Co-infections of Agrotis segetum granulovirus (AgseGV) and Agrotis segetum nucleopolyhedrovirus B (AgseNPV-B) do not enhance the mortality of cutworm larvae over single infections of either virus.

Running Title: Agrotis baculovirus co-infections

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Abstract

Mixed infections of insect larvae with different baculoviruses are occasionally found. They are of interest from an evolutionary as well as from a practical point of view when baculoviruses are applied as biocontrol agents. Here, we report mixed infection studies of neonate larvae of the common cutworm, *Agrotis segetum*, with two baculoviruses, *Agrotis segetum* nucleopolyhedrovirus B (AgseNPV-B) and *A. segetum* granulovirus (AgseGV). By applying qPCR analysis, co-infections of individual larvae were demonstrated and occlusion body (OB) production within single infected and co-infected larvae was determined in individual larvae. Mixtures of viruses did not lead to changes in mortality rates when compared with rates of single virus treatments, indicating an independent action within host larvae under our experimental conditions. AgseNPV-B infected larvae showed an increase in OB production during two weeks of infection, whereas the number of AgseGV OBs did not change from the first to the second week. In comparison with single infected larvae, fewer OBs of both viruses were produced in co-infections suggesting a competition of the two viruses for larval resources. Hence, no functional or economic advantage could be inferred from larval mortality and OB production from mixed infections of *A. segetum* larvae with AgseNPV-B and AgseGV.
1. Introduction

A large number of baculoviruses has been isolated and described from larvae of species of the insect orders Lepidoptera, Diptera and Hymenoptera (1). According to their occlusion body (OB) morphology, baculoviruses are distinguished into two morphological groups; the granuloviruses (GV) with a single virion in an ovo-cylindrical OB (granule) and the nucleopolyhedroviruses (NPV) with a few to many virions in a polyhedral OB (polyhedron) (2). Taxonomically, the family Baculoviridae is subdivided into four genera, the Alpha-, Beta-, Gamma- and Deltabaculovirus (2, 3). Alpha- and betabaculoviruses comprise lepidopteran-specific NPVs and GVs, respectively. Many lepidopteran species are susceptible to baculoviruses from different species. Even simultaneous infections, so-called co-infections or mixed infections, of two NPVs (4), two GVs (5), and NPVs and GVs (6, 7) have been observed.

Larvae of the turnip moth, Agrotis segetum (Dennis & Schiffermüller) (Lepidoptera: Noctuidae), so-called common cutworms, are important agricultural pests (8). Two different alphabaculovirus isolates, Agrotis segetum nucleopolyhedrovirus A (AgseNPV-A, also called Polish isolate) and A. segetum nucleopolyhedrovirus B (AgseNPV-B, also termed Oxford isolate) (9, 10, 11), as well as the betabaculovirus Agrotis segetum granulovirus (AgseGV) (12), were isolated and characterized from A. segetum larvae. AgseGV was tested extensively as a biocontrol agent for the control of A. segetum in the field (13–16) and AgseNPV-B has shown its potential as biocontrol agent under laboratory conditions (13).
Both viruses were found to infect *A. segetum* larvae simultaneously (17) but little is known about possible interaction in co-infections. Shvetsova and Ts’ai (17) reported an increased mortality when AgseGV and an *Agrotis* nucleopolyhedrovirus were simultaneously provided to larvae of *Apamea aniceps* and it was concluded from mortality data that these viruses did not show antagonistic behavior. In addition, both viruses were shown histologically to infect the same insect tissues of *A. segetum* larvae (17). However, in that study it was not distinguished whether the virus isolate used was AgseNPV-A or AgseNPV-B, since this nomenclature was later introduced (10). It is not known if there is a reciprocal influence of AgseGV and AgseNPV-B in co-infected larvae, and if so, which kind of effect occurs, and if this effect is of a functional and economic benefit when used as pest control agent. Both enhancing and inhibiting effects within common host larvae have been described for GV and NPV co-infections (18, 19). Enhancing effects were first observed for *Pseudaletia unipuncta* GV (PsunGV) and *P. unipuncta* (PsunNPV). In that study, the presence of a synergistic or enhancing factor associated with the occlusion body of increased the susceptibility of *P. unipuncta* larvae to PsunNPV (18). A contrary effect was observed for simultaneous treated larvae of *Heliocoverpa armigera* with *Helicoverpa* (*Heliothis*) armigera NPV (HearNPV) and H. armigera GV (HearGV) (19). The overall mortality decreased and HearGV appeared to suppress HearNPV infection (19). A similar observation was made for larvae of *Heliotris zea* infected with slow acting HearGV and fast-killing HearSNPV (6). When both viruses were fed to larvae at the same time, both viruses appeared to compete for larval resources. On the contrary, HearSNPV infected larvae that were post-treated with HearGV appeared to outcompete already established infections of the NPV. More
recently, an AgseNPV-B virus stock containing AgseGV revealed covert co-infections of AgseGV in virus propagations in *A. segetum* larvae (20). Within the same study, a PCR-based method for the identification and quantitation of *Agrotis* baculoviruses was established. Since a mixture of AgseGV and AgseNPV-B could be both beneficial and disadvantageous for one or both viruses, information about co-infections are considered important for a possible improvement of their application in the field.

