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Short Communication

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Comparative Pathogenesis of Generalist AcMNPV and Specific RanuNPV in Larvae of *Rachiplusia nu* (Lepidoptera: Noctuidae) Following Single and Mixed Inoculations

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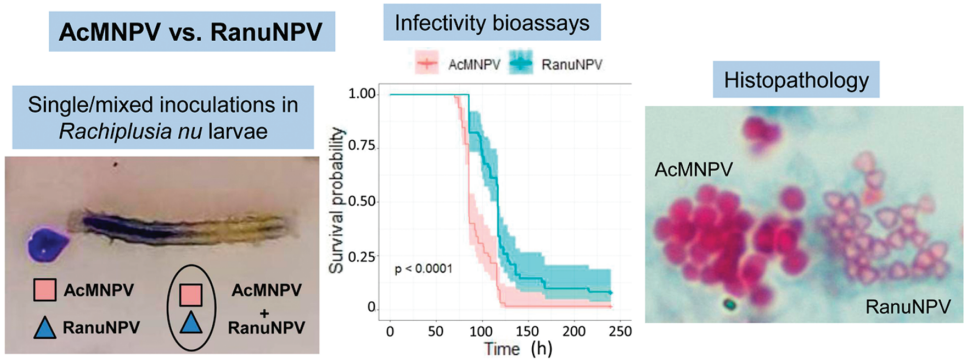
Abstract

The South American soybean pest, *Rachiplusia nu* (Guenée), is naturally infected by *Autographa californica multiple nucleopolyhedrovirus* (AcMNPV) and *Rachiplusia nu nucleopolyhedrovirus* (RanuNPV). We compared their pathogenicity to fourth-instar *R. nu* larvae, by evaluating time to death and virus spread throughout the tissues in single and mixed infections. Bioassays showed that generalist AcMNPV had a faster speed of kill than specific RanuNPV, while the mixed-virus treatment did not statistically differ from AcMNPV alone. Histopathology evidenced similar tissue tropism for both viruses, but co-inoculation resulted in mostly AcMNPV-infected cells. In sequential inoculations, however, the first virus administered predominated over the second one. Implications on baculovirus interactions and biocontrol potential are discussed.

Graphical Abstract



Key words: *Rachiplusia nu*, *Rachiplusia nu nucleopolyhedrovirus*, pathogenesis, mixed infections, viral insecticide

Rachiplusia nu (Guenée) is a major soybean defoliator in South America. During the last decades, soybean caterpillars in the region have been controlled using broad-spectrum insecticides and transgenic cultivars (Murúa et al. 2018). An exception to this was the development of a baculovirus-based product to manage *Anticarsia gemmatalis* in Brazil (Moscardi 1999). At a global scale, several

environmentally friendly, pest-specific baculoviral insecticides have been applied against Lepidoptera (Lacey et al. 2015).

Baculoviruses are dsDNA viruses that infect lepidopterans, hymenopterans, and dipterans. Virions consist of nucleocapsids enveloped (singly or in bunches) by a membrane and are occluded in protein structures called occlusion bodies (OBs). Based on the morphology and composition of the OBs, baculoviruses are either nucleopolyhedroviruses (NPVs) or granuloviruses (GVs). They are currently classified into four genera: *Alphabaculovirus* (lepidopteran NPVs), *Betabaculovirus* (lepidopteran GVs), *Gammabaculovirus* (hymenopteran NPVs), and *Deltabaculovirus* (dipteran NPVs) (Rohrmann 2019).

When ingested with the food, the OBs dissolve in the alkaline larval midgut releasing the occlusion-derived virions (ODVs). The primary infection initiates with a mutually dependent interaction of viral per os infectivity factors (PIFs) and receptors in the host midgut cells (Song et al. 2016). Eventually, the ODVs fuse with the membrane of the midgut epithelial cells; the nucleocapsids translocate to the nucleus and viral replication begins. Then, a second virus phenotype, the budded virus (BVs), exits the cells spreading the infection to other larval tissues via hemolymph and trachea. At late stages of infection, virions are occluded in newly formed OBs. Upon host death (usually 3- to 10-d postingestion [p.i.]), cadaver liquefies disseminating the OBs to restart the cycle (Rohrmann 2019).

