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Baculovirus resistance in the noctuid *Spodoptera exempta* is phenotypically plastic and responds to population density

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Parasite resistance mechanisms can be costly to maintain. We would therefore predict that organisms should invest in resistance only when it is likely to be required. Insects that show density-dependent phase polyphenism, developing different phenotypes at high and low population densities, have the opportunity to match their levels of investment in resistance with the likelihood of exposure to pathogens. As high population densities often precipitate disease epidemics, the high-density form should be selected to invest relatively more in resistance. We tested this prediction in larvae of the noctuid *Spodoptera exempta*. Larvae reared at a high density were found to be considerably more resistant to a nuclear polyhedrosis virus than those reared in isolation. A conspicuous feature of the high-density phase of *S. exempta* and other phase-polyphenic Lepidoptera is cuticular melanization. As melanization is controlled by the phenoloxidase enzyme system, which is also involved in the immune response, this suggests a possible mechanism for increased resistance at high population densities. We demonstrated that melanized *S. exempta* larvae were more resistant than non-melanized forms, independent of rearing density. We also found that haemolymph phenoloxidase activity was correlated with cuticular melanization, providing further evidence for a link between melanization and immunity. These results suggest that pathogen resistance in *S. exempta* is phenotypically plastic, and that the melanized cuticles characteristic of the high-density form may be indicative of a more active immune system.

Keywords: phase polyphenism; *Spodoptera exempta*; phenoloxidase; crowding; pathogen resistance; melanization

1. INTRODUCTION

Recent experiments with insects have demonstrated that resistance to parasites and pathogens may be costly to maintain (Fuxa & Richter 1989; Boots & Begon 1993; Sait *et al*. 1994; Kraaijeveld & Godfray 1997). We can therefore expect organisms to be under strong selection to invoke resistance mechanisms only when they are likely to be required. The threat of an individual coming into contact with pathogens often increases with population density, as the transmission process is usually density dependent (Anderson & May 1981). Therefore, insects that live at high population densities should be selected to invest relatively more in resistance than those that are accustomed to low densities. There is good evidence from a cross-species comparison that this is indeed the case among the Lepidoptera, with gregariously feeding species showing higher resistance levels than solitary feeding species. (Hochberg 1991*a*).

However, if population density fluctuates, then the optimal level of investment in resistance may differ markedly for individuals in successive generations (Wilson & Reeson 1998). Such fluctuations are particularly common among insects, owing to their high reproductive rates. If changes in population density are unpredictable from one generation to the next, then an individual has to rely on extrinsic cues to match its phenotype to its environment. This type of phenotypic plasticity, known as density-dependent phase polyphenism, occurs in locusts, planthoppers, aphids and some larval Lepidoptera. In these insects, the amount of tactile stimulation received during the early larval stages generally determines which of two extreme phenotypes an individual adopts (Ellis 1959; Kazimirova 1992). High-density forms differ from low-density conspecifics in a variety of features including colour, behaviour and development time (Long 1953; Tojo *et al*. 1985; Pener 1991; Gunn 1998). These changes are generally assumed to have evolved in response to increased intraspecific competition for food or density-dependent predation pressures. Here we examine a third possibility, that they are manifestations of a response to the increased risk from pathogens at high population densities. This hypothesis has rarely been tested experimentally (Wilson & Reeson 1998), but there is some evidence for a density-dependent increase in viral resistance from two species of Lepidoptera exhibiting

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phase polyphenism (Kunimi & Yamada 1990; Goulson & Cory 1995).

