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ORIGINAL CONTRIBUTION

Interactions between an ectoparasitoid and a nucleopolyhedrovirus when simultaneously attacking *Spodoptera exigua* (Lepidoptera: Noctuidae)

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Abstract

Insect pathogenic viruses and parasitoids represent distinct biological entities that exploit a shared host resource and have similar effects in suppressing host populations. This study explores the interactions between the ectoparasitoid *Euplectrus plathypenae* (Hymenoptera: Eulophidae) and the *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV) in larvae of *S. exigua* (Lepidoptera: Noctuidae). Parasitoid progeny failed to complete development in hosts that had been infected prior to parasitism. However, infection of *S. exigua* fourth instars at 48 h post-parasitism had no significant effects on the survival of parasitoid progeny. Larval and pupal development times of *E. plathypenae* that survived on virus-infected *S. exigua* did not differ significantly from that of parasitoids on healthy hosts. Virus-induced mortality and the production of occlusion bodies were very similar in parasitized and non-parasitized *S. exigua*. The virus was genetically stable over three passages in parasitized and unparasitized hosts. These results suggest that applications of SeMNPV-based insecticides are unlikely to disrupt pest control exerted by the parasitoid *E. plathypenae* in biological pest control programs as long as virus applications are timed not to coincide with parasitoid releases.

Introduction

The beet armyworm, *Spodoptera exigua* (Hübner), is a cosmopolitan pest that causes serious damage in a wide variety of crops. This pest has been successfully controlled with chemical insecticides although excessive use of these products has generated a number of concerns related to the development of resistance in some pest populations (Pérez et al. 2000), the presence of pesticide residues in human food (Hamilton et al. 2004) and the impact of pesticide applications on populations of non-target organisms (Desneux et al. 2007).

Baculoviruses have been demonstrated to be an efficient alternative to chemical control for a number of agricultural and forest pests, particularly Lepidoptera (Moscardi 1999). The multiple nucleopolyhedrovirus (NPV) of *S. exigua* (SeMNPV) has been successfully employed to control populations of this pest in greenhouse and field crops in the United States (Gelernter and Federici 1990), south-east Asia (Kolodny-Hirsch et al. 1997), northern Europe (Bianchi et al. 2001), and most recently in Spain (Lasa et al. 2007).

Insect pathogenic viruses and parasitoids represent distinct biological entities that exploit a shared host

resource and have similar effects in suppressing host populations (Hochberg et al. 1990; Begon et al. 1999). Such interactions have been reviewed in detail, mainly focusing on their safety and efficacy in biological control programs (Rosenheim et al. 1995; Begon et al. 1999; Wraight 2003). Although these natural enemies are considered to play an important role in pest population dynamics, their effective integration into pest management programs depends on a series of factors related to the insect stages attacked, duration of infection or parasitism, parasitoid discrimination between healthy and infected hosts, toxin production by the virus, parasitoid mediated manipulation of the host immune response and virus dispersal by parasitoids (Brooks 1993). The study of host–pathogen–parasitoid interactions may also be of value for the conservation of threatened species of Lepidoptera that are exploited by native or introduced natural enemies and for the design of effective pest management strategies that include both parasitoids and baculovirus-based insecticides (Hochberg et al. 1990).

The gregarious ectoparasitoid *Euplectrus plathypenae* (Howard) (Hymenoptera: Eulophidae) naturally attacks a range of noctuid species, including *S. exigua* larvae. During oviposition, *Eupletrus* spp. inject venoms that inhibit larval growth, weight gain and moulting, a process known as physiological paralysis (Coudron and Puttler 1988; Coudron et al. 1990; Coudron 1991), which results in a reduction in crop damage caused by the pest (Parkman and Shepard 1981). The parasitoid preferentially attacks newly moulted third, fourth and fifth instars and usually attaches between 10 and 40 eggs to the dorsal and lateral surfaces of the host larva, each in the presence of the other. Parasitoid larvae feed until ready to pupate whereupon they detach from the dead host and pupate in silk cocoons around the host corpse.

