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The influence of genes for melanism on the activity of the flour moth, *Ephestia kuehniella*

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ABSTRACT

We investigated the effects of genes controlling melanism on levels and patterns of activity, potential nonvisual components of fitness, of adult Mediterranean flour moths, *Ephestia kuehniella* Zeller (1879). Six genotypes of two melanic strains (Ala nigra and black) were used. We monitored continuously the walking or flight activity of 45 mated females per genotype during the third night of their lives, using automatic electrostatic techniques to carry out the measurements. Although there was high individual variation within genotypes, bb (melanic) females were more active than the two nonmelanic genotypes of the black strain (b+, ++) because they tended to show more bouts of activity. There were no differences in the average length of these bouts, or in the timing of initial and final activity. Overall, the females of the black strain were significantly more active than the females of the Ala nigra strain. The results are discussed in the context of the evolution of melanism in moths.

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The evolution of industrial melanism in *Biston betularia* and other moths is a classic example of an adaptation to a change in the environment (for reviews see: Kettlewell 1973; Bishop & Cook 1980; Lees 1981; Brakefield 1987). The black colour of the wings and body of melanic moths is determined by alleles at single gene loci in almost all species that have been investigated. The qualitative explanation of industrial melanism involves a change in the crypsis of different phenotypes owing to the blackening of the moth's resting background by air pollution. This results in changes in the predation rates of the phenotypes by birds (visual selection). The dark, melanic forms gain a selective advantage relative to the pale nonmelanics in regions influenced by air pollution. Recently the modelling approach of Mani (1982; Cook et al. 1986; Mani & Majerus 1993) has indicated that observed patterns of spatial or temporal variation in allele frequency can be explained fully only if the genes controlling melanism have effects on the phenotype other than the wing colour per se. Thus, estimates of visual selection and gene flow are not sufficient to explain the observed variation. Differences in fitness unrelated to visual selection and crypsis have been called nonvisual components of fitness.

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Melanin pigment is a nitrogen-rich polymer with a high molecular weight. Its biosynthetic pathway is based on the amino acid tyrosine with dopa and dopamine as intermediates (Jacobs 1985; Umebachi 1985; Riddiford & Hiruma 1988). One underlying hypothesis to account for nonvisual components of fitness is that the synthesis of melanin involves costs in terms of resource allocation for phytophagous insects which are nitrogen-limited (Brakefield 1987, 1988). There may also be more direct effects of melanization, however, since dopamine is a neurotransmitter (Livingstone & Tempel 1983) and melanism could directly influence activity patterns and, therefore, fitness. This possibility is supported by the study of Kyriacou et al. (1978) on the ebony polymorphism in *Drosophila melanogaster*. The ebony allele has pleiotropic effects on locomotor activity (see also Hall 1990). Melanic homozygote flies are hyperactive relative to the wild type. This difference in activity interacts with an increased wing vibration to affect mating success (Kyriacou 1981). Hodgetts (1972) and Hodgetts & Konopka (1973) found that at the time of eclosion, *ebony* mutants have elevated levels of free β-alanine and dopamine which return to normal during the first 2 days of adult life. Although it is noteworthy that the elevated levels precede the increase in wing vibration, there is insufficient evidence to support a causal relationship (Kyriacou et al. 1978).

We have found differences in adult longevity between mated females of different melanic genotypes of the

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flour moth, *Ephestia kuehniella* (M. D. Verhoog & P. M. Brakefield, unpublished data). Effects of the genes controlling melanism on adult activity and behaviour similar to those associated with *ebony* could contribute to the differences in longevity in *E. kuehniella*. In this study we compared the activity patterns of six genotypes of two melanic strains of *E. kuehniella* to detect and describe the types of nonvisual components of fitness that may be critical to the evolution of industrial melanism in other moths.

METHODS

Ephestia: Strains and Culture

Ephestia kuehniella (Lepidoptera; Pyralidae), the Mediterranean flour moth, is a cosmopolitan pest in flour mills (Brindley 1930; Ahmad 1936) and is of unknown origin (see reviews by Caspari & Gottlieb 1975; Leibenguth 1986). We used two melanic strains of *Ephestia* carrying either the dominant melanic allele An (Ala nigra; Cotter 1974) or the recessive melanic allele b (black). These alleles determine the body and wing colour of the moths. As far as we know, neither of these alleles occurs in natural populations; both were isolated from grain stores (W. B. Cotter, personal communication). Both laboratory strains have been maintained continuously at large population sizes. Before the cultures were established in Leiden, the melanic alleles were backcrossed in each of a large number of generations (about 75) to the wild-type strain B12 (W. B. Cotter, personal communication). Thus, any difference in activity, and hence fitness, can be interpreted as being due to the melanic alleles and not to linkage disequilibrium.

