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SHORT COMMUNICATION

Density-dependent polyphenism and baculovirus resistance in teak defoliator, *Hyblaea puera* (Cramer)

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Abstract. 1. Field populations of the teak defoliator larvae, *Hyblaea puera* Cramer exhibit colour polyphenism under different population densities: greyish-green with black- and orange-coloured dorsal bands in low-density endemic populations and uniformly black or intermediate colour during high-density population.

2. The density dependence of colour polyphenism was confirmed by field monitoring of *H. puera* populations during 2008–2010.

3. The above findings were later substantiated by rearing *H. puera* larvae under different densities (i.e. solitary and crowded in the laboratory). Ninety one per cent of the solitary reared laboratory population developed bright coloration whereas, 92% of the group reared larvae turned to black. Eight per cent of larvae from both the rearing densities were of intermediate colour.

4. Density-dependent resistance build-up against *H. puera* nucleopolyhedrovirus by *H. puera* were tested using the fifth instar larvae. The results showed three-fold increase of median lethal dose (LD_{50}) value for the group reared larvae (5332 polyhedral occlusion bodies/larvae) compared to the solitary reared ones (1727 polyhedral occlusion bodies/larva) and also significant difference for the mean time to death (3.6 and 3.3 days, respectively).

5. The study revealed the strong influence of larval density on *H. puera* larval melanism and resistance build-up against *H. puera* nucleopolyhedrovirus.

Key words. NPV, outbreak and endemic population, polyphenism, resistance, teak defoliator.

Introduction

Infectious diseases caused by viral, bacterial, protozoan, or helminth infections can be a reason for population cycles in forest insects. In such cases, the pathogen transmission rates are assumed to be proportional to the number of susceptible hosts and infectious pathogens (Anderson & May, 1981; Berryman, 1996). The earlier belief was that due to the increased stress associated with high population density, organisms may be more vulnerable to infectious diseases (Steinhaus, 1958). However, Wilson and Reeson (1998) hypothesised that in high-density fluctuating populations of organisms such as insects, natural selection will favour those that can allocate optimum resource on disease resistance.

The resistance mechanism in insects can be through morphological, behavioural, developmental, physiological, nutritional, biochemical, molecular genetic mechanisms, etc. (Narayanan, 2004). The external manifestations of such resistance build-up in high- and low-density forms may be through colour, behaviour, or developmental time (Reeson *et al*., 1998; Wilson, 2000; Lee *et al*., 2006). Density-dependent phase polyphenism, a type of phenotypic plasticity is common in larval Lepidoptera, in which the crowded forms tend to be darker (highly melanic) than the solitary. The density-dependent colour polyphenism have previously been explained in terms of strategies for antipredation (aposematism is selected for high density and crypsis is selected for

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low-density populations), camouflage (background matching), density-dependent prophylaxis, etc. (Hagen *et al*., 2003). Several studies have linked coloration to immune response. For example, the high-density forms developing more cuticular melanisation and resistance than the solitary forms were found in *Spodoptera exempta* (Reeson *et al*., 1998; Vilaplana *et al*., 2008), *S. littoralis* (Cotter *et al*., 2004a), and *Operophtera brumata* (Hagen *et al*., 2003). However, density-dependent phase polyphenism and resistance build-up was not found in the case of *Lymantria dispar* (Reilly & Hajek, 2008). The increased deposition of melanin in the cuticle at high population densities is an adaptive countermeasure to cope with the greater risk of disease transmission in crowded environments and is referred as density-dependent prophylaxis (Wilson & Reeson, 1998). Resistance development and the melanisation process are linked to high levels of phenoloxidase enzyme, which play a key role in the insect immune response, especially the encapsulation reaction occur in the haemolymph (Wilson & Reeson, 1998).

