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Immune function responds to selection for cuticular colour in *Tenebrio molitor*

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Cuticular colour in the mealworm beetle (*Tenebrio molitor*) is a quantitative trait, varying from tan to black. Population level variation in cuticular colour has been linked to pathogen resistance in this species and in several other insects: darker individuals are more resistant to pathogens. Given that cuticular colour has a heritable component, we have taken an experimental evolution approach: we selected 10 lines for black and 10 lines for tan adult cuticular phenotypes over at least six generations and measured the correlated responses to selection in a range of immune effector systems. Our results show that two immune parameters related to resistance (haemocyte density and pre-immune challenge activity of phenoloxidase (PO)) were significantly higher in selection lines of black beetles compared to tan lines. This may help to explain increased resistance to pathogens in darker individuals. Cuticular colour is dependent upon melanin production, which requires the enzyme PO that is present in its inactive form inside haemocytes. Thus, the observed correlated response to selection upon cuticular colour and immune variables probably results from these traits' shared dependence on melanin production.
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Introduction

Melanin is a ubiquitous pigment in invertebrates and vertebrates (Fox, 1979), and is particularly important in insect cuticle (Majerus, 1998). Melanin has been at the forefront of understanding adaptation since the first melanic peppered moth was discovered in 1859: subsequently, the functional and adaptive value of cuticular melanism in insects has been extensively examined (see Majerus, 1998; True, 2003).

In this paper, we take an experimental evolution approach to examine the relationship between cuticular melanisation and immune effector systems (including those involved in melanin production) in the haemocoel of the mealworm beetle, *Tenebrio molitor*. Cuticular colour is a continuous (Thompson *et al*, 2002), melanin-dependent and heritable (Rolff, pers. comm.) trait in *T. molitor*, varying from tan to black. In this species, a phenotypic correlation has been demonstrated between cuticular colour and resistance to an entomopathogenic fungus, *Metarhizium anisopliae*: black beetles were more resistant than tan beetles (Barnes and Siva-Jothy, 2000). Similar phenotypic correlations have been found in phase polyphenic species. For example, using phenotypic variation in cuticular colour in *Spodoptera exempta*, Wilson *et al* (2001) found positive correlations between melanism and cuticular and haemocoelic phenoloxidase (PO).

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Several studies have successfully employed selection techniques to examine correlated responses for resistance to pathogens (eg Kraaijeveld and Godfray, 1997; Fuxa and Richter, 1998; Webster and Woolhouse, 1999). The immune systems that are instrumental in providing resistance to parasites and pathogens are most probably involved in other physiological functions (Rolff and Siva-Jothy, 2003). Moreover, given that melanin is produced in the cuticle as a result of activation of the PO cascade, and that this cascade is also important in the humoral immune response (Söderhäll and Cerenius, 1998), it seems likely these two factors are linked. Indeed, in a quantitative genetics examination of insect immunity in *Spodoptera littoralis*, a positive genetic correlation was found between haemocyte density and cuticular melanisation (Cotter *et al*, 2004). Furthermore, correlated selection studies examining covariation in functionally related traits (in this case, putatively cuticular melanisation and immune effector systems) may aid our understanding of phenotypic integration of resistance traits (Pigliucci, 2003). Hence, we take an experimental evolution approach to determine whether the benefit associated with dark cuticle is correlated with haemocoelic immunity by selecting for cuticle colour over a number of generations, then examining the correlated responses in the immune system.

The invertebrate immune system is innate and evolutionarily ancient (Vasta and Lambris, 2002). Innate immunity is a shared feature of invertebrate and vertebrate immunity (Roitt, 1988; Vilmos and Kurucz, 1998) and is believed to show neither an anticipatory nature towards, nor specificity against, antigens (Carton and Nappi, 2001; but see also Kurtz and Franz, 2003). Important and commonly assayed aspects of invertebrate

immunity include blood-cell counts (haemocyte numbers/density), antibacterial activity in the haemolymph, and the activity of PO. Rather than examining a specific parasite/pathogen interaction, these measures provide a general index of innate immunity.

