Biocontrol Science and Technology (2001) 11, 649–662

Consequences of Interspecific Competition on the Virulence and Genetic Composition of a Nucleopolyhedrovirus in *Spodoptera frugiperda* Larvae Parasitized by *Chelonus insularis*

A. ESCRIBANO,[1] T. WILLIAMS,[2] D. GOULSON,[3] R. D. CAVE,[4] J. W. CHAPMAN[3] AND P. CABALLERO[1]

[1] *Departamento de Producción Agraria, Universidad Pública de Navarra, 31006 Pamplona, Spain;* [2] *ECOSUR, AP 36, Tapachula 30700, Chiapas, Mexico;* [3] *Biological Sciences, University of Southampton, Southampton, SO16 7PX, UK;* [4] *Escuela Agrícola Panamericana, AP 93, El Zamorano, Honduras*

(*Received for publication 14 August 2000; revised manuscript accepted 21 February 2001*)

*Nucleopolyhedroviruses (*Baculoviridae*) are virulent insect pathogens that generally show a high degree of host specificity and have recognized potential as biological insecticides. Whenever viruses are applied for pest control, a proportion of the infected insects will also be parasitized by hymenopteran or dipteran parasitoids and interspecific competition for host resources will occur; the severity of such competition is likely to be modulated to a large degree by the virulence of each type of parasite. We examined the impact of parasitism by the solitary egg-larval endoparasitoid* Chelonus insularis *(Hymenoptera: Braconidae) on the speed of kill of nucleopolyhedrovirus-infected* Spodoptera frugiperda *(Lepidoptera: Noctuidae) larvae and the pattern of host growth and virus production in infected and/or parasitized hosts. We also examined the effect of parasitism on the virulence, infectivity and genetic composition of serially passaged virus. Both parasitism and viral infection resulted in a marked reduction in host growth. When third instar larvae were dually parasitized and virus-infected, the growth rate was even more severely affected compared to parasitized larvae. There was a significant increase in virus production in larvae infected at later instars. Interspecific competition resulted in a substantial decrease in pathogen production in parasitized larvae infected at the fourth instar, but not in parasitized larvae infected at earlier instars. The serial passage experiment resulted in the appearance of four distinct genetic isolates of the virus detected by restriction endonuclease analysis. Of the three isolates that appeared in nonparasitized larvae, two showed increased virulence, expressed by mean time to death, and for one of these the infectivity, expressed as* LC_{50}*, was reduced. One isolate that appeared in parasitized larvae (isolate D) had increased virulence and infectivity. Southern blot analysis indicated that virus isolate D*

Correspondence to: Dr. P. Caballero. Tel: +34 948 169129; Fax: +34 948 169 732; E-mail: pcm92@unavarra.es. Present address for J.W. Chapman: Entomology and Nematology Department, IACR Rothamsted, Harpenden, Herts AL5 2JQ, UK.

ISSN 0958-3157 (print)/ISSN 1360-0478 (online)/01/050649-14
DOI: 10.1080/09583150120076193

was most likely generated by point mutation of a restriction site or by alterations such as duplications, deletions or by recombination of two or more genotypic variants present in the wild-type nucleopolyhedrovirus isolate. Our study provides clear evidence of interspecific competition within the host, since, depending on the timing of inoculation, adverse effects were observed upon both the parasitoid and the virus.

Keywords: Spodoptera frugiperda, Chelonus insularis, *SfMNPV, infectivity, lethal time, genetic isolates, interspecific competition*

INTRODUCTION

Insect baculoviruses (*Baculoviridae*) are highly virulent pathogens that generally show a high degree of host specificity (Miller & Lu, 1997; Blissard *et al*., 2000). As such, they have attracted considerable attention as biological insecticides in programmes of integrated pest control (Moscardi, 1999). Due to their narrow host range, baculoviruses are generally believed to be compatible with the action of other insect predators and parasitoids present in the crop habitat (Vasconcelos *et al*., 1996; Stark *et al*., 1999).

Baculovirus isolates are normally a mixture of strains that differ in their virulence (Maruniak *et al*., 1999; Muñoz *et al*., 1999). In addition, virulence can differ according to the geographical origin of the virus (Shapiro *et al*., 1991; Escribano *et al*., 1999), or origin, species and growth stage of the host (Boots & Begon, 1995; García-Maruniak *et al*., 1996; Milks, 1997). Virulence can also be manipulated artificially by selection (Pavan & Ribeiro, 1989; Kolodny-Hirsch & Van Beek, 1997) or by recombinant DNA technology employed to enhance the speed of kill of the virus (Bonning & Hammock, 1996).

Although baculoviruses do not infect insect natural enemies, an important aspect in the evaluation of baculovirus bioinsecticides is the possible impact on predators and parasitoids through competitive or indirect effects (Brooks, 1993). In situations where a host is simultaneously infected by virus and parasitized by an insect parasitoid, possible interactions include the death of the parasitoid due to virus-induced host mortality (Eller *et al*., 1988), or due to toxic factors produced by the virus in infected hosts (Hotchkin & Kaya, 1983). Virus production can also be impaired due to competition for host resources from the developing parasitoid (Hochberg, 1991a). Consequently, the degree of competition that occurs between baculoviruses and parasitoids in dually infected and parasitized hosts is likely to be modulated to a large degree by the virulence of each type of parasite.

Similarly, the pattern of pest mortality can be affected by changes in the susceptibility of parasitized hosts to viral infection (Beegle & Oatman, 1974; Santiago-Alvarez & Caballero, 1990). There are relatively few studies of the impact of virus infection on insect parasitoids (Brooks, 1993), and none, as far as we are aware, that examine the impact of the parasitoid on the genetic composition and phenotypic traits of viral progeny from dually infected hosts.

The solitary egg-larval endoparasitoid *Chelonus insularis* (Cresson) (Hymenoptera: Braconidae) is one of the most abundant parasitoids of the fall armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in the southern USA, Mexico and Central America (Ashley, 1986; Wheeler *et al*., 1989). The nucleopolyhedrovirus (NPV, *Baculoviridae*) of *S. frugiperda* has recognized potential as a biological insecticide (Moscardi, 1999) and is currently being developed for use by Mesoamerican maize growers (Williams *et al*., 1999).

Whenever viruses are applied as bioinsecticides, a proportion of the infected hosts will also be parasitized by hymenopteran or dipteran parasitoids (Martínez *et al*., 2000). The impact of parasitism on viral insecticidal characteristics will be particularly relevant when virus production involves the application of virus to field populations of host larvae, followed by harvesting of virus-infected larvae and the subsequent recycling of this inoculum for pest control throughout the growing season, as occurs in the control of *Anticarsia gemmatalis*, the major pest of soya in Brazil (Moscardi, 1989; Moscardi *et al*., 1997).

