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RESEARCH ARTICLE

Direct and correlated responses to laboratory selection for body melanisation in *Drosophila melanogaster*: support for the melanisation–desiccation resistance hypothesis

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SUMMARY

For *Drosophila melanogaster*, cuticular melanisation is a quantitative trait, varying from no melanin to completely dark. Variation in melanisation has been linked with stress resistance, especially desiccation, in *D. melanogaster* and other species. As melanism has a genetic component, we selected melanic and non-melanic phenotypes of *D. melanogaster* in order to confirm the association of desiccation resistance and rate of water loss with cuticular melanisation previously reported for this species. A bidirectional selection experiment for dark (D_1–D_4) and light (L_1–L_4) body colour in *D. melanogaster* was conducted for 60 generations. We observed a 1.6-fold increase in abdominal melanisation in selected dark strains and a 14-fold decrease in selected light strains compared with control populations. Desiccation resistance increased significantly in the dark-selected morphs as compared with controls. The observed increase in desiccation resistance appeared as a consequence of a decrease in cuticular permeability. Our results show that traits related to water balance were significantly correlated with abdominal melanisation and were simultaneously selected bidirectionally along with melanisation.

Key words: artificial selection experiment, abdominal melanisation, correlated responses, desiccation resistance, *Drosophila melanogaster*.

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INTRODUCTION

Several studies have shown evidence for the role of natural selection in maintaining phenotypic variation in body melanisation of diverse insect taxa (Majerus, 1998; True, 2003). In *Drosophila melanogaster*, body melanisation is a quantitative trait and shows significant levels of both within- and between-population variation (Parkash et al., 2008). Geographical populations of *D. melanogaster* from Africa (Pool and Aquadro, 2007), India (Parkash et al., 2008) and Australia (Telonis-Scott et al., 2011) have shown clinal variation in abdominal or thoracic melanisation, which suggest adaptations to local climatic conditions. In several other studies, clinal variation is evident for many stress-related traits that covary strongly with latitude or altitude (e.g. Parkash et al., 2008). However, it is not clear whether various ecologically relevant quantitative traits show independent or correlated selection responses. For example, associations between body melanisation- and desiccation-related traits have been observed in latitudinal as well as altitudinal populations of *D. melanogaster* from India but these studies provide indirect evidence (Parkash et al., 2008a). If desiccation resistance evolves through changes in cuticular permeability in *D. melanogaster*, the target of selection might be cuticular components (cuticular melanisation and/or cuticular lipids). A possible link between body melanisation and desiccation resistance can be shown if laboratory-selected strains for higher melanisation exhibit increased desiccation, but this hypothesis has not been tested so far in *D. melanogaster* or in other *Drosophila* species.

For wild populations, ecophysiological and morphological traits might coevolve according to their combined influence on fitness (Angilletta, 2009). For example, tropical habitats on the Indian subcontinent select for lighter body colour phenotypes as well as starvation tolerance (Parkash and Munjal, 2000), but it is not clear whether these traits are under independent selection or coevolve. Further, there is evidence in favour of the co-adaption hypothesis (Angilletta, 2009), e.g. behavioural thermoregulation and body colouration are co-adapted traits in the pygmy grasshopper (*Tetrix subulata*) (Forsman, 2000). Co-adapted traits are also represented by associations between body melanisation- and desiccation-related traits (Parkash et al., 2008a; Parkash et al., 2010a) and thermo-resistance traits in *D. melanogaster* (Parkash et al., 2010b). Cuticular hydrocarbons are also subject to natural selection, being important in providing desiccation resistance for many insect species (Gibbs and Rajpurohit, 2010). Several insect taxa have shown variable cuticular permeability due to changes in the composition or amount of cuticular lipids (Edney, 1977; Toolson, 1984; Hadley, 1994; Rourke, 2000). There are clines of water balance measures correlated with the amount of cuticular lipids (Rourke, 2000; Parkash et al., 2008a). Seasonal changes in the composition or amount of cuticular lipids also affect water loss in scorpions and tenebrionid beetles (Hadley, 1977; Toolson and Hadley, 1979; Hadley and Schultz, 1987). In contrast, analysis of water balance mechanisms in diverse *Drosophila* species has shown a lack of changes in cuticular traits for reduced body water loss (Gibbs et al., 1998; Gibbs et al., 2003; Gibbs and Matzkin, 2001; Parkash et al., 2008a). Further, there is lack of differences in melting temperature (T_m), composition and/or amount of surface lipids in laboratory selected desiccation-resistant and -sensitive strains of *D. melanogaster* (Gibbs et al., 1997). Within species, Indian populations of *D. immigrans* differ in water-loss rate, but not in surface lipid amounts (Parkash et al., 2008c). In a laboratory selection experiment, populations selected for desiccation resistance lost water ~50% less rapidly than unselected controls,

but the two groups exhibited minor differences in lipid composition and T_m (Gibbs et al., 1997). Thermal acclimation of the desert fly, *D. mojavensis*, results in substantial changes in HC composition, but relatively little change in water-loss rates (Gibbs et al., 1998). It must be noted that not all studies present negative findings (e.g. Toolson, 1982; Toolson and Kuper-Simbrón, 1989). If ecophysiological traits and cuticular traits (body melanisation and/or cuticular lipids) coevolve, we may expect correlated responses between body colour phenotypes and stress-related traits in *D. melanogaster*, but such trait associations have not yet been analysed.

Several studies have focused on laboratory selection of desiccation resistance (Hoffmann and Parsons, 1989a; Hoffmann and Parsons, 1989b; Hoffmann and Parsons, 1993; Gibbs et al., 1997; Chippindale et al., 1998; Djawdan et al., 1998; Telonis-Scott et al., 2006), thermal sensitivity (Huey et al., 1991; Gilchrist and Huey, 1999; Anderson et al., 2005) and starvation resistance (Chippindale et al., 1996; Harshman et al., 1999; Bubliy and Loeschcke, 2005). In contrast, a single study on laboratory selection of abdominal spot number in *Drosophila falleni* has shown that selection exerted by nematode parasites may influence pigmentation patterns (Dombeck and Jaenike, 2004). In most of the laboratory selection studies, replicate lines at the end of selection protocol were investigated for changes in the trait of interest as well as correlated selection responses, but the time course of evolutionary response in the selected lines has not been investigated. Although a time course analysis is time consuming, it can be helpful in better understanding the selected as well as correlated traits.