Establishment of the Six Genotypes

Because the two melanic phenotypes of Ala nigra (AnAn and An+), and also the two nonmelanic phenotypes of black (b+ and ++), were indistinguishable from each other, homozygote lines were developed from the melanic strains. We allowed 150 pairings of melanic Ala nigra moths, and of nonmelanic black moths, to mate and oviposit in jars containing medium made up in the proportions of 100 g of yeast to 0.1 litres of glycerol and 300 g of bran. Only nonsegregating families in which all F1 offspring were melanic (Ala nigra) or nonmelanic (black) were used to rear F2 families. We ensured that only fully homozygote F2 families were used to establish pure breeding stocks by scoring the first 50 moths to eclose. Furthermore, continuous full-sibling matings with more than 200 moths were then carried out for the next four generations. After that, we mixed the homozygote families (±30) of each line to create two large homozygote populations. The other two homozygote lines (of bb and ++(An)) were also reared from 30 inbred families and treated in the same way. We used these four homozygote lines to rear moths with the six genotypes.

We allowed at least 100 moths from each line to mate and oviposit in a plastic pot lined with netting and inverted over a funnel. We collected eggs in a jar below the funnel. Moths were reared in batches, each from up to 100 eggs placed in a glass jar with 100 g of medium. There is no detectable decrease in adult size from growth on 1 g of medium per larva. We obtained heterozygote genotypes by setting up the reciprocal crosses of the two homozygote lines within each melanic strain. The eggs of these reciprocal crosses were mixed before rearing.

Both rearing of the experimental animals and the experiments themselves took place in the same climate room. Conditions were kept constant throughout the experiment (see below). The temperature changed automatically with the light. The room was also kept free of vibration.

Measuring Locomotor Activity

We performed pilot experiments to develop the techniques for measuring activity. Both these and the main experiment consisted of numerous recording sessions, during which we collected activity data. Automatic electrostatic techniques were used (Luff & Molyneux 1970; Ball 1972; Hendrikse 1979; Snowball & Holmqvist 1994; R. W. van der Linden & A. Gluvers, unpublished data). The recording device consisted of three rows or 'plugs' of five copper tubes (height 4 cm, diameter 7 cm). Each tube was electrostatically shielded with a lid consisting of a copper screen, and there was no external electrostatical influence. The activity recordings made in each copper tube were independent of all others because of the use of resistance–capacitance-filters. All three plugs were connected to a Soliprot behaviour-recording device via an RS-237 Interface (R. W. van der Linden & A. Gluvers, unpublished data). The Soliprot recorded the activity and stored the information on an eprom (32 kb). Several eproms were used during each recording session with changing eproms taking about 18 s. After the recording session we transferred the data on each eprom to a PC for statistical analysis.

To standardize the measurements as far as possible, we worked only with mated female moths on night 3 (N3) of their life. Copulation occurred on night 1 or 2 after eclosion. We chose N3 as occurring within the peak of egg-laying activity and before any substantial adult mortality in the absence of predation (M. D. Verhoog & P. M. Brakefield, unpublished data). *Ephestia* do not feed as adults although they will drink. We offered no water during the experiment to ensure that feeding history did not influence activity patterns. Furthermore, the presence of such a feeding medium could also influence the sensitivity of the recording method. We counted the eggs laid on N3 and established their fertility by examining egg hatching after 1 week.

We recorded the activity of 15 female moths in each session (night). We compared only five of the six genotypes in a single session using three moths per genotype. One individual of each genotype was allocated at random to each plug according to a randomized block design. We placed each moth in a clear plastic petri dish (height 1 cm, diameter 5 cm) in one of the copper tubes (acclimatization, see Cole 1995). After 30 min we put the lids on

the tubes. Moths walking or flying in the petri dishes induced a change in capacitance, which was detected and amplified. We could measure only the occurrence of the activity (active or not active), not its intensity. In 19 days, we measured 45 females per genotype over 18 recording sessions. There was no recording session on day 14.

We checked the sensitivity of the recording device during the pilot experiments so that we recorded only walking or flight activity. The pilot experiments also showed the ‘noise’ (recording activity when it does not occur) to be around 0.001%. Furthermore, the sensitivity did not change over the course of the experiment.

The amount of time that an animal must be motionless to be judged inactive is also critical to the analysis of activity (see Cole 1995). We used a temporal scale of 0.1 s.

