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More than a colour change: insect melanism, disease resistance and fecundity

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A ‘dark morph’ melanic strain of the greater wax moth, *Galleria mellonella*, was studied for its atypical, heightened resistance to infection with the entomopathogenic fungus, *Beauveria bassiana*. We show that these insects exhibit multiple intraspecific immunity and physiological traits that distinguish them from a non-melanic, fungus-susceptible morph. The melanic and non-melanic morphs were geographical variants that had evolved different, independent defence strategies. Melanic morphs exhibit a thickened cuticle, higher basal expression of immunity- and stress-management-related genes, higher numbers of circulating haemocytes, upregulated cuticle phenoloxidase (PO) activity concomitant with conidial invasion, and an enhanced capacity to encapsulate fungal particles. These insects prioritize specific augmentations to those frontline defences that are most likely to encounter invading pathogens or to sustain damage. Other immune responses that target late-stage infection, such as haemolymph lysozyme and PO activities, do not contribute to fungal tolerance. The net effect is increased larval survival times, retarded cuticular fungal penetration and a lower propensity to develop haemolymph infections when challenged naturally (topically) and by injection. In the absence of fungal infection, however, the heavy defence investments made by melanic insects result in a lower biomass, decreased longevity and lower fecundity in comparison with their non-melanic counterparts. Although melanism is clearly correlated with increased fungal resistance, the costly mechanisms enabling this protective trait constitute more than just a colour change.

1. Introduction

The greater wax moth, *Galleria mellonella* (Lepidoptera), is an established model for innate immunity [1]. A strikingly dark morph of this insect was discovered with a high natural resistance to fungal infection [2]. This study compares the normal (non-melanic) morph and the dark (melanic) morph, challenged with the generalist entomopathogenic fungus *Beauveria bassiana*.

In insects, melanin pigments and their precursors are important as structural and protective components of the cuticle. Melanogenesis involves the formation of melanin pigments and toxic by-products from the action of phenoloxidase (PO) on quinone precursors, and occurs primarily in the cuticular structures, midgut epithelium and haemolymph [3], where it performs dual roles in hardening and darkening the cuticle [4] and in immune defence. Such defences include non-self-recognition and humoral encapsulation of invading organisms [5–7]. The presence of melanin and melanogenesis intermediates in the insect cuticle not only limits the growth of certain fungi, possibly by acting as a physical barrier, but

also suppresses synthesis of fungal cuticle-degrading enzymes, thus further impeding cuticle penetration [8,9].

Dark (or melanic) insect morphs exhibit an unusually high concentration of cuticular melanin, and there are reports of a positive correlation between melanism, PO activity and resistance to parasites and pathogens, including entomopathogenic fungi such as *B. bassiana* and *Metarhizium anisopliae* [6,10,11]. This often occurs in crowded insect populations as a form of density-dependent prophylaxis when the risk of infection is high [6,10–12]. To date, however, most melanism studies have been limited to investigations of the degree of melanization, as well as certain aspects of the insect immune response such as lysozyme, PO and haemocyte activity, and their trade-off with fecundity, lifespan and size. An artificial infection route (i.e. injection) is often used to gauge the response to fungi or other microbes.

The entomopathogen *B. bassiana* can infect diverse insect species and is widely used for the biocontrol of crop pests. Following inoculation of the insect cuticle, which is the primary and probably most important defence against natural pathogens, fungal penetration commences within 24 h. Cuticle-degrading enzymes (e.g. Pr1) and mechanical force enable the pathogen to penetrate the cuticle and gain access to the nutrient-rich haemolymph [13]. The cuticle is a powerful inducer of fungal proteases, which in turn may activate the insect's PO (melanization) cascade [14]. Melanin interferes with fungal development as well as the speed at which Pr1 and other enzymes can degrade the cuticle. Once inside the host, the fungus propagates, produces hyphal bodies or blastospores, and releases metabolites, which result in mycosis and host death [13].

During host–parasite coevolution, insects have acquired defences to protect them from fungal infections, but no study has yet properly addressed the question of whether insects can develop resistance and/or tolerance to entomopathogenic biocontrol agents in the field. We use the wider term 'resistance' to refer to traits that prevent or limit infection, whereas 'tolerance' refers to activities that reduce or mitigate the consequences of the invading fungal pathogen once it has gained access to the haemocoel [15]. We wished to understand the sort of trade-offs an insect might make to afford heightened resistance and tolerance to fungi, and what body systems would be recruited for this purpose. In turn, this will provide a greater insight into whether these factors would constitute a successful evolutionary strategy. Maintaining an effective fungal defence system is likely to be costly. Typical manifestations of immunity costs include the diversion of resources away from other immune responses or, commonly, from reproductive or other life-history traits [16–18]. Cotter *et al.* [19] showed that melanic lines of the phase-polyphenic lepidopteran *Spodoptera littoralis* had lower PO activity and higher lysozyme activity than non-melanic lines, suggesting a genetic trade-off between the two immune responses. In addition, lines with high PO activity had slower development rates, suggesting that investment in PO, rather than in melanism, is costly. It is unclear why some immune components are upregulated and others are downregulated, but these observations underscore the importance of measuring multiple traits when looking for fitness costs.

In this study, which is the first to identify and examine the melanic trait in *G. mellonella*, we hypothesized that increased fungal resistance is not just concerned with melanism and immunity, but is underpinned by a wider range

of anatomical, physiological and immunological traits in equilibrium with fitness costs. We determined the role and contribution of the cuticle, humoral and cellular defences in resisting and tolerating the fungus, and assessed a putative two-tiered antifungal system comprising constitutive defences (i.e. pre-formed and immediately available in the correct location during injury or attack) and inducible responses (recruited after damage or injury has been perceived). Trade-offs were also examined between the fungus-targeting defences, as were wider trade-offs between increased resistance and life-history traits. The study compared two inoculation methods: a natural topical infection route in which the fungus penetrates the cuticle, and injection where the fungus bypasses the cuticle to colonise the haemocoel. We further hypothesized that, in addition to PO and lysozyme activities that have provided the focus for earlier work [7,20], melanic insects might invest in a wider spectrum of defence-related products and genes, as well as management strategies to ameliorate stress during infection.

