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Impact of body melanisation on contrasting levels of desiccation resistance in a circumtropical and a generalist *Drosophila* species

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Abstract We investigated the role of cuticular lipids, body melanisation and body size in conferring contrasting levels of desiccation resistance in latitudinal populations of *Drosophila melanogaster* and *Drosophila ananassae* on the Indian subcontinent. Contrary to the well known role of cuticular lipids in water proofing in diverse insect taxa, there is lack of geographical variations in the amount of cuticular lipids per fly in both the species. In *D. ananassae*, quite low levels of body melanisation are correlated with lower desiccation resistance. By contrast, increased levels of desiccation resistance are correlated with quite high melanisation in *D. melanogaster*. Thus, species specific cuticular melanisation patterns are significantly correlated with varying levels of desiccation resistance within as well as between populations and across species. Role of body melanisation in desiccation resistance is further supported by the fact that assorted dark and light flies differ significantly in cuticular water loss, hemolymph and dehydration tolerance. However, similar patterns of body size variation do not account for contrasting levels of desiccation resistance in these two *Drosophila* species. Climatic selection is evidenced by multiple regression analysis with seasonal amplitude of thermal and humidity changes (Tcv and RHcv) along latitude on the Indian subcontinent. Finally, the contrasting levels of species specific distribution patterns are negatively correlated with RHcv of sites of origin of populations i.e. a steeper negative slope for *D. ananassae* corresponds with its desiccation sensitivity as compared with *D. melanogaster*. Thus, evolutionary changes in body melanisation impact desiccation resistance potential as well as distribution patterns of these two *Drosophila* species on the Indian subcontinent.

Keywords Body melanisation · Body size · Desiccation resistance · Cuticular lipids per fly · Cuticular water loss · *D. ananassae* · *D. melanogaster*

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Introduction

Several investigations have shown rapid response to laboratory selection for desiccation resistance in *Drosophila melanogaster* (Hoffmann and Parsons 1989; Gibbs et al. 1997; Telonis-Scott et al. 2006). Thus, genetic variation for desiccation resistance appears to be directly or indirectly under selection. Various studies on the mechanistic basis of desiccation resistance in various *Drosophila* species have shown that rate of cuticular water loss is negatively correlated with desiccation resistance (Kimura et al. 1985; Gibbs and Matzkin 2001; Parkash et al. 2008a). For ectothermic insects, more than 90% loss of body water involves cuticular transpiration under desiccation stress (Gibbs et al. 2003). If desiccation resistance evolves through changes in cuticular permeability, the target of selection might be cuticular components either cuticular lipids or cuticular melanisation. However, such a possibility has not been investigated in natural populations as well as through laboratory selection experiments.

For desiccation resistance, water proofing role of cuticular lipids has been reported for tenebrionid beetles, scorpions and grasshoppers (Edney 1977; Zachariassen 1988; Hadley 1994; Rourke 2000). Cuticular impermeability results due to changes in the amount and/or composition of cuticular lipids (Toolson 1984; Hadley 1994; Gibbs 1998). According to Gibbs (2002), most insect species produce lipids with higher ($T_m \geq 45°C$ due to higher proportion of alkanes) as well as lower melting points (due to alkenes, $T_m \leq 25°C$); and there is lack of correlation between T_m of cuticular lipids and cuticular water loss in several insect taxa from diverse habitats. Thus, the water proofing role of cuticular lipids may not be a universal mechanism for regulating water loss and thereby conferring desiccation resistance.

The role of body melanisation has been reported for thermoregulation in beetles and butterflies (de Jong et al. 1996; Ellers and Boggs 2004); and for conferring desiccation resistance in altitudinal as well as latitudinal populations of two *Drosophila* species (Parkash et al. 2008a, b). However, it is not clear whether species specific differences in body melanisation can impact their stress resistance levels. For generalist species, it is generally assumed that populations have abundant genetic variations in quantitative traits for adaptation (Hoffmann and Weeks 2007). By contrast, there are limited data on the evolutionary responses of stress related traits in tropical *Drosophila* species from humid habitats (Hoffmann et al. 2003).

Desiccation clines were earlier investigated only for male sex in latitudinal populations for *Drosophila melanogaster* and *Drosophila ananassae* (Parkash and Munjal 1999). This study did not consider evolutionary response of desiccation related traits in context of body melanisation. Most studies on clinal variations in various drosophilids have not analyzed mechanistic basis of desiccation resistance (Hoffmann and Parsons 1989; Parkash and Munjal 1999; Hoffmann et al. 2003; Hoffmann and Weeks 2007; Arthur et al. 2008). In the present investigation, we have analyzed populations of *D. melanogaster* and *D. ananassae* through common garden experiments for possible link between species specific differences in body melanisation and stress related traits. We have addressed the following questions: (a) whether contrasting levels of species specific desiccation resistance result due to variations in cuticular lipids, body melanisation and body size? (b) Do assorted lighter and darker flies from each geographical population show significant correlation with desiccation related traits? (c) Whether various desiccations related traits are under climatic selection on the Indian subcontinent?

There are no reports on simultaneous analysis of stress related traits in sympatric populations of different *Drosophila* species with contrasting distribution patterns across a

given continent. *D. ananassae* abounds in warm and humid tropical habitats but this species does not occur in higher latitudes as well as altitudinal localities. A comparison of stress related traits in sympatric populations of a generalist (*D. melanogaster*) and a warm adapted species (*D. ananassae*) may help in explaining their differential evolutionary potential. For circumtropical species (*D. ananassae*) body melanisation and desiccation resistance are significantly lower as compared with the generalist species *D. melanogaster*. However, analysis of ecophysiological traits in assorted darker and lighter flies for each of six populations of both species have evidenced clinal variation for desiccation related traits but there is lack of geographical variation for cuticular lipids. At intrapopulation level, body size changes are not correlated with desiccation resistance while there are significant positive correlations of body melanisation with desiccation resistance, dehydration tolerance and hemolymph in both the species. However, there is negative correlation between body melanisation and cuticular water loss. Contrasting levels of desiccation resistance in these two species can be explained on the basis of changes in body melanisation but not due to body size and cuticular lipids. Thus in *D. melanogaster* and *D. ananassae*, species specific differences in body melanisation impact desiccation related traits.