Most baculoviruses have a narrow host range (Rohrmann 2019). In contrast, *Autographa californica multiple nucleopolyhedrovirus* (AcMNPV), the *Alphabaculovirus* type species, infects multiple hosts in several lepidopteran families (Trudeau et al. 2001). Wild lepidopteran populations are naturally exposed to various pathogens. Especially during outbreaks, different virus strains or species may infect the same individual. Viruses can invade the host concurrently (co-infections) or successively (superinfections), modulating each other's fate and the resulting insect response (Da Palma et al. 2010). Interactions may range from synergistic to antagonistic or neutral (Rohrmann 2019). It has been shown that GVs generally enhance NPVs infections (Lepore et al. 1996, Mukawa and Goto 2007, Ishimwe et al. 2015, Cuartas-Otálora et al. 2019). Yet, little is known about disease development in lepidopteran larvae infected by two or more distinct NPVs. Such information is crucial to understand viral pathogenesis and evolution, and for establishing efficient and long-lasting control strategies.

Previous works reported the natural infection of *R. nu* by generalist AcMNPV and specific, phylogenetically distant *Rachiplusia nu nucleopolyhedrovirus* (RanuNPV) (Rodríguez et al. 2012, Jakubowicz et al. 2019). Here, we have assessed the speed of kill of either virus, alone or combined, against *R. nu* larvae. We have also examined their spread throughout the host tissues, following single or double (simultaneous and sequential) inoculations.

Materials and Methods

Insects, Viruses, and Inoculation

To initiate a healthy *R. nu* colony, adults were light-trapped in central Argentina during summer 2018/2019. Before use, insects were kept in quarantine for two generations to assess their pathogen-free status. Insects were reared on artificial diet at 26 ± 2°C and a photoperiod of 14:10 (L:D) h (Jakubowicz et al. 2019). Under these conditions, *R. nu* undergoes five larval instars (LI – LV), readily discriminated by their head-capsule widths (LI: 0.278 ± 0.007 mm, LII: 0.429 ± 0.022 mm, LIII: 0.723 ± 0.028 mm, LIV: 1.128 ± 0.035 mm, and LV: 1.692 ± 0.130 mm; n = 25 each). The original AcMNPV and RanuNPV isolates were obtained from two different groups of baculovirus-infected *R. nu* larvae collected in Santa Fe province (Argentina) in the 1990 decade. Both isolates have been characterized biologically and genetically (Rodríguez et al. 2012, Jakubowicz et al. 2019). Following multiplication in laboratory-reared larvae, the OBs of either virus were extracted using SDS, cheesecloth filtering and differential centrifugation, and quantified in a Neubauer chamber (Arneodo et al. 2018). To avoid unwanted mixtures, each inoculum was extensively checked by molecular and electron microscopic analyses. For all experiments hereafter, late LIII were starved for 16 h, and newly molted LIV were virus-inoculated by the 'droplet-feeding' method (OBs suspended in a colored sucrose solution; Hughes and Wood 1981). The mean ingestion in LIV was estimated to be 0.368 µl by weighing larvae before and after liquid intake (n = 95).

Time-Mortality Analyses

The speed of kill was determined in singly and co-infected fourth-instar *R. nu* larvae. In the first case, 3,000 OBs (AcMNPV or RanuNPV) per larva were administered, while dual, simultaneous inoculations consisted of 3,000 mixed OBs (AcMNPV + RanuNPV, 50:50) per larva. High virus doses were chosen to ensure near-complete mortality, based on previous assays. About 20 larvae per treatment (AcMNPV, RanuNPV, and AcMNPV + RanuNPV) were used in each replicate, along with 10 mock-inoculated controls (sucrose and dye solution only, no OBs). Three independent biological replicates were performed. Larvae were individually maintained in plastic cups at the above-mentioned conditions, and mortality was recorded regularly until death or pupation. A survival analysis was performed using Kaplan–Meier algorithm as implemented in 'Surv' function of the survival R-package (Therneau 2020). Dead larvae were first checked by optical microscopy. The distinctive shape of the OBs (cubic for AcMNPV and tetrahedral for RanuNPV), allowed rapid preliminary identification. Virus identity was further confirmed by specific PCRs. Total DNA from larvae was extracted with CTAB (Doyle and Doyle 1987) and diagnostic primers were designed to partially amplify the conserved *lef-8* gene of either virus. AcMNPV primers lef8AcMNPVFw (5′-CCAACGTGGACTACG AAATGG-3′) and lef8AcMNPVRv (5′-TGGTAAACCCGATACG CAAT-5′) were used with the following cycling parameters: initial denaturation at 95°C for 3 min, 30 cycles of 95°C for 1 min, 53°C for 1 min, 72°C for 30 s and a final extension at 72°C for 5 min. RanuNPV was detected with primers lef8RanuNPVFw (5′-GTAG CCGAAAAGTTTAAGTTGC-3′) and lef8RanuNPVRv (5′-AACT TGGGAACTGGTGTGTTG-3′) under identical conditions, except that annealing temperature was set at 54°C. Positive and negative controls were run in parallel. Amplicons of 548 bp (AcMNPV) and 440 bp (RanuNPV) were resolved by agarose gel electrophoresis and ethidium bromide staining.