Baculoviruses are capable of modulating host development to successfully complete the infection cycle (O'Reilly 1997). As the survival of insect parasitoids and baculoviruses depends on their ability to exploit a shared host species, these organisms must have the capacity to coexist at the population level, each in the presence of the other.

In this study, we examined the interactions between the parasitoid *E. plathypenae* and SeMNPV in *S. exigua* larvae. Specifically, we examined the concept that the impact of SeMNPV-based insecticide applications on the immature parasitoid population would depend on the interval between parasitism and infection. For this, host mortality responses to dual treatments with the parasitoid and the homologous virus were examined at different intervals between parasitoid oviposition and virus infection. The developmental times of the parasitoid larvae and pupae in virus-infected hosts and parasitism effects on the insecticidal properties of the virus were also studied. These findings provide insights into the interactions between distinct biological control agents in control pest programs involving both virus-based applications and parasitoid releases.

Material and Methods

Insects and viruses

Laboratory colonies of *S. exigua* were maintained at a constant temperature (25 ± 0.5°C), humidity (70 ± 5%) and photoperiod (16 h light: 8 h dark), in the insectary facilities of the Universidad Pública de Navarra, Pamplona, Spain. Larval stages were reared on a wheatgerm-based semi-artificial diet (Poitout and Bues 1974). The *E. plathypeanae* colony was started with parasitoids that emerged from parasitized *S. frugiperda* larvae collected from maize fields within a 20 km radius of Tapachula, Chiapas, Mexico. This colony was maintained using *Spodoptera frugiperda* (J. E. Smith) larvae from the laboratory population and had been in continuous culture for approximately 18 months prior to the start of this study.

The NPV used in this study was the wild-type strain of SeMNPV isolated from infected *S. exigua* larvae during an epizootic in vegetable greenhouses in El Ejido, Spain, named SeMNPV-SP2 (Se-SP2) (Caballero et al. 1992). The virus stock was produced in *S. exigua* early fourth instars that had been inoculated *per os* with SeMNPV occlusion bodies (OBs) and reared on diet until death. Virus-killed larvae were triturated and OBs were purified by filtration and centrifugation as described by Muñoz et al. (1998). Occlusion bodies were finally suspended in distilled water, counted in triplicate using a Neubauer Improved chamber (Hawsksley, Lancing, UK) and stored at 4°C prior to use.

Mortality and development of *Euplectrus plathypenae* on infected hosts

Host mortality and parasitoid developmental times on virus-infected and healthy hosts were compared on *S. exigua* third and fourth instars. A batch of 30 larvae were allowed to drink virus suspensions containing 10% sucrose and 0.001% Fluorella blue

following the droplet feeding method (Hughes and Wood 1981). Occlusion bodies concentrations administered to larvae were previously estimated to result in approximately 90% of mortality (2.4×10^6 and 2.3×10^7 OBs/ml for third and fourth instars, respectively). Larvae that consumed the suspension in a 10-min period were immediately exposed in groups to *E. plathypenae* females, in a ratio of 5 larvae to 10 parasitoids, until oviposition behaviour was observed. Parasitized larvae were individually transferred into 15-ml cups containing diet, incubated until virus-induced or parasitoid-induced mortality. Three additional batches of 30 moulting second or third instars of *S. exigua* were exposed to groups of *E. plathypenae* females overnight during which time moulting was completed and larvae became vulnerable to ovipositing parasitoids. The parasitized larvae, that by this time had reached the third and fourth instars, respectively, were allowed to drink a 90% lethal concentration of OB suspension at 0, 24 and 48 h post-parasitism (hpp). Parasitism did not reduce the number of larvae that drank OB suspensions. As controls, groups of 30 moulted larvae were parasitized and allowed to drink Fluorella blue with water instead of OB suspension following the same procedure, whereas another batch of 30 newly moulted insects were not exposed to parasitism but were inoculated with OB suspensions following the same procedure. In all cases, virus or parasitism induced host mortality and *E. plathypenae* development times were recorded at 12-h intervals. In the treatment in which infected larvae were exposed to *E. plathypenae* females, the numbers of eggs laid on healthy and infected hosts and the numbers of larvae that were not parasitized were recorded. The entire experiment was performed three times.

