Role of soil and larval excreta in the horizontal transmission of the baculovirus HpNPV and its implications in the management of teak defoliator *Hyblaea puera*

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In baculovirus–insect systems, a mixed-mode transmission strategy involving vertical transmission of virus from parent to offspring, and horizontal transmission from infected to susceptible or from the environment is well known. In this study, we examined the role of soil and excreta as alternative routes of horizontal transmission of *Hyblaea puera* nucleopolyhedrovirus (HpNPV) in the teak defoliator *H. puera* and how larval crowding influences these processes. The laboratory experiment failed to identify horizontal transmission of the virus from the soil during pupation or eclosion. However, the role of soil as a reservoir cannot be ignored as chances of transport of viral particles from soil to tree bark are expected through termite nests built on teak stems, which needs further examination. On the other hand, the experiments proved excreta as a major route of horizontal transmission and the rate of infectivity during crowding of larvae was significantly higher. Further research on other routes of horizontal transmission and host behaviour influencing the same are discussed here in the context of their role in managing of teak defoliator outbreaks.

Keywords: Baculovirus, horizontal transmission, *Hyblaea puera*, pest management, soil and larval excreta, teak.

THE nucleopolyhedroviruses (NPVs; family: Baculoviridae) have shown significant promise as practical insect control agents in agriculture and forestry due to their mixed-mode transmission strategy involving both vertical and horizontal transmission leading to population collapses in hosts[1–3]. Such mixed-mode transmission involving long-lived viral occlusion bodies (OBs) in the environment, and vertical transmission from infected adult insects to their offspring has key implications for the persistence and dispersal of baculoviruses and the management of insect pests[2]. Vertical transmission occurs from an infected adult to its offspring, while horizontal transmission occurs when vulnerable larvae ingest OBs persisting in their environment. Horizontal transmission occurs through the interactions between infected and susceptible individuals, and the rate of new infections depends on the larval density and their degree of contacts[2,4]. Typically, viral infection leads to overtly (i.e. observable symptoms of virus infection leading to death of the host) diseased hosts releasing OBs into the soil and foliage after disintegration of the cuticle[5,6]. High host population density and high pathogen replication rates increase infection prevalence and favour overt expression of the disease[7]. Earlier studies on horizontal transmission were based on the assumption that inoculum release occurs solely after the death of infected hosts, and this remained mostly untested[5]. However, there are a number of reports stating the existence of several alternate routes of horizontal transmission in insects[8], such as defecation and regurgitation by infected larvae[5,9,10], cannibalism[11], predators and scavengers[12], mating of infected adults[13], contaminated soil[1], etc.

In agriculture and forest systems, viral epizootics are typically triggered by inoculum in soil that acts as a reservoir for viral particles[1,14]. Several factors like wind, precipitation and soil conditions, and host plant characteristics, especially in forest systems, are known to affect the amount of NPVs transported from the soil reservoir to the foliage[15,16]. However, the dynamics of NPVs in the soil remains largely unknown in spite of the importance of this reservoir in triggering viral epizootics[1]. Similarly, horizontal transmission through excreta, regurgitation, cannibalism, predation and scavengers[9–13], which is influenced by the larval density, also remains untested for most of the baculovirus–insect systems.

The teak defoliator, *Hyblaea puera* is the most serious pest of teak throughout its range[17]. The discovery of the baculovirus *H. puera* nucleopolyhedrovirus (HpNPV) is considered a breakthrough in managing this pest[18]. The persistence of HpNPV in the soil was reported from the

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teak plantations of Nilambur, Kerala, India[19]. In *H. puera*, pupation and/or eclosion take place mostly in the soil under a thin layer of leaf litter, within a loosely built cocoon made of dry or decayed leaves, or soil particles held together with silk on the ground[17,20]. Therefore, viral transmission from the soil during this process is highly possible. During its annual cycle of occurrence, *H. puera* is known to occur in high densities, first during the fleshing of teak after pre-monsoon and then during the large-scale outbreaks[21]. In these stages of crowding, hundreds of larvae feed on each twig, providing the chance of horizontal transmission of the virus through the excreta and regurgitation. Understanding the dynamics of alternate routes of horizontal transmission can also help design effective control strategies for teak defoliator outbreaks.

This study aims to address the following questions: (a) Does HpNPV transmit directly from soil to pupa? (b) Does it transmit horizontally via larval excreta? If so, when does the infection manifest? (c) How does larval density influence horizontal transmission of HpNPV via alternative routes?