Materials and methods

Collections

The collections of sympatric populations of *D. melanogaster* and *D. ananassae* were made in a single trip during September, 2006 from twelve latitudinal sites (8.05–32.00°N) that also differ in altitude from (37–1,211 m). From each site, about 100–150 wild-caught individuals were obtained using net sweeping method. Wild-caught females were used to initiate 20 isofemale lines per population. For southern populations, T_{ave} varies between 20 and 25°C while the corresponding values are 18–22°C for northern populations. Based on T_{ave} data of the site of origin of populations, we maintained cultures at 21°C on cornmeal yeast agar medium. For all cultures, larval density was kept low by limiting the egg laying period to 8 h which resulted in 40–60 larvae per vial. All experiments were initiated soon after collections and performed with G_1 and G_2 generations. All assays were performed on 8 days old flies because the trait values did not vary as a function of age between 6 and 21 days (Gibbs and Matzkin 2001; Parkash et al. 2008c). For each isofemale line, we first analyzed body size (wing length) as well as body melanisation and this was followed by other assays such as desiccation resistance, water balance assays and cuticular lipid mass per fly at population level as well in assorted lighter and darker phenotypes. Climatic data for the sites of origin of populations were obtained from Indian Institute of Tropical Meteorology (IITM; www.tropmet.res.in).

Trait measurements

For both the species, wing length was measured from the thorax articulation to the tip of third longitudinal vein under Olympus SZ-11 microscope (Olympus, www. olympus.com) fitted with a micrometer. Body melanisation was measured in laboratory reared female individuals. Melanisation was estimated from dorsal view of the abdomen giving values ranging from 0 (no pigment) to 10 (complete darkness) for each of the six visible abdominal segments i.e. 2nd to 7th for both the species. The flies were scored by two independent persons and a high correlation ($r > 0.96 \pm 0.02$) between two sets of

observations ensured repeatability. Since the abdominal segments differ in size, relative sizes (i.e. 0.86, 0.94, 1.0, 0.88, 0.67, and 0.38 for 2nd to 7th segments, respectively, for both the species) were multiplied with segment wise pigmentation scores (Parkash et al. 2008d). In *D. ananassae*, segments 5th, 6th and 7th do not show melanisation. The data on % melanisation were calculated as (Σ observed melanisation scores of six abdominal segments per fly/Σ relative size of each segment × 10 per fly) × 100. The total body melanisation per fly was also estimated through Biowizard image analysis Software (Dewinter Optical Inc., www.dewinterindia.com).

To measure desiccation resistance, ten individuals from each line were isolated in a dry plastic vial, which contained 2 g of silica gel at the bottom and were covered with a disk of foam piece. Such vials with foam plugs were placed in a desiccation chamber (Secador electronic desiccator cabinet) which maintains 4–5% relative humidity. The vials were inspected every hour and the number of dead flies (completely immobile) was recorded. The survival curves as a function of desiccation hours were drawn and LT_{50} values (lethal tolerance time at which 50% flies die) were calculated by linear interpolation from such graphics.

Within population trait measurement

For *D. melanogaster*, individuals differ for the last three abdominal segments (5th, 6th and 7th) i.e. for dark flies, the sum of the last three segments is generally more than 20 out of maximum 30 for three segments (segment score × segments = 10 × 3). By contrast, light flies exhibit generally less than 10 score out of maximum of 30. Thus, assortment of dark and light flies is very convenient and rapid. While for *D. ananassae*, last three abdominal segments (5th, 6th and 7th) show no melanisation so dark and light flies were separated on the basis of (2nd, 3rd and 4th) abdominal segments i.e. for darker flies, the sum of the anterior three segments is generally more than 8 out of maximum 30 for three segments (segment score × segments = 10 × 3). By contrast, lighter flies exhibit generally less than 3 score out of maximum of 30.

Water balance analyses

For different physiological assays (total water content, cuticular water loss, hemolymph, dehydration tolerance and amount of cuticular lipids fly^{-1}), gravimetric methods were followed in 20 isofemale lines per population for both the species. Cuticular water loss due to short term desiccation (8 h) was done in groups of ten flies. Flies were weighed on a sartorius microbalance (CPA26P, www.sartorius.com) both before and after desiccation, and the cuticular water loss was calculated as mg h^{-1}: (initial body weight–body weight after 8 h desiccation stress)/initial body weight × 8. To measure possible differences in extracellular water (hemolymph), blotting assay was followed (Folk et al. 2001). Each fly was slightly anesthetized and weighed on a microbalance. The abdomen of each fly was pricked with fine surgical needle and hemolymph was blotted with Whatman filter paper. Hemolymph content was estimated from the reduction in mass following blotting (initial fly weight–weight after hemolymph removal/initial fly weight × 100). Dehydration tolerance was estimated as the percentage of total body water lost at death due to desiccation and was calculated by the formula (wet body weight–body weight at death)/(wet body weight–dry body weight) × 100.

For estimation of cuticular lipid mass per fly, individual flies in ten replicates per isofemale line were dried overnight at 60°C to get 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 in mg was estimated per unit surface area (surface area scales to 2/3 power of the wet body mass) as: difference between initial dry weight and dry weight after solvent treatment/initial dry weight × surface area (where area was expressed in cm^2 and wet body mass in mg). For all experiments on cuticular lipid mass per fly, estimates were based on ten replicates per isofemale line for populations as well as species (Parkash et al. 2008c). Further, in order to check possible errors in the estimates of cuticular lipid mass per fly, we performed three independent tests.

Statistical analyses

For all the traits (body melanisation, desiccation resistance, cuticular water loss, hemolymph, dehydration tolerance and amount of cuticular lipids/fly) population means ($n = 20$ lines × 10 individuals each) along with SE were used for illustrations and tabular data. Trait variability within as well as between populations was analyzed through ANCOVA with body size as a covariate; and percent of total variance explained by each variable was calculated as proportion of MS × degree of freedom out of the total sum of such values. Correlations between different traits were based on data from isofemale lines. Since within population correlations for melanisation and other traits were significantly high ($r > 0.88$) for all six populations; the data on *r*-values were represented as mean values across populations. Tcv for each locality was calculated as the coefficient of variation of monthly average temperatures across a year. Tcv values for 12 investigated localities varied from 3.15 to 33.40%. Likewise, RHcv was based on monthly data on relative humidity for each locality i.e. RHcv varied from 5.91 to 30.30% across twelve localities from Kanyakumari to Dharamshala. Since the investigated populations encounter significant seasonal variations in temperature and relative humidity, we used multiple regressions of trait values as a simultaneous function of Tcv and RHcv; and also for geographical variables (latitude and altitude) for the sites of origin of populations. Statistical calculations and illustrations were made with the help of Statistica 5.0.