Effect of parasitism and viral infection on host growth

To determine the effect of parasitism and viral infection on host larval growth, a batch of 120 newly moulted fourth instars were divided into four groups and randomly assigned to one of the following treatments: (i) 30 larvae were allowed to drink a 90% lethal concentration of OB suspension (2.3×10^7 OBs/ml) and then placed in a Petri dish arena with an *E. plathypenae* female and left overnight to oviposit on the larva; (ii) 30 healthy larvae were individually offered to single *E. plathypenae* females overnight in the Petri dish arena; (iii) 30 larvae were inoculated with OB suspension as described above but were not exposed to parasitism and (iv) 30 larvae were fed water without exposure to parasitism. Larvae were monitored daily for mortality and individually weighed at 0, 24, 48, 72 and 96 h post-treatment using an analytical balance. Cadavers were individually frozen at −20°C before weighing to avoid loss of OB material. The experiment was performed three times.

Viral production and genetic stability

To compare total OB production in parasitized and unparasitized larvae, a batch of 30 newly moulted fourth instars were randomly assigned to one of the following treatments: (i) droplet fed on OB suspension containing 2.3×10^7 OBs/ml (90% lethal concentration) as described above; and (ii) inoculated with the same concentration of OBs and then placed in an arena with female parasitoids in a proportion of 5 larvae to 10 parasitoids. A similar number of larvae were allowed to drink water and used as controls. All larvae were placed in 25-well tissue culture plates provided with diet and maintained at 25 ± 1°C until death or pupation. Larvae with clear signs of NPV disease were individually transferred to vials, and when dead, these insects were weighed and frozen at −20°C. Cadavers were individually homogenized in 1 ml distilled water, and OBs were purified by filtering through two layers of cheesecloth. The OB yield per larva was estimated by counting triplicate samples of a diluted OB suspension obtained from each larva using a Neubauer chamber under phase-contrast microscopy. The experiment was performed three times.

To assess the influence of parasitism on the genetic stability of the virus, OBs from 30 larvae, either non-parasitized or parasitized, were pooled and divided into two parts: half was used for DNA extraction and half was used to infect a cohort of 30 larvae by the droplet feeding technique as described above. Three successive passages were conducted; viral DNA from each passage (P1, P2 and P3) of OBs from parasitized and healthy larvae was analysed by restriction fragment length polymorphism (RFLP). For this, approximately 1 μg of viral DNA was incubated with the restriction enzyme *Bgl*II for 12 h. Reactions were stopped by addition of loading buffer (0.25% w/v bromophenol blue, 40% w/v sucrose in water), loaded in 0.7% TAE buffer (40 mM Tris-Acetate; 1 mM EDTA) agarose gels and electrophoresed at 40 V for 12 h. Ethidium bromide stained gels were then photographed on a UV transilluminator.

Occlusion bodies dose-mortality response and speed of kill bioassay

Dose-mortality response and speed of kill of OBs derived from parasitized and unparasitized larvae were compared in a droplet feeding bioassay (Hughes and Wood 1981) in *S. exigua* second instars. A cohort of newly moulted 30 s instars were allowed to drink from an aqueous suspension containing 10% (w/v) sucrose, 0.001% (w/v) Fluorella blue and one of five OB doses: 3, 9, 27, 81 or 243 OBs/larva. Larvae that ingested droplets within 10 min were individually transferred to 25-well tissue culture plates containing diet. A batch of 25 larvae was allowed to drink from an OB-free suspension and served as a control. Larvae were reared at 25°C, and mortality was recorded at 8-h intervals during 7 days. The experiment was performed three times.

