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Density-dependent prophylaxis in the mealworm beetle *Tenebrio molitor* L. (Coleoptera: Tenebrionidae): cuticular melanization is an indicator of investment in immunity

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If there are costs involved with the maintenance of pathogen resistance, then higher investment in this trait is expected when the risk of pathogenesis is high. One situation in which the risk of pathogenesis is elevated is at increased conspecific density. This paper reports the results of a study of density-dependent polyphenism in pathogen resistance and immune function in the mealworm beetle *Tenebrio molitor*. Beetles reared at high larval densities showed lower mortality when exposed to a generalist entomopathogenic fungus and a higher degree of cuticular melanization than those reared solitarily. The degree of cuticular melanization was a strong indicator of resistance, with darker beetles being more resistant than lighter ones regardless of rearing density. No differences were found between rearing densities in the levels of phenoloxidase, an enzyme key to the insect immune response. The results show that pathogen resistance is phenotypically plastic in *T. molitor*, suggesting that the maintenance of this trait is costly.

Keywords: *Tenebrio molitor*; phenoloxidase; density-dependent prophylaxis; cost of immunity; phenotypic plasticity

1. INTRODUCTION

Many organisms experience irregular and unpredictable fluctuations in population density (Begon *et al*. 1990). If the fitness costs and benefits of possessing a trait vary at different conspecific densities, then that trait should be phenotypically plastic with respect to density, provided that the organism is able to assess this (Via *et al*. 1995). Many insects are able to make such assessments during the larval stages (Dingle 1996) and make subsequent adjustments to their phenotype. A wide range of traits show such 'density-dependent phase polyphenism', including coloration, wing and sensilla morphology, developmental rate, hormone metabolism, pheromone production, reproductive potential and behaviour (see Applebaum & Heifetz (1999) for a review).

A potentially important life-history determinant showing density-dependent variation is the risk of pathogenesis. For most organisms, conspecifics are the main source of disease (Freeland 1983). Contact with increasing numbers of conspecifics raises the probability of infection (Steinhaus 1958) and, hence, the likelihood of needing to mount an immune response. Invertebrates have no specific (antibody–antigen-based) immunity, but mount a general response towards all material recognized as non-self (e.g. Ratcliffe *et al*. 1985). This contrasts with the vertebrate system in which previous exposure to a pathogen results in acquired immunity, largely in the form of an antibody producing memory B cells (Roitt 1988). Thus, in invertebrates, investment in a general immune response has the potential to increase resistance to all pathogen types and underinvestment at high densities can lead to loss of fitness due to the costs of pathogenesis (Råberg *et al*. 1998). However, mechanisms by which organisms resist parasites and pathogens can also be costly to maintain and express (Sheldon & Verhulst 1996; Kraaijeveld & Godfray 1997). Mounting an immune response involves the generation of toxic compounds and the risk of non-specific immune action damaging the host's own tissues (e.g. Råberg *et al*. 1998). In addition to autoimmune effects, immune function may also be traded off with other traits, such as larval competitive ability, as occurs in *Drosophila melanogaster* (Kraaijeveld & Godfray 1997). Consequently, insects which are able to assess population density should adjust their investment in general immunity according to the perceived risk of infection in order to balance these costs and benefits (density-dependent prophylaxis (DDP); Wilson & Reeson 1998).

Wilson & Reeson (1998) presented evidence that DDP can occur in a specifically coevolved system (baculoviruses and their lepidopteran hosts). It has been demonstrated that larvae of the noctuid moth *Spodoptera exempta* are more resistant to their baculovirus when reared at high population densities. The activity in the haemolymph of phenoloxidase (PO) and phase polyphenism in the level of cuticular melanization (black larvae were more resistant than green larvae) were positively correlated with resistance (Reeson *et al*. 1998). PO is a key enzyme in the synthesis of melanin and the ability to produce melanin is an important aspect of the insect immune response (Gillespie *et al*. 1997). PO is a key enzyme in resistance to a number of pathogen types (Chapman 1982). Internal parasites are usually isolated within a melanized layer of haemocytes (Gillespie *et al*. 1997) and insects with a greater ability to melanize their internal parasites are less susceptible to pathogenesis (Nappi *et al*. 1991). Thus, PO activity in the haemocoel