In the present study, we examine the impact of parasitism on the speed of kill of virus-

infected armyworm larvae and the pattern of host growth and virus production in infected and/or parasitized hosts. We also examine the effect of parasitism on the genetic composition and virulence of serially passaged virus in an attempt to elucidate the impact of parasitism on the virus infectivity and the virus virulence which are two key characteristics that affect the insecticidal potential of the virus.

MATERIALS AND METHODS

Biological Material

Larvae of *S. frugiperda* were obtained from a culture established in 1997 at the Universidad Pública de Navarra, Spain, maintained on semisynthetic diet in a growth chamber at 26 ± 1°C, 85% relative humidity (RH), with a 16 h day length. The braconid egg-larval parasitoid *Chelonus insularis* originated from a culture started with field-collected parasitized *S. frugiperda* larvae at El Zamorano, Honduras. *C. insularis* lay their eggs into eggs of *S. frugiperda*, but larval parasitoid development is completed much later when the parasitized host larvae begin metamorphosis in the fifth instar while nonparasitized larvae do so in the sixth instar. Parasitized larvae included in the experiments detailed below were obtained by placing 400 recently laid eggs of *S. frugiperda* in a plastic Petri dish (115 mm diameter, 45 mm high), during 24 h, together with four *C. insularis* females that had been observed to mate 48 h previously.

All experiments involved a multiple nucleopolyhedrovirus (MNPV) originally isolated from *S. frugiperda* larvae in Nicaragua and recently characterized (Escribano *et al.*, 1999). Viral occlusion bodies (OBs) for use in experiments were extracted from dead diseased larvae by trituration in water and purified by various steps of differential centrifugation as described elsewhere (Caballero *et al.*, 1992). The concentration of the resulting purified suspension was determined using a bacterial counting chamber under phase-contrast microscopy.

Mortality, Larval Growth and Virus Production

Newly moulted third and fourth instar larvae, nonparasitized or parasitized by *C. insularis*, were allowed to drink from an aqueous suspension containing 3.32×10^7 and 6.83×10^8 OBs ml^{-1} of virus, respectively, in a solution of 10% sucrose and 0.01% Fluorella blue dye. These concentrations represented the LC_{90} values for third and fourth instar larvae, respectively, (Escribano *et al.*, 1999). Control larvae, nonparasitized or parasitized by *C. insularis*, were treated with a solution of sterile water and food colouring. Thirty larvae that had completely ingested the solution within 10 min (as indicated by the blue coloration of the gut visible through the larval epidermis) were individually transferred into 25 ml cups containing diet and maintained at 26 ± 1°C, 85% RH, 16 h day length. Daily readings were taken on larval weight (± 0.1 mg) and mortality due to virus infection or parasitoid emergence.

A further 20 second, third, four and fifth instar larvae parasitized at the egg stage and nonparasitized larvae, were infected with their corresponding LC_{90} (Escribano *et al.*, 1999), and reared as above. Larvae that died of virus infection were individually transferred to a microfuge tube, and homogenized in 300 μl of distilled water. Viral OBs were purified according to the method of Muñoz *et al.* (1997) and yields of OBs per infected larva were determined by counting triplicate samples of diluted virus suspension under phase-contrast microscopy. In all cases, only intact insect cadavers were used to obtain virus yield data. The presence of a developing parasitoid in each *S. frugiperda* larva used in the parasitism treatments was confirmed by dissection under a binocular microscope.

Serial Passage of Virus in Nonparasitized and Parasitized Larvae

Groups of 30 newly moulted third instar nonparasitized or parasitized larvae were infected as above with an LC_{90} concentration of virus. Larvae were maintained on diet until they

died or pupated. Virus-killed larvae from each treatment were collected and frozen. Viral OBs were recovered from each pool of larvae and purified as indicated above. A portion of the purified virus was used to infect other batches of nonparasitized and parasitized larvae, for the following passage. The remaining virus was used for viral DNA purification and bioassay analysis (see below). This method of *in vivo* passaging was repeated using groups of 30 third instar larvae for a total of 10 passages. The experiment was performed three times.

DNA Analysis of Virus in Serial Passage Experiment

Virions were released from viral OBs by incubation with 30 mM-Na_2CO_3, 50 mM-NaCl, 30 mM-EDTA [pH 10.5] and purified by centrifugation in continuous sucrose gradient (Caballero *et al*., 1992). Purified virions were incubated with proteinase K (200 μg ml^{-1}) at 45–50ºC for 2.5 h and then with 1% sodium dodecyl sulfate for an additional 30 min. After phenol-chloroform extraction, the aqueous suspension containing the DNA was dialyzed against three changes of 10 mM-TE buffer at 4ºC for 48 h. DNA was incubated with *Pst*I or *Bgl*II (Amersham) at 37ºC for 4 h as described in the supplier's instructions. Reactions were stopped by the addition of loading buffer (0.25% bromophenol blue, 40% sucrose). Electrophoresis was performed using 0.8% agarose gel in TAE buffer (40 mM-Tris-acetate, 1 mM-EDTA [pH 8.0]) containing 0.25 μg ml^{-1} of ethidium bromide and photographed on a UV transilluminator.

Southern Blot Hybridization

For Southern blot analysis, the DNA from nonparasitized *S. frugiperda* larvae, parasitized *S. frugiperda* larvae, parasitized *S. frugiperda* larvae from which the parasitoid larvae had been removed, and adult female *C. insularis* was isolated using the procedure described by Heckel *et al*. (1995). DNA samples were digested with *Bgl*II, separated by electrophoresis and the gel was subjected to Southern blotting onto a Hybond-N + (Amersham) membrane overnight. A submolar DNA fragment that appeared in one DNA restriction profile in virus recovered from larvae parasitized by *C. insularis* was used as a probe. This submolar fragment was separated in low melting point agarose before being labelled with [$\alpha - ^{32}$P]dCTP by random priming (Sambrook *et al*., 1989). Labelled probe was allowed to hybridize at 42ºC in 5 × SSC (1 × SSC is 0.18 M NaCl, 10 mM-NaH_2PO_4, and 1 mM-EDTA, pH 7.7), 50% formamide, 20 mM-$NaPO_4$ (pH 7.0), 1 × Denhardt's solution, and 0.1 mg of herring-sperm DNA. After hybridization, the blots were washed twice, 15 min each, at 42ºC in 2 × SSC containing 0.1% SDS and exposed to X-ray film (Biomax, Kodax, Rochester, N.Y.) for 3 to 48 h at −80ºC with an intensifying screen.