This study was carried out to investigate the changes on mortality of *A. segetum* larvae treated with AgseGV and AgseNPV-B in mixed infections. Quantitative PCR (qPCR) was applied to determine the OB production of AgseGV and AgseNPV-B at the level of individual co-infected larvae to describe the effect of co-infection on progeny OB production.
2. Materials and methods

Insects and viruses

Rearing of *A. segetum* was performed at the Institute for Biological Control in Darmstadt (Julius Kühn Institute, JKI) as described (20). Neonate *A. segetum* larvae were used in all infection experiments. Stocks of *Agrotis segetum* nucleopolyhedrovirus B (AgseNPV-B) and *Agrotis segetum* granulovirus (AgseGV), which originated from the virus collection of Horticulture Research International (HRI) in Warwick (UK), were propagated in fourth instar of *A. segetum*.

Bioassay analysis

Infection studies were performed for AgseNPV-B and AgseGV in neonate *A. segetum* larvae feeding on different virus concentrations incorporated in semiartificial diet. For AgseNPV-B the final virus concentrations were $10^3$, $3.1 \times 10^3$, $10^4$, $3.1 \times 10^4$, $10^5$, $3.1 \times 10^5$, and $10^6$ OBs/ml. The bioassay for AgseGV was performed at final concentrations of $10^2$, $10^3$, $10^4$, $10^5$, $10^6$, $10^7$, and $10^8$ OBs/ml. Enumeration of virus OBs was performed by hemocytometer counting as described (21). The semiartificial diet (modified according to 20, 22) was mixed with OBs to their final concentrations prior to solidification at a semi fluid state (40 °C). Diet of the untreated control contained no virus. The diet of one concentration was filled in every well of a 50-well bioassay tray (LICEFA, Bad Salzuflen, Germany). Bioassay trays were kept open overnight to allow evaporation of excess humidity. On the following day, 50 neonate larvae per virus concentration (=treatment) were individually placed in each well of a bioassay tray.
larvae were used for the untreated control. After the bioassay trays were closed, they were kept at 22 °C with a 16/8 h light/dark photoperiod. Mortality was scored 1, 7 and 14 days post infection (dpi). Larvae that died within the first 24 h of the assay were assumed to have died from handling and were not included in the analyses. Mortality data were corrected according to Abbott (23). Median and 10% lethal concentrations (LC₅₀, and LC₁₀, respectively) were calculated for day 7 and day 14 for each virus by probit analysis using ToxRat software (ToxRat Solutions, Alsdorf, Germany). Parallelism of probit lines of 7 and 14 dpi was tested with potency estimation as provided by ToxRat software (ToxRat Solutions, Alsdorf, Germany).

