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Dietary protein-quality influences melanization and immune function in an insect

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Summary

1. In insects, cuticular melanization and immune function are strongly dependent on the quantity of dietary protein ingested. However, relatively little is known about the role played by the quality of nitrogenous resources in determining phenotypic variation in the degree of melanization and correlated immunological functions. We explored this issue in a generalist-feeding caterpillar, *Spodoptera littoralis*, by providing larvae with one of two semi-artificial diets differing in their quality of protein supplement (high-quality casein vs. low-quality zein).

2. Larvae given a high-quality protein diet had higher survival and faster growth rates than larvae on the low-quality protein diet; they also had more heavily melanized cuticles. Two components of constitutive immunity were assayed: lysozyme-like antibacterial activity and phenoloxidase (PO) activity. PO activity was not affected by diet quality, but antibacterial activity was higher for insects on the high-quality diet, providing a potential physiological mechanism for observed survival differences between the two dietary treatments.

3. Analysis of nitrogen conversion efficiency using chemically defined diets indicated that protein-quality had little effect on ingestion rates (i.e. nitrogen acquisition), but that post-ingestive utilization of nitrogen was reduced for larvae on the low-quality protein diet. This result implies that protein-quality had a significant influence on the nitrogen pool potentially available for investment in melanin production and immune function.

4. A split-plot, full-sib family breeding experiment was used to dissect the genetic control of cuticular melanization from the effects induced by dietary treatment. Estimates of broad-sense heritability indicated that the expression of melanization had a significant genetic basis, but there was little evidence for a genotype × environment interaction.

5. These results suggest that nutrition is a key factor that influences insect melanization and mediates its coupling with important physiological functions linked to survival.

Key-words: colouration, diet, heritability, immunocompetence, nutrition.

Introduction

The quality of dietary protein is an important determinant of key life-history traits in phytophagous insects (McNeill & Southwood 1978; Broadway & Duffey 1988; Karowe & Martin 1989; Felton 1996; Lee 2007). However, few studies have focused on the potential influence of protein-quality on morphological traits, such as colour patterns. One aspect of insect colouration that has received much attention from both evolutionary ecologists and physiologists is the expression of dark colouration, that is, melanization (see reviews by Kettlewell 1973; Brakefield 1987; Majerus 1998; True 2003). Melanization may confer benefits in terms of facilitating thermoregulatory heat gain (Kingsolver 1983; Guppy 1986; Ellers & Boggs 2002, 2004; Hazel 2002), promoting sexual selection or aposematic signalling (Wiernasz 1995; Sword 2002), and/or enhancing immunocompetence (Reeson *et al.* 1998; Barnes & Siva-Jothy 2000; Wilson *et al.* 2001; Cotter *et al.* 2004b; Armitage & Siva-Jothy 2005). In insects, especially in larval Lepidoptera, the genetic and environmental factors controlling the degree of melanization are well-documented (Goulson 1994; Goulson & Cory 1995; Gunn 1998; Hazel 2002; Solensky & Larkin 2003; Lee & Wilson 2006), but no previous studies appear explicitly to have examined the effects of diet quality on cuticular melanization in insects.

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Cuticular melanization occurs mainly through the deposition of melanin pigment, a nitrogen-rich quinone polymer, in the integument of insects. Therefore, the metabolic processes involved in melanin synthesis are assumed to incur a nitrogen cost (Brakefield 1987) and may be well susceptible to quantitative and qualitative variation in nitrogenous resources. Insect herbivores, especially food plant generalists, consume foods comprising a wide range of protein and amino acid concentrations as well as qualities. The quality of plant protein is primarily determined by its amino acid compositions, which is highly variable between plant species and between tissues within a plant (Felton 1996). It is expected that insects feeding on different dietary protein sources may differ in their nitrogen sink, which would translate into effects on melanic expression. One of the aims of the present study was to test for this diet-induced phenotypic variation in melanization by manipulating the dietary quality of protein and assessing the level of cuticular melanization in a generalist-feeding caterpillar, the Egyptian cotton leaf worm, *Spodoptera littoralis* (Boisduval). We explored heritable variation, as well as diet × genotypic interactions, by applying a full-sib, split-family breeding design.

Resource-dependence in animal colouration has been demonstrated most extensively for carotenoid-based colouration in vertebrates, in conjunction with its effect on immunity and traits related to sexual attractiveness (Blount *et al*. 2003; Faivre *et al*. 2003). There is a growing consensus that melanin may play a similar role as an indicator of immunological potency in insects (Barnes & Siva-Jothy 2000; Wilson *et al*. 2001). Thus, a second aim of the present study was to identify how nutritionally mediated affects on melanization correlated with aspects of immune function in *S. littoralis*. We measured two variables that have been commonly utilized for assessing immunocompetence in insects: phenoloxidase (PO) activity and lysozyme-like antibacterial activity (Moret & Schmid-Hempel 2001). These two activities are important components of the insect innate immune system that are constitutively expressed against potential pathogen attack (Schmid-Hempel 2005; Wilson 2005). Lysozyme is mainly produced in the fat body and haemocytes, but also in the intestinal tract especially during metamorphosis; it is most active against gram-positive bacteria. PO appears to have a much broader spectrum of activity against a range of parasites, including viruses, bacteria, fungi, and metazoan parasites (see Wilson *et al*. 2001). PO enzymes have been implicated in range of activities, including non-self recognition, wound healing, sclerotization, cytotoxic activities and both humoral and cellular encapsulation. PO and its precursor, prophenoloxidase (proPO), are key components of the proPO enzyme cascade, the end product of which is melanin and other quinones (True 2003). Thus, levels of PO and its substrates (including tyrosine and its derivatives) are important in the determination of cuticular melanization in *S. littoralis* and other insects (Wilson *et al*. 2001; Cotter *et al*. 2004b).

Finally, we have compared feeding and post-ingestive responses of caterpillars to diets that differed in protein-quality, to illustrate the mechanisms of diet-related changes in nutrient acquisition and utilization, which in turn determine the degree of cuticular melanization and its physiological correlates.

The main rationale for conducting this study was to emphasize the need to consider nutrition as a critical factor in investigations regarding the evolutionary ecology of insect melanization and immunity.