Biological Activity of Virus in Serial Passage Experiment

The biological activity of the virus isolates produced in the different passages was determined in terms of infectivity and virulence. The infectivity, defined as the ability of a microorganism to produce infection, was expressed by means of LC_{50}, and the virulence, defined as the disease producing power of a microorganism, was expressed by the mean time to death. The median lethal concentrations (LC_{50}) and the mean times to death of the original wild type isolate and the virus samples collected after 1, 3, 6 and 10 passages were determined by droplet bioassay (Hughes & Wood, 1981). First instar *S. frugiperda* larvae were selected at the moment they began to moult as determined by head capsule slippage. Selected larvae were starved for 8 h at 25ºC and were then offered an aqueous suspension containing virus at concentrations of 9.6×10^3, 4.8×10^4, 2.4×10^5, 1.2×10^6, or 6×10^6 OBs ml^{-1}. This range of concentrations was found to kill between 5 and 95% of the test larvae in bioassays previously performed with the original isolate. Larvae that ingested the solution within 10 min were transferred to individual cells of a 25-compartment plastic dish with diet and maintained at 25 ± 2ºC. Larval mortality was recorded every 12 h until larvae had either died or pupated. Bioassays with 25 larvae per virus concentration plus 25 larvae as a control were performed three times.

Data Analysis

Virus yield and time-mortality data were analyzed for each host instar at time of virus infection and larval condition (nonparasitized or parasitized by *C. insularis*) using analysis of variance (ANOVA) models in the generalized linear modelling package of the program SPSS (v. 7.5). The data were obtained as one piece of information for each larva and, therefore, each data point was independent of the others. Model-checking showed that these data satisfied the assumptions of normality required by ANOVA. Differences between individual treatments were assessed using independent contrasts. To perform these contrasts, data in different treatments were grouped together, and the old treatment variable was replaced in the ANOVA models by the new grouping. If this replacement caused a non-significant change in the variation explained by the model, there was no significant difference between the grouped treatments. Concentration-mortality regressions for the different virus inocula bioassayed were calculated with the probit option (Finney, 1971) of the computer program POLO-PC (Le Ora Software, 1987).

RESULTS

Mortality, Larval Growth and Virus Production

Virus mortality for both nonparasitized and parasitized *S. frugiperda* larvae was in all cases greater than 95%. The mean time to death of virus-infected parasitized *S. frugiperda* larvae (98.4 h ± 3.33) did not differ significantly from that observed in virus-infected nonparasitized larvae (100.8 h ± 4.61) ($F_{1,48} = 0.29$; NS) of the third instar. In contrast, mortality due to parasitism in parasitized but not infected larvae occurred at 149.3 h (± 5.8 h) after the start of the experiment in larvae parasitized by *C. insularis* (all figures are means ± 95% CL).

Parasitism by *C. insularis* resulted in diminished host growth compared to the nonparasitized *S. frugiperda* larvae. Virus infection also severely reduced host growth from 48 h or 72 h onwards compared to mock-infected control larvae. Weight gain was most severely compromised in larvae that were dually virus infected and parasitized by *C. insularis* (Figure 1).

There was a highly significant increase in virus production with larval instar ($F_{2,54} = 78.8$; $P < 0.001$); the effect of parasitism was also highly significant ($F_{1,54} = 19.2$; $P < 0.001$) and there was a significant interaction between these factors ($F_{2,54} = 19.8$; $P < 0.001$). In nonparasitized larvae there was a significant positive relationship between virus yield and the stage of the larva at infection (Table 1). In parasitized larvae, the number of viral OBs produced in infected larvae was significantly reduced compared to nonparasitized larvae only in the fourth instar, not in the preceding instars. Since parasitoid emergence occurred prior to the fifth instar, data on virus yield in parasitized fifth instar larvae could not be obtained.

DNA Analysis of Virus in Serial Passage Experiment

In addition to the wild-type (WT) restriction endonuclease profile described previously (Escribano *et al*., 1999), DNA extracted from viral OBs after passages 1, 3, 6 and 10 from virus-killed nonparasitized and parasitized larvae showed four isolates when subjected to restriction endonuclease analysis using *Bgl*II or *Pst*I (Figure 2). These isolates, hereafter referred to as A, B, C and D were characterized by the presence of a particular submolar fragment different for each isolate. Isolates A, B, and C, showed a submolar fragment at 6.1 kb, 8.1 kb, and 6.5 kb, respectively, when treated with *Pst*I but were indistinguishable from the WT when treated with *Bgl*II. Isolate profile D was characterized by a submolar fragment at 14.5 kb following treatment with *Bgl*II, but did not differ from the WT restriction endonuclease profile when treated with *Pst*I.

Profile isolates A, B, and C were only detected in virus produced in nonparasitized *S. frugiperda* larvae while profile isolate D was only detected in virus recovered from parasitized larvae (Table 2). Profile isolate A was detected in experiments I and III at

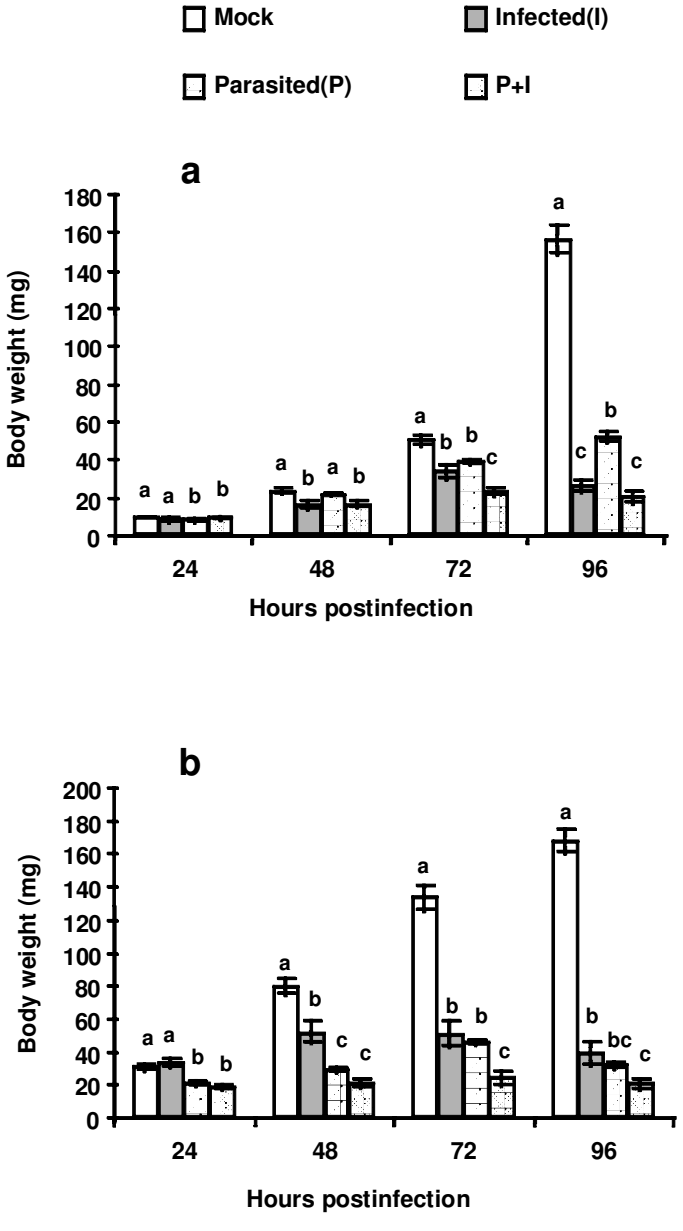

FIGURE 1. Body weight of *Spodoptera frugiperda* third (a) and fourth (b) instars infected with nucleopolyhedrovirus and/or parasitized by *Chelonus insularis*. Data were obtained as one piece of the information for each larva and timepoint and, therefore, each data point was independent of the others. Columns represent means ± SE.

passage 6 and 10, respectively, but in experiment I isolate A was replaced by isolate B at passage 10. Profile C appeared in experiment II at passage 6 and remained unchanged until passage 10. Profile D was detected in experiment II with larvae parasitized by *C. insularis* at passage 3. After appearing, this isolate remained unchanged in subsequent passages suggesting that it represents some consistent advantage for the virus in parasitized hosts.