Melanic *G. mellonella* adopt a different evolutionary strategy to deal with fungal pathogens; they sacrifice fecundity, size and longevity to prioritize defences on the 'front line', targeting the likely sites of infection or damage. Timing and location are critical to this strategy, and protective measures are also in place to deal with the aftermath of infection and to prevent secondary infection.

2. Material and methods

(a) Insects

Two separate geographical populations of the greater wax moth, *G. mellonella*, were used: the melanic (M) morph, and a non-melanic (NM) form with normal cuticular pigmentation (figure 1a). All insects were maintained at 28°C on an artificial medium. Full rearing details are provided in the electronic supplementary material, §S1.2.

(b) Fungal infections

Beauveria bassiana isolate Sar-31 was used for all experiments. Unless otherwise stated, insects infected by topical application or injection were sixth instar larvae raised in the same cohort, and sampled at 12, 24 and 48 h post-infection (pi; topical) or at 6 and 12 h pi (injection). Three topical doses of *B. bassiana* (5×10^5 , 5×10^6 and 5×10^7 conidia ml⁻¹) were used to determine the susceptibility of M and NM larvae to fungal infection over 10 days. For all other topical infection studies, the dose was 5×10^7 conidia ml⁻¹. All microinjection infections used a 3 µl suspension of 750 blastospores per larva, and were monitored over 5 days. Control larvae received 3 µl sterile PBS. The experimental sampling times reflected the optimal intervals to observe the acute stages of mycosis (when germination and penetration should be peaking in susceptible insects) and concomitant insect defences, to assess likely determinants of fungal defence. Full details of fungus culture, inoculation methods and the detection of fungal particles in the haemolymph are provided in the electronic supplementary material, §§S1.3 and S1.4.

(c) Conidial adhesion, germination and penetration

The insect epicuticular waxes are the first point of contact for fungal conidia; these can interfere with adhesion, germination and (subsequently) host susceptibility [21]. Therefore, conidial adhesion to the surface of the M and NM larval cuticles

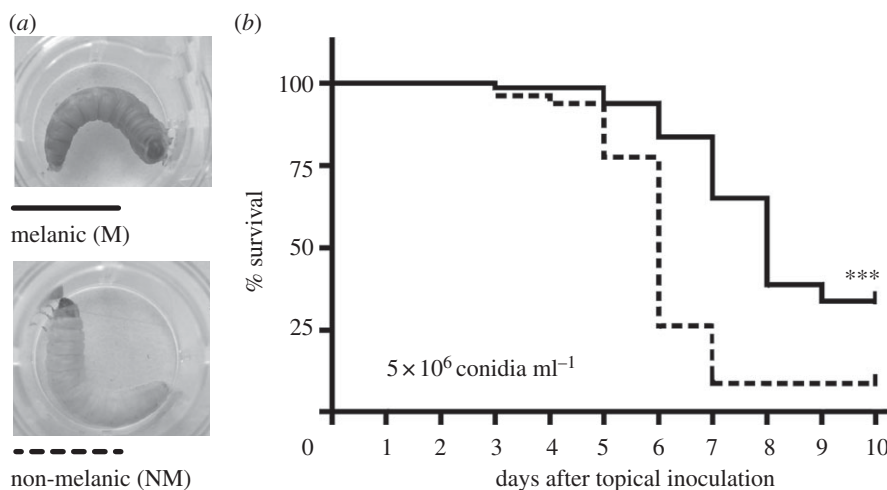


Figure 1. (a) Cuticular melanism and colour forms of *G. mellonella* larvae and (b) mortality rates following topical treatment with the fungus *B. bassiana* at a dose of $5 \times 10^6 \text{ ml}^{-1}$ (see the electronic supplementary material, figure S1 for responses to other doses). *** $p < 0.001$ compared with NM larvae ($n = 80\text{--}140$).

in vivo, and conidial germination on solvent extracts of the epicuticular waxes *in vitro*, were assessed using methods adapted from Ment *et al.* [22]. Full details are provided in the electronic supplementary material, §S1.5.

(d) Cuticle thickness

The average cuticle thickness was calculated from the eighth sternite region of each of 20 M and NM uninfected sixth instar larvae. Full details are provided in the electronic supplementary material, §S1.6.

(e) Phenoloxidase activity in plasma and cuticle

Larvae were topically infected as described above. At 12, 24 and 48 h pi, cell-free haemolymph plasma samples and homogenized cuticle fragments were prepared for spectrophotometric analysis of PO activity using the substrate L-DOPA, and expressed as a change in absorbance per minute per mg protein. Control (uninfected) animals were sampled at 0 h. The experiment was repeated independently three times. Full details are in the electronic supplementary material, §S1.1 and S1.7.

(f) Cellular and humoral defences

Cellular responses to natural (topical) fungal infection, haemocyte encapsulation and total haemocyte counts (THCs) were investigated from haemolymph of M and NM larvae, before and after infection. The extent of melanic encapsulation was assessed from nylon monofilament implants retrieved from the haemocoel. The experiments were repeated three times. Haemolymph plasma lysozyme-like activity was determined by a zone-of-clearance assay using freeze-dried *Micrococcus lysodeikticus* as a substrate suspended in agarose. The radius of the digested zone was compared with a standard curve made with egg white lysozyme (EWL) [23] and expressed as an EWL equivalent (mg ml^{-1}). The experiment was repeated independently three times. Full details are provided in the electronic supplementary material, §S1.8 and S1.9.