Setup of Mixed Infection Experiments
Neonate *A. segetum* larvae were individually exposed to AgseGV and AgseNPV-B in different concentrations and combinations. Two final OB concentrations of AgseNPV-B were used in this experiment: a low (NPVL = 0.8 x 10³ OBs/ml) and a high (NPVH = 37 x 10³ OBs/ml) concentration. AgseGV was used in a single final concentration (GV = 900 x 10³ OBs/ml). The treatments were set up as follows: (i) GV, (ii) NPVL, (iii) NPVH, (iv) GV+NPVL, (v) GV+ NPVH, and (vi) untreated control. Preparation of bioassay trays and diet containing the different combinations of virus concentrations was done as described for bioassay analysis. Twenty-five neonate *A. segetum* larvae were added to each virus and mixed virus treatment, whereas the control contained 50 neonate larvae. Larvae mortality was scored and analyzed separately for the first (days 2-7) and second (days 8-14) week. Cadavers that died by viral infection were collected entirely without contaminating the sample with artificial diet. Samples were stored individually at -20 °C.
The larval mortality of each infection experiment was corrected according to Abbott (23). Test for significant variation in mean mortality of treatments was performed by one-way analysis of variance (ANOVA) followed by Tukey’s test for pairwise comparisons between treatments in RStudio (version 0.97.551) statistical software.

**Viral DNA preparation**

Viral DNA was purified separately from individual larvae. Frozen larval remains of mixed infection experiments were prepared for OB purification by thawing and centrifugation at 13,000 × g for 30 sec without additional buffers. By this initial step, the integrity of most infected larval bodies was broken up. Larval tissues that remained intact were homogenized within the tubes using mini-pestles followed by an additional centrifugation step. Liquid supernatants were discarded and the pellets composing of cell debris and OBs, were subjected to DNA isolation using Ron’s tissue DNA Mini Kit (Bioron, Ludwigshafen, Germany). In short, disintegrated larvae were resuspended in 250 µl tissue lysis buffer and treated with proteinase K (250 µg/ml) (Thermo Fisher Scientific, Waltham, MA, USA). Samples were incubated overnight at 52 °C. Subsequently, 30 µl of 1 M Na₂CO₃ were added to the sample to dissolve OBs under alkaline conditions for 30 min. Samples were neutralized by adding the same volume of 1 M HCl. Larval debris was pelleted at 12,000 × g for 1 min and the brownish, cloudy supernatant was transferred to a fresh centrifugation tube. Herring sperm DNA (3 µg) was added to the samples in order to saturate the kit’s silica membrane in the later course of the DNA isolation protocol. DNA solutions were processed according the kit’s protocol and...
eventually eluted from the membrane by adding 400 µl of 10 mM TE buffer. For reasons of standardization and reproducibility, all steps of viral DNA extraction and isolation were adjusted to previously described protocols for qPCR standard sample generation (20).

Quantitation of larval OB production

Quantitation of AgseNPV-B and AgseGV OBs of single larvae was performed by qPCR (20). This technique allowed standardized quantitation of OBs produced by even small larval cadavers. To assure the reliability of the experiment, a minimum of five single larvae was used to quantify median OB production in single and co-infected larvae per treatment. Therefore, quantitative analyses on single infected larvae were performed for the GV and GV+NPV$_L$ treatments for AgseGV as well as the NPV$_H$ and GV+NPV$_H$ treatments for AgseNPV-B. OB production in co-infections was analyzed from larvae of the GV+NPV$_H$ treatment. Quantitation standards for qPCR, serial ten-fold dilutions of OBs of AgseNPV-B (in the range of $10^9$ to $10^4$ OBs/ml) and AgseGV ($10^{11}$ to $10^6$ OBs/ml) in triplicate were prepared. DNA was isolated from all dilution steps as previously described (20). The lower limit of determination (LOD) for AgseGV and AgseNPV-B quantitation was $10^5$ OBs of AgseGV and $10^3$ OBs of AgseNPV-B. For a single qPCR, 2 µl of standard or sample DNA were mixed with 1 µl 0.2 pM of each forward and reverse primer (prAsBpolh-f/prAsBpolh-r and prAsGVgran-f/prAsGVgran-f, respectively) 12.5 µl 1 x Maxima® SYBR Green/Rox qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) and 8.5 µl ddH$_2$O to a total reaction volume of 25 µl (20). The non-target controls contained 2 µl ddH$_2$O instead of DNA sample. Each larval DNA sample was tested twice for AgseNPV-B and AgseGV in separate qPCR runs along with...
their corresponding standard samples. Each run included non-target controls. QPCR parameters and melting curve settings were adjusted as previously described (20). Data were processed with Bio-Rad CFX Manager 2.0 (Bio-Rad, Hercules, CA, USA). Cq values were obtained by single threshold analysis. Statistical differences in OB production between treatments in single and co-infected larvae were revealed by Kruskal-Wallis test. Comparisons between groups were shown by pairwise Wilcoxon rank sum test and p value adjustment according to Holm-Bonferroni method (24). Statistical analyses were performed by using RStudio (version 0.97.551) software.

