Insecticidal Characteristics of Two Commercial *Spodoptera exigua* Nucleopolyhedrovirus Strains Produced on Different Host Colonies

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ABSTRACT The insecticidal characteristics of two *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV) strains produced on two different *S. exigua* colonies were measured using the same two host colonies. These strains constitute the active ingredients of the biological insecticides Vir-ex and Spexit and were produced on insect colonies from Spain and Switzerland. Demographic characteristics of insects from each colony were examined before infection. Larval developmental time, larval survival, and adult sex ratio did not differ between the colonies, whereas mean pupal weight was significantly higher in the Spanish colony insects. After infection, susceptibility to virus occlusion bodies (OBs), time to death, larval weight at death, and total production of OBs/larva varied significantly depending on virus strain and the colony used. Vir-ex OBs produced in Spanish colony larvae had improved insecticidal characteristics in terms in lethal dose and speed of kill metrics than other strain-colony combinations. OB production was significantly higher in Spanish colony insects infected with Spexit compared with Vir-ex infected insects from the Swiss colony, with intermediate values for the other two strain-colony combinations. Virus strain and host colony origin were highly influential in determining the insecticidal characteristics of OBs and should be considered as key parameters that require optimization during the production of SeMNPV-based insecticides.

KEY WORDS SeMNPV, Spexit, Vir-ex, production, insect colony effect

The beet armyworm, *Spodoptera exigua* (Lepidoptera: Noctuidae) is a major polyphagous pest of a wide variety of crops worldwide (Hill 2008). The intensive use of chemical insecticides has resulted in the development of resistance in populations of this pest (Brewer and Trumble 1989, Ahmad and Arif 2010). This, and increasing consumer concerns regarding the presence of pesticide residues in food, means that biological insecticides are well positioned for use in the integrated management of this pest.

Baculoviruses comprise a family of arthropod specific viruses with recognized applications in the development of biological insecticides (Moscardi 1999). Because of their high specificity, baculovirus-based insecticides are compatible with insect natural enemies such as parasitoids, predators or other pathogens.

Several isolates of the *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV) (Baculoviridae) have been characterized and developed as the active ingredients of insecticidal products such as Spod-X (Certis Europe BV, Maarsen, Netherlands), Spexit (Andermatt Biocontrol AG, Grossdietwil, Switzerland), and Vir-ex (Biocolor SA, Almería, Spain). The efficiency of these products has been demonstrated under greenhouse (Smits et al. 1987, Bianchi et al. 2002, Lasa et al. 2007b) and field conditions (Gelernter et al. 1986, Kolodny-Hirsch et al. 1997).

The commercial viability of baculovirus insecticides largely depends on their efficacy compared with chemical pesticides and their production costs (Szewczyk et al. 2006). Large scale baculovirus production requires mass-rearing and infection of host larvae. Understanding the factors that modulate variation in host susceptibility and virus occlusion body (OB) productivity per larva is important for optimizing the virus production process. This is particularly significant for SeMNPV, given its absolute specificity for *S. exigua* (Gelernter and Federici 1986) and high genotypic variation present within and between isolates (Hara et al. 1995).

Genotypic heterogeneity in SeMNPV isolates is reflected in marked phenotypic differences in key insecticidal traits, including OB pathogenicity measured as dose-mortality metrics, speed of kill, and OB productivity (Bianchi et al. 2000, Muñoz and Caballero 2000, Murillo et al. 2006). As such, the SeMNPV isolate and the host colony used as a biofactory, must be carefully selected to achieve maximum yields of highly insecticidal strains.

The aim of this study was to characterize the insecticidal characteristics of two SeMNPV strains produced in two different *S. exigua* colonies. The SeMNPV strains selected constitute the active ingre-

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dients of Vir-ex and Spexit, and were tested against two *S. exigua* colonies that differed in their geographical origin. This information will be of clear value when selecting host colonies for the production of effective SeMNPV-based insecticides.