Results

Clines for quantitative traits

Population means for four quantitative traits (body size, % body melanisation, desiccation resistance hours and cuticular water loss mg h^{-1}) form linear clines with latitude of origin and the data are given in Table 1 and illustrated in Fig. 1. For body size variations, results are interesting because regression analysis shows similar values of intercept as well as slope values for both the species (intercept = 2.26 mm; slope = 0.013; Fig. 1a). However, the two species differ significantly for % body melanisation (for *D. melanogaster*, intercept = 23.08%; Slope = 0.42; Fig. 1b) and corresponding values for *D. ananassae* are significantly low (intercept = 0.64% but a similar slope value = 0.42; Fig. 1b). Likewise, we obtained contrasting values of intercept but similar slope values for desiccation resistance across species (for *D. melanogaster* vs. *D. ananassae*: intercept values are 14.66 vs. 6.02 h but similar slope values = 0.31; Fig. 1c). There is a negative relationship

Table 1 Comparative data on population mean ± SE for three ecophysiological traits (% melanisation, desiccation hour and cuticular water loss) in 12 latitudinal populations of *D. melanogaster* (Dm) and *D. ananassae* (Da) grown at 21°C

Sites/altitude (meters)	Lat. °N	% Melanisation		Desiccation hours		Cuticular water loss (mg h−1)	
		Dm	Da	Dm	Da	Dm	Da
Kanyakumari (37)	8.05	23.2 ± 1.59	5.1 ± 0.60	16.1 ± 1.05	9.0 ± 0.50	0.032 ± 0.0017	0.054 ± 0.0027
Madurai (133)	9.50	27.8 ± 1.06	5.2 ± 0.76	18.0 ± 1.25	9.4 ± 0.56	0.031 ± 0.0016	0.050 ± 0.0026
Bangalore (921)	12.58	30.1 ± 1.60	5.5 ± 0.80	19.3 ± 1.35	10.1 ± .65	0.025 ± 0.0011	0.045 ± 0.0028
Anantpur (350)	14.41	31.5 ± 1.56	6.0 ± 0.90	20.0 ± 1.45	10.4 ± 0.68	0.023 ± 0.0010	0.044 ± 0.0030
Pune (559)	18.30	34.1 ± 1.78	7.3 ± 0.95	20.5 ± 1.55	11.4 ± 0.72	0.026 ± 0.0012	0.046 ± 0.0021
Nagpur (310)	21.09	32.2 ± 1.80	7.1 ± 1.00	21.0 ± 1.68	11.7 ± 0.74	0.025 ± 0.0011	0.043 ± 0.0019
Bhopal (523)	24.10	33.6 ± 1.90	8.8 ± 1.21	21.5 ± 1.72	12.0 ± 0.80	0.023 ± 0.0010	0.041 ± 0.0032
Jhansi (251)	25.70	30.3 ± 2.01	8.5 ± 1.25	22.0 ± 1.82	12.4 ± 0.78	0.022 ± 0.0009	0.040 ± 0.0021
Gorakhpur (77)	26.45	33.7 ± 2.27	9.6 ± 1.28	20.4 ± 1.64	11.0 ± 0.82	0.024 ± 0.0012	0.043 ± 0.0032
Rohtak (219)	28.94	36.4 ± 2.67	10.8 ± 1.58	22.8 ± 1.80	13.9 ± 0.90	0.019 ± 0.0014	0.036 ± 0.0023
Kalka (600)	30.51	40.5 ± 3.56	11.8 ± 1.68	24.3 ± 1.94	14.2 ± 0.9	0.017 ± 0.0015	0.032 ± 0.0024
Dharamshala (1,211)	32.00	46.6 ± 3.07	12.3 ± 1.70	28.5 ± 2.04	15.8 ± 1.01	0.015 ± 0.0016	0.027 ± 0.0018
c.v.	–	11.59	22.25	13.02	20.66	21.25	14.28

For each trait and species, coefficient of variation (c.v.) across populations is also given

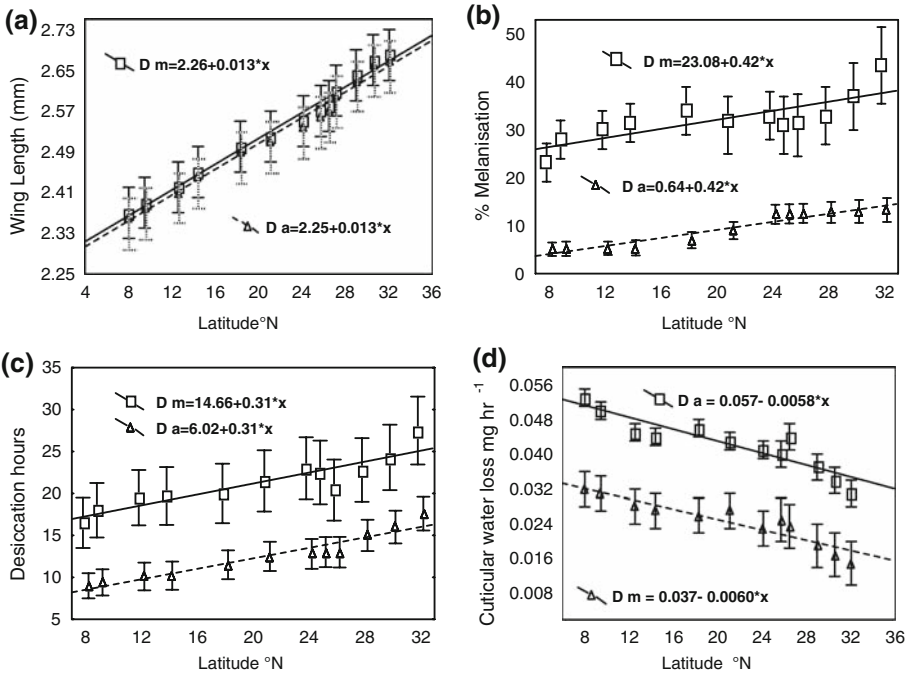

Fig. 1 Geographical variation in body size **a** % body melanisation; **b** desiccation resistance; **c** and cuticular water loss h^{-1}; **d** in twelve sympatric populations of *D. melanogaster* and *D. ananassae*. The *slope values* for each trait are quite similar across species but *intercept values* differ significantly

between cuticular water loss mg h^{-1} and latitude of origin. Thus, we obtained significant clines for various traits in both the species, differing in intercept values (Fig. 1a–d).

Further, for all the populations of both the species, data on three other characteristic measures of desiccation resistance (% hemolymph, % dehydration tolerance and cuticular lipids mg fly^{-1} cm^{-2}) are given in Table 2. For cuticular lipids, there are no geographical variations (i.e. lack of clinal variation) in both the species. However, for hemolymph and dehydration tolerance, there are much shallower but linear clines (slope for dehydration tolerance = 0.36; slope for hemolymph = 0.18) which are similar for both the species but intercept values differ significantly for each trait ($P < 0.001$). Such data suggest contrasting levels of species specific variations for desiccation related traits. However, total water content ($\sim$70%) did not vary among populations. Except body size and cuticular lipids per fly, we obtained significantly higher trait values for *D. melanogaster* as compared with *D. ananassae*.