Haemocyte numbers are commonly assayed because these cells are used to phagocytose and/or encapsulate pathogens: the more cells there are, the faster and larger the encapsulation response is likely to be (eg Eslin and Prévost, 1998). Antibacterial peptides are produced within a few hours after entry of the fungus or bacteria into the insect (Söderhäll and Cerenius, 1998): antibacterial activity can be induced in the haemolymph by injecting bacterial cell wall components (eg lipopolysaccharides (LPS)) into an individual, then taking a haemolymph sample and testing its effectiveness at killing live bacteria (Moret and Schmid-Hempel, 2000). Finally, the enzyme PO is involved in the pathway of melanin production: this biochemical pathway is critical in a number of physiological functions including immune responses (Shiao *et al*, 2001) and the formation of melanised cuticle (Sugumaran, 2002). The activity of this enzyme is known to be correlated with refractoriness to pathogens (eg Nigam *et al*, 1997) and its expression in the exocuticle is responsible for the hardening and darkening of the insect integument (Andersen, 1974; Sugumaran, 1991), factors which contribute to the effectiveness of the exoskeleton as a barrier to pathogens.

The aim of this study was to examine whether experimental selection for cuticle colour resulted in concomitant alterations in allocation to immune function. We produced constant larval density selection lines for tan or black *T. molitor* adult cuticle phenotypes, and examined key adult immune system parameters. Included in the experimental design was a bacterial-cell wall component (LPS) immune challenge, aimed at determining whether animals of the two cuticle colours upregulate their immune system in different ways given the threat of disease. Given the advantage that black beetles have under parasite selection (Barnes and Siva-Jothy, 2000), we predicted selection for black cuticle would result in a correlated positive response in immune effector systems that protect the insect from pathogen invasion.

Materials and methods

Beetle cultures: selection protocol

Colour selected lines were produced by selecting cuticular colour (assayed on the dorsal side of the beetle – see Thompson *et al*, 2002) to be either light (tan) or dark (black). To initiate the colour-selected lines, virgin males and females, from out-bred stock cultures, with extremes of cuticular phenotype (black and tan) were allowed to mate monogamously. The cuticular colour of the offspring of each monogamous pairing was assessed 7-days post imaginal eclosion; if the line was selected for black cuticular colour, the darkest three male and female offspring of that line were allowed to mate monogamously to continue the line (and *vice versa* with the lightest offspring for tan lines). Three brother-sister pairs were allowed to mate at each generation. The offspring from the pair producing the most extreme phenotype were allowed to produce the next generation. This selection protocol resulted in one pair contributing to the next generation in each line. This may lead to inbreeding but this method of selection was chosen in order to reduce the amount of variation amongst individuals within a selection line, thus enabling us to control for as many other factors as possible, for example size etc. Most lines were maintained for six or seven generations (except three black and two tan lines which were maintained for 11 generations for logistical reasons), each consisted of ca. 100+ individuals. The generation time in *T. molitor* is ca. 4 months.

Although selected lines can have potential shortcomings in the form of inbreeding depression, genetic drift, and spurious genetic correlations, our use of 20 replicate lines (10 for each cuticular colour) was intended to overcome most of these potential problems. Stock beetles acted as a cuticular colour reference for the selection lines: colour diverged away from the reference line in both selection lines (reference line mean weighted average luminance (WAL – see below for a description) = 73; black selection lines mean WAL = 66; tan selection lines mean WAL = 87).

Beetle cultures: experimental animals

Up to 100 first to third instar larvae were removed from each of 10 lines selected for black cuticles and 10 lines selected for tan cuticles. The larvae from each line were allocated to a plastic box containing 4 g of rat diet per larvae, *ad libitum* access to water, and weekly apple supplementation throughout larval development. The boxes were maintained at 26 ± 1°C in a LD 12:12 h photo cycle. Upon pupation individuals were removed from the cultures and sexed and weighed; a minimum of five males and five females were retained from each of the colour-selected lines within the weight range of 93–113 mg. The pupae were kept in individual grid box containers until imaginal eclosion, whereupon they were transferred to individual petri dishes containing approximately 5 g of rat diet, water, a small chunk of apple, and a piece of paper. The water and apple were replenished 7 days later.