Materials and Methods

Insect Colonies and Biological Characteristics. Larvae of *S. exigua* were obtained from two laboratory colonies, one originating from field-collected adults in Almería, Spain, designated as the Almerian colony, and the other established from larvae supplied by Andermatt Biocontrol AG, designated here as the Swiss colony. The geographical origin of the Swiss colony is uncertain, but this colony had been maintained in Andermatt Biocontrol AG laboratories in Switzerland for at least 5 yr. These colonies were maintained continuously for five generations on an artificial diet that had been adapted for *S. exigua* mass-rearing (Elvira et al. 2010). Colonies were maintained in different but identical rearing chambers at 25 ± 1°C, 75 ± 5% RH, and a photoperiod of 16:8 (L:D) h. Biological parameters of each colony were compared by calculating larval developmental time, pupal sex ratio, and pupal weight. Groups of 30 neonate larvae of each colony were placed individually in 25-ml plastic cups containing 6-ml blocks of diet. The experiment was performed eight times for each colony and results were subjected to *t*-test or analysis of variance (ANOVA) followed by means separation by Tukey test in SPSS version 15.0 (SPSS Inc., Chicago, IL).

Virus Stocks and Molecular Identification. Samples of SeMNPV OBs were obtained from the commercial products Vir-ex and Spexit. OBs were amplified in *S. exigua* early fourth instars from the Swiss colony. Viral DNA was extracted and digested with the restriction endonuclease *Bgl*II for virus identification. DNA samples were obtained by dissolving OBs in a one-third volume of an alkaline solution (0.3 M Na_2CO_3, 0.5 M NaCl, 0.03 M EDTA; pH 10.5), incubated with proteinase K, followed by phenol/chloroform extraction and alcohol precipitation. The extracted DNA was washed with 70% ethanol, resuspended in sterile milli-Q water, and stored at 4°C until use. For analysis, a 2-μg sample of viral DNA was incubated with Bgl*II* for 4 h at 37°C. Reactions were stopped by addition of loading buffer (0.25% [wt:vol] bromophenol blue, 40% [wt:vol] sucrose), and digestions were loaded in 0.7% TAE buffer (40-mM Tris-acetate; 1-mM EDTA) agarose gels, and electrophoresed at 20 V for 12 h. Ethidium bromide stained gels then were photographed on a UV transilluminator by using the Gel-doc software (Bio-Rad, Madrid, Spain).

OB Production. The number of OBs produced in individual larvae was determined in fourth instars from each colony that had molted in the previous 6-h period. Groups of 25 larvae from each colony were starved for 12 h, individually weighed, and allowed to drink from aqueous suspensions (Hughes and Wood 1981) containing 10% (wt:vol) sucrose, 0.001% (vol:vol) Fluorella Blue food dye, and 6.4×10^3 OBs/ml, a concentration that previously was estimated to result in 90% mortality. Four virus × insect colony treatment combinations were considered: Vir-ex × Almerian, Vir-ex × Swiss, Spexit × Almerian, and Spexit × Swiss. Groups of control larvae were treated identically but consumed sucrose solution without OBs. Larvae that drank from the OB suspension during a 10-min period were reared individually in 25-ml plastic cups with diet and examined daily for signs of polyhedrosis disease until death. Larvae that had died recently and had not disintegrated were carefully weighted, frozen individually at −20°C, and stored until further use.

For OB counting, cadavers were thawed, individually homogenized in 1-ml distilled water, filtered through fine steel gauze to remove insect debris, and diluted in distilled water. The number of OBs recovered from each larva was determined using a Neubauer improved counting chamber under phase contrast microscopy at ×400 and calculated as the mean number of OBs counted in three different samples of each suspension. The relationship between OB production and larval weight (OB/mg) was estimated based on insect weight recorded postmortem, when the integument was still intact. The entire experiment was performed three times. Larval weights, numbers of OBs per larva, and OBs per milligram larval weight were analyzed by *t*-test for comparisons involving two samples and ANOVA when more than two samples were compared, in SPSS version 15.0. *Bgl*II restriction profiles were examined using batches of DNA extracted from OBs of each repetition to confirm the identity and replication fidelity of each virus × colony combination.

