Divergent strategies for stress adaptations in *D. punjabiensis* in context of global warming.

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Abstract

In subtropical parts of the Indian subcontinent, autumn is a cold and dry season, while spring is wet and humid, and ectothermic *drosophilids* are expected to evolve desiccation resistance to cope with drier climatic conditions. Previous studies have described that in tropical populations of *D. punjabiensis*, body color polymorphism is maintained through humidity changes as opposed to thermal melanism, and seasonal changes in the frequency of body color morphs in this tropical species supports the melanism-desiccation hypothesis. But the mechanistic bases of such climatic adaptations in two body color morphs of *D. punjabiensis* are largely unknown. We tested the hypothesis that divergence in the physiological basis of desiccation-related traits is consistent with body color morph-specific adaptations to climatic conditions, for which we examined the response of water balance to relative humidity (RH), temperature, and their interaction in *D. punjabiensis*, using two body color morphs that had been allowed to rear at low or high humidity at 17°C and 25°C. We found, at low RH, dark body color morph had significantly greater trait values than light body color morph at both the temperatures. A comparative analysis of water budget of the two body color morphs showed that higher water content, reduced rate of water loss, and greater dehydration tolerance confer higher desiccation resistance in dark morph of *D. punjabiensis* at low RH. We found that carbohydrates act as metabolic fuel during desiccation stress in both the morphs, but a higher level of stored carbohydrates was evident in dark morph at low humidity. Further, total energy budget differs significantly between these two body color morphs at two humidities. Thus, body color morph-specific divergence in water-balance-related traits in *D. punjabiensis* is consistent with their adaptations to wet and dry habitats.

Introduction

Seasonal variations in quantitative traits are generally assessed by differences in thermal conditions (Kingsolver and Wiernasz, 1991; Holloway *et al*., 1997; Lee and Denlinger, 1991; Loeschcke *et al.*, 1994; Addo-Bediako *et al*., 2000; Hoffmann *et al.*, 2003; Overgaard and Sorensen, 2008), but evolutionary responses due to other environmental factors have received lesser attention (Tauber *et al*., 1998; Chown and Nicolson, 2004; Danks, 2007; Brakefield *et al*., 2007). Tropics experience relatively consistent temperatures, but there are significant seasonal variations in precipitation. Seasonal variations have been reported for body size in different *Drosophila* species (Tantawy, 1965; Kari and Huey, 2000) and for desiccation resistance in a single population of *D. simulans* (Mckenzie and Parsons, 1974). In tropical species of butterflies, wet season forms (wsf) are lighter, while dry season forms (dsf) are darker and such differences correspond with local climatic adaptations (Brakefield and Reitsma, 1991; Roskam and Brakefield, 1999). However, there are limited data on seasonal adaptations in different insect species (Majerus, 1990; Osawa and Nishida, 1992; Aldridge *et al*., 1993; Kari and Huey, 2000; Torres and Madi-Ravazzi, 2006).

Heritable changes in body melanisation with possible fitness consequences constitute a suitable model for phenotypic evolution in different insect species (Majerus, 1998; Hollocher *et al*., 2000a, b; Llopart *et al*., 2002; True, 2003; Wittkopp *et al*., 2003; Dombeck and Jaenike, 2004). In different insect taxa, there are diverse patterns of body melanisation, *i.e*. (a) several black species of Collembola occur in temperate regions, *i.e*., Pyrenees, Swiss Alps, and Himalayas (Mani, 1968; Rapoport, 1969); (b) in *D. melanogaster*, a cosmopolitan species, the extent of melanism varies with geographical location (Pool and Aquadro, 2007; Parkash *et al*., 2008a, b); (c) discrete melanic and non-melanic morphs occur as genetic polymorphism in species of montium species subgroup (Ohnishi and Watanabe, 1985). Such diversity of melanisation patterns might correspond with contrasting changes in thermal and/or humidity conditions in the temperate *vs*. tropical regions. In temperate localities, melanics can warm up faster and become active as compared with non-melanics (Watt, 1968; Brakefield and Willmer, 1985; Berry and Willmer, 1986; True, 2003). Thus, the increase in the frequency of melanic morph in temperate regions is in agreement with thermal melanism hypothesis.

Besides thermal effects, body melanisation can control water balance in ectothermic insect species (Rajpurohit *et al*., 2008). The role of body melanisation in conferring desiccation resistance has been shown in a generalist species – *D. melanogaster* (Parkash *et al*., 2008b), in a cold-adapted species – *D. immigrans* (Parkash *et al*., 2008c), and a tropical species – *D. polymorpha* (Brisson *et al*., 2005). These studies have shown that assorted darker and lighter flies from a given population differ in their desiccation resistance level and show differential rates of cuticular water loss, which are negatively correlated with body melanisation (Parkash *et al*., 2008a, b, c). In order to test which of the climatic variables could be a selective agent in the tropics, we may raise the following hypothesis: (a) changes in phenotypic frequencies of dark and light morphs may occur through thermal effects and/or humidity changes; (b) humidity changes (dry *vs*. wet) may impact water balance for different body color morphs; (c) if body melanisation confers desiccation resistance, the dark morph is expected to prevail under dry seasons, while the reverse may occur for light morph under humid conditions.

Seasonal climatic changes might be mild or harsh in different geographical regions. This is supported by significantly steeper clines for desiccation resistance in various *Drosophila* species on the Indian subcontinent (Parkash and Munjal, 1999; Parkash *et al*., 2005), but a lack of clinal variation on the Australian continent (Hoffmann and Harshman, 1999; Hoffmann and Weeks, 2007). Subtropical countries usually receive significant rainfall during only some part of the year leading to substantial variation in the level of humidity in many parts of the tropics. So the variation in temperature and humidity has profound effect on the properties of water balance. Therefore, water balance in tropical climate needs to be given special attention to incorporate the variation in its properties.

