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Fatal attraction

– Do virus-infected cadavers of *Spodoptera littoralis* larvae attract healthy conspecifics?

Dödlig attraktion

– Lockas friska bomullsflylarver till virusinfekterade larvkadaver?

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Abstract

Baculoviruses manipulate insect larval behaviour by inducing increased activity and by enhancing the movement to the top of the host plant. The *Spodoptera littoralis* nucleopolyhedrovirus (SpliNPV) may have a large impact on larval populations and is currently used as a control method. Dispersal of NPVs is fairly limited since foliage or infected larvae need to be ingested for transmission. *S. littoralis* is cannibalistic under high population densities or low food availability conditions. We hypothesized that healthy larvae recognize and are attracted to virus-infected cadavers. Dual choice experiments in petri dish arenas, involving infected and uninfected larval cadaver suspensions revealed no significant differences in neonate larval attraction. In contrast, fourth instar larvae fed significantly more on infected larval cadavers than on cadavers of uninfected larvae. Although mortality of larvae that fed on infected cadavers was increased, high mortality in both groups suggests vertical transmission of the virus in this species. Our results show that that virus-killed cadavers increase attraction and feeding by healthy larvae. Consumption of conspecific cadavers increases viral dissemination. Our results suggest that larvae killed by NPV infection emit chemical cues, which make them more attractive to conspecific larvae, thereby promoting in viral transmission.

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Introduction

The Baculoviridae are one of the major families of insect pathogenic viruses and are the most commonly found and well studied (Inceoglu et al., 2006). The family Baculoviridae has been isolated mainly from insects, including Lepidoptera, Hymenoptera, Diptera, Coleoptera, Neuroptera, Trichoptera, Thysanura, but has also been described in Crustaceae (Possee et al., 1997; Moscardi, 1999; King et al., 2000). Due to their congenital host killing activity, naturally occurring baculoviruses have been developed as safe and effective tools for the protection of crop and forest in Europe, America and Asia (Moscardi, 1999, Lacey et al., 2001, Inceoglu et al., 2006).

Baculoviruses are viruses with circular double-stranded DNA genomes and consists of two types: nucleopolyhedroviruses (NPVs) and granuloviruses (GVs). The viruses mainly enter and infect the host after ingesting food contaminated with viral occlusion bodies (OBs). The OBs dissolve in the alkaline midgut lumen, allowing occlusion-derived viruses (ODVs) to pass through the insect peritrophic membrane and infect the gut cells before transmitting to other tissues, leading to insect death and liquefaction of tissues (Ikeda et al., 2015).

Both types of baculoviruses have been applied for use as biopesticides (Ikeda et al., 2015). NPVs are being produced and successfully applied for the protection of soybean in Brazil (Moscardi, 1999) and many crops in China (Yang et al., 2012). However, baculoviruses present some limitations such as long killing times, narrow host specificity, short field stability, and high cost of production (Inceoglu et al., 2006).

The Egyptian cotton leafworm, *Spodoptera littoralis* (Lepidoptera: Noctuidae) is a destructive pest that is distributed throughout the Middle East, Africa and in Mediterranean Europe (CABI & EPPO, 2011). Larvae can cause damage to many economically important plants such as cotton, flax, groundnuts, jute, lucerne, maize, rice, soybeans, tea, tobacco, and many vegetables (CABI & EPPO, 2011). Economical losses associated with *S. littoralis* damage can indirectly result from larvae feeding on foliage. Cotton foliage feeding by *S. littoralis* larvae can be conducive to reductions in yield greater than 50% (Russell et al., 1993).