Experiments of artificial selection in *Drosophila* have recently been used as an experimental evolutionary tool to identify the relevant traits that are most likely to be involved in adaptation to environmental temperature in ectotherms (Hoffmann et al., 2003; Bowler and Terblanche, 2008). One of the main advantages of artificial selection experiments is the possibility to evaluate not only direct, but also correlated responses to selection (Harshman and Hoffmann, 2000). Artificial selection experiments have revealed moderate to relatively high levels of heritability for resistance to high-temperature stress in *D. melanogaster* (Hoffmann et al., 2003; Reusch and Wood, 2007). Remarkably, most artificial selection programmes were mainly performed in *D. melanogaster*, though recent studies have also addressed the question of whether results in *D. melanogaster* are consistent across species (Hoffmann and Willi, 2008). Further, artificial selection on thermal-stress traits was generally performed in only a single direction (mainly for increased resistance), but studies have shown that selection for decreased resistance to heat stress can also be informative as the selection response can often be asymmetrical for thermal-stress traits (Gilchrist and Huey, 1999; Folk et al., 2006; Norry et al., 2007; Mori and Kimura, 2008; Gomez et al., 2009; Bertoli et al., 2010).

Body melanisation is one of the most common types of phenotypic variations in insects (Majerus, 1998). Phenotypic variation of body melanisation in some lepidopterans and coelopterans are represented by discrete morphs (melanic and non-melanic) consistent with a major locus (Da Cunha, 1949; Martinez and Cordeiro, 1970). Several studies have shown changes in the frequencies of two or more allelic variants in response to temporally or spatially variable climatic conditions (Umina et al., 2005; Parkash et al., 2009; Parkash et al., 2012). In contrast, variation in body melanisation in a *D. melanogaster* population follows a bell-shaped curve and such a quantitative trait is expected to respond quickly to laboratory as well as field selection. Such pigmentation differences are polygenic and interact with abiotic factors of the environment (Wittkopp et al., 2003). In *D. melanogaster*, melanisation varies continuously across geographical gradients on different continents and such clines linked with body melanisation reflect adaptations to local climatic conditions (David et al., 1985; Capy et al., 1988; Munjal et al., 1997; Pool and Aquadro, 2007; Clusella Trullas et al., 2007; Telonis-Scott et al., 2011). Several studies have considered plastic change in melanisation scores at different growth temperatures in laboratory populations of *D. melanogaster* (Das et al., 1994; Ottenheim et al., 1999; Gibert et al., 1996; Gibert et al., 2000; DeWitt and Scheiner, 2004).

Melanin patterns are involved in diverse aspects of insect ecology (Majerus, 1998; True, 2003). For example, increased melanisation has been associated with higher fitness under thermal as well as aridity stresses in *D. melanogaster*, i.e. a darker cuticle may improve thermoregulation as well as reduce cuticular water loss (Parkash et al., 2008). Changes in body melanisation are associated with thermal- and/or water-stress-related traits but the target of selection is not clear. Laboratory selection of desiccation and starvation resistance shows parallel responses in *D. melanogaster* while field population show opposite clines. Such a mismatch between field and laboratory selection may be due to selection acting on some other associated trait that may impact resistance to starvation and desiccation in different ways. There is ample empirical support for the thermal melanism hypothesis (Watt, 1969; David et al., 1985; David et al., 1990; Jong et al., 1996; Majerus, 1998; Rajpurohit et al., 2008). Several studies have demonstrated a direct influence of melanisation on body temperature by increasing solar absorption under cool conditions (Jong et al., 1996; Ottenheim et al., 1999; Ellers and Boggs, 2004). Furthermore, the higher body temperature in more melanised females also increases egg maturation rate (Ellers and Boggs, 2004) and, in ladybird beetles, dark-coloured individuals benefit from increased mating success and earlier emergence in spring (Ellers and Boggs, 2004). In copper butterflies, pupal and wing melanisation increased with increasing altitude (Karl et al., 2009). Conversely, lighter individuals may be better protected against overheating in warm environments (Munjal et al., 1997; Ellers and Boggs, 2002; Pereboom and Biesmeijer, 2003). Accordingly, selection is expected to favour darker phenotypes in colder environments. However, a longitudinal cline for body colour in *D. americana* is not associated with desiccation resistance (Wittkopp et al., 2011). Thus, it is not clear whether change in melanisation can play a role in multiple abiotic stressors. If body melanisation is the target of selection, we may expect consistent changes in correlated traits. Laboratory-selected darker and lighter lines may clarify whether such traits coevolve.

The primary aims of the present study are to establish laboratory-selected replicate lines with high and low abdominal melanisation and analyse change after every five generations in abdominal melanisation due to a direct selection response as well as correlated selection responses in physiological traits related to water stress. We assessed traits related to water conservation, i.e. body water content, rate of water loss, haemolymph content and dehydration tolerance level, in dark- and light-selected replicate lines of *D. melanogaster*. We further tested whether desiccation resistance shows covariation with selected darker and lighter body colour lines. We also provide data on realised heritability based on laboratory-selected lines. This paper presents the results of a long-term artificial selection experiment with three major strengths: (1) selection was maintained for 60 generations, a greater length than most experiments of this type; (2) the trait under selection varies clinally in the field, and a suite of correlated responses were also measured, giving the work a good grounding in the biology of the organism in the field; and (3) the genetic variation in the trait was

measured every five generations, a rarely implemented addition to a selection experiment.

MATERIALS AND METHODS

Selection of lines for dark and light body colour

Wild-living flies of *Drosophila* species (*N*=150–200 flies per site) were collected from six localities along an altitudinal transect in the western Himalayas: two lowland, 500–600 m (Kalka and Parwanoo); two midland, 1200–1400 m (Kandaghat and Solan); and two highland, 2000–2200 m (Kasauli and Shimla). On average, approximately 40% were *D. melanogaster* out of each sample of wild-caught flies. From each population, 10 pairs of wild-caught flies were pooled to make a mass-bred population that was grown for seven generations (2 week cycle on standard *Drosophila* medium) at a constant growth temperature of 21±0.05°C and 65±1% relative humidity in a temperature- and humidity-controlled incubator. This mass-bred population was maintained in a population cage (*N*=5000–6000 flies) in the laboratory for seven generations before onset of the selection protocol. Four stocks (P_1–P_4), each with 250–300 pairs of flies, were derived from this mass-bred population. For each stock, two replicate lines (control and selected) were established. Thus, we had four control lines and the respective four selected (S_1–S_4) lines, each with approximately 300–350 pairs of flies.