D. punjabiensis belongs to the montium species subgroup of *melanogaster* group and has been reported to be widely spread in India as well as south-east Asian regions (Parshad and Paika, 1964). This species exhibits color dimorphism for the last two abdominal segments in females, and light morph is dominant over dark morph (Ohnishi and Watanabe, 1985; Parkash *et al.*, 2009). On the Indian subcontinent, geographical populations of *Drosophila* species of the subgenus Sophophora and mesic have been investigated for water conservation (Parkash *et al.*, 2008a, b, 2010, 2012), but there are no studies on montium species of subgenus *Drosophila* despite their abundance on the Indian subcontinent.

It would be interesting to find the significance of light and dark morph in context or varying climatic condition in India. Seasonally varying environments impose strong selection and can cause rapid phenotypic changes in quantitative traits (Shaipro, 1976; Tauber, 1981), but there is a dearth of

field data in *D. punjabiensis*, which is widely spread in the Indian subcontinent.

Water availability is an important determinant of the range position of many species and is also a significant correlate of species richness in many areas. Several comparative studies of water balance in *Drosophila* have been performed (Hoffmann and Harshman, 1999); unfortunately, *D. punjabiensis* and its body color morphs are not yet explored in the Indian subcontinent. Water can be lost through the spiracles during respiration, by transpiration through the cuticle, or by excretion from the mouthparts or feces. Reductions in any or all of these routes could be responsible for lower overall water-loss rates. Excretory water loss accounts for a small fraction, no more than 6%, of total losses in either laboratory or natural populations (Gibbs *et al.*, 1997). This leaves cuticular component water losses as the main sites of water conservation. This indicates towards the vital role of cuticular lipid in terms of water balance related traits. It would be amusing to relate phenotypic plasticity with energy metabolite and how it contributes to the spread of *D. punjabiensis* in extensive space.

We find *D. punjabiensis* behavior very engrossing and one of the untouched species of the Indian subcontinent. Thus in this study we will strive to find solutions to the following questions. (i) Whether seasonal variation in frequency of body color morphs results due to thermal and/or humidity changes? (ii) Whether body color polymorphism in *D. punjabiensis* is ecologically relevant in tropics? (iii) Whether there is variation in water conservation strategy of two body color morphs and, if any, which morph performs better in which conditions? (iv) Does melanisation derive the morphological traits (water balance) on dominance of light morph over dark commands irrespective of the climate and environmental conditions?

Materials and Methods

Collections and cultures

Wild individuals of *D. punjabiensis* (n = 100–120) were collected from six latitudinally (11°02', 12°59', 17°27', 27°09', 28°35', 31°06') varying localities from tropical and subtropical parts of the Indian subcontinent. The collections were made in spring (July–August) and in autumn (October–November) seasons with net sweeping and bait traps from fruit markets and godowns (Table 1). Wild-caught females were used to initiate isofemale lines (20 lines per population). All cultures were maintained at low density (60–70 eggs per vial of 40 mm × 100 mm size) on cornmeal-yeast-agar medium at 17°C-40% RH, 17°C-80% RH and 25°C-40% RH, 25°C-80% RH. All experiments were performed with G_6 and G_7 generations on six days old mated female flies. Climatic data for thermal variables of origin of populations were obtained from Indian Institute of Tropical Meteorology (IITM; www.tropmet.res.in), but data on relative humidity were obtained from 'Climatological Tables' published by the Indian Meteorological Department, Govt. of India, New Delhi.

Trait analysis

We used 10 individuals of each replicate (10 replicates × 20 isofemale line each) of *D. jambulina* to quantify body melanisation, epicuticular lipid mass, desiccation resistance, multiple measures of water balance, and levels of energy metabolites. For flies grown at low and high humidity each of low and high temperature, we tested desiccation related traits at their respectively growth conditions, *i.e.*, 17°C-40% RH, 17°C-80% RH and 25°C-40% RH, 25°C-80% RH. Therefore, growth condition and experimental conditions were the same in our experimental setup. For analysis we use the mean of ten replicates for each isofemale line of *D. punjabiensis.*

Table 1. Geographical and seasonal climatic data of the sites of origin of *Drosophila punjabiensis* populations.

Populations	Lat.(°N)	Spring season				Autumn season			
		T_{min}(°C)	T_{max}(°C)	T_{ave}(°C)	RH(%)	T_{min}(°C)	T_{max}(°C)	T_{ave}(°C)	RH(%)
Simla	31º06'	14.8	21	17.9	85	10.4	17.2	13.8	42
Dehi	28º35'	26.8	34	30.97	58.16	20	30	24.88	21.54
Agra	27º09'	22.6	31.4	27	79	15.8	30.6	23.2	60
Hyderabad	17º27'	22.6	31.4	27	70	19.7	30	24.8	62
Banglore	12º59'	19.3	28.2	23.7	79	18.4	27.6	23	75
Coinbatore	11º02'	21.8	31.5	26.6	77	21.4	31.1	26.2	74

For each season, T_{min}, mean monthly minimum temperature; T_{max}, mean monthly maximum temperature; T_{ave}, mean monthly average temperature; RH (%), relative humidity

Analysis of body melanisation

Body melanisation of individual female flies was visually scored with an Olympus stereo-zoom microscope SZ-61 (www.olympus.com) from the dorsal and lateral views of the female abdomen, giving values ranging from 0 (no melanisation) to 10 (complete melanisation) for six abdominal segments. Further, the relative size of each abdominal segment was calculated in proportion to the largest (fourth) abdominal segment, which was assigned a value of 1.0. Because the abdominal segments differ in size, these relative sizes (*i.e*., 0.86, 0.78, 0.92) were multiplied with segment-wise melanisation scores. Data on percent melanisation were calculated as (Σ observed weighted melanisation scores of abdominal segments per fly/Σ relative size of each abdominal segment × 10 per fly) × 100 (parkash *et al.*, 2008a).