OB Pathogenicity and Virulence. The median lethal dose (LD_{50}) and mean time to death (MTD) were estimated in second instars from each colony by using the droplet-feeding technique. Four virus inocula were assayed: the Vir-ex amplified in the Almerian or Swiss colony insects and Spexit amplified in insects from each colony. Late first instars were starved at 25 ± 2°C and allowed to molt to the next instar over a period of 10 h. Groups of second instars were allowed to feed on sucrose plus food dye droplets containing one of six concentrations of OBs: 3.03×10^3, 9.09×10^3, 2.72×10^4, 8.18×10^4, 2.45×10^5, or 7.36×10^5 OBs per milliliter, previously calculated to result in mortalities between 10 and 90%. Control larvae were treated identically but fed on a solution of sucrose and food dye alone. Twenty-five larvae that ingested the OB suspension within 10 min were placed individually in the cells of a 25 well tissue culture plate each containing a cube of diet and were incubated at 25 ± 2°C. Mortality was registered at 12-h intervals for 7 d. The bioassay was performed on three occasions by using different batches of insects. Dose-mortality results were subjected to logit regression by using the GLIM 4 program (Numerical Algorithms Group 1993) with a binomial error distribution specified. OB pathogenicity was expressed as the median lethal dose (LD_{50}) based on an average ingested volume of 0.33 μl per larva in this instar, estimated in previous studies (Smits and Vlak 1988a, b). LD_{50} values were compared

by *t*-test. Time-mortality results were subjected to Weibull analysis in GLIM 4. The validity of the Weibull model was determined using the Kaplan–Myer procedure (Crawley 1993). The OB concentration used for the time-mortality analysis was 2.5×10^5 OBs per milliliter, equivalent to 81 OBs per larva, that resulted in 65–85% mortality in all treatments.

Determination of OB Size. For calculation of OB size, purified OBs were prepared in epoxy resin, sectioned, and stained with 1% (wt:vol) OsO_4 for transmission electron microscopy. OB diameters were obtained by measuring the longest diagonal distance between two polyhedral vertices of 40 OBs in microphotographs. This procedure was performed three times with different batches of OBs, and compared with data obtained for purified OBs from the original inocula, which were considered as controls. Results were subjected to Kruskal–Wallis analyses in SPSS version 15.0.

Results

Biological Comparison of *S. exigua* Colonies. No differences were observed in larval development time between insect colonies ($t = 0.656$, df = 30, $P = 0.517$) or according to sex ($t = 0.076$, df = 30, $P = 0.940$). The mean (±SE) development time from egg to pupa was 11.3 ± 0.3 d for males and 11.6 ± 0.5 d for females. No significant differences were observed in adult sex ratio between colonies ($t = 0.680$; df = 14; $P = 0.507$), although the proportion of males tended to be slightly male-biased in both colonies (0.544 in Almerian colony, 0.566 in Swiss colony). Pupal weight (mean ± SEM) differed significantly between colonies ($F_{3,31} = 17.81$, $P < 0.001$); males (103.07 ± 3.80 mg); and females (125.60 ± 4.20 mg) from the Almeria colony were significantly heavier than males (93.40 ± 1.67 mg) and females (105.42 ± 2.41 mg) from the Swiss colony (males $t = 4.416$, df = 14, $P = 0.001$; females $t = 2.308$, df = 14, $P = 0.037$).

Identification of Viruses Amplified in *S. exigua* Colonies. Viral DNAs obtained from the two original inocula and from the OBs amplified in larvae from each colony were analyzed using *Bgl*II. The isolates differed in their restriction fragment profiles and the presence of submolar bands indicating that both isolates comprised mixtures of different genotypes (Fig. 1). However, restriction profiles of the amplified viruses did not differ from those of the original inocula, indicating that each virus had replicated correctly and had not become contaminated in either insect colony.