Materials and methods

STUDY ORGANISMS

Spodoptera littoralis is a polyphagous noctuid moth, feeding on various plant families, which include Solanaceae, Gramineae, Malvaceae, Rosaceae and others (Brown & Dewhurst 1975). More than 100 individual larvae of *S. littoralis* were originally collected in Egypt in July 2002, and the laboratory culture had been established in the UK for *c.* 15 generations at the start of this study. To maintain genetic variation in this laboratory culture, in excess of 200 full-sib families were routinely bred by randomly crossing unrelated male–female pairs. This culturing method has proved to maintain significant genetic variation in this caterpillar (Cotter & Wilson 2002; Cotter, Kruuk & Wilson 2004a; Lee & Wilson 2006; Cotter *et al*. 2008). The laboratory culture was maintained on a wheat-germ based semi-artificial diet supplemented by casein (see below). Insects were kept at 25 °C under a 12 : 12 light : dark photoperiod.

DIETS

Two wheat-germ based semi-artificial diets differing in their nutritional quality were prepared according to the method described by Hoffman, Lawson & Yamamoto (1966). Both comprised the same quantity of wheat-germ (42·4% by dry mass), sucrose (17·2%), brewer's yeast (8·4%), various micronutrients and antibiotic substances (12·5%) and protein supplement (19·5%). However, the two diets differed in the quality of their protein. The high-quality diet contained casein as the protein supplement, whereas in the low-quality diet, the casein was replaced with zein. Zein is a maize-derived protein considered to be low in nutritional quality for its deficiency of certain essential amino acids, such as lysine (Broadway & Duffey 1988; Karowe & Martin 1989; Lee 2007). Diets were moist, with the dry components thoroughly mixed into a 2% agar solution at a ratio of five parts agar solution to one part dry ingredients.

EFFECTS OF DIETARY QUALITY AND GENOTYPE ON MELANIZATION: BREEDING EXPERIMENT

A total of 10 full-sib families were established by pairing 10 randomly chosen, unrelated males and females from the culture described above. Since insects were reared in the same environment over generations, possible confounding effects due to parental environment were eliminated. For each family, we randomly split the brood into the two dietary regimes (high- and low-quality protein diet), so allowing genotype × environment interactions to be determined. For logistical reasons, the experiment was run in two blocks using different sets of families. The first experimental block included 150 caterpillars from 5 full-sib families (block 1, 30 insects per family) and the second consisted of 200 insects from another 5 full-sib families (block 2, 40 individuals per family). In both experimental blocks, newly hatched caterpillars from each full-sib family were reared on a casein-based

wheat-germ (high-quality) diet under crowded conditions until the third larval stadium (*c.* 3–4 days after hatching). Upon moulting to the third larval stadium, larvae were placed solitarily in 25 mL plastic polypots, either containing 'high-quality protein' or 'low-quality protein' dietary treatment (15 and 20 replicates for each diet per family in block 1 and 2, respectively). They were then kept in an incubator at 25 °C under a 12 : 12 light : dark photoperiod until they reached the final larval stadium. The degree of cuticular melanization was measured for individual caterpillars two days after their ecdysis to the final stadium, using an *AvaSpec-2048* fibre optic spectrometer and an *AvaLight-HAL* Tungsten Halogen light source (Avantes, Eerbeek, the Netherlands). Measurements were taken using a 2-mm diameter bifurcated fibre optic probe that was positioned at a 90° angle to the integument surface of each insect. A cylindrical plastic tube was attached to the probe to maintain a constant distance (2 mm) from the sample. Before the measurement, we took two individual caterpillars from the maintenance culture to establish standard references. One was a late fifth-instar larva with exceptionally pale cuticle and the other was a newly moulted sixth- (final) instar larva exhibiting extreme melanization. These were used as the pale and dark standards, respectively. Based on these standards, relative reflectance (%) was measured for each experimental animal, with the value of 0% and 100% representing dark and pale standards, respectively. Thus, lower reflectance indicated a greater degree of cuticular melanization. For each individual caterpillar, triplicate reflectance values were recorded at 575 nm wavelength from randomly chosen points at its dorsal longitudinal band (it is over this part of the spectrum that colour differences between individuals were maximized; Lee & Wilson 2006). Our earlier study showed high repeatability among these triplicate measurements, as well as positive correlations of the mean reflectance values measured between the three different cuticular regions (Lee & Wilson 2006). We ensured that all insects weighed more than 500 mg at the point of the measurement, to minimize the effects of larval body mass on cuticular melanization (see Lee & Wilson 2006). Results from an ANOVA, including block and dietary treatment as fixed factors and family as a random factor, revealed that there was neither a significant effect due to block ($F_{1,297} = 1{\cdot}10$, $P = 0{\cdot}296$) nor a significant block × diet interaction ($F_{1,297} = 0{\cdot}16$, $P = 0{\cdot}689$) on the measured reflectance value. Consequently, data from the two blocks were pooled and 'block' was not included as a factor in subsequent analyses.

During this experiment, a total of 301 individual caterpillars were scored for their degree of cuticular melanization, with an average of 15 replicates per family per diet. Of the 301 caterpillars, 131 and 170 individuals were measured for their melanization on high- and low-quality protein diet, respectively. This difference in sample size was due to the fact that many caterpillars on high-quality diet had already entered into their wandering stage, at which point reflectance measurement was considered to be inappropriate because of shrinkage occurring at larval cuticle surface. The effects of family and diet were tested with a two-way ANOVA using the general linear model options (PROC GLM) in SAS version 9·1 (SAS Institute, Cary, NC), allowing for an unbalanced design. Dietary treatment was included as a fixed factor and family as a random factor. First, the full model was fitted to determine whether there was a significant family × diet interaction term (genotype × environment interaction). If not, the model was reduced by subsequently removing any non-significant two-way interaction terms from the full model. Where significant family effects were detected, the heritability of cuticular melanization was calculated as twice the intra-class correlation (i.e. the proportion of the total variance contributed by the among-family component). Heritability was also calculated separately for caterpillars in each dietary treatment by performing one-way ANOVAs with family as a random factor, followed by variance components analysis using REML iterations in the PROC VARCOMP option. Because a full-sib breeding design was used, our estimation is considered as 'broad-sense heritability', which does not distinguish the contributions to the total phenotypic variation of the additive and non-additive variance components (e.g. dominance and epistasis) (Roff 1997). This heritability estimate from a full-sib analysis should be treated as an upper limit to the actual value. Analyses were performed using untransformed data.