(g) Life-history traits

The larval development time (from egg hatching to pupation), male and female pupal weight, and adult fecundity (mean egg production over 5 days per female) were monitored in M and NM insects. Full details are provided in the electronic supplementary material, §S1.10.

(h) QRT-PCR analysis of insect immunity-related genes

The expression of a range of immunity-related genes was quantified. Fat body was dissected from larvae at 24 and 48 h after topical infection, and 12 h after blastospore injection. Gene expression was measured by quantitative RT-PCR (QRT-PCR) using normalized cDNA samples, relative to two reference genes, *18S rRNA* and *elongation factor 1- α* (*EF1*). Eight immune-related genes were investigated, coding for the defensin-like antimicrobial peptides [24] gallerimycin and galiomicin (both of which have strong antifungal activity [25]), the moricin-like gloverin, cecropin D (an AMP upregulated by bacteria and fungi), an atypical defensin-derived immune-related peptide called 6-tox, the toll receptor 18-wheeler, the siderophore transferrin and an insect metalloproteinase inhibitor (IMPI). Primers were designed from published *G. mellonella* sequences (NCBI) or from coding sequence where high-homology protein sequences could be identified from an expressed sequence tag (EST) library [26]. Full details are provided in the electronic supplementary material, §S1.11–S1.12 and table S1.

(i) QRT-PCR analysis of putative stress management genes

It is possible that AMPs work in concert with other factors that contribute to increased tolerance to *B. bassiana*, such as stress tolerance and detoxification, as well as inflammatory mediators. To study putative stress-management genes, a single time point of 48 h after topical infection (or 12 h after injection) was used, which reflected the times of greatest expression for other assayed genes. Eight target loci were investigated. Two loci are involved with the function of molecular chaperone heat-shock proteins (*Hsp-90* and *7GM contig 21310*), whose activities ameliorate stress [27], and one codes for an enzyme dealing with oxidative stress (*contig 17373*). Five loci were used as indicators of stress, being associated with G-protein coupled receptor activity and stress response (*contig 20595*), haemocyte migration and cell proliferation (*contigs 704* and *233*), anti-apoptosis activity (*6GM contig 5976*), and receptor activity for inflammatory mediators (*2GM contig 20004*). These genes were selected following pre-screening, and primers were designed from published *G. mellonella* sequences (NCBI), from a *G. mellonella* EST library [26] and from lepidopteran *Hsp-90* primers described by Xu *et al.* [28]. Full details are in the electronic supplementary material, §S1.11–S1.12 and table S1.

(j) Data analyses

Electronic supplementary material, table S2 details sample sizes, number of independent repeats, normality tests and choice of

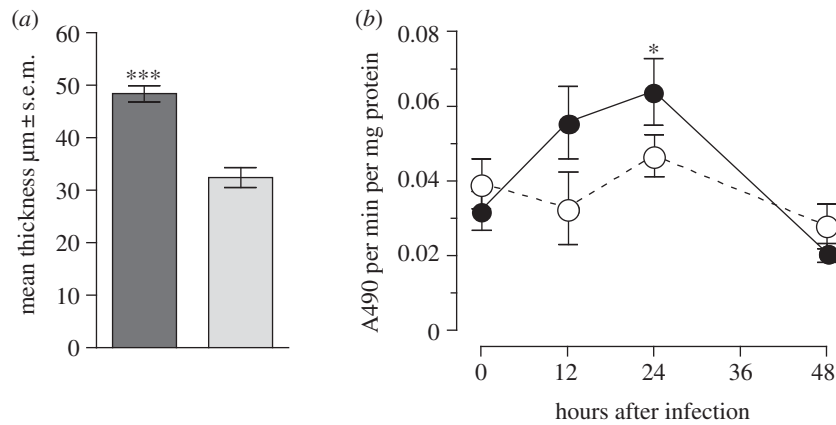


Figure 2. (a) Mean cuticular thickness of *G. mellonella* larvae, showing significantly thicker cuticles of M than NM larvae \pm s.e.m. ($n = 20$, $***p < 0.0001$). (b) Cuticular PO activity in M (dark grey bars and filled circles) and NM larvae (light grey bars and open circles) topically inoculated with *B. bassiana*. Activity was significantly enhanced in M larvae but not NM larvae 24 h pi relative to the uninfected controls ($*p < 0.05$; $n = 9$).

Table 1. Development of mycosis in melanic (M) and non-melanic (NM) forms of *G. mellonella* larvae after topical inoculation with *B. bassiana*.

time (h) pi	% germination-positive ^a		% penetration-positive ^b		% with haemolymph colonization ^c	
	M	NM	M	NM	M	NM
9	0	0	0	0	n.a.	n.a.
12	33	36	27	23	n.a.	n.a.
24	83	55	45	45	0	0
48	100	100	53 ($p < 0.01$)	90	10 ($p < 0.001$)	65

^aGermination-positive larvae on which more than 50% of the conidia germinated ($n = 30$).

^bPercentage of larvae in which penetrating hyphae are observed in 10 or more sites on the cuticle ($n = 30$).

^cPercentage of larvae in which fungal blastospores are detected in haemolymph ($n = 20$).

statistical analyses. Data analyses were performed using GRAPHPAD PRISM v. 4.0 (GraphPad Software, USA) and STATISTIC v. 6.0 (StatSoft Inc., USA). Data were checked for Gaussian distribution using the Agostino–Pearson omnibus test, and if abnormal a conservative non-parametric analysis was applied. In data with Gaussian distribution, Grubbs's extreme studentized deviate test was used to exclude extreme outliers. The data from three independently repeated experiments (blocks) were pooled for analysis after confirming (by two-way ANOVA) that 'experiment', treated as a variable, had no significant effect on the outcome. Differences between M and NM larvae, or between treated and control samples, were considered significant when $p < 0.05$.