TABLE 1. Viral occlusion body (OB) production in different instars of *Spodoptera frugiperda* larvae nonparasitized or parasitized by *Chelonus insularis*

Larval condition	Production of virus at each instar[a] (Mean number OBs/larva $\times 10^8 \pm$ SE)			
	L_2	L_3	L_4	L_5
Nonparasitized	0.31 ± 0.04a	1.26 ± 0.15b	3.37 ± 0.47c	15.10 ± 1.60d
Parasitized, *C. insularis*	0.36 ± 0.02a	1.21 ± 0.11b	1.55 ± 0.12b	ND

ND: not determined as parasitoid emergence occurred in fifth instar.

[a]Means followed by the same letter are not significantly different, Fisher's LSD test ($P > 0.05$). Data were obtained as one piece of the information for each larva and, therefore, each data point was independent of the others.

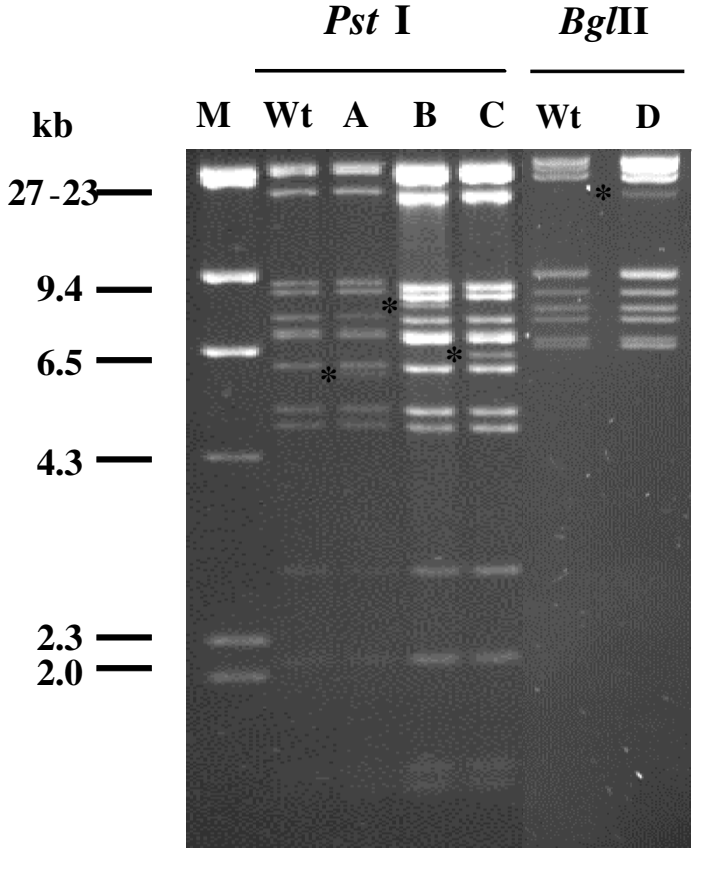

FIGURE 2. Restriction endonuclease profiles of viral DNA, amplified in *Spodoptera frugiperda* larvae nonparasitized, parasitized by *Chelonus insularis* following digestion with *Pst*I or *Bgl*II. M (Marker), wt (wild-type virus), A, B and C are virus isolates that appeared in nonparasitized larvae, D is a virus isolate that appeared only in parasitized larvae. A *Hin*dIII digest of λ DNA was used as molecular marker. Asterisks indicate the presence of distinct submolar fragments in each isolate.

TABLE 2. Restriction endonuclease profiles generated after serial passage of wild-type (WT) virus in nonparasitized and parasitized *Spodoptera frugiperda* larvae

Treatment and passage number	*Pst*I profile[a]			*Bgl*II profile[a]		
	I	II	III	I	II	III
Nonparasitized						
P_1	WT	WT	WT	WT	WT	WT
P_3	WT	WT	WT	WT	WT	WT
P_6	A	C	WT	WT	WT	WT
P_{10}	B	C	A			
WT	WT	WT				
Parasitized *C. insularis*						
P_1	WT	WT	WT	WT	WT	WT
P_3	WT	WT	WT	WT	D	WT
P_6	WT	WT	WT	WT	D	WT
P_{10}	WT	WT	WT	WT	D	WT

[a]WT, A, B, C, and D represent the different virus isolates identified by their DNA restriction endonuclease profiles presented in Figure 2. I, II and III represent the results of three replicate experiments.

Southern blot analysis of the profile D indicated that the submolar fragment at 14.5 kb derived from the viral genome, since hybridization signals were detected in the DNA isolated from virus isolate D and from wild-type virus but not with the DNA isolated from nonparasitized *S. frugiperda* larvae, *S. frugiperda* larvae parasitized by *C. insularis* with or without the parasitoid larva, and adult female *C. insularis* (Figure 3). The probe showed