EFFECT OF DIET QUALITY ON LIFE-HISTORY TRAITS

Caterpillars from the first experimental block (starting with 150 caterpillars from 5 full-sib families) were tested for the effect of diet on various aspects of larval performance. Larval mortality was inspected daily for caterpillars on both high- and low-quality protein diets from the start of the third stadium until pupation. One larva on high-quality protein diet escaped from the rearing polypot on the fourth day of the experiment and was discarded from all analyses. For surviving insects, the duration of larval development was determined as the number of days between the beginning of the third larval stadium and pupation. These pupae were then weighed to the nearest 0·1 mg (fresh mass) and returned to the incubator. The duration of pupal development was measured to within 6 h of adult eclosion.

EFFECT OF DIETARY QUALITY ON IMMUNE FUNCTION

Insects in the second block (consisting of 200 individuals from 5 full-sib families) were used to determine the effects of dietary protein-quality on two immune function traits: lysozyme-like antibacterial activity and haemolymph phenoloxidase (PO) activity. Two days after ecdysis to the final larval stadium, individual insects from each dietary treatment were measured for their cuticular melanization (see above). Immediately afterwards, haemolymph was collected from individuals by piercing the final proleg with a sterile needle. In order to prepare for the measurement of PO activity, 8 μL of haemolymph was added to 400 μL of ice-cold phosphate-buffered saline (PBS, pH 7·4) in a plastic Eppendorf tube. Undiluted blood samples were also collected for measuring lysozyme-like antibacterial activity. Both buffer-diluted and undiluted samples were frozen in a –85 °C freezer until lysozyme-like antibacterial, PO activity and the amount of protein were measured following the standard protocol designed for this insect (see the detailed procedures in Wilson *et al*. 2001; Cotter *et al*. 2004b). In brief, for the PO activity assay 100 μL of 10 mM L-Dopa (substrate) was added to 100 μL of buffered haemolymph and the absorbance of the mixture was measured at 492 nm on a Versamax tunable microplate reader (Molecular Devices, Sunnyvale, CA) after 20 min. of incubation at 25 °C. PO activity is expressed as PO units, in which one unit represents the amount of enzyme required to increase the absorbance by 0·001 min^{-1}. Haemolymph protein content was quantified by Bradford's method (Bradford 1976), which involved adding 200 μL of dye reagent to 10 μL buffer-diluted blood sample, followed by reading the absorbance change, using the plate reader with a standard curve created from a BSA standard. Triplicate samples per individual insect were used for examining PO activity and protein level.

A lytic zone assay was performed to determine the lysozyme-like antibacterial activity of undiluted haemolymph. Holes (2 mm diameter) were punched into agar plates containing 10 mL of 1·5% agar mixed with *c.* 50 mg of the gram-positive bacterium *Micrococcus lysodeikticus*.

Each hole was filled with 1 μL of ethanol saturated with phenylthiourea (PTU), inhibiting the melanization of the haemolymph sample. When the ethanol had evaporated, 1 μL of undiluted haemolymph was transferred into the holes, two replicates per individual. Agar plates were then incubated for 24 h at 33 °C. After this period, the plates were digitally photographed and the diameter of the clear zones determined using Image Pro-Plus software (Media Cybernetics, Silver Spring, MD). The relative difference in the lysozyme-equivalent antibacterial lytic activity of haemolymph was indicated by the size of diameter for each sample (Moret & Schmid-Hempel 2001).

EFFECT OF DIETARY QUALITY ON NITROGEN CONVERSION EFFICIENCY

To determine whether the nitrogen conversion efficiency of ingested nitrogen to body nitrogen growth differed between the two dietary treatments, we prepared two chemically defined diets, comprising 21% protein and 21% carbohydrate, following the methods described in Simpson & Abisgold (1985). Such diets allow more accurate assessment of the efficiency with which ingested nutrients are allocated to growth than is possible with semi-defined diets. The diet composition was chosen to emulate the protein : carbohydrate ratio of the wheat-germ based semi-artificial diets, which is *c.* 34% : 28% protein : carbohydrate. Similar to the wheat-germ based diets in the first experiment, we manipulated the composition of the protein source. The protein content of high-quality diet was composed entirely of a 3 : 1 : 1 mixture of casein : peptone : albumen, which provides a similar amino acid profile to that of seedling wheat (Simpson & Abisgold 1985). For generating low-quality diet, 50% of this casein-based protein mixture was replaced by a corresponding amount of zein.

Upon moulting to the final larval stadium, 20 insects from the laboratory culture were weighed to the nearest 0·1 mg and each was assigned to its own experimental arena, a 9-cm diameter Petri dish (Lee 2007). Caterpillars received a single block of one of the two diets. Each block was weighed to the nearest 0·1 mg before being presented to the insects, and after each 24 h any remaining food was collected and replaced with a fresh, pre-weighed block. Removed blocks were dried to constant mass at 50 °C, and weighed to the nearest 0·1 mg. This procedure continued daily until each insect pupated. The duration of this final larval stadium was similar between the insects on the two diets (accelerated failure-time analysis: $\chi^2 = 0{\cdot}04$, d.f. = 1, $P = 0{\cdot}853$), with *c.* 6·0 and 6·2 days for insects on high- and low-quality diet, respectively. Food consumption was determined as the difference between the initial dry mass of the food (estimated from the initial fresh mass of the food using regression equations) and the final dry mass of the food. Protein consumption was calculated based on diet composition, and the amount of nitrogen consumed was estimated as the amount of protein consumed divided by a conversion factor of 6·25 (AOAC 1980). Experimental insects were kept in an incubator at 25 °C under a 12 : 12 light : dark photoperiod throughout the experiment.

When insects pupated, they were killed by freezing and dried at 50 °C to constant mass in a desiccating oven. Dried carcasses were weighed and lipid-extracted using the method described in Lee (2007). Analysis of nitrogen content was performed for these lean carcasses using the micro-Kjeldahl procedure (AOAC 1980). A sample of 20 freshly moulted, final-instar larvae were sacrificed for constructing a linear regression equation allowing the estimations of the starting nitrogen content for each experimental insect. Nitrogen growth (i.e. the amount of nitrogen accumulated in the body tissues over the stadium) was obtained by subtracting these starting values from the pupal content.