Materials and methods

Insects and HpNPV inoculum

The teak defoliator population maintained in the Entomology Laboratory of Kerala Forest Research Institute at Nilambur was used for the laboratory experiment on horizontal transmission of HpNPV[18,21,22]. The virus used for horizontal transmission experiment was originally isolated from diseased larvae collected from the teak plantations of Nilambur[18] and maintained in the above Laboratory following standard methods[22].

Viral transmission through soil

The experiment consisted of aluminium trays (35 × 35 × 7 cm) containing equal volumes of sterilized soil and decaying leaf spread over the soil maintained at a temperature of 28° ± 4°C and a relative humidity of 60% ± 10%. Next, 25 ml of HpNPV solution containing 6.4×10^{11} OBs/ml was sprayed over the soil surface using a chromatographic sprayer. Late fifth instar larvae were allowed to crawl over the contaminated soil surface and the trays were covered with a muslin cloth to prevent escape of the larvae and ensure sufficient aeration. Controls were set similarly, in which 25 ml distilled water replaced the viral inoculum. Five replications were maintained and each replication contained seven larvae. Following pupation (two days after treatment), the pupae with their cocoon covering were transferred into glass bottles (20 × 10 cm) separately. After emergence, the moths were paired and transferred to glass bottles for mating and subsequent egg-laying. The new generations were reared up to pupal stage to determine larval death, if any, due to HpNPV infection. Data on pupation, pupal weight, adult emergence, fecundity, hatchability and viral incidence were recorded. Data on parent pupation, pupal weight, adult emergence, fecundity and per cent hatchability of eggs were subjected to one-way ANOVA followed by LSD post hoc tests[23].

Transmission through excreta of infected host

Fifty healthy, fourth instar larvae of *H. puera* from the laboratory population were infected with a lethal dose of HpNPV solution using leaf disc bioassay method[24]. Next, 10 µl of NPV solution was placed on a 0.5 cm[2] tender teak leaf disc and fed to fifth instar larvae which were starved for 3 h. After 2–3 h, the larvae that had eaten the whole leaf disc were transferred to plastic rearing tubes (5.5 × 2.5 cm) containing a semi-synthetic diet. Excreta samples were collected randomly from ten larvae each at 0, 6, 12, 16, 24, 30, 36, 42, 48, 54 and 60 h post inoculation. Faeces collected were soaked in 1 ml distilled water in separate Eppendorf tubes and all the larvae were transferred to new rearing tubes at each time point above to avoid contamination. Controls consisted of an equal number of faecal samples of larvae fed on artificial diet at each time point. The viral OBs present, if any, in the excreta samples were isolated and quantified following standard methods[5,25]. The samples were filtered through four layers of muslin cloth. The filtrate was then subjected to centrifugation at 1500 rpm for 2 min to remove the faecal matter. The supernatant was again centrifuged at 10,000 rpm for 7 min to sediment the NPV particles. The supernatant was discarded and the pellet thus obtained was made up to a known volume using distilled water and enumeration of OBs was done using an improved Neubauer's haemocytometer.

In order to test the infectivity of OBs, one sample was randomly selected from those derived from the excreta collected at different time-intervals. Next, 10 µl of virus suspension was delivered to fifth instar larvae by the leaf disc method. Six replicates with ten larvae per replicate for each time point were used for the experiment. The treated larvae were reared on artificial diet and the culture room was maintained at a temperature of 28° ± 4°C and relative humidity of 60 ± 10%. Mortality due to infection was recorded until death or pupation. Mortality due to viral infection was confirmed using differential Giemsa staining. Data on the OBs obtained from the faecal samples and the percentage of NPV mortality at different time points were compared using one-way ANOVA with multiple comparison tests[23].

Influence of larval density on spreading viral infection

The influence of larval density on HpNPV infection spreading rate was examined under more realistic conditions using

Table 1. Comparison of pupal weight, fecundity, and percentage of pupation, adult emergence and hatchability of F1 generation larvae of *Hyblaea puera* retrieved from soil treated with HpNPV and control

	Pupation (%)	Pupal weight (g)	Adult emergence (%)	Fecundity (no. of eggs)	Hatchability (%)
Treatment	85.71 ± 10.1	0.23 ± 0.04	90.48 ± 8.75	433 ± 131.05	84.33 ± 13.77
Control	91.43 ± 7.82	0.23 ± 0.05	77.14 ± 19.24	512.33 ± 208.25	87.36 ± 8.17

