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Intra- and Intergenerational Persistence of an Insect Nucleopolyhedrovirus: Adverse Effects of Sublethal Disease on Host Development, Reproduction, and Susceptibility to Superinfection▽

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Sublethal infections by *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV) are common in field populations of the beet armyworm (*S. exigua*, Hübner) in the Almerian horticultural region of Spain. Inoculation of second, third, and fourth instars with occlusion bodies (OBs) of an isolate (VT-SeAl1) associated with vertically transmitted infections resulted in 15 to 100% of sublethal infection in adult survivors, as determined by reverse transcription-PCR (RT-PCR) detection of viral DNA polymerase transcripts, and quantitative PCR (qPCR) targeted at the DNA polymerase gene. The prevalence of adult sublethal infection was positively related to the inoculum OB concentration consumed during the larval stage. Sublethal infections persisted in OB-treated insects for at least five generations. Viral transcripts were more frequently detected in adult insects than in third instars. qPCR analysis indicated a consistently higher prevalence of sublethal infection than RT-PCR. Sublethal infection was associated with significant reductions in pupal weight, adult emergence, fecundity, and fertility (egg hatch) and significant increases in larval development time and duration of the preoviposition period. Insects taken from a persistently infected experimental population were significantly more susceptible to the OB inoculum than control insects that originated from the same virus-free colony as the persistently infected insects. We conclude that OB treatment results in rapid establishment of sublethal infections that persist between generations and which incur costs in the development and reproductive capacity of the host insect.

Nucleopolyhedroviruses (NPVs; *Baculoviridae*) are used as the basis for biological insecticides for the control of lepidopteran pests of forests and field and greenhouse crops in developed and developing countries (23). Horizontal transmission of NPVs occurs when susceptible larvae consume plants contaminated by viral occlusion bodies (OBs) and often results in the death of the infected insect and liberation of massive numbers of OBs that are subsequently consumed by other larvae.

A growing body of molecular evidence indicates that insect pathogenic viruses can persist in adult insects that were sublethally infected as larvae (2, 17, 39). Molecular techniques have been based on the detection of viral DNA or mRNA transcripts (3, 11–13, 32) or, more recently, proteomics-based techniques (1). The mechanisms by which the virus persists in the cells of sublethally infected insects without triggering apoptosis remains unclear, although advances in the field of microRNA research may soon shed light on this issue (33). Covert infections are believed to persist in one of two forms: as a latent infection in which the virus is not replicating and transcriptional activity is minimal, or as a sublethal infection in which the virus is transcriptionally active and replication occurs at a low level (2, 3, 9). Compared to healthy conspecifics, sublethally infected insects can experience slower development rates, lower pupal and adult body weights, reduced reproductive capacity, or altered preoviposition period (26, 30, 38). Sublethal infection also makes possible opportunities for the vertical transmission of infection from parents to offspring, which can be an important mechanism of virus survival when opportunities for horizontal transmission are low (31). However, when subjected to periods of stress, triggered by poor diet, crowding, or the presence of other pathogens, latent and sublethal infections can be activated into a lethal overt infection that produces OBs for subsequent horizontal transmission (8).

The *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV) forms the basis for a number of bioinsecticidal products for control of the beet armyworm, *S. exigua*, that are marketed in the United States, parts of Europe, and several southern Asian countries (6, 18, 34). Recently, an SeMNPV isolate was successfully commercialized for use in biological pest control programs for the extensive greenhouse horticultural crops grown in Almería, southern Spain (20, 21). Field-collected adults from natural populations of *S. exigua* collected in and around Almerian greenhouses show evidence of sublethal SeMNPV infection by analysis of the presence of viral gene transcripts. Additionally, adults from a virus-free labora-

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TABLE 1. Percentage of larval mortality due to NPV disease and other causes, pupation rates, and prevalence of sublethal infection in the second, third, and fourth instars[a]