DATA ANALYSES

Analyses on immune function parameters were undertaken using two-way ANOVA and ANCOVA with larval body mass at immune function measurement as a covariate. Dietary treatment was included as a fixed factor and family as a random factor. One-way ANCOVA was performed to analyze the nitrogen conversion efficiency, with the amount of nitrogen eaten as covariate and body nitrogen as response variable (Raubenheimer & Simpson 1992). Prior to these parametric analyses, the data were checked for normality and homogeneity of error variance using Kolmogorov–Smirnov and Bartlett's test, respectively. Where necessary, data were transformed. Effect of diet on larval survival was tested using non-parametric Kaplan–Meier tests. Any effects due to diet and family on the duration of larval development were tested using the accelerated failure-time analysis (using Weibull distribution) in the PROC LIFEREG option in SAS version 9·1.

Results

EFFECTS OF DIETARY QUALITY AND GENOTYPE ON CUTICULAR MELANIZATION

There was a clear effect of dietary quality on cuticular melanization, with the insects reared on casein-supplemented diet displaying significantly darker colouration (i.e. lower relative reflectance value) relative to those on zein-supplemented diet (Fig. 1a; Table 1). This dietary effect on melanization was observed consistently across all full-sib families, as evidenced by a non-significant family × diet interaction term (Table 1). The absence of a genotype × environment interaction became even more apparent when reaction norms were inspected for the 10 full-sib families (Fig. 1b). On both diets, there was a significant difference in the degree of cuticular melanization between the families (Table 1), suggesting the presence of additive genetic variation in this trait. Removal of the non-significant two-way interaction in this model did not affect the significance of the two main factors (diet: $F_{1,290} = 168{\cdot}34$, $P < 0{\cdot}001$; family: $F_{9,290} = 6{\cdot}48$, $P < 0{\cdot}001$). The broad-sense heritability (h^2) of the cuticular melanization was 0·31, which was estimated from the reduced ANOVA model using the pooled data set in which the non-significant diet × family interaction term was removed. To disentangle any condition-dependence of the heritability estimate, we performed one-way ANOVAs separately for insects in each dietary treatment. These revealed that there was highly significant between-family variation in cuticular melanization in both dietary treatments

Table 1. Two-way factorial ANOVA of the full-sib breeding design for cuticular melanization in final-instar *S. littoralis* caterpillars, with diet as a fixed factor and family as a random factor. Untransformed data were used for the analysis

Source	d.f.	SS	MS	*F*	*P*
Family	9	8 986·30	998·48	5·94	< 0·001
Diet	1	26 428·60	26 429·60	157·13	< 0·001
Family × Diet	9	2 575·12	286·12	1·70	0·088
Residual	281	47 263·76	168·20		
Total	300	85 253·78			

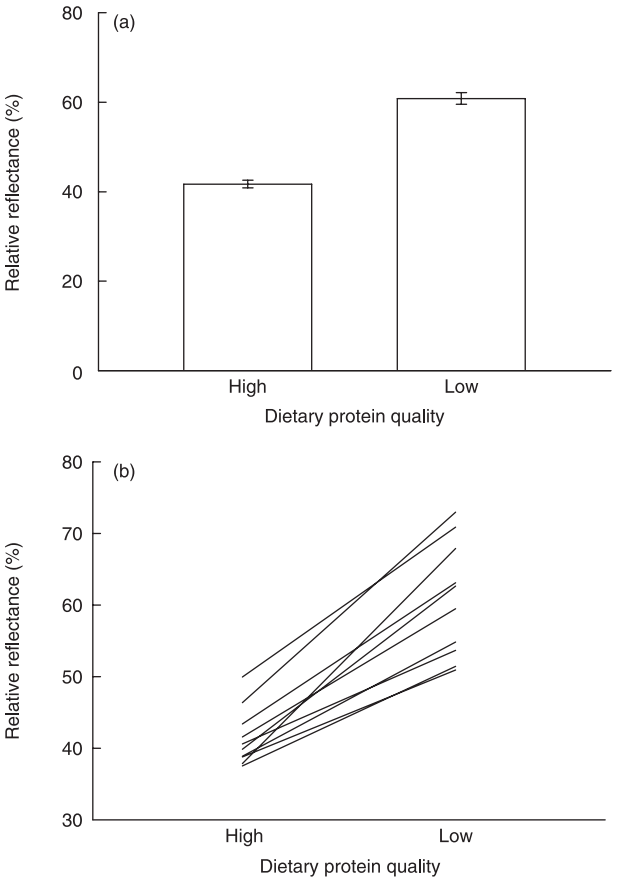

Fig. 1. The degree of cuticular melanization for final-instar *S. littoralis* caterpillars reared on high- and low-quality protein diet. Relative reflectance value (%) was measured from the dorsal part of the cuticle of individual caterpillars. The lower the reflectance, the darker the insects. (a) Mean relative reflectance value (%) (± 1 SEM) of insects on the two diets pooled over the 5 full-sib families. (b) Plot of family means across the two diets for relative reflectance value (%), demonstrating non-significant genotype × environment interaction (see text).

(Table 2), with broad-sense heritability estimates of 0·24 and 0·39 for high- and low-quality dietary treatments, respectively. The genetic correlation of melanization across the dietary treatments was approximated using the family means (Via 1984). This showed that the genetic correlation was positive (r_g = 0·708) and significantly different from zero (P = 0·022).

EFFECT OF DIETARY QUALITY ON LIFE-HISTORY TRAITS

Survival from the beginning of the third larval stadium to the pupal stage was higher for insects on high-quality diet than those on the low-quality diet (log-rank test: χ^2 = 14·63, d.f. = 1, P < 0·001; Fig. 2a), and this diet-related difference in survival was consistent across five full-sib families (Fig. 2b). It should be noted that the patterns of survival varied between the families (Fig. 2b), suggesting possible genetic variation in survival rates. Among surviving insects, larvae on the low-quality diet took significantly longer to reach pupation than those on high-quality diet (accelerated failure-time analysis: χ^2 = 18·61, d.f. = 1, P < 0·001; Fig. 2c). The duration of larval development also varied between families (χ^2 = 49·48, d.f. = 4, P < 0·001), but there was no significant interaction between diet and family (χ^2 = 5·65, d.f. = 4, P = 0·227). The fresh mass of pupae was unaffected by any of the main factors (ANOVA, diet: $F_{1,75}$ = 0·22, P = 0·644; family: $F_{4,75}$ = 0·86, P = 0·490; diet × family interaction: $F_{4,75}$ = 0·52, P = 0·723). The percentage of pupae that eclosed as adult moths was *c*. 52% (28 out of 54 pupae; mean pupal duration: 10·1 days) and *c*. 13% (4 out of 31 pupae; mean pupal duration 10·3 days) for those insects on high- and low-quality diet, respectively. Within each diet treatment, there were no significant phenotypic correlations between larval colouration (% reflectance) and either of the two life-history traits of surviving insects (i.e. pupal mass and larval development) (all P > 0·067; see Table 3).