OB Production in *S. exigua* Colonies. Mortality registered in all treatments was between 90 and 95%, with no virus mortality registered in the mock-infected controls. Larvae from each colony had a similar weight immediately before inoculation ($F_{3,23} = 0.046$, $P = 0.940$). The weight of larvae postmortem varied significantly between the four treatments ($F_{3,23} = 7.41$, $P = 0.002$) (Fig. 2). Almerian larvae infected with Spexit were 25.1% ($t = 2.764$, df = 10, $P = 0.01$) and 31.7% ($t = 2.485$, df = 10, $P = 0.02$), heavier respectively, than Almerian or Swiss larvae treated with Vir-ex. Swiss larvae inoculated with Spexit had intermediate weight values (Fig. 2).

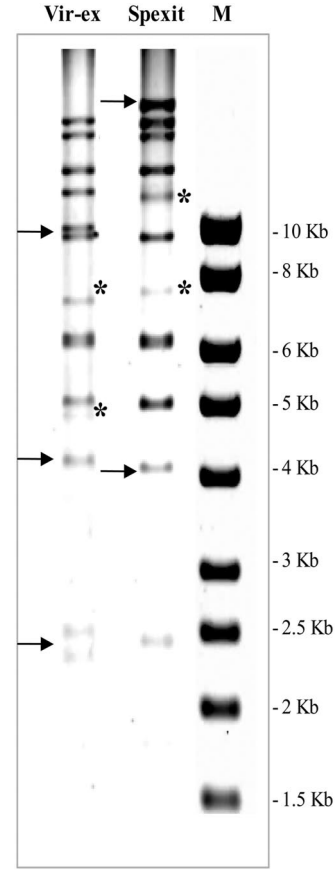

Fig. 1. *Bgl*II restriction endonuclease profiles of viral DNA extracted from the original virus inocula obtained from the commercial formulations Vir-ex and Spexit. Arrows on the left indicate restriction fragment length polymorphisms. Asterisks on the right indicate submolar fragments. A molecular weight marker (M), hyperladder I (Bioline, London, United Kingdom) was included.

Total OB production per larva differed significantly between virus × colony treatments ($F_{3,23} = 4.06$, $P = 0.021$) (Fig. 3A) and also in terms of OBs per milligram larval weight ($F_{3,23} = 3.68$, $P = 0.029$) (Fig. 3B). In the Almerian insects infected by Spexit, OB yields, expressed as total OBs per larva and OBs per milligram larval weight, were 2.3- and 1.9-fold higher, respectively, than those of the Swiss insects infected by Vir-ex. No significant differences were observed between insect colonies for the same virus inoculum when comparing total OBs per larva (Vir-ex: $t = 1.217$, df = 10, $P = 0.252$; Spexit: $t = 0.922$, df = 10, $P = 0.378$) and OBs per milligram larval weight (Vir-ex: $t = 0.343$, df = 10, $P = 0.739$; Spexit: $t = 0.478$, df = 10, $P = 0.473$). No significant interactions were detected between virus and insect colony for postmortem body weight ($F_{1,24} = 1.109$, $P = 0.305$); total OBs per larva ($F_{1,24} = 0.104$, $P = 0.751$); or OBs per milligram larval weight ($F_{1,24} = 0.280$, $P = 0.602$).

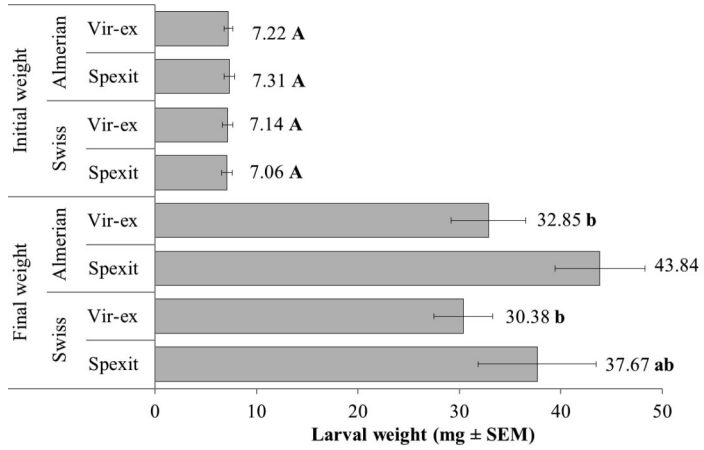

Fig. 2. Mean (±SEM) larval weight in milligrams at inoculation (initial weight) and postmortem (final weight). Columns headed by different letters differ significantly (ANOVA, Tukey test, $P < 0.05$) for comparisons before inoculation (uppercase letters) and after death (lowercase letters).