Results

Biological activity

Bioassay analyses were performed to compare the virulence of AgseGV and AgseNPV-B to neonate A. segetum larvae. At 7 dpi the LC$_{10}$ and LC$_{50}$ of AgseGV could not be calculated due to low mortality rates (Table 1). After 14 days, the LC$_{10}$ of AgseGV was 2.60 x 10$^3$ OBs/ml and the LC$_{50}$ was 8.33 x 10$^5$ OBs/ml. The 14-day LC$_{50}$ of AgseGV was 254 times higher than the LC$_{50}$ of AgseNPV-B with 3.28 x 10$^3$ OBs/ml. The higher virulence of AgseNPV-B was also reflected by the LC$_{10}$ (7.7 x 10$^2$ OBs/ml) and LC$_{50}$ (3.70 x 10$^4$ OBs/ml) at 7 dpi and LC$_{10}$ (3.4 x 10$^2$ OBs/ml) at 14 dpi (Table 1).

Mortality of mixed infections
Single and mixed infections of *A. segetum* larvae were performed with a single concentration of $9.0 \times 10^5$ OBs/ml for AgseGV, which equaled a LC$_{50}$ at 14 dpi, and two concentrations of AgseNPV-B, NPV$_L = 8.0 \times 10^2$ OBs/ml (LC$_{10}$ at 7 dpi) and NPV$_H = 3.7 \times 10^4$ OBs/ml (LC$_{50}$ at 7 dpi) (Table 1). Differences between mortality results of mixed virus experiments were analyzed separately for 7 and 14 dpi by one-way ANOVA analysis. Differences between treatments were scored by post hoc Tukey’s test (level of significance $p < 0.05$). At 7 dpi, significant differences between treatments were observed (One-way ANOVA, $F(4,25) = 11.13$, $p < 0.001$). The lowest mortality was observed for the GV treatment (6.17% ± 4.97%) and NPV$_L$ (3.11% ± 3.97%) treatment. Within the first week (days 2-7) only a few larvae died and no significant difference between GV and NPV$_L$ treatment was observed ($p = 0.934$) (Fig. 1A). The highest mean mortality of single virus treatments was found for NPV$_H$ (22.7% ± 9.07%) where the mortality was statistically different from GV ($p = 0.002$) and NPV$_L$ treatment ($p < 0.001$).

In all single virus treatments the mortality increased from the first to the second week (Fig. 1B), where significant differences were detected (One-way ANOVA, $F(4,25) = 14.21$, $p < 0.001$). At 14 dpi NPV$_L$ treatment exhibited the lowest increase in mortality (5.87% ± 5.37%) stating that the fewest additional larvae had died within the second week. The NPV$_L$ mortality was statistically different from GV (36.8% ± 11.3%) ($p = 0.029$) and NPV$_H$ (60.1% ± 20.6%) ($p < 0.001$) (Fig. 1B).

The mortality of the GV+NPV$_L$ treatment was 9.50% ± 8.97% and 50.1% ± 22.6% after the first and second week, respectively, but did not differ statistically from the mortality
found in the GV treatment of the first (p = 0.913) and second week (p = 0.648). The
GV+NPV_H treatment caused a mortality of 22.6% ± 5.25% and 73.7% ± 17.8% after the
first and second week, respectively, which were similar to the results of the NPV_H
treatments (p = 0.999 and p = 0.632) (Fig. 1A and 1B). Thus, both mixed virus treatments
equaled either the mortality caused by AgseGV (GV) or AgseNPV-B (NPV_H/NPV_L)
treatment that induced the higher mortality, when provided to larvae alone.