Values shown are mean ± SD.

potted teak plants. Fourth-instar larvae were infected with 2.5×10^6 OBs/larva using the leaf disc method and single larvae were introduced to each teak sapling planted in polybags (70 cm height with eight leaves). Plants and infected larvae were maintained at 28° ± 4°C and 60% ± 10% relative humidity and the larvae were removed after 36 h. Healthy larvae at four rearing densities, i.e. 3, 5, 7 and 10, were introduced on the leaves of potted plants contaminated (with excreta or oral secretions) by the infected larvae. Twelve replications were set for each density. Controls were set in the same way in which healthy larvae replaced the infected ones. Once the leaves on the potted plants were eaten, the larvae were transferred individually to plastic rearing tubes (5.5×2.5 cm) containing a semi-synthetic diet. Observations were taken until death or pupation of the larvae, whichever occurred first. The mortality rates at different rearing densities were compared using one-way ANOVA. Where necessary, data were transformed ($\log_{10}$ or arcsine square root) to meet the underlying assumptions[23]. All statistical analyses were carried out using the statistical package SPSS 16.0.

Results

Transmission through soil

There was no significant difference in the percentage pupation (one-way ANOVA: $F_{1,10} = 1.00$, $P > 0.05$) and pupal weight ($F_{1,52} = 0.165$, $P > 0.05$) of larvae between the treated and control sets. The number of adults that emerged from the pupae of the treated set and untreated control was also not different ($F_{1,10} = 0.29$, $P > 0.05$). The number of eggs laid ($F_{1,10} = 0.541$, $P > 0.05$) and hatchability ($F_{1,10} = 0.206$, $P > 0.05$) were similar among the treated and untreated groups. There was no significant variation in the pupation rate, pupal weight, adult emergence, fecundity, hatchability and viral incidence between the treated and control sets (Table 1). There were no viral deaths among the larvae of the progenies of both groups, indicating an absence of HpNPV transmission through the soil.

Transmission through excreta of infected host

The presence of OBs in the excreta samples was first observed at 24 h post inoculation and recorded from the rest of samples collected thereafter. There was a significant difference in the average number of OBs at different time

intervals (one-way ANOVA: $F_{10,78} = 2.139$, $P < 0.05$) and it increased with time (Figure 1; linear regression: $R^2 = 0.83$). The mean number of OBs/ml was 3.2×10^5, 7.3×10^6, 4.2×10^6, 1.9×10^7, 4.4×10^6, 7.5×10^6 and 2.9×10^7 at 24, 30, 36, 42, 48, 54 and 60 h post inoculation respectively.

The results of bioassay using the virus extracted from the excreta indicate the start of HpNPV infection at about 18 h post inoculation. There was a significant difference in the percentage mortality of larvae after ingestion of OBs derived from the faeces collected at different time points post-inoculation (one-way ANOVA: $F_{10,65} = 115.89$; $P < 0.001$). The infection increased gradually (Figure 2; logarithmic regression: $R^2 = 0.69$) and maximum mortality (0.77%) was found at 54 h post inoculation. No viral deaths were observed in the control treatment.

Effect of larval density on spreading viral infection

There was significant variation in the mortality of the larvae fed with teak leaves previously inhabited by HpNPV contaminated larvae at different rearing densities (one-way ANOVA: $F_{3,47} = 14.54$, $P < 0.0001$). The mortality of larvae increased with increasing rearing density (Figure 3; linear regression: $R^2 = 0.89$). No viral deaths were observed in the control treatment.

Discussion

Soil to pupa transmission

Soil can act as a major reservoir for the baculovirus and the viral particles are known to remain viable for over 40 years in forest systems[1,14]. In teak, which is a deciduous species, soil could be the substratum that will hold all the virus particles due to their washing away from the leaf surfaces during rainfall and leaf fall. In the teak plantations at Nilambur, where the virus was sprayed for the control of *H. puera*, persistence of NPV in the soil was found from the Valluvassery plantation after three years of application, while no virus could be recovered from the Kariem–Muriem plantation after eight years of application[19]. We could not find any HpNPV incidences in the generations of *H. puera* pupae collected and reared from these plantations (author's unpublished data). The chances of contamination at the time of cocoon formation or eclosion are high in *H. puera*, since the pre-pupal and pupal

