Ecological Entomology (2006) **31**, 196–205

Melanism in a larval Lepidoptera: repeatability and heritability of a dynamic trait

KWANG PUM LEE and KENNETH WILSON Department of Biological Sciences, Lancaster University, U.K.

Abstract. 1. Although it is well established that the deposition of melanin pigment in the cuticle of larval Lepidoptera is influenced by both environmental and genetic factors, few studies have examined intra-individual regional variation in the degree of melanism or the ontogenetic dynamics of this trait. Here, heritable and density-dependent effects on within-individual and stage-specific variation in melanism were examined in caterpillars of the Egyptian cotton leafworm, *Spodoptera littoralis* (Boisduval).

2. Using quantitative spectrometric methods, it is shown that cuticular melanism changes dramatically within larval stadia, showing the highest and lowest levels of melanism early (first day) and late (final day) in each larval stadium respectively. However, solitary-reared caterpillars were significantly paler than those reared gregariously at all stages of development and maintained greater levels of variation in melanism. This variation in melanism was repeatable and exhibited a significant heritable component (narrow sense heritability based on offspring–parent regressions: $h^2 = 0.18$–0.30).

3. The degree of melanism was correlated negatively with larval body weight in solitary caterpillars, but not gregarious ones. Melanism also varied spatially, with the lateral longitudinal band being consistently darker than the dorsal or dorso-lateral bands. Crowd-rearing increased melanism in all regions of larval cuticle, but the extent of crowding-induced melanism was more pronounced in the dorsal and dorso-lateral bands than in the lateral one.

4. These results indicate that although cuticular melanism is a highly dynamic trait, ontogenetic changes in relative cuticular melanism are both predictable and repeatable within individuals and genotypes. This has implications for our understanding of the evolution of melanism and for applying artificial selection on the basis of colour.

Key words. Caterpillar, cuticular melanism, density dependence, development, phenotypic integration, phenotypic plasticity.

Introduction

Melanism (the occurrence in a species of dark or black forms) has intrigued evolutionary biologists for over a century (Kettlewell, 1973; Majerus, 1998). Numerous hypotheses have been proposed to explain the origin and maintenance of melanic phenotypes. Melanism has been particularly well studied in insects, and industrial melanism in the peppered moth (*Biston betularia*) has now become a classic textbook example of rapid evolution in the field (summarised in Majerus, 1998). Melanism in this species is the result of a simple, genetically based polymorphism, but in many insects it is determined by a sometimes complex interaction between genetic and environmental factors. Melanism in larval Lepidoptera has been particularly well studied, and previous work has shown that the expression of melanism may be affected by a range of environmental

Correspondence: Dr Kwang Pum Lee, The Research Institute of Basic Sciences, Seoul National University, Seoul 151-747, Republic of Korea. E-mail: kwangpumlee@hotmail.com

factors, including temperature (Goulson, 1994; Gunn, 1998; Hazel, 2002; Solensky & Larkin, 2003), light (Faure, 1943), humidity (Goulson, 1994), diet (K. P. Lee & K. Wilson, unpublished data), and population density (Long, 1953; Kazimirova, 1992; Goulson & Cory, 1995; Gunn, 1998). For example, in the African armyworm (*Spodoptera exempta*), larvae are generally pale green or brown at low population densities and jet black under crowded conditions, but the magnitude of the response to population density also has a significant genetic component (Wilson *et al*., 2001).

The adaptive significance of density-dependent melanism remains to be established. However, in many insects, melanism is due to the deposition of melanin, a nitrogen-rich quinone polymer that is known to have potent antimicrobial activity (St Leger *et al*., 1988; Nappi & Vass, 1993; Ourth & Renis, 1993). It has therefore been suggested that the increased deposition of melanin in the cuticle at high population densities is an adaptive countermeasure to cope with the greater risk of disease transmission in crowded environments ('density-dependent prophylaxis'; Wilson & Reeson, 1998; Reeson *et al*., 1998; Barnes & Siva-Jothy, 2000; Cotter *et al*., 2004a). Other hypotheses for the evolution of density-dependent melanism invoke possible thermoregulatory benefits of melanism (Goulson, 1994; Gunn, 1998) and a potential role for melanism in aposematic signalling (Iwao, 1968; Wilson, 2000).

Regardless of the precise mechanisms maintaining density-dependent melanism in the field, the potential for either natural or artificial selection to result in realised changes in the expression of melanism depends critically on the heritability of the propensity of individuals to become melanic in response to appropriate cues. This, in turn, is constrained by the repeatability of the trait. In previous studies of melanism in insects, it has generally been assumed that the expression of melanism is a fixed trait or one that essentially changes monotonically with age (e.g. Thompson *et al*., 2002). However, in larval Lepidoptera and other soft-bodied insects, it is likely that melanism is a dynamic trait both within and between larval instars. This is because, following ecdysis, the surface area of the cuticle increases markedly as the insect feeds and grows (e.g. during the final instar of *Manduca sexta* larvae, the cuticle surface area increases by approximately fourfold; Chapman, 1998). Therefore, because melanin is deposited in the cuticle just *prior* to ecdysis (Curtis *et al*., 1984; Hiruma *et al*., 1984), the expression of melanism is likely to change following moulting as cuticular melanin is dispersed within the expanding cuticle as the insect grows. This means that melanism is likely to be a highly dynamic and plastic trait, and this has potential implications not only for quantifying melanism in individual insects, but also for understanding the adaptive value of melanism and the selection pressures associated with it.

Spodoptera littoralis (Boisduval) (Lepidoptera: Noctuidae) is a polyphagous moth (Brown & Dewhurst, 1975), and larvae of this species exhibit a form of density-dependent polyphenism similar to that described for *S. exempta*: under low density conditions the larvae are generally pale brown or grey whereas under crowded conditions they may become dark brown or black, due to the deposition of melanin in the cuticle (Hodjat, 1970; Altstein *et al*., 1994; Cotter *et al*., 2004a). Thus, the aim of the present study was to use a quantitative spectrometric technique to examine density-dependent effects on developmental and regional variation in cuticular melanism in *S. littoralis* caterpillars, as well as to determine the repeatability and heritability of melanism in this species. The motivation behind the present study was to determine some of the developmental and genetic constraints acting on the evolution of melanism in the field, and to identify any potential problems associated with applying artificial selection on the basis of colour in the laboratory. These sorts of issues are at the heart of the study of phenotypic integration, an area of growing interest amongst evolutionary biologists (Pigliucci, 2003; Pigliucci & Preston, 2004).