Instar	OB concn/ml	*n*	% larval mortality (*n*)		Pupation rate (*n*)	% sublethal infection in adults (*n*)	
			NPV	Other causes		RT-PCR	qPCR
L_2	3.8×10^4	120	79.2 (95)	12.5 (15)	8.3 (10)	100 (2)	
	3.8×10^3	120	63.3 (76)	12.5 (15)	24.2 (29)	87.5 (16)	
	3.8×10^2	120	41.7 (50)	15.0 (18)	43.3 (52)	90 (20)	100 (10)
	3.8×10^1	120	17.5 (21)	15.8 (19)	66.7 (80)	40 (20)	
	Control	120	0 (0)	20.0 (24)	80.0 (96)	0 (5)	0 (10)
L_3	3.7×10^4	120	77.5 (93)	12.5 (15)	10.0 (12)	80 (5)	
	3.7×10^3	120	60.8 (73)	16.7 (20)	22.5 (27)	78.6 (14)	
	3.7×10^2	120	40.0 (48)	13.3 (16)	46.7 (56)	45 (20)	60 (10)
	3.7×10^1	120	16.7 (20)	18.3 (22)	65.0 (78)	20 (20)	
	Control	120	0 (0)	17.5 (21)	82.5 (99)	0 (5)	0 (10)
L_4	7.8×10^4	144	78.5 (113)	11.8 (17)	9.7 (14)	80 (5)	
	7.8×10^3	144	61.1 (88)	13.9 (20)	25.0 (36)	84.2 (19)	
	7.8×10^2	144	44.4 (64)	9.0 (13)	46.5 (67)	40 (20)	60 (10)
	7.8×10^1	144	13.2 (19)	13.2 (19)	73.6 (106)	15 (20)	
	Control	144	0 (0)	15.2 (22)	84.7 (122)	0 (5)	0 (10)

[a] Shown are the percentage of larval mortality due to NPV disease and other causes, pupation rates, and prevalence of sublethal infection (RT-PCR and qPCR) in adults that survived treatment with one of four OB concentrations, or a water control, in the second, third, and fourth instars.

tory colony that survived a virus challenge in the larval stage also proved positive for viral mRNA by reverse transcription-PCR (RT-PCR) (4). However, the relationship between inoculum concentration, host developmental stage, and the transgenerational persistence of sublethal disease is poorly understood, as are the effects of infection on host demographic parameters and susceptibility to subsequent viral infection.

In this study, we addressed the following four principal questions. (i) Does the prevalence of sublethal infection vary with host larval stage and OB dose? (ii) Does the prevalence of sublethal infection vary over successive generations? (iii) What are the costs associated with OB treatment in larval growth and adult reproduction in surviving insects? (iv) Do covertly infected insects differ in their susceptibility to peroral infection by OBs compared to healthy conspecifics?

MATERIALS AND METHODS

Insects and viruses. A virus-free *S. exigua* culture was obtained from Andermatt Biocontrol AG (Grossdietwil, Switzerland) and reared in continuous culture for four generations from the moment the experiments were started. Insects were maintained at a constant temperature (25 ± 1°C), relative humidity (RH; 50% ± 5%), and photoperiod (16-h/8-h light-dark cycle) on artificial diet (7) in the insectary facilities of the Universidad Pública de Navarra, Pamplona, Spain.

The SeMNPV isolate VT-SeAl1 containing a single genotype confirmed by restriction enzyme (REN) analysis and PCR, was used in all the experiments. VT-SeAl1 was isolated from spontaneous nucleopolyhedrovirus (NPV) infections in the laboratory-reared larval progeny of *S. exigua* females collected in and around the greenhouses of Almería (Spain) in the summer of 2007 (4). Consequently, this isolate was considered to have been vertically transmitted (VT) from parents to offspring and was assigned the "VT" prefix. VT-SeAl1 OBs were grown in *S. exigua* fourth instars that were frozen until needed. OB suspensions were prepared by homogenizing infected cadavers in distilled water, straining them through a fine wire gauze, and washing them twice in 0.1% SDS and distilled water, followed by counting in triplicate with a Neubauer chamber.