Trait comparisons across species

A comparison of trait variability (body melanisation and desiccation resistance) in assorted darker and lighter flies of one southern (Kanyakumari) and one northern population (Dharamshala) are shown in Fig. 2. For each species, mean values for body melanisation vary twofold to threefold between dark and light flies. For *D. ananassae*, the southern flies are quite lighter in body melanisation (m $\pm$ SD: Dark = 7.6% $\pm$ 1.7; light = 2.2% $\pm$ 1.6) while northern flies demonstrate about twofold increase for darker flies and about fourfold

Table 2 Data on mean ± SE on three quantitative traits (% hemolymph, % dehydration tolerance and cuticular lipids per fly) in 12 latitudinal populations of *D. melanogaster* (Dm) and *D. ananassae* (Da) grown at 21°C

Sites	% Hemolymph		% Dehydration tolerance		Cuticular lipids mg fly^{-1} cm^{-2}	
	Dm	Da	Dm	Da	Dm	Da
Kanyakumari	14.01 ± 0.12	8.52 ± 0.08	40.1 ± 1.21	34.5 ± 0.52	0.0109 ± 0.004	0.0122 ± 0.005
Madurai	15.12 ± 0.10	8.57 ± 0.09	40.4 ± 1.25	34.6 ± 0.56	0.0108 ± 0.005	0.0123 ± 0.006
Bangalore	15.25 ± 0.15	8.65 ± 0.09	41.5 ± 1.35	35.2 ± 0.75	0.0107 ± 0.005	0.0121 ± 0.007
Anantpur	15.52 ± 0.25	8.43 ± 0.10	41.8 ± 1.25	35.6 ± 0.80	0.0108 ± 0.006	0.0121 ± 0.006
Pune	16.01 ± 0.45	8.85 ± 0.12	43.2 ± 1.45	38.5 ± 0.84	0.0109 ± 0.005	0.0121 ± 0.007
Nagpur	15.95 ± 0.50	8.72 ± 0.18	42.5 ± 1.50	37.5 ± 0.75	0.0108 ± 0.005	0.0121 ± 0.006
Bhopal	16.52 ± 0.45	9.52 ± 0.25	45.2 ± 1.58	39.2 ± 0.95	0.0109 ± 0.004	0.0123 ± 0.005
Jhansi	16.05 ± 0.52	9.21 ± 0.35	44.1 ± 1.85	38.5 ± 1.02	0.0107 ± 0.003	0.0122 ± 0.005
Gorakhpur	17.10 ± 0.65	9.45 ± 0.40	46.2 ± 1.95	40.1 ± 1.12	0.0106 ± 0.004	0.0121 ± 0.006
Rohtak	18.12 ± 0.70	10.48 ± 0.41	47.5 ± 2.01	40.5 ± 1.21	0.0105 ± 0.004	0.0122 ± 0.006
Kalka	19.52 ± 0.75	11.21 ± 0.35	48.3 ± 2.42	41.2 ± 1.48	0.0106 ± 0.006	0.0121 ± 0.005
Dharamshala	20.27 ± 0.85	12.25 ± 0.45	50.2 ± 2.52	42.1 ± 1.52	0.0108 ± 0.005	0.0122 ± 0.006
c.v.	9.70	14.82	7.30	7.09	1.22	0.64

For each trait and species, coefficient of variation (c.v.) across populations is also given

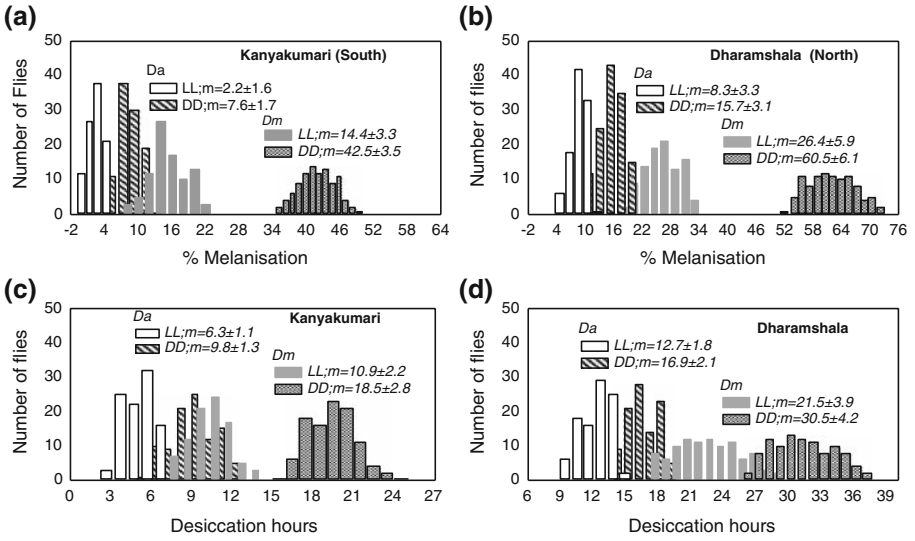

Fig. 2 Within population variations in % body melanisation (**a**–**b**) and desiccation resistance (**c**–**d**) in assorted light (LL) and dark (DD) flies of southern tropical (Kanyakumari) and northern subtropical (Dharamshala) populations of *D. melanogaster* and *D. ananassae*. *Scales* on the x-axis vary about twofold for both the traits. The criteria for assortment are given in the method section

for lighter flies (Fig. 2). By contrast, for *D. melanogaster*, the values for % melanisation are significantly higher i.e. trait values for assorted dark and light for southern population (Dark = 42.5% ± 3.5; light = 14.4% ± 3.3) and northern population (Dark = 60.5% ± 6.1; light = 26.4% ± 5.9) are significantly higher (fourfold to sixfold) when compared with corresponding values for *D. ananassae*. These contrasting patterns of body melanisation across species have been shown in Fig. 2a and b. We have observed similar levels of changes in desiccation resistance across species and populations (Fig. 2c, d). *D. ananassae* is a desiccation sensitive species both in south (Dark = 9.8 ± 1.3 h; light = 6.3 ± 1.1 h) as well as for north (Dark = 16.9 ± 2.1 h; light = 12.7 ± 1.8 h). However, *D. melanogaster* has shown higher desiccation resistance for southern as well as northern populations. The corresponding values for desiccation resistance in *D. melanogaster* are twofold higher as compared with *D. ananassae*. Thus, there are parallel changes in percent body melanisation and desiccation resistance for both the species.