Materials and methods

Insects

Spodoptera littoralis larvae were originally collected in Egypt in July 2002, and a culture had been established in the laboratory for approximately 12 generations at the start of this study. Upon moulting to the third larval instar ($\approx$3–4 days after hatching), caterpillars were placed in 25-ml plastic polypots, either individually ('solitary' reared) or in groups of two ('crowd' or 'gregariously' reared). Rearing in groups of two stimulates larvae to express the 'gregarious' phenotype, whilst minimising any stresses associated with crowding, such as food limitation. In both rearing treatments, larvae were provided with *ad libitum* wheatgerm-based semi-artificial diet (Hoffman *et al*., 1966), and were kept at 25 °C under LD 12:12 h photoperiod.

Effects of rearing density on melanism

Measurement of melanism. The degree of cuticular melanism was measured for both solitary and crowd-reared caterpillars 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 in order to maintain a constant distance of 2 mm from the sample. A late fifth instar *S. littoralis* caterpillar with conspicuous pale coloration was used to set the white standard reference, while the dark standard was established by eliminating light from the probe. These standards allowed the quantification of the relative paleness of a sample compared with the white standard reference, which was

expressed as a reflectance value (%). Thus, 100% reflectance was equivalent paleness to the white standard, while 0% reflectance was equivalent to the dark standard. Triplicate reflectance values were recorded at 575 nm wavelength from the dorsal longitudinal band (Fig. 1) of each individual at different stages of development as follows: late fourth (last day of fourth instar), early fifth (first day of fifth instar), late fifth, early sixth, and mid-sixth (second day of sixth instar). These triplicate measurements showed high repeatability at each larval stage (e.g. for mid-sixth instar larvae, $r = 0.89$, $n = 139$ larvae, $P < 0.001$). Reflectance was measured at 575 nm, rather than integrated across the visible spectrum, because preliminary analyses indicated that it was over this part of the spectrum that colour differences between individuals were maximised.

Caterpillars displayed distinct regional differences in cuticle coloration, which were particularly pronounced in the older (mid-sixth instar) caterpillars. To determine the extent of these differences, the reflectance was measured from randomly chosen points at each of following locations for these caterpillars: dorsal band, lateral band, and the region between the two, which is referred to as the dorso-lateral band (Fig. 1). All caterpillars were weighed to the nearest 0.1 mg immediately after colour measurement.

Data analysis. Prior to any statistical data analyses, the three replicated measurements of reflectance were averaged to generate a mean reflectance value. These data were then log-transformed to comply with the assumptions of the parametric tests. However, all graphical representations depict untransformed data. Repeated-measures analysis of variance (ANOVA) was used to test for the within-subject effect of the developmental stage of larvae on the mean reflectance and on larval body weight. The same repeated-measures method was applied for analysis of the colour differences between the three regions of cuticle within individuals, but this time the region was used as a within-subject factor. The effect of rearing condition (solitary or gregarious) was the between-subject factor in these analyses. To account for any violations of sphericity assumptions of the variance–covariance matrix, significance levels corrected by Huynh–Feldt epsilon (ε) adjustments were used when examining the within-subject factor (Quinn & Keough, 2002). Simple least-squares linear regression lines were fitted to describe any potential effect of larval weight on insect colour. The relationships between larval colour measured at different developmental stages were examined with parametric Pearson's correlations and repeatabilities. Repeatability (r) is the intra-class correlation coefficient, and in the present context is defined the proportion of total variation in a trait that can be explained by differences between individuals. They were estimated here using the methods of Lessells and Boag (1987), and approximate standard errors for these estimates were determined via permutation. All statistical analyses were performed using SAS v. 8.2 (SAS Institute, Cary, North Carolina) or S-PLUS v. 6.2 (Insightful Corp., Seattle, Washington).

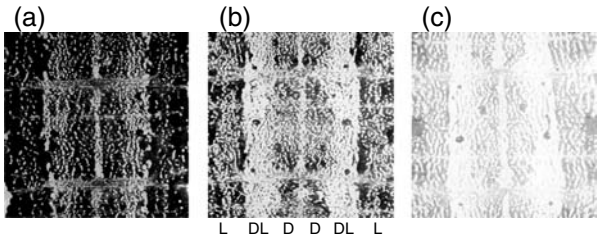

Fig. 1. Cuticular melanism in *Spodoptera littoralis*. Photographs show the isolated cuticles from: (a) dark, (b) intermediate, and (c) pale individuals of the mid-sixth instar caterpillars. Three cuticle regions are indicated: dorsal band (D), dorso-lateral band (DL), and lateral band (L).

Heritability

The heritability of cuticular melanism was estimated using 204 full-sibling solitary-reared larvae from 22 families (a mean of 9.67 offspring per family). As described above, newly moulted third instar caterpillars from each family were assigned singly to individual diet pots and the degree of melanism (% reflectance) was measured from the dorsal longitudinal band of each insect 2–3 days after reaching the final larval instar. At measurement, the body weight of all larvae was within the range of 500–1000 mg in order to minimise the effects of body weight on cuticular melanism (see Results). By using the components of variance extracted from a one-way ANOVA, with family as a random factor in a full-sibling analysis, heritability was initially calculated as twice the intra-class correlation (i.e. the proportion of the total variance attributable to the among-family component). The estimate from this full-sibling design is generally considered as 'broad sense heritability' because it does not distinguish between the contributions to total phenotypic variation of additive and non-additive genetic (e.g. dominance and epistasis) variance components (Roff, 1997).

In order to avoid possible overestimation, an additional analysis was conducted based on offspring–parent regression, from which the ratio of additive genetic variance to total phenotypic variance were calculated (i.e. 'narrow sense heritability'). The mean offspring values of reflectance (%) for each family were regressed against the mid-parent values (the average of the parents), and against those of their father (sire), and mother (dam). The slope coefficient (b) was estimated by least-square linear regression weighted for family size and bootstrapped 2500 times. The exact probability estimates for these regression analyses were calculated via 2500 sample permutations (e.g. Smith *et al.*, 1999). In offspring–parent regressions, the heritability estimate derived from the offspring vs. mid-parent relationship is calculated as the slope coefficient of the regression, while the heritability estimate based on the regression of the mean offspring value on each parent's

value is calculated as twice the slope of the regression line (Falconer & Mackay, 1996; Roff, 1997).