Desiccation and starvation resistance

Desiccation resistance was measured as the function of time to lethal dehydration (LT 100) under dry air. Ten batches of 10 female individual were isolated in individual dry plastic vials (40 mm × 100 mm) with 2 g silica gel at the bottom and were covered with a foam disc. The vials were then placed in a respective growth condition (humidity chambers). The numerical value for desiccation and starvation is obtained by the ratio of water left after death to the difference of initial weight to weight of dried fly after dehydration protocol. A fly is announced dead when it does not show any motion. We pooled data on isofemale lines for survival curve analysis. Similarly, starvation stress was given in the same set up except silica gel is replaced with cotton ball dipped in 2 ml of distilled water to provide moisture.

Basic measures of water balance

To estimate total body water content and dehydration tolerance (%), 10 flies of each isofemale line were used. First, individual flies were weighed on Sartorius microbalance (model CPA26P, 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 drying at 60°C. Further, after mild anesthesia (1 min) with solvent ether, flies were weighed on a Sartorius microbalance both before and after desiccation stress until death.

Dehydration tolerance was estimated as the percentage of total body water lost until death due to desiccation and was calculated by the formula: (wet body mass – body mass at death) / (wet body mass – dry body mass) × 100 (Gibbs *et al.*, 1997). For calculation of the rate of water loss in *D. punjabiensis*, we followed the method of Wharton (1985), modified by Benoit *et al.* (2005) and

Yoder *et al.* (2009). Total body water content (*m*) was calculated as the difference between wet or fresh (*f*) and dry mass (*d*), *i.e.*, $m = f - d$. Individual flies were weighed and placed at ave (average percent relative humidity/100) for a specified time at 1 h intervals (1 to 8 h) and reweighed. The rate of water loss was derived from the slope of regression line on a plot of $\ln(m_t/m_0)$ against time according to Wharton's exponential equation (1985) $m_t = m_0 e^{-kt}$, where m_t is the water lost at time *t*, and m_0 is the initial water content. Rate (k_t) is the slope of the regression line and is expressed as % per hour. To verify comparison between control and organic solvent treated flies, students *t* test is applied, which gives significant difference between them.

Assessment of extractable hemolymph content

Individual flies were carefully pinned to a microdissection dish at its anterior and posterior ends with microdissection pins, and a narrow incision was made through the cuticle with a third pin while visually observing through a stereo-zoom microscope. The leaking extractable hemolymph was absorbed with an absorbent tissue moistened with an isotonic saline solution (Folk *et al.*, 2001). Extractable hemolymph content was estimated as reduction in mass following hemolymph blotting (Cohen *et al.*, 1986; Hadley, 1994). Further, tissue water was estimated after subtracting exsanguinated mass before and after drying. From the same data, we also calculated hemolymph water content by subtracting tissue water from total body water content.

Lipid content

Individual 8 day old mated female flies were dried in 2 ml Eppendorf tubes (www.tarson.in) at 60°C for 48 h and then weighed on a Sartorius microbalance (model CPA26P, 0.001 mg precision). Thereafter, 1.5 ml di-ethyl ether was added in each eppendorf tube and kept for 24 h under continuous shaking (200 r/min.) at 37°C. Finally, the solvent was removed and flies were again dried at 60°C for 24 h and reweighed. Lipid content was calculated per individual fly by subtracting the lipid free dry mass from initial dry mass per fly (Hoffmann *et al.*, 2001).

Assay sensitivity for cuticular lipids and total body lipids

We tested the assay sensitivity by measuring cuticular lipids as well as total body lipids in the replicate samples. We followed 1 h treatment with hexane without shaking for cuticular lipids. However, for total body lipids, we first removed cuticular lipids followed by 24 h treatment with di-ethyl ether with continuous shaking at 200 r/min. We did not find a difference in the estimates of either cuticular lipids or total body lipids under our assay conditions.

Trehalose and glycogen estimation

For trehalose and glycogen content estimation, 10 flies of each isofemale line were homogenized in a homogenizer (Labsonic M, www.sartorius.com) with 300 µL Na_2CO_3 and incubated at 95°C for 2 h to denature proteins. An aqueous solution of 150 µL acetic acid (1 mol/L) and 600 µL sodium acetate (0.2 mol/L) was mixed with the homogenate. The homogenate was subsequently centrifuged (Fresco 21, ThermoFisher Scientific, USA) at 12,000 r/min for 10 min. This homogenate was used for independent estimations of trehalose and glycogen as given below.

For trehalose estimation, aliquots (200 µL) were placed in two different tubes; one was taken as a blank whereas the other was digested with trehalase at 37°C using the Megazyme trehalose assay kit (K-Treh 10/10, www.megazyme.com). In this assay, released D-glucose was phosphorylated by hexokinase and ATP to glucose-6- phosphate and ADP, which was further coupled with glucose-6-phosphate dehydrogenase and resulted in the reduction of nicotinamide adenine dinucleotide (NAD). The absorbance by NADH was measured at 340 nm (UV-2450-VIS, USA). The pre-existing glucose level in the sample was determined in a control reaction lacking trehalase and subtracted from total

glucose concentration. For estimation of glycogen, a quantity of 50 µL aliquots were incubated with 500 µL *Aspergillus niger* glucoamylase solution (8.7 U/mL in 200 mmol/L of acetate buffer) for 2 h at 40°C with constant agitation, and the suspension was centrifuged at 4000 r/min for 5 min. It mainly hydrolyzed alpha-(1,4) and alpha-(1,6) glycosyl linkages and was suited for breakdown of glycogen. Glucose concentration was determined with 20 µL of supernatant from the suspension and added to 170 µL of a mixture of G6-DPH (0.9 U/mL), ATP (1.6 mmol/L), and NADP (1.25 mmol/L) in triethanolamine hydrochloride buffer (380 mmol/L TEA–HCl and 5.5 mmol/L of MgSO4) and 10 µL of hexokinase solution (32.5 U/mL in 3.2 mol/L ammonium sulphate buffer), and absorbance was measured at 340 nm.