Chemical control of *S.littoralis* faces difficulty such as the development of resistance towards chemical insecticides, inimical effects on the natural enemies, and other non-target insects, and serious toxic effects on humans (Hegazi and Abd-Allah, 2004; Ghoneim et al., 2015). One of the most promising insect biocontrol agents are baculoviruses. A number of baculoviruses with improved insecticidal activities have been developed against lepidopteran pests. Since baculoviruses are species or genus specific, viral insecticides allow controlling pest insects without damaging beneficial insects or the ecosystem (Moscardi, 1999; Fuxa, 2004). *Spodoptera littoralis* nucleopolyhedroviruses (SpliNPVs) are a member of the genus *Alphabaculovirus*, part of the Baculoviridae family, which may have single or multiple nucleocapsids per envelope (Rohrmann, 2014). There has been considerable research on the use of SpliNPV as a microbial insecticide (Toprak et al., 2007; Hatem et al., 2011). Commercial formulations of the virus have been developed as Spodopterin® (Natural Plant Protection S.A., Nogueres, FR) (Moscardi, 1999). However, SpliNPV is not effective enough as a stand-alone product. The use of SpliNPV and other NPVs in the field has shown that low persistence on leaf surfaces is a limiting factor since ultraviolet radiation inactivates the viral particles (Fuxa, 2004). Additionally, larvae need to ingest viral particles in early larval stages in order to avoid economical damage in crops. As such, baculovirus dispersal on a spatio-temporal scale is one of the main considerations when optimizing their use as biological control agents.

Insect-assisted spread of pathogens is a key component of horizontal transmission of viruses between generations. Insect pathogenic viruses and insect hosts are considered to have coevolved over millions of years (Thézé, 2011). The modes of transmission within insect populations are not well understood, but may provide insight in the development of insect management strategies.

The main route of baculovirus transmission is caterpillar feeding on contaminated foliage. Baculoviruses manipulate host larvae behaviour by inducing increased activity and by enhancing their movement to the top of the host plants. Dangling cadavers, leaking viral OBs from the top of plants accelerate the rain-driven dissemination onto the lower plants parts (Rebolledo et al., 2015; van Houte et al., 2014). Alternately, cannibalism may also play a role in horizontal transfer of viral OBs from larvae to larvae.

Cannibalistic behaviour is advantageous from an evolutionary perspective. Cannibalistic species may have increased survival, growth rate, fecundity or a reduction of conspecific antagonists (Fox, 1975; Polis, 1981; Meffe and Crump, 1987; Church and Sherratt, 1996). However, Chapman et al (1999) have proposed three disadvantages to cannibalism. First, larvae attacked by conspecifics may defend themselves causing injury or death to its attacker (Fox, 1975; Polis, 1981). Second, cannibalism reduces inclusive fitness (Polis, 1981), and lastly, cannibalism may increase virus transmission by consuming infected conspecifics (Chapman et al., 2000; Williams and Hernandez, 2006). Cannibalism in *S.littoralis* is observed in laboratory populations during later larval instars, or in cases of limited food availability or at high population densities (Reeson et al., 2000; Richardson et al., 2010).

Behaviour involved in an uninfected host's avoidance or attraction to infected conspecifics has yet to be thoroughly studied (Hawley et al., 2011). Both avoidance and attraction behaviours alter the risk of infection by pathogens (Eakin et al., 2015). Avoidance behaviour to virus-infected conspecifics has only been demonstrated in the gypsy moth (*Lymantria dispar* L.) (Capinera et al.; 1976; Parker et al., 2010). In contrast, our previous work has shown that larval suspensions of larvae infected with SpliNPV OBs are attractive to first instar *S. littoralis* larvae (unpublished data). This suggests that *S. littoralis* larva may be able to distinguish and navigate toward conspecific cadavers or fluids, potentially, for use as a protein source.

Spodoptera littoralis larvae emit specific odours profiles that may have an ecological function. Larval odour profiles may vary between infected and uninfected larvae, mediated by OB production and tissue liquefaction. Changes in odour profiles could be of importance in altering larval behaviour. We hypothesised that infected larval cadavers may become more attractive to uninfected individuals as a route to enhance viral transmission. Alternately, uninfected larvae could avoid infected cadavers as an adaptive measure to avoid coming into contact with pathogens. We here show that healthy larvae *S. littoralis* differentiate and navigate towards virus-infected conspecific corpses. Larval orientating and feeding behaviour affect larval mortality caused by consuming SpliNPV via conspecific necrophagy.

Materials and Methods

Insects

S.littoralis were reared on artificial diet (Hinks and Byers, 1976) at 25°C ± 2°C, 65% ± 2% RH with a 18:6 LD cycle at SLU Alnarp. Adult moths of both sexes were kept in plastic jars and provided with a 10% honey solution on a cotton ball and allowed to mate. Mated females oviposit on waxed-paper. Waxed-paper oviposition sheets were placed in acrylic boxes with moistened paper towels to maintain a humid environment.