Effect of instar and OB concentration on the prevalence of covert infection. To determine whether the prevalence of covert infection varied with larval instar and inoculum concentration, second, third, and fourth instars were treated with OB concentrations estimated to result in 20 to 80% mortality. Groups of premolt larvae of each instar were starved overnight for approximately 12 h and, having molted, were offered one of four OB concentrations in 10-fold increments, in the second (3.8×10^4 to 3.8×10^1 OBs/ml), third (3.7×10^4 to 3.7×10^1 OBs/ml), and fourth (7.8×10^4 to 7.8×10^1 OBs/ml) instars (Table 1). For this, OB suspensions were diluted in sterile distilled water with 10% sucrose and 0.001% (wt/vol) Fluorella blue food dye. The first 24 larvae that ingested the OB suspension within 10 min were individually transferred to 24-well tissue culture plates containing semisynthetic diet. Equal numbers of control larvae of each instar were allowed to drink from an OB-free suspension. Bioassays were performed five times for second and third instars and six times for fourth instars. Larvae were reared at 25 ± 1°C at 50% ± 5% RH and checked daily until death or pupation. To confirm the identity of OB progeny from virus-killed larvae, viral DNA was extracted from OBs and subjected to restriction endonuclease profile analysis (24). Adult survivors were individually frozen at −80°C for subsequent analysis as described below. The effects of larval stage and inoculum concentration on larval mortality and adult emergence were estimated by fitting generalized linear models with a binomial error structure in GLIM 4 (Numerical Algorithms Group, Oxford, United Kingdom).

Detection of sublethal SeMNPV infections. Specific viral genes were targeted by PCR-based methods to detect viral DNA or viral transcripts. Specific primers DNApol.F (5′-ATGACTTCTTCGTCGTCGTC-3′) and DNApol.R (5′-TAGC ACGTCGTGTTAGCGTG-3′) were designed to amplify a 320-bp region of the highly conserved DNA polymerase gene based on the full sequence of the SeMNPV-US1 strain (15).

To detect viral transcripts, total RNA was TRIzol extracted (Invitrogen, United Kingdom) from individual adult survivors that had consumed an OB inoculum in each instar and for each OB concentration, as described by Cabodevilla et al. (4). RT-PCR targeted at DNA polymerase transcripts was performed in two steps. First, cDNA synthesis was performed from 4 μl of total insect RNA (160 to 400 ng) previously treated with DNase, using the ImProm-II reverse transcriptase (Promega) and the DNApol.R reverse primer (described above) according to the manufacturer's instructions. An aliquot of the reaction (1/4 volume) was then subjected to PCR amplification with *Taq* DNA polymerase (Bioline) and the two internal primers for the DNA polymerase gene (DNApol.F and DNApol.R). The amplification conditions were 94°C for 2 min, followed by 30 cycles of 94°C for 45 s, 50°C for 45 s, and 72°C for 45 s, and a final cycle ending with a 10-min incubation at 72°C. PCR products were electrophoresed in 1% agarose gel with ethidium bromide as the dye. A Bioline Hyper-Ladder I size marker was used for size determination. DNA fragments were visualized in a UV transilluminator, photographed, and examined with the molecular analysis program Chemi doc (Syngene).

The identity of the PCR amplification products was confirmed by sequencing. For this, a subset of three RT-PCR-positive samples was selected at random and amplified by PCR under the same conditions described above. The amplicons were purified with a QIAquick PCR amplification kit (Qiagen), and the resulting DNA samples were sequenced in an ABI PRISM 377 automated DNA se-

quencer (Sistemas Genómicos, Valencia, Spain). Sequencing was performed from both ends of the amplicons using the DNApol.F and DNApol.R primers. Sequence data were subjected to pairwise comparison for nucleotide identity and aligned against the VT-SeAl1 complete genome sequence (O. Cabodevilla and E. Herniou, unpublished data), using the ClustalW program.

For the detection of viral genomic DNA, total DNA was extracted from adult control moths and from the adult survivors of OB treatments. Insects were killed and stored at −80°C. The abdomen of each adult was individually dissected, placed in a 1.5-ml microcentrifuge tube, and ground in liquid nitrogen. A 1-μl volume of 50 μg/μl proteinase K was diluted in 300 μl of tissue and cell lysis solution C (MasterPure complete DNA and RNA purification kit; Epicentre Biotechnologies, Madison, WI) according to the manufacturer's recommended protocol for tissue samples. This mixture was added to each sample and incubated at 65°C for 15 min. DNA was treated with RNase for 30 min at 37°C. Protein precipitation reagent was added to lyse the samples, and the debris was pelleted by centrifugation. Isopropanol was used to precipitate nucleic acids. Samples were washed with ethanol and resuspended in sterile Milli-Q water. Blank extraction samples containing only water were processed in parallel to control for cross-contamination during the extraction.