Significant correlations between larval weight gain during the infection period and log total OB yields were detected for the Spexit × Almerian, Vir-ex × Almerian, and Spexit × Swiss treatments, but not for the Vir-ex × Swiss treatment (Fig. 4).

Pathogenicity and Virulence of OBs Produced in Different Colonies. Vir-ex OBs produced in the Almerian colony (Vir-ex × Almerian treatment) had the lowest LC_{50} value in second instars from the Almerian colony (Table 1). All the other inocula were less pathogenic for the Almerian colony insects. In contrast, no significant differences were observed in OB pathogenicity between the four inocula against larvae from the Swiss colony. No virus-induced mortality was registered in mock-infected controls.

The mean time to death (MTD) in second instars ranged from 95.1 h postinfection (hpi) for Almerian larvae infected with Vir-ex × Almerian inoculum to 121.7 hpi for Swiss larvae infected with Spexit × Swiss inoculum (Table 1). Larvae from both colonies treated with Spexit × Almerian or Spexit × Swiss survived significantly longer than Almerian larvae infected in the Vir-ex × Almerian treatment. The instantaneous risk of death increased over time in all treatments (Weibull shape parameter $\alpha = 5.1070$). Time mortality distribution curves showed mortality peaks at 84 and 96 hpi for Vir-ex × Almerian and Vir-ex × Swiss treatments, respectively, and at 84 and

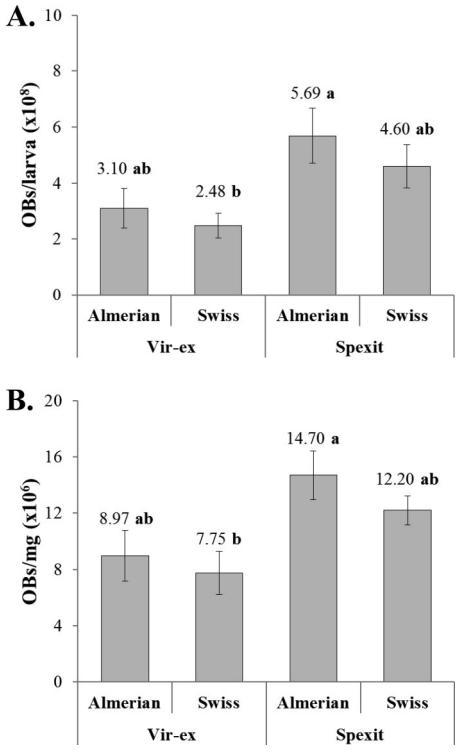

Fig. 3. Occlusion body production per larva (A) and per milligram of larval body weight (B). Columns headed by different letters differ significantly (ANOVA, Tukey test, $P < 0.05$).

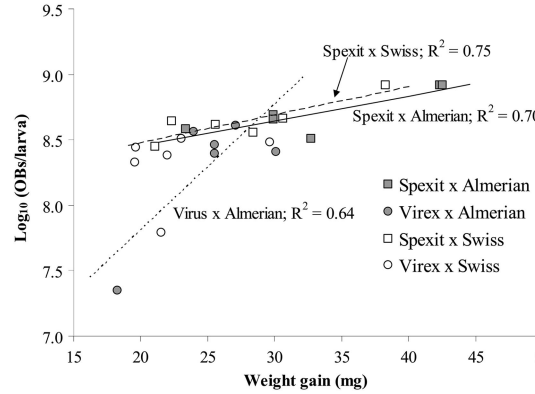

Fig. 4. Linear regression of weight-gain response (milligrams) in infected fourth instars and log [OB production per larva]. No significant relationship was observed in the Vir-ex × Swiss treatment.