Protein assay

Protein levels were determined using the bicinchoninic acid (BCA) method as followed by Marron and coworkers (Marron *et al.*, 2003). For the protein assay, 10 mated female flies per isofemale line were homogenized in 3 mL distilled water and centrifuged at 10,000 r/min for 5 minutes. Further, a 50 µL aliquot was taken from the supernatant and treated with 2 mL BCA reagent (Sigma-Aldrich,) and incubated at 25°C for 12 h. Absorbance was recorded at 562 nm, and protein concentration was determined by comparison with a standard curve.

Energy metabolites

We measured each metabolite (carbohydrates, lipids, or proteins) in 10 replicate sets of 20 isofemale lines. Total energy budget was calculated using standard conversion factors following Schmidt- Nielsen (Schmidt-Nielsen, 1990).

Statistical analysis

For each trait, population means (20 isofemale lines, 10 replicates each) are presented ± s.e.m. Effects of morph on desiccation-related traits, energy metabolites, body weight, basic measures of water balance, and dehydration and starvation effect were compared with mixed model ANOVA (morph and humidity: fixed effect; temperature: random effect) in *D. punjabiensis*. Effects of different organic solvents on rate of water loss was compared with student's *t* test (Zar, 1999). Total energy budget in *D. punjabiensis* due to differential storage of energy metabolites was calculated using standard conversion factors (Schmidt-Nielsen, 1990; Marron *et al.*, 2003). For multiple comparison, we adjusted the alpha value at 0.05 significance level; asterisks denote a significant difference (*$P < 0.1$; **$P < 0.01$; ***$P < 0.001$). Statistica (release 5.0, Statsoft Inc., Tulsa, OK, USA) was used for calculations as well as figures.

Results

Humidity of the sites selected for collection varies according to the seasonal change. Autumn is characterized by more dry conditions and spring describes more humid conditions. Such variation in the field condition facilitates governance of light and dark body color morph. As shown in Figure 1A, dark body color flies more profoundly appeared in autumn, and the reverse happens in spring with more light body colored flies captured while collecting.

Effect of seasonal changes in humidity on body color polymorphism of *D. punjabiensis*

Data on percent abundance of wild caught flies of *D. punjabiensis* from 6 different sites as a function of relative humidity of origin of population are shown in Figure 1. *Drosophila punjabiensis* dark body color morph is more abundant in autumn season, whereas the lighter morph of *D.*

punjabiensis is more abundant in rainy/spring season of the same site (Figure 1A). The autumn season of India is characterized by cold and dry conditions (dark: $y = 61.60 + 0.26x$; and light: $y = 38.40 - 0.26x$; Figure 1B), while the monsoon put forth comparatively hot and humid climatic conditions (dark: $y = 72.21 - 0.54x$; and light: $y = 27.78 + 0.54x$; Figure 1C). Therefore, significant reduction in T_{ave} as well as relative humidity along an elevation gradient may act as selection factors for abundance of dark morph. Thus, darker morph of *D. punjabiensis* is better adapted to cold and dry climatic condition as compared to lighter morph.

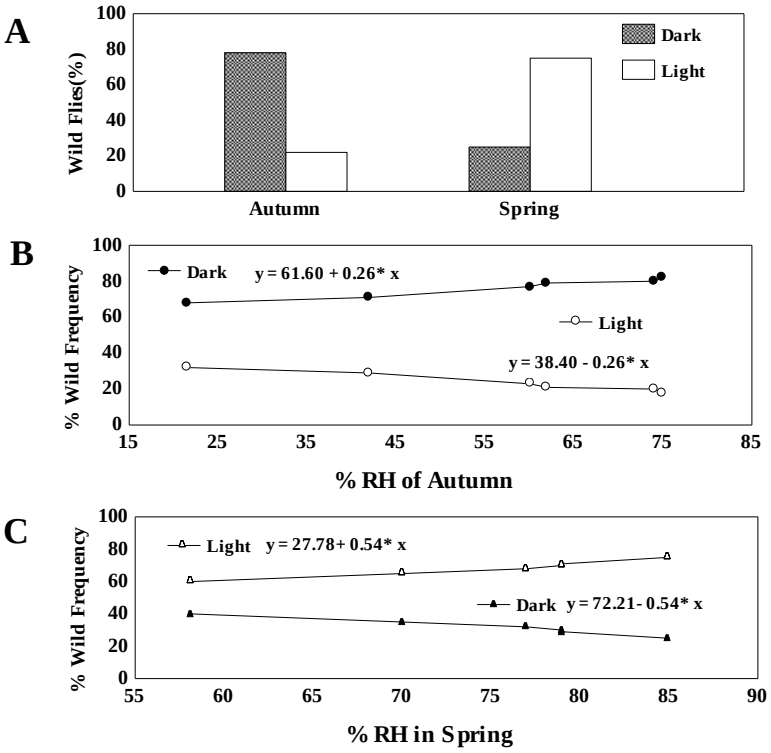

Figure 1. Field data on seasonal changes in the % frequency of dark and light morphs in (A) autumn & rainy population; comparison of light and dark morph frequency in humidity of the six sites selected across the latitude (B & C) in autumn and rainy season, respectively, of *D. punjabiensis*. The number of wild-caught individuals was 200–250 for each season as well as locality.

Body melanisation

We observed melanisation in relation with the humidity condition on the growth temperature (Figure 2). It was startling to find constant melanisation (74 ± 1.23: mean ± S.E) score of dark morph at both the humidity condition of low temperature (17°C) (Figure 2E), but decreases at high humidity (80% RH) of high temperature (25°C), *i.e.*, 60 ± 1.45 in comparison to low humidity (40% RH) of high temperature (25°C), which is 62.4 ± 1.53 (Figure 2F), whereas melanisation scores of light morph is approximately equal irrespective of the humidity and growth temperature. Melanisation at low and high humidity at both temperatures 17°C and 25°C is ~32% (Figure 2E, F).