The Recording Sessions

There was a fixed time period (start 1330 hours, end 2030 hours) during which continuous time measurements were taken (T=252 000 recording time units of 0.1 s; i.e. a total of 7 h). Since the pilot experiments showed moths were inactive in light, the measurements were centred around a 6-h dark period. Immediately before and after the dark period was a scotophase of 15 min (dusk and dawn, 0.6 W/m^2) and a 15-min period of daylight (4.6 W/m^2). Temperature varied with the light conditions (24.2 ± 0.5°C).

Censoring (not recording start and/or end times of an activity bout) occurred as the inevitable result of a fixed observation time and because of the limited capacity of the eproms. Since the loss of information was slight, never exceeding 1% of the total observation time, we discarded censored observations.

Activity Variables

The variables analysed were: (1) total amount of activity (S_a after Haccou & Meelis 1994); (2) number of activity bouts, N_a; (3) mean bout length; (4) latency (the first occurrence of an activity bout); (5) cessation of activity (final activity bout); and (6) within-individual variation of bout lengths (i.e. standard deviation of bout length). Data on fertility, number of eggs laid and the dry weight of the moths was also recorded (moths were killed in a freezer and then dried to constant weight at 50°C).

Data Analysis

Almost all variables (per genotype) departed from normality, usually because of extreme outliers. We transformed the data for S_a by taking the power 1/4, yielding S_a'. Subsequent application of Hartley’s test of homogeneity of variances did not yield any significant results. The S_a' data, were, therefore, analysed with both parametric and nonparametric tests. We analysed other variables using the Kruskal–Wallis test (Sokal & Rohlf 1995). Only scale-invariant tests, which did not depend on the recording time unit, were carried out.

Of 268 females measured, 18 were infertile (AnAn: 6; An+: 2; bb: 3; b+: 3; and ++(b): 4). These females were significantly heavier than fertile females (medians 30.5 and 21.8, respectively, with Kruskal–Wallis test: H_1=9.30, P<0.01), and were excluded from the analysis. As a result, female dry weight did not vary significantly between the six genotypes.

We compared individuals at the levels of: (1) genotype at each gene locus (three genotypes per gene); (2) between genes (An and b); and (3) phenotype (melanic and nonmelanic, per gene).

For data analysis we used the statistical package MINITAB 9.2.

RESULTS

The moths usually became active just after the onset of either the recording session, dusk, or dark period, with the dusk period as median. They became quiescent just after the onset of dawn.

Table 1 gives the results of comparisons of the genotypes at each gene for each variable. There were no differences between the three Ala nigra genotypes in patterns and levels of activity. For the black gene locus, the bb females were significantly more active (S_a) with more activity bouts (N_a) than the b+ and ++ females.

The same pattern of results is given for the analysis of phenotypes (nonmelanic and melanic; Table 2). Again there was no difference in patterns or levels of activity for the Ala nigra locus. For the black locus, the melanics (bb) were more active (S_a) with more activity bouts (N_a) than the nonmelanic phenotypes (b+ and ++). Although the black melanic females had significantly more activity bouts, their average bout length was the same as for the black nonmelanic females (Fig. 1).

In the comparison made across the two gene loci, the black females were significantly more active than the Ala nigra females, having more activity bouts per observation period (Table 3). This result is mainly due to the bb females.

The overall result for bb is also apparent for the data viewed over individual recording sessions (Fig. 2). The bb females can be compared with the other black genotypes by taking the average activity rank of each genotype per day and applying a sign test (N=13, P<0.05; Siegel & Castellan 1988). There was substantial variation in activity from day to day (Kruskal–Wallis test: H_{17}=57.36, P<0.001). This is mainly due to recording sessions 15–19, as a Kruskal–Wallis analysis of the data using only the first 13 recording sessions yielded no significant heterogeneity (Kruskal–Wallis test: H_{12}=19.03, NS). We have no explanation for the difference between recording sessions 1–13 and 15–19. However, each of the differences between the genotypes or phenotypes for the black gene remained significant when only the data for the first 13 days were analysed (Tables 1, 2, 3).

DISCUSSION

Melanic bb females were more active with more activity bouts than the nonmelanic genotypes of the black strain.

