Comparison of the Activities of Three LdMNPV Isolates in the Laboratory Against the Chinese Strain of Asian Gypsy Moth

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Abstract: Three isolates of gypsy moth [*Lymantria dispar* (L.)] nucleopolyhedrovirus (LdMNPV), from China (LdMNPV-H), Japan (LdMNPV-J) and the registered strain from North America (LdMNPV-D), were bioassayed in the Inner Mongolia Autonomous Region, People's Republic of China, in 2004, using a modified version of the diet plug method, to determine their pathogenicity and virulence based on the dose- and time-response of second-instar larvae of the Chinese strain of the Asian gypsy moth. Results showed that LdMNPV-H and LdMNPV-D did not differ significantly in dose-response. The LD_{50} and LD_{95} for LdMNPV-H were 211 and 1414 OBs $larva^{-1}$, respectively, while LdMNPV-D had a somewhat lower LD_{50} (194 OBs $larva^{-1}$) and a higher LD_{95} (1705 OBs $larva^{-1}$). LdMNPV-J was the least pathogenic, with a LD_{50} of 940 OBs $larva^{-1}$ and LD_{95} of 11 457 OBs $larva^{-1}$. Overall, LdMNPV-H and LdMNPV-D had similar pathogenicity against second-instar larvae of the Chinese strain of the Asian gypsy moth, and were considerably more pathogenic than LdMNPV-J. Time-responses were not significantly different among the three virus strains.

Keywords: Baculovirus, isolates, pathogenicity, virulence, *Lymantria dispar*.

INTRODUCTION

The gypsy moth, *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae), is a polyphagous defoliator of Eurasian origin known to feed on over 300 species of trees and shrubs. Oaks (*Quercus* spp.) are the favoured hosts [1]. Two strains of gypsy moth are commonly recognized in the literature, the European strain, originating from Europe (including European Russia) and North Africa, and the Asian strain from central and eastern Asia [2].

The European strain of the gypsy moth (EGM) was brought to North America in 1869 in an attempt to improve silk production. However, a few specimens accidentally escaped during a storm and became established in the north-eastern United States (US) [3]. The spread of the EGM in the US is well-documented, and this strain now occurs throughout the north-eastern US [4, 5]. EGM has also spread into Canada and is now found from Nova Scotia to mid-western Ontario, causing light to severe defoliation over large areas in Ontario and Quebec [6].

Several methods and various control agents have been used in the US, first to control it, then to slow down the spread of the gypsy moth [4, 7, 8]. During its periodic outbreaks, widespread defoliation (an average of 1.2 million ha annually) occurs when control measures are not applied [9]. It is estimated that approximately $11 million (US) is spent annually in the US on gypsy moth control [10].

Recently, a revised nomenclature for gypsy moths was proposed based on wing pattern and the ability of the female to fly [2]. According to the proposed nomenclature, the sub-genus *Porthetria* includes several closely related *Lymantria* spp. The common name "gypsy moth", *L. dispar dispar* (L.), now denotes only the winged but flightless EGM and the nearly wingless gypsy moth occurring in India, while the term "Asian gypsy moth", *L. dispar asiatica* Vnukovskij (AGM), refers to the strain that has winged females capable of flight. *L.d. asiatica* is found in Russia east of the Ural Mountains, the northern two-thirds of China, and Korea. In addition, four species of gypsy moth in the subgenus *Porthetria*, *L. albescens* Hori and Umeno, *L. postalba* Inoue, *L. umbrosa* (Butler), and *L. xylina* Swinhoe, and one subspecies, *L.d. japonica* (Motschulsky), occur in Japan. All of the females of gypsy moths occurring in the Japanese archipelago and nearby islands have functional wings. Of these, the Japanese gypsy moth, *L.d. japonica*, has the widest distribution throughout the Japanese mainland.