Desiccation and starvation resistance

We found significant differences in desiccation survival hours of dark morph of *D. punjabiensis* at both the temperatures of low humidity (40% RH), ($F = 10.28$, $P < 0.001$); Figure 2C, Table 2, in comparison both temperature of high humidity (80% RH) ($F = 19.01$; $P < 0.001$) (Figure 2D, Table 2). In contrast, we found rather different results in starvation resistance, here light morph produced a better result and survived more hours in comparison to dark morph at low temperature of high humidity, *i.e.*, ~158.88 hrs at 17°C of 80% RH ($F = 331600$; $P < 0.01$, Table 2).

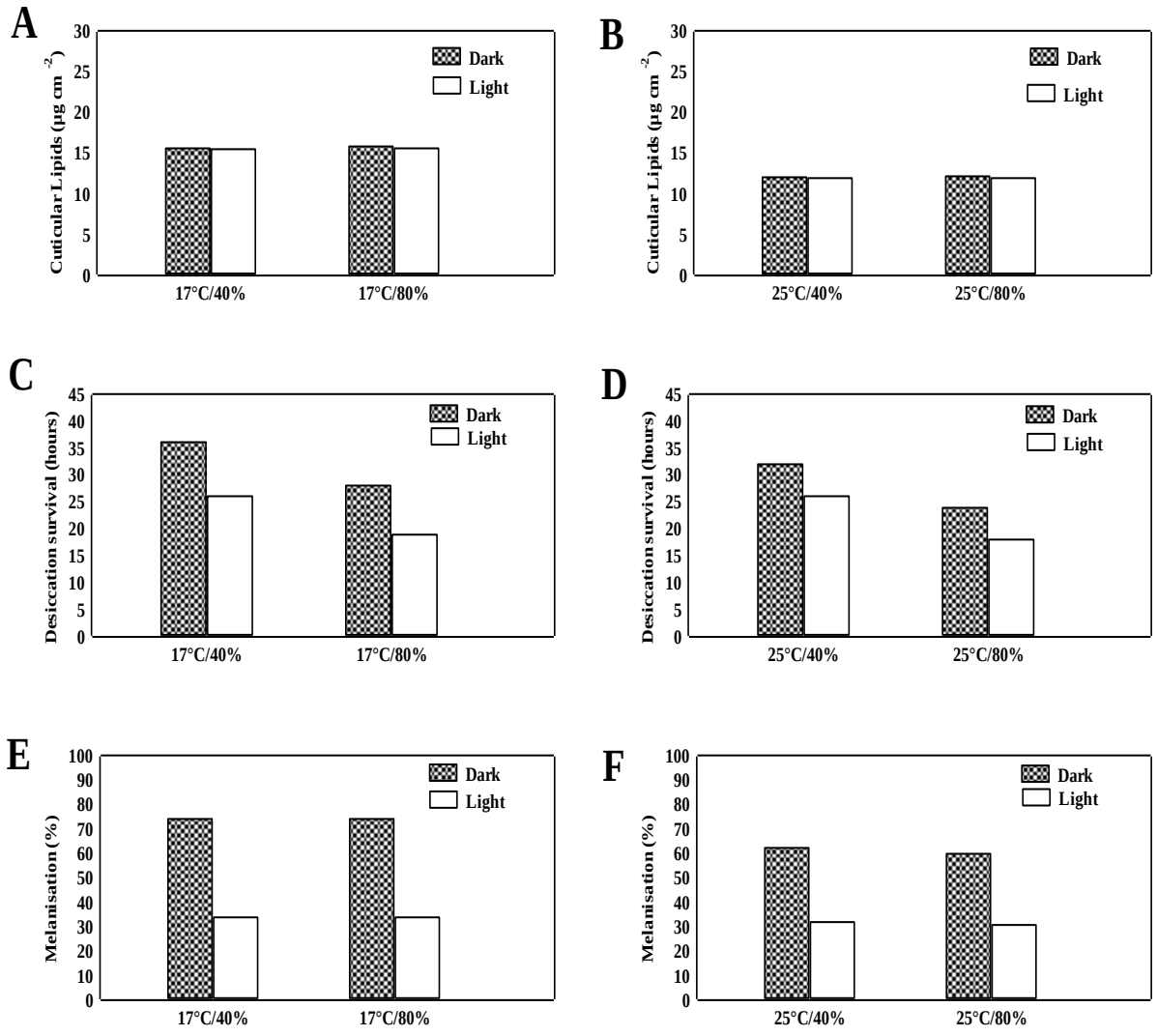

Figure 2. Mean ± s.e. bars of cuticular components (% melanisation E, F, and cuticular lipids A, B) along with desiccation survival hours (C, D) for low (40% RH) and high humidity (80% RH) maintained in a cold (17ºC) and warm temperature (25ºC), respectively.

Dehydration tolerance

Dark morph of *D. punjabiensis* happens to be more tolerant to dehydration conditions at high as well as low humidity at both growth temperatures in comparison with light morph, thus lost more water in the process (Table 2).

Basic measures of water balance and tissue water

Data on morph-specific differences in water balance traits, desiccation hours, and starvation hours due to high and low relative humidity at two different growth temperatures (17°C and 25°C) in adult stage of *D. punjabiensis* are shown in Table 2, Figure 3A, and Figure 3B. In flies reared at low humidity of 17°C, we observed a significant increase in wet and dry mass as well as total body water

content and tissue water content (~1.5 fold) of dark morph in comparison to light morph (wet mass: $F = 168.2$, $p < 0.01$; dry mass: $F = 3411$, $p < 0.01$; total body content: $F = 3572$, $p < 0.01$; tissue water content: $F = 1851$, $p < 0.01$; Table 2). Similar inclination was observed with dark morph reared at different temperatures of high humidity 80% (wet mass: $F = 554$, $p < 0.01$; dry mass: $F = 6004$, $p < 0.01$; total body content: $F = 7336$, $p < 0.01$; tissue water content: $F = 2178$, $p < 0.01$; Table 2).

