34: 84-100.

Webb, R. E., G. B. White, T. Sukontarak, J. D. Podgwaite, D. Schumacher, A. Diss and R. C. Reardon. 2004b. Biological efficacy of Gypchek against a low- density leading-edge gypsy moth population. North. J. Appl. For. 21: 144-149.

Whiteman, R.L. and K. Felton. 2003. Gypsy moth suppression project summary at Beltsville Agricultural Research Center, Goddard Space flight Center, James J. Rowley Training Center, Baltimore-Washington Parkway, Greenbelt Park, National Plant Germplasm and Biotechnology Lab, National Plant Materials Center, Patuxent Research Refuge, Catoctin Mountain Park, Monocacy National Battlefield. April-May, 2003. Forest Health Protection, USDA Forest Service, Morgantown, WV.

Wieber, A. M., R. E. Webb, R. L. Ridgway, K. W. Thorpe, R. C. Reardon, D. M. Kolodny Hirsch and K. M. Tatman. 1995. Effect of seasonal placement of *Cotesia melanoscela* (Hymenoptera: Braconidae) on its potential for effective augmentative release against *Lymantria dispar* (Lepidoptera: Lymantriidae). Entomophaga 40: 281-292.

Woods, S. and J. S. Elkinton. 1987. Bimodal patterns of mortality from nuclear polyhedrosis virus in gypsy moth (*Lymantria dispar*) populations. J. Invertebr. Pathol. 50: 151-157.