As a result of increased global commerce, repeated accidental introductions of the exotic Asian strain have occurred in North America, but so far all of these introductions (mostly from Siberia and possibly also from China) have been successfully eradicated [8, 11]. The AGM poses a more serious threat than the EGM for three reasons: the adult females of the Asian subspecies can fly, unlike the winged but flightless European females; the larvae of the Asian subspecies also feed on conifers [12]; and the Asian subspecies is one of the most widespread defoliators of deciduous and larch forests in China, where it periodically reaches outbreak levels [13]. In addition, AGM and EGM can hybridize, and many of the resulting females can also fly [14, 15]. The ability of both hybrid and purebred females to fly will likely allow the AGM to spread throughout North

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America much more rapidly than the European strain has so far, and cause more devastating economic losses [16]. Thus, the introduction of the Asian subspecies presents a far greater threat to the coniferous forests of Canada and the US than that posed by its European relative.

The gypsy moth has several naturally occurring infectious diseases, one of which, is a type of baculovirus known as a multi-capsid nucleopolyhedrovirus (LdMNPV). It is a major factor controlling *L. dispar* populations in North America [17]. LdMNPV has been observed to reach epizootic proportions as larval densities increase [18]. During the latter half of the last century, research on NPV in several countries culminated in the commercial development of baculovirus insecticides for control of gypsy moth in the US, Canada and the former Soviet Union.

With the increase in international trade, it is possible that the AGM will likely become established. Russia, China, South Korea and Japan are four likely sources for these introductions because of increasing trade between Asia and North America. To prepare for the eventual potential establishment of the AGM in North America, three geographic isolates of LdMNPV were tested against laboratory colonies of the EGM and the AGM strain from Siberia, Russia (AGM-R) [16]. Restriction enzyme digestion profiles indicated that these three virus strains were similar yet distinct, and the isolates can be easily distinguished [16]. One of the three virus strains was isolated from the AGM from Heilongjiang (LdMNPV-H) Province, P.R. China. The second field-collected strain tested (LdMNPV-J) was probably obtained from *L.d. japonica*, the most widely distributed gypsy moth in Japan. The third strain tested was the LdMNPV used in Disparvirus® (LdMNPV-D), a baculovirus product registered for gypsy moth control in Canada [19]. This is the same strain as the one registered in the US under the trade name Gypchek [20].

Ideally, it would have been best to simultaneously bioassay all three virus strains with the three insect strains (EGM, AGM-R and AGM-C) at the same time in the same laboratory. However, quarantine requirements, as well as lack of finances and manpower, forced us to do this comparison in two stages. In the first stage, the three virus strains (D, H and J) were tested in Canada in 2001 and 2002 against EGM and AGM-R, respectively. In the second stage, the same three virus strains were tested against the AGM from China using the same laboratory rearing conditions. This second set of bioassays was done in the Inner Mongolia Autonomous Region, People's Republic of China, where the insect is native.

In this paper, we report on the results of the second stage of the investigation. We compare the pathogenicity and virulence of the same three geographic isolates of LdMNPV against the Chinese strain of the Asian gypsy moth (AGM-C) in the laboratory in Huhhot, Inner Mongolia Autonomous Region, People's Republic of China in 2004.

MATERIALS AND METHODOLOGY

Test Larvae

Egg masses of AGM were collected in March, 2004, from the main host tree, Prince Rupprecht's larch, *Larix principis-rupprechtii* Mayr, in He-Lin County, Inner Mongolia Autonomous Region (150 km south of Huhhot, P.R. China) and stored at 5°C for 2 months.

Starting May 1, eggs were gently separated from the egg masses by hand. Surface decontamination was performed by wrapping the eggs (approximately 500 eggs per package) in a single layer of cheesecloth and immersing them in a 0.05% sodium hypochlorite solution for 30 seconds, followed by rinsing in five distilled water rinses for 1 min each. The eggs were then placed on filter paper to air-dry at room temperature. Approximately 500 eggs were placed into each of 30 fluted containers [6 oz. (177 ml) OZ6-XE6] and covered with cardboard lids (DS306) (Sweetheart Cup Co., Owing Mills, Maryland). The eggs were reared at 25ºC, 60% RH, and 16L:8D photoperiod, i.e. the same conditions used in the first stage in Canada [16]. After hatching, 100 larvae were transferred to each of 150 cups (described above) containing a modified artificial gypsy moth diet [21, 22] and reared at 25ºC, 60% RH, and 16L:8D photoperiod. Newly moulted (less than 24h old) second-instar larvae were starved for 18-24 h before being inoculated with a virus-contaminated diet plug.