Table 2. Data on trait values (mean and ANOVA, *F**) of adult flies (6 days post eclosion) for basic measures of water balance (a), dehydration tolerance (b), desiccation (c), starvation (d) in dark and light body color morph of *D. punjabiensis* for low (40%RH) and high (80%RH) humidity maintained at cold (17°C) and warm (25°C) temperature.

Traits	Low humidity				F value	High humidity				F value
	17°C		25°C			17°C		25°C		
	Dark	Light	Dark	Light		Dark	Light	Dark	Light	
a) Basic measures of hemolymph and tissue water										
Wet mass (mg fly $^{-1}$)	1.83	1.28	1.05	0.70	168.2**	1.60	1.06	0.97	0.65	554.0**
Dry mass (mg fly $^{-1}$)	0.549	0.384	0.32	0.21	3411**	0.48	0.32	0.29	0.20	6004**
Total water content (mg fly $^{-1}$)	1.281	0.896	0.73	0.49	3572**	1.12	0.74	0.68	0.45	7336**
Hemolymph content (mg fly $^{-1}$)	0.46	0.19	0.26	0.11	3078**	0.40	0.17	0.24	0.10	3690**
Wet mass after hemolymph removal (mg fly $^{-1}$)	1.37	1.09	0.79	0.59	5228**	1.20	0.89	0.73	0.55	7748**
Hemolymph water content (mg fly $^{-1}$)	0.322	0.133	0.177	0.077	4212**	0.28	0.21	0.17	0.07	2064**
Tissue water content (mg fly $^{-1}$)	0.959	0.763	0.553	0.413	1851**	0.84	0.53	0.51	0.38	2178**
b) Dehydration tolerance (%)	98.59	82.77	96	78	212510**	91.93	70.78	76	59	3170**
c) Desiccation survival (hours)	36	26	28	19	10.28***	32	26	24	18	19.01***
d) Starvation (hours)	78.42	116.12	60.25	89.25	3010**	118.12	158.88	87.70	122.6	331600**

*F** value here is the interaction of morph and humidity conditions; **p< 0.01; ***p<0.001

Hemolymph content

Hemolymph content was found to be higher in dark morph at both humidities in comparison to light morph (~2 fold, Table 2). Dark morph of both low and high growth temperature at low humidity showed 58% more hemolymph content than light morph, whereas light morph of both low and high growth temperature at high humidity showed 42% less hemolymph then dark morph (Table 2).

Analysis of energy budget (J/mg) of carbohydrates, lipids, and proteins of adult

Analysis of energy budget (J/mg) of carbohydrates, lipids, and proteins of adult flies (6 days post eclosion) in dark and light body color morph of *D. punjabiensis* for low (40%) and high (80%) humidity maintained at cold (17°C) and warm (25°C) temperature is shown in Table 3. The storage level of proteins was similar in both the morphs (0.712 J/mg). Based on standard conversion factors (Schmidt-Nielsen, 1990; Marron *et al.*, 2003), we compared the energy budget due to each metabolite (carbohydrates or lipids or proteins) in both body color morphs of *D. punjabiensis* (Table 3). Dark morph has greater carbohydrate storage level, while light morph has greater triglyceride level for both humidities and at both temperatures (Figures 3,C, D, respectively).

Discussion

For ectothermic insects, spatial variations in quantitative traits are well documented, but temporal variations have received lesser attention (Bijlsma and Loeschcke, 1997; Hoffmann and

Table 3. Analysis of energy budget (J/mg) of carbohydrates, lipids and proteins of adult flies (6 days post eclosion) in dark and light body color morph of *D. punjabiensis* for low and high humidity at 17°C and 25°C.

Metabolites	Low humidity				High humidity			
	17°C		25°C		17°C		25°C	
	Dark	Light	Dark	Light	Dark	Light	Dark	Light
Carbohydrates	9.28	5.62	7.10	4.32	8.92	5.60	6.82	4.30
Lipids	5.84	7.68	4.36	5.58	5.99	9.05	4.44	6.70
Proteins	0.712	0.712	0.712	0.712	0.712	0.712	0.712	0.712
Total	15.83	13.42	12.17	10.61	15.62	15.36	18.38	11.71

Conversion factors: 17.6 J/mg for carbohydrates; 39.3 J/mg for lipids; and 17.8 J/mg for proteins. (Schmidt-Nielsen 1990; Marron *et al*., 2003