For the selection regime, 40 dark and 40 light female flies were selected from each of the four selected (S_1–S_4) lines to initiate the next generation, while the remaining flies were discarded. Each generation, approximately 300 emergent female flies per replicate were aged for 7 days prior to establishment of the next generation. It was observed that melanisation of flies did not change after 2 days. The flies with >45% and <30% body melanisation were sorted as darker and lighter flies for the first generation of selection. These selected female flies correspond to a selection intensity of approximately 1.40 (Falconer, 1981); this selection intensity depends only on the proportion of the population included in the selected group, provided the distribution of phenotypic values is normal. This selection procedure was followed independently for each of the five mass-bred stocks. The selection protocol was followed for 60 generations, resulting in a dark-selected line (D_1, D_2, D_3 and D_4) and a light-selected line (L_1, L_2, L_3 and L_4).

A replicate of each selected line (D_1–D_4 and L_1–L_4) was maintained without further selection after the 60th generation, i.e. from the 61st through the 65th generation. These replicate lines were maintained with the same number of flies without any further selection regime. For relaxed lines, i.e. the 61st through the 65th generation, a sample of 100–150 flies of each dark- and light-selected line was again scored for changes in abdominal melanisation, if any.

Quantification of melanisation: direct response to selection

General scoring method

Body melanisation was estimated under a stereo zoom microscope (Olympus, www.olympus.com) from a lateral view of the female and male abdomen, giving values ranging from 0 (no melanisation) to 10 (complete melanisation) for each of the six visible (second to seventh) abdominal segments (David et al., 1990). Because the abdominal segments differ in size, relative sizes (i.e. 0.86, 0.94, 1.0, 0.88, 0.67 and 0.38 for the second to seventh segments, respectively) were multiplied by segment-wise melanisation scores. Abdominal melanisation scores were weighted to the relative sizes of the respective segments. The abdomen of each fly minus the viscera was mounted on a slide and total body melanisation per fly was also estimated using Biowizard image analysis software (Dewinter Optical, www.dewinterindia.com). Data on percent melanisation were calculated as: (observed weighted melanisation scores of six abdominal segments per fly/relative size of each abdominal segment × 10 per fly) × 100.

Response to selection

The selection response was measured every fifth generation. Selection of abdominal melanisation was performed on female flies only, but in the time course of selection, males were also analysed. For both sexes and each replicate line of control as well as dark- and light-selected flies, 60 flies were scored for abdominal melanisation every fifth generation.

Correlated responses to selection: analysis of stress resistance/assay

We analysed correlated changes in body water content, hemolymph content, desiccation resistance, rate of water loss and dehydration tolerance in selected darker and lighter replicate lines of *D. melanogaster*. We further analysed changes in epicuticular lipids in control and selected replicates, if any. For analysis of stress resistance and epicuticular lipids, we analysed 10 replicates from each of the control (C_1–C_4), dark-selected (D_1–D_4) and light-selected (L_1–L_4) lines of *D. melanogaster*.

Desiccation resistance

Desiccation resistance was measured as time to lethal dehydration (LT_{100}) under dry air in 10 replicates of female and male individuals of each of the control (C_1–C_4), dark-selected (D_1–D_4) and light-selected (L_1–L_4) lines. Ten male and female individuals were isolated in dry plastic vials that contained 2 g of silica gel at the bottom of each vial that was covered with a disc of plastic foam piece. The vials were placed in a desiccator chamber (Secador electronic desiccators cabinet; www.tarson.com) that maintained 6–8% relative humidity and the number of immobile flies was counted at 1 h intervals. The LT_{100} effect in dry air was recorded.

Basic measures of water balance

In order to estimate total body water content, rate of water loss and dehydration tolerance (%), 10 flies of each of the control, dark-selected and light-selected lines were used. First, the flies were weighed on Sartorious microbalance (Model CPA26P; with 0.001 mg precision) and then reweighed after drying at 60°C overnight. Total body water content was estimated as the difference between masses before and after desiccation stress of 8 h at 6–8% relative humidity, and water loss was calculated as: (initial body mass – body mass after 8 h desiccation stress)/initial body mass × 8 ($\mu g\,h^{-1}$). Dehydration tolerance was estimated as the percentage of total body water lost at death due to desiccation (until death) and was calculated by the formula: (wet body mass – mass at death)/(wet body mass – dry body mass) × 100.

To measure hemolymph volume, blotting assays were conducted. Ten replicates of 7-day-old male and female individuals were anaesthetised and weighed as a group. The abdomen of each was gently torn with surgical forceps, and haemolymph was blotted from the opening with a piece of Kimwipe slightly moistened with isotonic saline. Within a maximum of 10 min, 10 blotted flies were reweighed as a group and dried for 1 h at 60°C and weighed a third time. Hemolymph volume was estimated from the reduction in mass following blotting.

Response of cuticular lipids to selection

For estimation of cuticular lipid mass per fly, individual flies in 10 replicates per replicate line were dried overnight at 60°C to obtain

constant dry mass, i.e. devoid of body water. Such dried flies were kept in HPLC-grade hexane for 1 h; thereafter the flies were removed from the solvent and were again dried at room temperature and finally reweighed. The Sartorius microbalance (CPA26P, www.sartorius.com) with precision up to 0.001 mg ensured accuracy. For each individual fly, cuticular lipid mass (mg) was estimated per unit surface area (surface area scales to 2/3 power of the wet body mass) as: difference between initial dry mass and dry mass after solvent treatment/initial dry mass × surface area (where area was expressed in cm^2 and wet body mass in mg).

Statistical analyses

The response to selection was analysed by computing realised heritability over every fifth generation of selection in both dark- and light-selected replicate lines. The realised heritability for cuticular melanisation was calculated for each line by plotting the mean melanisation score for each generation against the cumulative selection differential. The expected selection differential was calculated as the deviation of the mean cuticular melanisation score of the selected individuals in each generation from the population mean before selection. This was then summed each generation to give the cumulative selection differential. The realised heritability (h^2) was then calculated from the slope of the regression of mean colour score (R) against the cumulative selection differential (S), as $h^2=R/S$ (Falconer and Mackay, 1996). ANOVA was used to compare control and selected dark and light lines, with four replicates each control, dark and light group × 10 replicates each. Replicate values are presented as means ± s.d. Statistica (Statsoft, Release 5.0, Tulsa, OK, USA) was used for calculations as well as illustrations.