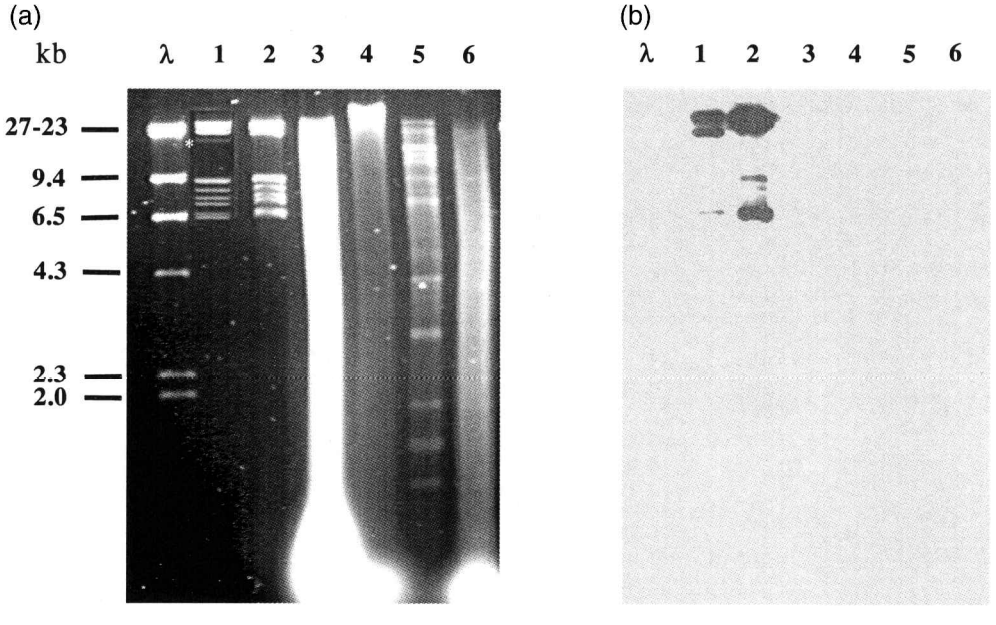

FIGURE 3. (a) Restriction endonuclease profiles of *Bgl*II-digested DNA from: virus isolate D (lane 1), wild-type virus (lane 2), *Spodoptera frugiperda* larvae (lane 3), *S. frugiperda* parasitized larvae (lane 4) *S. frugiperda* parasitized larvae with parasitoid larva removed (lane 6) and adult female *C. insularis* (lane 5). (b) Southern blot analysis of gel shown in (A) using the 14.5 kb submolar *Bgl*II fragment of virus isolate D as probe. Lane λ, lambda DNA digested with *Hin*dIII.

strong hybridization with the corresponding submolar fragment at 14.5 kb in the virus isolate D profile but no hybridization was detected in the wild-type virus profile at the 14.5 kb migration point indicating that the submolar fragment was not present in detectable quantities in the wild-type isolate. Hybridization signals of different intensities were also detected in various equimolar restriction fragments of both virus isolate D and wild-type virus DNA.

Biological Activity of Virus in Serial Passage Experiment

The infectivity of each virus isolate (WT, A, B, C, and D) was determined by bioassay and, in all cases, virus induced mortality increased with virus concentration. The probit analysis regression line slopes were fitted in parallel with a common slope of 0.76 ($\chi^2 = 7.52$, df $= 4$, $P = 0.479$) (Table 3). The relative potencies of the four novel virus isolates was calculated as the ratio of the probit regression of the wild-type and each novel isolate, with the corresponding 95% fiducial limits (Finney, 1971). Virus produced in parasitized larvae (virus isolate D) was 5.5 times more potent than the original WT inoculum. No significant differences were found between the virus profiles A and B compared to wild type virus, while the virus isolate C was significantly less infective.

The profile isolates also differed in their virulence as determined by mean time to death of insects infected in bioassays; only isolate A was not significantly different from the wild-type virus. Of the remaining isolates, isolate D showed a 10.8% reduction in mean lethal time and isolate C was still more virulent, with a 16.8% reduction in mean lethal time compared to the wild type virus. Isolate B was intermediate between C and D (Table 3).

DISCUSSION

Both parasitism and viral infection resulted in a marked reduction in host growth. When third instar larvae were dually parasitized and infected, the growth rate was even more severely affected compared to parasitized larvae. There was a significant increase in virus production in larvae infected at later instars, reflecting the correlation between log body weight and log virus production reported in baculovirus infections of other species of Lepidoptera (Evans *et al*., 1981; Kunimi *et al*., 1996). Interspecific competition for host resources resulted in a substantial decrease in pathogen production in parasitized larvae infected at the fourth instar, but not in parasitized larvae infected at earlier instars. This effect may be related to the stage of development of the parasitoid and its effects on the rate of growth of the host larvae; the yield of viral occlusion bodies is positively correlated with host growth rate (Shapiro, 1986). Almost no host growth was observed in dually parasitized and infected fourth instar larvae during the 96 h study period (Figure 1(b)) whereas the weight of parasitized + infected third instar larvae more than doubled in the same period (Figure 1(a)). The survival of *C. insularis* was not possible in *S. frugiperda* larvae that ingested a lethal dose of the nucleopolyhedrovirus during the second, third or early fourth larval instars confirming the results previously reported by Escribano *et al*. (2000).

The serial passage experiment resulted in the appearance of distinct genetic isolates. Of the three isolates from nonparasitized larvae, two showed increased virulence and for one of these (isolate C), the infectivity was reduced. One isolate arose in parasitized larvae which had increased virulence and infectivity.

There are several possible explanations for the origins of these genetic isolates. DNA hybridization analysis revealed that the 14.5 kb submolar fragment comprised virus genomic sequences; hybridization signals being detected in the *Bgl*II digest of virus isolate D and the wild-type isolate, but not in DNA from unparasitized or parasitized *S. frugiperda* larvae (with or without the parasitoid larvae) or adult female *C. insularis* wasps. Since the Southern hybridization experiment was performed three times, using a different probe preparation on each occasion, the most likely explanation for the hybridization signals detected in the wild-type isolate is that regions homologous to probe sequences exist in other parts of the virus

TABLE 3. Response of second instar *Spodoptera frugiperda* larvae to wild-type virus and four virus isolates (A–D) harvested after serial passage in *S. frugiperda* larvae nonparasitized and parasitized by *Chelonus insularis*

Virus variant	Regression equation	SE of slope	LC_{50} (OBs ml^{-1})	Relative potency	95% CL		Mean time to death (h)	95% CL	
					lower	upper		lower	upper
WT	$y = 0.76x + 0.84$	0.209	2.87×10^5	1	—	—	83.5	75.8	91.19
A	$y = 0.76x + 0.77$	0.208	3.65×10^5	0.78	0.274	2.259	89.2	82.08	96.36
B	$y = 0.76x + 1.09$	0.199	1.38×10^5	2.07	0.738	5.885	72.6	67.23	77.86
C	$y = 0.76x + 0.45$	0.193	9.46×10^5	0.30	0.128	0.701	69.5	65.64	73.41
D	$y = 0.76x + 1.41$	0.179	5.22×10^4	5.51	2.227	13.819	74.4	72.44	76.36

Regression lines, LC_{50} values, and relative potency with respect to the wild-type (wt) virus, were calculated using the POLO-PC program (Le Ora Software, 1987). LC_{50} values of virus isolates A and B did not differ significantly from the wt isolate, whereas isolates C and D were significantly less and more infective, respectively. The time-mortality data were subjected to analysis of variance using the generalized linear modelling package of the program SPSS (v. 7.5). Isolates A and B did not differ significantly from the wt isolate, whereas isolates C and D were significantly less and more virulent, respectively.

genome. These results indicate that virus isolate D did not originate via insertion of a host or parasitoid transposon. It also appears unlikely that a latent virus infection was involved, as has been identified in a laboratory culture of *Mamestra brassicae* (Hughes *et al*., 1997), due to the fact that isolate D appeared only in parasitized hosts and not in unparasitized conspecifics from the same culture. Parasitism, however, may have caused immunosupression as *Chelonus* species have an associated polydnavirus (Stoltz & Whitfield, 1992), and this might have permitted the expression of a latent virus, although we saw no signs of baculovirus disease in parasitized hosts that were not inoculated with virus.