Cuticular melanization is a common feature in the crowded form of many phase-polyphenic Lepidoptera. The process of melanization involves phenoloxidase (PO) enzymes, which oxidize tyrosine derivatives to quinones, which can then form melanin (Gotz & Boman 1985). The phenoloxidase enzyme system is also believed to play a key role in the insect immune response, especially the encapsulation reaction (Ashida & Brey 1997) that occurs in the haemolymph in response to invasion by a range of parasites and pathogens, including nematodes, parasitoids, fungi, bacteria, protozoa and viruses (Poinar 1974; Gotz 1986; Hung & Boucias 1992; Walters & Ratcliffe 1983; Paskewitz *et al*. 1988; Washburn *et al*. 1996). Several different types of phenoloxidase have been isolated from the haemolymph and cuticle of various insects (Sugumaran & Kanost 1993), but no consensus has been reached as to the relationship between these enzymes. Hiruma & Riddiford (1988) found that the granular phenoloxidase responsible for cuticular melanization in *Manduca sexta* was distinct from cuticular wound-healing PO and haemolymph PO. However, Aso *et al*. (1985) suggest that haemolymph prophenoloxidase may be a precursor to both haemolymph and cuticular phenoloxidase, and genetic evidence suggests that in *Drosophila* the same structural locus codes for both haemolymph and cuticular PO (Pentz *et al*. 1986). In the silkworm *Bombyx mori*, cuticular prophenoloxidase is synthesized in the haemocytes before being actively transported into the cuticle (Ashida & Brey 1995). This apparent link between phenoloxidase enzymes in the cuticle and the haemolymph raises the possibility that the cuticular melanization that occurs in high-density populations of some Lepidoptera may be an indication of a more active immune system (Wilson *et al*. 1998).

We can therefore make a number of predictions about pathogen resistance in insects that show density-dependent phase polyphenism. First, because resistance to pathogens will be at a greater premium in high-density populations, (i) the high-density phase will show greater resistance to pathogens than the low-density phase. Second, the observation that high population densities trigger cuticular melanization in such species, suggests that any increases in resistance may be as a result of higher levels of phenoloxidase activity. If this is the case, we would predict that (ii) after controlling for larval density, resistance will be positively correlated with cuticular melanization. This second prediction assumes that there is a link between cuticular melanization and phenoloxidase activity in other parts of the body. We can test this assumption by making a third prediction that (iii) cuticular melanization will be positively correlated with haemolymph phenoloxidase activity. The system used to test these predictions was the African armyworm *Spodoptera exempta* (Lepidoptera: Noctuidae) and its nuclear polyhedrosis virus (NPV). *S. exempta* exhibits extreme density-dependent phase polyphenism, with individuals reared at a high density becoming heavily melanized during the later larval instars. The high-density phase is also associated with differences in behaviour and development time (Simmonds & Blaney 1986). Larvae reared in isolation typically exhibit green or brown coloration (and will henceforth be referred to as green larvae). However, some individuals reared solitarily have melanized cuticles (and will be referred to as black larvae), and this provides an opportunity for examining the effects of melanization independently of rearing density.

2. METHODS

(a) *Rearing of larvae*

For the purposes of our experiments, *S. exempta* larvae from an inbred laboratory stock were reared, within 24 h of hatching, at densities of either one (solitary) or six (crowded) per 12-ml plastic pot. During this time they were fed on freshly cut wheat seedlings. All the larvae used in each experiment had hatched on the same day. Larvae reared in crowds were black by the third instar, and although most of those reared in isolation developed the green/brown coloration characteristic of low-density field populations, a small proportion (15%) turned black.

(b) *Virus bioassays*

Larvae were exposed to a virus within 24 h of the onset of the fourth instar (about 8 d after hatching). The virus was administered by providing the larvae with small plugs of an artificial diet containing 1 µl of virus solution. The diet-plug method was used as it ensures that all larvae consume the same amount of virus; there may be differences in the time taken to consume the virus, but this is unlikely to affect susceptibility (Milks 1997). Five different doses were used, 3.5×10^4, 1.1×10^4, 3.5×10^3, 1.1×10^3, and 3.5×10^2 polyhedral inclusion bodies (PIBs) per larva, as well as a control in which 1 µl of water was added to the diet plugs. Any larva that did not consume the whole diet plug within a 24 h period was discarded. At each dose, 40 crowded, 40 solitary, green and 20 solitary, black larvae were used. They were then reared in individual pots containing the artificial diet, until death or pupation. Viral death was diagnosed by smearing the dead larvae onto a slide and staining with Giemsa. These slides were examined at ×1000 magnification for the presence of PIBs. Two replicates of this procedure were done.