RESULTS

Response to selection: changes in abdominal melanisation in dark and light selected lines

Selection was initiated from mass-bred populations with high variability for abdominal melanisation (mean ± s.d. = 41.39±13.12; Fig. 1A). Selection for abdominal melanisation in *D. melanogaster* for 60 generations resulted in an approximately 14-fold decrease in abdominal melanisation (ANOVA; females, $P<0.0001$; males, $P<0.0001$; Table 1) in light-selected strains and a 1.6-fold increase in dark-selected strains (ANOVA; females, $P<0.0001$; males, $P<0.0001$; Table 1). A plot of the response to selection as a function of cumulative selection differential (Fig. 1B) indicated that the response was asymmetric: divergence from unselected controls was faster and greater in light-selected lines than in dark-selected lines.

Realised heritabilities were estimated from the regression of selection response on cumulative selection differential. The regression of cumulative selection differential on response was highly significant for each of the dark- and light-selected lines, giving heritability estimates (h^2±s.e.m.) for cuticular melanisation of 0.46±0.03 for dark-selected lines (mean value of five replicates) and 0.39±0.02 for light-selected lines (Fig. 1B). For abdominal melanisation, females responded quickly to selection. The laboratory selection was performed on females only but males were also found to respond to selection for changes in abdominal melanisation (Fig. 2A). Dark-selected females showed ~87% melanisation, while males exhibited ~88% melanisation (Fig. 2A). However, in light-selected strains, females and males were very light (approximately 3% melanisation only; Fig. 2A). The replicate selected lines reached significantly different final percent melanisation, and the complete lack of response to relaxed selection. Further, the variability in each dark- and light-selected strain was quite low (mean ± s.d.; dark, 87.12±0.79; light, 3±0.21).

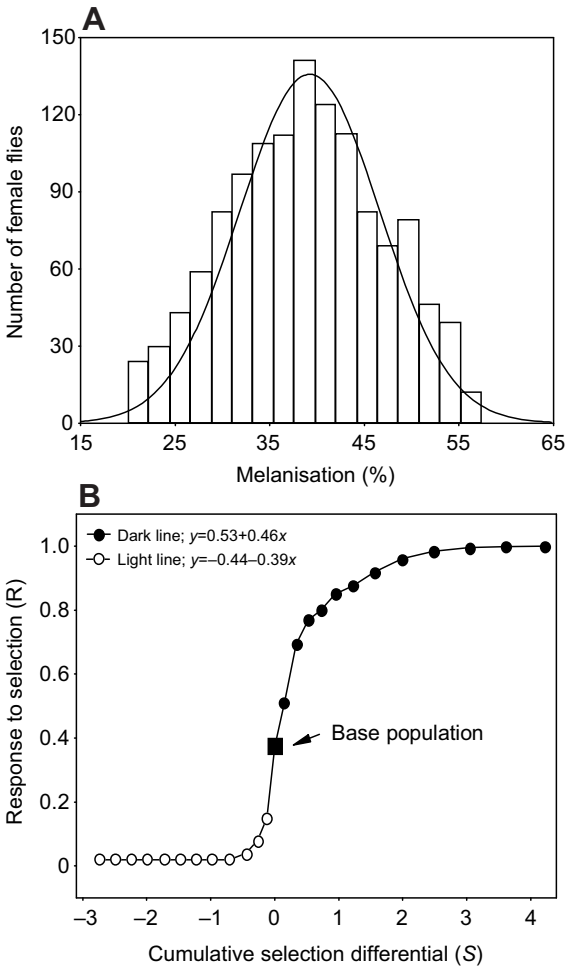

Fig. 1. (A) Variation (mean) of abdominal melanisation in a sample of flies (N=1236) after mass breeding for seven generations in the laboratory at 21°C and before onset of laboratory selection. Percent melanisation of the base population was 41.39±13.12%. (B) Regression between response to selection (R) and cumulative selection differential (S) for estimation of realized heritability of laboratory-selected dark and light lines of *D. melanogaster*.

Physiological assays

Male and female flies were tested for changes in body water content (Table 1) and the analysis showed a significant line effect in the ANOVA ($P<0.001$). Darker selected lines had approximately 10.0% more water content, whereas in light-selected replicates body water content decreased by ~13.0% as compared with their unselected controls. Fig. 2B–D illustrates a comparison of desiccation-related traits in selected darker and lighter body colour strains as compared with unselected control lines. For control and selected strains of *D. melanogaster* grown at 21ºC, there is a lack of difference in cuticular lipid mass. In contrast, we found a significant difference in the desiccation resistance of these two selected darker and lighter strains (Table 2, Fig. 2B) and a significant difference in their rate of water loss, i.e. 0.3% h^{-1} (1.5% h^{-1} in darker *versus* 1.8% h^{-1} in lighter strains; Fig. 2C). However, the level of dehydration tolerance was much higher in darker (~82.32%) than lighter strains (~22%) as compared with control lines (~50%; Table 2, Fig. 3D).

Correlated responses to selection

For any trait, consistent bidirectional changes in selected lines relative to their unselected control lines are due to the effect of selection

Table 1. Changes in body melanisation, desiccation resistance and rate of water loss in four replicate lines of dark- and light-selected lines as compared with control lines of *Drosophila melanogaster*

	Melanisation (%)		Desiccation resistance (h)		Rate of water loss (mg fly^{-1})	
Replicate lines	♂	♀	♂	♀	♂	♀
Control						
C_1	47.21±5.21	43.50±4.25	26.01±3.21	29.1±3.10	19.51±4.25	21.23±5.33
C_2	48.32±7.02	38.21±4.98	29.53±4.29	34.4±4.02	20.21±3.98	23.21±4.22
C_3	47.25±3.25	42.57±5.65	25.55±4.71	32.8±4.21	18.14±5.36	21.50±3.65
C_4	46.36±4.98	39.57±6.21	21.22±3.89	26.5±3.29	20.30±4.22	22.11±4.78
Dark selected						
D_1	90.00±0.65	87.25±0.33	52.21±1.22	54.4±1.12	6.30±1.20	7.21±1.00
D_2	86.58±0.36	88.26±0.18	53.11±2.00	56.7±2.45	7.12±0.89	7.56±0.89
D_3	89.14±0.12	88.56±0.14	50.98±1.89	51.6±1.30	6.23±1.01	6.39±1.77
D_4	87.98±0.14	86.00±0.54	54.22±1.25	56.8±1.89	6.89±1.00	7.14±0.77
Light selected						
L_1	3.60±0.21	4.22±0.54	14.00±1.70	16.5±0.56	36.21±1.13	37.54±1.32
L_2	1.10±0.13	3.23±0.36	13.74±0.99	16.5±0.49	35.98±1.02	36.87±1.00
L_3	2.25±0.19	3.21±0.25	16.04±1.74	18.0±0.51	36.89±0.98	37.00±1.89
L_4	2.32±0.17	5.00±0.52	12.36±1.12	16.0±0.61	36.11±1.00	36.98±1.00
Mean squares for ANOVAs (df)						
Control *vs* dark selected						
Lines (7)	17512.7***	19854.3***	3264.47***	2963.52***	546.61***	547.85***
Error (72)	4.141	3.012	0.723	0.954	0.519	0.405
Control *vs* light selected						
Lines (7)	12242.5***	10254.1***	3065.28***	2732.96***	629.46***	759.78***
Error (72)	5.323	4.901	0.712	0.623	0.73	0.619

Data are means ± s.e.m. For each trait, ANOVA values represent statistical differences between selected (dark or light) and control lines. Each value is based on analysis of 10 replicates of 10 individual flies of each sex.

