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Infection with Spodoptera litura NPV Reduces Food Consumption and Weight Gain of *Spodoptera litura* Larvae

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Authors' Contribution
GA carried out the experiments and wrote the paper. WW assisted in the experimental design and statistical analysis. JMVdesigned the study and edited the manuscript.

Key words
Spodoptera litura nucleopolyhedroviruses, Food consumption, Weight gain, *Spodoptera litura.*

ABSTRACT

Insect-pathogenic baculoviruses have established potential as biological insect pest control agents. However, slow speed of kill relative to chemicals and continued feeding following application limit their effectiveness in preventing crop losses. Here we studied the food consumption and weight gain of the cotton leafworm *Spodoptera litura* following application of two viral doses of Spodoptera litura nucleopolyhedrovirus (Pakistan isolate SpltNPV-Pak-BNG). Infected larvae with final polyhedrosis (disease resulting in dissolution of larval tissues and accumulation of viral occlusion bodies) exhibited reduced food intake and weight gain relative to uninfected larvae. The unexposed larvae and the larvae that were exposed but survived exhibited the same food consumption and weight gain. This study did thus not reveal any sub-lethal effects of exposure to the virus on food consumption and weight gain. There was no viral dose dependency observed in food intake or weight gain by infected larvae, suggesting there is no increased crop damage upon virus treatment but rather a decrease to be expected.

INTRODUCTION

The *Baculoviridae* (King *et al*., 2012) are a large family of invertebrate viruses that are found ubiquitously in insects (notably members of the orders Lepidoptera, Hymenoptera and Diptera) in natural habitats. They are attractive biological control agents for lepidopteran insect pests due to their insecticidal properties, host specificity, safety to non-target animals and lack of toxic residues in the environment (Inceoglu *et al*., 2006; Szewczyk *et al*., 2006). However, baculoviruses have limitations, which are restricting their use for insect control in practice. Research is thus required to mitigate or eliminate these limitations.

A key limitation is a relatively slow speed of kill as compared to chemical insecticides (Moscardi *et al*., 2011; Beas-Catena *et al*., 2014; Knox *et al*., 2015). It may take several days to a few weeks before infected larvae die or stop feeding (Vasconcelos *et al*., 2005). Feeding may continue until the insects eventually die (Glass, 1958; Subrahmanyam and Ramakrishnan, 1981). Crop losses following the application of baculoviruses may therefore still be substantial (Bonning and Hammock, 1994; Bianchi *et al*., 2000a). To eliminate this drawback, fast-acting baculoviruses can be selected for or can be genetically modified to improve the speed of kill and reduce crop losses (Inceoglu *et al*., 2006; Szewczyk *et al*., 2006; Sun *et al*., 2009; Cai *et al*., 2010; Georgievska *et al*., 2010). However, these recombinant viruses have reduced within-host fitness compared to their parent wild-type virus (Cory, 2000; Zwart *et al*., 2009; Georgievska *et al*., 2010). It is also difficult to produce recombinant viruses *in vivo* because the yield per cadaver is lower than for wild-type viruses (Sun *et al*., 2005). In addition, such recombinant viruses, being GMO, meets public resistance and is not compatible with organic production methods. So, selection for fast-acting baculovirus strains is an attractive option.

Feeding behaviour of infected larvae determines the extent to which baculoviruses can protect crops against feeding damage (Hoover *et al*., 1995). Baculoviruses delay the moult of larval hosts (reviewed by Clem and Passarelli, 2013). Normally, insect larvae cease feeding during larval-larval and larval-pupal moults during their life span (O'Reilly and Miller, 1989). However, infection with baculoviruses interferes with this feeding arrest, resulting in a prolonged feeding period after viral infection and increased weight gain by infected larvae to maximise virus yield (O'Reilly and Miller, 1991). For instance, Subrahmanyam and Ramakrishnan (1981) demonstrated

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that *Spodoptera litura* larvae that are exposed to and infected with Spodoptera litura nucleopolyhedrovirus (SpltNPV) continue feeding until death and consumed more food than the unexposed larvae. On the other hand, Vasconcelos *et al*. (2005) reported that baculovirus infected *Mamestra brassicae* larvae caused significantly less defoliation in cabbage plants than uninfected larvae.