Day 8: Cuticular colour analysis, haemolymph collection, and LPS injection

Cuticular colour was analysed 8 days after imaginal eclosion. In short, beetles were anaesthetised on ice, and a digital image of the elytra was captured. The degree of cuticular darkness was analysed from the captured image using Optimas 6® software, which produces a WAL on a grey scale between 0 and 255 (0 darkest, 255 lightest; see Thompson *et al*, 2002). Beetles were kept cold anaesthetised for immediate haemolymph collection. The ventral pleural membrane between the thorax and abdomen and surrounding area were swabbed with 70% ethanol and allowed to air dry, a fine-gauge (30 G) syringe needle was inserted into the pleural membrane and 2 µl of haemolymph was collected into a capillary. The haemolymph was added to 20 µl of ice-cold sodium cacodylate buffer (0.01 M sodium cacodylate, 0.005 M calcium chloride, pH 7.4) in a 0.5 ml centrifuge tube and frozen immediately at −90°C for the pre-immune challenge PO assay (PO_{pre} assay). Freezing at −90°C disrupted the haemocytes and allowed stable storage of the haemolymph.

The beetles were then injected with 5 μl of LPS dissolved in ice-cold Ringer solution (0.5 mg/ml) (Moret and Schmid-Hempel, 2000). Injections were carried out using sterilised 10 μl glass capillary tubes that had been pulled to a fine tip. The beetles were subsequently replaced in their petri dishes. The presence of LPS will activate zymogenic proPO which in turn becomes the active PO (Cerenius and Söderhäll, 2004), thus a challenge with LPS should upregulate the immune system.

Day 9: Haemolymph removal and haemocyte density

Beetles were cold anaesthetised on ice and haemolymph was removed as for PO_{pre}, except that a 4 μl sample was taken and added to 40 μl of ice-cold sodium cacodylate buffer in a 0.5 ml centrifuge tube. The solution was vortexed and split in three ways. A volume of 10 μl of the sample was added to 20 μl of ice-cold sodium cacodylate buffer and vortexed for haemocyte counting. The solution was pipetted onto a haemocytometer and the haemocytes counted in three 1 mm × 1 mm squares, from which a mean value was obtained.

Another 10 μl of the sample was added to a chilled 0.5 ml centrifuge tube treated with 1-phenyl-2-thiourea to inhibit melanisation. The sample was vortexed and frozen immediately at –90°C, until used in the antibacterial activity assay. The remaining 10 μl of sample was frozen for the post-immune challenge PO assay (PO_{post} assay).

Antibacterial assay

Petri dish test plates (9 cm diameter) were prepared by adding 0.05 ml of live *Arthrobacter globiformis* bacterial suspension (10^5 cells/ml) to 5 ml of sterile broth medium (10 g bactotryptone, 5 g yeast extract, 10 g sodium chloride, 1000 ml distilled water, pH 7), with 1% of bacto-agar at 45°C. The plates were swirled to disperse the bacteria and left to set in a sterile flow cabinet at room temperature. The plates were stored upside down at 4°C until needed (maximum storage time was 3 days).

In all, 10 equally spaced 2 mm diameter wells were made per plate. A volume of 2 μl of each haemolymph sample was added into each well. One well in each plate contained 2 μl of a positive control (made by experimentally activating the immune system of eight non-colour-analysed beetles from the stock population with LPS for 24 h). The plates were incubated upside down overnight at 30°C. During this period, the antibacterial activity of the haemolymph, which had been induced by LPS injection, inhibited bacterial growth of the *A. globiformis*. This resulted in clear circular zones around each of the holes. These clear zones were measured by placing an acetate sheet onto an inverted petri dish and drawing the perimeter of each of the zones. The area of the drawn zone was then measured from a digitised image obtained from a video camera (PULNiX TM/765) and analysed using Optimas 6® software, and standardised according to the standard on the plate.