Virus Inoculum

The activity of three geographic isolates of gypsy moth virus was tested in bioassays in the laboratory. One isolate, LdMNPV-D (Disparvirus®), was produced and supplied by Dr. John C. Cunningham, Natural Resources Canada, Canadian Forest Service (CFS), Great Lakes Forestry Centre, Sault Ste. Marie, Ontario, Canada, from the registered virus product, Disparvirus® (Pest Management Regulatory Agency, Health Canada, Registration number 24869). The other two virus isolates were originally collected from field populations of gypsy moths. One of these two isolates, LdMNPV-H, was collected in Heilongjiang Province of China near Harbin (courtesy of Professor Yue Shukui, Northeastern Forestry University, Harbin). The other isolate, LdMNPV-J, was collected in Japan (Ibaraki Prefecture, Honshu Island; courtesy of Dr. Shimazu Mitsuaki, Forestry and Forest Products Research Institute) [16].

All three LdMNPV strains were amplified twice *in vivo* and mass-produced under quarantine conditions at the Pacific Forestry Center (PFC), Natural Resources Canada, Canadian Forest Service, using freshly moulted fourth instar AGM-R larvae. The laboratory colony of AGM-R used for the amplification and mass-production were obtained from the Otis Methods Development Center, USDA, APHIS, PPQ, Massachusetts (courtesy of Dr. Vic Mastro and Mr. John Tanner). The mass-produced virus was stored for 2 years in insect cadavers in 50 ml centrifuge tubes at -20ºC. The insect cadavers were shipped to China for processing in early 2003, among freezer packs in a Styrofoam cooler. Virus occlusion bodies (OBs) were purified following the procedure described previously [16]. The stock suspension of OBs for each LdMNPV strain was prepared 1 month before the bioassays were conducted, and stored at 4°C until use. OBs were quantified using a haemocytometer.

Dose-Response

Freshly moulted second-instar AGM-C (*L.d. asiatica*) larvae were starved for 24 h and then inoculated with 1 µl of

virus suspension of one of the three strains of LdMNPV using a modified version of the contaminated diet-plug method [23, 24] to determine the dose and time-response to each LdMNPV isolate. Control larvae were fed a diet plug inoculated with distilled water (dH_2O). Virus inoculations targeted 5, 30, 50, 80, 85, 90 and 95% mortality (determined by preliminary bioassays), thereby giving the narrowest confidence limits for both the LD_{50} and LD_{95} [25].

During the preliminary bioassays of all three LdMNPV strains, a concentration close to the LD_{50} for LdMNPV-D was determined. However, the results of the Probit analysis for the other two virus strains either did not fit the Probit curve or the replicates differed substantially from each other, so an analysis of the pooled data could not be performed and no doses approximating the LD_{50}s could be determined for either LdMNPV-H or LdMNPV-J in 2003. Because of the possibility that the outbreak would collapse (depriving us of a source of larvae for the bioassay) and monetary constraints, it was decided not to repeat this preliminary bioassay, but instead to add two extra doses to improve the chances of calculating the LD_{50} and LD_{95} dose for LdMNPV-H or LdMNPV-J. Therefore, for the full bioassay, for LdMNPV-D, five concentrations were used to determine the LD_{50}, while for LdMNPV-H and LdMNPV-J two extra doses were used (total of seven concentrations) to determine the LD_{50} and LD_{95} in 2004.