There are thus contradictory observations in previous studies regarding food consumption and crop damage by baculovirus infected larvae as compared to healthy larvae. A detailed understanding of food consumption following infection by baculoviruses is needed to design effective use strategies for baculoviruses against insect pests. Therefore, a case-by-case approach is required to study this aspect, in the current case the leafworm *S. litura* and its baculovirus SpltNPV.

This study addresses three questions: (i) are food intake and weight gain of larvae reduced or not when they are infected with SpltNPV? (ii) if there is a reduction in food intake and weight gain, when does it set in following exposure to the baculovirus? (iii) is there any difference in food intake and weight gain between unexposed larvae and those that exposed to the virus but survived infection, *i.e.* are there sublethal effects on food intake and weight gain in those larvae that do not get lethally infected but have been exposed? These issues need to be addressed in order to design an optimal spraying regime to achieve minimal crop damage.

MATERIALS AND METHODS

Insect rearing and the SpltNPV-Pak-BNG isolate

A *S. litura* colony was maintained in the insectary of the National Agricultural Research Centre (NARC), Islamabad, Pakistan. Insects were reared at 26±2°C, 50-60% relative humidity and 14h:10h day-night photoperiod on a semi-synthetic diet as described in Ali *et al*. (2018b). We used newly moulted 4th instar larvae of *S. litura* to evaluate the feeding and weight gain response by the infected and healthy larvae.

The SpltNPV-Pak-BNG isolate used in this experiment was collected from cotton in the Bahawalnagar district of Punjab, Pakistan, and was amplified *in vivo* in 3rd instar larvae of *S. litura*. Occlusion bodies (OBs) were purified from dead larvae according to Ali *et al*. (2018b). The viral concentrations were prepared by counting the OBs in the virus suspension using a haemocytometer (Neubauer) using phase contrast light microscopy. The SpltNPV-Pak-BNG isolate has a median lethal dose (LD_{50}) of 1.88x10^4 OBs / larva and a median survival time (ST_{50}) of 108 h post infection (hpi) in 4th instar larvae of *S litura* (Ali *et al*., 2018b).

Bioassay

Two viral stock concentrations, 3×10^6 and 3×10^7 OBs/ml, respectively, were prepared for conducting leaf disc bioassays to monitor feeding reduction and/or weight gain. In each assay, 45 newly moulted 4th instar larvae were individually placed in 24-well tissue culture plates (bottom diameter of 15.5 mm) to avoid cannibalism, and starved for 15 h. The larvae were then fed using leaf discs inoculated with OBs. Fresh tender leaves of young *Ricinus communis* plants were cut into 3 mm^2 pieces and placed on a 1% plant agar-solidified solution in the 24-well tissue culture plates. The plant agar kept the tender leaves moist and prevented them from drying out. A 3.33 µl volume of viral suspension was placed on each leaf disc and allowed to air-dry. Fifteen leaves were inoculated with the 3×10^6 OBs/ml solution, while fifteen other leaves were inoculated with the 3×10^7 OBs/ml solution. The 15 remaining leaves were mock inoculated with distilled water. The resulting viral doses (dose=D) on the leaf disks were D1=10^4, D2=10^5, and D0=0 OBs, respectively. The overnight-starved larvae were exposed to the leaf discs for 24 h and kept at 26±2°C overnight in a climate chamber. Ten inoculated larvae that had eaten the whole leaf disc (randomly chosen) were weighed and transferred to 6-well tissue culture plates (bottom diameter of 35 mm) having a weighed amount of artificial diet plugs. The 6-wells plates were then covered with parafilm, tissue paper and by the original lid to prevent larval escape. Larvae were incubated at 26±2 °C and a 14h:10h day-night photoperiod in a climate chamber.

Food consumed and weight gain by individual larvae were determined every 24 h during the first three days and subsequently every 12 h until death or pupation using a PW 214 Analytical Balance (USA) with a readability range of 0.1 mg to 210 g. The remaining diet plugs were weighed to assess the food consumed by the larvae. The larvae were also weighed to determine increase/loss in body weight. The remaining diet plugs and excrements were removed and replaced with fresh, weighed plugs of artificial diet. This process was continued for each larva until death or pupation. The experiment was conducted in three replicates.