In order to estimate the prevalence of latent infections, quantitative PCR (qPCR) was performed using SYBR fluorescence in 96-well reaction plates in a 7900HT sequence detection system (Applied Biosystems). Amplifications were performed in a total reaction volume of 20 μl containing 10 μl of SYBR Premix Ex *Taq* (2×), 0.4 μl of ROX reference dye (50×), 0.4 μl of both DNApol.F and DNApol.R primers (10 pmol/μl) and 5 μl of DNA template. Six nontemplate reactions were included in each run. The concentration of SeMNPV genomic DNA was estimated in a spectrophotometer (A_{260}), and DNA was serially diluted in UV-irradiated Milli-Q water, across the range 5×10^{-1} to 5×10^{-10} ng, for construction of the standard curve in triplicate. The qPCR protocol consisted of an initial denaturation at 95°C for 30 s, followed by 45 amplification cycles of 95°C for 5 s and 60°C for 30 s, and finally a dissociation stage of 95°C for 15 s, 60°C for 15 s, and 95°C for 15 s. Data acquisition and analysis were performed with Sequence Detector version 2.2.2 software (Applied Biosystems). Reaction parameters and conditions were optimized initially on SeMNPV Cl-Cs-purified DNA. The specificities of PCR products were monitored by analyzing amplification profiles and the corresponding dissociation curves. The detection limit was considered the lowest dilution showing the correct amplification curve and a single peak at the expected melting temperature (84°C) for the dissociation curve. The frequencies of insects that tested positive for sublethal infection by qPCR in each OB treatment were compared by Fisher's exact test.

In order to assess the specificity of the method, the DNApol.F and DNApol.R primers were tested against 5×10^{-1} to 5×10^{-10} ng genomic DNA from four different NPVs, including, *S. frugiperda* MNPV, *Autographa californica* MNPV, *Spodoptera littoralis* NPV, *Helicoverpa armigera* NPV, and host genomic DNA.

Transgenerational persistence of sublethal infections. Groups of 30 premolt *S. exigua* fourth instars were starved overnight and then during a 10-min period allowed to drink an OB suspension containing 9×10^3 OBs/ml by the droplet-feeding method (14). Fourth instars ingested an average volume of 3.3 μl (35), equivalent to a dose of 30 OBs per larva that was previously shown to result in ~50% mortality. Control insects consumed droplets that did not contain OBs. All procedures were performed in triplicate. Inoculated larvae were individually placed in 25-ml plastic cups perforated for ventilation and provided with artificial diet. Larvae that did not succumb to polyhedrosis disease were reared through to pupation at 25 ± 1°C and 50% ± 5% RH. Pupae were sexed, and 20 male and female pupae were selected randomly from each replicate, placed in paper bags, and allowed to mate. Eggs were collected daily, and offspring were reared in groups in 300-ml plastic containers provided with artificial diet until the fourth instar. A total of 100 larvae were randomly selected across all offspring and were reared individually until pupation, whereupon they were sexed and assigned to 20 pairs to produced the following generation. A total of six successive generations were reared on artificial diet. Between 10 and 30 adults were selected at random from the second to fifth generations and were frozen at −80°C for subsequent analysis by RT-PCR and qPCR. Similarly, groups of 10 to 30 randomly selected third-instar larvae were subjected to RT-PCR analysis to estimate the prevalence of sublethal infection during larval development. As the prevalence of sublethal infection did not vary over time, the results were summed across generations 2 to 5 and the effects of stage (larva versus adult) were compared by Fisher's exact test.