The starved larvae were reared in darkness at 25 ± 1 ºC, 60% RH and allowed to feed for 24 h on the treated diet. Larvae from laboratory colonies are used to eating this artificial (substitute) diet and readily accept this diet. After 24 h, larvae that had consumed the entire plugs were transferred individually to Solo PL1 plastic souffles (29.6 ml cups, Solo Cup Company, Urbana, IL), containing untreated fresh diet and capped with Solo PL1 paper lids, and reared at 25 ± 1ºC, 60% RH, and 16L:8D photoperiod. Larvae that did not consume the entire plug were discarded. Bioassay of each viral strain dose and a corresponding experimental control group was replicated three times with 72 larvae per replicate, for a total of 216 larvae per concentration, for each viral isolate. Food was changed weekly and the larvae were monitored daily for mortality. Moribund larvae were considered as dead and gently prodded with a probe. Mortality caused by virus was diagnosed from typical virus disease characteristics (soft, flaccid body) and confirmed by microscopic examination. Larval mortality was analyzed using Probit analysis [26] to estimate the LD_{50}, LD_{95}, and 95% confidence limits.

Time-Response

Second-instar AGM-C larvae were treated using virus-inoculated diet plugs and then reared on non-contaminated diet. The virus doses were standardized to produce similar mortality rates in order to facilitate comparisons among the three virus strains. Equally effective doses causing less than 50% mortality were chosen, because the ST_{50}s generated from these data are relatively insensitive to dose differences [27]. Data obtained from the dose-response bioassays for each viral isolate were analyzed to determine ST_{50} using ViStat [28].

RESULTS

Dose-Response

There were no significant differences in dose-response (LD_{50} and LD_{95}) among the three replicates for each of the three LdMNPV strains, as determined by overlapping 95% confidence intervals [26]. Therefore, the three replicates for each virus strain were pooled and the combined data were used for the Probit analysis of the viral strains' dose-response. Mortality in the control groups was very low (2.8%). Natural response was estimated [26] for the regression of data sets in which control mortality occurred. The experiment was terminated 21 d post-inoculation, and no pupation occurred within this observation period.

The dose-response bioassays of larvae of AGM-C showed that LdMNPV-H and LdMNPV-D had very similar activity against the second-instar AGM-C larvae. The median lethal doses (LD_{50}) for LdMNPV-D and LdMNPV-H were 194 and 211 OBs $larva^{-1}$, respectively, and were not significantly different (i.e. there was overlap of 95% confidence limits of LD_{50}). Similarly, LD_{95}s were not significantly different at 1705 and 1414 OBs $larva^{-1}$, respectively. LdMNPV-J was significantly less pathogenic against the AGM-C (the 95% confidence limits did not overlap), having the highest LD_{50} and LD_{95} (940 and 11 457 OBs $larva^{-1}$, respectively) (Table **1**).

The slope of the regression line in Probit analysis has generally been interpreted as a measure of variability in host susceptibility to the virus [29], with less variation among hosts producing steeper slopes. Slopes of the dose responses for the three isolates (Table **1**) indicate that AGM-C is equally susceptible to LdMNPV-H and LdMNPV-D and least susceptible to the infection by LdMNPV-J. Both LdMNPV-H and LdMNPV-D isolates caused higher mortality of AGM-C larvae infected in the second instar, than LdMNPV-J isolate. Regression lines for the dose-response of AGM-C larvae to the three virus strains were parallel, and the ratio of relative potency of LdMNPV-H and LdMNPV-D to LdMNPV-J was estimated [25, 26] to be 4.5 and 4.8 fold, respectively.

Time-Response

Virulence, best determined by the time-response, is the length of time it takes for the virus to cause mortality and is an important measure of the effectiveness of the virus as a biopesticide [27]. The virulence of the three LdMNPV strains to second-instar AGM-C larvae was compared in terms of median time response, ST_{50} (time required to kill 50% of the larvae), at the equally effective dose. There were no significant differences in the time-response (ST_{50}) among the replicates (receiving doses causing somewhat less than 50% mortality) within each of the three virus strains bioassayed. Therefore, data sets were pooled for each of the virus strains prior to analysis with ViStat [28].