Statistical analyses

The mortality response of virus-infected larvae was subjected to one-way analysis of variance (ANOVA) and Tukey's test for pairwise multiple comparisons. Parasitoid-induced mortality of experimental and control groups was compared by *t*-test. Virus-induced mortality in different instars was compared by *z*-test. The mean number of eggs laid, parasitoid larval and pupal development times on healthy and virus-infected larvae and OB production in parasitized and healthy larvae were analysed by Mann–Whitney rank sum test. The effect of parasitism and viral infection on host larval growth was analysed by Kruskal–Wallis one-way analysis of variance on ranks. Each treatment was compared to the others by pairwise multiple comparison procedures (Dunn's Method). Dose-mortality results were subjected to Probit analysis using Polo-PC (LeOra Software 1987). Time-mortality results of individuals that died in the highest concentration treatment (1×10^6 OBs/ml) were subjected to Weibull survival analysis in Generalized Linear Interactive Modelling 3.77 (GLIM; Numerical Algorithms Group, Oxford, UK).

Results

Mortality of dually parasitized and virus-infected *Spodoptera exigua* larvae

The great majority (approximately 97%) of third and fourth instars that were first infected and then exposed to parasitism died from polyhedrosis disease (table 1). Similarly, third and fourth instars that were first parasitized and then infected at 0 and 24 h post-parasitism almost all succumbed to virus (table 1), as did larvae that were only inoculated with OBs.

Virus mortality was significantly reduced in insects that were first parasitized and then infected at 48 h post-parasitism compared to the mortality in larvae exposed to parasitism at earlier time points in both third ($F_{4,10}$ = 125.4, P < 0.001) and fourth instars ($F_{4,10}$ = 326.8, P < 0.001). Virus-induced mortality of third instars was significantly higher than that of fourth instars in this treatment, suggesting a greater susceptibility to systemic virus infection in third over that of fourth instars (*z* = 2.87, P = 0.004), because of interstadial developmental resistance. Finally, the percentage of parasitism in the parasitism-only treatment was almost identical in third and fourth instar hosts (P > 0.05) (table 1).

Developmental time of *Euplectrus plathypenae* larvae and pupae on virus-infected hosts

The developmental time (egg to pupa) of *E. plathypenae* on healthy *S. exigua* larvae or larvae that were infected at 48 h post-parasitism averaged 5.5 ± 0.1 days (table 2) and was similar for both third (*U* = 1790, P = 0.584) and fourth instars (*U* = 3178, P = 0.936). The duration of the parasitoid pupal stage averaged 18.6 ± 0.1 days and was also similar between healthy and infected hosts for third (*U* = 1744, P = 0.855) and fourth (*U* = 1799, P = 0.637) instars.

Effect of parasitism and viral infection on host larval growth

Both parasitism and viral infection significantly affected host growth from 24 h post-infection (hpi) onwards (Kruskal–Wallis, *H* = 31.544 for 24 hpi, *H* = 98.706 for 48 hpi, *H* = 90.762 for 72 hpi, *H* = 106.005 for 96 hpi, P < 0.001; fig. 1). A similar reduction in larval weights in the parasitized and dually infected and parasitized larvae was observed in the 24 hpi treatment compared to control and virus-infected treatments (P < 0.05, pairwise multiple comparisons Dunn's Method). At 48, 72 and 96 hpi, the weight of larvae from virus-infected and parasitized treatments were similar to one to another at each timepoint (P > 0.05) but differed significantly from that of the control larvae (P < 0.05). Dually infected and parasitized larvae had the lowest weight (P < 0.05) at 24 h, but at 72 and 96 h, larval weights did not differ significantly from those of infected non-parasitized larvae (P > 0.05).