Virus-infected Larvae

Death due to baculovirus infection was identified by a clear indication of the integument liquefaction. Larvae infected with SpliNPV were collected from laboratory population and frozen immediately after death to avoid liquefaction.

Preparation of Larval Suspensions

Uninfected larval suspensions were made from fourth instar larval cadavers. Groups of 10 fourth instar larvae were separated, starved for 24h, frozen for 2h, defrosted and then macerated. Infected larval suspension was directly obtained from virus-killed cadavers as described above. Both larval suspensions were filtered to remove coarse sediments. Uninfected ground larvae were mixed with blue food colouring (1:10) (Dr.Oetker Sverige AB, Göteborg, SE) to indicate the larval ingestion while infected larvae were not mixed with colouring due to infected larvae suspension typically displayed a dark brown/black colour.

Neonate *S.littoralis* larvae attraction towards larval suspensions

Bioassays consisted of two 50µl drops, which were pipetted onto opposite ends of a plastic petri dish (92×16mm, No.82.1472, Sarstedt AG & Co., Nümbrecht, DE). Ten neonate larvae were collected between 24-48h post-hatching and used under starved conditions. The larvae were placed in the petri dish with a fine brush, which was then covered with a clear plastic lid to prevent the larvae from escaping, and larvae were observed for a period of 30 minutes while they made a choice. Larvae were then left to feed for one hour and a half and were then inspected under a dissecting microscope to determine the colour of the gut. A total of 20 independent replicates were performed. In all replicates half of dishes had the side of the treatments switched to avoid a position effect.

Fourth instar *S.littoralis* larvae attraction towards larval cadavers

SpliNPV-infected cadavers were produced as described above. Virus-free fifth instar larvae were frozen for 2h. Defrosted cadavers were placed on opposite side of a 14–cm-diameter glass petri dish. A single, fourth instar larva, starved for 24h, was released at the centre of the glass petri dish and was monitored for physical contact and feeding behaviour during 30 min. Larval feeding was determined when they perforated the integument and actively chewed the cadaver. After the observation time, each larva was transferred to a 30 ml plastic cup with a piece of artificial diet and kept in a rearing room until pupation or death. The larvae were checked every day to make sure the diet had not desiccated. Dry diet was replaced with a fresh block. The test was performed a total of 70 times. In all replicates half of dishes had the side of the treatments switched to avoid a position effect.

Statistical analysis

Neonate larval orientation to infected vs. uninfected larval suspension were statistically analysed using a binomial test.

Proportion of fourth instar larvae attraction was carried out using a binomial test. Mortality percentage after fed or contact cadaver was analysed using a Chi-squared test. Significance was determined at $\alpha = 95\%$. All statistical analysis were performed using R (R Core Team, 2015).

Results

Neonate larval attraction to larval suspensions

Dual choice assay were performed to investigate the orientation of neonate larvae to infected larval cadaver suspension versus uninfected larval suspension. 57.0 % of the larvae were first attracted to virus-infected larval internal liquid and 42.5% of the larvae chose uninfected liquid (Figure 1A). These results were not significant ($P = 0.98$). 0.5 % of larvae that did not make a choice were excluded from analysis. After one hour and a half, 55.5% of the larvae consumed the infected larval suspension, 43.5% of the larvae incepted uninfected larval suspension (*P*=0.96) (Figure 1B). 1.0% of larvae that did not make a choice were excluded from analysis.