In lepidopteran–virus systems due to the maintenance of group feeding, individuals are always at a risk of infection, as the normal way of infection is through ingesting contaminated food. Such gregarious feeding can lead to increased levels of resistance in crowded forms, which has been observed in the case of 13 temperate lepidopterans (Hochberg, 1991). The teak defoliator, *Hyblaea puera* Cramer (Lepidoptera: Hyblaeidae) is a serious pest of teak in the Asia-Pacific region and becoming increasingly important in Latin America. Normally, the larvae are greyish-green with white, black, and orange-coloured dorsal longitudinal bands. During population outbreaks uniformly black-coloured larvae predominate (Fig. 1). The presence of both types in the same population is also a common phenomenon (Nair, 2007). The endemic phase of the population cycle of the pest is characterised by sparse, very low population density found during the natural defoliation of teak (November to January). Pest outbreaks (late February to August) witness millions of similar aged caterpillars feeding on teak canopy, where each tender leaf of the tree may harbour 50–100 larvae. With an average total life span of 20–21 days, five to six outbreak peaks were observed within a season. The population collapse of the pest was often associated with a baculovirus infection caused by *Hyblaea puera* nucleopolyhedrovirus (HpNPV) (Sudheendrakumar *et al*., 1988). Insect baculoviruses are known to transmit both horizontally (within generation) and vertically (parents to offspring). In high-density host populations viral transmission occurs horizontally between infected and susceptible larvae, and through alternatives routes such as excreta, oral secretion, regurgitation, cannibalism, and mating (Cory & Myers, 2003). In this study, we investigated (i) whether phenotypic plasticity in larval colour of *H. puera* is density-dependent in the field as well as in the laboratory population, and (ii) density-dependent changes in virus resistance levels in the HpNPV system.

Fig. 1. Colour polyphenism in the fifth instar larvae of the teak defoliator *Hyblaea puera*. The orange-coloured larvae are typical of the low-density population and the black-coloured larvae are typical of the outbreak population.

Materials and methods

To assess the density-dependent colour polyphenism of field populations of *H. puera*, insect sampling was carried out during endemic (January to February) and outbreak phases (March to April) in 2008, 2010, and 2011. Sampling was done in 12 teak plantations (Kariem-Muriem, Nellikkutha, Muttikkadavu, Valluvassery, Aruvakkode, Aravallikkavu, Panayangodu, Nedungayam, Emangad, Old Amarambalam, Naripoyil, and Chathanpurai) in Nilambur North and South Forest Divisions, Kerala. The plantations were spatially separated at an average distance of 5–8 km and with an age class range of 4–30 years. Trees (15–30 per plantation) were randomly selected and three samples (twigs with four leaves) were collected from the top, middle, and bottom tiers of each tree. During the endemic phase, sampling was restricted to wet/shady places with tender leaves. For each sample the number, stage, and colour of the collected larvae were recorded. As the colour variation was more prominent in late instars, younger instars collected during the endemic phase were brought to the laboratory. In the laboratory, they were reared individually on teak leaf to the pupal stage in plastic rearing tubes (5.5 × 2.5 cm). The culture room was maintained at a constant temperature of 28 ± 4 °C, relative humidity of 60 ± 10%, and on a LD photoperiod 12 : 12. High-density populations were sampled only from the first outbreaks in different plantations (five, four, and three outbreaks during 2008, 2010, and 2011, respectively) as the successive outbreaks were presumably originated from these populations.

The sampling period was adjusted to when the larvae were fourth or fifth instars. Based on the intensity of melanism, the larvae were grouped into three categories: melanic (uniformly black coloured larvae), non-melanic (bright coloured with orange and black longitudinal bands running over the dorsal side), and intermediate (black coloured with light orange longitudinal stripes running over the dorsal surface).

To substantiate density-dependent colour polyphenism of field populations of *H. puera* larvae, laboratory evaluation was carried out using larvae obtained from the continuous culture maintained at the Entomology Laboratory, Kerala Forest Research Institute (KFRI) Subcentre, Nilambur. It was ensured that parent moths of such larvae had originated from dark-coloured larvae of the same generation. The density of young instars during the high-density phase range between 50 and 100 larvae per leaf. Therefore, one set of larvae were reared in groups of 100 to the third instar on teak leaves in 20 glass bottles (20 × 10 cm). From the third instar onwards, larvae were reared individually on a semi-synthetic diet (Mathew *et al*., 1990) in plastic rearing tubes (5.5 × 2.5 cm). Another set of larvae was reared individually. The larvae were transferred within 24 h of hatching and fed with teak leaf until the third instar. Thereafter, they were reared on semi-synthetic diet and laboratory conditions were maintained as above.

Dosage–mortality bioassays using HpNPV were carried out in the laboratory to assess the resistance levels (LD_{50} values) of solitary and group reared *H. puera* larvae against viral infections. The virus used in the study was the NDM strain collected originally from the Nedungayam teak plantation. Fifth instar larvae of *H. puera* were starved for 3 h and then fed with 10 µl of HpNPV (amplified and purified from laboratory stock) solution on a tender teak leaf disc (0.5 cm^2). After 2–3 h, the larvae that ate the whole leaf disc were transferred to an artificial diet. Six different viral doses made up in distilled water were used, 2.5×10^1, 9.2×10^2, 5×10^3, 4.8×10^4, 2.5×10^5, and 1.3×10^6 polyhedral occlusion bodies (POBs) per larvae to calculate the LD_{50} values. Control insects were fed with teak leaf discs treated with distilled water. At each dose, 90 larvae each were used for both solitary and group reared forms. They were reared on artificial diet until death or pupation. Mortality due to viral infection was recorded at every 24 h. Viral deaths were confirmed by smearing the haemolymph on a slide and differential Giemsa staining.