Results

Cuticular melanism across larval stadia

There was a significant effect of larval rearing condition on cuticle colour (repeated-measures ANOVA: between-subject factor, $F_{1,194} = 60.11$, $P < 0.001$), with the gregarious caterpillars exhibiting darker coloration (lower reflectance) than those reared in isolation (Fig. 2). However, caterpillars in both rearing treatments showed rapid colour changes within each larval stadium, as indicated by a significant within-subject effect of developmental stage ($F_{4,776} = 440.04$, $P < 0.001$, Huynh – Feldt $\varepsilon = 0.8587$). During the pre-moult periods (late stage of instar), caterpillars were pale but became markedly darker when they had just moulted to the next larval instar (Fig. 2). A significant rearing density-by-development stage interaction term ($F_{4,776} = 7.87$, $P < 0.001$) indicated that such patterns of colour change differed between insects reared at the two larval densities. Subsequent profile analysis of contrasts revealed that the effect of rearing density on colour was most pronounced during the first 2 days after moulting to the final instar ($F_{1,194} = 9.75$, $P = 0.002$, significance level Bonferroni adjusted; Fig. 2). When the frequency distributions of cuticle reflectance values were compared for solitary and gregarious larvae at this stage, solitary caterpillars not only had a higher mean reflectance (35.3% vs. 21.6%) but also exhibited greater variation in reflectance than crowded insects (variance: 145.4 vs. 52.9) (Fig. 3a). Within solitary larvae, the darkest 25% of individuals (i.e. those in the lowest quartile of the reflectance distribution) displayed a distinctively dark cuticle, indistinguishable from that of gregariously reared caterpillars (Fig. 3b), while those with highest reflectance were paler in colour.

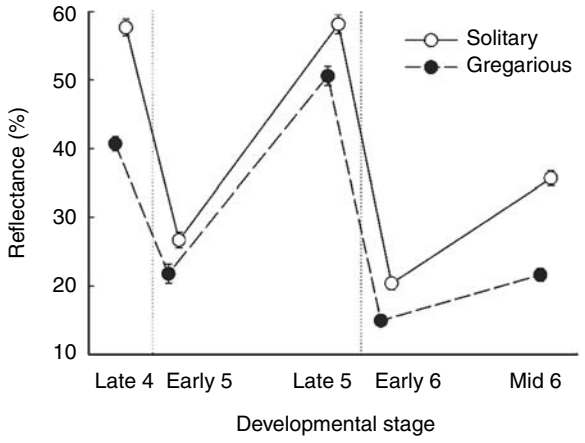

Fig. 2. Temporal dynamics of cuticular melanism for solitary and gregarious caterpillars across five successive developmental stages from late fourth to mid-sixth instar. Reflectance value (%) was measured from the dorsal longitudinal band of the cuticle of individual caterpillars (mean ± SE). The lower the reflectance, the darker the insects.

As a consequence of the extensive colour variation between larval stages, the repeatability of melanism within individual larvae between late fourth and mid-sixth instar was low and non-significant for both solitary and gregarious caterpillars (Table 1). However, after accounting for this variation due to larval stage (by subtracting the average reflectance for a given stage from the individual reflectance values), the repeatability of cuticular melanism increased markedly (Table 1). Thus, although individuals varied in colour during development, after accounting for larval stage variation in colour, individuals were consistently relatively pale or dark. Among the solitary caterpillars, the degree of cuticular melanism was significantly positively correlated across all five developmental stages examined (Table 2). In contrast, caterpillars reared gregariously exhibited relatively weak, and mostly non-significant, correlations across developmental stages, and the overall variation in colour was much lower (Bartlett's test of homogeneity of variance using the untransformed data pooled over the entire larval developmental stages, $\chi^2 = 21.67$, $P < 0.001$).

Melanism in different regions of cuticle

Two days after moult to the final instar, caterpillars displayed distinct dark longitudinal bands in the dorsal and lateral parts of their body (Fig. 1). Between these bands, there was a paler dorso-lateral band. Caterpillars reared in isolation were significantly paler (higher reflectance) in all three major regions of cuticle than those reared gregariously (between-subject factor, $F_{1,194} = 64.21$, $P < 0.001$; Fig. 4). In both solitary and gregarious insects, the lateral band was the darkest, the dorso-lateral band was the lightest, and dorsal band was intermediate between the two (within-subject factor, $F_{2,388} = 319.66$, $P < 0.001$, Huynh–Feldt $\varepsilon = 0.8459$). A significant rearing density-by-cuticle region interaction ($F_{2,388} = 10.87$, $P < 0.001$) indicates that the effect of crowding was manifested differently across the three cuticle regions, as seen by a much smaller phase difference observed in the lateral bands of the two phenotypes compared with the other regions (Fig. 4). Despite this, the regional variations in melanism were correlated strongly and positively with each other for both solitary and gregarious insects (Table 3), and between-bands repeatability was high after accounting for differences in the average darkness of the three bands (Table 1).

Larval weight and melanism

During larval development, caterpillars increased their body weight in a near-exponential manner (within-subject factor, $F_{4,768} = 1861.73$, $P < 0.001$, Huynh–Feldt