PO assay

A commonly used method of PO quantification is the measurement of conversion by PO, of the substrate L-dopa into dopachrome. This enzymatic reaction results in a darkening in the test solution from the production of dopachrome; the change in absorbency of the solution can be quantified spectrophometrically at 490 nm, and hence the V_{max} (the velocity of the enzyme reaction when the substrate is at saturating concentration; Thompson, 2002) can be determined (eg Barnes and Siva-Jothy, 2000; Gillespie *et al*, 2000). The greater the rate of conversion of L-dopa to dopamine, the higher the activity of PO. The methodology used herein for quantifying PO activity in *T. molitor* conforms to Michaelis-Menten kinetics and is highly repeatable (Thompson, 2002).

A sterile 96-well plate culture cluster (Costar) was placed on ice and 140 μl of distilled water and 20 μl of phosphate-buffered saline (10 mM sodium phosphate, pH 5.5) added to each of the 96 wells. In all, 20 μl of each sample for PO analysis (one individual's PO_{pre} or PO_{post} sample) was added to each well, with one well per plate remaining as a blank. A volume of 20 μl of L-DOPA/sodium cacodylate buffer (4 mg/ml) was added to each well, including the blank. The 96-well plate was put immediately into a pre-warmed (30°C) tunable microplate reader (Versamax, Molecular Devices). The reaction was allowed to proceed at 30°C (the plate-reader had a controlled temperature plate) for 30 min. Softmax PRO 4.0 software calculated the absorbency of the samples every 11 s. After 30 min the enzyme activity was determined by examining the slope of the linear phase of the reaction. As the rate during the linear phase of the reaction is proportional to the amount of enzyme present in the sample, the value given by the Softmax PRO software's autoslope function was used as the units for the activity of enzyme concentration.

Microsporidia

Some cultures were infected with microsporidia, which are common parasites in insects (Weiser, 1963; Canning, 1989). We examined whether infection with microsporidia had any effect upon the immune parameters measured; microsporidia burdens were noted when haemocyte counts were made. In total, 87% of the beetles had a microsporidian infection and the mean burden of microsporidia over all individuals was 85.3 ± 13.7 ($\pm$standard error) per microlitre of haemolymph (c.f. a mean haemocyte density across all individuals of 5136.6 ± 337.4 ($\pm$standard error) cells per microlitre of haemolymph). A multivariate analysis of covariance (MANCOVA) was used to examine the effect of mean microsporidia burden per selected line (covariate, ln transformed) on the four response variables associated with immunity: PO_{pre} (ln transformed), PO_{post}, haemocyte density (ln transformed), and antibacterial response. None of the dependent variables were related to microsporidia burden (MANCOVA: Wilks' Lambda $\Lambda = 0.837$, $F_{4,35} = 0.729$, $P = 0.586$), suggesting that the microsporidians were immunologically benign (see also Dunn *et al*, 1993), although we cannot rule out the possibility that microsporidia have some unmeasured affect upon the beetles.

Statistical analyses

All statistical analyses were conducted using either StatView version 5.0 or SPSS version 10 for Macintosh. As some of the analyses involved multiple measurements from the same beetles (ie, haemocyte count, antibacterial activity, PO_{pre}, and PO_{post} samples), where

