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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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Abstract. In baculovirus-insect systems, a mixed-mode transmission strategy involving vertical transmission of the virus from parent to offspring, and horizontal transmission of the virus 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 teak defoliator and how the larval crowding influence these processes. The laboratory experiment failed to identify horizontal transmission of virus from soil during pupation or eclosion. However, the role of soil as reservoir cannot be ignored as the chances of transport of viral particles from soil to tree bark is expected through termite nests build on teak stems, which need 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 needs on other routes of horizontal transmission and host behaviour influencing the same are discussed in the context of their role in management of the teak defoliator outbreaks.

Keywords: baculovirus, *Hyblaea puera*, teak defoliator, HpNPV, horizontal transmission, pest management

Introduction

The nucleopolyhedroviruses (NPV); (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 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 host release OBs onto soil and foliage after disintegration of the cuticle[5,6]. High host population density and high pathogen replication rates increases the infection prevalence and

44 favours the overt expression of the disease[7]. Earlier studies on horizontal transmission were
45 based on the assumption that inoculum release occurs solely after the death of infected hosts
46 and this belief remained mostly untested[5]. However, there are a number of reports stating
47 the existence of several alternate routes of horizontal transmission in insects[8] such as
48 defecation and regurgitation by infected larvae[5,9-10], cannibalism[11], predators and
49 scavengers[12], mating of infected adults[13], contaminated soil[1], etc.

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

58 The teak defoliator, *Hyblaea puera* is the most serious pest of teak throughout its
59 range[17]. The discovery of the baculovirus *Hyblaea puera* nucleopolyhedrovirus (HpNPV) is
60 considered as a major breakthrough in the management of this pest[18]. The persistence of
61 HpNPV in the soil was reported from the teak plantations of Nilambur[19]. In *H. puera* the
62 process of pupation and/or eclosion takes place mostly in soil under a thin layer of leaf litter,
63 within a loosely built cocoon made of dry or decayed leaves, or soil particles held together
64 with silk on the ground[17,20] and therefore, viral transmission from soil during this process is
65 highly possible. During its annual cycle of occurrence, *H. puera* is known to occur in high

66 densities, first during fleshing of teak after pre-monsoon and second during the large-scale
67 outbreaks[21]. In these stages of crowding, hundreds of larvae feed on each twig, providing
68 the chance of horizontal transmission of the virus via the excreta and regurgitation.
69 Understanding the dynamics of alternate routes of horizontal transmission can also help to
70 design effective control strategies for teak defoliator outbreaks.

71 This study aims to address the following questions: a) do HpNPV transmi directly
72 from soil to pupa? b) do HpNPV transmit horizontally via larval excreta? If so, when can
73 the infection manifest? and c) how does larval density influence horizontal transmission of
74 HpNPV via alternative routes?

75 **Materials and methods**

76 *Insects and HpNPV inoculum*

77 The teak defoliator population maintained in the Entomology Laboratory of KFRI at
78 Nilambur, Kerala were used for the laboratory experiment on the horizontal transmission of
79 HpNPV[18,21-22]. The HpNPV used for horizontal transmission experiment was originally
80 isolated from diseased larvae collected in the teak plantations of Nilambur[18] and maintained
81 in the above Laboratory following standard methods[22].

82 *Viral transmission through soil*

83 The experimental arenas consisted of Aluminium trays (35 x 35 x 7 cm) containing equal
84 volumes of sterilized soil and decaying leaf spread over the soil maintained at a temperature
85 of 28±4 °C and a relative humidity of 60±10%. 25 ml of HpNPV solution containing 6.4 x
86 10^{11} OBs/ml 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 muslin cloth to prevent the escape of larvae and ensure sufficient aeration. Controls were set similarly, in which 25 ml distilled water replaced the viral inoculum. Five replications were kept and each replication contained seven larvae each. Following pupation (two days after the treatment), the pupae with their cocoon covering were transferred into glass bottles (20 x 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 upto pupa to find out 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 percent hatchability of eggs were subjected to One-Way ANOVA followed by LSD *post hoc* tests[23].

Transmission through excreta of infected host

50 healthy fourth instar larvae of *H. puera* from the lab population were infected with a lethal dose of HpNPV solution using leaf disc bioassay method[24]. 10 µl of NPV solution were placed on a 0.5 cm^2 tender teak leaf disc and fed up to fifth instar larvae which were starved for 3 hrs. After 2-3 hrs, larvae that had eaten the whole leaf disc were transferred to plastic rearing tubes (5.5 x 2.5 cm) containing semi-synthetic diet. Excreta samples were collected randomly from 10 larvae each at 0, 6, 12, 16, 24, 30, 36, 42, 48, 54 and 60 hours post inoculation. Feces 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 to avoid contamination. Controls consisted of an equal number of fecal samples of larvae fed on artificial diet at each time point. The viral inclusion bodies (OBs) present if any in the excreta

samples, were isolated and quantified following standard methods[5,25]. The samples were filtered through 4 layers of muslin cloth. The filtrate was then subjected to centrifugation at 1500 rpm for 2 min to remove the fecal matter. The supernatant 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 were done using improved Neubauer's haemocytometer.