Table 1 Percentage of mortality induced by nucleopolyhedrovirus infection or parasitism by *Euplectrus plathypenae* in *Spodoptera exigua* third and fourth instars exposed to parasitism and/or treated with virus occlusion bodies at different intervals

	Third instar		Fourth instar	
	Virus infection	Parasitism	Virus infection	Parasitism
Infected then parasitized	97.3 ± 1.4a	2.7 ± 1.4a	97.2 ± 1.4a	2.8 ± 1.4a
Parasitized then infected (0 hpp)	98.7 ± 1.3a	1.3 ± 1.3a	100 ± 0.0a	–
Parasitized then infected (24 hpp)	98.6 ± 1.4a	1.4 ± 1.4a	98.6 ± 1.4a	1.4 ± 1.4a
Parasitized then infected (48 hpp)	45.0 ± 2.9b	55.0 ± 2.9b	19.9 ± 3.6b	80.1 ± 3.6b
Parasitized only	–	89.0 ± 1.7c	–	90.4 ± 2.4b
Infected only	97.1 ± 2.9a	–	97.2 ± 1.4a	–

Hpp, hours elapsed post-parasitism.
Mean values followed by different letters are significantly different for treatment comparison within columns (*t*-test; $P < 0.05$).

Table 2 Mean (±SE) duration of larval and pupal development of *Euplectrus plathypenae* on healthy or virus-infected at 48 h post-parasitism *Spodoptera exigua* third and fourth instars

	Larval development time (days)[1]		Pupal development time (days)[1]	
Instars treated	Infected	Control	Infected	Control
Third	5.54 ± 0.08a	5.47 ± 0.07a	18.49 ± 0.13a	18.43 ± 0.15a
Fourth	5.49 ± 0.06a	5.48 ± 0.07a	18.81 ± 0.15a	18.71 ± 0.12a

[1]Means followed by the same letter are not significantly different by the Mann–Whitney–test for comparison within instars.

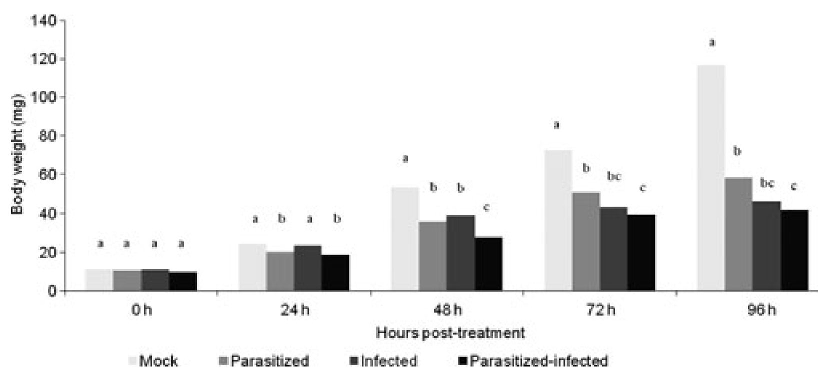

Fig. 1 Weight of *Spodoptera exigua* fourth instars infected with *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV) and/or parasitized by *Euplectrus plathypenae* at 0, 24, 48, 72 and 96 h post-infection. Columns headed by different letters differ significantly between treatments (Dunn's multiple comparison test, $P < 0.05$).

Effect of parasitism on the production, genetic stability and infectivity of OBs

The total OB production, genetic stability, and infectivity of OBs obtained from parasitized larvae were assessed to determine whether parasitism affected the insecticidal properties of OBs. Mean (±SE) OB production was similar in parasitized ($8.40 \times 10^8 \pm 1.03 \times 10^8$) and non-parasitized larvae ($9.25 \times 10^8 \pm 1.06 \times 10^8$) ($U = 2593$, $P = 0.112$). Occlusion bodie production was also similar between these groups in terms of OB/mg larval body weight at death with a mean production of $2.73 \times 10^7 \pm 0.04 \times 10^7$ OBs/mg in parasitized larvae and $2.49 \times 10^7 \pm 0.21 \times 10^7$ OB/mg in unparasitized larvae ($U = 2004$, $P = 0.301$).