(c) *Phenoloxidase assay*

Haemolymph (5 µl) was extracted from larvae at the start of the fifth instar, and placed into 200 µl of phosphate-buffered saline in a plastic Eppendorf tube. PO activity was assayed spectrophotometrically using L-DOPA as a substrate, a method modified from Ashida & Soderhall (1984): 900 µl of 10 mM L-DOPA was added to the buffered haemolymph and the mixture was incubated for 20 min at 25 °C. The mixture was then transferred to a plastic cuvette and the absorbance at 475 nm measured. A control group had 50 µl of 10% phenylthiourea (PTU) (which inhibits PO) added to the buffered haemolymph.

(d) *Data analysis*

Data were analysed using the generalized linear interactive modelling (GLIM) program (McCullagh & Nelder 1989). Data from the two replicates of the viral bio-assays were combined, with replicates included as a factor in the analysis. All terms were initially included in the model, along with all interactions. Terms that did not contribute to the fit of the model were then removed. Mortality was analysed using binomial errors and logit link, whereas time until death and PO activity were log-transformed and analysed with normal errors. Inspection of

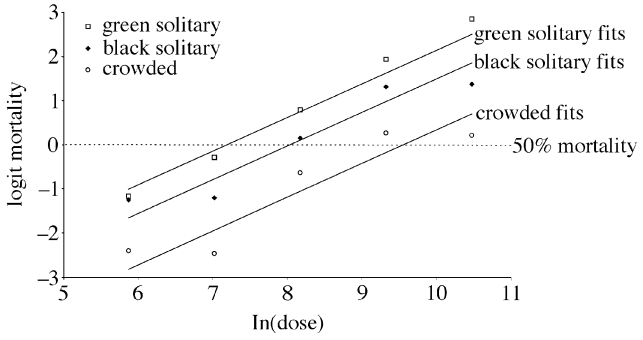

Figure 1. The levels of mortality experienced by each colour and rearing-density treatment across the range of doses used, showing data (points) and fitted values (solid lines). Solitary larvae suffered significantly higher mortality than those reared in crowds. Among the solitary larvae, green larvae suffered significantly higher mortality than black larvae.

residuals confirmed that all models conformed to the assumptions of the general linear model.

3. RESULTS

(a) *Virus bioassays*

Susceptibility to NPV was found to vary considerably with both larval colour and rearing density (figure 1); across the range of doses used, overall mortality was 70% for green solitaries compared with 59% for black solitaries and 42% for crowd-reared larvae. Statistical analysis showed the larvae reared in isolation suffered significantly higher mortality than those reared in crowded conditions ($\chi^2_2 = 48.6$, $p < 0.001$). Among the larvae reared in isolation, those that were green at the start of the fourth instar were significantly more susceptible to NPV than black individuals ($\chi^2_1 = 7.06$, $p < 0.01$). The LD_{50}s for solitary green, solitary black and crowded larvae were 1325, 3082 and 14 188 PIBs per larva, respectively. There was a significant correlation between dose (total number of PIBs consumed) and mortality ($\chi^2_1 = 150.7$, $p < 0.001$). There was no significant interaction between dose and larval colour or rearing density. Mean larval weight for each group was tested for inclusion in the model but was found to be non-significant ($\chi^2_1 = 0.82$, $p > 0.05$). There were no viral deaths among any of the larvae in the control groups.

Considering only larvae that died from viral infection, time until death also varied with rearing density (figure 2). Larvae reared in crowded conditions took significantly longer to die than those reared in isolation ($F_{2,540} = 74.3$, $p < 0.0001$). Among the solitary larvae, green individuals died sooner than black larvae ($F_{1,381} = 4.21$, $p < 0.05$). The mean times to death were 8.3, 7.8 and 7.4 days for crowded, solitary black and solitary green larvae, respectively. Speed of kill was positively correlated with dose ($F_{1,540} = 48.8$, $p < 0.0001$) and negatively correlated with larval weight at the time of exposure to the virus ($F_{1,540} = 79.9$, $p < 0.0001$). All interaction terms were non-significant.