Table 1. Kruskal–Wallis results of the comparisons made between the genotypes at the within-gene level on each of the six variables listed (median ×0.1 s)

	Ala nigra				black			
	Genotype	Median	*N*	Result	Genotype	Median	*N*	Result
Total amount of activity (S_a)*†	AnAn	19 476	39	NS	bb	38 376	40	H_2=10.48, P<0.01§
	An+	18 852	43		b+	25 703	42	
	++	21 951	45		++	26 218	41	
Number of activity bouts (N_a)‡	Anan	1355	39	NS	bb	2903	40	H_2=14.81, P<0.01**
	An+	1380	43		b+	1838	42	
	++	1480	45		++	1786	41	
Mean bout length	AnAn	14.11	39	NS	bb	13.42	40	NS
	An+	13.23	43		b+	15.49	42	
	++	13.84	45		++	14.08	41	
Latency	AnAn	10 182	39	NS	bb	9637	40	NS
	An+	9583	43		b+	9662	42	
	++	9730	45		++	9785	41	
Cessation	AnAn	242 513	39	NS	bb	242 987	40	NS
	An+	243 081	43		b+	243 121	42	
	++	243 707	45		++	243 395	41	
Standard deviation bout length	AnAn	19.96	39	NS	bb	16.65	40	NS
	An+	16.90	43		b+	20.67	42	
	++	19.58	45		++	19.34	41	

*S_a′ ANOVA for black: $F_{1,120}$=4.50, P<0.05.
†Dunn–Šidák (Sokal & Rohlf 1995): bb/b+ H_1=10.25, P<0.01; bb/++ H_1=5.05, NS; b+/++ H_1=0.44, NS.
‡Dunn–Šidák: bb/b+ H_1=11.91, P<0.01; bb/++ H_1=10.26, P<0.01; b+/++ H_1=0.09, NS.
§Kruskal–Wallis test for days 1–13: H_2=8.71, P<0.05.
**Kruskal–Wallis test for days 1–13: H_2=12.70, P<0.01.

Table 2. Kruskal–Wallis results of the comparisons made between phenotypes (M: melanic; N: nonmelanic) at the within-gene level on each of the six variables listed (median ×0.1 s)

	Ala nigra				black			
	Phenotype	Median	*N*	Result	Phenotype	Median	*N*	Result
Total amount of activity (S_a)*	M	19 461	82	NS	M	38 376	40	H_1=9.91, P<0.01†
	N	21 951	45		N	26 072	83	
Number of activity bouts (N_a)	M	1367	82	NS	M	2903	40	H_1=14.74, P<0.001‡
	N	1480	45		N	1828	83	
Mean bout length	M	14.09	82	NS	M	13.42	40	NS
	N	13.84	45		N	15.01	83	
Latency	M	9642	82	NS	M	9637	40	NS
	N	9730	45		N	9729	83	
Cessation	M	243 075	82	NS	M	242 987	40	NS
	N	243 707	45		N	243 345	83	
Standard deviation bout length	M	18.59	82	NS	M	16.65	40	NS
	N	19.58	45		N	20.07	83	

*S_a′ ANOVA for black: $F_{1,121}$=6.15, P<0.05.
†Kruskal–Wallis test for days 1–13: H_1=8.70, P<0.01.
‡Kruskal–Wallis test for days 1–13: H_1=12.70, P<0.001.

A possible explanation for the difference in activity is an influence of the monoamine dopamine. Dopamine is not only an important intermediary in the melanization and sclerotization of the insect cuticle, but also a neurotransmitter and can, therefore, influence behaviour and activity in insects (Hodgetts & Konopka 1973; Kyriacou et al. 1978; Hall 1990; Restifo & White 1990). As predicted by Brakefield (1987, 1988), we have found differences between genes in their effect on activity: while bb moths were more active than b+ or ++, there was no evidence for comparable effects of the corresponding alleles at the Ala nigra gene. Predicting the type or direction of an influence on behaviour through allelic variation at any particular gene for melanism will be impossible, at least without any detailed knowledge of the gene's mode of biochemical activity in the melanization pathway (see Kyriacou et al. 1978; Riddiford & Hiruma 1988). Several enzymes are involved in the pathway including dopa decarboxylase and phenoloxidases. The consequences for nonvisual components of

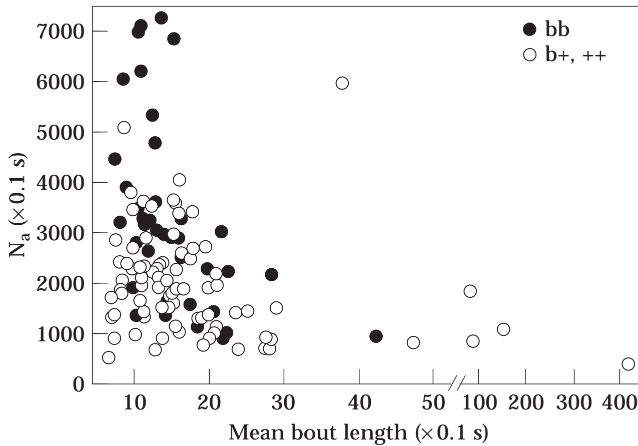

Figure 1. Number of activity bouts (N_a) for individual moths of the black phenotypes per recording session plotted against mean bout length.