Costs of surviving OB treatment on host development and reproduction. To assess the cost of having survived OB treatment on larval development and adult reproduction, two insect lines were established. The first line consisted of 360 second instars inoculated with 3.8×10^2 OBs/ml, estimated to resulted in ~40% NPV mortality and ~90% sublethal infection in surviving insects (estimated by RT-PCR). The second line of healthy insects consisted of 120 second instars that were mock-infected and used as a control. Both insect lines were reared individually in 24-well tissue-culture plates containing semisynthetic diet at 25 ± 1°C and 50% ± 5% RH. All insects that developed to the pupal stage were weighed and their sexes were determined. Pupae were examined daily until adult emergence. Eighteen pairs of emerging adults from treated lines and 10 pairs from control lines were placed in single pairs in a paper bag provided with water in a small container with a cotton wick. The following variables were compared for insects from virus-treated and control lines: larval development time (from inoculation to pupation), pupal weight, duration of the pupal stage, sex ratio, adult longevity, preoviposition period, fecundity, and fertility (egg hatch). Developmental times, pupal weight, adult longevity, preoviposition period, and fertility results were not normally distributed and were analyzed by nonparametric Mann-Whitney test (SPSS, version 15.0). Fecundity results were analyzed by fitting generalized linear models in GLIM 4 with a normal error distribution specified; treatment means were compared by *t* test (Numerical Algorithms Group 1993).

Susceptibility of healthy and sublethally infected insects to OB inoculum. The susceptibility of sublethally infected second instars to the OB inoculum was compared with that of control insects. For this, groups of 24 premolt second instars were selected from the sixth generation of insects in the transgeneration persistence of infection experiment. Larvae were starved overnight and when molted were fed with one of five OB concentrations: 2.43×10^5, 8.1×10^4, 2.7×10^4, 9×10^3, and 3×10^3 OBs/ml. An additional group of 24 larvae was mocked infected and included in each bioassay as a control. After inoculation, larvae were individually transferred to 24-compartment plates containing artificial diet and reared at 25 ± 1°C and 50% ± 5% RH. Larvae were monitored daily for virus mortality for up to 7 days postinfection. Two and three replicates were performed for the healthy and OB-treated insect lines, respectively, according to the number of available larvae. Dose-mortality data were submitted to a Probit regression analysis using the POLO-PLUS statistical program (Le Ora Software 2002–2008). Median lethal concentrations (LC_{50}s) were estimated, and parallelism in regression slopes was examined, whereas treatment effects were determined by examination of OB potency values in each line of insects (28).

RESULTS

Effect of instar and OB concentration on the prevalence of covert infection. NPV mortality increased significantly with OB concentration ($\chi^2 = 348.1$, $df = 1$, $P < 0.001$) and varied to a significant but small degree with larval instar ($\chi^2 = 7.7$, $df = 2$, $P = 0.021$) (Table 1). Restriction endonuclease analysis using BglII confirmed that the virus responsible for the infections was VT-SeAl1 (data not shown). The frequency of sublethal infection as estimated by RT-PCR appeared to be positively influenced by the OB concentration ingested by the larvae (Table 1). The ability to examine this hypothesis statistically was limited by the low number of insects that survived following consumption of large quantities of OBs. A *post hoc* analysis was therefore performed in which the results from the two highest OB concentrations that consistently resulted in >50% mortality were compared with the results from the two lowest OB concentrations that consistently resulted in <50% mortality. Comparisons within each instar revealed that a higher frequency of transcript-positive insects were present in the survivors that consumed high OB concentrations in the third instar (Fisher's exact test, $P < 0.001$) and fourth instar (Fisher's exact test, $P < 0.001$), whereas the difference was borderline significant in the second instar (Fisher's exact test, $P < 0.054$).

A subset of 10 samples corresponding to the medium concentration for each larval instar was analyzed by qPCR targeted at the viral DNA polymerase gene (Table 1). The proportions of adults that were positive for viral DNA by qPCR were consistently higher than those obtained by detection of viral transcripts (RT-PCR). None of the control larvae gave

TABLE 2. Numbers of adults and third instars that proved positive for SeMNPV DNA polymerase genomic DNA or gene transcripts in control and SeMNPV-treated insect lines in the second to fifth generations

Generation	No. of positive insects/no. tested per generation[a]			
	Controls		Virus treated	
	qPCR	RT-PCR	qPCR	RT-PCR
3rd instars				
G2		0/10		1/30
G3		0/10		0/30
G4		0/10		1/20
G5		0/10		2/20
Total		0/40		4/100
Adults				
G2		0/10	10/10	5/30
G3	0/10	0/10	7/10	4/30
G4	0/10	0/10	10/10	3/20
G5		0/10	10/10	3/20
Total	0/20	0/40	37/40	15/100

[a] Genomic DNA results were determined by qPCR, and gene transcript results were determined by RT-PCR.

positive results by either method, confirming that the laboratory colony was virus free.