An alternative explanation is that isolate D was generated by selection of a genotypic variant present in the wild-type isolate at a very low concentration. Natural baculovirus isolates typically represent a mixture of individual genotypes that may be cloned *in vitro* by plaque purification (Maruniak *et al*., 1984; Ribeiro *et al*., 1997) or *in vivo* by limiting dilution assays in host larvae (Smith & Crook, 1988). These variants can exist at such low concentrations that their presence cannot be detected using DNA hybridisation techniques (Muñoz *et al*., 1999). We suggest this possibility is also unlikely as isolate D appeared in only one of three replicate experiments using parasitized larvae run in parallel under the same conditions, and would therefore have experienced identical pressures of selection resulting in the appearance of this isolate in all three replicates.

Finally, we suggest that isolate D was most likely generated by point mutation of a restriction site or by alterations such as duplications, deletions or by recombination of two or more genotypic variants present in the wild-type nucleopolyhedrovirus isolate. Each of these mechanisms has been identified in the formation of genotypic variants of the nucleopolyhedroviruses of *Anticarsia gemmatalis* (Croizier & Ribeiro, 1992; García-Maruniak *et al*., 1996; Maruniak *et al*., 1999) and *Spodoptera exigua* (Muñoz *et al*., 1998; 1999). Genotypic variants present in one wild type SfMNPV isolate have been cloned and mapped (Maruniak *et al*., 1984) and many others have been detected by the presence of submolar bands in restriction endonuclease profiles (Shapiro *et al*., 1991; Escribano *et al*., 1999). This possible origin of virus isolate D is compatible with the observed results in that it may have only been generated in one of the three replicates, and due to its increased virulence, would have been selected favourably over other virus variants.

Differences in infectivity or virulence between genetic variants present within an isolate have been reported before. Genotypic variants cloned from wild-type *A. gemmatalis* nucleopolyhedrovirus (Ribeiro *et al*., 1997) exhibited marked differences in infectivity while variants from *Autographa californica* nucleopolyhedrovirus appeared to be phenotypically uniform in terms of infectivity and virulence (Andrews *et al*., 1980). Differences in the infectivity of certain nucleopolyhedrovirus variants towards heterologous host species have also been reported (Weitzman *et al*., 1992; García-Maruniak *et al*., 1996). Recently, Muñoz *et al*. (1998) presented evidence that two genotypic variants present in a nucleopolyhedrovirus wild-type isolate were naturally-occurring deletion mutants. These variants were parasitic; for replication, they required the presence of genes present in other virus variants. Furthermore, the presence of deletion mutants was associated with a reduction in the insecticidal activity of the wild-type isolate and of other variant mixtures in which they were present.

If genetic variants present within an isolate differ in their biological activity, they are likely to compete intraspecifically within the host. Serial passage is likely to lead to dominance of whatever strain(s) are favoured by the prevailing conditions. For example, under field conditions, serial passage of *A. gemmatalis* NPV (AgMNPV) over the course of six years by soya growers in Brazil resulted in an increase in genetic heterogeneity that was associated with greater heterogeneity in the virulence of variants cloned out of recycled inoculum (Maruniak *et al*., 1999). Abot *et al*. (1995) reported an overall decrease in viral infectivity following recycling of AgMNPV inoculum. In contrast, a high degree of stability of phenotypic traits has previously been reported following 20 serial passages of isolates of *Helicoverpa zea* NPV (McIntosh & Ignoffo, 1986). In the present study, no changes were detected in the infectivity or virulence of the wild-type SfMNPV material following ten

passages, but in several replicates previously undetectable variants with differing phenotypes became more abundant.

Our study provides clear evidence of interspecific competition within the host, since, depending on the timing of inoculation, adverse effects were found upon both the parasitoid and the virus. Direct consequences on parasitoids developing in virus-infected hosts have been reported including the production of virus-induced toxic factors that inhibit parasitoid development (Kaya & Tanada, 1971, 1973; Hotchkin & Kaya, 1983), premature death of the host (Brooks, 1993) and the preclusion of parasitoid persistence in virus-infected host populations (Bird & Elgree, 1957). The body size of a braconid endoparasitoid was reduced in *Heliothis virescens* larvae infected with a recombinant baculovirus expressing an insect neurotoxin gene, probably due to a reduced development time in recombinant virus infected hosts compared to larvae infected with the wild-type virus (McCutchen *et al*., 1996).

Conversely, the presence of a developing parasitoid may have detrimental effects on the production of progeny virus (Caballero *et al*., 1990; and this study), but parasitoids that emerge from baculovirus-infected hosts have also been shown to be responsible for virus transmission during the act of oviposition (Young & Yearian, 1990; Hochberg, 1991b; Caballero *et al*., 1991) and as such, parasitoids may be important agents for the dispersal of these viruses.

This study presents the first evidence of the consequences of parasitism on characteristics pivotal to the insecticidal potential of the virus. The virus isolate that appeared during serial passage of virus exclusively in parasitized hosts showed increase virulence and infectivity compared to wild-type virus. It seems unlikely that this was a purely chance observation as none of the three isolates that appeared in non-parasitized hosts were observed in dually parasitized and infected larvae.

Within-host intraspecific competition in serial passage experiments drives the evolution of virulence; the pathogen strain with the highest replication rate will achieve the greatest numerical representation in the inoculum used to infect the following generation of insects and will thereby have a selective advantage over competitor strains (Ebert, 1998). In parasitized hosts infected by virus, the situation is slightly more complicated as all strains of the pathogen also experience interspecific competition for host resources with the developing parasitoid larvae. As yet, we understand very little about the processes that maintain genetic and phenotypic diversity within pathogen populations. Presumably, changes in virus infectivity and virulence carry associated costs in some other component(s) of virus fitness but for the moment these remain unknown.

ACKNOWLEDGEMENTS

This research was funded by the European Commission (contract number IC18-CT96–0097). A. Escribano received a grant from the Universidad Pública de Navarra. T. Williams received additional support from SIBEJ9901015047.

REFERENCES

Abot, A.R., Moscardi, F., Fuxa, J.R. Sosa-Gómez, D.R. & Richter, A.R. (1995) Susceptibility of populations of *Anticarsia gemmatalis* from Brazil and the United States to a nuclear polyhedrosis virus. *Journal of Entomological Science* **30**, 62–69.

Andrews, R.E., Spence, K.D. & Miller, L.K. (1980) Virulence of cloned variants of *Autographa californica* nuclear polyhedrosis virus. *Applied and Environmental Microbiology* **39**, 932–933.