Within population analysis

Data on six quantitative traits in assorted dark and light flies from six out of twelve populations of both the species are given in Table 3 and Figs. 3 and 4. For all the traits, values are significantly higher for *D. melanogaster* as compared with *D. ananassae*. Assorted groups of dark and light flies show regular linear clinal variation for all the traits except cuticular lipids per fly in both the species. For each trait, values are significantly lower for lighter flies as compared with darker flies i.e. statistical comparisons are significant on the basis of '*t*' test, $P < 0.01$ or $P < 0.001$ (Table 3). For assorted dark and light phenotypes, correlations of body melanisation with desiccation resistance($r = 0.89 \pm 0.05$; $P < 0.001$) or with cuticular water loss mg h^{-1} ($r = -0.90 \pm 0.03$; $P < 0.001$) are highly significant (Fig. 3). The correlations based on within population trait variability suggest correlated

Table 3 Statistical comparisons of mean ± (SD) for six ecophysiological traits in assorted dark and light morphs in each of six latitudinal populations of *D. melanogaster* and *D. ananassae*

Traits	Kanyakumari (8.05)	Bangalore (12.58)	Nagpur (21.09)	Jhansi (25.70)	Rohtak (28.94)	Dharamshala (32.00)
% Melanisation						
Dm (Dark)	42.5 ± 3.52	44.2 ± 3.27	46.3 ± 3.74	48.2 ± 4.59	55.3 ± 4.95	60.4 ± 5.92
(Light)	14.2 ± 3.26	16.5 ± 3.22	18.8 ± 4.01	20.5 ± 4.17	23.3 ± 4.52	26.3 ± 5.85
t-test	***	***	***	***	***	***
Da (Dark)	7.66 ± 1.55	8.02 ± 2.06	8.85 ± 2.11	9.62 ± 2.61	13.20 ± 2.76	15.65 ± 3.28
(Light)	2.22 ± 1.59	3.25 ± 1.77	4.75 ± 1.68	5.52 ± 2.39	7.12 ± 2.95	8.20 ± 3.05
t-test	***	***	***	***	***	***
Desiccation hours						
Dm (Dark)	19.5 ± 2.75	20.1 ± 3.10	20.5 ± 3.25	23.6 ± 3.85	25.2 ± 3.95	30.5 ± 4.21
(Light)	10.9 ± 2.20	12.2 ± 2.75	13.3 ± 2.72	15.2 ± 3.15	16.8 ± 3.58	21.5 ± 3.96
t-test	***	***	***	***	***	***
Da (Dark)	9.8 ± 1.25	11.3 ± 1.35	12.5 ± 1.65	13.5 ± 1.80	14.9 ± 1.95	15.8 ± 2.05
(Light)	6.3 ± 1.02	7.3 ± 1.12	8.4 ± 1.40	9.8 ± 1.52	10.8 ± 1.70	11.7 ± 1.85
t-test	***	**	***	***	***	***
Cuticular water loss (mg $h^{-1)}$						
Dm (Dark)	0.026 ± 0.0006	0.025 ± 0.0005	0.022 ± 0.0007	0.017 ± 0.0008	0.015 ± 0.0009	0.013 ± 0.0010
(Light)	0.039 ± 0.0002	0.037 ± 0.0003	0.035 ± 0.0005	0.031 ± 0.0006	0.029 ± 0.0006	0.025 ± 0.0007
t-test	***	***	***	***	***	***
Da (Dark)	0.045 ± 0.0002	0.043 ± 0.0002	0.039 ± 0.0003	0.035 ± 0.0003	0.032 ± 0.0003	0.030 ± 0.0003
(Light)	0.068 ± 0.0001	0.066 ± 0.0001	0.061 ± 0.0002	0.057 ± 0.0002	0.056 ± 0.0001	0.054 ± 0.0002
t-test	***	**	**	**	**	**
% Hymolymph						
Dm (Dark)	19.63 ± 1.95	20.35 ± 2.05	21.24 ± 2.12	21.82 ± 2.53	22.58 ± 2.95	23.12 ± 3.18
(Light)	11.52 ± 1.90	12.12 ± 2.01	12.52 ± 2.05	13.21 ± 2.50	13.69 ± 2.90	14.12 ± 3.28
t-test	***	***	***	***	***	***

Table 3 continued

Traits	Kanyakumari (8.05)	Bangalore (12.58)	Nagpur (21.09)	Jhansi (25.70)	Rohtak (28.94)	Dharamshala (32.00)
Da (Dark)	10.78 ± 1.28	10.58 ± 1.32	11.39 ± 1.49	12.01 ± 1.90	12.82 ± 1.97	13.16 ± 2.50
(Light)	7.15 ± 1.25	7.58 ± 1.28	8.05 ± 1.45	9.38 ± 1.85	9.49 ± 2.01	10.01 ± 2.52
t-test	*	*	*	*	*	*
% Dehydration tolerance						
Dm (Dark)	43.81 ± 1.85	44.42 ± 1.99	48.50 ± 2.09	49.21 ± 2.31	49.82 ± 2.42	51.82 ± 2.52
(Light)	37.52 ± 1.65	39.32 ± 1.70	42.15 ± 1.85	43.35 ± 1.93	44.40 ± 2.01	45.65 ± 2.12
t-test	***	***	***	***	***	***
Da (Dark)	35.25 ± 1.20	37.81 ± 1.25	40.12 ± 1.30	42.12 ± 1.35	43.31 ± 1.45	43.33 ± 1.52
(Light)	32.18 ± 1.12	34.50 ± 1.18	37.85 ± 1.22	39.07 ± 1.30	40.20 ± 1.35	40.02 ± 1.42
t-test	*	*	*	*	**	*
Cuticular lipids mg fly^{-1} cm^{-2}						
Dm (Dark)	0.0078 ± 0.0005	0.0077 ± 0.0005	0.0078 ± 0.0005	0.0080 ± 0.0004	0.0079 ± 0.0004	0.0077 ± 0.0005
(Light)	0.0158 ± 0.0006	0.0162 ± 0.0007	0.0157 ± 0.0006	0.0158 ± 0.0007	0.0157 ± 0.0007	0.0155 ± 0.0006
t-test	***	***	***	***	***	***
Da (Dark)	0.0121 ± 0.0002	0.0119 ± 0.0002	0.0122 ± 0.0003	0.0120 ± 0.0003	0.0128 ± 0.0004	0.0121 ± 0.0003
(Light)	0.0138 ± 0.0001	0.0139 ± 0.0002	0.0139 ± 0.0002	0.0138 ± 0.0002	0.0141 ± 0.0002	0.0141 ± 0.0001
t-test	*	*	*	*	*	*