***P<0.001; ns, non-significant. ANOVAs on melanisation were carried out on arcsine-transformed data.

(Lynch, 1980) and indicate that abdominal melanisation and the co-responding traits are influenced by some of the same genes [i.e. they show genetic covariance (Falconer, 1981)]. Sixty generations of artificial selection on abdominal melanisation produced consistent bidirectional selection. Correlated responses in body water content, desiccation resistance, rate of water loss, dehydration tolerance (Table 1, Fig. 2B–D) and mean trait values increased significantly for body water content, desiccation resistance and dehydration tolerance in dark-selected replicate lines, whereas rate of water loss showed a negative correlated response. However, in light-selected replicate lines, body water content, desiccation resistance and dehydration tolerance decreased significantly, and rate of water loss showed a positive correlated response (Table 1, Fig. 2B–D).

Direct selection of cuticular melanisation impacted desiccation resistance significantly (Fig. 2). Comparison of direct and indirect selection responses provides a clearer view of the melanism–desiccation correlation hypothesis. Replicates selected for higher melanisation showed higher desiccation resistance and reduced rate of water loss, whereas desiccation survival decreased and rate of water loss increased significantly in replicates selected for low cuticular melanisation compared with controls (Table 2). Selection for cuticular melanisation had a significant effect on water balance and desiccation resistance. Darker lines had significantly higher desiccation resistance (P<0.0001) than control flies, whereas lighter lines showed twofold decreases in desiccation survival. Similar effects were observed for rate of water loss (P<0.0001). For body water content, there were non-significant changes in selected *versus* control replicates (P<0.23). Further, we observed no changes in wing length (as a measure of body size) due to selection ($F_{7,72}$=5.01, P>0.05).

Flies were analysed for cuticular lipids in control as well as selected replicates to examine their response towards selection and relation with desiccation resistance. Cuticular lipids did not change in response to selection (Fig. 3D, Table 2). There was a non-significant difference for cuticular lipids between the control lines (P<0.07) and also between the selected lines (P<0.20) when tested by ANOVA. Changes in desiccation and changes in water loss rate were significantly correlated with selection in cuticular melanisation (Fig. 4, Table 3). Cuticular lipids did not respond to selection and were not correlated with increasing desiccation resistance and water balance (Table 3).

DISCUSSION

Drosophila melanogaster exhibits considerably high variation in abdominal melanisation in natural populations. Laboratory-selected (for dark and light body colour) strains of *D. melanogaster* differ significantly in abdominal melanisation. Our study shows that there is a great deal of genetic variation in abdominal melanisation in *D. melanogaster*, as evidenced by consistent, rapid and substantial response to selection for high and low melanisation phenotypes. For body melanisation, we found a rapid response to laboratory selection. Interestingly, selection produced both light females as well as light males, in contrast to the well-known sexual dimorphism of body melanisation in *D. melanogaster*. The replicate selected lines reached significantly different final percent melanisation, and the complete lack of response to relaxed selection suggests that different combinations of alleles affecting abdominal melanisation may have become fixed in the different selected lines. Although the external appearance of our selected strains is different from that of typical wild populations, we detected no statistically significant effects of melanisation on basic morphometric traits such as wing length, thorax length and wing width (P<0.12). However, dark- and light-selected strains differed significantly in wet body mass (P<0.001).