Table 2. One-way ANOVA of the effect of family on cuticular melanization tested separately for final-instar *S. littoralis* caterpillars reared on high- and low-quality protein diet

Source	d.f.	SS	MS	F	P
(a) High-quality protein diet					
Family	9	2 181·97	242·44	2·76	0·006
Residual	121	10 647·26	87·99		
Total	130	12 829·23			
(b) Low-quality protein diet					
Family	9	10 421·30	1157·92	5·06	< 0·001
Residual	160	36 616·50	228·85		
Total	169	47 037·80			

EFFECT OF DIETARY QUALITY ON IMMUNE FUNCTION

Total protein levels in the haemolymph of larvae reared from the third larval stadium on high-quality protein diet were significantly higher than in that of larvae on the low-quality diet (ANOVA: $F_{1,160}$ = 8·18, P = 0·005; Fig. 3a). Haemolymph protein levels differed between families ($F_{4,160}$ = 2·60, P = 0·038) and there was a significant interaction between the two factors (diet × family interaction: $F_{4,160}$ = 3·79, P = 0·006). However, when larval body mass at immune measurement was

Table 3. Phenotypic correlations between the degree of melanization (% reflectance) and two life-history traits for surviving *S. littoralis* caterpillars (pupal mass and larval developmental time) on high- and low-quality protein diet. Pearson's correlation coefficient (r) was used

Dietary protein quality	Sample size	Pupal mass vs. Reflectance		Larval developmental time vs. Reflectance	
		r	P	r	P
High	49	–0·264	0·067	0·101	0·453
Low	29	0·005	0·978	–0·050	0·796

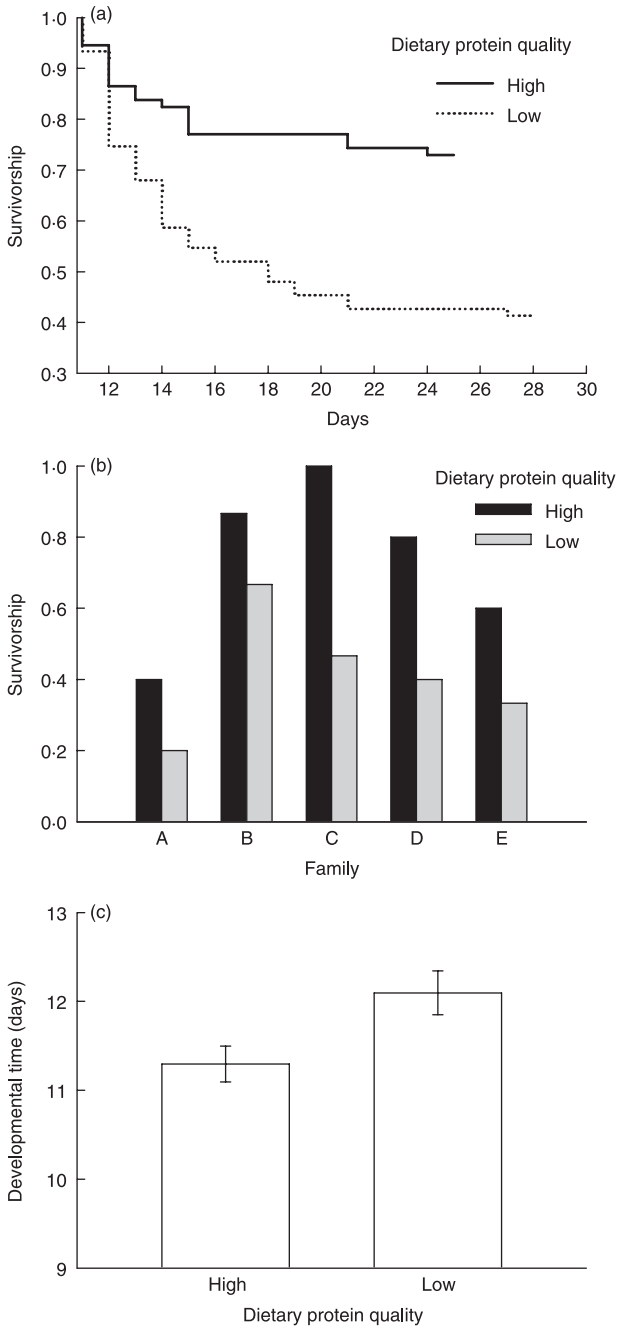

Fig. 2. Effects of the dietary quality on various life-history traits in *S. littoralis* caterpillars. (a) Patterns of survivorship for insects reared on high- and low-quality protein diet. (b) A plot illustrating family differences in survivorship for 5 full-sib families (each of five families was arbitrarily coded A–E). (c) Mean developmental time (± 1 SEM) for insects on high- and low-quality protein diet (measured from 54 and 31 surviving individuals, respectively). The duration of larval survival and development was determined as the number of days from the beginning of the third larval stadium (day 0) to death and to pupation, respectively.