Parasitism did not appear to affect the genetic stability and gross genotypic composition of the virus when larvae were first infected and then parasitized. *Bgl*II restriction profiles of viral DNA of the progeny

derived from parasitized larvae across three serial passages and three replicates did not present obvious differences in restriction enzyme profile compared to that of the wild-type isolate (fig. 2).

The pathogenicity and speed of kill of OBs from parasitized larvae were compared with the wild-type isolate by bioassay in terms of LD_{50} and mean time to death (MTD) in second instars. LD_{50} values were estimated by fitting regressions with a common slope for both inocula ($\chi^2 = 0.33$, d.f. = 1, $P = 0.567$). No significant differences were observed between OBs from healthy larvae (Se-SP2wt) and OBs from parasitized larvae (Se-SP2p) based on the relative potency fiducial limits (Robertson and Preisler 1992) (table 3). Mean times to death were similar for insects inoculated with OBs from parasitized and unparasitized hosts ($t = 0.255$, d.f. = 4, $P = 0.806$) (table 3).

Discussion

In this study, we aimed to assess the interaction of two different biological agents, one NPV and one parasitoid, that exploit the crop pest, *S. exigua*. Parasitism is determined to a great extent by parasitoid selection of hosts that are suitable for immature parasitoid development (Vinson 1976; Vinson and Iwantsch 1980). Infection with a lethal pathogen may reduce the value of an otherwise suitable host, depending on whether there are costs to the parasitoid associated with attacking these hosts and the resulting competition between the macro and microparasite. Costs may include loss of progeny that fail to complete their development, reduced adult size, and increased development time or time wasted in handling infected hosts (Irabagon and Brooks 1974; Beegle and Oatman 1975; Levin et al. 1983; Caballero et al. 1990; Hochberg 1991). Therefore, when oviposition incurs a cost and larval parasitoids are unable to develop in infected hosts, host selection models predict that the adult female parasitoid should reject these individuals (Versoi and Yendol 1982). We previously observed evidence of host discrimination in *E. plathypenae* in that fewer parasitoid eggs were laid on infected third instar *S. exigua* compared to healthy larvae, whereas no such difference was seen in fourth instars (Stoianova et al. 2007).

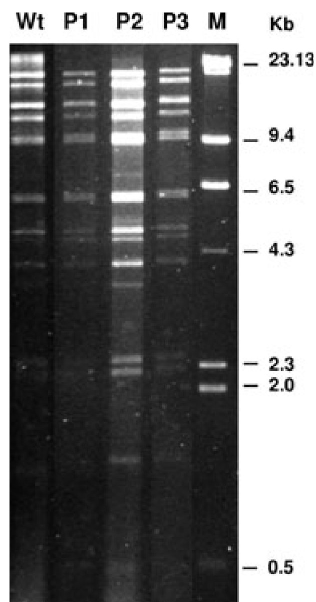

Fig. 2 *Bgl*II REN profiles of DNA extracted from larvae inoculated with Se-SP2 wild type (lane 1) and Se-SP2p (lane 2, 3 and 4) across three serial larval passages (P1, P2, and P3). Molecular weight marker (M) and its fragment sizes in kb are indicated to the right of the picture.