stages occur mostly in the soil. However, such modes of transmission during these processes could not be detected in the experiments. There was no viral death and no significant differences in pupation, pupal weight, adult emergence, fecundity and hatchability of the F1 generation of insects originating from the larvae that were allowed to pupate in soil inoculated with virus compared to control. Though horizontal transmission of NPV from the soil to the insect hosts is well known from agricultural systems[1], factors such as spatial distribution of the virus on the plant, and transport between plants and the soil are expected to significantly influence virus transmission at a local scale[1,4]. Fuxa and Richter[26] showed that physical forces like rainfall and/or wind are required to transport of NPV onto cotton plants, as no virus was detected on the plants in the controls in the precipitation or wind experiments. In this context, vertical transport of the virus from soil to leaves in taller forest plantation trees like teak may be extremely difficult. Raymond *et al.*[27] have reported that in deciduous trees like oak and Sitka spruce, the persistence of NPV was more on stems than on leaves. This could be possible in the case of teak also, as it is a common phenomenon in teak plantations that termites carry soil to

Figure 1. Occlusion bodies (OBs) obtained from HpNPV-infected host excreta samples at different time intervals. Error bars represent standard deviation of OBs/ml at each time point ($n = 10$).

Figure 2. Mortality of fifth instar larvae of *Hyblaea puera* infected with foecal suspension obtained from HpNPV-infected host excreta samples at different time intervals. Error bars represent standard deviation of the difference between mean mortalities ($n = 6$).

different heights of the stems for colony build-up and there will be a potential transport of viral particles during this process, especially in young plantations. Therefore, future research on the transport of HpNPV should focus on this aspect as well.

Transmission through excreta of infected host

The presence of OBs in the excreta of infected host found in the experiment suggests that it could act as an alternative route of viral transmission. Similar observations were also reported in *Mamestra brassicae* larvae after the fourth day of virus inoculation[5]. The detection of OBs in the excreta only after 24 h post inoculation indicates that the virus could have originated from replication within the host larvae, rather than voided by the host from the original inoculum. The presence and regular increase of free OBs in the haemolymph of infected *H. puera* larvae during haemocyte counts after 4 h post inoculation of the virus by Biji[28], also substantiate this result. The reduction in the number of excreta samples after 42 h post inoculation may be due to the reduction in the metabolism rate, as cessation of feeding was reported after virus infection[29].

Though free OBs were found in the excreta only at 24 h, infection due to viral attack could have started well before this. In a previous study[28], free OBs were detected in the haemolymph at 4 h post infection, but infectivity started from 2 h post inoculation itself causing 33% mortality. These results were contrary to those obtained in the case of *M. brassicae*, where viral particles were present in the faeces only on the fourth day of viral infection and no viral deaths were recorded before this[5]. The mean number of OBs found among the excreta samples collected at different time points in the experiment was higher than the LD_{50} values recorded for the fifth instar larvae of *H. puera*[28]. The maximum mortality rate of *H. puera* infected with OBs extracted from the excreta was 77%.

Much of the work on horizontal transmission has focused on transmission occurring primarily via the ingestion of
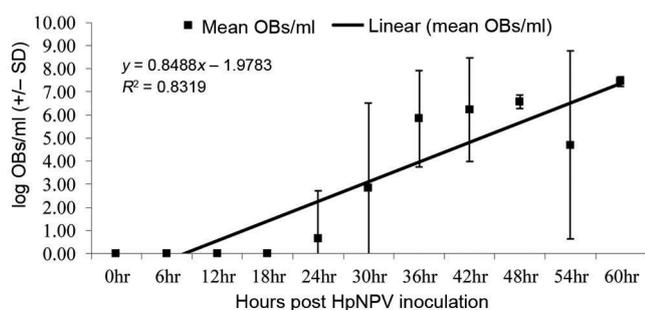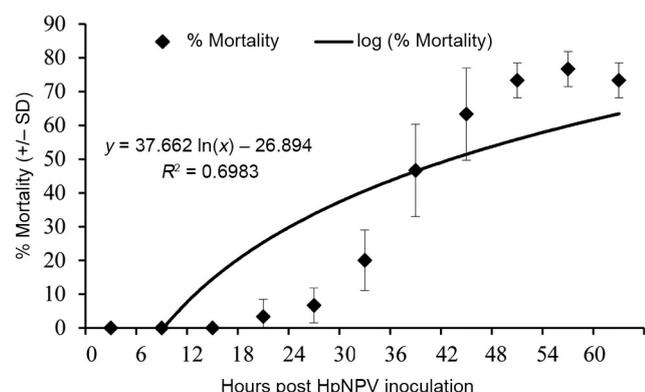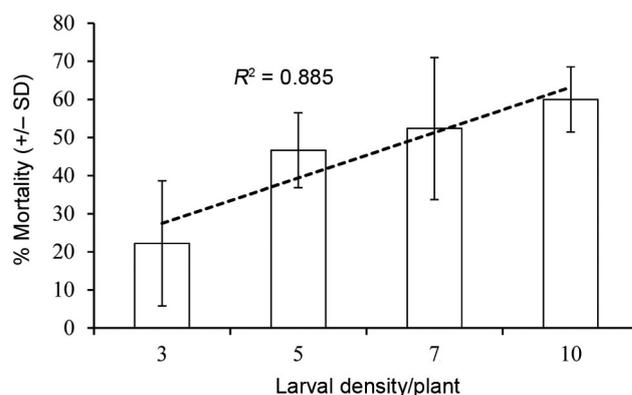