In order to test the infectivity of OBs, one sample selected randomly and individually from samples derived from the excreta collected at different time intervals. 10 µl of virus suspension was delivered to fifth instar larvae by leaf disc method. Six replicates with 10 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 facilitated with a temperature of 28±4 °C and a 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. The data on the OBs obtained from the fecal samples and the percentage NPV mortality of different time points were compared using One-Way ANOVA with multiple comparison tests[23].

Influence of larval density on spreading viral infection

Influence of larval density on HpNPV infection spreading rate was examined under a more realistic condition using potted teak plants. Fourth instar larvae were infected with 2.5 X 10^6 OBs/larva by leaf disc method and single larvae were introduced on each teak saplings planted in polybags (70 cm height with 8 leaves). Plants and infected larvae were kept at 28±4 °C and 60±10% relative humidity and the larvae were removed after 36 hrs. Healthy

larvae at four rearing densities *i.e*, 3, 5, 7 and 10 were introduced over 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 the plastic rearing tubes (5.5 x 2.5 cm) containing semi-synthetic diet. Observations were taken until death or pupation of the larvae whichever happens first. The mortality rates at different rearing densities were compared using One-Way ANOVA. Where necessary, the data were transformed (log_{10} or arcsine square root) to meet 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 the control sets. The number of adults emerged from the pupae of the treated set and untreated control were also not differed ($F_{1,10} = 0.29$, $P > 0.05$). The number of eggs laid ($F_{1,10} = 0.541$, $P > 0.05$) and the 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 rates, pupal weight, adult emergence, fecundity, hatchability and viral incidence between treated and control sets (Table 1). There were no viral deaths among the larvae of the progenies of both groups indicating absence of HpNPV transmission through soil.

Table 1 here

153 *Transmission through excreta of infected host*

154 The presence of OBs in the excreta samples was first observed at 24 hours post inoculation
155 and recorded from the rest of samples collected after that. There was a significant difference
156 in the average number of OBs at different time intervals (One-way ANOVA: $F_{10,78} = 2.139$,
157 $P < 0.05$) and increased as the time progresses (Figure 1; linear regression: $R^2 = 0.83$). The
158 mean numbers of OBs per ml were 3.2×10^5, 7.3×10^6, 4.2×10^6, 1.9×10^7, 4.4×10^6, $7.5 \times$
159 10^6 and 2.9×10^7 at 24, 30, 36, 42, 48, 54 and 60 hours post inoculation, respectively.

160 The results of the bioassay using the virus extracted from the excreta indicates the
161 start of HpNPV infection at about 18 hours post inoculation. There was a significant
162 difference in the percentage mortality of larvae after ingestion of the OBs derived from the
163 feces collected at different time points post inoculation (One-way ANOVA: $F_{10,65} = 115.89$;
164 $P < 0.001$). The infection increased gradually (Figure 2; logarithmic regression: $R^2 = 0.69$)
165 and maximum mortality (c.77%) was found at 54 hours post inoculation. No viral deaths
166 were observed in the control treatment.

167 *Effect of larval density on spreading viral infection*

168 There was a significant variation in the mortality of the larvae fed on teak leaves previously
169 inhabited by HpNPV contaminated larvae at different rearing densities (One Way ANOVA:
170 $F_{3,47} = 14.54$, $P < 0.0001$). The mortality of larvae increased with increasing rearing densities
171 (Figure 3; linear regression: $R^2 = 0.89$). No viral deaths were observed in the control
172 treatment.

173 Figure 1-3 here

Discussion

Soil to pupa transmission

Soil can act as a major reservoir for 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 washing off them from leaf surfaces during rain and leaf fall. In the teak plantations at Nilambur, Kerala, where the virus has sprayed for the control of *H. puera*, persistence of NPV in the soil has 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]. However, 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 is high in *H. puera,* since the pre-pupa and pupa stages occurs mostly in soil. However, such mode of transmission during these processes could not be detected in the experiments as well. There was no viral death and no significant differences in pupation, pupal weight, adult emergence, fecundity and hatchability of the F1 generation of insects originated from larvae that were allowed to pupate in soil inoculated with virus compared to control. Though, horizontal transmission of NPV from soil to the insect hosts are well known from the agricultural systems[1], a number of factors such as the spatial distribution of virus on the plant, and 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 rain and/or wind are required for the transport of NPV onto cotton plants, as no virus was detected on plants in the controls in the precipitation or wind experiments. In this context, vertical transport of 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 the leaves. This could be possible in the case of teak also, as it is a common phenomenon in the teak plantations that termites carry soil to 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 transport of HpNPV should focus on this direction also.