3. Results

(a) Insect survival following *Beauveria bassiana* topical treatment

Under natural (topical) treatment, and at all three tested inoculum doses, the M form of *G. mellonella* larvae (figure 1a) was significantly more resistant to the fungus *B. bassiana* compared with the NM form, as measured by survival rate and total mortality (see figure 1; electronic supplementary material, figure S1; for 5×10^5 ml, hazard ratio = 2.05, Wald = 7.45, d.f. = 1, $p = 0.0083$; for 5×10^6 ml⁻¹, hazard ratio = 5.38, Wald = 34.18, d.f. = 1, $p < 0.0001$; for 5×10^7 ml⁻¹, hazard ratio = 2.32, d.f. = 1, Wald = 20.45, $p < 0.001$). This was particularly evident at the higher doses (figure 1b; electronic supplementary

material, figure S1), when LT₅₀ values were extended by 2 days in M larvae. Furthermore, at every dose tested, the overall number of larvae surviving to the end of the experiment was always greater in the M group compared with the NM group (figure 1b; electronic supplementary material, figure S1).

(b) Conidial adhesion and germination

No significant differences were noted between the M and NM larvae regarding conidial adhesion to the cuticle, germination *in vitro* on epicuticular extracts or germination on live insects (see the electronic supplementary material, figure S2). Indeed, all M and NM larvae had more than 50 per cent germination 48 h following topical treatment (table 1). Fungal cuticular penetration and haemolymph invasion, however, were significantly impeded in M compared with NM larvae. Penetration was 1.7 times more frequent in NM larvae ($\chi^2 = 9.93$, d.f. = 1, $p = 0.0016$; table 1), and active fungal colonization of the haemolymph was observed in significantly more NM than M larvae (65% versus 10%) by 48 h ($\chi^2 = 12.91$, d.f. = 1, $p = 0.0003$; table 1).

(c) Mechanical and chemical defences of the cuticle following topical inoculation

The cuticle of M larvae was significantly thicker than that of NM insects at the same developmental stage ($48.4 \mu\text{m}$ s.e. ± 1.5 versus $32.4 \mu\text{m}$ s.e. ± 1.9 ; $t_{6,664}$, d.f. = 19, $p < 0.0001$; figure 2a).

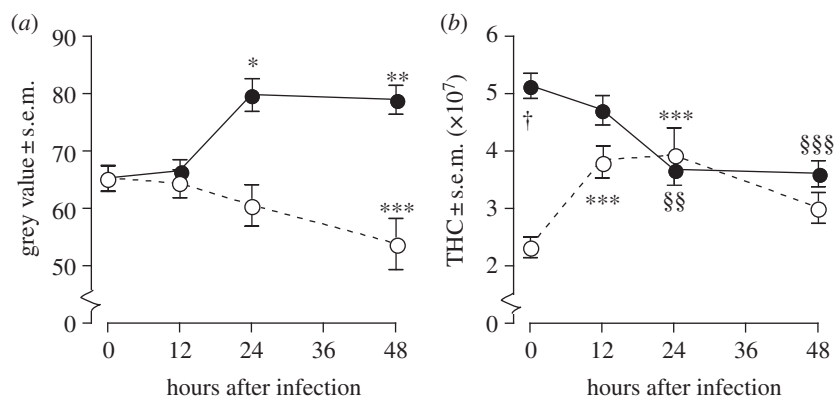


Figure 3. (a) Melanotic encapsulation response and (b) haemocyte numbers in M (solid line) and NM (dashed line) *G. mellonella* larvae following topical application of *B. bassiana* \pm s.e.m. For encapsulation, melanization of each implant was assessed by image analysis and is represented by the grey value, with greater melanization being observed in M than NM larvae, $n = 32\text{--}45$, $*p < 0.05$, $**p < 0.01$ compared with uninfected control larvae; $***p < 0.001$ compared with M larvae at 48 h. Total haemocyte counts (THCs), $n = 15\text{--}27$, $\dagger = p < 0.001$ (comparison between M and NM larvae), $*** = p < 0.001$ (NM larvae compared with NM larval control), $\S\S = p < 0.01$ (M larvae compared with M larval control), $\S\S\S = p < 0.001$ (M larvae compared with M larval control).

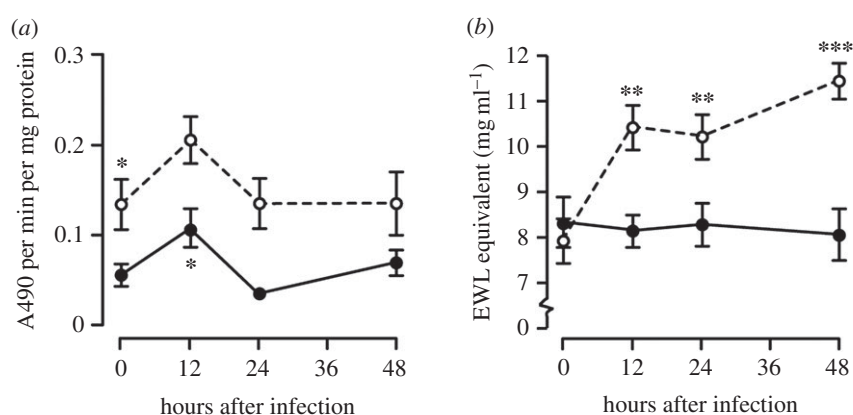


Figure 4. (a) PO and (b) lysozyme-like activity in haemolymph of *G. mellonella* M (solid line) and NM (dashed line) larvae topically infected with *B. bassiana*, showing greater activity in NM than M larvae for PO ($*p < 0.05$) and lysozyme ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$) compared with uninfected control (time zero) larvae. Each graph shows the mean of at least 26 samples \pm s.e.m. The PO activity in uninfected larvae was also higher in NM than M larvae ($*p < 0.05$).