All the assays were made at 21°C

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

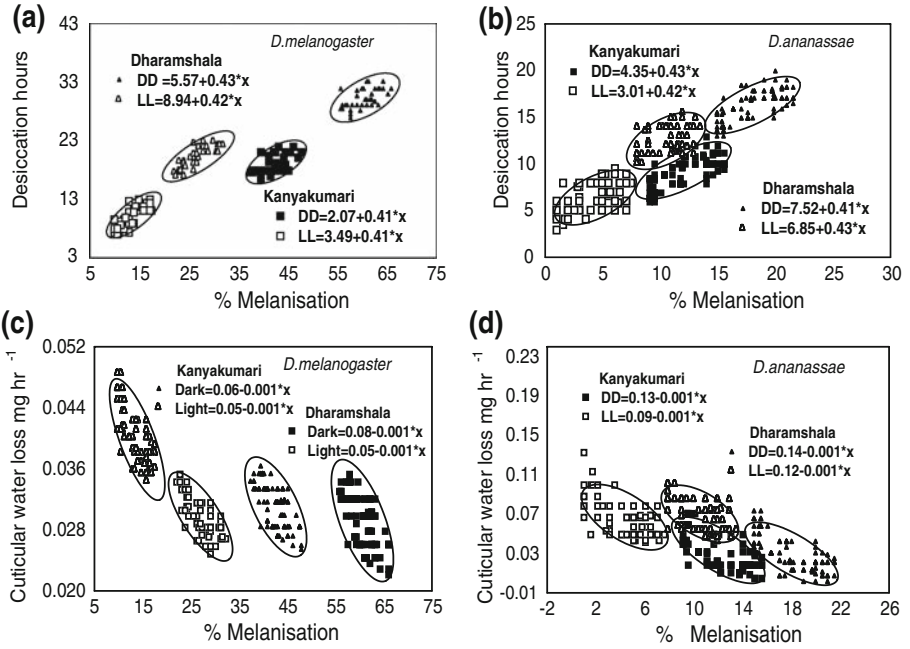

Fig. 3 Regression analysis of within population trait variability (desiccation resistance and cuticular water loss mg h^{-1}) as a function of body melanisation in assorted dark (DD) and light (LL) flies from northern (Dharamshala) and southern (Kanyakumari) populations of *D. melanogaster* (**a**–**b**) and *D. ananassae* (**c**–**d**). For the two traits, *x* and *y scale* are different for the two species. For all traits, correlations with % melanisation are highly significant ($r > 0.93 \pm 0.05$)

changes in these traits. The results on % dehydration tolerance, % hemolymph and cuticular lipids per fly in northern vs. southern populations are illustrated in Fig. 4. For all these traits, values differ significantly between assorted dark and light morphs in both the species. Such analysis was extended for correlations between body size variations and % body melanisation (Fig. 4d) as well as with desiccation resistance; and the results showed lack of correlations. Thus, body size changes which are quite similar across these two species are not correlated with body melanisation and desiccation stress.

Results of ANCOVA, with body size as a covariate have shown significant trait variability due to species and morphs (dark and light assorted flies) and their interactions for four ecophysiological traits (% body melanisation, desiccation resistance, cuticular water loss and hemolymph) and the data are shown in Table 4. For these quantitative traits, there are significant levels of genetic variability i.e. % variance due to species ($\sim$50%), morphs (22–29%) and their interactions (12–14%) are highly significant. Thus, these traits vary mainly due to species and morph specific differences. For % dehydration tolerance, trait variability results due to species (21%), population (36%), morph (18%) and interaction effects between species and morphs (11%). By contrast, cuticular lipids do not vary between species and populations while there is significant variance due to morphs (68%) and species $\times$ morph interaction (31%). Thus, the results of ANCOVA suggest species and morph specific differences in body melanisation might impact trait variability in desiccation related traits.

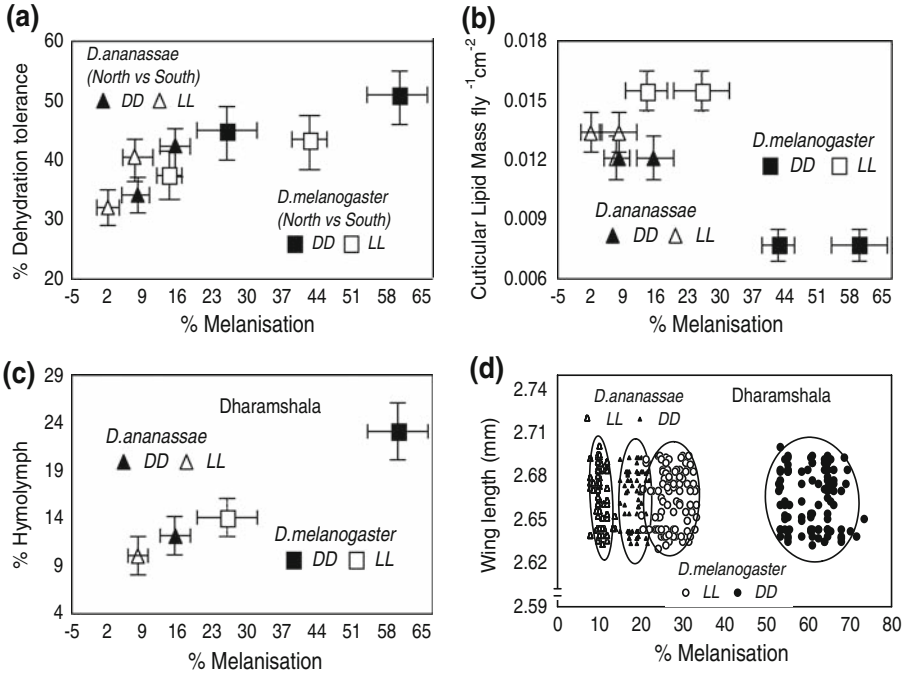

Fig. 4 Comparison of trait variability (mean ± SD) of dehydration tolerance (**a**), cuticular lipids (**b**) as a function of body melanisation in assorted dark (DD) and light (LL) northern vs. southern population of *D. melanogaster* and *D. ananassae*; hemolymph (**c**) and body size (**d**) data on a single northern population have been shown there is lack of correlation between body melanisation and body size (**d**)

Climatic data analysis

Variations in desiccation resistance across species are often assumed to be under direct or indirect selection pressure due to climatic conditions. The magnitudes of variations for body melanisation are consistent with levels of desiccation resistance in these *Drosophila* species. Thus, body melanisation could be the target of natural selection pressure due to environmental conditions of the origin of species populations. Multiple regression analysis of trait variability as a simultaneous function of variations along latitude and altitude of origin of populations are given in Table 5. There are clear cut differences in intercept value across species for different traits. Slope values due to latitude as well as altitude are significant for all the traits in both the species. Thus, the impact of climatic variables associated with latitude and altitude seem to be significant for all the ecophysiological traits. Data on multiple regression analysis with seasonal changes in temperature and RH (i.e. Tcv and RHcv) are given in Table 5 and illustrated for RHcv in Fig. 5a. The regression slope values are quite similar across species for a given trait (% melanisation, $b = 0.52 \pm 0.08$ or 0.55 ± 0.09; desiccation resistance, $b = 0.30 \pm 0.04$ or 0.33 ± 0.08; Fig. 5a) as a function of RHcv. Data on Tcv are given in Table 5 and the trends are quite similar.