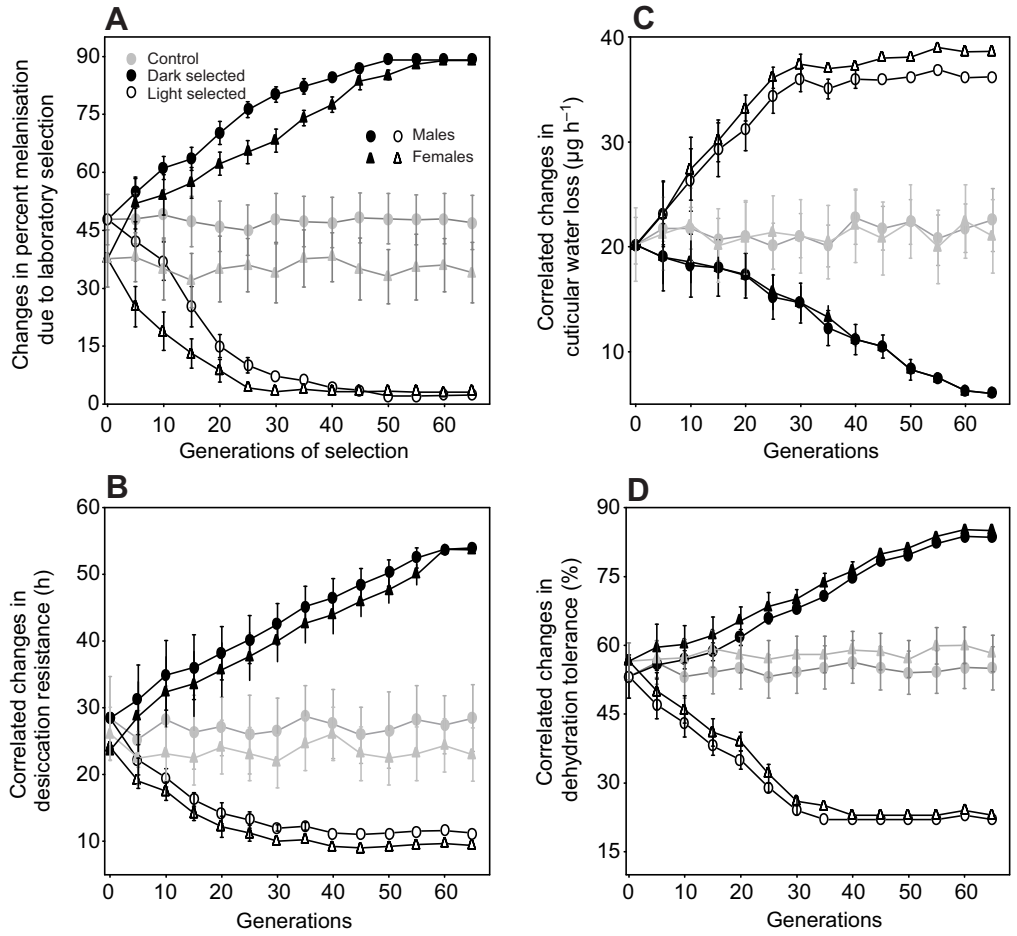

Fig. 2. (A) Results of laboratory selection up to the 60th generation for changes in total body melanisation per fly in control and selected dark and light lines of *D. melanogaster*. (B–D) Correlated changes in desiccation resistance, cuticular water loss and dehydration tolerance are shown after every five generations of laboratory selection in dark and light lines along with their respective control lines. For each trait, data are shown as means of five replicate lines. Selection was made on the melanisation of female flies but corresponding changes in males are also shown.

We found an asymmetric response to selection in lines selected for dark and light abdominal melanisation in *D. melanogaster* (Fig. 1B). Asymmetric responses to selection are a common finding in selection experiments. The reasons given by Falconer and Mackay (Falconer and Mackay, 1996) involve experimental artefacts (random drift, inbreeding depression and unmeasured natural or sexual selection acting during the experiment) as well as a multitude of potential genetic causes (genetic asymmetry, presence of major genes, scalar asymmetry). Random drift is unlikely to explain the present results because of the consistency of responses between replicates. Inbreeding depression is also unlikely because the mean of the unselected control line did not decline. However, we cannot at this point rule out the genetic causes (major genes, directional dominance or genetic asymmetry); it is possible that the asymmetric response is explained by scalar asymmetry: high phenotypic values might be particularly subject to environmental influence (e.g. condition-dependency, rearing conditions) and the extreme values an artefact of laboratory rearing. One interesting possibility is that the asymmetry results from the previous action of selection in the base population. Favourable alleles are expected to have frequencies above their symmetrical points (Falconer and Mackay, 1996).

Artificial selection experiments are longer in duration and correlations are limited to the trait being selected, but they have the advantage of directly revealing patterns of responses and co-responses to a specified selection regimen. Thus, they can provide an independent test for the presence of significant patterns of genetic variation and co-variation in a given environment. Because of the physiological relationship between melanisation and desiccation resistance, we predicted that selection for melanisation would result in a correlated increase and decrease in desiccation resistance of *D. melanogaster*. We found that desiccation resistance was higher in dark-selected strains and was significantly lower in light-selected strains (Fig. 1C). For adaptations to drier habitats, the function of cuticular lipids in reducing cuticular water loss is well known in different insect taxa from deserts (Hadley, 1994). However, some studies have shown the role of melanisation in reducing cuticular water loss in *Drosophila* species (Parkash et al., 2008b; Parkash et al., 2008c). Associations between pigmentation and desiccation resistance were initially proposed by Kalmus (Kalmus, 1941). In the present work, we have investigated effects of bidirectional selection of body melanisation selection on correlated traits. For *D. melanogaster*, rapid response to direct selection of melanisation confirms the existence of substantial additive genetic variation for this trait.

Table 2. Changes in body water content, hemolymph volume and dehydration tolerance in four replicate lines of dark- and light-selected lines as compared with control lines of *D. melanogaster*

	Water content (mg fly^{-1})		Hemolymph (%)		Dehydration tolerance (%)	
Replicate lines	♂	♀	♂	♀	♂	♀
Control						
C_1	1.23±0.04	1.25±0.04	13.43±3.21	15.25±2.21	50.01±3.21	56.66±2.32
C_2	1.19±0.04	1.22±0.05	12.00±2.21	13.54±3.21	49.53±4.29	54.32±3.21
C_3	1.18±0.03	1.19±0.04	12.12±2.98	14.12±2.36	50.55±4.71	52.01±2.98
C_4	1.20±0.04	1.21±0.03	12.56±2.13	14.00±2.07	51.22±3.89	53.65±3.14
Dark selected						
D_1	1.29±0.02	1.32±0.01	18.19±1.01	20.21±0.89	82.21±1.22	84.20±1.21
D_2	1.30±0.01	1.34±0.02	19.30±0.98	21.32±1.11	81.11±2.00	83.11±1.58
D_3	1.31±0.01	1.33±0.02	20.11±1.00	22.32±0.85	81.98±1.89	81.21±1.05
D_4	1.28±0.02	1.33±0.01	20.21±0.88	21.27±0.99	82.22±1.25	82.11±0.97
Light selected						
L_1	1.03±0.02	1.06±0.01	10.11±0.87	11.67±0.21	21.00±1.70	22.23±1.23
L_2	1.05±0.02	1.09±0.01	10.00±1.03	10.99±0.80	21.74±0.99	23.13±2.51
L_3	1.02±0.01	1.05±0.02	11.21±0.56	9.78±0.74	22.04±1.74	22.98±1.58
L_4	1.03±0.02	1.06±0.02	10.88±0.87	10.23±0.81	21.36±1.12	22.19±1.21
Mean squares for ANOVAs (df)						
Control *vs* dark selected						
Lines (7)	0.406**	0.038**	0.019***	0.015***	0.2843***	0.3013***
Error (72)	0.002	0.003	0.00004	0.00001	0.0007	0.0004
Control *vs* light selected						
Lines (7)	0.191**	0.291**	0.0061	0.0026***	0.6943***	0.7427***
Error (72)	0.001	0.002	0.0002	0.0001	0.002	0.001

Data are means ± s.e.m. For each trait, ANOVA values represent statistical differences between selected (dark or light) and control lines. Each value is based on 10 replicates of 10 individual flies. **P<001; ***P<0.001. ANOVAs on percent data were carried out on arcsine-transformed data.

Therefore, populations of *D. melanogaster* have the potential to undergo rapid genetic changes when they are exposed to one or the other selective environment. The correlated responses to selection indicate that at least some of the genes that contribute to variation in abdominal melanisation have pleiotropic effects on other traits. Specifically, body melanisation shares positive additive genetic covariance with body water content, hemolymph content desiccation resistance and dehydration tolerance, and it shares negative genetic covariance with rate of water loss. Epicuticular lipids, however, do not respond to selection.