Histopathology

To examine virus spread throughout the host tissues, OBs of AcMNPV and/or RanuNPV were inoculated alone (single infection), simultaneously (co-infection), and sequentially (superinfection). Single inoculations consisted of 3,000 OBs of either virus per fourth-instar larva. In simultaneous inoculations, larvae were fed with 3,000 OBs of virus mixture (50:50). For sequential inoculations, 24 h after exposure to the first virus (1,500 OBs), the second virus was administered (1,500 OBs). To promote ingestion, larvae were starved again for 6 h prior to droplet feeding with the new virus suspension. Thus, five treatments were assayed: AcMNPV, RanuNPV, AcMNPV

+ RanuNPV, AcMNPV followed by RanuNPV, and RanuNPV followed by AcMNPV. At 4-d p.i. (first inoculation, in the case of superinfections), larvae were fixed in Bouin–Dubosq–Brasil and embedded in paraffin. Transversal sections (ca. 5 µm) were stained with Azan (Hamm 1966) and observed in a Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan). At least three specimens per treatment were processed, together with healthy controls. Supplementary to the histopathological preparations, the cadavers of 13 co-infected and six superinfected larvae (three per combination, i.e., AcMNPV first and RanuNPV first) were individually homogenized in distilled water for direct inspection in a Neubauer chamber. For each specimen, different fields (chamber squares of 0.004-µl volume) were randomly examined: 5–16 squares per larva in the case of simultaneously infected specimens, and 4 or 5 squares/larva for sequentially infected specimens. The observed OBs were categorized by shape and counted. Then, the relative proportion of cubic and tetrahedral OBs was estimated. A generalized mixed linear model for a binomial count was fitted to the proportion of cubic (AcMNPV) OBs depending on the inoculation procedure (simultaneous, AcMNPV first, and RanuNPV first). The model was fitted using function glmer from lme4 package (Bates et al. 2015) of R language (R Core Team 2020) under the user interface provided by InfoStat statistical software (Di Rienzo et al. 2020). A DGC-posteriori test (Di Rienzo 2002) was used to separate mean proportions.

Results and Discussion

Speed of Kill of AcMNPV and RanuNPV in *R. nu* Larvae Following Single and Simultaneous Inoculations

As expected, all treatments caused high mortality rates: 98.5% (AcMNPV), 91.9% (RanuNPV), and 100% (mixed inoculation). Larvae liquefaction, typical of most baculovirus infections, was a common feature. Healthy controls developed normally. The disease etiology was confirmed by microscopic and molecular analyses of the cadavers. In singly inoculated larvae, either cubic or tetrahedral polyhedra were observed under light microscopy, and the predicted DNA fragments of AcMNPV or RanuNPV were amplified by PCR. Instead, OBs of both shapes were recovered from co-inoculated larvae (though AcMNPV-like OBs clearly predominated). Intense PCR bands for both AcMNPV and RanuNPV (see Supp Fig. 1 and Supp Materials [online only]) confirmed the dual infection of *R. nu* larvae with these phylogenetically distant NPVs. The speed of kill varied markedly depending on the treatment. The survival time of AcMNPV-infected larvae was 3.87 ± 0.62 d (the first death was as early as 70-h p.i.), whereas RanuNPV-infected larvae survived 4.76 ± 1.01 d (first death at 85.5-h p.i.). Mixed-virus treatment killed the larvae in 3.73 ± 0.57 d (first death also at 70-h p.i.). Comparisons of RanuNPV versus AcMNPV alone, RanuNPV versus AcMNPV + RanuNPV, and AcMNPV versus AcMNPV + RanuNPV were made using ad hoc contrasts. Statistically significant differences were found between RanuNPV treatment and the other two (AcMNPV alone and AcMNPV + RanuNPV; $P < 0.0001$). The virus mixture had a slightly increased insecticidal performance compared with AcMNPV alone, but this was not statistically significant ($P = 0.091$). Mortality curves are shown in Fig. 1.