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and, hence, melanin synthesis is potentially a key determinant of resistance to parasites. The synthesis of melanin invokes a specific cost because it involves the generation of reactive oxygen species and quinones in the haemocoel which are harmful to the host's tissues (e.g. Nappi *et al*. 1995). Consequently, this trait is likely to exhibit phenotypic plasticity dependent on the risk of parasite encounter and, as such, it is a trait which would be expected to show density-dependent polyphenism.

This study aims to test the predictions of the theory of DDP by studying the mortality of the mealworm beetle *Tenebrio molitor* (Coleoptera: Tenebrionidae) when exposed to the entomopathogenic fungus *Metarhizium anisopliae* (strain Fl42). Tenebrionids are stored product pests and frequently undergo changes in population density (Tschinkel & Willson 1971). A number of traits show density-dependent polyphenism. At high density, larvae have been observed to experience extra larval moults (Weaver & Macfarlane 1990; Connat *et al*. 1991; Kotaki & Fujii 1995), increased mortality and cannibalism (Savvidou & Bell 1994). Immature phases of tenebrionids appear to be able to assess the density of conspecifics using both mechanical and chemical cues (Tschinkel & Willson 1971; Kotaki & Fujii 1995).

In this study, investment in immunity was assessed by measuring the mortality of adult beetles reared at different densities when exposed to *M. anisopliae*. This is a generalist insect pathogen to which the study population of beetles has never been exposed (60+ generations) and, as such, is ideal for studying investment in non-specific immunity. This is therefore the first test of DDP in a non-coevolved insect–pathogen system. In coevolved systems in which a population has had prior exposure to a specific pathogen, it is possible for individuals to become more reactive to that pathogen (Dularay & Lackie 1987) or for the pathogen to evolve a way to evade the host immune response (Huxham *et al*. 1988). Thus, using a novel generalist pathogen ensures that any plasticity in host susceptibility cannot be attributed to differential investment in coevolved host responses, but rather to investment in a general prophylactic response. Fungus is also suitable because the host's immune system responds to infection by melanizing the fungal hyphae (Hajek & St Leger 1994) which invade the haemocoel through the cuticle (Goettel & Inglis 1997) and, once inside, activate the host's PO (Söderhäll *et al*. 1979).

The central aim of this study was to examine whether investment in immunity shows phenotypic plasticity with respect to rearing density in *T. molitor*. Two measures of immunity were used: a direct measure of the mortality of beetles to an entomopathogenic fungus and an assay of an important immune effector system (PO) known to correlate with DDP in another insect order (Reeson *et al*. 1998). Cuticular colour, being commonly plastic with respect to population density in a number of insects (Applebaum & Heifetz 1999) and correlated with resistance in a lepidopteran (Reeson *et al*. 1998), was also assessed.

2. METHODS

(a) *Beetle cultures*

The beetles were from a single genetically depauperate inbred line maintained at the University of Sheffield, UK. Experimental cultures were set up using larvae less than one week old (and less than 4 mm in length) which had hatched from a single cohort of eggs. The larvae were assigned to either 'solitary' or 'gregarious' cultures. Solitary cultures ($n = 215$) consisted of a single animal in *ca.* 130 cm^3 of rat chow, whilst gregarious cultures ($n = 16$) consisted of 15 individuals in *ca.* 400 cm^3 of rat chow. This amount of feed was *ad libitum*. As a source of fluids, 0.5 g of lettuce per beetle per culture was provided biweekly.

(b) *Preparation of fungal inocula*

Spore preparations and assays were performed as described by Goettel & Inglis (1997). Fungus was cultured on potato dextrose agar (PDA) plates and doses were prepared by scraping fungal spores into a 0.05% Triton X-100 solution. The spore concentration was determined by counting spores with the aid of a compound microscope (Leitz Diaplan) using a haemocytometer. Only intact spores were counted. Inoculates were never older than 24 h and were stored at 4 °C when not in use.