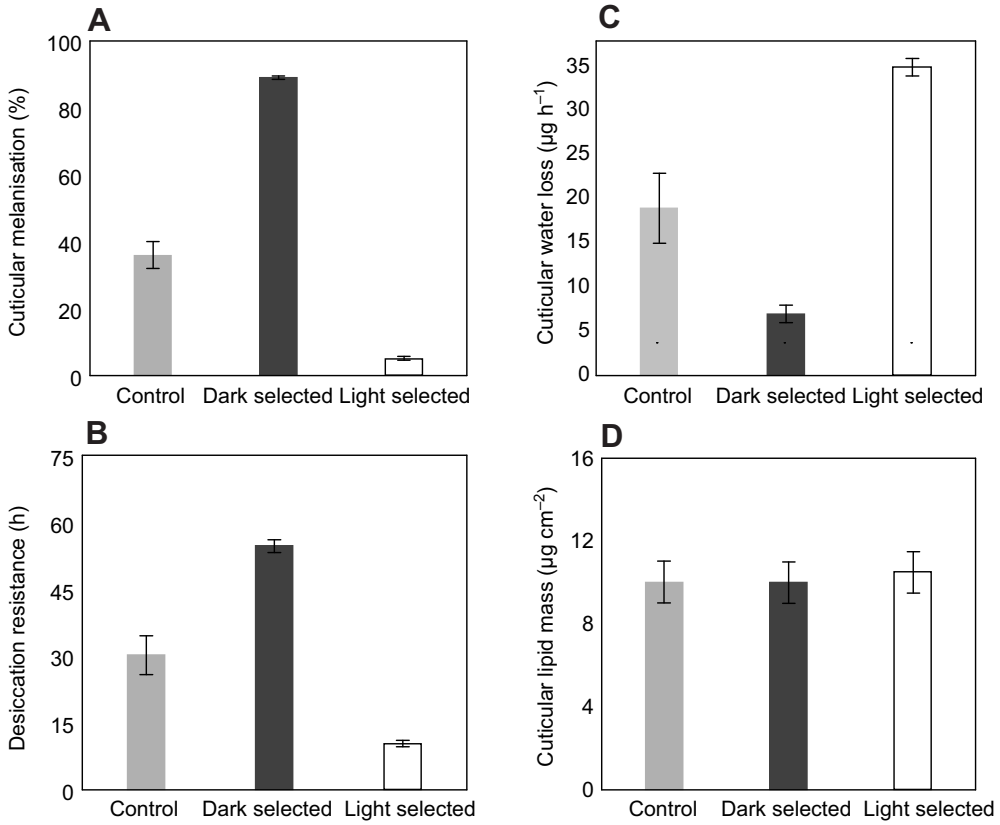

Fig. 3. Changes in (A) cuticular melanisation, (B) desiccation resistance, (C) cuticular water loss and (D) cutitcular lipid content in dark- and light-selected lines of *D. melanogaster* compared with control lines. Trait values represent data from the 60th generation of selection. Each value is based on analysis of 10 replicates of 10 individual flies from each of five replicate lines. Data are means ± s.d.

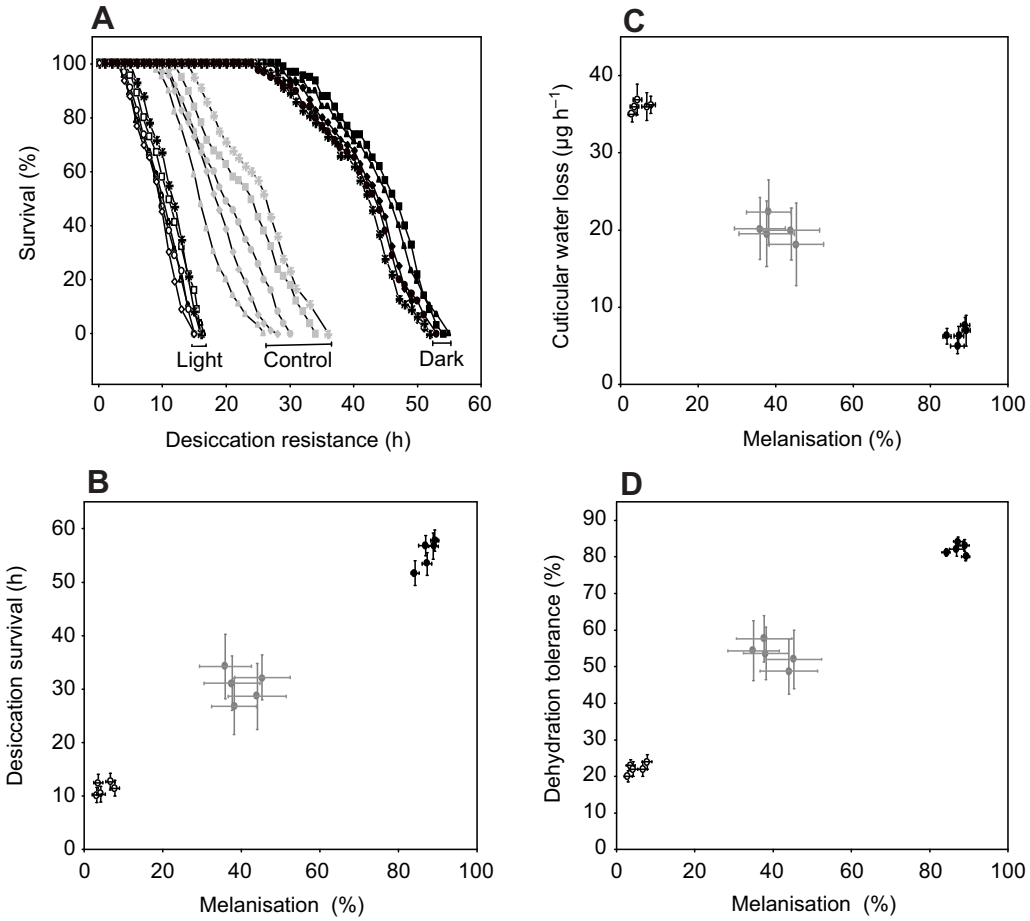

Fig. 4. (A) Desiccation survival curves of control and laboratory-selected dark and light body colour lines of *D. melanogaster* (*N*=5 each; values are shown as average percentage survival per hour) when stressed in groups of 10 female flies of the 60th generation. Correlated changes in desiccation resistance, cuticular water loss and dehydration tolerance in control and five selected (dark and light) lines. Between-line variability (mean ± s.d.) is shown for desiccation-related traits as a function of changes in body melanisation of control and selected lines.