Dose–mortality regressions were calculated to determine LD_{50} values and slopes, by analysing the data using probit analysis, including likelihood ratio tests of parallelism and equality (χ^2) with Polo Plus (LeOra Software, 2002). All other statistical tests were conducted in SPSS 16. Where appropriate, treatments were compared by one-way ANOVA and χ^2-tests and data are presented as mean ± SD values unless otherwise stated.

Results

Density of endemic populations collected during 2008, 2010, and 2011 were 0.05 ± 0.22, 0.06 ± 0.24, and 0.02 ± 0.12 larvae/twig (range: 0–1 larvae/twig and $n = 600$ twigs/year), respectively. The proportions of non-melanic fifth instar larvae over the three sampling years were 40%, 11%, and 22%, respectively. Among the younger instars, which were reared in the laboratory to pupal stage, both non-melanic (44%, 53%, and 71%, respectively) and intermediate forms (56%, 47%, and 29%, respectively) were present. The density of outbreak populations of *H. puera* was considerably higher with an average of 9.96 ± 11.46 (range: 0–56; n = 243 twigs), 14.28 ± 11.01 (range: 0–46; n = 180 twigs), and 5.71 ± 4.14 (range: 0–25; n = 153 twigs) larvae/twig during 2008, 2010, and 2011, respectively. All the fifth instar larvae found in the samples (871 of 2421, 36%; 1144 of 2571, 45%, and 544 of 874, 62%, respectively) had uniform black colour.

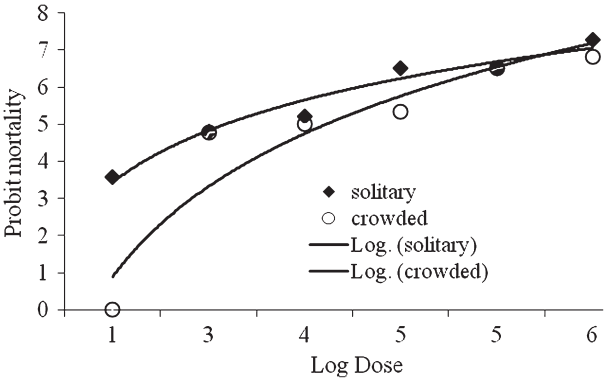

Fig. 2. The mortality faced by larvae of different rearing densities against different doses used. Linear regression: $r^2 = 0.96$ (solitary) and $r^2 = 0.89$ (crowded).

The effects of rearing density on larval colour as observed in the laboratory were highly significant ($\chi^2_3 = 87.99$, $P < 0.0001$). The percentage of melanic and non-melanic forms were 1 and 91, respectively, for solitary forms ($n = 210$) while there was 92% melanic forms and no non-melanic forms in group reared larvae ($n = 270$). Both rearing densities resulted in 8% intermediate forms developing.

Susceptibility of teak defoliator larvae to HpNPV was found to differ with rearing densities; the overall mortality was higher for solitary larvae (66%, 355 of 540) compared with 57% (310 of 540) for crowded larvae (likelihood ratio test of equality: $\chi^2 = 16.15$, $P < 0.05$). The mortality of both solitary and crowded larvae were increased with dose (Fig. 2; linear regression: $R^2 = 0.96$ and $R^2 = 0.89$ for solitary and crowded forms, respectively). The LD_{50} value for larvae reared in groups (5332 POBs/larva) was more than three-fold compared to larvae reared in solitary (1727 POBs/larva; Table 1). The time to death after inoculation of virus was significantly higher in the crowded treatment (3.6 days) compared to the solitary treatment (3.3 days; $F_{1,660} = 39.0$, $P < 0.0001$).

Discussion

In field populations of *H. puera*, colour polyphenism has been observed (Nair, 2007), but its density dependence has not been tested so far. This study has provided direct

Table 1. Dose–mortality regression showing slope and LD_{50} values and its 95% confidence limits.