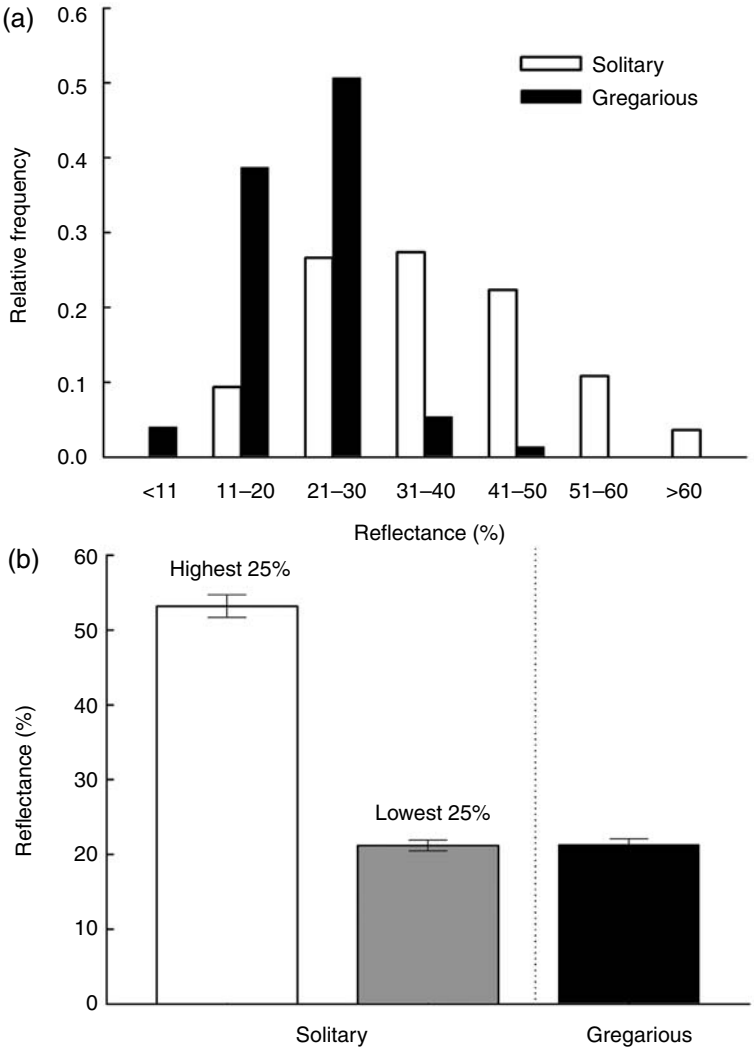

Fig. 3. Detailed comparison of cuticular melanism in the dorsal longitudinal band of mid-sixth instar caterpillars at different rearing densities. (a) Distributions of the relative frequency of solitary and gregarious caterpillars across the reflectance values (%). (b) Mean reflectance values (± SE) of the two groups of solitary caterpillars, palest 25% and darkest 25% in the population in (a), and of gregarious insects. The lower the reflectance, the darker the insects.

$\varepsilon = 0.3443$). Solitary caterpillars were significantly heavier (by roughly 12%) than those reared in groups (between-subject factor, $F_{1,194} = 64.21$, $P < 0.001$), and this relationship persisted in successive developmental stages, as indicated by the non-significant within-subject interaction term ($F_{4,768} = 1.95$, $P = 0.157$). To examine the effect

Table 1. Repeatability (± approximate SE) for the degree of melanism (% reflectance) in (a) the dorsal cuticle band across the developmental stages of individual caterpillars from late fourth to mid-sixth instar, and (b) dorsal, lateral, and dorso-lateral bands within individual mid-sixth instar caterpillars. Repeatabilities (r) were estimated using the methods of Lessells and Boag (1987) and approximate standard errors were determined by randomisation. Adjusted reflectance was calculated by subtracting the average reflectance for a given stage or region from the individual reflectance values.

	All larvae (n = 194 larvae)	Solitary only (n = 131 larvae)	Gregarious only (n = 63 larvae)
(*a*) *Temporal repeatability*			
Reflectance	0.015 ± 0.024 NS	−0.016 ± 0.026 NS	−0.078 ± 0.030 NS
Adjusted reflectance	0.260 ± 0.034***	0.284 ± 0.042***	0.204 ± 0.058***
(*b*) *Spatial repeatability*			
Reflectance	0.211 ± 0.046***	0.047 ± 0.052 NS	0.262 ± 0.082***
Adjusted reflectance	0.509 ± 0.041***	0.459 ± 0.052***	0.621 ± 0.062***

NS, $P > 0.05$, ***$P < 0.001$.

Table 2. Pearson's correlation coefficients (r) between the degree of melanism (% reflectance) in the dorsal cuticle band across the developmental stages of individual caterpillars.

Stage	Early fifth	Late fifth	Early sixth	Mid-sixth
Solitary				
Late fourth	0.340***	0.290***	0.250**	0.216*
Early fifth		0.446***	0.424***	0.267**
Late fifth			0.397***	0.176*
Early sixth				0.480***
Gregarious				
Late fourth	0.202 NS	0.170 NS	0.259*	−0.043 NS
Early fifth		0.208 NS	0.243 NS	0.266*
Late fifth			0.110 NS	0.178 NS
Early sixth				0.441***

NS, $P > 0.05$, *$0.01 < P < 0.05$, **$0.001 < P < 0.01$, ***$P < 0.001$.

Table 3. Pearson's correlation coefficients (r) between the degree of melanism (% reflectance) measured from the three different cuticle bands (dorsal, dorso-lateral, and lateral) of individual caterpillars at the mid-sixth instar.

Region	Dorso-lateral	Lateral
Solitary		
Dorsal	0.641***	0.547***
Dorso-lateral		0.337***
Gregarious		
Dorsal	0.567***	0.782***
Dorso-lateral		0.623***

NS, $P > 0.05$, ***$P < 0.001$.

of body weight on larval coloration, cuticle reflectance was regressed against body weight for both solitary and gregarious individuals (Fig. 5). For solitary mid-sixth instar caterpillars there was a significant positive relationship between body weight and cuticle reflectance ($F_{1,129} = 20.92$, $P < 0.001$), i.e. melanism declined as larval body weight increased. However, no such relationship was found between larval body weight and colour for the gregarious caterpillars ($F_{1,61} = 0.03$, $P = 0.858$). After accounting for weight differences between solitary and gregarious larvae, solitary larvae were generally paler than those reared in groups (Fig. 5), as indicated by a significant interaction between larval rearing-density and body weight (ANCOVA with body weight as covariate: $F_{1,190} = 4.06$, $P = 0.045$). Similar results were obtained from equivalent regression analyses for each developmental stage.