Statistical analysis

Initially, food consumption and weight gain of individual replicates was analyzed for five groups of larvae: exposed infected larvae, exposed but not infected (exposed surviving) larvae for each of the two dose treatments and the unexposed larvae. No significant differences were detected in food consumption and weight gain among the replicates. The data from three replicates therefore were combined to determine food consumption and weight

gain for all the three treatments, through comparing the mean and by using a univariate generalized linear model in SPSS (IBM Corp, 2012). Pairwise comparisons were conducted using t-tests in R to find differences between the treatments means at $P \leq 0.05$.

RESULTS

Fourth instar larvae of *S. litura* were infected with SpltNPV in a leaf disk bioassay. Two doses were applied with 10^4 and 10^5 OBs per larva, respectively (Table I). Mortality increased with viral dose. No mortality was observed in mock-infected larvae (unexposed). The number of larvae dying at the lower dose of 10^4 OBs per larva was 4, 3 and 3 in replicates 1, 2 and 3, respectively. The number of larvae dying at the higher dose of 10^5 OBs per larva was 7, 7 and 10 in replicates 1, 2 and 3, respectively (Table I). The overall mortality was 33% and 80%, respectively, for the lower and higher dose.

Food consumption

Exposed larvae that became infected and eventually died ate 59% (dose: $1x10^4$ OBs/larva) or 58% (dose: $1x10^5$ OBs/larva) less for than exposed insects that did not die following OB exposure. There was thus no viral dose dependency in food intake by exposed infected larvae, but the difference between exposed infected and unexposed (control) larvae was highly significant ($P \leq 0.05$; Table II).

Table I.- Mortality of 4th instar larvae of *S. litura* challenged with different viral doses (OBs/larva).

Replicates	Control	Low dose (10^4 OBs/larva)		High dose (10^5 OBs/larva)	
	Alive	Dead	Alive	Dead	Alive
Replicate 1	10	4	6	7	3
Replicate 2	10	3	7	7	3
Replicate 3	10	3	7	10	0
Total	30	10	20	24	6