However, there was no significant difference in basal (time zero) PO activity in the cuticle of M versus NM larvae (figure 2b). During the early stages of infection (12–24 h, which is the peak penetration time), the cuticular PO activity in M larvae became elevated above control (time zero uninfected) larvae, peaking at 24 h pi ($F_{5.58}$, d.f. = 7, $p < 0.05$; figure 2b), but dropped by 48 h pi to a level below that of the time zero (uninfected) controls (figure 2b). Although minor fluctuations were recorded in NM larvae during the course of the experiment, these were not statistically significant.

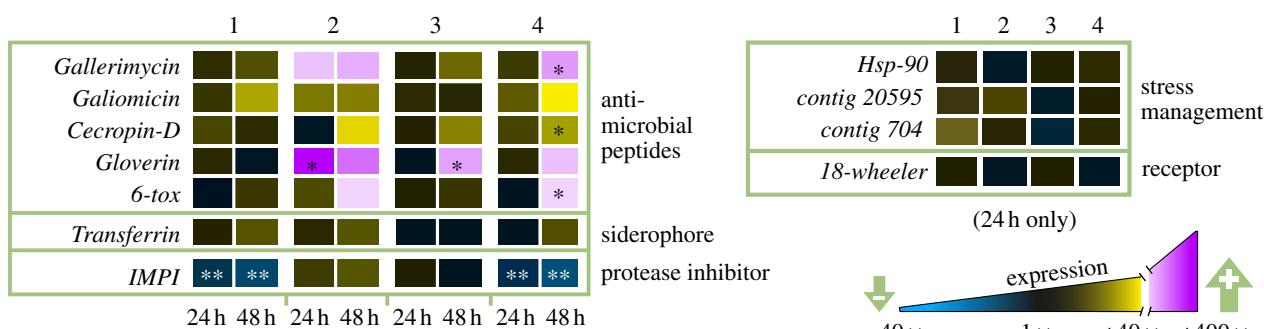
(d) Cellular and humoral immune defences following topical inoculation

Melanin encapsulation of nylon implants was similar for both uninfected M and NM larval controls (figure 3a). However, at 24 and 48 h pi with *B. bassiana*, this response was enhanced in M larvae (one-way non-parametric ANOVA; Kruskal–Wallis with Dunn's post-test; K–W statistic = 60.67, d.f. = 7, $p < 0.01$ and $p < 0.001$, respectively) but diminished in NM larvae, relative to the respective controls (d.f. = 7, $p < 0.01$ at 48 h, and also compared with uninfected M larvae; d.f. = 7, $p < 0.01$ at 24 h and d.f. = 7, $p < 0.001$ at 48 h; figure 3a).

Uninfected control M larvae compared with NM larvae had significantly higher THCs ($5.1 \times 10^7 \text{ ml}^{-1} \pm 2.2 \times 10^6 \text{ ml}^{-1}$ versus $2.3 \times 10^7 \text{ ml}^{-1} \pm 1.8 \times 10^6 \text{ ml}^{-1}$, respectively; figure 3b;

$t_{9.04}$, d.f. = 7, $p < 0.001$). However, the overall difference in the THCs was significantly affected both by the strain of the larvae and by the time point (two-way ANOVA; strain effect $F_{26.4}$, d.f. = 1, $p < 0.0001$; time effect $F_{3.62}$, d.f. = 3, $p = 0.0148$; interaction $F_{13.74}$, d.f. = 3, $p < 0.0001$). Post hoc analyses (one-way ANOVA with Bonferroni's post-test) showed that the THC of infected M larvae was significantly lower than uninfected controls at 24 and 48 h pi ($t_{3.979}$, d.f. = 7, $p < 0.01$ at 24 h; $t_{4.152}$, d.f. = 7, $p < 0.001$ at 48 h; figure 3b), but still higher than the THC of uninfected NM larvae. NM larvae exhibited an opposite pattern, with a significant rise in haemocyte numbers following topical fungal infection peaking at 12–24 h ($t_{4.107}$ at 12 h, $t_{4.337}$ at 24 h, d.f. = 7, $p < 0.001$). The haemolymph PO activity in uninfected M larvae was significantly lower than that of NM larvae (K–W statistic = 61.26, d.f. = 7, $p < 0.05$; figure 4a). While both types of larvae responded rapidly and transiently to fungal infection by elevating haemolymph PO activity (in both cases peaking at 12 h, d.f. = 7, $p < 0.05$), this response was always stronger in NM compared with M larvae. At later time points (24 and 48 h), PO activity returned to levels approximately equivalent to those of the respective uninfected larvae.

Uninfected M and NM larvae had haemolymph lysozyme-like activities of identical intensity (figure 4b). Interestingly, topical fungal infection did not increase lysozyme-like activity in the haemolymph of M larvae, whereas infected



bars: (1) M ctrl cf. NM ctrl ; (2) NM inf cf. NM ctrl ; (3) M inf cf. M ctrl ; (4) M inf cf. NM ctrl

Figure 5. Expression of antimicrobial peptide genes and other immunity/stress-linked genes in M and NM larvae after topical fungal infection. Expression of *Cecropin-D*, *Gallerimycin*, *Galiomycin*, *Gloverin*, *IMPI*, *Transferrin* and *6-tox* was assayed in fat body tissue by QRT-PCR in uninfected animals, and at 24 h and 48 h after topical infection. Expression of *18-wheeler*, *Hsp-90* and *contigs 20595* and *704* was assayed in uninfected controls and at 48 h. Basal expression in uninfected M larvae (column 1) is calculated as a fold change relative to NM uninfected larvae. Fold change in infected NM larvae (column 2) is also relative to NM uninfected larvae. Fold change in infected M larvae is relative to the M uninfected expression (column 3) and to NM uninfected baseline to indicate overall expression (column 4). The colour gradient indicates fold changes in gene expression. Basal expression in uninfected NM larvae is represented by black ($1 \times$ fold change). Increasing blue intensity indicates gene downregulation, whereas yellow and purple represents upregulation in the range 1–40-fold and 41–400-fold, respectively. The mean $\Delta\Delta C_t$ value of three independent blocks is reported. * $p < 0.05$; ** $p < 0.01$. Further details can be found in the electronic supplementary material, S51.12.