Transmission through excreta of infected host

The presence of OBs in the excreta of the infected host found in the experiment suggest that it could act as an alternative route of viral transmission. Similar observations were also reported in species such as *M. brassicae* larvae after the fourth day of virus inoculation[5]. The detection of OBs in the excreta only after 24 hours post inoculation indicate that the virus could be originated from the replication within the host larvae rather than voided by the host from the original inoculum. The presence and the regular increase of free OBs in the haemolymph of infected *H. puera* larvae during haemocyte counts after four hours post inoculation of virus by Biji[28], also substantiate this result. The reduction in the number of excreta samples after 42 hours 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 hrs, infection due to viral attack could be started well before this. In a previous study[28], the free OBs were detected in the haemolymph at 4 hours post infection, but the infectivity started from two hours post inoculation itself causing 33% mortality also support this view. These results were contrary

to those obtained in the case of *M. brassicae,* where the viral particles were present in the feces 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 were 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 excreta was 77%.

Much of the work on horizontal transmission focused on transmission occurring primarily via the ingestion of OBs released from the dead and decayed NPV-infected larvae[8]. However, the deposition of the oral secretions and excreta by the infected host to a shared food source was reported as the major route of natural MdSGHV transmission among adult house flies[10]. In the case of the gypsy moth, *Lymantria dispar*, exclusion of feces from the rearing cages resulted in a 58% decrease in horizontal transmission[9]. All these observations along with the results of the present study showing the release of infective viral particles within 24 hours post inoculation suggests that excreta can act as an alternate route for density-dependent horizontal transmission by increasing the inoculum pool available in the ecosystem leading to viral epizootic.

Influence of larval density on spreading HpNPV inoculums

Studies on baculovirus transmission at high host densities are focused on horizontal transmission and in such cases, the pathogen transmission rates are 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 baculovirus, which occur when larvae consume foliage contaminated by the cadavers of virus-infected conspecific larvae.

However, apart from this, several alternative routes of transmission through defecation and regurgitation, cannibalism, predators and scavengers, etc, which are density-dependent are also reported in insect-baculovirus systems[1,9-11]. Results of this study shows 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 death of infected larvae. This confirms the occurrence of alternative routes of HpNPV transmission through the excreta as proved in this 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 densities. 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 larger numbers of individuals which result in an increased pool of inoculums.

Implications for teak defoliator management

The alternative routes of horizontal transmission described here seems to 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 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 the pre-monsoon resulting patchy epicentre populations. The OB's released through the excreta of the infected larvae, if any, will contaminate the leaves in the lower strata which is occupied by the following populations. In epicentre populations, up to 40 larvae/twig are known to occur, which lead to higher rates of interaction among larvae and favourable for the horizontal transmission of HpNPV. Further, the increased mobility of the infected larvae at the initial stages of infection could also help in spreading the inoculum pool and increase

the chances for 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, the modifications in the feeding behaviour and ability to detect and avoid virus-infected cadavers by the older larvae also have strong effect on the infection risk or disease spread[31,32] and therefore infection in early stage by alternative routes are important in viral spread.

Seeding of HpNPV in the high density epicenter populations of the teak defoliator is known as an economical and environment friendly method for the management of the pest[33]. The horizontal transmission of OBs in *H. puera* through excreta proved here augment spread of disease after the seeding of HpNPV and this mixed-mode of transmission of the virus have 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 dosages of HpNPV application to control the teak defoliator outbreaks.