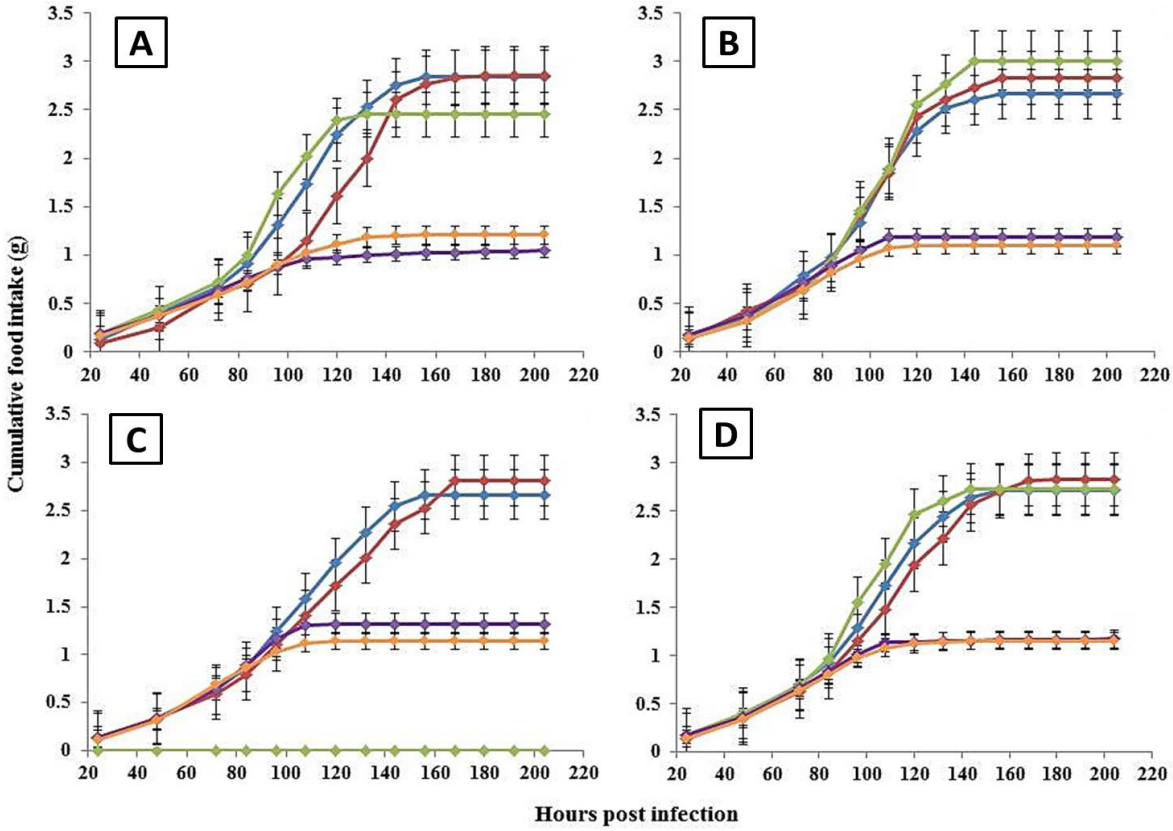

Fig. 1. Effect of different viral doses (D0=0, D1=10^4 and D2=10^5 OBs/larva) on cumulative food consumption of unexposed and exposed infected larvae of *S. litura* over time. D0-unexposed (control) presented in blue colour, D1-exposed surviving in red colour, D1-exposed infected in purple colour, D2-exposed surviving in green colour and D2-exposed infected in orange colour. The experiment was completed in three replicates. **A**, replicate 1; **B**, replicate 2, **C**, replicate 3; **D**, replicates combined. The data were pooled in panel d to show the combined effect of replicates. Error bars represent standard error of the mean.

There was no significant difference between the virus doses. In addition, there was also no significant difference in food intake between larvae of the unexposed group and exposed surviving of OBs exposure. Differences in cumulative food consumption between exposed infected larvae and those that are either unexposed or that exposed surviving emerge between 72 and 96 hpi (Fig. 1). There was no viral dose-food intake dependency among the exposed infected larvae. The feeding of exposed infected larvae stopped completely at ~120 hpi, whereas feeding of noninfected or exposed surviving larvae stopped at ~140 hpi, prior to moulting.

Table II.- Food consumption by 4th instar larvae of *S. litura* till pupation or death upon challenge with different viral doses.

Replicates	Mean food consumed (g±SE)				
	Control	Low dose (10^4 OBs/larva)		High dose (10^5 OBs/larva)	
		Dead	Alive	Dead	Alive
Replicate 1	2.84 ± 0.16^a (n = 10)	1.04 ± 0.26^b (n = 4)	2.85 ± 0.2^a (n = 6)	1.20 ± 0.19^b (n = 7)	2.45 ± 0.30^a (n = 3)
Replicate 2	2.66 ± 0.27^a (n = 10)	1.18 ± 0.49^b (n = 3)	2.82 ± 0.32^a (n = 7)	1.09 ± 0.32^b (n = 7)	3.01 ± 0.49^a (n = 3)
Replicate 3	2.66 ± 0.22^a (n = 10)	1.32 ± 0.39^b (n = 3)	2.80 ± 0.26^a (n = 7)	1.14 ± 0.22^b (n = 10)	00±00 (n = 0)
Pooled data	2.83 ± 0.12^a (n = 30)	1.17 ± 0.21^b (n = 10)	2.83 ± 0.15^a (n = 20)	1.15 ± 0.14^b (n = 24)	2.73 ± 0.27^a (n = 6)

S. litura larvae were exposed to different viral doses through leaf disc bioassay (T0=0, T1=10^4 and T2=10^5 OBs/larva). The control was fed with distilled water on the leaf disc. All larvae were given a pre-weighed piece of food. The mean food consumed by infected and healthy larvae is presented. SE represents the standard error of the mean. The food consumed with the same letter is not significantly different at $P \leq 0.05$. Here *n* is number of larvae.