NM larvae exhibited significantly elevated lysozyme activity compared with the controls ($t_{3.716}$, d.f. = 7, $p < 0.01$ at 12 h; $t_{3.339}$, d.f. = 7, $p < 0.01$ at 24 h; $t_{5.135}$, d.f. = 7, $p < 0.001$ at 48 h; figure 4b).

(e) Gene expression before and after topical infection

Expression of a range of immunity-related genes was investigated to establish basal (control uninfected) expression levels reflecting immune ‘surveillance’ capacity, and after topical application of *B. bassiana* to gauge additional acute responses to fungal infection. In general, the basal expression was slightly elevated in M compared with NM larvae, with the notable exception of *IMPI* (downregulated in M larvae, $t_{9.525}$, d.f. = 3, $p < 0.01$; figure 5; electronic supplementary material, figure S3). Analysis of all the AMP genes by two-way ANOVA revealed a significant overall trend towards upregulated basal expression in uninfected M relative to NM insects (at 24 and 48 h pi; $F_{5.13}$ and 9.85, respectively, d.f.n = 1, d.f.d = 24 and 25, respectively, $p < 0.05$; electronic supplementary material, table S3).

The NM larvae responded rapidly to topical infection, engendering a strong upregulation of most genes by 24 h, whereas expression in M insects was close to basal levels. For example, expression of the AMP *Gloverin* in NM larvae was elevated by 363-fold at 24 h, but still at basal levels in M larvae. By 48 h, however, *Gloverin* expression had risen significantly in M larval fat body (70-fold above M basal expression, $t_{20.09}$, d.f. = 1, $p < 0.05$; figure 5; electronic supplementary material, figure S3).

The AMP genes *Gallerimycin*, *Cecropin-D* and *Gloverin* were all highly upregulated by 48 h (between 11- and 363-fold above control levels, although in NM larvae *Cecropin-D* was transiently downregulated at 24 h). *Galiomycin*, *6-tox* and *transferrin* were modestly upregulated (two- to 12-fold), but the expression of *18-wheeler* was unaffected (figure 5; electronic supplementary material, figure S3). Although M larvae appeared to upregulate gene expression to a lesser extent than NM larvae over 48 h, the overall effect was very similar, because M larval basal expression was already

higher than NM insects. *IMPI* exhibited differential responses in infected insects; it was strongly upregulated in NM larvae, but unaltered in M larvae.

Several of the genes linked with stress management were expressed at higher basal levels in M larvae compared with the NM larvae (approx. threefold, but 17.4-fold in the case of *contig 704*), and this trend was highly significant overall as assessed by two-way ANOVA ($F_{34.46}$, d.f. = 1, $p < 0.0001$; electronic supplementary material, table S3). Some of these genes were also upregulated 48 h after topical infection in M larvae (see figure 5; electronic supplementary material, figure S3), but overall NM insects exhibited a greater induced upregulation of putative stress-management genes ($F_{4.6}$, d.f. = 1, $p < 0.05$; electronic supplementary material, table S3). Several genes were unaffected, suggesting constitutive expression. See the electronic supplementary material for more data on all these genes.

(f) Insect survival, cellular and humoral responses after fungal injection

The NM larvae were significantly more susceptible to injected *B. bassiana* blastospores than M larvae in terms of survival time (hazard ratio = 2.03, d.f. = 1, Wald = 11.70, $p < 0.001$; figure 6a) and overall mortality (d.f. = 1, $p < 0.001$; figure 6b). There were no differences, however, in the encapsulation and lysozyme-like responses in the haemolymph of M and NM larvae 6 and 12 h post injection (see the electronic supplementary material, table S4). The plasma PO response in NM larvae was significantly elevated relative to M larvae at 6 h post injection (see the electronic supplementary material, table S4, K–W statistic = 22.17, d.f. = 7, $p < 0.05$).

Gallerimycin, *Cecropin-D*, *Gloverin*, *Galiomycin*, *6-tox*, *18-wheeler* and *contig 20595* all underwent modest (two- to 12-fold) upregulation 12 h post injection with the trend for greater upregulation in M than NM larvae (see figure 7; electronic supplementary material, figure S4 and table S3; two-way ANOVA, $F_{10.85}$, d.f. = 1, $p < 0.01$). The magnitude of AMP gene induction at 12 h was relatively low compared

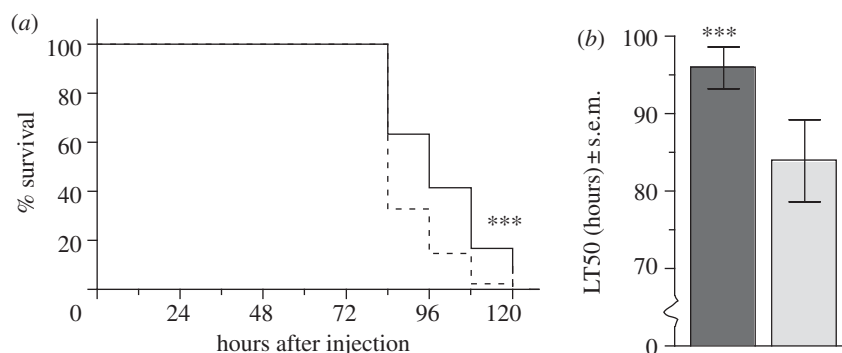


Figure 6. (a) Mortality rate and (b) median LT₅₀ of M (dark grey bars, solid line) and NM larvae (light-grey bars, dashed line) after injection with 750 *B. bassiana* blastospores per larva. *** $p < 0.001$ in comparison with NM larvae.