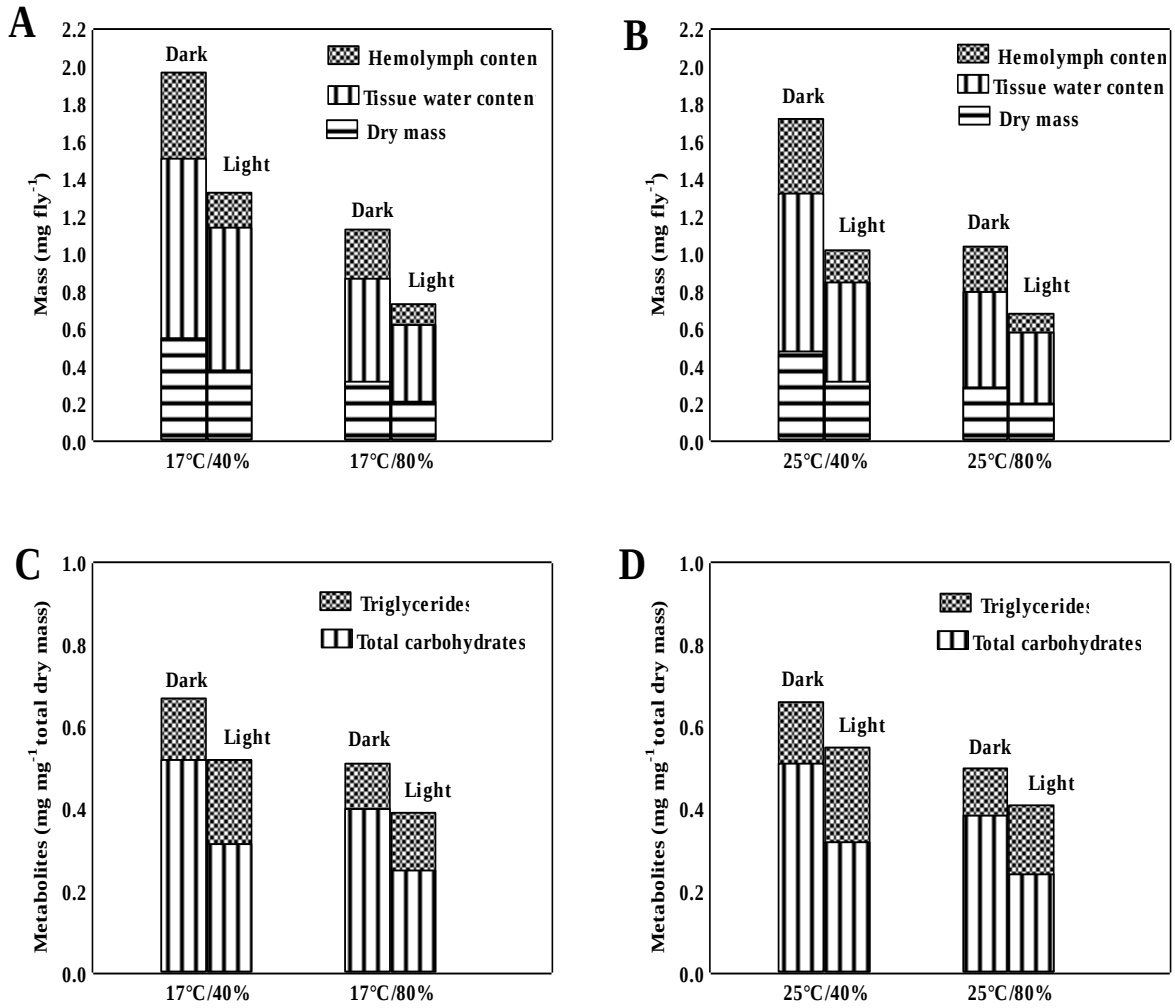

Figure 3. Water balance related traits of dark and light body color morphs at variable humidity condition of 17ºC and 25ºC. Comparison of hemolymph content, tissue water content, and dry mass of dark and light body color morph (A & B, respectively) and triglycerides and total carbohydrates of dark and light morph (C & D, respectively) for low and high humidity at 17°C and 25°C.

Weeks, 2007). In temperate regions, several studies have shown huge variations in body size for different *Drosophila* species. By contrast, there are few studies on the analysis of desiccation related traits (which show high heritability) in wild populations living under seasonally varying conditions. A single study on Australian populations of *D. melanogaster* has shown lack of temporal variation in desiccation resistance (Mckenzie and Parsons, 1974). However, on the Indian subcontinent, there are significant seasonal variations in the montane localities (Table 1). Our data have evidenced considerable changes in body melanisation and desiccation related traits across two seasons (autumn and spring). Further, there is lack of changes in cuticular lipids which do not account for variations in desiccation resistance across seasons. To the best of our knowledge, adaptive significance of body color plasticity to cope with seasonally varying desiccation stress has not been previously analyzed.

D. punjabiensis falls under the cosmopolitan category, which simply means it is accessible throughout the year at various sites. Our collection along a constant longitude and constantly variable latitude rightfully signifies the survival of this very species at varying climatic condition. Melanism is common in insect taxa, but its evolutionary causes are quite diverse (True, 2003; Wittkopp *et al*., 2003). Ohnishi and Watanabe in 1985 reported the presence of body color polymorphism in *D. punjabiensis.* As the Figure 1 implies, availability of the species is constant all over the year but the frequency of the body color morph varies according to season. We discovered that the frequency of the morph is subject to temperature and humidity conditions, in which survival of darker morph is enhanced in autumn conditions and monsoon/ rainy is suitable for lighter morph. Thus the seasonally varying environment puts a selection within the species. The case seems to be similar to the study of pepper moths (Tutt, 1896; Kettlewell, 1955, 1956). To examine the significance of melanisation or any other mechanism, we tried to mimic the nature's condition in laboratory scale and performed the earlier described test along with help of various literatures.

On the Indian subcontinent, T_{ave} does not vary along latitude. There is lack of correlation between T_{ave} and latitude ($r = 0.19 \pm 0.44$; ns). By contrast, humidity changes are significant along latitude ($r = 0.92 \pm 0.10$). Field data have shown significant seasonal variation in the percent frequency of dark and light morphs, *i.e*., greater frequency of melanic morph in autumn as compared to rainy/monsoon season (Figure 1). Data on varying humidity conditions under laboratory set-up have also shown changes in the frequencies of dark and light morphs. Thus, seasonally varying humidity seems to be the principle selective agent for adaptive changes in the frequencies of dark and light body color morphs.

Our study comes up with some new and interesting nitty-gritty about *D. punjabiensis*. Talking in terms of dominance, Watanabe (1985) and Parkash *et al.* (2009) have already proved that light morph is prevailing over dark body color morph, but high frequency of dark morph in the autumn season indicates different selection criteria. Our study signifies that dark morph flourish better at low humidity and low temperature, whereas high temperature and high humidity condition suits better to light morph. This is in relation to circumstances found in nature. Genetically light morph dominates over dark, but when it comes to survival at low temperature and humidity condition, dark morph performs better. Melanisation provides the required resistance and hence makes it more adaptive. This is perfectly shown by constant melanisation score of dark morph at variable humidity (74 ± 1.23: mean ± S.E at both 40% and 80% RH of low temperature - 17°C). In conclusion, melanisation derives the elevated morphometric traits (water balance) in the dark body color morph, irrespective of it being recessive, than light morph. Water balance traits and performance in extreme condition are not the deciding factor for dominance. It settles on the appearance of a particular trait in the subsequent progeny, when a cross is made between two extremely contrasting traits.