(b) *Phenoloxidase assays*

Phenoloxidase activity in the haemolymph varied according to the colour of the larvae from which it

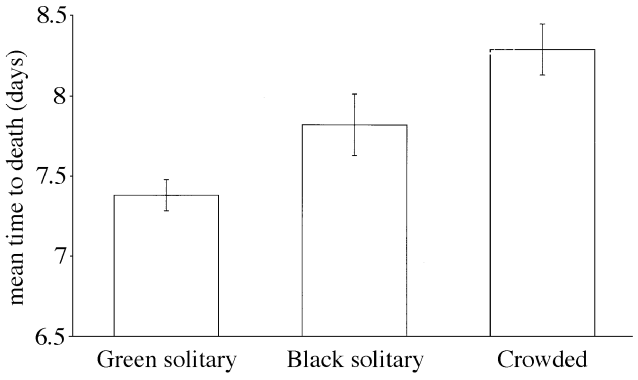

Figure 2. The time elapsed between exposure to virus and viral death, showing means and standard errors. The differences between the groups are significant. Crowded larvae took on average around a day longer to die than solitary green forms. Solitary black larvae were intermediate.

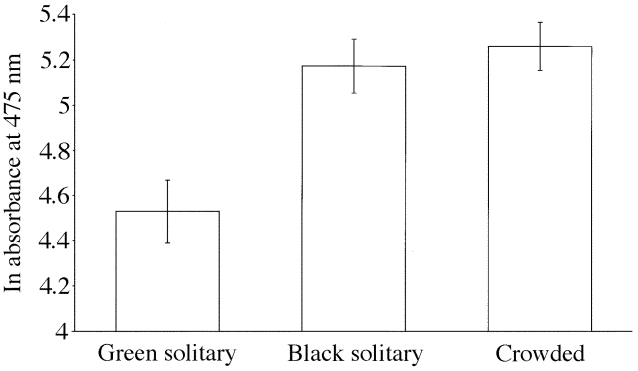

Figure 3. Haemolymph phenoloxidase activity (measured by melanin production), showing means and standard errors. There was significantly more phenoloxidase activity among both the crowded and the solitary/black larvae than in the solitary green larvae. There were no differences between solitary black and crowded larvae.

was extracted (figure 3). Model simplification resulted in solitary black and crowded larvae being combined ($F_{1,110} = 0.06$, $p > 0.05$). PO activity was significantly higher in solitary black and crowded (black) larvae than in green larvae ($F_{1,110} = 30.5$, $p < 0.0001$). When PTU was added to the reaction mixture, no melanization occurred. This supports the assumption that the melanization observed in the absence of PTU is a result of phenoloxidase activity.

4. DISCUSSION

The first prediction, that the high-density phase of a polyphenic species should show increased resistance to pathogens, is supported by this study. Larvae reared in crowds were found to have an LD_{50} more than ten times that of the typical solitary forms. The magnitude of this difference suggests that the high-density phase has been selected for increased resistance. Pathogens, and in particular NPV, are known to be an important mortality factor in outbreak populations of *S. exempta* in the field (Persson 1981; Odindo 1983). There are differences in development time between the two phases, with larvae reared in crowds tending to pupate earlier than those

reared in isolation (Simmonds & Blaney 1986). However, we believe that this is unlikely to have caused the differences in susceptibility observed in this experiment, as the larvae were dosed at the start of the fourth (penultimate) instar before development time differences become apparent, and the viral deaths occurred before any larvae entered the pre-pupal stage. Furthermore, the longer time to death among crowd-reared larvae would not be expected to result from a faster development rate.

Most models of insect–pathogen dynamics assume that the rate of transmission of a pathogen is proportional to the number of encounters between susceptible hosts and infectious pathogens (e.g. Anderson & May 1981). The transmission coefficient, which describes the probability of each encounter resulting in a new infection, is assumed to be constant. The increase in resistance seen at high host densities in *S. exempta* violates this assumption, and if repeated in field conditions would result in lower than expected rates of transmission among high-density populations. This could have the effect of destabilizing the host–pathogen interaction (Liu *et al*. 1986; Hochberg 1991*b*) and may contribute to the boom-and-bust nature of the population dynamics of *S. exempta* and other outbreak Lepidoptera.