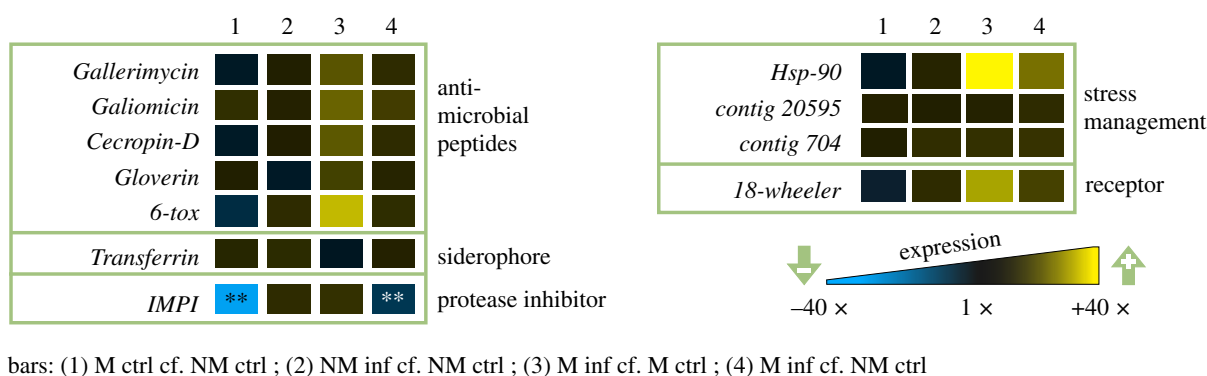


Figure 7. Expression of antimicrobial peptide genes and other immunity/stress-management genes in M and NM larvae 12 h after injection of fungal blastospores. Expression was assayed in fat body tissue by QRT-PCR in injected and sham-injected control animals. Expression in M controls (bar 1) is calculated as a fold change relative to NM controls. Fold changes in infected NM (bar 2) are also relative to NM controls, whereas fold changes in infected M larvae are relative to the uninfected M (bar 3) and uninfected NM to indicate overall expression (bar 4). The colour gradient indicates fold changes in expression, which in NM controls is represented by black (1 × fold change), while blue and yellow indicate downregulation and upregulation (1–40-fold), respectively. The mean $\Delta\Delta Ct$ value of three independent experiments is reported. ** $p < 0.01$. Further details can be found in the electronic supplementary material, S51.12.

with topical infection, and the expression of *Transferrin* was unaffected by injection at this time point. The exception was *Hsp90*, which exhibited a 38-fold upregulation in M larvae following injection, compared with just twofold in NM larvae (see figure 7; electronic supplementary material, figure S4). As with topical infection, the greatest morph-type disparity occurred with *IMPI*, which was mildly upregulated in NM larvae. In M larvae, *IMPI* expression remained completely unchanged from the basal level, which, in turn, was considerably lower in M than NM larvae ($t_{10.38}$, d.f. = 2, $p < 0.01$). The largest upregulation was of *contigs* 20595 and 704 following injection with the fungus in the M larvae, and minor upregulation of *contig* 704 in the NM larvae (see figure 7; electronic supplementary material, figure S4). The expression of the other putative stress-management genes was unchanged in both morphs (see the electronic supplementary material for data).

(g) Cost of melanism on life-history traits

Insects with enhanced melanism had significantly reduced pupal biomass in both males and females compared with NM insects (27.8% reduction for females and 34.7% reduction for males; K–W statistic = 143.9, d.f. = 3, $p < 0.001$; electronic supplementary material, figure S5b), probably as a result of shorter average larval development times ($25.7 \pm$ s.e. 0.25 days in M insects compared with $26.4 \pm$ s.e. 0.07 days in NM insects; $U = 9954$, d.f. = 1, $p < 0.001$; electronic supplementary material, figure S5a). Adult fecundity was also severely curtailed, with the average M moth laying

fewer than half the eggs of her NM counterpart ($284.5 \pm$ s.e. 28.5 eggs versus $588.4 \pm$ s.e. 32.2 eggs; $t_{7.052}$, d.f. = 78, $p < 0.001$; electronic supplementary material, figure S5c).

4. Discussion

A melanic (dark morph) form of *G. mellonella* was found to have increased resistance to the entomopathogenic fungus *B. bassiana*. This resistance is both heritable and multifactorial, comprising several different physiological and anatomical traits. These traits constitute a costly but effective 'be prepared/rapid response' strategy, reflecting augmented pre-formed frontline defences, increased immune surveillance and possibly a greater capacity to deal with stress.

The M larvae exhibit longer larval survival times, slower fungal penetration of the cuticle and a lower propensity to develop haemolymph infections when challenged naturally (topically) and by injection, compared with an NM geographical variant of *G. mellonella*. There appear to be no special features of the M larval cuticle that can prevent attachment or germination of fungal conidia, because these proceed identically on the cuticles and in the cuticular wax extracts from both M and NM insects. However, the M insect cuticle is substantially thicker and it can generate a short burst of enhanced cuticular PO activity coinciding with early mycosis (as the fungus attempts to enzymatically breach the cuticle). This ability appears to be lacking in the NM insects. A thicker cuticle is not ultimately impenetrable, but fungal penetration is delayed, and these extra hours may allow sufficient time for the insect

to orchestrate its antifungal defences, such as the formation of melanotic capsules around fungal infection structures and increased transcription of antifungal peptides. Presumably melanin and upstream precursors can, respectively, form a tough physical barrier, and exert a fungi-toxic or fungi-static effect [5,29]. Melanin could also be involved with wound healing after fungal penetration, as it is in mosquito midguts following penetration by the malaria parasite [30].