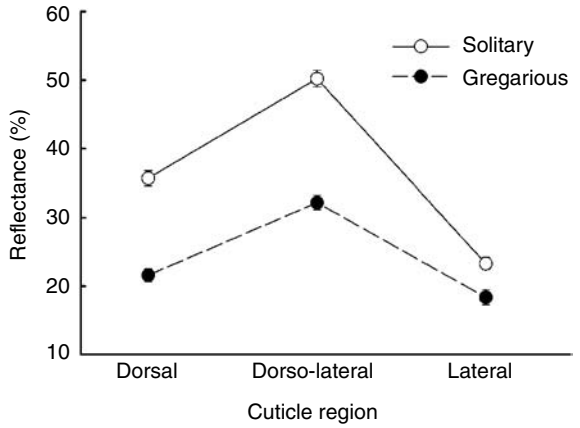

Fig. 4. Spatial variation in cuticular melanism within solitary and gregarious caterpillars. Reflectance values (%) were measured from the three different cuticle regions (dorsal band, dorso-lateral band, and lateral band; see Fig. 1) of mid-sixth instar caterpillars (mean ± SE). The lower the reflectance, the darker the insects.

Heritability of melanism

Results from the one-way ANOVA indicated a significant effect of 'family' on the melanism of the dorsal cuticle in mid-sixth instar caterpillars ($F_{21,182} = 3.11$, $P < 0.001$), with an estimated broad sense heritability (h^2) of 0.37 ± 0.14 (standard error, SE). When the mean offspring values for each family ($n = 22$) were regressed against the mid-parent values, the male parent (sire) values, and the female parent (dam) values, narrow sense heritability estimates were as follows: $h^2_{\text{mid-parent}} = 0.18 \pm 0.08$ ($P = 0.026$), $h^2_{\text{sire}} = 0.30 \pm 0.14$ ($P = 0.042$), and $h^2_{\text{dam}} = 0.18 \pm 0.11$ ($P = 0.092$) respectively (Fig. 6).

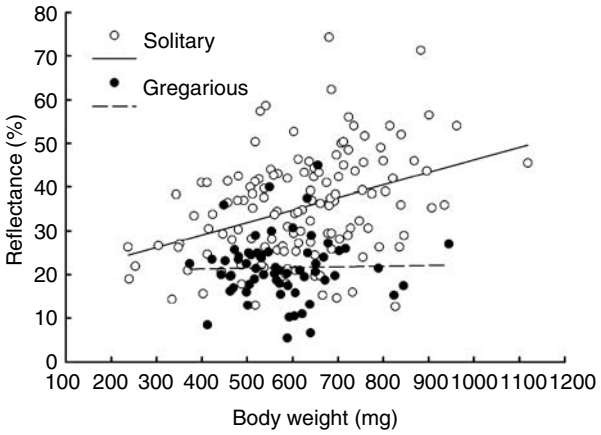

Fig. 5. Relationship between larval body weight and cuticular melanism in solitary and gregarious caterpillars. Reflectance values (%) were measured from the dorsal cuticle band of mid-sixth instar caterpillars. Low reflectance indicates dark coloration. Least-squares regression lines were fitted to demonstrate the effects of rearing condition on the relationship between larval body weight and melanism. The slope coefficients for solitary and gregarious insects are 0.028 (± 0.006, SE; $n = 131$) and 0.001 (± 0.009; $n = 63$) respectively.

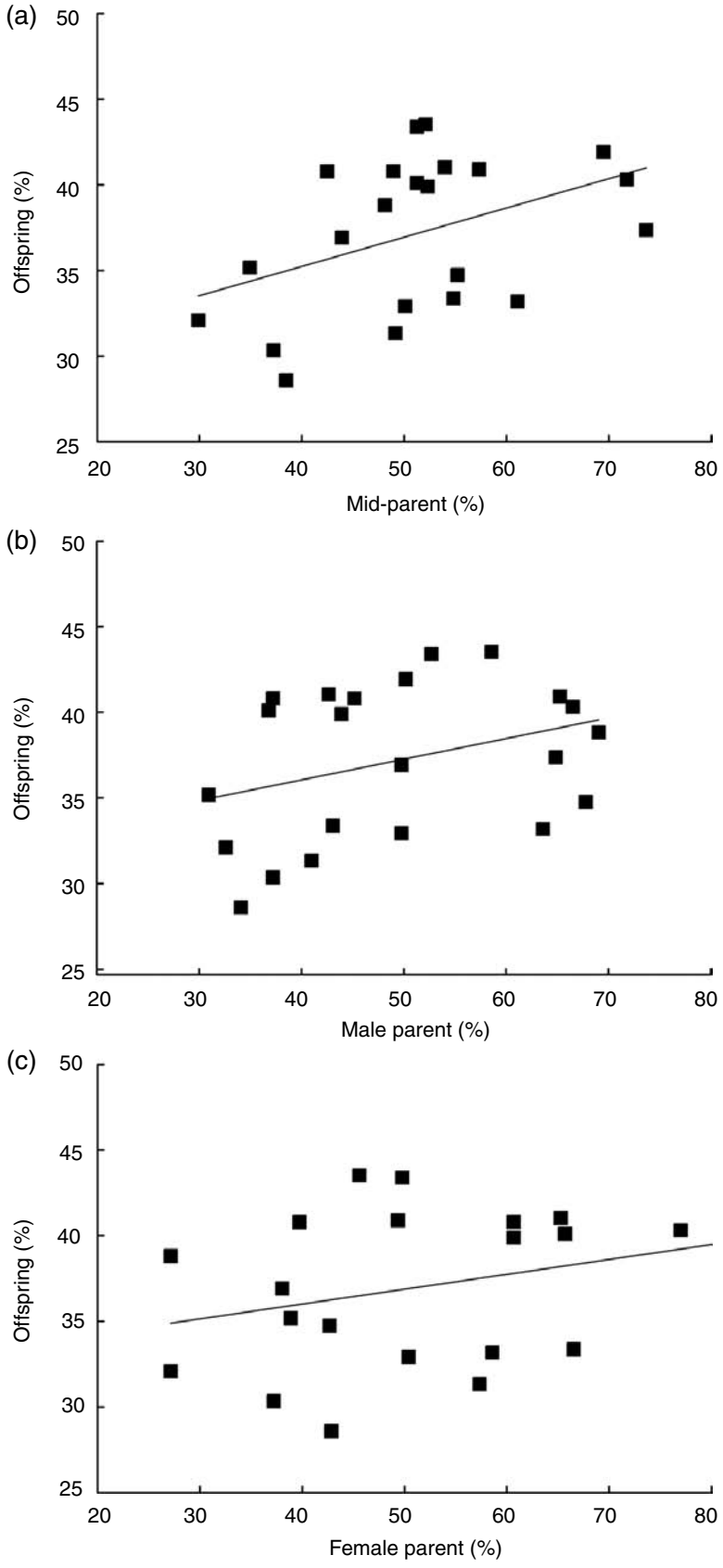

Fig. 6. Least-squares regressions of the mean offspring against (a) mid-parent (slope coefficient, $b = 0.180 \pm 0.081$, SE), (b) male parent ($b = 0.149 \pm 0.071$), and (c) female parent ($b = 0.088 \pm 0.054$) for the degree of melanism in the dorsal cuticle band of mid-sixth instar caterpillars. Each axis denotes the mean reflectance value (%), with lower values indicating greater degrees of melanism. Regression lines were estimated by bootstrapped linear regression.