(c) *Assignment to treatments*

The beetles were removed from their cultures upon pupation, sexed and then randomly assigned to either the fungal or PO assays. Sixty males and 60 females from each rearing density were assigned for use in the fungal assay. Twenty males and 20 females from solitary cultures and two beetles per sex per gregarious culture were randomly assigned to the PO assay. On imaginal emergence, the beetles were weighed and the date of emergence noted. Beetles failing to emerge properly (defined by failure of the elytra to meet posteriorly) were excluded from the experiment.

(d) *Phenoloxidase assay*

Twenty-four hours after emerging as adults, haemolymph extracts were taken by washing the abdomen through with 2 ml of ice-cold sodium cacodylate buffer (0.01 M Na-coc and 0.005 M $CaCl_2$). Samples were immediately frozen at −30 °C to disrupt the haemocyte membranes.

The frozen samples were thawed for 3 min at 30 °C followed by centrifugation (2800 *g* at 4 °C for 15 min). The supernatants were removed and vortexed after which 500 µl of supernatant was mixed with 1 ml of 3 mM L-DOPA in sodium cacodylate solution and the reaction allowed to proceed at 30 °C in a spectrophotometer (Pharmacia Biotech Ultraspec 2000) for 20 min. Readings were taken every minute at 490 nm and analysed using Swift II software (Pharmacia Biotech). The enzyme activity was measured as the slope of the reaction curve during the linear phase of the reaction (between 5 and 15 min after the reaction mix was made; A. I. Barnes, personal observation).

(e) *Fungal assay*

The beetles were assayed on the second day after imaginal emergence. Twenty beetles from each rearing density (ten males and ten females) were assigned to each of one of six doses of fungus (0.0, 1.0×10^7, 2.5×10^7, 5.0×10^7, 7.5×10^7 and 1.0×10^8 spores ml^{-1}). The doses were adjusted for beetle size by determining the surface area of the beetle from its weight using the following conversion: surface area (cm^2) = 10 (weight (g))$^{0.67}$ (Schmidt-Nielsen 1984).

The fungal doses listed above were then defined as the doses for an average beetle (0.116 g in weight and 2.38 cm^2 surface area; A. I. Barnes, personal observation). Doses for beetles deviating from this measurement were scaled using the following formula: actual dose = (2.38/s.a.) assigned dose.

Table 1. *Traits exhibiting density-dependent phase polyphenism in* T. molitor

(Errors (where given) are standard deviations. *χ^2-test, $p < 0.001$; **t-test, $p < 0.001$.)

	elytra colour*					
rearing density	black	brown	tan	mean larval period (days)*	adult weight (mg)**	egg-to-adult mortality*
solitary	8%	43%	49%	140 ± 7.7	103 ± 14.2	3%
gregarious	15%	59%	26%	115 ± 8.5	117 ± 18.2	24%

The beetles were exposed to the fungus by dipping them in 1 ml of spore solution for 5 s. Control beetles were dipped in 0.05% Triton X-100 with no fungal spores. The beetles were individually incubated at 26 °C and 70% relative humidity for six days after which mortality was scored.

(f) *Assessment of colour*

On the fourth day after eclosion, the elytra colour of the beetles was subjectively assessed under an 11 W fluorescent white light. Fully developed adult beetles were defined as either 'black', 'brown' or 'tan' using the following criteria. The elytra of 'tan' beetles were light brown in colour and this was clearly visible under normal, diffuse lighting. The elytra of 'brown' beetles were not easily distinguishable from 'black' beetles except under direct illumination where they appeared dark brown. 'Black' beetles showed no obvious brown coloration to their elytra, even under strong, direct illumination.