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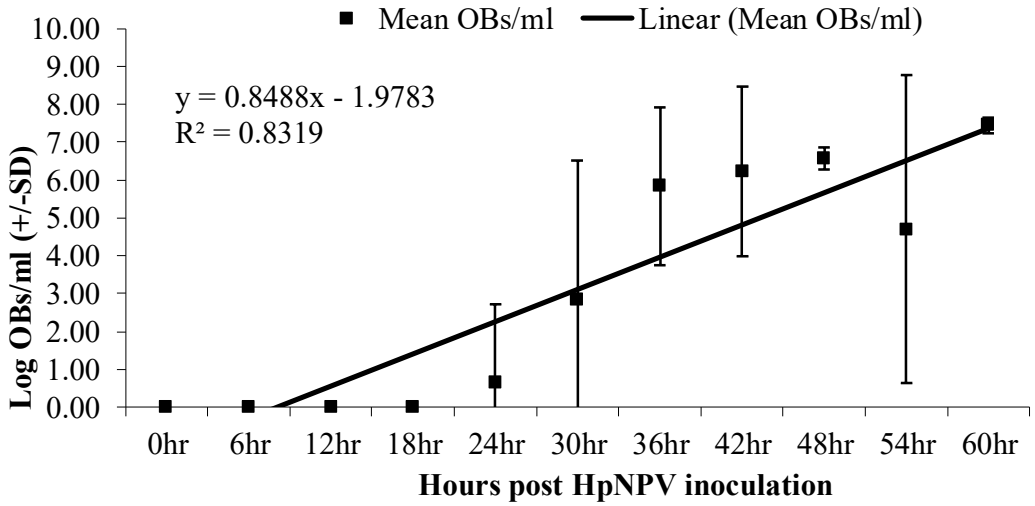
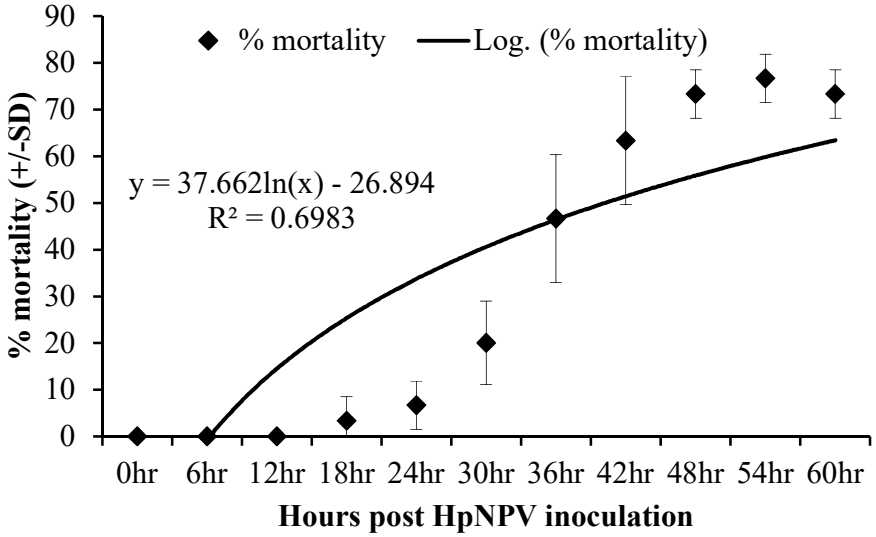
Table 1. Comparison of pupal weight, fecundity and percentages of pupation, adult emergence and hatchability of F1 generation larvae of *H. puera* retrieved from soil treated with HpNPV and control. Shown are mean±SD.

	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



372

373 **Figure 1.** Occlusion bodies obtained from HpNPV infected host excreta samples at different
374 time intervals. Error bars represent standard deviation of the OBs/ml at each time point
375 (n=10).
376
377



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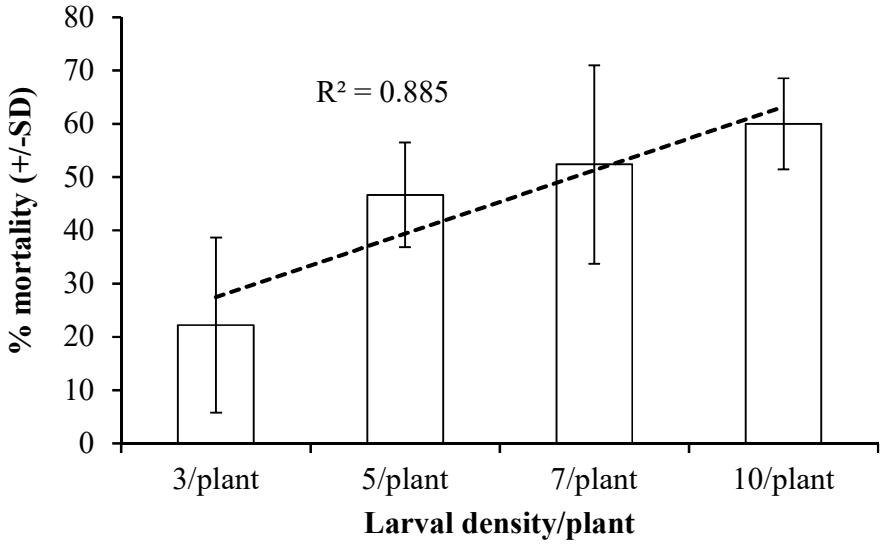
379 **Figure 2**. Mortality of fifth instar larvae of *H. puera* infected with fecal suspension obtained
380 from HpNPV infected host excreta samples at different time interval. Error bars represent
381 standard deviation of the difference between mean mortality (n= 6).



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