Discussion

Previous studies examining cuticular melanism in live insects have generally relied on categorising the degree of melanism by qualitatively scoring colour by eye (Tojo, 1991; Kazimirova, 1992; Goulson, 1994). However, in using this subjective approach, not only are subtle differences in colour between individuals likely to be overlooked (including the presence of colour variation in the ultraviolet end of the light spectrum, e.g. Church *et al*., 1998), but also statistical analyses will generally be restricted to crude category-based tests. In contrast, the present study collected objective, quantitative, and repeatable data on larval colour using a fibre optic spectrometer. Although reflectance spectrometry has been used fairly extensively over the last two decades by biologists interested in colour variation in other animal taxa, especially birds and fishes (e.g. Endler, 1990; McNaught & Owens, 2002; Grether *et al*., 2005), few studies have used it to examine colour variation in insects (Church *et al*., 1998). This methodology turned out to be a powerful tool for tracing the colour changes of individual caterpillars over the course of their development and for quantifying colour variation within and between individuals. Moreover, the continuous distribution of the spectrometric data allowed the use of more powerful (parametric) statistical tests than would have been possible using a crude qualitative measure of colour score.

In line with previous studies on this species (Hodjat, 1970; Altstein *et al*., 1994; Cotter *et al*., 2004a) and on other Lepidoptera (e.g. Faure, 1943; Long, 1953; Iwao, 1962; Tojo, 1991), the results showed that crowd-rearing significantly increases cuticular melanism in larval *S. littoralis*. Moreover, spectrometric data establish, for the first time, that despite regional variation in colour (Fig. 1), this density-dependent increase in cuticular melanism was expressed in all regions of the cuticle simultaneously, but more so in the dorsal and dorso-lateral bands than in the lateral band. Even though the rearing density treatments were imposed only at the start of the third instar, differences in cuticle colour were apparent in all developmental stages, from late fourth to mid-sixth instar. This indicates that just two caterpillars per arena were sufficient to induce melanism, probably through physical contact between the larvae (Kazimirova, 1992; Gunn, 1998). Also, the darker colour of fourth instar larvae that had been reared gregariously from the start of the previous instar, indicated that the response to crowding was rapid, as was also reported for a closely related species, *Spodoptera litura* (Tojo, 1991).

Besides being paler than their gregarious conspecifics, solitary caterpillars were also more variable in the measured reflectance compared with those insect reared gregariously over the development. The significantly lower variability among the latter suggests a directional phenotypic response by caterpillars to crowding. A similar phenomenon was observed in populations of the phase polyphenic grasshopper *Schistocerca emarginata*, with greater levels of melanic variation being observed in solitary-reared than crowd-reared insects (Sword, 2002). Sword (2002) has argued that within species exhibiting density-dependent phase polyphenism, the degree of colour variation in the low- and high-density phenotypes may reflect the history of selection acting on the population in the wild (see also Pigliucci *et al*., 1995; Hoffman & Merila, 1999). Thus, the reduced levels of colour variation in crowded larvae would suggest that natural selection in the field may have occurred more frequently for melanic coloration at high local population density than at low. Although the adaptive significance of melanism at high density has yet to be conclusively demonstrated, there is good evidence from a range of species, including *Spodoptera* caterpillars and *Schistocerca* locusts (Reeson *et al*., 1998, 2000; Wilson *et al*., 2001, 2002), that cuticular melanism is associated with enhanced resistance to pathogens. Given that most entomopathogens are probably transmitted in a density-dependent manner, it has been argued that density-dependent melanism may be a widespread prophylactic response to a predictable infection risk (Wilson & Reeson, 1998; Wilson *et al*., 2002; Wilson & Cotter, 2006).

It seems likely that the high degree of phenotypic variation observed in these solitary insects reflects genetic differences between individuals (Tojo, 1991; Goulson, 1994; Cotter *et al*., 2004b), and the offspring – parent regressions clearly support the existence of an additive genetic component to melanism in this population. The heritability estimates obtained in the present study ($h^2 = 0.18$–0.30) are slightly lower than those obtained in an earlier study, based on a visual score of cuticular melanism ($h^2 = 0.36 \pm 0.08$; Cotter *et al*., 2004b). However, the two estimates are not directly comparable, as they are based on two different insect populations originating from Egypt, and were derived using different methods (full-sibling design and offspring–parent regression vs. half-sibling design and variance-components analysis).

One of the most interesting observations made during this study is that there is a large cyclical increase and fall in cuticular melanism (reflectance) across the developmental stages. Thus, both solitary and gregarious caterpillars displayed dark cuticle colour (low reflectance) on the first day after they moulted into a new instar, but subsequently became gradually paler until they reached the pre-moult stage (Fig. 2). This decrease in melanism (increase in reflectance) is probably due to the rapid expansion of the exocuticle with growth, and the dispersion of melanin granules in the cuticle (Chapman, 1998). In this regard, it is pertinent to note that, for mid-sixth instar solitary caterpillars, the paleness of the cuticle tended to increase with larval weight, presumably because the *density* of pigment granules in the cuticle was lower for heavier caterpillars with greater surface area. This considerable developmental variation in cuticular melanism completely masked all other sources of variation in colour, and resulted in small and non-significant repeatabilities within individuals (Table 1). Despite this, individual caterpillars generally remained *relatively* pale or *relatively* dark throughout their larval development, especially those caterpillars that

were reared solitarily. This is reflected in the larger, and statistically significant, repeatabilities that were observed after accounting for the average colour of individuals at each larval stage (Table 1). Thus, although cuticular melanism is both repeatable and heritable, and so may respond to both natural and artificial selection, the response to selection is likely to be relatively slow unless the timing of the selection process is consistent across generations with respect to larval development.