The time-response data indicated that all three strains of the LdMNPV have similar ST_{50}s, as determined by the overlapping 95% confidence limits. LdMNPV-D, LdMNPV-H and LdMNPV-J had ST_{50}s of 8.9, 8.3 and 8.6 d, respectively. However, for two of the three virus strains tested (LdMNPV-H and LdMNPV-J), the observed χ^2 values were

Table 1. Response of Second-Instar Larvae of the Chinese Strain of *Lymantria dispar asiatica* to Three Geographic Isolates (LdMNPV-D, LdMNPV-H, and LdMNPV-J) of Nucleopolyhedrovirus in Laboratory Bioassay, Huhhot, P.R. China, 2004

Isolate	Dose Response				Time Response		
	LD_{50}[a] (OBs $larva^{-1}$)	LD_{95}[a] (OBs $larva^{-1}$)	Slope±SE	χ^2/df[b]	ST_{50}[c] (day)	Slope±SE	χ^2/df[d]
LdMNPV-D	194 (157 - 235)	1705 (1254 – 2541)	1.74 ± 0.14	12.72 / 13	8.85 (8.45 - 9.25)	10.31 ± 1.17	9.54 / 9
LdMNPV-H	211 (175 - 255)	1414 (1066 – 2011)	1.99 ± 0.14	6.25 / 11	8.33 (8.02 - 8.65)	10.89 ± 1.10	28.36 / 11
LdMNPV-J	940 (748 – 1167)	11 457 (7768 – 19 366)	1.51 ± 0.13	6.17 / 10	8.61 (8.19 - 9.03)	8.18 ± 0.80	23.88 / 12

[a] calculated using Polo PC [26]; numbers in parentheses indicate 95% confidence limits.
[b] the degrees of freedom are less than 14 and differ among the analyses because data points considered "outliers" (dose responses that were abnormally high or low) were excluded from the final determination of the LDs for each virus.
[c] determined using pooled replicates causing less than 50% mortality; calculated using ViStat [28]; numbers in parentheses indicate 95% confidence limits and were calculated as twice the standard error of the ST_{50}.
[d] degrees of freedom differ because of the number of days that virus-induced mortality occurred varied among the three LdMNPV strains.

higher than the predicted χ^2 values (Table **1**), indicating a greater deviation of the response data from the regression lines, i.e. the experimental data for these two strains were a poor fit for the model used by ViStat [16].

DISCUSSION

Making direct comparisons between the results obtained in this paper and those reported in previous studies is somewhat problematic, because the bioassay methodologies used vary considerably. Unlike many other studies of gypsy moth virus strains, in which the lethal concentrations (LC) for different isolates of LdMNPV were determined, this current and one of the previous studies [16] determined the more accurate lethal doses (LD) of three isolates of LdMNPV. Interpreting the results of some of the earlier studies using LC is made even more difficult because, in some cases, cytoplasmic viruses were also tested at the same time [30, 31]. Earlier studies also reported variations in the biological activities of different geographic isolates of gypsy moth virus [30-35], and variation in the responses of gypsy moth larvae from different geographic locations to the same virus strain. The age or phase of the gypsy moth outbreak, the isolation, preparation and storage of the strains are also thought to influence the biological activity (both pathogenicity and virulence) of the virus [33].

A previous study [16], using the contaminated diet plug method, compared the dose and time-responses of second-instar larvae of the EGM and AGM-R to three gypsy moth virus isolates. Both experiments used almost identical methodologies, the only difference being that the field-collected larvae used for the bioassay in China were starved for 24 h prior to inoculation, whereas the larvae used in the Canadian study were from laboratory colonies and were not starved. Because we tested the same three virus strains against second-instar AGM-C larvae using an almost identical methodology, most of the comparisons will be between the results we report in this paper and the previous study. Hence, we presented our data using the same format (Table **1**) as in Ebling *et al.* [16] to facilitate comparison of the bioassay results.