Furthermore, regression analyses of % species distribution of wild populations with RHcv are shown in Fig. 5b. There are significant differences in the intercept as well as

Table 4 ANCOVA for explaining variability in six ecophysiological traits due to species, populations and assorted body color morphs and their interactions

	Species(1)	Population (2)	Morphs (3)	1 × 2	1 × 3	2 × 3	1 × 2 × 3	Error
df	1	5	1	5	1	5	5	2,376
% Melanisation								
MS	365,079***	6,391***	215,073***	631***	102,219***	331**	267**	11.0
% Var.	48.94	4.28	28.83	0.42	13.70	0.22	0.17	3.41
Desiccation hours								
MS	361,173***	6,024***	213,637***	1,207***	97,682***	134**	109**	4.01
% Var.	50.14	4.18	29.65	0.84	13.56	0.094	0.077	1.45
Cuticular water loss (mg $h^{-1)}$								
MS	1.058***	0.038***	0.56***	0.016***	0.248***	0.015***	0.0066**	0.00004
% Var.	45.38	8.28	24.07	3.43	10.64	3.24	1.41	3.43
% Hemolymph								
MS	32622.6***	452***	13300.1***	56.5***	7147.7***	215.5***	90.8**	1.6
% Var.	53.42	3.70	21.78	0.46	11.70	1.76	0.74	6.41
% Dehydration tolerance								
MS	8,166***	2,763***	6,993***	241***	4,193***	102***	49**	1.01
% Var.	21.35	36.13	18.29	3.14	10.96	1.33	0.64	8.12
Cuticular lipids mg fly^{-1} cm^{-2}								
MS	0.000003*	0.000001NS	0.01292***	0.000001NS	0.00596***	0.000001NS	0.000001NS	0.000001
% Var.	0.015	0.00052	67.55	0.00052	31.11	0.00052	0.00052	1.27

NS non significant

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Table 5 Multiple regression analysis of trait variability as a simultaneous function of latitude and altitude and with climatic variable Tcv and RHcv of the origin of populations of *D. melanogaster* and *D. ananassae*

Traits	$a \pm$ SE	Latitude		Altitude		R^2
		r_1	$b_1 \pm$ SE	r_2	$b_2 \pm$ SE	
D. melanogaster						
% Melanisation	22.09 ± 1.50	0.60 ± 0.13***	0.33 ± 0.07**	0.54 ± 0.13**	0.007 ± 0.001***	0.87
Desiccation hours	14.19 ± 0.62	0.78 ± 0.08***	0.26 ± 0.027**	0.40 ± 0.08**	0.0031 ± 0.0006***	0.92
Cuticular water loss mg h^{-1}	0.037 ± 0.001	−0.85 ± 0.08***	−0.00051 ± 0.00005***	−0.30 ± 0.08**	−0.000004 ± 0.000001***	0.87
D. ananassae						
% Melanisation	1.94 ± 0.63	0.88 ± 0.08***	0.31 ± 0.029***	0.20 ± 0.08**	0.0015 ± 0.0006***	0.94
Desiccation hours	5.96 ± 0.84	0.75 ± 0.11***	0.23 ± 0.037***	0.37 ± 0.12**	0.0028 ± 0.0008***	0.86
Cuticular water loss mg h^{-1}	0.057 ± 0.001	−0.81 ± 0.09***	−0.0006 ± 0.00006***	−0.34 ± 0.09**	−0.000006 ± 0.000002***	0.90
Traits	$a \pm$ SE	Tcv		RHcv		R^2
		r_1	$b_1 \pm$ SE	r_2	$b_2 \pm$ SE	
D. melanogaster						
% Melanisation	20.54 ± 1.45	0.41 ± 0.15***	0.22 ± 0.08***	0.59 ± 0.15**	0.43 ± 0.11***	0.93
Desiccation hours	14.40 ± 0.82	0.49 ± 0.15***	0.21 ± 0.062***	0.52 ± 0.15**	0.14 ± 0.042***	0.85
Cuticular water loss mg h^{-1}	0.35 ± 0.015	−0.61 ± 0.16***	0.00030 ± 0.00008***	−0.38 ± 0.16	0.00028 ± 0.0001***	0.90
D. ananassae						
% Melanisation	2.89 ± 1.01	0.48 ± 0.21***	0.13 ± 0.05***	0.49 ± 0.21**	0.19 ± 0.08***	0.87
Desiccation hours	6.20 ± 1.07	0.46 ± 0.20***	0.17 ± 0.077***	0.50 ± 0.20**	0.13 ± 0.05***	0.84
Cuticular water loss mg h^{-1}	0.056 ± 0.001	−0.60 ± 0.16***	−0.0004 ± 0.0001**	−0.39 ± 0.16**	−0.0035 ± 0.0001***	0.94

** $P < 0.01$; *** $P < 0.001$

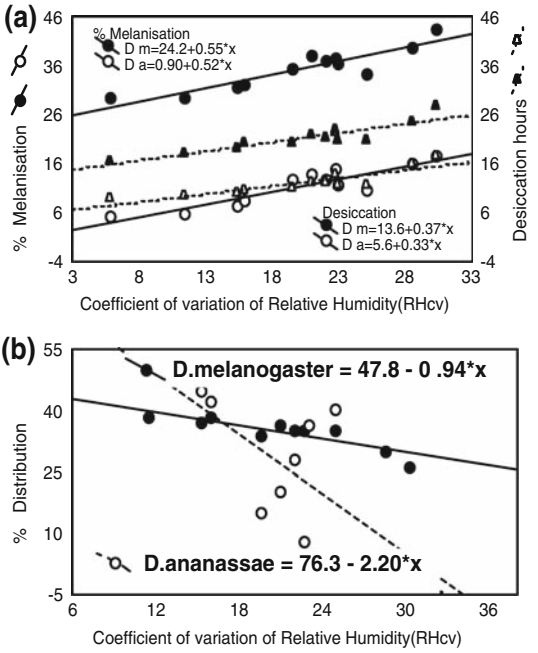

Fig. 5 Regression analysis of changes in % melanisation and desiccation resistance (**a**); % species distribution (**b**); as a function of RHcv of site of origin of populations

slope values (for *D. melanogaster* intercept = 47.8%, slope = −0.94; and for *D. ananassae*, intercept = 76.3%, slope = −2.20). Such data suggest that significant reduction in % distribution of *D. ananassae* along latitude matches with its desiccation sensitivity. However, for *D. melanogaster*, the changes in % distribution are significantly lower which correspond with its high desiccation resistance level.