Based on the observed mortality of mixed virus treatments the hypothesis was tested
that AgseGV and AgseNPV-B exhibit an additive effect in mortality on A. segetum
larvae. For testing, the survival rates (1 - mortality rate) of the GV and NPV_L, as well as
the survival rates of GV and NPV_H treatments of each replicate were multiplied. The
products were retransformed to mortality rates (expected mortality = 1 - survival rate)
and compared with their corresponding mixed virus treatments (= observed mortality) by
Student’s t-test. This was performed separately for the mortality observed in the first (7
dpi) (Fig. 2A) and second week (14 dpi) (Fig. 2B). At 7 dpi, observed and expected
values did not significantly differ for GV+NPV_L (t = 0.084, df = 10, p = 0.0935) and
GV+NPV_H (t = 1.115, df = 10, p = 0.291) treatments (Fig. 2A). At 14 dpi, no significant
differences were found for GV+NPV_L (t = 0.941, df = 10, p = 0.369) and GV+NPV_H (t =
0.244, df = 10, p = 0.812) (Fig. 2B). This indicated that AgseGV and AgseNPV-B acted
independently within A. segetum larvae.

Ratio of GV and NPV infected larvae
Though no effect of co-infections was observed in terms of mortality, a possible influence on virus progeny production (= OB production) was tested. Larvae exposed to AgseGV only produced solely AgseGV proving the purity of the AgseGV stock (Fig. 1A and B).

In NPVL, where only four larvae of the first week were subjected to qPCR analysis, two cadavers contained AgseNPV-B and two were virus free (Fig. 1A). A single larval cadaver contained both AgseNPV-B and AgseGV when collected from the NPVL treatment (second week) (Fig. 1B). The latter finding was unexpected as only AgseNPV-B was used as an inoculum. In NPVH only treatments the occurrence of co-infections with both AgseNPV-B and AgseGV was also confirmed. Four (14.3%) larval cadavers collected in the first week (Fig. 1A) and two (20%) larval cadavers obtained in the second week (Fig. 1B) contained both AgseNPV-B and AgseGV. The remaining larvae that were collected from NPVH and died by viral infection contained AgseNPV-B only. Larvae with only AgseGV were not observed in NPVL and NPVH treatments (Fig. 1A and B).

Co-infected larvae were found in both GV+NPVL and GV+NPVH mixed virus treatments. The ratio of co-infected larvae in GV+NPVL reached 18.8% and 14.3% within the first (Fig. 1A) and second week (Fig. 1B), respectively. By this, the ratio of co-infections was similar to those of NPVH treatment. In the GV+NPVH treatment the ratio of co-infected larvae reached 52.9% for larvae that died within the first week (Fig. 1A). Cadavers that were not scored as co-infected were found to contain only AgseNPV-B (35.3%) or AgseGV (5.9%) (Fig. 1A). Within the second week of the GV+NPVH treatment, a mixed infection occurred in 76.2% of dead larvae, whereas 23.8% of the dead larvae were...
infected by AgseNPV-B only (Fig. 1B). According to the qPCR based ratios, all larvae of the GV and GV+NPVt treatments that died by viral infection were infected by AgseGV, in single as well as co-infections (Fig. 1A and B). The contrary was observed for GV+NPVh treatment where most larval cadavers contained AgseNPV-B only (Fig. 1A and B).

Larval occlusion body production

The larval OB production in single and co-infections per treatment was separately analyzed for AgseGV and AgseNPV-B. Since the logarithmically transformed amounts of larval OB production in single and co-infections were not normally distributed (data not shown), non-parametric Kruskal-Wallis analysis was conducted. Significant differences in single and co-infected larvae were detected for AgseGV ($H(5) = 35.80, p < 0.001$) and AgseNPV-B ($H(5) = 31.35, p < 0.001$).