Melanin *G. mellonella*, in contrast to the NM larvae, have higher basal concentrations of circulating haemocytes, which decline concomitant with enhanced melanotic encapsulation of fungal elements, suggesting that the haemocytes are playing an active role in the encapsulation process. It should be emphasized that this response is only elicited when the infection route is via cuticular penetration, implying that critical factors in the cuticle must be activated by the fungus and then communicated to the circulating haemocytes.

The M and NM morphs use divergent strategies for tissue-specific (fat body) expression of immunity-related genes, and respond differently depending on the infection route. However, the transcriptional differences between specific AMPs do not readily explain the differences in fungal tolerance, considering that after natural infection the final maximal expression of these genes is often similar in both morphs. The more important difference, therefore, may lie in the kinetics of those responses or in the ultimate target of the AMPs, which may be secondary infections rather than *B. bassiana*. The slightly elevated basal expression of AMPs in M larvae relative to NM larvae could ensure that mature antimicrobial peptides are already in circulation in anticipation of an infection. By contrast, NM larvae upregulate AMPs more rapidly after topical infection, which may simply reflect the faster rate of fungal penetration through a thinner cuticle. Although AMP gene expression may be triggered by fungi, most of the AMPs we examined are not confirmed to exert activity against filamentous fungi, though gallerimycin is known to have some effect against *M. anisopliae* [25]. The potency of antifungal peptides depends on the fungal species, and possibly even the strain [31,32], with genes of the broad-spectrum AMPs being activated by a disparate range of elicitors [33]. Nevertheless, our data confirm that increased expression of *Gallerimycin*, *Galiomicin* and *Cecropin* occurs in (non-melanin) *G. mellonella* larvae naturally infected with *B. bassiana* [33,34]. *Gloverin* was highly upregulated by topical fungal infection in both *G. mellonella* morphs, which is in agreement with its recently discovered activity against bacteria and fungi [35]. It is worth noting that insects infected with entomopathogenic fungi do not develop septicaemia, and that some fungal-derived toxins have putative antibacterial activity, which may augment host AMPs in preventing colonization by opportunistic pathogens [36].

Considering that the expression of immunity-related genes does not sufficiently explain fungal tolerance, and acknowledging that fungal infection must create several sources of stress for the insect, we hypothesized that AMPs might work in concert with factors linked to stress adaptation and detoxification, and inflammatory-type responses. Although the activity of most of the genes examined in this context appeared to be constitutive, the stress-response genes *Hsp90*, *contig 20595* (with a conserved Methuselah-like G-protein receptor functional domain) and *contig 704* (putatively involved in cell repair and migration, with a conserved pleiotrophin-like domain)

were strongly upregulated in M larvae, suggesting the fungus-tolerant insects may also be better equipped to deal with diverse stressors arising from damage and infection.

Some immune components did not appear to play an obvious role in fungus tolerance, such as haemolymph lysozyme, which was not activated in M larvae infected by either topical or injected routes. The lack of a strong cellular and humoral response following injection emphasizes the valuable role of the cuticle in the fungal tolerance strategy. Melanin and its phenolic precursors are only beneficial to the insect in the correct concentration and location, so overproduction of these toxic compounds could prove deleterious/stressful to the host, and may explain why haemolymph PO activity does not exceed a certain threshold in M larvae both during and in the absence of fungal infection. Our findings contrast with reports [6,7,11] that insects with density-induced melanism exhibit a gamut of enhanced lysozyme and haemolymph PO activity as well as increased encapsulation responses and cuticular PO activity. Cotter *et al.* [19], conversely, suggest that diminished lysozyme activity is a trade-off for enhanced PO activity in the melanin form of the lepidopteran *S. littoralis*.

The very low *IMP* expression in M insects is in line with the known role of the protein to counteract melanization caused by either microbial or endogenous enzymes [37]. High-resolution melt analysis of the M insect amplicons, and subsequent sequencing, indicated three separate silent point mutations in a 296 bp cDNA fragment of the *IMPI* gene (representing 65% of the coding sequence; figure 5), which were observed consistently in all M samples but not in the NM samples. Although such synonymous modifications have not altered protein functionality, they do imply that the Siberian (dark morph) *G. mellonella* has undergone a relatively long period of divergent evolution from the UK (NM or pale) morph. Although it cannot be ruled out that the observed traits arose as a result of another selective force across the two geographical regions, this is unlikely because of the beehive-dwelling lifestyle of *G. mellonella*, which means the insect is largely sheltered from obvious pressures acting on melanin alleles, such as industrial pollution and UV radiation [38].

Melanin *G. mellonella* have adopted a different strategy to a fungal entomopathogen by investing in defence, but sacrifice fecundity and pupate as smaller individuals than their larger, fecund but infection-prone non-melanin counterparts. Similar observations were made with melanin *S. littoralis* [19], but it is important to note that compromised fecundity is not an inevitable outcome of enhanced immunity [39,40]. It therefore remains to be seen whether, if placed under constant selective pressure from exposure to *B. bassiana*, a stable melanin *G. mellonella* population would ever evolve with full fungal resistance if confronted with an increasing drain on its reproductive resources. The implications for biological control implementation and contingencies are obviously of great significance.

In conclusion, the successful fungal tolerance mechanism of melanin *G. mellonella* larvae targets the post-germination steps of mycosis through a combination of energetically costly cellular and humoral immune responses, tougher mechanical barriers, and strategies to prevent secondary infection and ameliorate stress. By limiting their fecundity, melanin insects have been able to adopt an effective 'be prepared' strategy that slows the progress of invading fungi and ensures a strong defence is mounted, but is also better equipped to deal with the aftermath of infection or

damage. Melanism is just one of many traits that contribute to this striking and somewhat troubling phenotype.

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