appropriate a MANCOVA or a multivariate analysis of variance (MANOVA) was used. When there are several dependent variables, there is potential for inflated Type I errors (Zar, 1999) due to multiple tests of (likely) correlated dependent variables (Tabachnik and Fidell, 1996). A MANCOVA or MANOVA allows for comparison of the population means of all variables of interest at the same time (multivariate response), rather than considering multiple responses as a suite of univariate responses (Zar, 1999). This reduces the magnitude of Type I errors. The statistical significance of the MANCOVA/MANOVA can be determined in a variety of ways. We used the most widely used test statistic, Wilks' Lambda (Λ) (Zar, 1999) (although alternative test statistics (eg Pillai's trace) gave similar results). When the MANCOVA or MANOVA were statistically significant, subsequent univariate ANOVAs were performed to elucidate which responses contributed to the significant multivariate response. All analyses were performed upon mean values for line, or mean values for line split by gender. Data were tested for normality using Kolmogorov–Smirnov and Shapiro–Wilk tests. Distributions that were significantly non-normal were transformed (where possible, and as indicated). Where not possible, the appropriate nonparametric test was performed. Means are presented with $\pm$ one standard error.

Results

Response to selection for cuticular colour

Lines selected for black cuticles were significantly darker than those selected for tan cuticles (Mann–Whitney *U*-test: $U' = 100$, $n_1 = 10$, $n_2 = 10$, $P = 0.0002$; means: black 66.3 ± 0.71 (lower values indicate darker cuticle), tan 87.4 ± 2.39). There was no significant difference in pupal weight (ln transformed) between black and tan selected lines (two-sample *t*-test: $t = -0.068$, df $= 18$, $P = 0.946$).

The effect of colour code and gender upon the immune variables

A two-way MANOVA was used to determine the effect of colour code (ie black or tan), gender, or an interaction between the two, upon the four response variables associated with immunity: PO_{pre} (ln transformed), PO_{post}, haemocyte density (ln transformed), haemocyte density, and antibacterial activity.

The dependent variables associated with immunity were significantly related to colour code (MANOVA: Wilks' Lambda $\Lambda = 0.575$, $F_{4,33} = 6.092$, $P = 0.001$). Black selected lines had significantly higher PO_{pre} activity than tan selected lines (Figures 1 and 2 (showing mean PO activity for each line), Table 1), and significantly higher haemocyte density than tan beetles (Figure 3, Table 1). However, PO_{post} and antibacterial activity did not differ between black and tan lines, and thus did not contribute to the significant multivariate response (Table 1). To exclude the possibility that the lines that had been selected for 11 generations were driving the statistical significance in this analysis, we performed the MANOVA excluding these lines: there was still a significant effect of colour code upon the response variables (MANOVA: Wilks' Lambda $\Lambda = 0.592$, $F_{4,23} = 3.962$, $P = 0.014$). As with the analysis including these lines, PO_{pre} (ANOVA: $F_{1,29} = 5.243$, $P = 0.030$) and haemocyte density (ANOVA: $F_{1,29} = 11.642$, $P = 0.002$) were significantly elevated in the black selected lines.

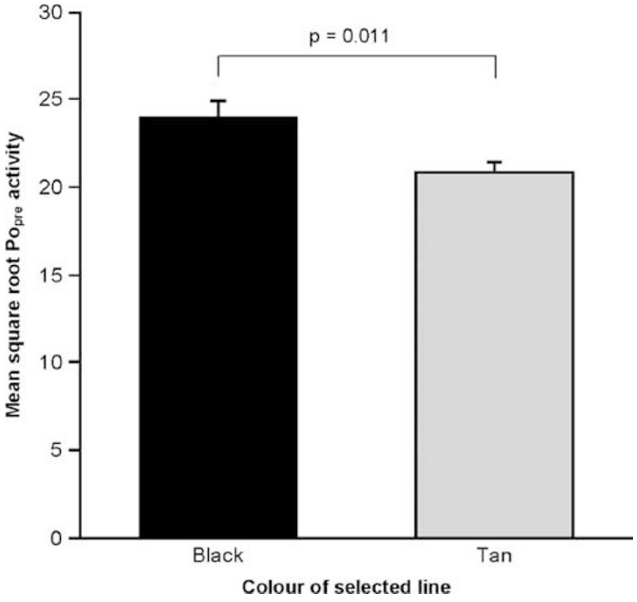

Figure 1 Mean difference in square root Po_{pre} activity between lines selected for black and tan cuticle. Black selected lines had significantly higher mean square root PO_{pre} activity than tan selected lines (see Table 1). Error bars represent one standard error.