Density	n	LD_{50} (95% confidence interval)	Slope (± SE)	t ratio	χ^2(df)
Solitary	540	1.73×10^3 (8.08×10^2 – 3.34×10^3)	0.79 (±0.06)	13.60	5.23 (4)
Crowded	540	5.33×10^3 (9.71×10^2 – 2.05×10^4)	0.74 (±0.06)	13.39	20.62 (4)

evidence for an increasing degree of cuticular melanism (bright orange to black) in response to increasing population density in the field and laboratory. The density-dependent colour polyphenism have previously been explained in terms of strategies for antipredation, camouflage, density-dependent prophylaxis, etc. (Hagen *et al*., 2003). In several density-dependent polyphenic species, the high-density forms are aposematic (warning coloured and unpalatable). It has been hypothesised that density-dependent melanism might have evolved as an antipredator strategy in which aposematism is selected for high density and crypsis is selected for low-density populations (Wilson *et al*., 2001). However, this is not true in the case of *H. puera*, as the outbreak population is darker in colour rather than the warning colorations exhibited by high-density aposematics. In contrast, the low-density, endemic forms exhibit much brighter coloration (bright orange/red and black stripes) than outbreak individuals. Moreover, large assemblages of avian insectivores exclusively feeding on *H. puera* larvae are commonly found during the outbreak period. According to the second prediction, if larval coloration is an adaptation to blend with the background, the melanic form should provide better camouflage at outbreak period whereas the non-melanic form should provide better camouflage at low population density. The dark larvae on dark green leaves during *H. puera* outbreaks can provide some extent of masking from the predators but the bright coloration of the endemic population in the same background leaf coloration is in contrast with the background matching hypothesis (Hagen *et al*., 2003). Therefore, crypsis and aposematism as an antipredation mechanism, and camouflage may not be a likely explanation for the density-dependent colour polyphenism in *H. peura*.

The third possibility, the density-dependent prophylaxis hypothesis, which states that polyphenism is an external manifestation of resistance build-up against density-dependent pathogen transmission (Wilson & Reeson, 1998). If the density-dependent pathogen transmission exists, it is possible that insects will use the contact rate with other individuals as a cue to match their levels of investment in immune function to the perceived risk of transmission (Wilson & Reeson, 1998; Wilson *et al*., 2001; Hagen *et al*., 2003). In outbreak populations of *H. puera*, the crowded situation during the younger instars may serve as the cue for being melanic. The larvae reared gregariously in laboratory conditions and later singly after attaining the third instar stage developed darker coloration, whereas isolation of the larvae within 24 h post-hatching resulted in bright coloration. It was also found that the group reared *H. puera* forms exhibiting a three-fold higher resistance (in terms of LD_{50} values) towards HpNPV infection than solitary. The co-occurrence of melanism and increased levels of disease resistance (8–10-fold) in the high-density forms were reported for several phase polyphenic insects such as *S. littoralis* (Cotter *et al*., 2004a), *O. brumata* (Hagen *et al*., 2003), *Mythimna separata* (Kunimi & Yamada, 1990), and *S. exempta* (Reeson *et al*., 1998, 2000; Vilaplana *et al*., 2008). However, the reverse effect was found in *L. dispar* against *Lymantria dispar* multicapsid nuclear polyhedrosis virus (LdMNPV) (Reilly & Hajek, 2008). The co-occurrence of melanism and increased resistance in the outbreak populations of *H. peura* suggest larval contact rate as a proximate mechanism for developing melanism as observed in other studies (Hagen *et al*., 2003), but require further experimental evidences.

The physiological mechanism behind the density-dependent prophylactic response was not tested in this study. However, it is known that melanin enhances disease resistance in insects by improving both physical and chemical properties of insect cuticle (Wilson *et al*., 2001). The process of melanisation involves phenoloxidase enzyme, which is also involved in various immune responses directed against parasites and pathogens, including encapsulation reactions (Reeson *et al*., 1998; Wilson *et al*., 2001). Previous studies have shown a link between the larval densities, production of melanin, and elevated phenoloxidase activity, which led to increased resistance (Reeson *et al*., 1998; Wilson *et al*., 2001, 2002; Cotter *et al*., 2004a). Moreover, trade-offs of immune function and life-history traits were also recorded in phase polyphenic insects (Cotter *et al*., 2004a,b, 2008; Vilaplana *et al*., 2008). In contrast, other studies indicate that higher levels of phenoloxidase may not account for larval survival or for every case of resistance to baculovirus infection (Saejeng *et al*., 2010). High cuticular melanisation in *H. puera* is related to larval density, and disease resistance is theoretically linked to larval density, but it is not clear whether the cuticular melanisation is linked to disease resistance, which deserves further studies.

In conclusion, this study revealed the existence of density-dependent colour polyphenism and resistance build-up against invading baculovirus by *H. puera* larvae. As the population dynamics of the insects are associated with density-dependent pathogen transmission, which is strongly linked with the disease resistance level of the pests, caution must be taken while considering the success and economics of biological control programmes.

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