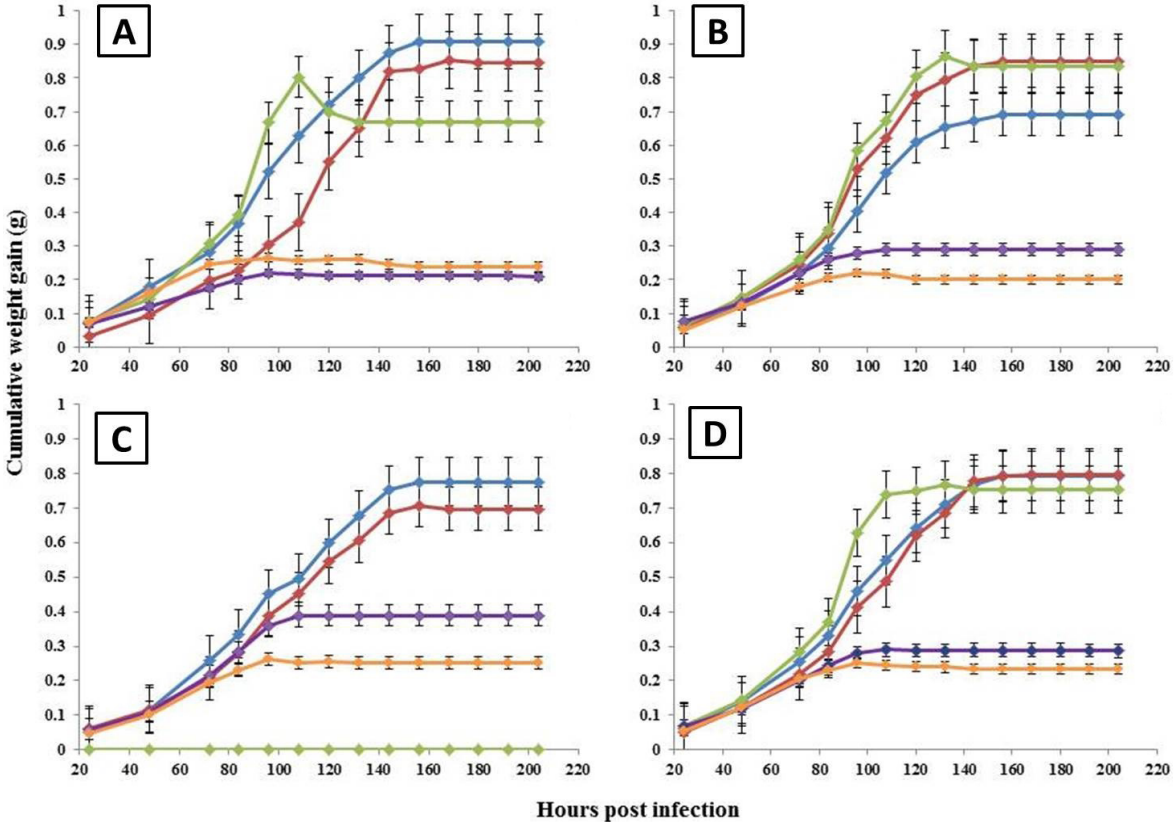

Fig. 2. Effect of different viral doses (D0=0, D1=10^4 and D2=10^5 OBs/ml/larva) on cumulative weight gain in unexposed and exposed infected larvae of *S. litura* over time. The control (D0-unexposed) is presented in blue colour, D1-exposed surviving in red colour, D1-exposed infected in purple colour, D2-exposed surviving in green colour and D2-exposed infected in orange colour. The experiment was completed in three replicates. **A**, replicate 1; **B**, replicate 2; **C,** replicate 3; **D**, replicates combined. The data were pooled to show the combined effect of replicates in panel d. Error bars indicate the standard error of the mean.

Table III.- Weight gain by 4th instar larvae of *S. litura* challenged with different viral doses.

Replicates	Mean weight gain (g±SE)				
	Control	Low dose (10^4 OBs/larva)		High dose (10^5 OBs/larva)	
		Dead	Alive	Dead	Alive
Replicate 1	0.91±0.06^a (n = 10)	0.21±0.09^b (n = 4)	0.85±0.08^a (n = 6)	0.24±0.07^b (n = 7)	0.67±0.11^a (n = 3)
Replicate 2	0.69±0.07^a (n = 10)	0.29±0.13^b (n = 3)	0.85±0.08^a (n = 7)	0.20±0.08^b (n = 7)	0.84±0.13^a (n = 3)
Replicate 3	0.78±0.06^a (n = 10	0.39±0.10^b (n = 3)	0.70±0.07^a (n = 7)	0.25±0.06^b (n = 10)	00±00 (n= 0)
Pooled data	0.79+0.04^a (n = 30)	0.29+0.06^b (n = 10)	0.80+0.04^a (n = 20)	0.23+0.04^b (n = 24)	0.75+0.08^a (n = 6)

S. litura larvae were exposed to different viral doses through a leaf disc bioassay (T0=0, T1=10^4 and T2=10^5 OBs/larva). The control was fed with distilled water on leaf disc. The mean weight gain by infected and healthy larvae is presented. SE represents the standard error of the mean. Weight gains with the same letter are not significantly different at $P \leq 0.05$.