Discussion

Indian subcontinent (8–32°N) provides localities with diverse climatic conditions which favor adaptations of specific phenotypic variations. However, unlike temperature, relative humidity shows no consistent patterns along latitude on different continents. Seasonal changes in India provide a regular but changing climatic pattern according to latitude. By contrast, such a climatic pattern does not occur on the Australian continent. Thus, we may expect different evolutionary responses to climatic selection for various quantitative traits on different continents. For desiccation resistance, clinal variations along altitude or latitude have been reported in various *Drosophila* species from the Indian subcontinent (Parkash and Munjal 1999; Parkash et al. 2008a, b, c, d) while there are no geographical variations for this trait in many *Drosophila* species on the Australian continent (Hoffmann and Weeks 2007; Arthur et al. 2008). However, these studies have not considered the mechanistic basis of desiccation resistance in natural populations. In the present studies, we have shown that changes in body melanisation can explain changes in desiccation resistance levels while there is no role of cuticular lipids and body size for these two species.

Adaptive mechanisms for desiccation resistance have considered reduction in cuticular permeability due to changes in the quantity and physical properties of cuticular lipids

(Hadley 1994; Gibbs 2002). Several studies have shown that changes in amount of cuticular lipids across species are associated with desiccation resistance (Hadley 1994). However, only two studies have shown geographical variations in the amount of cuticular lipids per fly i.e. altitudinal populations of grasshopper *Melanoplus sanguinipus* from California (Rourke 2000) and latitudinal populations of *Zaprionus indianus* (Parkash et al. 2008c). For other insect taxa, there are no data on changes in the amount of cuticular lipids in geographical populations. For two *Sophophoran* species (*D. melanogaster* and *D. ananassae*), we did not find variations in cuticular lipids across geographical populations. Thus, lack of changes in the amount of cuticular lipids per fly can not account for contrasting levels of desiccation resistance in both the species along latitude. However, cuticular hydrocarbons are a chemically heterogeneous class. It is possible that subsets of cuticular hydrocarbon are important for desiccation resistance while other cuticular hydrocarbons may function primarily as pheromones and these aspects needs future investigations.

The magnitude of desiccation resistance varies across different *Drosophila* species (Gibbs and Matzkin 2001). Varying resistance levels among species are often linked with their distribution patterns but the target of natural selection is not clear. Unlike desiccation resistance, there are no reports on laboratory selection of body melanisation except one study which has shown differences in pathogen resistance among dark and light strains of *D. falleni* (Dombeck and Jaenike 2004). Laboratory selected dark and light strains in *D. melanogaster* might differ significantly in their desiccation resistance level but this aspect has not been analyzed so far. However, a positive correlation between body melanisation and desiccation resistance is evident from the analysis of yellow body color mutant strain of *D. melanogaster* (Kalmus 1941); and on the basis of assorted darker and lighter flies in populations of different *Drosophila* species (Rajpurohit et al. 2008). In *D. melanogaster*, we found significant differences in desiccation resistance levels between assorted dark and light flies from a given population (Parkash et al. 2008a, d). Present study has suggested that varying levels of body melanisation affect cuticular permeability and reduce cuticular water loss. In *D. ananassae*, quite lower body melanisation levels are linked with reduced levels of desiccation resistance while opposite is the case for *D. melanogaster*. Significant differences occur for cuticular water loss, dehydration tolerance and hemolymph in assorted darker and lighter flies from each population. Such data have supported the role of body melanisation in conferring desiccation resistance in these species.

Positive correlations between desiccation resistance and body size are evident for tenebrionid beetles, scorpions and grasshoppers etc. (Zachariassen 1988; Edney 1977; Hadley 1994; Addo-Bediako et al. 2001); and for desert *Drosophila* species (Gibbs and Matzkin 2001). However, a comparative analysis of three mycophagous *Drosophila* species showed that the smallest species (*D. putrida*) was the most desiccation resistant as compared with *D. falleni* and *D. tripunctata* (Worthen and Haney 2002). In the present studies, body size changes are not correlated with desiccation resistance. In these species, body size is quite similar but there are contrasting levels of desiccation resistance. Thus, on the basis of within population analysis, body size changes can not account for desiccation resistance in these two species.

A comparison of geographical variations for body melanisation, desiccation resistance and cuticular water loss per fly in *D. melanogaster* as well as in *D. ananassae* suggests non overlapping species specific clinal variation. The most resistant northern populations of *D. ananassae* are comparable to the least resistant southern populations of *D. melanogaster* for all the desiccation related traits as well as body melanisation. Thus, the baseline

resistance levels are significantly different across these two species. For sympatric populations of these species, selection pressures due to climatic conditions might differ if they vary in their behavioral evasion of desiccation stress under wild conditions. This is possible because both the species are linked with domestic habitats which may offer protection from desiccation stress. In this respect, these species may encounter differential exposure to climatic stress conditions resulting in different slope values for desiccation resistance across species. Alternatively, if there are direct selection pressures on body melanisation which differ significantly across species, we may expect similar response to selection (similar slope values) but with significant differences in intercept or baseline resistance level which correspond to equatorial regions.

Ectothermic insect taxa may be thermal generalists, or high or low temperature thermal specialists. Sensitivity to temperature and humidity has the potential to influence the ecology, behavior and adaptive fitness of different insect species. Thus, the extent to which ectotherms can tolerate changes in their ambient thermal environments can be critical in determining their distribution and abundance. On the Indian subcontinent, southern tropical habitats and northern subtropical regions have impacted distribution patterns of *D. melanogaster* and *D. ananassae*. However, it is not known which ecophysiological traits are influenced by varying climatic conditions. The contrasting levels of body melanisation correspond with species specific desiccation resistance i.e. body melanisation could be the target of climatic selection. For *D. ananassae*, inability to colonize high latitudes as well as altitudes may be limited by its quite lower levels of body melanisation. Thus, for these two *Drosophila* species, variability for body melanisation may be a limiting factor for ecological success under variable climatic conditions. This is supported by distribution of *D. ananassae* along latitude which has shown a steeper negative slope ($b = -2.20 \pm 0.21$) as compared with *D. melanogaster* ($b = -0.94 \pm 0.11$).

In conclusion, present studies have shown that changes in desiccation resistance and cuticular water loss are a consequence of changes in species specific magnitude of body melanisation as well as its plasticity potential under varying climatic conditions along latitude on the Indian subcontinent. Low levels of body melanisation in *D. ananassae* are associated with its lower desiccation resistance. *D. ananassae* occurs abundantly in tropical habitats reflecting high humidity conditions in such habitats and its distribution declines along increasing latitude. On the contrary, *D. melanogaster* with high levels of body melanisation can confer cuticular impermeability for water loss under drier climatic conditions and thus, occurs as a widespread species under tropical and subtropical habitats all along the Indian subcontinent. We may suggest that variable levels of body melanisation across *Drosophila* species can impact their desiccation resistance level.

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