Comparison of the Pathogenicity of Three LdMNPV Isolates Against AGM-C and AGM-R

When second instar AGM-C larvae were challenged with the three virus isolates, we found that the pathogenicity of LdMNPV-D and LdMNPV-H against AGM-C was not significantly different. However, both LdMNPV-D and LdMNPV-H were significantly more pathogenic against AGM-C than LdMNPV-J.

In comparison, Ebling *et al.* [16] reported that LdMNPV-H was more pathogenic against AGM-R than LdMNPV-J, and significantly more pathogenic than LdMNPV-D. LdMNPV-H had a significantly lower LD_{50} (648 OBs $larva^{-1}$) than LdMNPV-J (1260 OBs $larva^{-1}$) and LdMNPV-D (1904 OBs $larva^{-1}$). The LD_{95} of LdMNPV-H (8540 OBs $larva^{-1}$) was significantly lower than that of LdMNPV-D (208 600 OBs $larva^{-1}$), but not for LdMNPV-J (20 841 OBs $larva^{-1}$). Comparing the pathogenicity of the three virus isolates against AGM-C (this study) and AGM-R [16], LdMNPV-D and LdMNPV-H were significantly more pathogenic against the AGM-C than to the AGM-R, while LdMNPV-J was somewhat more pathogenic against the AGM-C than to the AGM-R, but not significantly.

Comparison of the Virulence of Three LdMNPV Isolates Against AGM-C and AGM-R

Interesting patterns emerged when we compared the time-responses (virulence) of the same three virus isolates against AGM-C and AGM-R. There were no significant differences among the median time-responses (ST_{50}) of the three virus isolates (LdMNPV-D, LdMNPV-H and LdMNPV-J) when second-instar AGM-C larvae were challenged (Table **1**). Similarly, there were no significant differences among the three virus isolates in the time-responses when second-instar AGM-R larvae were challenged [16]. The ST_{50} of AGM-R to the three virus isolates varied from 10.4 to 11.5 d [16]. These ST_{50} values are about 1.5 to 2 d longer than what we obtained for AGM-C, which varied from 8.3 to 8.9 d (Table **1**).

Possible Sources of Variability in Virus Activity

LdMNPV-D, the active ingredient in the registered virus products in North America (Gypchek and Disparvirus®), was significantly more pathogenic against the EGM than the two strains of AGM bioassayed. It has been postulated that this virus strain was accidentally introduced into North America, either with its host or with one of the introduced parasitoids of gypsy moth [36]. Thus, it is highly likely that LdMNPV-D virus strain has always been associated with the EGM.

LdMNPV-H and LdMNPV-D were equally pathogenic against AGM-C, and significantly more than LdMNPV-J. The fact that LdMNPV-D and LdMNPV-H were equally effective was unexpected, because one would have expected that LdMNPV-H, isolated in Heilongjiang Province in China, to be more pathogenic to *L.d. asiatica*. Likewise, it was somewhat surprising that LdMNPV-J was the least pathogenic strain against AGM-C (Table **1**), and only moderately pathogenic against AGM-R [16], unless it was originally isolated from a species of lymantriid other than the Japanese gypsy moth (*L.d. japonica*).

Previous studies (using LC_{50}) have revealed differences in pathogenicity among geographic isolates of LdMNPV. One study [37] found that the LdMNPV isolate from France was less active compared with a North American and a Korean isolate. In a different study [38], six geographic isolates of LdMNPV from China were tested against EGM established in eastern North America. None of these virus strains were found to be as effective as the strain used in the currently registered products (Gypchek and Disparvirus®) for gypsy moth control in North America. In a third study [34], 19 different strains of LdMNPV were bioassayed against a laboratory colony of the introduced EGM, including one strain from Japan. The median lethal concentration (LC_{50}) values of the geographical isolates against the colonized strain of *L. dispar* in the US varied from 1.7×10^3 to more than 5×10^6 OBs ml^{-1}. It was also found that the North American virus isolate was generally the most active against gypsy moths, and the virus from Japan was the least active of the isolates tested. Unfortunately, the origin of the Japanese virus strain tested was not indicated [34], and when they did their work the gypsy moth complex was less understood.