Weight gain

The difference in weight gain between the virus exposed infected larvae that died and those that exposed surviving was highly significant ($P \leq 0.05$; Table III). There was no viral dose dependency in weight gain by the exposed infected larvae. There was also no significant difference in weight gain by larvae of the unexposed and exposed surviving that were exposed but did not get overt infection (polyhedrosis). Differences in cumulative weight gain between exposed infected larvae and those that are either unexposed or that exposed surviving emerged between 72 and 96 hpi (Fig. 2).

DISCUSSION

The most important parameter of successful control using insecticides is speed of action and the time at which exposed insects stop feeding. In the case of SpltNPV-Pak-BNG and *S. litura*, there was a highly significant difference in food intake and weight gain between unexposed and exposed infected larvae. Viral infection impaired food intake and reduced weight gain. There was no dose dependency in the effects of baculovirus infection on food consumption or weight gain and there was no difference in food consumption and weight gain between unexposed and exposed infected larvae during the first 48-72 h after OB ingestion. A significant difference between the exposed infected and unexposed larvae in food consumption and weight became apparent between 72 and 96 hpi. Exposed infected larvae consumed less food than the exposed larvae that did not become infected (exposed surviving). We found no significant difference in food intake between unexposed and exposed larvae surviving infection. It is possible that in the latter a latent or persistent infection was established (viral presence), but then this did not affect the feeding behaviour of these exposed but surviving larvae. Such infection, if present, can contribute to long-term management of pest populations, as latent or persistent infections can turn into overt infections (polyhedrosis) through biological and environmental stress factors (Milks *et al*., 1998; Myers *et al*., 2000; Duan and Otvos, 2001).

The timing of divergence in food consumption and weight gain between exposed surviving and exposed infected larvae in our study agree with the data of Vasconcelos *et al*. (2005) for *M. brassicae*. One to two days after exposure is the time when the larval host tries to overcome the viral infections by shedding infected midgut epithelial cells (Keddie *et al*., 1989). The infected host deploys its resources in this elimination process that would otherwise be used for larval host growth and this may reduce food consumption (Keddie *et al*., 1989; Cory *et al*., 1997; Cory and Meyers, 2003).

Earlier studies demonstrated that a limitation of naturally occurring wild-type baculovirus is their low speed of kill, which allows the insect pests to significantly damage the crops before they are controlled (Bonning and Hammock, 1994; Bianchi *et al*., 2000a). This is due to expression of the *egt* gene in baculovirus, which blocks moulting and interferes with normal feeding arrest during larval-larval and larval-pupal moulting (O'Reilly, 1995). This interference results in prolonged feeding and hence greater food consumption in infected larvae within the instar (O'Reilly and Miller, 1991). Subrahmanyam and Ramakrishnan (1981) reported that SpltNPV-infected *S. litura* larvae consumed 66% more food than healthy larvae. It could be that the SpltNPV-Pak-BNG isolate we used resulted in a more reduced survival time (Ali *et al*., 2018b) and hence reduced food intake. Differences in survival time have been noted among SpltNPV-Pak isolates (Ali *et al*., 2018a), but survival times could not be devised from Subrahmanyam and Ramakrishnan (1981) and hence not compared with the data presented here.

Paradoxically, our results reveal that, in spite of the relatively low speed of kill, the food intake and weight gain by viral infected larvae is quite low compared to healthy larvae, in agreement with the findings of Vasconcelos *et al*. (2005). The results demonstrate that the viral applications induce impaired feeding in infected larvae and can

significantly lower the food consumption and weight gain in infected compared to healthy larvae, and thereby reduce crop damage. In a previous study (Ali *et al*., 2018a) four isolates of SpltNPV-Pak were found that were faster than SpltNPV-Pak-BNG, while having the same median lethal dose. It would be of interest now to determine whether these faster acting SpltNPVs further reduce the food uptake and hence further limit crop damage.

In conclusion this paper shows reduced food consumption and weight gain of baculovirus infected *S. litura* larvae, provided the larvae are infected leading to mortality (high enough dose). A field experiment should reveal whether this all results in reduced crop damage and to what extent.

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Statement of conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this article.

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