In order to test whether these subjective colour classes represented real differences in cuticular colour, ten elytra representative of each colour class were analysed using images (collected using a Sony TR2000E camera under 11 W fluorescent illumination) digitized in Optimas 6 software. The elytra darkness was measured as a greyscale value between 0 and 255. There was a significant effect of colour on the darkness greyscale values obtained (ANOVA, d.f. = 2, $F = 179.537$ and $p < 0.0001$). The greyscale values were significantly different between each colour class (Tukey multiple comparison test $p < 0.001$).

(g) *Analysis*

The mortality and colour data were analysed using logistical regression analysis in Statview 5.0 (SAS Institute, Inc.). The PO data were transformed to normality by the following formula: PO = log(slope + 0.0001). These data were then analysed using ANOVA in Statview 5.0 (SAS Institute, Inc.).

3. RESULTS

(a) *Phenotypic plasticity in egg-to-adult development*

Gregarious larvae reached adulthood significantly earlier (d.f. = 338, $t = 27.78$ and $p < 0.001$) and gave rise to significantly heavier adults (Welch's unequal t-test d.f. = 336, $W = 53.91$ and $p < 0.001$) than solitary larvae (table 1). Gregarious larvae suffered significantly higher mortality (d.f. = 1, $\chi^2 = 26.42$ and $p < 0.0001$) than solitary larvae (table 1).

Gregarious beetles were also significantly darker than solitary beetles (d.f. = 2, $\chi^2 = 14.21$ and $p < 0.001$) (table 1). When rearing density, sex, weight and development time were included as terms in a logistical regression for the model 'colour', only rearing density contributed significantly to the fit of the model (d.f. = 2, $\chi^2 = 14.41$ and $p < 0.001$).

(b) *Fungal bioassay*

There was no mortality in the control groups, so all mortality in the fungus-treated groups was assumed to be due to the action of the fungus alone. It was not possible to assign specific LD_{50} values because the doses resulted in very high mortality. However, the mortality differed significantly between the two rearing densities across the range of doses (d.f. = 1, $\chi^2 = 5.88$ and $p = 0.015$) (figure 1). When the terms rearing density, colour, dose, sex and adult weight were included in a logistical regression for the model 'mortality' only two contributed to the fit: dose (d.f. = 1, $\chi^2 = 7.23$ and $p = 0.007$) and colour (d.f. = 1, $\chi^2 = 35.61$ and $p < 0.001$).

The percentage mortality (irrespective of dose) was significantly different between colour morphs (black = 29%, brown = 65% and tan = 91%) (d.f. = 2, $\chi^2 = 37.09$ and $p < 0.001$) (figure 2). A logistical regression for the model 'mortality' performed within each colour morph including the terms development time and weight indicated that neither of these contributed significantly to the fits of the models.

(c) *Phenoloxidase assay*

There was no significant difference in the haemolymph PO activity between the gregarious (mean = 0.006 ± 0.010) and solitary (mean = 0.009 ± 0.014) beetles (d.f. = 1, $F = 2.532$ and $p = 0.115$).

4. DISCUSSION

Investment in immunity shows density-dependent phase polyphenism in the mealworm beetle *T. molitor*: animals reared at higher density were more resistant to the entomopathogenic fungus *M. anisopliae* (figure 1). This resistance was correlated with the level of cuticular melanization in the beetles, with darker beetles being more resistant (figure 2). Thus, gregariously raised beetles tended to be darker than those raised solitarily and darker beetles were more resistant than lighter ones. There was no difference in the haemolymph PO activity between the two rearing densities.