To confirm the identity of RT-PCR amplifications, PCR products were sequenced and compared to the corresponding sequences for VT-SeAl1 and Se-US1 isolates. The alignments revealed 100% nucleotide identity between the PCR product and the corresponding sequence from VT-SeAl1, compared to 93% nucleotide identity in the corresponding sequence of Se-US1 (data not shown).

Transgenerational persistence of sublethal infections. The persistence of sublethal infections through successive generations was estimated by RT-PCR and/or qPCR (Table 2). The prevalence of RT-PCR-positive adults varied between 3/20 (13%) and 5/30 (17%). The frequencies of transcript-positive adults were consistently higher in all generations (total, 15/100) than those of third larval instars (total 4/100), and this effect was highly significant when summed over the four generations tested (Pearson's $\chi^2 = 7.04$, $df = 1$, $P = 0.008$). The results of qPCR studies indicated that the prevalence of sublethal infection detected by this technique was consistently higher than values obtained by RT-PCR, reaching 100% for three out of four generations analyzed, albeit based on small sample sizes of 10 insects per generation (Table 2). The prevalence of qPCR-positive insects summed over generations (37/40) was significantly higher than the prevalence of transcript-positive insects (15/100) during the same period (Pearson's $\chi^2 = 73.5$, $df = 1$, $P < 0.001$).

Quantities as low as 1×10^{-6} ng SeMNPV DNA could be consistently detected by qPCR. The genome of *S. exigua* is estimated to be 135.6 kb in length (15), equivalent to the detection of 7 genome copies, with positive detection as low as 0.7 genome copy on occasions. The DNA polymerase primer specificity was confirmed by the absence of positive amplifications when tested against other NPVs. Lack of interference by host DNA was tested by amplification of the SeMNPV DNA dilutions in the presence of 100 ng of *S. exigua* DNA from virus-free adults (data not shown). Also, no amplification was detected in controls containing only host (*S. exigua*) genomic DNA, indicating the absence of cross-reactivity. No amplifications were obtained in the insect control line by RT-PCR or by qPCR (Table 2).

Costs of surviving OB treatment on host development and reproduction. Larval developmental time was ~2 days longer (Table 3) for insects that survived the OB inoculum compared to healthy insects (Mann-Whitney U = 3,717, $n = 94$ and 146, $P < 0.001$), whereas the duration of the pupal stage was not affected (Mann Whitney U = 956, $n = 94$ and 146, $P = 0.721$) (Table 3). Pupal weight was significantly lower from the survivors of OB treatment than for healthy insects (Mann-Whitney U = 4,249, $n = 94$ and 146, $P < 0.001$). The pupal sex ratio did not differ significantly for control and virus-treated groups, being 38% ($n = 94$) and 43% ($n = 146$) males for control and OB treatments, respectively ($\chi^2 = 0.556$, $df = 1$, $P = 0.456$). Adult emergence was also reduced in the OB-treated insects ($\chi^2 = 17.15$, $df = 1$, $P < 0.001$; virus group, 62.3%, $n = 146$; control, 75.5%, $n = 94$), but differed according to sex; the rate of nonemergence of males (53.9%, $n = 63$) was significantly higher than that of females (25.3%, $n = 83$) in the survivors of the OB treatment ($\chi^2 = 4.119$, $df = 1$, $P = 0.042$).

Adult longevity (both sexes) did not differ significantly between the survivors of OB treatment and controls (Mann-Whitney U = 427, $n = 20$ and 59, $P = 0.061$) (Table 3). However, the preoviposition period was extended by an average of 1.2 days (Mann-Whitney U = 28, $n = 10$ and 18, $P = 0.002$), and fecundity was reduced by ~50% in OB treatment survivors (mean ± standard error [SE] = 331.1 ± 30.3, $n = 10$) compared to controls (mean ± SE = 687.9 ± 29.1, $n = 18$) ($F = 59.67$, $df = 1, 26$, $P < 0.001$). Finally, fertility measured by percentage of egg hatch was significantly lower in OB-treated survivors (median ± interquartile range = 59.0 ± 61.0) than in controls (median ± interquartile range = 74.5 ± 9.9) (Mann-Whitney U = 32.5, $n = 10$ and 18, $P = 0.004$).