Table 1. Logit regression and time-to-death analysis on *S. exigua* second instars from the Almerian and Swiss colonies after consumption of SeMNPV OBs of two commercial products (Vir-ex and Spexit) produced in fourth instars from each colony

Virus × colony treatment	Host colony	Logit regression[a]				Time to death[b]		
		Intercept (± SE)	LD_{50} (OBs/larvae)	95% CI		MTD (hpi)	95% CI	
				Low	High		Low	High
Vir-ex × Almerian	Almerian	1.47 ± 0.16	7.4a	5.6	9.7	95.1a	89.6	100.9
	Swiss	1.82 ± 0.14	12.8ab	9.7	16.8	103.8ab	97.7	110.3
Vir-ex × Swiss	Almerian	1.79 ± 0.14	15.0b	11.4	19.8	105.5ab	98.6	112.7
	Swiss	1.98 ± 0.14	11.9ab	9.0	15.6	105.8ab	99.8	112.3
Spexit × Almerian	Almerian	2.14 ± 0.14	13.9b	10.9	19.2	115.2b	108.3	122.5
	Swiss	1.98 ± 0.14	17.7b	13.4	23.3	117.9b	110.6	125.7
Spexit × Swiss	Almerian	2.14 ± 0.14	20.4b	15.5	27.0	118.6b	111.6	126.0
	Swiss	1.65 ± 0.13	19.4b	14.7	25.6	121.7b	114.7	129.0

[a] Logit regressions were fitted in GLIM with a common slope of 0.75 ± 0.04 (SE) for all virus inocula. The test of nonparallelism was not significant ($\chi^2 = 5.12$; df = 7; $P = 0.65$). LD_{50} values followed by identical letters did not differ significantly (*t*-test, $P > 0.05$).
[b] Mean time to death (MTD) values labeled with different letters differed significantly (*t*-test, $P < 0.05$, Weibull analysis, GLIM).

108 hpi for Spexit × Almerian and Spexit × Swiss treatments, respectively (data not shown).

Size of OBs Amplified in Different Colonies. Mean OB diameters ranged between 1.0 and 1.2 μm (Table 2). Vir-ex × Almerian and Vir-ex × Swiss OBs were significantly larger than the original control inoculum (Kruskal–Wallis $\chi^2 = 79.125$, df = 5, $P < 0.001$) and similar to that of Spexit × Almerian or Spexit × Swiss OBs. No significant variation in size was detected in Spexit × Almerian or Spexit × Swiss OBs with respect to the original inoculum (Kruskal–Wallis $\chi^2 = 23.21$, df = 2, $P < 0.001$).

Discussion

Efficient mass-production of baculoviruses involves a balance between total OB production per infected cell, the extent and pathological effects of the infection within each larva, and the pathogenic properties of the OBs produced in those cells. Although virus production seems straightforward, its success largely depends on environmental and biotic factors such as the virus strain and the host insect population (Shapiro 1986). Indeed, different strains of the same virus and even different genotypes within a single virus strain may vary significantly in their insecticidal properties (Cory et al. 2005). Likewise, different host species or geographically distinct populations may display distinct susceptibilities to particular virus strains (Milks 1997, Ribeiro et al. 1997). In this respect, the results of the current study underline the importance of achieving a suitable virus-host combination to optimize SeMNPV production in vivo. Unlike some other viruses, such as *Autographa californica* multiple nucleopolyhedrovirus (MNPV) or *Helicoverpa zea* single nucleopolyhedrovirus (SNPV) that can infect several species of Lepidoptera (Moscardi 1999), no other host species can be used as biofactories for SeMNPV production because of the absolute host specificity of this virus (Gelernter and Federici 1986).

Table 2. Mean (± SEM) OB diameter of Vir-ex and Spexit passaged in Almerian and Swiss larvae or from the original inocula (original)

Virus inocula	*S. exigua* colony	Diameter (μm± SE)
Vir-ex	(Original)	1.06 ± 0.04b
	Almerian	1.14 ± 0.03a
	Swiss	1.21 ± 0.03a
Spexit	(Original)	0.99 ± 0.04b
	Almerian	1.07 ± 0.04ab
	Swiss	1.05 ± 0.04ab

Values followed by different letters differed significantly (Kruskal–Wallis, $P < 0.05$).