These results support the theory that investment in innate immunity is mediated by the probability that an immune challenge will be encountered, specifically based on the number of conspecifics present (Wilson & Reeson 1998) and that this redistribution of resources is adaptive. An alternative non-adaptive explanation (that gregariously raised beetles are simply in better condition and have more total resources to invest in immunity) may seem to be supported by the fact that the solitary beetles were lighter and slower in developing (table 1). However, if size and development time were indicators of condition

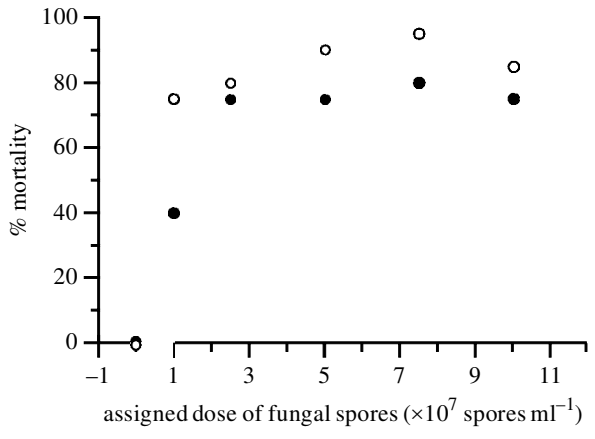

Figure 1. Percentage mortality of gregariously (closed circles) and solitarily (open circles) raised adult *T. molitor* exposed to *M. anisopliae* over a range of fungal doses. The mortality is significantly different between rearing densities ($\chi^2 = 5.88$ and $p = 0.015$).

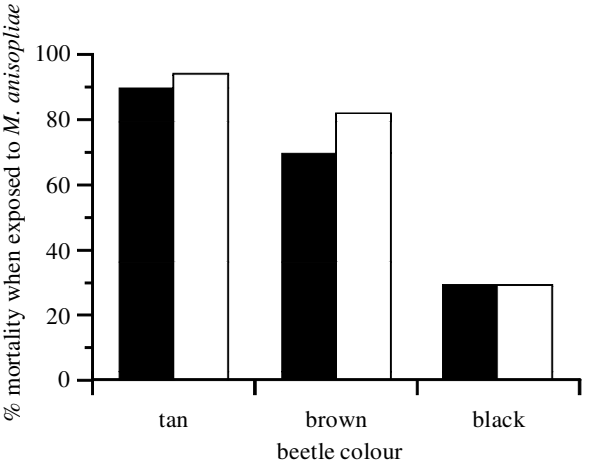

Figure 2. Overall mortality (regardless of dose) of each elytra colour type of *T. molitor* split by their rearing densities. Open bars, solitarily raised beetles; filled bars, gregariously raised beetles. Lighter beetles were more susceptible to *M. anisopliae* than darker ones (d.f. = 1, $\chi^2 = 35.61$ and $p < 0.001$). Thus, because the gregarious beetles were significantly darker than the solitary ones (d.f. = 2, $\chi^2 = 14.21$ and $p < 0.001$), mortality is significantly higher in beetles raised solitarily than those raised gregariously (d.f. = 1, $\chi^2 = 5.88$ and $p = 0.015$).

in the study population, then they should contribute to the fit of the model 'mortality' in a logistic regression. This was not the case, either for the whole data set or when each colour morph was analysed separately. Thus, a small black beetle is equally as likely to be susceptible to the fungus as a large black beetle and likewise for brown and tan beetles. Second, both rearing densities had an equal amount of food and liquid, so the only environmental difference was the presence or absence of conspecifics. Resistance to the fungus was not obligately dependent on conspecifics being present, because some solitary individuals showed high resistance to the fungus (i.e. belonged to the darker colour morphs). Thus, despite the fact that weight and development time differed significantly between rearing densities, rearing density itself was not a significant contributor to mortality. This means that at least some of the solitarily raised individuals were able to invest in immunity to the same level as the gregariously raised beetles, suggesting that equal resources were available to both. Finally, differences in development time (Tschinkel & Willson 1971; Connat *et al.* 1991; Savvidou & Bell 1994; Kotaki & Fujii 1995) and weight (Weaver & McFarlane 1990) are frequently observed in tenebrionids when the population density is manipulated. These have been interpreted as being adaptive for the avoidance of cannibalism (tenebrionids are highly cannibalistic) which occurs following larval moults and during pupation (see the discussions in Tschinkel & Willson (1971), Weaver & McFarlane (1990) and Savvidou & Bell (1994)). Thus, differences in life-history variations in tenebrionids are often due to alternative strategies for the minimization of larval mortality rather than being simply condition dependent and an adaptive explanation (DDP; Wilson & Reeson 1998) for the variation in fungal resistance found in this study is favoured.