In AgseNPV-B infected larvae of NPVh and GV+NPVh, a temporal change in the median OB generation was observed from the first to the second week (Fig. 3A). Dead larvae of the first week showed no significant difference in progeny OB production ($W = 94, p = 0.896$) with $2.3 \times 10^6$ OBs/larva (NPVh, single infected), $5.44 \times 10^7$ OBs/larva (GV+NPVh, single infected) and $1.6 \times 10^6$ OBs/larva (GV+NPVh, co-infected). At a higher level, the same observation was made for OB production in the second week ($W = 17, p = 1.000$). Here, similar amounts of $5.44 \times 10^7$ OBs/larva (NPVh, single infected), $89.7 \times 10^6$ OBs/larva (GV+NPVh, single infected) and $1.09 \times 10^7$ OBs/larva (GV+NPVh, co-infected) were measured. The temporal increase in progeny OB production was...
significant for single infected larvae of NPV<sub>H</sub> and GV+NPV<sub>H</sub> but not co-infected larvae of GV+NPV<sub>H</sub>. A different situation in larval OB production was measured for AgseGV. Here, a temporal change in the amount of produced OBs from the first to the second week was absent (Fig. 3B). Single infected larvae contained $2.89 \times 10^9$ OBs/larva (GV, first week), $1.47 \times 10^9$ OBs/larva (GV, second week), $4.66 \times 10^8$ OBs/larva (GV+NPV<sub>L</sub>, first week), and $1.64 \times 10^9$ OBs/larva (GV+NPV<sub>L</sub>, second week). Only co-infected GV+NPV<sub>H</sub> larvae produced significantly fewer amounts of OBs with $2.36 \times 10^8$ OBs/larva (first week) and $1.61 \times 10^8$ OBs/larva (second week). In pairwise comparison, the produced amounts of AgseGV OBs in co-infected GV+NPV<sub>L</sub> larvae were significantly lower than in single infections of the GV+NPV<sub>L</sub> (second week) and GV treatment (Fig. 3B) ($p < 0.05$).

**Ratio of infection on the individual level**

As AgseNPV-B infection appeared to cause an adverse effect on the AgseGV production in co-infected larvae (Fig. 3B), we tested whether this effect was also seen in individual larvae. For this reason, the individual production of AgseNPV-B and AgseGV OBs was correlated in co-infections (Fig. 4A and B). When applying Spearman’s rank correlation, the coefficient ($r_s$) was negative ($r_s < 0$) but no significant correlation was observed in OB production for larvae that died within the first ($r_s(18) = -0.329; p = 0.182$) and second week ($r_s(16) = -0.244; p = 0.361$).
Discussion

Personal observations in our laboratory and published results on simultaneous infections of *A. segetum* larvae by AgseGV and AgseNPV (17, Gürlich and Huber, personal communication) provided little information about potential interactions of both viruses. In the present study, not only mortality data were evaluated from single and mixed virus treatments but also qPCR analyses were performed to identify and to quantify the production of AgseGV and AgseNPV-B OB progeny in infected larvae on an individual level.

The mortality rates of AgseNPV-B bioassay experiments were consistent with previously published bioassay studies obtained in 10-day bioassays (25). With a LC$_{50}$ (10 dpi) of 10 $\times$ 10$^3$ OBs/ml, the result lay between the newly determined LC$_{50}$ (7 and 14 dpi). The AgseGV isolate used in the present study showed the same slow activity as previously described (13, 26).

The present study focused primarily on co-infections with defined concentrations of AgseGV and AgseNPV-B OBs. The observed mortality data indicate that the virus, which led to the highest mortality in its corresponding single virus treatment, also dominated the overall mortality in an AgseGV and AgseNPV-B co-infection. Neither a significant increase nor a decrease in mortality was observed in mixed virus treatments that could give hints for a co-operative or inhibiting effect of both viruses. The assumption of an independent virus infection was underlined by the comparison of the observed and expected mortality of mixed virus treatments. The qPCR based identification and quantitation of produced viruses of single and co-infected larvae revealed that larvae mainly died from the virus that was applied in the higher lethal
concentration (LC). When the 14-day LC50 of AgseGV was mixed with the high concentration of AgseNPV-B (7-day LC50), as done in the GV+NPV_H treatment, the ratio of co-infected larvae was at maximum. Furthermore, it could be concluded that with an increasing concentration of AgseNPV-B (GV > GV+NPV_L > GV+NPV_H) the ratio of co-infections increased. A GV induced enhancement of the AgseNPV-B infection, as it was described for PsunGV and PsunNPV (7), could not be concluded.