Ashley, T.R. (1986) Geographical distributions and parasitization levels for parasitoids of the fall armyworm, *Spodoptera frugiperda*. *Florida Entomologist* **69**, 516–524.

Beegle, C.C. & Oatman, E.R. (1974) Differential susceptibility of parasitized and non-parasitized larvae of *Trichoplusia ni* to a nuclear polyhedrosis virus. *Journal of Invertebrate Pathology* **24**, 188–198.

Bird, F.T. & Elgree, D.E. (1957) A virus disease and introduced parasites as factors controlling the European spruce sawfly, *Diprion hercyniae* (Htg.) in Central New Brunswick. *Canadian Entomologist* **89**, 371–378.

Blissard, G.W., Black, B.N., Crook, N.E. Keddie, R., Possee, R.D., Rohrmann, G.F., Thielmann, D.A. & Volkman, L.E. (2000) Baculoviridae, in *Virus Taxonomy: Seventh Report of the International Committee on Taxonomy of Viruses* (Van Regenmortel, M.H.V., Fauquet, C.M., Bishop, D.H.L., Carstens, E.B., Estes, M.K., Lemon, S.M., Maniloff, J., Mayo, M.A., McGeoch, D.J., Pringle, C.R. & Wickner, R.B., Eds). Academic Press, New York, pp. 195–202.

Bonning, B.C. & Hammock, B.D. (1996) Development of recombinant baculoviruses for insect control. *Annual Review of Entomology* **41**, 191–210.

Boots, M. & Begon, M. (1995) Strain differences in the Indian meal moth, *Plodia interpunctella*, in response to a granulosis virus. *Researches on Population Ecology* **37**, 37–42.

Brooks, W.M. (1993) Host-parasitoid-pathogen interactions, in *Parasites and pathogens of insects, Vol. 2: Pathogens* (Beckage, N.E., Thompson, S.N. & Federici, B.A., Eds). Academic Press, San Diego, pp. 231–272.

Caballero, P., Vargas-Osuna, E. & Santiago-Alvarez, C. (1990) Development of *Apanteles telengai* (Hym.: Braconidae) and *Campoletis annulata* (Hym.: Ichneumonidae) in granulosis virus (GV) infected *Agrotis segetum* (Lep.: Noctuidae) larvae. *Journal of Applied Entomology* **110**, 358–364.

Caballero, P., Vargas-Osuna, E. & Santiago-Alvarez, C. (1991) Parasitization of granulosis-virus infected and noninfected *Agrotis segetum* larvae and the virus transmission by three hymenopteran parasitoids. *Entomologia Experimentalis et Applicata* **58**, 55–60.

Caballero, P., Zuidema, D., Santiago-Alvarez, C. & Vlak, J.M. (1992) Biochemical and biological characterization of four isolates of *Spodoptera exigua* nuclear polyhedrosis virus. *Biocontrol Science and Technology* **2**, 145–157.

Croizier, G. & Ribeiro, C.P. (1992) Recombination as a possible major cause of genetic heterogeneity in *Anticarsia gemmatalis* nuclear polyhedrosis virus populations. *Virus Research* **26**, 183–196.

Ebert, D. (1998) Experimental evolution of parasites. *Science* **282**, 1432–1435.

Eller, F.J., Boucias, D.G. & Tumlinson, J.H. (1988) Interactions between *Microplitis croceipes* (Hymenoptera: Braconidae) and a nuclear polyhedrosis virus of *Heliothis zea* (Lepidoptera: Noctuidae). *Environmental Entomology* **17**, 977–982.

Escribano, A., Williams, T., Goulson, D. Cave, R.D., Chapman, J.W. & Caballero, P. (1999) Selection of a nucleopolyhedrovirus for control of *Spodoptera frugiperda* (Lepidoptera: Noctuidae): structural, genetic, and biological comparison of four isolates from the Americas. *Journal of Economic Entomology* **92**, 1079–1085.

Escribano, A., Williams, T., Goulson, D. Cave, R.D. & Caballero, P. (2000) Parasitoid-pathogen-pest interactions of *Chelonus insularis*, *Campoletis sonorensis*, and a nucleopolyhedrovirus in *Spodoptera frugiperda* larvae. *Biological Control* **19**, 265–273.

Evans, H.F., Lomer, C.J. & Kelly, D.C. (1981) Growth of nuclear polyhedrosis virus in larvae of the cabbage moth, *Mamestra brassicae* L. *Archives of Virology* **70**, 207–214.

Finney, D.J. (1971) *Probit Analysis*. Cambridge University Press, London, UK.

García-Maruniak, A., Pavan, O.H.O. & Maruniak, J.E. (1996) A variable region of *Anticarsia gemmatalis* nuclear polyhedrosis virus contains tandemly repeated DNA sequences. *Virus Research* **41**, 123–132.

Heckel, D.G., Gahan, L.J., Tabashnik, B.E. & Johnson, M.W. (1995) Randomly amplified polymorphic DNA differences between strains of diamondback moth (Lepidoptera; Plutellidae) susceptible or resistant to *Bacillus thuringiensis*. *Annals of the Entomological Society of America* **88**, 531–537.

Hochberg, M.E. (1991a) Intra-host interactions between a braconid endoparasitoid, *Apanteles glomeratus*, and a baculovirus for larvae of *Pieris brassicae*. *Journal of Animal Ecology* **60**, 51–63.

Hochberg, M.E. (1991b) Extra-host interactions between a braconid endoparasitoid, *Apanteles glomeratus*, and a baculovirus for larvae of *Pieris brassicae*. *Journal of Animal Ecology* **60**, 65–77.

Hotchkin, P.G. & Kaya, H.K. (1983) Pathological response of the parasitoid, *Glyptapanteles militaris*, to nuclear polyhedrosis virus-infected armyworm hosts. *Journal of Invertebrate Pathology* **42**, 51–61.

Hughes, P.R. & Wood, H.A. (1981) A synchronous peroral technique for the bioassay of insect viruses. *Journal of Invertebrate Pathology* **37**, 154–159.

Hughes, D.S., Possee, R.D. & King, L.A. (1997) Evidence for the presence of a low-level, persistent baculovirus infection of *Mamestra brassicae* insects. *Journal of General Virology* **78**, 1801–1805.

Kaya, H.K. & Tanada, Y. (1971) Properties of a viral factor toxic to the parasitoid *Apanteles militaris*. *Journal of Insect Physiology* **17**, 2125–2138.

Kaya, H.K. & Tanada, Y. (1973) Hemolymph factor in nuclear polyhedrosis virus toxic to *Apanteles militaris*. *Journal of Invertebrate Pathology* **21**, 211–214.

Kolodny-Hirsch, D. & Van Beek, N.A.M. (1997) Selection of a morphological variant of *Autographa californica* nuclear polyhedrosis virus with increased virulence following serial passage in *Plutella xylostella*. *Journal of Invertebrate Pathology* **69**, 205–211.