Another problem is to differentiate the waterproofing role of cuticular melanisation from that of cuticular tanning (hardening) in *D. melanogaster*. Different cuticular components may affect cuticular transpiration in insects (Chapman, 1998). Darker cuticle achieves its colour as a result of deposition of melanin granules [polymers of dopa and other tyrosine derivatives (True, 2003)]. Like cuticular lipids, melanin is also hydrophobic and therefore may reduce cuticular permeability. It has been suggested that the darkening and hardening of cuticle result from cross-linking of cuticular proteins with melanin (Pryor, 1940; Fraenkel and Rudall, 1940; Hopkins and Kramer, 1992). Thus, melanisation and sclerotization pathways (tanning) could be related because in the insects, harder body parts are generally darker. In the present study, we have not considered the role of sclerotization. However, further studies are needed to differentiate the effects of cuticular melanisation and cuticular tanning for waterproofing function in *Drosophila* species and in insects in general.

Insect cuticle is a complex structure and its components vary greatly between populations and species (Rajpurohit et al., 2008). Changes in body melanisation have been shown to affect cuticular permeability in some *Drosophila* species (Parkash et al., 2008b; Parkash et al., 2008c). According to the melanisation–desiccation hypothesis, darker flies of *D. melanogaster* are abundant in cooler uplands while lighter flies are predominant in foothills (Parkash et al., 2008c). The laboratory-selected desiccation-resistant and -sensitive strains of *D. melanogaster* have shown similar amounts of cuticular lipid mass (Gibbs et al., 1997), and there is also a lack of differences in the amount of cuticular lipid mass in northern *versus*

Table 3. Correlation values (±s.e.m.) for different ecophysiological traits with changes in cuticular melanisation and cuticular lipid mass in selected lines for dark and light body colour of *D. melanogaster*

	% Melanisation		Cuticular lipids	
Trait	Dark	Light	Dark	Light
1. Melanisation (%)	–	–	–0.24±0.28 ns	0.33±0.27 ns
2. Cuticular lipid mass (µg cm^{-2})	–0.24±0.28 ns	0.18±0.31 ns	–	–
3. Body water content	0.89±0.07**	0.85±0.08**	–0.21±0.21 ns	0.28±0.31 ns
4. Hemolymph (%)	0.91±0.03***	0.89±0.07***	–0.35±0.19 ns	0.31±0.27 ns
5. Desiccation (h)	0.98±0.04***	0.99±0.03***	–0.25±0.27 ns	0.32±0.27 ns
6. Cuticular water loss (µg h^{-1})	–0.96±0.07***	–0.97±0.05***	0.19±0.23 ns	–0.30±0.27 ns
7. Dehydration tolerance	0.96±0.05***	0.90±0.12***	–0.21±0.23 ns	0.15±0.28 ns

**P<0.01; ***P<0.001; ns, non-significant.

southern populations of *D. melanogaster* (Parkash et al., 2008a). However, in two cases (*Melanoplus sanguinipes* and *Z. indianus*), there are significant intrapopulation differences in the amount of cuticular lipid mass per cm^2 (Rourke, 2000; Parkash et al., 2008a; Parkash et al., 2012).

The present work provides good evidence that the associations between both desiccation resistance and the rate of water loss with cuticular melanisation in *D. melanogaster* populations from India have a genetic basis, which has been suggested by comparisons in field and laboratory populations. This is the first experiment to explore these associations with a long-term artificial selection experiment. However, pigmentation clines have been reported in *Drosophila* (Parkash et al., 2008), and desiccation resistance is often proposed as an adaptive benefit of increased melanisation. We tested the association between pigmentation and desiccation in *D. melanogaster* using selection and found that there was a causative relationship between melanisation and rate of water loss, as selection for both high and low melanin resulted in a correlated response to selection in both desiccation resistance and water balance [however, note that Wittkopp et al. (Wittkopp et al., 2011) performed a similar experiment using *D. americana* and *D. novamexicana*, and found no effect of pigmentation genotype on desiccation resistance].

We observed a substantial increase in both male and female survival to desiccation stress following selection for abdominal melanisation. There was a sexual dimorphism for abdominal melanisation as well as desiccation resistance at the onset of our selection regime, but at the 60th generation of selection these differences vanished. The presence of similar resistant levels in males and females implies that there is a common genetic basis between the sexes underlying the selection responses. Fig. 2 represents a genetic association between traits involved in correlated responses to selection. Selection was associated with an increase in wet mass. This observation is consistent with lines directly selected for desiccation resistance (Gibbs et al., 1997) and lines selected indirectly in response to very mild desiccation stress (Kennington et al., 2003), but not other directly selected lines (Bubliy and Loeschcke, 2005; Hoffmann and Parsons, 1989b). The changes in correlated traits such as desiccation survival with melanisation of selected replicates were independent of body size. The absence of changes in body size suggests that changes in the surface to volume ratio were not involved in increased desiccation tolerance. There were also correlated responses in the mass loss of flies, suggesting that selected strains had a different rate of water loss through the cuticle.

Selection experiments are vital tools of evolutionary biology and the results of nearly a century's worth of selection experiments have helped establish the genetic component of evolutionary theory (Provine, 1971; Falconer, 1992). In addition, selection experiments have provided stocks that have been useful for many other topics, from estimating mutation rates to understanding the molecular, biochemical and physiological foundations of trait variation (Hill and Caballero, 1992; Mackay, 2001; Conner, 2003; Garland, 2003). The association between melanisation and other correlated traits has been well documented in natural populations of *D. melanogaster* (Parkash et al., 2008), but such associations have not been analysed following artificial selection experiments of abdominal melanisation. Selection experiments in other drosophilids and insects have detected patterns of genetic covariance between traits (Pitnick and Miller, 2000; Nunney, 1996; Beldade et al., 2002). The results should be especially informative, as the application of quantitative-genetic theory to the evolution of phenotypic differences between populations assumes the pattern of genetic variance and covariance remains relatively constant across evolving populations (Lande, 1982; Arnold, 1981).

Conclusions

Through the analysis of correlated responses to direct selection on abdominal melanisation, we detected a pattern of genetic co-variation among desiccation related traits. A direct selection for cuticular melanisation has selected correlated traits (water balance related traits) in *D. melanogaster*. The evidence for correlated traits is based on trait correlation analysis, i.e. darker flies have lower rate of water loss, which confers greater desiccation resistance. In contrast, higher rate of water loss in lighter flies sustains lower desiccation tolerance. The results of this study therefore indicate the potential for abdominal melanisation evolution to facilitate or constrain the evolution of desiccation resistance in *D. melanogaster*. Still, the generality of these results deserves further investigation.

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