The observation that NPV_L and NPV_H alone resulted in AgseGV infected larvae indicated that either the AgseNPV-B inoculum contained AgseGV or *A. segetum* larvae harbored a latent AgseGV infection. The latter could be excluded since no larvae of the control showed any symptoms or died by viral infection. Therefore, a slight contamination of the AgseNPV-B stock was likely. The amounts of AgseGV and AgseNPV-B OBs per larva were regarded as realistic of what an *A. segetum* larva could be capable to produce. With a range from 4.66 x 10^8 to 2.89 x 10^9 AgseGV OBs per single infected larva the median amounts were within the expected range. In a study on different *Cydia pomonella* GV (CpGV) mutants replicating in *Cydia pomonella*, virus offspring production was calculated to vary between 2.0 to 3.6 x 10^10 OBs/larva (27). A comparison with the present results is difficult because fifth instar *C. pomonella* larvae, thus larger caterpillars were infected with CpGV (28). For AgseNPV-B, the median OB offspring production matched that of PsunNPV (18). There, fifth instar larvae of the armyworm, *P. unipuncta* were infected with PsunNPV produced about 10^4 - 10^7 OB/larva and was in a similar range (2.1 x 10^6 to 8.97 x 10^7 OBs/larva) as observed in our study for *A. segetum* larvae infected with AgseNPV-B. Our results on the production of AgseGV and AgseNPV-B OB progeny suggest the potential capacity OB production,
which neonate *A. segetum* larvae are able to produce during the infection period. It is not known, if later larval stages were able to produce higher amounts of OBs when exposed to other virus concentrations. However, the co-infection experiments clearly indicated that both viruses should be propagated separately and contaminations should be avoided, since mixtures led to a decrease in yields of OBs.

In our case, a prolonged larval life induced by viral infection was not observed as described for HearGV infected *H. armigera* larvae (29). Due to the extended survival time, larvae were doubled their size and mass by a longer feeding time, providing the virus more host resources for replication. For AgseGV and AgseNPV-B, this assumption could be excluded because of similar mortality in single and mixed virus treatments. The baculovirus encoded *ecdysteroid glucosyltransferase* (*egt*) gene that was shown to play a major role in extending larval life (30) is encoded by AgseGV (31), AgseNPV-B (9), AgseNPV-A (32) and AgipNPV (33). If deleted in naturally occurring Δ*egt* genotypes of AgipNPV infected *A. ipsilon* larvae died significantly faster (34).

Infected larvae have an upper limited in OB production, set by the availability of host resources that can be utilized for production of progeny OBs. In case of co-infected larvae, this upper limit could either be shifted in favor of AgseGV or AgseNPV-B, but both viruses could also replicate equally. AgseNPV-B exhibited a steady and significant increase in median OB formation in single infected larvae between the first and second week, whereas AgseGV showed no such temporal increase. This observation may rely on tissue tropism of AgseGV. An early infection of the complete tissue or organ might prevent an increase in OB formation. On the contrary, AgseNPV-B may spread within
the whole larval body resulting in a temporal increase of progeny OBs. It is not known, if tissue tropism occurs for AgseGV.

In co-infections, however, AgseNPV-B production appeared to be less influenced by the presence of AgseGV than *vice versa*. This was clearly observed by a significantly lower median OB production of AgseGV in co-infections than in single infections.

Furthermore, it co-infected larvae contained less AgseGV and AgseNPV-B than larvae infected by one of these viruses alone. However, a significant negative correlation on AgseNPV-B and AgseGV OB production was not observed. A possible explanation for a missing correlation but a negative mutual interference could lay in the nature of the less pathogenic AgseGV and the more virulent AgseNPV-B to *A. segetum* larvae, and in independent infection processes that start in different cells for both viruses. In this case, AgseNPV-B could replicate normally within co-infected larvae within the first week, but interfere with the less virulent AgseGV in the later states.

Cells, infected by one baculovirus, lose their susceptibility to a secondary infection, has been demonstrated for two genotype variants of AcMNPV, as well as for AcMNPV and SfMNPV infections (35). So called superinfections of a single cell were shown to be temporarily possible shortly after the first viral infection (35). Whether AgseGV and AgseNPV-B are able to superinfect *A. segetum* cells is unknown, but a reciprocal exclusion can be an explanation for reduced progeny generation.

In conclusion, an synergistic effect of AgseGV and AgseNPV-B within co-infected larvae was not observed. But, the results suggest a certain competition of AgseGV and AgseNPV-B for larval resources.
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Table 1. Lethal concentration (LC50) for AgseNPV-B, AgseGV and AgipNPV determined for neonate A. segetum larvae. Mortality rates were scored at 7 and 14 days post infection (dpi) (n = total number of individuals per bioassay; CL = confidence limits calculated by normal approximation; df = degrees of freedom; nd = not defined due to low mortality rates of AgseGV within the first week).