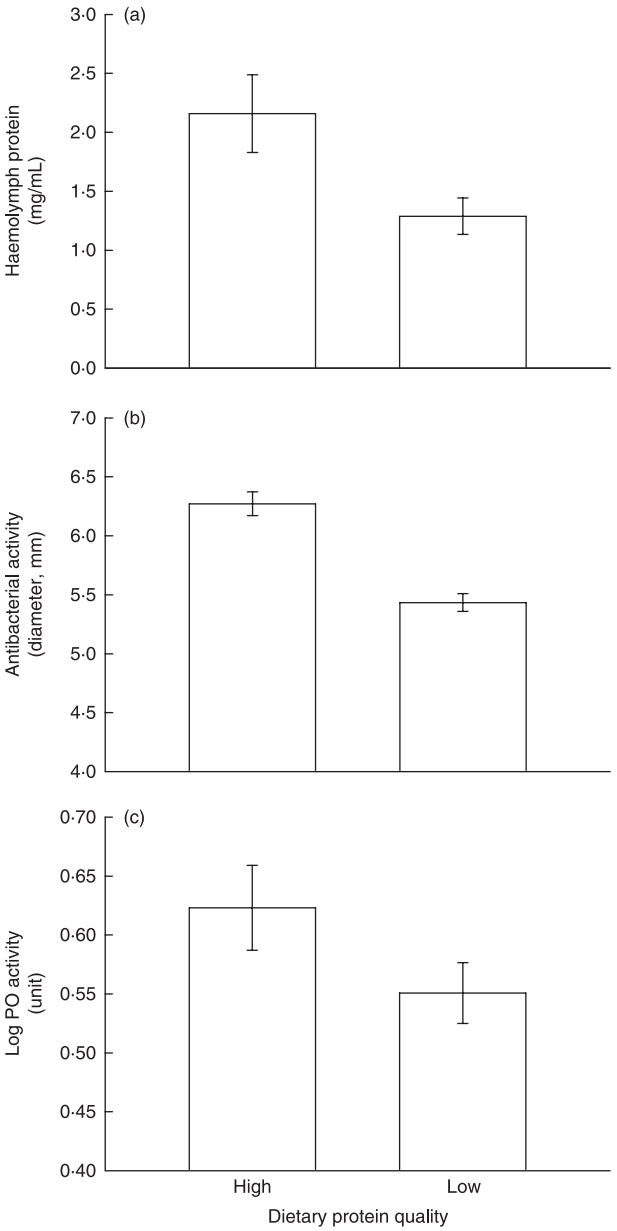

Fig. 3. Effects of the dietary quality on (a) haemolymph protein content, (b) lysozyme-like antibacterial activity, represented here as diameter of cleared zone in a lytic zone assay, and (c) phenoloxidase (PO) activity (log-transformed) of final-instar *S. littoralis*. The values are means ± 1 SEM.

included in the ANCOVA model as a covariate, the significant main effect of diet on total protein levels became non-significant ($F_{1,159} = 1{\cdot}65$, $P = 0{\cdot}201$). This was due to a significant covariate effect ($F_{1,159} = 84{\cdot}59$, $P < 0{\cdot}001$), showing increasing total haemolymph protein with larval mass, as well as insects being heavier on high-quality diet relative to those on low-quality diet (mean ± 1 SEM: 605·30 ± 13·16 mg and 551·14 ± 10·73 mg for high- and low-quality diet, respectively; *t*-test: $t = 3{\cdot}22$, d.f. = 168, $P = 0{\cdot}002$). The significance levels of the family effect and the diet × family interaction remained unchanged in the ANCOVA (family: $F_{4,159} = 5{\cdot}45$, $P < 0{\cdot}001$; diet × family interaction: $F_{4,159} = 7{\cdot}07$, $P < 0{\cdot}001$). Lysozyme-like antibacterial activity was higher for insects reared on high-quality diet (ANOVA: $F_{1,160} = 52{\cdot}72$, $P < 0{\cdot}001$; Fig. 3b). Families also varied markedly in their levels of antibacterial activity ($F_{4,160} = 3{\cdot}74$, $P = 0{\cdot}006$), suggesting a significant additive genetic component to this trait, while there was little

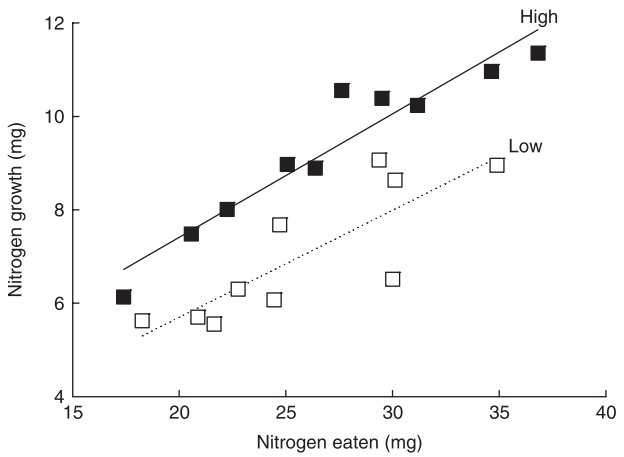

Fig. 4. Utilization plot illustrating the conversion efficiencies of ingested nitrogen to body nitrogen growth for final-instar *S. littoralis* caterpillars on high- and low-quality protein diet. Each square represents an individual insect that pupated. Filled and open squares represent individuals feeding on high- and low-quality diet, respectively. Least-square regressions are fitted to demonstrate the patterns of nitrogen utilization.

indication of a diet × family interaction ($F_{4,160} = 2{\cdot}06$, $P = 0{\cdot}088$). When larval mass was taken into account as a covariate in ANCOVA, antibacterial activity also increased with larval mass ($F_{1,159} = 15{\cdot}53$, $P < 0{\cdot}001$) but inclusion of the covariate did not change the significance of the main factors and their interaction (diet: $F_{1,159} = 41{\cdot}66$, $P < 0{\cdot}001$; family: $F_{4,159} = 4{\cdot}30$, $P = 0{\cdot}003$; diet × family interaction: $F_{4,159} = 1{\cdot}81$, $P = 0{\cdot}129$). PO activity was slightly higher for larvae on the high-quality protein diet, but this difference was non-significant (ANOVA: $F_{1,160} = 2{\cdot}94$, $P = 0{\cdot}088$; Fig. 3c). There was a significant difference between families in their PO activity levels, again suggesting a possible additive genetic component to this aspect of insect immunity ($F_{4,160} = 2{\cdot}77$, $P = 0{\cdot}029$); the diet × family interaction was non-significant ($F_{4,160} = 1{\cdot}42$, $P = 0{\cdot}230$). Further ANCOVA results revealed a non-significant covariate effect on PO activity (larval mass: $F_{1,159} = 0{\cdot}00$, $P = 0{\cdot}999$), with almost identical significance levels to those from ANOVA for the remaining effects (diet: $F_{1,159} = 2{\cdot}77$, $P = 0{\cdot}098$; family: $F_{4,159} = 2{\cdot}75$, $P = 0{\cdot}030$; diet × family interaction: $F_{4,159} = 1{\cdot}40$, $P = 0{\cdot}236$).