As previously mentioned, it is also possible that the nucleopolyhedrosis virus from Japan was not isolated from *L.d. japonica*, but from one of the other species of *Lymantria* that are known from Japan. Recent taxonomic work [2] proposed that what was once considered a single species (*L. dispar*) actually consists of several species and subspecies, five of which occur in the Kurile Islands, Japan, and Ryukyu Islands. Interestingly, the AGM, *L.d. asiatica*, was not listed as one of the gypsy moths occurring in Japan [2]. Despite repeated attempts, we could not precisely pinpoint either the location or the scientific name of the host from which the LdMNPV-J strain we used was obtained. However, we think that it is highly probable that the virus strain we used was obtained from *L.d. japonica* (Motschulsky), the most widely distributed subspecies of gypsy moth in Japan.

These variances may also be due to differences in larval strains of AGM used in the two studies. The AGM-R larvae tested during the previous study [16] were reared from a laboratory colony of a Russian strain obtained from the Otis Methods Development Center, USDA, APHIS, PPQ, Massachusetts, that had been in rearing for over 51 generations (John Tanner, USDA, personal communication), while the AGM-C larvae tested in our experiment were collected from the field in He Ling County near Huhhot in north-western China. Thus, the differences in the experimental results may also be due either to changes in resistance in the AGM-R somehow induced by prolonged breeding under laboratory conditions, compared with the field-collected AGM-C, or simply to comparing different strains of the same insect. It would be interesting to see if the biological activity of these same three geographic strains of virus would be different if tested (using the same methodology) against a field-collected Russian and Japanese strains of Asian gypsy moths.

It has been shown by this study and others that both the geographic isolate and the gypsy moth larval strain tested influence the effectiveness of the virus as a biopesticide. Therefore, it is very important to select the most virulent isolate for a given host population, first by laboratory bioassay, then by confirmation in small-scale field tests, before seeking registration or proceeding with large-scale field use as a biopesticide. However, this is not always practical in operational gypsy moth, or other defoliator, control programs, if the virus product is not already registered.

The AGM-R tested in the previous study [16] and the AGM-C in our current study occur in the two geographic areas from which the introduction of *L.d. asiatica* to North America is most likely to originate due to the ever-increasing trade with these two countries. There are two other potential sources for introduction from Asia, Japan and Korea. Given the differences in the dose and time responses observed in the AGM-R challenged in the previous study [16] and in this study (AGM-C), it would be highly desirable to test these same three gypsy moth virus strains against the Japanese gypsy moths identified as native to Japan [2], or at the very least against *L.d. japonica* (the most common subspecies). This subspecies, with its wide distribution in Japan, may be a third possible source of introduction of AGM into North America.

Recent DNA work on lymantriid moths [39] generally agrees with the earlier work by Pogue and Schaeffer [2], but also states that *L.d. asiatica* and *L.d. japonica* cannot always be easily separated. Increasing trade among Asian countries, with its potential for introductions could have further complicated the distinction between *L.d. asiatica* and *L.d. japonica* (LM Humble 2010, personal communication). It is recommended that the same three virus strains be bioassayed against recently collected strains of *L.d. asiatica* from the Russian Far East, China, and Korea, as well as *L.d. japonica* from Japan. DNA barcoding of the field-collected AGM strains being bioassayed would also be desirable, especially in light of possible past misidentifications of some *Lymantria* spp. [40]. The other, less widely distributed gypsy moths of the subgenus *Porthetria* in Asia, *Lymantria albescens, L. postalba*, *L. umbrosa,* and *L. xylina*, could be studied at a later date. Bioassaying the species or subspecies of gypsy moth occurring in Japan could be done cooperatively with researchers in Japan to avoid quarantine concerns and accelerate this research.

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