The insecticidal activity of AcMNPV varies from host species to host species, even among closely related taxa. For instance, AcMNPV was highly pathogenic to *Heliothis virescens*, whereas it showed reduced mortality and extended lethal time in another heliothinae,

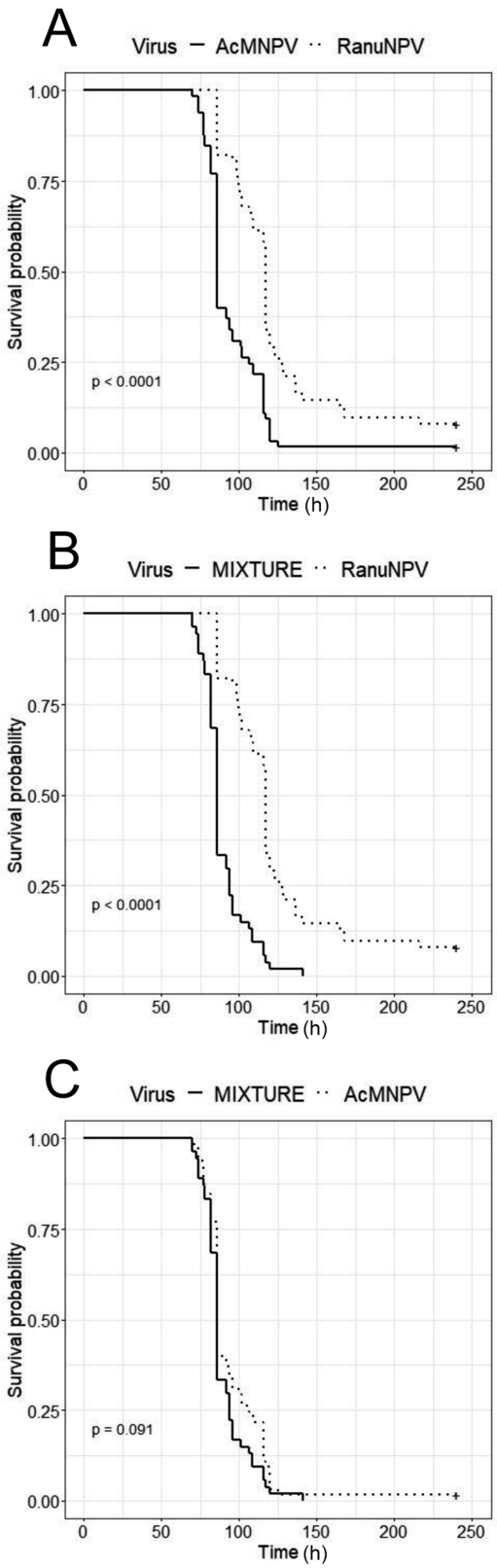


Fig. 1. Mortality curves of fourth-instar *Rachiplusia nu* larvae fed with 3,000 OBs of either AcMNPV or RanuNPV (single inoculations) and 1,500 OBs of AcMNPV + 1,500 OBs of RanuNPV (co-inoculations). (A) AcMNPV versus RanuNPV. (B) Virus mixture versus RanuNPV. (C) Virus mixture versus AcMNPV.

Helicoverpa zea (Trudeau et al. 2001). In turn, heliothinae-restricted *Helicoverpa armigera nucleopolyhedrovirus* (HearNPV) has proven efficient to control all the main pests in the subfamily (Rowley et al. 2011). Interestingly, we found that generalist AcMNPV was more virulent to *R. nu* than specific RanuNPV under the described conditions. This was not completely unexpected, since *R. nu* is a natural host of both viruses, the OBs of AcMNPV are larger than those of RanuNPV (thus possibly occluding more virions), and AcMNPV contains multiple nucleocapsids per virion compared to singly enveloped RanuNPV. Differential virulence factors could additionally explain differences in pathogenicity, especially regarding the notably dissimilar speed of kill.