EFFECT OF DIETARY QUALITY ON NITROGEN CONVERSION EFFICIENCY

The conversion efficiency of ingested nitrogen to body nitrogen growth was investigated by constructing an 'utilization plot' (Raubenheimer & Simpson 1994), in which nitrogen growth (the amount of nitrogen gained in body tissues over the final stadium) was plotted against nitrogen consumption across the final stadium for caterpillars reared on the two chemically defined diets (Fig. 4). When regressions were fitted for larvae on the high- and low-quality diets, we found parallel and positive linear relationships between body nitrogen growth and nitrogen consumption (Fig. 4; ANCOVA with nitrogen consumption as a covariate, covariate effect: $F_{1,17} = 75{\cdot}16$, $P < 0{\cdot}001$). Dietary treatment had a significant effect on nitrogen conversion efficiency ($F_{1,17} = 38{\cdot}03$, $P < 0{\cdot}001$), being consistently greater for larvae on high-quality protein diet compared to low-quality protein diet (Fig. 4). When food consumption rates were compared, larvae on the two diets differed little in their nitrogen consumption (ANOVA: $F_{1,18} = 0{\cdot}32$, $P = 0{\cdot}580$), suggesting that diet quality had little impact on feeding behaviour. Nitrogen growth was significantly higher for insects on the high-quality diet vs. low-quality diet ($F_{1,18} = 10{\cdot}62$, $P = 0{\cdot}004$).

Discussion

Our aim was to explore the causal relationships between dietary protein-quality and two coupled physiological traits: cuticular melanization and immunocompetence. The main finding of the present study is our demonstration that dietary protein-quality determines both the extent of cuticular melanization and the insect's physiological mechanisms for resisting infection. Given the significance of melanization and immunocompetence to numerous aspects of the biology of insects, this finding has important implications for understanding the links between nutrition, organismal functions and ecology in insects.

Eating a diet with low-quality protein has been associated with high mortality, reduced growth, delayed development, and low reproductive output in insect herbivores (Broadway & Duffey 1988; Karowe & Martin 1989; Felton 1996; Lee 2007). Our results reveal that, in addition to its impacts on demographic traits, the quality of dietary protein also influences the phenotypic expression of colour, with caterpillars reared on low-quality protein diet (i.e. zein-supplemented) exhibiting paler colouration than those reared on high-quality protein diet (i.e. casein-supplemented). Diet-induced polyphenism in colouration has been reported occasionally in larval Lepidoptera (Clarke, Dickson & Sheppard 1963; Greene 1989). However, the main focus of those studies was on the role of the host-plant (or plant-derived) defensive chemicals in eliciting a morph that provides better protection against predators. Here, we report the first case of colour variation (melanization) arising from variation in nutritional quality of the diet.

The question arises as to why and how nutrition affects the expression of melanization. As mentioned earlier, melanin is a nitrogen-rich compound, which may require substantial nitrogen or protein investment for its production (Blois 1978). It is therefore predicted that eating diets with poor nutritive quality reduces the internal nitrogen pool destined for melanin production and subsequent deposition in the insect integument. In accordance with this prediction, we observed that insects on the low-quality protein diet had a lower haemolymph protein pool than those on the high-quality protein diet. There are two possible explanations for this observation. The first is that larvae on the low-protein diet have reduced intake rates, i.e. nutrient acquisition; the second is that they have reduced nitrogen utilization efficiency post-ingestively. Our data provide little support for the first

possibility, as the difference in the amount of nitrogen eaten was negligible between the two treatments. In agreement with this finding, an earlier study demonstrated that there was little effect of moderately low-quality protein diet on feeding by *S. littoralis* caterpillars, as long as the ratio of protein to carbohydrate in the diet was ≥ 1 (Lee 2007). In the present study, we provided caterpillars with diets comprising a near equal ratio of protein to carbohydrate. In contrast, compared to larvae on the high-quality diet, those on the low-quality diet were much less efficient at converting nitrogen intake to body nitrogen, again confirming an earlier observation (Lee 2007). This could be due to lower digestion and absorption, or to an increased rate of catabolism of all but the deficient amino acids in nutritionally imbalance protein (Horie & Watanabe 1983; Karowe & Martin 1989; Felton *et al*. 1992). Whatever the mechanism, it is likely that the lower protein pool resulted in fewer resources being available for provisioning the substrates (tyrosine) or enzymes (phenoloxidase, PO) required for melanin synthesis. Our measurement of PO activity revealed little difference between the two treatments, suggesting that the latter is unlikely and that eating low-quality protein may have limited the supply of substrate for melanin production.

As well as a dietary effect, the present data also indicate that the phenotypic expression of cuticular melanization has a heritable component, with a significant broad-sense heritability estimate (h^2) of 0·31 when data from both diet treatments were pooled. This value is comparable to a previous estimate based on the analysis of 22 full-sib families (h^2 = 0·37; Lee & Wilson 2006). The absence of a genotype × environment interaction (family × diet interaction, here) suggests that the relative degree of cuticular melanization expressed by the different families is independent of their nutritional environment. A strong and positive between-treatment genetic correlation suggests that the same sets of genes are responsible for a given trait in both environments (Via 1984). Broad-sense heritability was also calculated separately for each dietary regime to examine possible differences in genetic variation between the animals under nutritionally favourable and stressful conditions (Hoffmann & Parsons 1991; Hoffmann & Merilä 1999). Our results showed that the heritability estimate was higher for the insects reared on low-quality diet (h^2 = 0·39) than for those on the high-quality diet (h^2 = 0·24). However, it should be noted that, before this study, *S. littoralis* caterpillars had been cultured on high-quality diet for *c.* 15 generations. Thus, during this time, there may have been selection on canalization leading to a reduction in phenotypic variation on the high-quality diet (Hoffmann & Merilä 1999). Alternatively, it is possible that the limitation on resources resulting from eating low-quality protein might have unmasked variation in phenotypic expression that would have otherwise have been hidden under favourable nutrient conditions (Hartl, Dykhuizen & Dean 1985; Hoffmann & Merilä 1999).