Susceptibility of covertly infected insect to a superinfection. To compare the susceptibility of insects from a sublethally infected line compared to the healthy insect line, second-instar larvae from the sixth generation were inoculated with a range of OB concentrations. NPV mortality increased with increasing OBs ingested for both infected and healthy lines, although regression slopes could not be fitted in parallel. Relative potency was, therefore, calculated as the ratio of LC_{50}s (28). Insects from the sublethally infected line were 1.9-fold more susceptible to VT-SeAl1 OBs than healthy insects from the virus-free line (Table 4).

DISCUSSION

The frequency of sublethal infection in *S. exigua* adults was dependent on larval stage at inoculation and the OB dose ingested. Baculovirus transcripts have been found in high frequencies of adults that survived after OB treatments administered in the larval stage (2, 39). However, very little is known about the factors involved in the establishment and persistence of subethal infections. A previous study of the *S. exigua*-SeMNPV pathosystem revealed that different genotypes differed in their abilities to persist as sublethal infections in the

TABLE 3. Development time, pupal weight, adult emergence, sex ratio, longevity, and preoviposition period in *S. exigua* survivors of SeMNPV OB treatment in the second instar compared to those in mock-infected controls[a]

Insect group	Median development time (days)		Median pupal wt (mg)	Pupal sex ratio (% male)	Adult emergence (%)	Median adult longevity (days)	Median preoviposition period (days)
	Larvae	Pupae					
Controls	10 (3; $n = 94$) A	8 (2; $n = 94$) A	84.8 (11.7; $n = 94$) A	38 ($n = 94$) A	75.5 ($n = 94$) A	9 (3; $n = 20$) A	3 (1; $n = 10$) A
OB treatment survivors	11 (3; $n = 146$) B	8 (1; $n = 146$) A	80.3 (12.4; $n = 146$) B	43 ($n = 146$) A	62.3 ($n = 146$) B	10 (2; $n = 59$) A	4 (2; $n = 18$) B

[a] Means or medians followed by different letters are significantly different ($P < 0.05$) for treatment comparisons within columns. Values in parentheses indicate interquartile range and sample size (n).

TABLE 4. Probit analysis of occlusion body concentration-mortality response in second-instar *S. exigua* larvae from a healthy or sublethally infected population at the sixth generation postinoculation

Population	LC_{50} (10^4 OBs/ml)	Slope (mean ± SE)	Intercept (mean ± SE)	Potency	95% fiducial limits for potency
Healthy	2.83	0.82 ± 0.09	−3.67 ± 0.43	1	
Covertly infected	1.49	1.49 ± 0.14	−6.20 ± 0.60	1.90	1.06–3.40

adult stage, suggesting certain genotypes may be better adapted than others to vertical transmission (4). VT-SeAl1 was selected for the present study as it was consistently observed to produce ~100% of the sublethal infection in the adult survivors of OB treatments.

Inoculation of three instars with increasing concentrations of OBs resulted in NPV mortality that increased from 13% to 79%. High-concentration OB treatments were also associated with a higher prevalence sublethally infected survivors, compared to the survivors of the low-concentration OB treatments. The trend was consistent for any given larval stage, although this finding was less well supported statistically in the case of larvae treated in the second instar.

Examination of transgeneration persistence of sublethal infection provided strong support for vertical transmission of VT-SeAl1 in *S. exigua* cultures. Once established, the sublethal infection was maintained for at least five successive generations. Field-collected or laboratory-reared adults, suspected of carrying a sublethal infection, have previously been reared through several generations in the laboratory (3, 39). Contrary to this approach, we used a virus-free population of *S. exigua* in which sublethal infections were induced. By doing so, a high prevalence of sublethal infection (70 to 100%) was observed by qPCR for detection of viral DNA, whereas the prevalence of adults that tested positive for viral transcripts was notably lower (6 to 13%). Similarly, Burden et al. (3) detected nearly 100% of sublethal infection in 10 different wild populations of *Mamestra brassicae*, three of which were reared in the laboratory under clean conditions and tested in the fifth and eighth generations, demonstrating that the virus was transmitted vertically and was continuously replicating at low levels. In contrast, others have reported a decreasing prevalence of *S. exempta* virus transcript-positive adults through sequential generations of laboratory rearing (39).