Kunimi, Y., Fuxa, J.R. & Hammock, B.D. (1996) Comparison of wild type and genetically engineered nuclear polyhedrosis viruses of *Autographa californica* for mortality, virus replication and polyhedra production in *Trichoplusia ni* larvae. *Entomologia Experimentalis et Applicata* **81**, 251–257.

Le Ora Software (1987) *POLO-PC a User's Guide to Probit or Logit Analysis*. Berkeley, CA, USA.

Martínez, A.M., Goulson, D., Chapman, J.W. Caballero, P., Cave, R. D. & Williams, T. (2000) Is it

feasible to use optical brightener technology with a baculovirus bioinsecticide for resource-poor maize farmers in Mesoamerica? *Biological Control* **17**, 174–181.

Maruniak, J.E., Brown, S.E. & Knudson, D.L. (1984) Physical maps of SfMNPV baculovirus DNA and its genomic variants. *Virology* **136**, 221–234.

Maruniak, J.E., Garcia-Maruniak, A., Souza, P. Zanotto, M.A. & Moscardi, F. (1999) Physical maps and virulence of *Anticarsia gemmatalis* nucleopolyhedrovirus genomic variants. *Archives of Virology* **144**, 1991–2006.

McCutchenn, B.F., Herrmann, R., Heinz, K.M. Parrella, M.P. & Hammock, B.D. (1996) Effects of recombinant baculoviruses on a nontarget endoparasitoid of *Heliothis virescens. Biological Control* **6**, 45–50.

McIntosh, A.H. & Ignoffo, C.M. (1986) Restriction endonuclease cleavage patterns of commercial and serially passaged isolates of *Heliothis Baculovirus. Intervirology* **25**, 172–176.

Milks, M.L. (1997) Comparative biology and susceptibility of cabbage looper (Lepidoptera: Noctuidae) lines to a nuclear polyhedrosis virus. *Environmental Entomology* **26**, 839–848.

Miller, L.K. & Lu, A. (1997) The molecular basis of baculovirus host range, in *The Baculoviruses* (L.K. Miller, Ed.). Plenum Press, New York, pp. 217–236.

Moscardi, F. (1989) Use of viruses for pest control in Brazil: the case of the nuclear polyhedrosis virus of the soy bean caterpillar, *Anticarsia gemmatalis. Memorias Instituto Oswaldo Cruz* **84**, 51–56.

Moscardi, F. (1999) Assessment of the application of baculoviruses for control of Lepidoptera. *Annual Review of Entomology* **44**, 257–289.

Moscardi, F., Leite, L.G. & Zamataro, C.E. (1997) Production of nuclear polyhedrosis virus of *Anticarsia gemmatalis* Hübner (Lepidoptera: Noctuidae): effect of virus dosage, host density and age. *Annals of the Entomological Society of Brasil* **26**, 121–32.

Muñoz, D., Vlak, J.M. & Caballero, P. (1997) *In vivo* recombination between two strains of the genus *Nucleopolyhedrovirus* in its natural host *Spodoptera exigua. Applied and Environmental Microbiology* **63**, 3025–3031.

Muñoz, D., Castillejo, J.I. & Caballero, P. (1998) Naturally occurring deletion mutants are parasitic genotypes in a wild-type nucleopolyhedrovirus population of *Spodoptera exigua. Applied and Environmental Microbiology* **64**, 4372–4377.

Muñoz, D., Murillo, R., Krell, P.J. Vlak, J.M. & Caballero, P. (1999) Four genotypic variants of a *Spodoptera exigua* nucleopolyhedrovirus (Se-SP2) are distinguishable by a hypervariable genomic region. *Virus Research* **59**, 61–74.

Pavan, O.H.O. & Ribeiro, H.C.T. (1989) Selection of a baculovirus strain with a bivalent insecticidal activity. *Memorias Instituto Oswaldo Cruz* **84**, 63–65.

Ribeiro, H.C.T., Pavan, O.H.O. & Muotri, A.R. (1997) Comparative susceptibility of two different hosts to genotypic variants of the *Anticarsia gemmatalis* nuclear polyhedrosis virus. *Entomologia Experimentalis et Applicata* **83**, 233–237.

Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA.

Santiago-Alvarez, C. & Caballero, P. (1990) Susceptibility of parasitized *Agrotis segetum* larvae to a granulosis virus. *Journal of Invertebrate Pathology* **56**, 128–131.

Shapiro, D.I., Fuxa, J.R., Braymer, H.D. & Pashley, D.P. (1991) DNA restriction polymorphism in wild isolates of *Spodoptera frugiperda* nuclear polyhedrosis virus. *Journal of Invertebrate Pathology* **58**, 96–105.

Shapiro, M. (1986) *In vivo* production of baculoviruses, in *The Biology of Baculoviruses. Vol 2: Practical Application for Insect Control* (Granados, R.R. &. Federici, B.A., Eds). CRC Press, Florida, pp. 31–62.

Smith, I.R.L. & Crook, N.E. (1988) *In vivo* isolation of baculovirus genotypes. *Virology* **166**, 240–244.

Stark, D.M., Mills, N.J. & Purcell, A.H. (1999) Interactions between the parasitoid *Ametadoria misella* (Diptera: Tachinidae) and the granulovirus of *Harrisina brillians* (Lepidoptera: Zygaenidae). *Biological Control* **14,** 146–151.

Stoltz, D.B. & Whitfield, J.B. (1992) Viruses and virus-like entities in the parasitic Hymenoptera. *Journal of Hymenoptera Research* **1**, 125–139.

Vasconcelos, D.S., Williams, T., Hails, R.S. & Cory, J.S. (1996) Prey selection and baculovirus dissemination by carabid predators of Lepidoptera. *Ecological Entomology* **21**, 98–104.

Weitzman, M.D., Possee, R.D. & King, L.A. (1992) Characterization of two variants of *Panolis flammea* multiple nucleocapsid nuclear polyhedrosis virus. *Journal of General Virology* **73**, 1881–1886.

Wheeler, G.S., Ashley, T.R. & Andrews, K.L. (1989) Larval parasitoids and pathogens of the fall armyworm in Honduran maize. *Entomophaga* **34,** 331–340.

Williams, T., Goulson, D., Caballero, P. Cisneros, J., Matínez, A.M., Chapman, J.W., Roman, D.X. & Cave, R.D. (1999) Evaluation of baculoviruses bioinsecticide for small-scale maize growers in Latin America. *Biological Control* **14**, 67–75.

Young, S.Y. & Yearian, W.C. (1990) Transmission of nuclear polyhedrosis virus by the parasitoid *Microplitis croceipes* (Hymenoptera: Braconidae) to *Heliothis virescens* (Lepidoptera: Noctuidae) on soybean. *Environmental Entomology* **19**, 251–256.