Mixed baculovirus infections can result in different types of virus interactions. Research on this topic was mainly devoted to dual GVs–NPVs infections. In most cases, GVs produced a synergistic effect on NPVs infectivity (Mukawa and Goto 2007, Ishimwe et al. 2015, Cuartas-Otálora et al. 2019). This is not, however, a universal pattern. Hackett et al. (2000) found that a GV isolated from *H. armigera* inhibited the replication of HearNPV in *H. zea* larvae. Independent action was also reported: neither an increase nor a decrease in mortality was observed in mixed-virus treatments of *Agrotis segetum* larvae with *Agrotis segetum granulovirus* and *Agrotis segetum nucleopolyhedrovirus* B (Wennmann et al. 2015). Concerning NPVs–NPVs interactions in vivo, studies focused on different strains (wild-type and/or genetically modified) of the same NPV species (Georgievska et al. 2010, Virto et al. 2014). Heterologous NPVs infections in cell cultures will be discussed later. In our study, the effect of the mixed-virus treatment was close to that of the most virulent NPV alone (i.e., AcMNPV). The use of high viral doses to calculate the mean times to death could have masked subtle differences among treatments, especially in terms of final mortality. Therefore, additional experiments with reduced doses are needed to explore possible synergic effects. In any case, our data suggest that co-inoculation with AcMNPV and RanuNPV does not impact negatively on pathogenicity. Considering that both viruses can occur in wild *R. nu* populations, this issue is particularly relevant in view of their potential single or combined field application for pest control.

Spread of AcMNPV and RanuNPV in *R. nu* Tissues Following Single and Mixed (Simultaneous and Sequential) Inoculations

At 4-d p.i. (when samples were fixed), AcMNPV and RanuNPV displayed similar tissue tropism in singly infected, fourth-instar *R. nu* larvae. At this late stage of the disease progress, a widespread systemic infection was observed. In both cases, fat bodies, epidermis, and tracheal matrix were the most affected organs, as previously reported for RanuNPV (Jakubowicz et al. 2019). Bright red polyhedra (cubic or tetrahedral, according to the virus species) were observed inside enlarged nuclei. In mixed-infection experiments, the histopathology varied with the order in which the viruses were administered to the larvae. Four days after simultaneous feeding with AcMNPV and RanuNPV, the overwhelming majority of OBs in the infected organs were cubic-shaped, AcMNPV-like OBs. Nevertheless, a few cell nuclei filled with tetrahedral, RanuNPV-like OBs were also detected (Fig. 2A). These infrequent RanuNPV-infected cells were distributed in an apparently random manner, surrounded by predominantly AcMNPV-infected tissue, providing evidence that AcMNPV is a stronger competitor compared with specific RanuNPV. The proportion of both types of OBs in homogenates of dead larvae, estimated by Neubauer