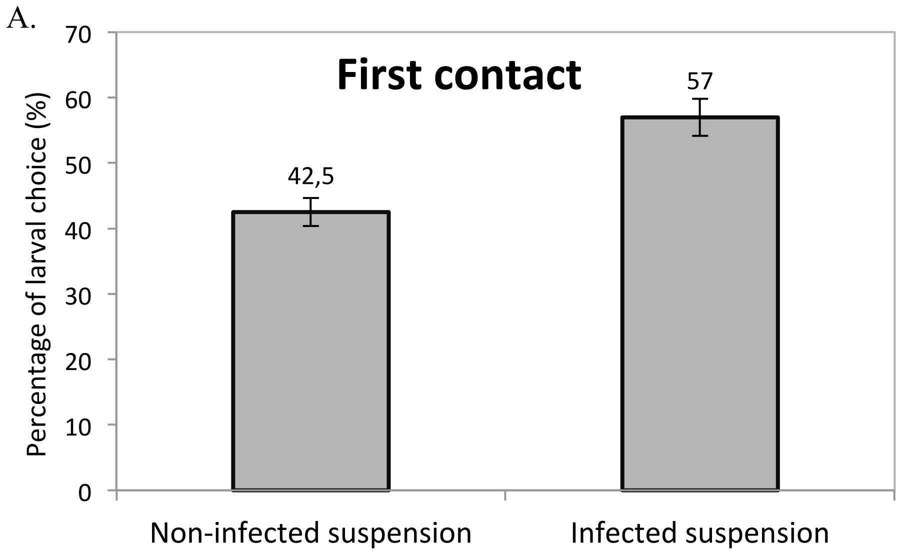

Figure 1. (A) Percentage of attraction of *S. littoralis* neonate larvae that stimulated by uninfected larval suspension or SpliNPVs-infected suspension. (B) Percentage of ingestion of *S. littoralis* neonate that offered uninfected larval suspension or SpliNPVs-infected suspension.

Fourth instar *S.littoralis* larvae attraction towards larval cadavers

Dual-choice feeding experiment show that a significantly higher amount of larvae chose to feed on infected conspecifics (31) than uninfected conspecifics (14) (binomial test, $P = 0.008$). Surprisingly, mortality was not significantly different between larvae that fed on uninfected (42.9%) and infected (54.8%) cadavers ($x^2 = 0.55$, $df = 1$, $P = 0.46$) (Figure 2A). 5 larvae fed on both types of cadaver, while 20 larvae did not feed on either cadaver.