The dependent variables were not significantly related to gender (MANOVA: Wilks' Lambda $\Lambda = 0.961$, $F_{4,33} = 0.333$, $P = 0.854$), and there was no significant interaction between gender and colour code (MANOVA: Wilks' Lambda $\Lambda = 0.938$, $F_{4,33} = 0.542$, $P = 0.706$). Consequently, we exclude gender from further analyses.

PO_{pre} *versus* PO_{post} activity

There was a significant difference in PO_{pre} and PO_{post}, with higher PO activity after immune challenge (Wilcoxon signed rank: $z = -3.211$, $n = 20$, $P = 0.0013$; mean $PO_{pre} = 510.4 \pm 30.6$, mean $PO_{post} = 636.2 \pm 32.4$). If PO activity is examined separately for black and tan lines, there is significant increase in PO_{post} activity in tan lines (Wilcoxon signed rank: $z = -2.701$, $n = 10$, $P = 0.0069$; mean tan $PO_{pre} = 437.1 \pm 25.5$, mean tan $PO_{post} = 633.1 \pm 33.0$), but not in black lines (Wilcoxon signed rank: $z = -1.478$, $n = 10$, $P = 0.139$; mean black $PO_{pre} = 583.8 \pm 45.9$, mean black $PO_{post} = 639.2 \pm 57.7$).

Inbreeding coefficients

The number of generations of selection varied between lines. In order to exclude the possibility that the varying degrees of inbreeding may have accounted for the correlation between immune function and cuticular colour, a MANCOVA was performed with the immune parameters (PO_{pre} (square-root transformed), PO_{post}, haemocyte density (ln transformed) and antibacterial response) as dependent variables, colour code as a factor, and inbreeding coefficients (Falconer and Mackay, 1996) for each line as a covariate. The variables associated with immunity were not significantly related to the inbreeding coefficient (MANCOVA: Wilks' Lambda $\Lambda = 0.912$, $F_{4,14} = 0.339$, $P = 0.847$).

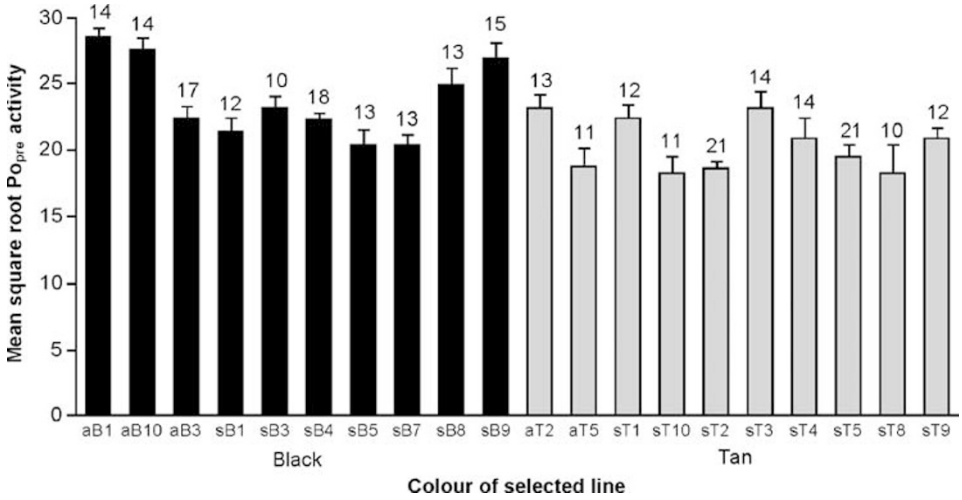

Figure 2 Mean values of square root PO_{pre} activity for all black and tan selected lines. Black bars indicate black selected lines and grey bars indicate tan selected lines; 'a' refers to the 11 generation lines. Error bars represent one standard error, and numbers above bars indicate the number of beetles from each line.