Figure 3. Mortality caused by HpNPV in teak defoliator larvae reared at different densities. Values represent mean ± SD ($n = 12$).

OBs released from the dead and decayed NPV-infected larvae[8]. However, deposition of oral secretions and excreta by the infected host to a shared food source was reported as the major route of natural Musca domestica Salivary Gland Hypertrophy Virus transmission among adult houseflies[10]. In the case of the gypsy moth, *Lymantria dispar*, exclusion of faeces from the rearing cages resulted in a 58% decrease in horizontal transmission[9]. The results of the present study and previous studies discussed above suggest that the excreta can act as an alternative route for density-dependent horizontal transmission by increasing the inoculum pool available in the ecosystem.

Influence of larval density on spreading HpNPV inoculums

Studies on baculovirus transmission at high host densities have focused on horizontal transmission and in such cases, the pathogen transmission rates were assumed to be proportional to the number of susceptible hosts and infectious pathogens[4,30]. Majority of these studies have focused on the transmission of baculoviruses, which occurs when the larvae consume foliage contaminated by the cadavers of virus-infected conspecific larvae.

Apart from this, several alternative routes of transmission through defecation and regurgitation, cannibalism, predators and scavengers, etc. which are density-dependent, have also been reported in insect–baculovirus systems[1,9–11]. Results of the present study show that exposure to foliage used by the virus-infected larvae can cause HpNPV infection in conspecific larva at an early stage through the virus released before the death of the infected larvae. This confirms the availability of alternative routes of HpNPV transmission through the excreta, as proved in the present study, and also through oral secretion or regurgitation of infected *H. puera* larvae. The mortality due to HpNPV infection in healthy larvae released on the leaves exposed to virus-infected larvae increased from 30% to 60% with increasing rearing density. Similar density-dependent mortality due to horizontal transmission was also found in other laboratory studies[5,9–11]. Such a pattern may be due to the cumulative amount of virus released by a larger number of individuals, which results in an increased pool of inoculums.

Implications for teak defoliator management

The alternative routes of horizontal transmission described here play a key role in the spread of HpNPV infection in high-density field populations since mixed-aged larval populations are not uncommon in *H. puera* during the outbreak season. In *H. puera*, the population build-up begins by laying eggs on the tender leaves in the upper strata of the trees after pre-monsoon, resulting in patchy epicentre populations. The OBs released through the excreta of the infected larvae, if any, will contaminate the leaves in the lower strata which are occupied by the following populations. In the epicentre populations, up to 40 larvae/twig are known to occur, which leads to higher rates of interaction among the larvae and favours horizontal transmission of HpNPV. Further, the increased mobility of the infected larvae at the initial stages of infection could help in spreading the inoculum pool and increase the chances of viral epizootic[31]. This is particularly important as the susceptibility of older instars of *H. puera* is much lower than the early instars[28]. Moreover, modifications in the feeding behaviour and ability to detect and avoid virus-infected cadavers by the older larvae also have a significant effect on the infection risk or disease spread[31,32], and therefore infection in the early stages by alternative routes is important in viral spread.

Seeding of HpNPV in the high-density epicentre populations of the teak defoliator is known as an economical and environment-friendly method for management of the pest[33]. The horizontal transmission of OBs in *H. puera* through the excreta proved here augments the spread of the disease after the seeding of HpNPV and this mixed-mode of transmission of the virus has significant implications in the management of the teak defoliator. However, along with understanding the role of different routes of horizontal transmission, further information on the stage structure and host behaviour is important in deciding the timing and dosage of HpNPV application to control teak defoliator outbreaks.

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