Several studies on Lepidoptera have reported negative correlations between melanization and various life-history traits (e.g. growth and development), suggesting potential trade-offs between resource allocation to melanin synthesis and somatic growth, which could constrain the evolution of melanization (Brakefield 1987; Windig 1999; Cotter *et al*. 2004b; Talloen, Van Dyck & Lens 2004). In the present study, we found no evidence for such trade-offs within the two dietary treatments. However, when diet-induced variation was taken into account, we found that the increasing degree of melanization was associated with improved larval fitness, with the darker caterpillars from the high-quality diet treatment exhibiting faster larval development, higher eclosion rate, and, most notably, higher survival compared to the paler caterpillars from the low-quality diet treatment. It is likely that the larger protein pool of larvae fed the high-quality diet allowed them simultaneously to allocate protein to melanin synthesis and to survival and development (Van Noordwijk & de Jong 1985).

As noted previously, insects on the high-quality protein diet enjoyed higher survival. A potential mechanism generating this relationship is the effect of diet quality on immune function (Sheldon & Verhulst 1996; Rolff & Siva-Jothy 2003; Schmid-Hempel 2005; Siva-Jothy, Moret & Rolff 2005; Wilson 2005). Evidence from a range of insect species shows that environmentally induced cuticular melanization is associated with enhanced immune responsiveness or resistance to pathogens (Reeson *et al*. 1998; Barnes & Siva-Jothy 2000; Wilson *et al*. 2001; Cotter *et al*. 2004b; Armitage & Siva-Jothy 2005; Wilson & Cotter 2008). In line with these findings, our data showed enhanced immune function, particularly in terms of lysozyme-like antibacterial activity, for dark insects reared on high-quality protein diet. Maintaining or activating the immune system costs protein (Lee *et al*. 2006). Consistent with this finding, it is perhaps not surprising that those larvae feeding on the low-quality diet experienced reduced internal protein reserves and a concomitant reduction in the strength of pathogen resistance mechanisms. In combination with protein deficiency, it is also likely that high metabolic costs for processing low-quality diet might have limited energy reserves (Karowe & Martin 1989), which would have otherwise been utilized for maintaining and activating the immune system. Although the cause of the elevated mortality in the low-quality diet treatment was not ascertained, it is pertinent to note that these larvae appeared to grow normally on zein-based diet until they reached the final-instar wandering stage, after which mortality in these larvae increased upon entering the pre-pupal stage. At this point, most of the dying insects displayed darkening of their entire integument, a symptom similar to that observed in larvae dying of bacterial infection. This suggests that the high mortality suffered by insects on low-quality diet was not due to malnutrition, but may have resulted from weakened resistance to opportunistic bacteria or other microparasites during metamorphosis; this hypothesis is currently under investigation. Interestingly, the baseline mortality rates were rather high even on the high-quality diet for some families (see Fig. 2b) and also those that died displayed darkening of the entire integument as described above, perhaps suggesting a transitory pathogenic infection persisting in the culture at

this time. Unlike antibacterial activity, there was little difference in PO activity between insects on the two diets. It is unclear why this latter component of immune function was less influenced by diet than was lysozyme-like antibacterial activity. Despite expressing the same PO activity, the insects on the two diets may vary in the amount of proPO (the inactive precursor of PO). However, previous studies have shown a positive correlation between levels of spontaneously activated PO and proPO (activated using chymotrypsin) in this species ($r = 0{\cdot}845$, $P < 0{\cdot}001$; S.C. Cotter, unpublished data).

Results from the current study confirm the earlier suggestion that there is a significant protein or nitrogen requirement for the expression of melanization (Brakefield 1987). Until recently, the nutrient-dependence of melanization had rarely been investigated, relative to other ecological effectors such as population density (Reeson *et al*. 1998; Barnes & Siva-Jothy 2000; Cotter *et al*. 2004b) or temperature (Goulson 1994; Hazel 2002; Solensky & Larkin 2003). Our results are also consistent with the idea that the degree of melanin deposition is an indicator of an individual's nutritional status influencing physiological functions linked to survival (Barnes & Siva-Jothy 2000; Wilson *et al*. 2001; Cotter *et al*. 2004b; Armitage & Siva-Jothy 2005). The fact that the coupling between cuticular melanization and immune defence is mediated via protein-quality has a number of ecological implications for insect herbivores living under a wide range of nutritional environments in nature. The quality of protein varies across different plant families and plant parts (Felton 1996) and generalist herbivores are likely to encounter diverse protein sources due to their broad host–plant range. The impact of this natural variability in host-plant protein-quality on the expression of immune responses in insects has largely been neglected, but may well play an important part in determining the dynamics of plant × herbivore × pathogen interactions (Price *et al*. 1980; McVean *et al*. 2002; Cory & Hoover 2006). For instance, feeding on a plant tissue with either low- or high-quality protein may result in reduced or improved host resistance against invading pathogens, thus leading to changes in demographic variables (e.g. population growth). Studies to date have investigated the impact of diet on immunity by reducing the amount of food (Siva-Jothy & Thompson 2002), by controlling the quantity of specific nutrients in the diet (Stoehr 2007) and the balance of essential macronutrients (Lee *et al*. 2006), and by providing plant materials with potentially differing nutritive quality (Ojala *et al*. 2005; Kapari *et al*. 2006; Klemola *et al*. 2007). As far as we are aware, this study is the first to show that qualitative aspects of protein nutrition are also important in determining immune responses. It would be interesting to determine whether altered cuticular melanization as a consequence of dietary quality impacts on other ecological functions of melanization, such as thermoregulation, sexual attraction, and aposematism.

Finally, the present study challenges the existing view regarding the role of food in influencing animal colouration. It has been largely accepted that in vertebrates the expression of carotenoid-based colour is more labile to dietary changes than melanin-based colours. This is because animals are unable to synthesize carotenoids metabolically and so must obtain them from their food, whereas the melanin pigment can be synthesized *de novo* (Hill & Brawner 1998; Badyaev & Hill 2003). Contrary to this general belief, our results indicate that, in insects at least, melanin is limited by dietary protein-quality and that melanic expression may function as an 'honest' indicator of insects' physiological condition, as is the case for the carotenoid-based colouration in vertebrates (Owens & Wilson 1999).

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