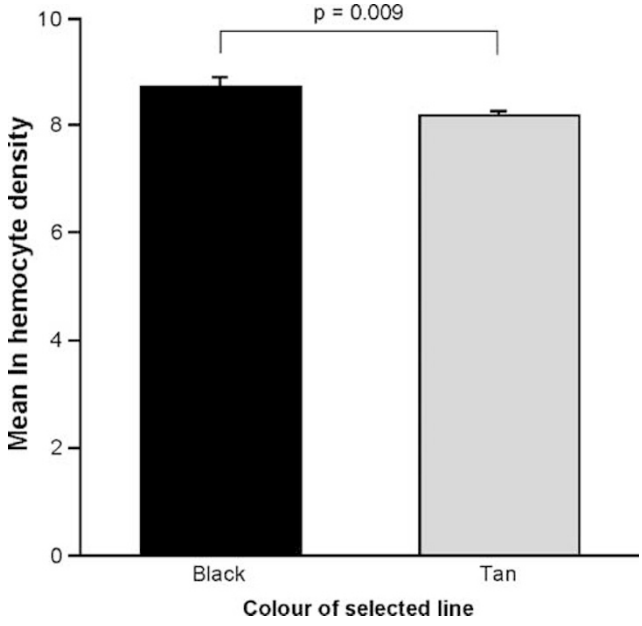

Figure 3 Mean difference in ln haemocyte density between lines selected for black and tan cuticle. Black selected lines had a significantly higher mean ln haemocyte density than tan selected lines (see Table 1). Error bars represent one standard error.

Table 1 Univariate ANOVA results for the effect of colour (black and tan) upon each of the individual four response variables associated with immunity (PO_{pre} (ln transformed), PO_{post}, haemocyte density (ln transformed), and antibacterial response)

Dependent variable	*Sum of squares*	F	*df*	P
PO_{pre}	0.777	11.849	1,39	0.001
Haemocyte	3.901	15.087	1,39	0.000
PO_{post}	7.744	0.000	1,39	0.987
Antibacterial	10.201	0.116	1,39	0.735

Discussion

This study demonstrates a correlated response of selection for cuticular colour with a cellular and a humoral immune component in *T. molitor.* Beetles selected for black cuticles had significantly higher PO_{pre} activity and higher haemocyte density post-immune challenge than beetles selected for tan cuticles. However, there was no difference in PO_{post} or antibacterial activity between black and tan lines. The basis of the correlation is not known, but it is likely to be pleiotropic since cuticular colour and the correlated immune traits are underpinned by PO activity, which ultimately resides in haemocytes (Hoffmann, 1995). Although the PO that produces melanin in the cuticle is different from that which produces immunity in the haemolymph (Ashida and Brey, 1998), the precursor (proPO) is shared by both pathways (Aso *et al*, 1985) and haemocytes play an important role in transporting proPO to the cuticle (Ashida and Brey, 1995). Contrary to predictions (see Rolff (2002) for overview and rationale), we found no effect of gender upon the immune traits we measured across our selection lines.

Haemocytes and selection for cuticular colour

Haemocyte number has been found to positively correlate with encapsulation ability against parasitoid eggs in six species of *Drosophila* from the *melanogaster* subgroup (Eslin and Prévost, 1998). Furthermore, a major immune response against fungal infection in insects is cellular encapsulation (Hajek and St. Leger, 1994; see also Gillespie *et al*, 2000). The higher haemocyte density demonstrated in this study is likely to be indicative of a heightened immune response, and may be instrumental in the observation that *black* beetles have higher resistance to *M. anisopliae* (Barnes and Siva-Jothy, 2000) because of their increased capacity to produce an encapsulation response. A higher haemocyte density may also indicate increased immunity against other pathogens as well.