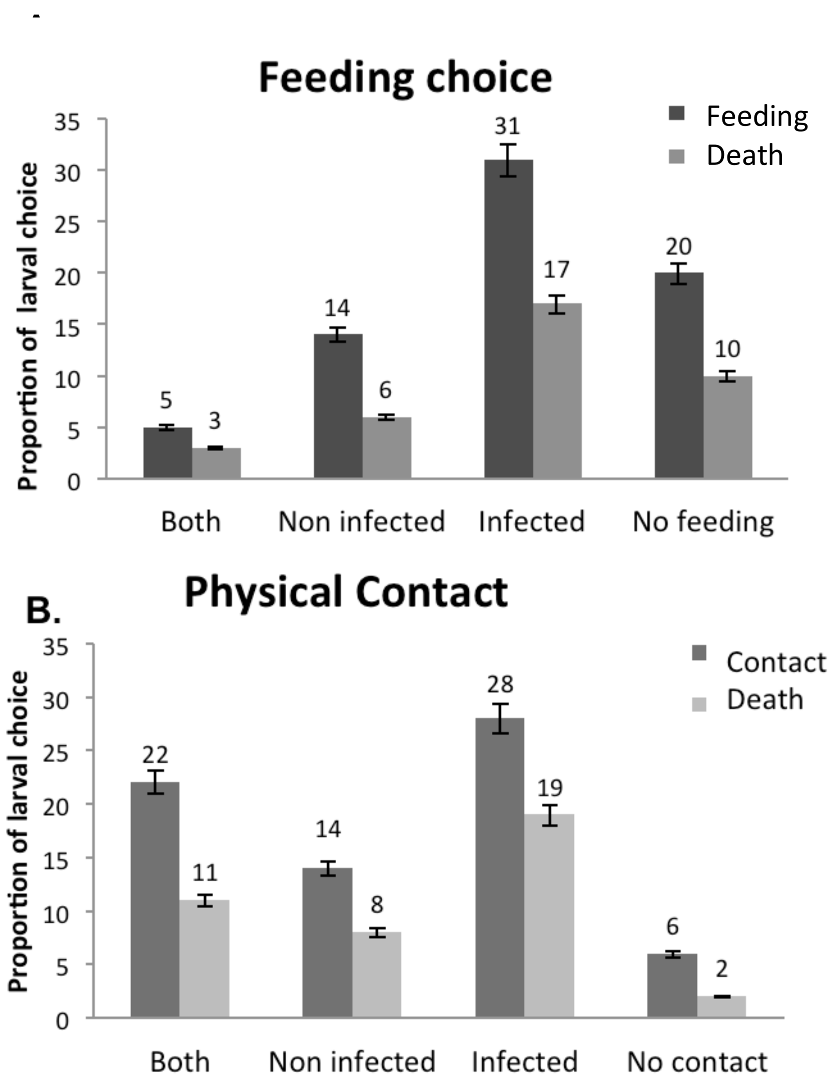

Figure 2. Larval choice between uninfected cadavers and infected cadavers (n= 70). (A) Dark grey, the number of larvae showed feeding behaviour. Light grey, the number of dead larvae after feeding on each cadaver. (B) Dark grey, the number of larvae made physical contact uninfected cadavers vs. infected cadavers. Light grey, the number of dead larvae after making physical contact.

Contact with infected cadavers by fourth-instar was twice as high than those that came into contact with uninfected cadavers (*P*=0.008) (Figure 2B). There is 57.2% larval mortality from making contact with healthy cadavers and 67.9% mortality from contact with infected cadaver(x^2 = 0.47, *df* = 1, *P* = 0.49). The six larvae that did not contact either cadaver were eliminated from the analysis.

Discussion

Our results show that attraction to larval cadavers and liquefied remains may increase larval necrophagy. This behaviour also contributes to virus dispersal and propagation. Neonate larval attraction to infected larval or uninfected larval suspensions suggests that neonate larvae are attracted to larval remains whether they contain viral OBs or not. Contrary to what has been reported in *Lymantria dispar*, *S. littoralis* larvae were not seen to avoid infected cadavers or remains. In feeding assays, *L. dispar* avoids the consumption of virus-contaminated leaves, suggesting that infected cadavers might metabolise volatile or gustatory cues detected by larvae (Capinera et al., 1976; Parker et al., 2010).

Feeding choice tests with fourth instar *S.littoralis* show that larvae are strongly attracted to and feed on virus-infected conspecifics. The mortality of larvae that consumed infected larval cadavers was not different from those that fed on uninfected cadavers, suggesting that the virus may be transmitted directly from parent to progeny in our colony. Vertically transmitted viruses are predicted to be less lethal than viruses acquired by ingestion (Lipsitch et al., 1996) since they are completely reliant on the survival of their hosts for transmission (Vilaplana et al., 2010). The trade-off hypothesis of virus evolution predicts that there is equilibrium between virulence (host mortality caused by virus) and effective transmission of the virus (Day, 2003). If vertically transmitted strains have too high virulence it would lead to a failure of transmission to offspring. Manipulating host's survival is especially important for viruses. Maximizing virulence without it becoming a limitation to be evolved through natural selection (Dennehy, 2014).

NPV infection may lead to metabolic changes in infected larvae and cadavers, ending in the release of volatile cues that increase attraction of healthy larvae. In humans, malaria infected hosts are more attractive to mosquitos due to the production of chemical signals (Lacroix et al., 2005). A number of plant pathogenic viruses are also known to release chemical compounds that attract insect vectors (Mauck et al., 2010), however, this has yet to be shown for insect pathogenic viruses). Furthermore, chemical signals emitted by infected cadavers that may be affecting larval behaviour have yet to be identified and we cannot exclude without tactile or gustatory effects.

Our results show that NPV infection enhances larval attraction and feeding in *S. littoralis*. However, the precise mechanism for the increased attraction and ingestion we observed, is as yet not well known and requires further research. High selective pressure and low levels of virulence favours the development of resistance (Day, 2003). Additionally the short persistence of viral formulations on plant foliage can be improved by adding a wide range compounds to the virus agents. Some studies have reported that larval attractants or phagostimulants can improve viral efficiency such as sugar, yeasts and pear ester (Arthurs et al., 2007; Knight and Witzgall, 2013). Biological control based on baculoviruses has advantages over of conventional strategies with synthetic pesticides. Our research may contribute to the improvement of baculoviruses use under field conditions by elucidating how baculoviruses affect larval behaviour to either enhance viral transmission or avoid infection.

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