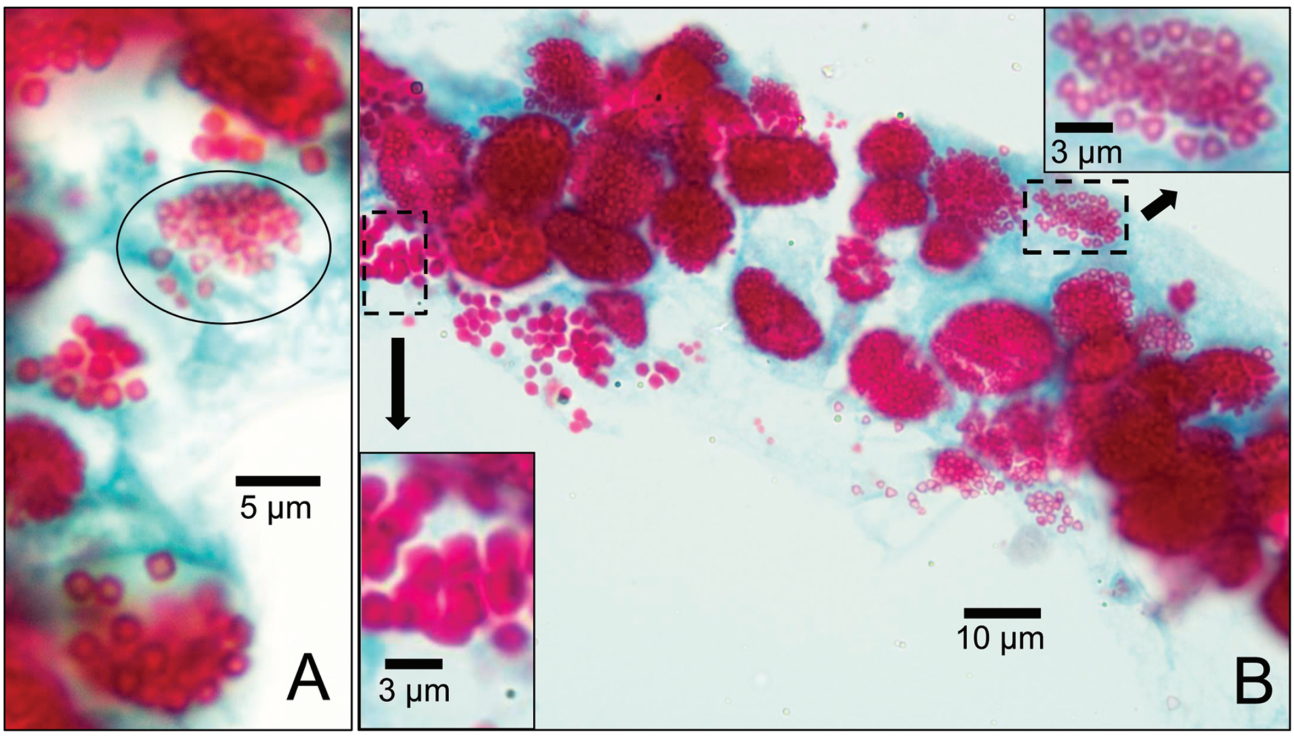


Fig. 2. Azan-stained sections of fourth-instar *Rachiplusia nu* larvae, showing heavily baculovirus-infected fat bodies. Bright-red OBs are seen filling hypertrophied cell nuclei or scattered across the tissue after disruption of the nuclear membrane (see online version for color figures). (A) Simultaneous AcMNPV/RanuNPV inoculation, resulting in mostly cubic (AcMNPV-like) OBs production. Tetrahedral (RanuNPV-like) OBs are highlighted by an oval. (B) Sequential inoculation (RanuNPV followed by AcMNPV). The OBs corresponding to the first virus administered clearly predominate. Enlarged views of either OB morphology are shown. See online version for color figure.

Table 1. Proportion of cubic (AcMNPV-like) OBs versus total OBs in the cadavers of mixed-infected *Rachiplusia nu* larvae

Inoculation sequence	Total no. of OBs counted (both shapes)	Proportion of AcMNPV-like OBs	SE	
AcMNPV first	1203	0.99	0.01	A
Simultaneous	27,638	0.98	0.01	A
RanuNPV first	902	0.11	0.09	B

Common letters imply nonsignificant differences among proportions ($P > 0.05$). [Single column width]

chamber counting, supported this observation. AcMNPV-like OBs markedly outnumbered RanuNPV-like OBs (summarized in Table 1). In 2 out of 13 co-inoculated larvae examined, only AcMNPV-like OBs were detected. In successive inoculations, the first-administered virus exhibited a higher proliferation throughout the larval tissues than the second one (Fig. 2B). Nevertheless, mixed infections progressed with both inoculation sequences. Even the apparently less aggressive RanuNPV still managed to infect a few fat body and epidermis cells when inoculated 24 h after AcMNPV. Again, viruses appeared to co-infect the same tissues but not the same cells. The share of AcMNPV- or RanuNPV-like OBs in larval homogenates further evidenced the former findings. When AcMNPV was inoculated first, cubic OBs were far more abundant than tetrahedral OBs. In contrast, first inoculation with RanuNPV yielded considerably more tetrahedral than cubic OBs (Table 1).

The absence of co-infected cells might be attributed to the superinfection exclusion phenomenon, in which an established virus interferes with a second virus infection. Such interactions have been studied in vitro, owing to a better control of the experimental variables regarding the virus entry into the cell. For NPVs, this effect has been demonstrated in Sf9 cells infected with different genotypes of AcMNPV. Also, infection with AcMNPV protected the cells from a subsequent infection with *Spodoptera frugiperda nucleopolyhedrovirus* and vice versa (Beperet et al. 2014). Assuming that each OB shape corresponds to a single baculovirus species, our in vivo experiments are in line with this report. We could not perform further in vitro assays, because RanuNPV BVs failed to infect Sf9 and Tn5B1-4 cell cultures.

Overall, our study demonstrated that both specific RanuNPV and generalist AcMNPV were highly pathogenic to *R. nu* larvae, but the latter virus had a better insecticidal performance. When simultaneously inoculated, AcMNPV appeared to outcompete RanuNPV for available host tissue. In sequential inoculations, however, the extent of tissue colonization by either virus depended on the order in which the viruses were administered. These data provide new insights into the still poorly explored field of NPVs interactions in vivo and should be taken into account for the planning of biological control strategies.

Supplementary Data

Supplementary data are available at *Journal of Economic Entomology* online.

Acknowledgments

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Conflict of interest

None.

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