PO activity and selection for cuticular colour

Selecting for cuticular colour resulted in a significant increase in PO_{pre} in beetles from black lines compared to tan lines. PO is an important component of the insect immune response. Braun *et al* (1998) found that a

Drosophila mutant devoid of activatable ProPO had reduced survival when challenged with *Beauveria bassiana* fungal spores, and Söderhäll and Ajaxon (1982) showed that products derived from reactions catalysed by PO were inhibitory to fungal growth. Furthermore, tsetse flies bred for refractoriness to midgut infection with *Trypanosoma brucei rhodesiense* showed significantly higher haemolymph PO activity than susceptible flies (Nigam *et al*, 1997). Therefore, increased PO_{pre} activity may enable black beetles to produce a more effective immune response against pathogens.

Studies using other species exhibiting dark/light variation in phenotype and measuring their immune components have shown equivocal results. For example, melanic *Spodoptera exempta* had higher PO activity in the cuticle, haemolymph, and midgut, as well as melanising a greater proportion of ectoparasitoid eggs than non-melanic larvae (Wilson *et al*, 2001); in contrast, melanic *Hemideina maori* demonstrated a lower melanotic encapsulation response than the yellow banded morph (Robb *et al*, 2003). More recently, Cotter *et al* (2004) showed elevated PO and encapsulation in dark *Spodoptera exempta*, but lower levels of antibacterial activity. Our results suggest that PO_{pre} activity is genetically correlated with cuticular colour in *T. molitor* but, once upregulated after immune challenge, there is no correlation. This could be due to similar PO_{post} between black and tan selected lines, or it could be due to the sampling method: differences in PO_{post} may only be apparent before or after the point, 24 h in this study, at which the measurement of PO_{post} was made.

Challenging the beetles with bacterial cell wall components (LPS) resulted in a significant increase in PO_{post} compared to PO_{pre}, suggesting that PO activity is upregulated in response to bacterial infection in *T. molitor* (see also Kim *et al*, 2000). However, this change is almost certainly due to effects in the tan lines: there was a significant difference between PO_{pre} and PO_{post} in tan lines, but not in black lines. Black beetles therefore maintain a higher PO_{pre} than tan beetles, suggesting that black beetles maintain a prophylactic level of PO_{pre}, a potentially costly strategy (see Siva-Jothy and Thompson, 2002). Indeed, Kumar *et al* (2003) found that *Anopheles gambiae* genetically selected to be refractory to *Plasmodium* melanise and encapsulate this parasite, concurrent with being in a chronic state of oxidative stress when compared to susceptible *A. gambiae* strains. This could suggest that the high constitutive PO_{pre} activity in black beetles may bear a cost in terms of oxidative stress, whereas the induced PO activity in tan beetles may only produce oxidative stress as and when this enzyme is activated.

Antibacterial response and selection for cuticular colour

Selecting for cuticular colour resulted in no significant difference in haemolymph antibacterial activity between black and tan lines. Antimicrobial peptides are produced by haemocytes (Hoffmann, 1995), but are not involved in cuticular darkening. This lack of a correlated response supports the notion that PO is the physiological link that underpins the relationship between cuticular colour and a heightened immune response (see also Siva-Jothy, 2000).

Conclusions

We have demonstrated that selecting for cuticular colour in *T. molitor* results in a correlated change in the expression of two immune parameters. Pre-immune challenge activity of PO showed increased expression in lines selected for black cuticle, and, after an immune challenge with LPS, haemocyte density also showed increased expression in lines selected for black cuticle. The relationship is probably the result of the dependence of melanin formation upon PO, and the fact that the inactive precursor of PO (proPO) resides in the haemocytes (Hoffmann, 1995). The association helps to explain why darker insects are more refractory to pathogens that invade the hemocoel via the cuticle (Barnes and Siva-Jothy, 2000; Wilson *et al*, 2001). However, we cannot exclude the possibility that differences in cuticular architecture between black and tan beetles also play a part. Melanin and sclerotin incorporation into exocuticle increases its darkness and strength (Sugumaran 1991) and so may enable darker insects to present a tougher primary barrier to entomopathogens. Furthermore, inbreeding is unlikely to be the causal explanation for the differences in immunity between the two lines, given that the inbreeding coefficient had no significant effect upon the immune variables measured.

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