Gregarious caterpillars were significantly lighter in weight than solitary ones across the larval stadia, but their darker cuticle seemed not to be determined entirely by their smaller size. A comparison of the relationship between reflectance level and larval body weight at colour measurement for the two phenotypes (Fig. 5) clearly demonstrated consistently lower reflectance (greater melanism) for gregarious insects relative to solitary ones of an equivalent larval weight, suggesting that crowded rearing had a major impact on the expression of melanism independent of body weight. A possible explanation for the smaller body weight of melanic, gregarious larvae is that it is a consequence of a potential trade-off between resource allocation to the synthesis of nitrogen-rich melanin pigment and somatic growth, which is often limited by nitrogen in phytophagous insects (Brakefield, 1987; Windig, 1999; Talloen *et al*., 2004).

In summary, the present study presents a quantitative analysis of the effects of environmental and genetic factors on the expression of melanism in Lepidopteran larvae. This work shows that melanism is a highly dynamic trait that varies dramatically during larval development, and in response to population density and body weight. However, melanism is also a highly repeatable trait within individuals, both spatially (i.e. between cuticle regions) and temporally (relatively dark larvae remain *relatively* dark throughout larval development), and so may be considered a single, integrated trait (Pigliucci, 2003; Pigliucci & Preston, 2004). These results have implications for understanding the selection pressures that have moulded phenotypic variation in melanism, because selection is acting on a highly plastic trait. Thus, in absolute terms, a given individual may be considered melanic at one point in time, yet non-melanic at another, whilst relative to other members of its cohort may be considered consistently melanic or non-melanic. This makes understanding the strength and direction of selection operating on such traits extremely difficult, and has implications for applying artificial selection on the basis of larval colour.

Acknowledgements

We would like to thank Professor Esmat Hegazi for supplying *S. littoralis* and Clare Benskin for assistance in insect rearing. The work was conducted under licence from DEFRA (U.K.) and with financial support to K.W. from the Natural Environment Research Council (U.K.).

References

Altstein, M., Ben-Aziz, O. & Gazit, Y. (1994) Pheromone biosynthesis activating neuropeptide (PBAN) and colour polymorphism: an immunochemical study in *Spodoptera littoralis*. *Journal of Insect Physiology*, **40,** 303–309.

Barnes, A.I. & Siva-Jothy, M.T. (2000) Density-dependent prophylaxis in the mealworm beetle *Tenebrio molitor* L. (Coleoptera: Tenebrionidae): cuticular melanization is an indicator of investment in immunity. *Proceedings of the Royal Society of London B*, **267,** 177–182.

Brakefield, P.M. (1987) Industrial melanism: do we have the answers? *Trends in Ecology and Evolution*, **2**, 117–122.

Brown, E.S. & Dewhurst, C.F. (1975) The genus *Spodoptera* (Lepidoptera, Noctuidae) in Africa and the Near East. *Bulletin of Entomological Research*, **65**, 221–262.

Chapman, R.F. (1998) *The Insects: Structure and Function*, 4th edn. Cambridge University Press, Cambridge.

Church, S.C., Bennett, A.T.D., Cuthill, I.C., Hunt, S., Hart, N.S. & Partridge, J.C. (1998) Does lepidopteran larval crypsis extend into the ultraviolet? *Naturwissenschaften*, **85**, 189–192.

Cotter, S.C., Hails, R.S., Cory, J.S. & Wilson, K. (2004a) Density-dependent prophylaxis and condition-dependent immune function in Lepidopteran larvae: a multivariate approach. *Journal of Animal Ecology*, **73**, 283–293.

Cotter, S.L., Kruuk, L.E.B. & Wilson, K. (2004b) Costs of resistance: genetic correlations and potential trade-offs in an insect immune system. *Journal of Evolutionary Biology*, **17**, 421–429.

Curtis, A.T., Hori, M., Green, J.M., Wolfgang, W.J., Hiruma, K. & Riddiford, L.M. (1984) Ecdysteroid regulation of the onset of cuticular melanization in allatectomized and *black* mutant *Manduca sexta* larvae. *Journal of Insect Physiology*, **30**, 597–606.

Endler, J.A. (1990) On the measurement and classification of color in studies of animal color patterns. *Biological Journal of the Linnean Society*, **41**, 315–352.

Falconer, D.S. & Mackay, T.F.C. (1996) *Introduction to Quantitative Genetics*, 4th edn. Longman, Harlow, Essex.

Faure, J.C. (1943) Phase variation in the armyworm, *Laphygma exempta* (Walk.). *Scientific Bulletin of the Department of Agriculture and Forestry of the Union of South Africa*, **234**, 2–17.

Goulson, D. (1994) Determination of larval melanization in the moth, *Mamestra brassicae*, and the role of melanin in thermoregulation. *Heredity*, **73**, 471–479.

Goulson, D. & Cory, J.S. (1995) Responses of *Mamestra brassicae* (Lepidoptera: Noctuidae) to crowding: interactions with disease resistance, colour phase and growth. *Oecologia*, **104**, 416–423.

Grether, G.F., Cummings, M.E. & Hudon, J. (2005) Countergradient variation in the sexual coloration of guppies (*Poecilia reticulata*): drosopterin synthesis balances carotenoid availability. *Evolution*, **59**, 175–188.

Gunn, A. (1998) The determination of larval phase coloration in the African armyworm, *Spodoptera exempta* and its consequences for thermoregulation and protection from UV light. *Entomologia experimentalis et applicata*, **86**, 125–133.

Hazel, W.N. (2002) The environmental and genetic control of seasonal polyphenism in larval color and its adaptive significance in a swallowtail butterfly. *Evolution*, **56**, 342–348.