The second prediction was that pathogen resistance would be correlated with cuticular melanization, independent of rearing density. Again this was supported by the data, with solitary black larvae having an LD_{50} 2.5 times greater than that of solitary green larvae. This suggests that the higher phenoloxidase levels associated with cuticular melanization in crowd-reared larvae may be responsible for the differences in resistance between the phases. However, there were still considerable differences in resistance between solitary black and crowded (black) larvae, even though there were no apparent differences in colour. In addition to differences in resistance, rearing density and colour were also correlated with the time taken for larvae to die from NPV infection. Larvae reared in crowds took, on average, a day longer than solitary green larvae to succumb, while the time-length before the deaths of solitary black larvae was intermediate. This may be a result of a more active immune response in crowded and melanized larvae slowing down the initial spread of the virus through the tissues. The shorter time before death observed in solitary larvae would allow a pathogen to spread more rapidly from one host to another, and may therefore be expected to reduce the threshold host-population level necessary to maintain a pathogen (Anderson & May 1981). However, among Lepidoptera and their NPVs, larvae that die sooner tend to release fewer infectious pathogens (P. Hernandez-Crespo, personal communication), which would increase the threshold host density.

The third prediction, that cuticular melanization would be correlated with phenoloxidase levels, was also supported, with solitary black larvae showing significantly higher phenoloxidase activity than green larvae. There were no differences in phenoloxidase activity between solitary black and crowded (black) larvae. Variation in phenoloxidase levels may therefore provide an explanation for differences in resistance between solitary green and solitary black larvae, but not for the differences between solitary black and crowded (black) larvae. This link between phase polyphenism and haemolymph phenoloxidase activity provides further support for the hypothesis that the colour variation in phase-polyphenic species is related to phenotypic plasticity in pathogen resistance. Because the route of infection of the NPV used in these experiments is through the midgut, higher phenoloxidase levels in the cuticle should not in themselves affect susceptibility. For most lepidopteran NPVs, initial viral replication occurs in the cells lining the midgut and the virus then spreads to other tissues via the haemolymph (Federici 1997), so phenoloxidase levels in the midgut cells and haemolymph will have a greater bearing on the chances of an infection taking hold. (However, cuticular phenoloxidases may have an important role in resisting pathogens that penetrate through the cuticle, such as entomopathogenic fungi.) The results of this experiment suggest that, whatever the exact relationships of the different enzymes involved, haemolymph phenoloxidase activity is linked to cuticular melanization. These higher levels of phenoloxidase in the haemolymph could lead to a more rapid and vigorous melanization–encapsulation reaction in response to exposure to a pathogen.

The results presented here strongly suggest that insects may respond in an adaptive manner to cues that predict an increased threat of pathogen attack later in life. Crowding during the early larval instars may be the cue that triggers an increase in phenoloxidase levels, enabling a larva to mount a more vigorous response to a future infection. In addition to baculoviruses, this mechanism may also confer increased resistance to other natural enemies such as parasitoids, which are common among wild *S. exempta* populations (Merrett 1986). These higher phenoloxidase levels may also be responsible for the cuticular melanization that is characteristic of the high-density forms of many polyphenic species. The observation that solitary black larvae are still considerably less resistant than crowded (black) larvae, despite having similar haemolymph phenoloxidase levels, suggests that crowding must also stimulate some other, as yet unknown, mechanism of resistance. We believe that an increased investment in prophylactic resistance in response to high population densities is a widespread phenomenon (Wilson & Reeson 1998). However, it is likely to be most clearly manifested in species, such as *S. exempta*, that exhibit density-dependent phase polyphenism. Indeed, many of the adaptations associated with phase-polyphenic species must now be reassessed in terms of their potential role in defence against pathogens.

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