Immature parasitoids often cannot complete their development if their hosts are infected with a virus because of premature death of the host (Brooks 1993; Nakai et al. 1997; Nakai and Kunimi 1998). Some baculoviruses and an entomopoxvirus induce the infected host to produce a substance that adversely affects the development and survival of immature parasitoids (Brooks 1993; Kyei-Poku and Kunimi 1998; Kunimi et al. 1999). However, we found that juvenile *E. plathypenae* could develop successfully on infected larvae, although parasitoid development depended on the time elapsed between parasitism and virus infection. When larvae were treated first with virus and then parasitized, host mortality was attributed to the virus; the parasitoid larvae managed to complete their development only when the virus was inoculated 48 h post-parasitism. These results are similar to those reported for *M. pallidipes* on *S. exigua* larvae, in which a high proportion of parasitoids could complete their development in hosts that were infected by SeMNPV at 1–3 days post-parasitism (Jiang et al. 2011). In contrast, when hosts were treated first with virus and subsequently parasitized, parasitoid progeny died prior to completing their development (Murray et al. 1985; Matthews et al. 2004; Jiang et al. 2011). Our results indicate no evidence of virus toxic proteins that kill the developing parasitoid larvae (Kaya 1970; Kaya and Tanada 1972; Kunimi et al. 1999) but rather parasitoid mortality occurred as a result of premature death of the infected host. The dose of virus consumed can also affect the survival of the parasitoid. For example, emergence of *Habrobracon hebetor* adults was negatively correlated with the dose of

Table 3 LD_{50} values, potency and mean time to death (MTD) in *Spodoptera exigua* second instars inoculated with viral occlusion bodies (OBs) obtained after three passages in parasitized larvae (Se-SP2p) compared to wild-type OBs passaged in nonparasitized larvae (Se-SP2 wt)

Treatment	LD_{50} (OB)[1]	Potency[2]	Fiducial limits (95%)		MTD (h)[3]	Fiducial limits (95%)	
			Lower	Upper		Lower	Upper
Se-SP2wt	5.28	1	–	–	94.6	92.3	97.0
Se-SP2p	5.94	1.2	0.8	1.4	98.7	96.2	101.1

[1]Probit regression curves were fitted with a common slope of 1.50 ± 0.14

[2]Potency was calculated as the ratio of LD_{50} values.

[3]MTD was estimated by Weibull analysis.

Mamestra brassicae NPV (MbMNPV) that *S. exigua* larvae had consumed (Rabie et al. 2010).

The presence of virus in the host may affect the development of the parasitoid through competition for resources or by disrupting the host endocrinological system. In this study, no significant changes in parasitoid developmental times were observed in infected *S. exigua*. However, in other host–pathogen–parasitoid systems, immature parasitoid developmental times were either significantly extended (Hotchkin and Kaya 1983; Nakai and Kunimi 1997; Nakai et al. 1997), reduced (Caballero et al. 1990) or were unchanged by virus infection (Beegle and Oatman 1975; Jiang et al. 2011).

The results of this study indicate that the combination of SeMNPV infection and *E. plathypenae* parasitism resulted in additive effects on weight gain in *S. exigua* larvae. Similar weight gain studies of the parasite *Cotesia congregata* and the *Autographa californica* NPV on *Manduca sexta* larvae have reported reduced larval growth for larvae treated with both agents with respect to those only infected or parasitized (Washburn et al. 2000). Viral genomic DNA extracted from OB progeny from parasitized larvae did not undergo important changes in restriction endonuclease profiles or additional variables such as total OB production, OB infectivity or speed of kill, as reported for the *Spodoptera frugiperda* NPV–host system involving parasitism by *Campoletis sonorensis* (Escribano et al. 2000).

In summary, parasitism did not alter the insecticidal properties of OBs produced in dually infected and parasitized hosts, indicating pest control programs based on the use of SeMNPV-based bioinsecticides may be compatible with the use of *E. plathypenae* in integrated control programs. However, control programs involving virus insecticides and parasitism of *S. exigua* should consider careful timing of virus applications and should examine the effect of virus dose on immature parasitoid survival (Stoianova et al. 2007; Rabie et al. 2010). Specifically, detrimental effects on parasitoid populations may be reduced by scheduling parasitoid releases that do not coincide with OB applications targeted at *S. exigua* control during the critical developmental period of the immature parasitoid. Suitable timing and use of correct virus application rates should also provide economic benefits from improved pest control provided by both these types of natural enemies.

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