Hiruma, K., Matsumoto, S., Isogai, A. & Suzuki, A. (1984) Control of ommochrome synthesis by both juvenile hormone and

melanization hormone in the cabbage armyworm, *Mamestra brassicae*. *Journal of Comparative Physiology B*, **154**, 13–21.
Hodjat, S.H. (1970) Effects of crowding on colour, size and larval activity of *Spodoptera littoralis* (Lepidoptera: Noctuidae). *Entomologia Experimentalis et Applicata*, **13**, 97–106.
Hoffman, J.D., Lawson, F.R. & Yamamoto, R.T. (1966) Tobacco hornworms. *Insect Colonization and Mass Production* (ed. by C. N. Smith), pp. 479–486. Academic Press, London.
Hoffman, A.A. & Merila, J. (1999) Heritable variation and evolution under favourable and unfavourable environmental conditions. *Trends in Ecology and Evolution*, **14**, 96–101.
Iwao, S. (1962) Studies on the phase variation and related phenomena in some lepidopterous insects. *Memorial of the College of Agriculture, Kyoto University*, **84**, 1–80.
Iwao, S. (1968) Some effects of grouping in lepidopterous insects. *Colloques International du Centrenational de la Recherche Scientifique*, **173**, 185–210.
Kazimirova, M. (1992) The role of physical contact in the induction of phase polyphenism of *Mamestra brassicae* (Lepidoptera, Noctuidae). *Acta Entomologia Bohemoslovaca*, **89**, 87–95.
Kettlewell, H.B.D. (1973) *The Evolution of Melanism: the Study of a Recurring Necessity*. Clarendon Press, Oxford.
Lessells, C.M. & Boag, P.T. (1987) Unrepeatable repeatabilities: a common mistake. *Auk*, **104**, 116–121.
Long, D. (1953) Effects of population density on larvae of Lepidoptera. *Transactions of the Royal Entomological Society of London*, **104**, 543–585.
Majerus, M. (1998) *Melanism: Evolution in Action*. Oxford University Press, Oxford.
McNaught, M.K. & Owens, I.P.F. (2002) Interspecific variation in plumage colour among birds: species recognition or light environment? *Journal of Evolutionary Biology*, **15**, 505–514.
Nappi, A.J. & Vass, E. (1993) Melanogenesis and the generation of cytotoxic molecules during insect cellular immune-reactions. *Pigment Cell Research*, **6**, 117–126.
Ourth, D.D. & Renis, H.E. (1993) Antiviral melanization reaction of *Heliothis virescens* against DNA and RNA viruses *in vitro*. *Comparative Biochemistry and Physiology*, **105B**, 719–723.
Pigliucci, M. (2003) Phenotypic integration: studying the ecology and evolution of complex phenotypes. *Ecology Letters*, **6**, 265–272.
Pigliucci, M. & Preston, K. (2004) *Phenotypic Integration: Studying the Ecology and Evolution of Complex Phenotypes*. Oxford University Press, Oxford.
Pigliucci, M., Schlichting, C.D. & Whitton, J. (1995) Reaction norms of *Arabidopsis*. II. Response to stress and unordered environmental variation. *Functional Ecology*, **9**, 537–547.
Quinn, G.P. & Keough, M.J. (2002) *Experimental Design and Data Analysis for Biologists*. Cambridge University Press, Cambridge.
Reeson, A.F., Wilson, K., Cory, J.S., Hankard, P., Weeks, J.M., Goulson, D. et al. (2000) Effects of phenotypic plasticity on pathogen transmission in the field in a Lepidoptera – NPV system. *Oecologia*, **124**, 373–380.
Reeson, A.F., Wilson, K., Gunn, A., Hails, R.S. & Goulson, D. (1998) Baculovirus resistance in the noctuid *Spodoptera exempta* is phenotypically plastic and responds to population density. *Proceedings of the Royal Society of London B*, **265**, 1787–1791.
Roff, D.A. (1997) *Evolutionary Quantitative Genetics*. Chapman & Hall, New York.
Smith, J.A., Wilson, K., Pilkington, J.G. & Pemberton, J.M. (1999) Heritable variation to gastrointestinal nematodes in an unmanaged mammal population. *Proceedings of the Royal Society of London B*, **266**, 1283–1290.
Solensky, M.J. & Larkin, E. (2003) Temperature-induced variation in larval coloration in *Danaus plexippus* (Lepidoptera: Nymphalidae). *Annals of the Entomological Society of America*, **96**, 211–216.
St Leger, R.J., Cooper, R.M. & Charnley, A.K. (1988) The effect of melanization of *Manduca sexta* cuticle on growth and infection by *Metarhizium anisopliae*. *Journal of Invertebrate Pathology*, **52**, 459–470.
Sword, G.A. (2002) Plasticity and the evolution of warning coloration. *Proceedings of the Royal Society of London B*, **269**, 1639–1644.
Talloen, W., Van Dyck, H. & Lens, L. (2004) The cost of melanization: butterfly wing coloration under environmental stress. *Evolution*, **58**, 360–366.
Thompson, J.J.W., Armitage, S.A.O. & Siva-Jothy, M.T. (2002) Cuticular colour change after imaginal eclosion is time-constrained: blacker beetles darken faster. *Physiological Entomology*, **27**, 136–141.
Tojo, S. (1991) Variation in phase polymorphism in the common cutworm, *Spodoptera litura* (Lepidoptera: Noctuidae). *Applied Entomology and Zoology*, **26**, 571–578.
Wilson, K. (2000) How the locust got its stripes: the evolution of density-dependent aposematism. *Trends in Ecology and Evolution*, **15**, 88–90.
Wilson, K. & Cotter, S.C. (2006) Density-dependent prophylaxis in insects. *Phenotypic Plasticity in Insects: Mechanisms and Consequences* (ed. by D. W. Whitman and T. N. Ananthakrishnan), pp 381–420. Science Publishers, Plymouth, U.K.
Wilson, K., Cotter, S.C., Reeson, A.F. & Pell, J.K. (2001) Melanism and disease resistance in insects. *Ecology Letters*, **4**, 637–649.
Wilson, K. & Reeson, A.F. (1998) Density-dependent prophylaxis: evidence from Lepidoptera – baculovirus interactions? *Ecological Entomology*, **23**, 100–101.
Wilson, K., Thomas, M.B., Blanford, S., Doggett, M., Simpson, S.J. & Moore, S.L. (2002) Coping with crowds: density-dependent disease resistance in desert locusts. *Proceedings of the National Academy of Sciences of the United States of America*, **99**, 5471–5475.
Windig, J.J. (1999) Trade-offs between melanization, development time and adult size in *Inachis io* and *Araschnia levana* (Lepidoptera: Nymphalidae)? *Heredity*, **82**, 57–68.

Accepted 8 November 2005