However, based on the ability to detect between 7 and 0.7 genome copies, it is clear that the detection of viral genomic DNA by qPCR represents a far more sensitive technique for diagnosis of sublethal infection by baculoviruses than nested or conventional PCR techniques (3, 39) or RT-PCR-based detection of viral gene transcripts. Alternatively, the higher prevalence of viral DNA detection than mRNA detection could be related to differences in the replication activities of the virus in different life stages of the host.

Baculovirus sublethal infections appear to be frequent in natural populations of lepidopteran species, and this implies that the host must invest resources in the suppression of virus replication following consumption of OBs and the initial infection process and subsequently during insect growth and

development to the adult stage. Consequently, we examined whether surviving the OB inoculum in the larval stage was associated with biological costs to the insect host. Specifically, we found evidence for increased larval developmental time and significant reductions in pupal weight and adult emergence. Pupal development time and adult longevity were not significantly affected, but the preoviposition period, fecundity, and egg fertility were all adversely affected in the survivors of VT-SeAl1 OB treatment.

Prior to the development of molecular techniques, studies on the effects of OB treatments on survivors were prone to doubt concerning the infection status of the treated insects (10, 22, 25, 37, 40, 41), but the varied nature of their findings and lack of consistent patterns suggested that sublethal effects of OB treatments varied considerably, depending on the host-pathogen system under study. One exception to this generalization is that almost all studies report that the survivors of OB treatments experience adverse effects on their reproductive capacity. For survivors of the VT-SeAl1 treatment, pupal weight and fecundity were significantly reduced compared to those of mock-infected conspecifics, and correlations between pupal weight and female egg production have been established in other lepidopteran species (16, 38), including *S. exigua* (35).

Rothman and Myers (29) proposed that reductions in fecundity may be due to pathological damage to the reproductive tissues of infected females. Histopathology studies have confirmed the presence of virions in rudimentary spermatocytes and oocytes in the developing gonads in late-instar *Bombyx mori* larvae, and mating between healthy and infected adults resulted in reduced fecundity, possibly due to improper oocyte vitellogenesis (17). As observed in the present study, egg hatchability (fertility) was also adversely affected following OB treatments in other pest moth species (22, 27), which together with reduced fecundity, further lowers the reproductive capacity of these insects.

The extended preoviposition period of OB-treated insects has clear biological significance for migratory insects, such as the species belonging to the genus *Spodoptera*, since this period determines the distances that females can reach before starting to lay eggs. The extended preoviposition period observed in OB-treated female *S. exigua* insects and other species of Lepidoptera (36, 38), may therefore influence the distance that infected females are able to migrate before they begin to lay eggs, although no empirical evidence for this exists to date.

Finally, insects from the sixth generation of a sublethally infected line proved to be ~2-fold more susceptible to superinfection by OBs of the same isolate than control insects that originated from a virus-free colony of identical genetic composition. By taking this approach, we ensured that biological differences in susceptibility could be attributed to the effects or costs associated with sublethal disease and were not due to genetic differences between populations. The finding that sublethal infection increases susceptibility to OB superinfection is in line with a previous study indicating that larvae from an infected *S. exigua* colony derived from field-collected adults were consistently more susceptible to a range of SeMNPV isolates than insects from a virus-free colony (4).

A plausible explanation for the increased susceptibility of sublethally infected insects is that the external virus reactivates the endogenous infection. The genetic factors associated with latent or sublethal infection are poorly understood in baculoviruses, although a number of examples in the published literature have provided evidence that infection with a heterologous NPV can trigger a sublethal infection into a lethal state of disease (5, 12, 13, 19). Further experiments are required to elucidate the conditions under which sublethal infections can be activated to kill the host, but this strategy may be clearly advantageous to the resident virus when it detects the presence of a competing pathogen.

From a practical point of view, sublethal infections may benefit pest control programs, given that insects that do not die from overt infection after consuming contaminated food have a high probability of reduced reproduction. Moreover, the sublethal infection is likely to be transmitted to their offspring, which in turn are more likely to succumb to patent NPV disease following application of biopesticide viral OBs to crops than healthy conspecific larvae. As a result, vertically transmitted infections are likely to result in improved levels of pest control from one generation to the next.

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