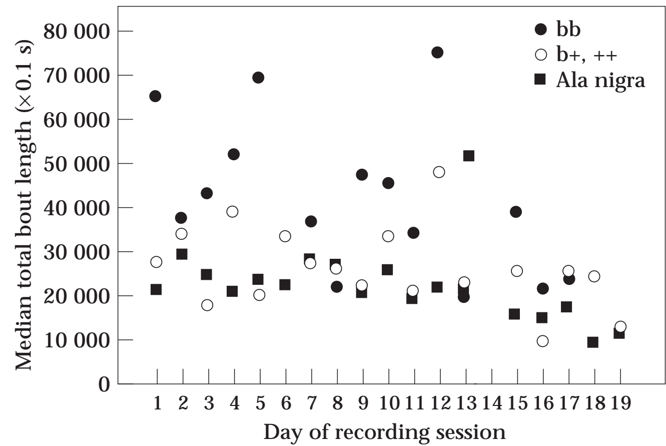

Figure 2. Median total bout length per recording session for the black, melanic, genotype (bb), the black, nonmelanic, genotypes (b+ and ++(b)), and the Ala nigra genotypes pooled (AnAn, An+ and ++(An)). There was no recording session on day 14.

fitness will depend on the precise way in which an allele specifying melanism regulates the pathway of melanization. They may also depend on any pleiotropic effects on the linked pathway of cuticle sclerotization and tanning (see Hodgetts 1972; Hodgetts & Konopka 1973). Such effects are possible because dopamine can be shifted from the melanization pathway towards the formation of sclerotization compounds during cross-linking of the cuticle.

Since the level and pattern of adult activity could readily influence the reproductive success of moths in nature, our data show the potential for genes controlling melanism to affect nonvisual components of fitness that are unrelated to the colour pattern and visual selection on crypsis. The potential for effects of activity on fitness is illustrated by our finding that nonmelanic black females (b+ and ++) live significantly longer than the melanic black genotype (bb) (M. D. Verhoog & P. M. Brakefield, unpublished data). The higher activity of the bb females found in this study could readily account for a shortened life span.

The modelling studies by Mani (Mani 1980, 1982, 1990; Cook et al. 1986; Mani & Majerus 1993) of spatial and temporal variation in the frequency of the carbonaria allele which controls the fully black melanic phenotype of *B. betularia* show that visual selection by predators cannot fully account for the observed patterns in allele frequency. Incorporating differences between genotypes for nonvisual components of fitness can produce convincing fits between observed data and predictions from the models which also use field estimates of visual selection and details of population biology, including migration rates. The indirect evidence for such nonvisual differences in preadult fitness came mainly from analyses of segregation ratios for melanic and nonmelanic genotypes in laboratory-rearing experiments (Lees 1981). Observations in other insects demonstrate a number of other ways in which genes controlling melanism might yield nonvisual differences in fitness between genotypes (references in Brakefield 1987, 1988; and see Washburn et al. 1996). Our results clearly demonstrate the potential

Table 3. Kruskal–Wallis results of the comparison made between the two genes on each of the six variables listed (median ×0.1 s)

	Genes	Median	*N*	Result
Total amount of activity (S_a)*	Ala nigra	19 906	127	H_1=34.77, P<0.001†
	black	29 097	123	
Number of activity bouts (N_a)	Ala nigra	1405	127	H_1=28.14, P<0.001‡
	black	2016	123	
Mean bout length	Ala nigra	14.07	127	NS
	black	14.32	123	
Latency	Ala nigra	9642	127	NS
	black	9700	123	
Cessation	Ala nigra	243 238	127	NS
	black	243 204	123	
Standard deviation bout length	Ala nigra	18.78	127	NS
	black	18.73	123	

*S_a′ ANOVA result for black: $F_{1,248}$=38.05, P<0.001.
†Kruskal–Wallis test for days 1–13: H_1=18.97, P<0.001.
‡Kruskal–Wallis test for days 1–13: H_1=21.98, P<0.001.

for such differences acting through the effects of genes controlling melanism on adult activity and behaviour in moths.

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