Restriction endonuclease profiles of the original inocula Vir-ex and Spexit, and from samples taken upon passage in each host colony remained unchanged indicating fidelity in the replication of each virus. The presence of submolar fragments in the restriction profiles is indicative of mixed genotype populations in each of these bioinsecticide products. Indeed, the Vir-ex product was specifically designed to include a mixture of specific genotypes that together resulted in high insecticidal activity (Caballero et al. 2009b). Genotypic diversity in baculovirus populations is a widespread phenomenon (Erlandson 2009, Redman et al. 2010), and may facilitate rapid adaptation to variation in biotic and abiotic conditions, including variation in host characteristics (Ribeiro et al. 1997, Espinel-Correal et al. 2010).

We hypothesized that larger OBs may occlude a greater number of occlusion derived virions (ODVs) resulting in an increased probability of infection per OB, but differences in OB pathogenicity were not correlated with differences in the size of OBs. The number of virions occluded within OBs is genetically determined, at least in *S. frugiperda* MNPV that is closely related to SeMNPV (Simón et al. 2008).

Host weight gain during the infection period has been described as a key factor for OB production along with the host response to OB dose, or the duration of the infection, and is directly correlated with OB yield (Sherman 1985, Shapiro 1986). However, the OB yields obtained were influenced not only by larval growth rates (weight gain), but also by the insecticidal properties of the virus (Fig. 4). OB yields were significantly higher in the Almerian colony insects infected by Spexit compared with the Swiss colony infected with Vir-ex. These differences were correlated

with larval postmortem weights. OB yield values of Vir-ex in Almerian insects were not significantly different to those of Spexit in the same host, even though the final weight of Almerian larvae infected by Vir-ex was significantly lower than that of Almerian larvae infected by Spexit. This is important because the most suitable virus strain and host colony combination to kill Almerian insects was that of Vir-ex in the Almerian insects, as indicated by pathogenicity and virulence parameters. Similarly, improved insecticidal performance of native over foreign strains of SeMNPV was reported previously for virus strains grown in, and tested on, Spanish *S. exigua* populations (Muñoz and Caballero 2000, Murillo et al. 2006).

The maximum amount of virus produced per larva was $\approx 6 \times 10^8$ OBs, which is comparable to that reported by Shapiro (1982), but was ≈40% lower than yields obtained using different inoculation methods and doses (Smits and Vlak 1988b, Cherry et al. 1997). Overall, Vir-ex produced in Almerian insects appeared to represent a suitable combination for both OB production and pathogenicity against Almerian larvae. In fact, this combination is currently being used to produce Vir-ex for use in Almerian greenhouse crops with considerable success (Caballero et al. 2009a). Slower killing nucleopolyhedrovirus (NPVs) have a longer period for viral replication and tend to produce a greater number of OBs in each larva than faster killing isolates (Cory and Myers 2003). Our results are consistent with this tendency because in the least virulent treatments, those involving Spexit, the final larval weights and OB yields were higher than observed in the faster-killing Vir-ex treatments (Fig. 4).

The results of this study support previous observations on the varying susceptibility of host populations to geographically distinct isolates (Ribeiro et al. 1997, Escribano et al. 1999, Erlandson et al. 2007). Viruses appear to retain high infectivity toward local host populations, presumably as a result of continuous host-pathogen coevolution. Moreover, in certain cases, the use of exotic NPV isolates has the potential to impact negatively on the biological activity of native populations (Muñoz and Caballero 2000).

Previous studies on OB production in NPVs have focused on optimizing factors such as OB dose, infection method, larval stage at infection, incubation period, rearing density, diet, rearing temperature, and harvesting techniques (Shapiro 1986, Cherry et al. 1997) and more recently, hormone-mediated manipulation of larval development (Lasa et al. 2007a). The results presented here highlight the importance of selecting a combination of highly insecticidal isolates and a productive insect colony for the commercial production of SeMNPV-based insecticides.

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