<table>
<thead>
<tr>
<th>Virus</th>
<th>n</th>
<th>LC10 (95% CL) [x10³ OBs/ml]</th>
<th>LC50 (95% CL) [x10³ OBs/ml]</th>
<th>Slope*</th>
<th>χ²</th>
<th>df</th>
<th>LC10 (95% CL) [x10³ OBs/ml]</th>
<th>LC50 (95% CL) [x10³ OBs/ml]</th>
<th>Slope*</th>
<th>χ²</th>
<th>df</th>
</tr>
</thead>
<tbody>
<tr>
<td>AgseGV</td>
<td>3.054</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.30 a</td>
<td>111.7</td>
<td>5</td>
<td>2.63</td>
<td>(0.05 – 128.0)</td>
<td>0.51 a</td>
<td>212.5</td>
<td>5</td>
</tr>
<tr>
<td>AgseNPV-B</td>
<td>1.256</td>
<td>0.77</td>
<td>37.0</td>
<td>0.76 b</td>
<td>18.39</td>
<td>5</td>
<td>0.34</td>
<td>(0.22 – 0.52)</td>
<td>1.30 b</td>
<td>9.56</td>
<td>5</td>
</tr>
</tbody>
</table>

*Different letters indicate no parallelism of probit lines at either day 7 or 14. The criteria of parallelism was rejected when p < 0.05.
Figure Legends

Figure 1: Mean mortality rates of (A) first week (day 2-7) and (B) second week (day 8-14) of single (GV, NPV_L, and NPV_H) and mixed (GV+NPV_L and GV+NPV_H) virus treatments. Different letters above columns indicate significant differences between treatments (One-way analysis of variance (ANOVA) followed by Tukey HSD test for pairwise comparisons between treatments, significance at p < 0.05). Vertical lines represent standard deviations. Pie charts above each column represent the ratio of non-infected (white), co-infected (black), AgseGV only (light grey) and AgseNPV-B only (dark grey) infected larvae of each treatment (n = number of larvae measured in qPCR).

Figure 2: Comparison of the observed and expected mortality rates of GV+NPV_L and GV+NPV_H treatments observed in the (A) first and (B) second week post infection. Expected mortality values were calculated from survival rates of replicates of GV, NPV_L and NPV_H treatments assuming independent action (for details see Results). Statistical analyses were conducted separately for the observed and expected mortality of each treatment. Different letters indicate significant differences (student’s t-test, significance at p < 0.05). Vertical lines represent standard deviations.

Figure 3: Median OB production of (A) AgseNPV-B and (B) AgseGV within single and co-infected larvae, which succumbed during the first (2-7 days) and second (8-14 days) week post infection. Minimum of y-axes represent the lower limits of detection (LOD) for AgseGV (10^5 OBs/larva) and AgseNPV-B (10^3 OBs/larva). Different letters
indicate significant differences (Wilcox rank-sum test, significance at p < 0.05) between treatments.

Figure 4: Correlation analysis (Spearman’s rank correlation coefficient = $r_s$) of larval AgseGV and AgseNPV-B OB production within co-infected A. segetum larvae. Only larvae of the GV+NPV$_H$ treatment that died within the (A) first and (B) second week were considered. Vertical lines = median AgseGV OB production; horizontal lines = median AgseNPV-B OB production; solid lines = mixed GV+NPV$_H$ treatment; dashed lines = single GV and NPV$_H$ treatment are drawn as reference.
Figure 2

![Graph A](image)

- **GV + NPVL**
- **GV + NPVH**

![Graph B](image)

- **GV + NPVL**
- **GV + NPVH**
Figure 3

A

NPV/larva

2-7 days | 8-14 days
single infected | co-infected

NPVH

GV+NPVH

B

GV/larva

2-7 days | 8-14 days
single infected | co-infected

GV

GV+NPVL

GV+NPVH
Figure 4

A

\( r = -0.2441 \)
\( p = 0.3609 \)

B

\( r = -0.3292 \)
\( p = 0.1821 \)