Hence, this paper is the first demonstration, to our knowledge, that DDP occurs against a non-specific pathogen in a host from a population not previously exposed to it. The similarity of the results presented here with those of Reeson *et al.* (1998) (i.e. insects reared at high density were found to be more pathogen resistant) and the fact that the system used is non-specific suggests that plasticity in immune investment is a common response to fluctuations in population density, at least in insects exhibiting other density-dependent polyphenisms. Supporting this is the observation that, in both studies, greater resistance was correlated with increased cuticular melanization; indeed, colour was a better predictor of resistance than rearing density for *T. molitor*. The function of cuticular melanization in resistance (if there is one at all) is not known. Cuticular melanization may be a sink for harmful quinones recruited for but not used in sclerotization (Chapman 1982). Sclerotized proteins are impermeable to many fungal secretions, resistant to enzyme degradation and mechanically prevent hyphal penetration (Hajek & St Leger 1994). Hence, it is not clear whether cuticular melanin is a preformed barrier which actively contributes to fungal resistance or whether it is merely a by-product of increased sclerotization and has no direct role in resistance. However, if cuticular melanization is functionally or developmentally linked to investment in immunity then the fact that high-density phases of insects are frequently more heavily melanized (Applebaum & Heifetz 1999) suggests that DDP may be a widespread phenomenon in the Insecta. Further evidence that the degree of melanization correlates with pathogen resistance comes from studies of two lepidopterans: *Ephestia kuhniella* Zeller, in which parasitoids develop more slowly in the black form (Verhoog *et al.* 1996) and *Antheraea pernyi* Guérin-Méneville, in which the melanic form is more resistant to a nuclear polyhedrosis virus (Gershenzon 1994).

Larval mortality was found to be significantly higher in the gregariously raised *T. molitor* (table 1). The higher mortality in gregarious larvae compared to solitary larvae may be attributable to cannibalism. This could explain at least one of the selective forces behind the existence of DDP

in *T. molitor*, because cannibalism (or high mortality) is invariably observed at high larval densities (Tschinkel *et al*. 1967; Savvidou & Bell 1994) and is known to increase the risk of pathogenesis (Pfennig *et al*. 1998). At high larval densities cannibalistic interactions become more frequent (Weaver & McFarlane 1990), potentially increasing the need for greater investment in immunity.

There was no difference in the activity of PO in the haemolymph of beetles reared at different densities. On this point *T. molitor* seems to differs from *S. exempta* (Reeson *et al*. 1998), although in the latter case it is not clear whether the haemolymph PO levels are a causal factor of the prophylactic response. This result is surprising given that haemolymph PO activation is one of the primary responses when non-self material is encountered in the haemocoel, including fungal components (Söderhäll 1981, 1982) and that, in this study, resistance to a pathogen was correlated with a melanic trait. However, haemolyph PO, cuticular melanization and pathogen resistance may be independently and complexly regulated (Ashida & Brey 1998). It should also be noted that, in this study, colour and not rearing density was the better predictor of susceptibility and that the experimental design meant that assessing differences in PO between colour morphs was impossible. Such data would have helped to elucidate the relationship between haemolymph PO and pathogen resistance.

Tenebrio molitor is capable of mounting a density-dependent prophylactic response to a generalist entomopathogen (*M. anisopliae*). This is the first demonstration, to our knowledge, of this phenomenon outside the Lepidoptera and the first to use a generalist pathogen. The nature of the results suggests a widespread applicability of the theory of DDP, at least among insects, where density-dependent polyphenisms are common. The results may also be significant because they provide indirect support for the theory that there is a cost associated with the maintenance of immune function (Følstad & Karter 1992; Wedekind & Følstad 1994), an important life-history trait in metazoan organisms.

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