Unlike temperature, the role of humidity as a selection agent has not been considered for different quantitative traits in various *Drosophila* species so far. A single study has used laboratory

selection to determine the evolutionary effects of relative humidity, temperature, and their interactions on adult wing area (body size) in *D. melanogaster* (Kennington *et al*., 2003). In this study, after 20 weeks of laboratory selection, low RH lines had significantly greater wing area than high RH lines. Thus, body size in *D. melanogaster* can evolve rapidly in response to humidity selection. Thus, it may be argued that evolutionary responses for desiccation stress related traits might result due to humidity selection. Based on evidence in the present studies, we find that changes in the frequencies of dark and light morphs correspond with humidity selection under field conditions.

Possible role of body melanisation in conferring desiccation resistance was initially demonstrated for ebony mutant strains of *D. melanogaster* (Kalmus, 1941). Subsequently, darker and lighter laboratory strains of *D. polymorpha* were shown to differ in desiccation resistance (Brisson *et al*., 2005). For altitudinal populations of *D. melanogaster*, assorted dark and light flies exhibited significant differences in desiccation resistance and cuticular water loss (Parkash *et al*., 2008b). In correlation with the previous study, we also come up with the same result that dark morph has higher survival under desiccation condition which indicates towards melanisation as the underlying mechanism of resistance towards water loss.

Insects can enhance their desiccation resistance by increasing their total body water content, reducing the rate of body water loss, and tolerating a larger proportion of overall water loss from the body (Hadley, 1994; Gibbs *et al.*, 1997). Dark body color morph of *D. punjabiensis* contains the highest body water content at low humidity and temperature condition, thus showing higher desiccation resistance. At the same condition on the other hand lighter morph does not show any such relation with the temperature and humidity conditions suitable to it. This makes us think the requirement of desiccation resistance when a species is enjoying better temperature and humidity conditions. This indicates lighter morph of *D. punjabiensis* does not require any such resistance mechanism thus did not perform in desiccating conditions.

In the present study, we tested the role of cuticular lipids to support desiccation resistance, as expected amount of cuticular lipid and survival in desiccation stress are highest for dark morph in low humidity and low temperature condition. On the other hand such mechanism seems to be missing in light morph; therefore, no better performance at favorable conditions but slight hint of developing such mechanism at adverse condition is foreseen. This is advent with significantly better survival of light morph at low humidity and temperature condition, which is certainly not suitable for their survival in nature.

In the present study seasonal data on wild living flies have shown frequency changes of dark and light morphs in *D. punjabiensis*, which correspond with varying humidity levels at six different latitudinal sites. Change in humidity level is the principle agent of natural selection, which modifies the frequencies of dark and light morphs. The melanism-desiccation hypothesis finds support from studies on body color mutant strains (Kalmus, 1941), use of assorted dark and light individuals from a given population (Parkash *et al*., 2008b). In tropical regions, seasonal changes in precipitation cause desiccation stress in autumn. *D. punjabiensis* has adapted to wet (spring season) and dry (autumn) seasons by modifying the frequencies of color morphs through assortative matings. Laboratory data on *D. punjabiensis* show evidence in favor of melanism-desiccation hypothesis. Like *D. punjabiensis*, other species of montium species subgroup are expected to provide similar evidence. Thus, seasonally varying humidity conditions in the tropics can maintain body color polymorphism and desiccation resistance in *D. punjabiensis.*

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References: Addo-Bediako, A., S.L. Chown, and K.J. Gaston 2000, Proceedings of the Royal Society London 267: 739–745; Aldridge, D., C.W. Jones, E. Mahar, and M.E.N. Majerus 1993,

Entomol. Record and J. Variat. 105: 203–214; Berry, R.J., and P.G. Willmer 1986, Ecol. Entol. 11: 251–260; Bijlsma, S., and V. Loeschcke 1997, Env. Stress Adaptation Evol.; Brakefield, P.M., and P.G. Willmer 1985, Heredity 54: 9–14; Brakefield, P.M., J. Pijpe, and B.J. Zwaan 2007, J. Bioscie. 32: 465–475; Brakefield, P.M., and N. Reitsma 1991, Ecol. Entom. 16: 291–303; Brisson, J.A., D.C. De Toni, I. Duncan, and A.R. Templeton 2005, Evolution 59: 1046-1059; Chown, S.L., and S.W. Nicolson 2004, *Insect Physiological Ecology: Mechanisms and Patterns*. Oxford University Press, Oxford; Cohen, A.C., R.B. March, and J.D. Pinto 1986, Comp. Biochem. Physiol. A. 85:743–746; Danks, H.V., 2007, The Canadian Entomol. 139: 1–44; Dombeck, I., and J. Jaenike 2004, Evolution. 58: 587–596; Folk, D.G., C. Han, and T.J. Bradley 2001, J. Exp. Biol. 204: 3323–3331; Gibbs, A.G., A.K. Chippendale, and M.R. Rose 1997, J. Exp. Biol. 200: 1821–1832; Hadley, N.F., 1994, CA: Academic Press; Hoffmann, A.A., R. Hallas, C. Sinclair, and P. Mitrovski 2001, Evolution 55: 1621–1630; Hoffmann, A.A., and L.G. Harshman 1999, Heredity 83: 637–643; Hoffmann, A.A., J.G. Sorenson, and V. Loeschcke 2003, J. Therm. Biol. 28: 175–216; Hoffmann, A.A., and A. Weeks 2007, Genetica 129: 133–147; Hollocher, H., J.L. Hatcher, and E.G. Dyreson 2000a, Evolution 54: 2046–2056; Hollocher, H., J.L. Hatcher, and E.G. Dyreson 2000b, Evolution 54: 2057–2071; Holloway, G.J., C.G. Marriott, and H.J. Crocker 1997, Ecol. Entol. 22: 425–432; Kalmus, H., 1941, Proc. Roy. Soc. London Ser. B. 130: 185–201; Kari, J.S., and R.B. Huey 2000, J. Therm. Biol. 25: 267–272; Kennington, W.J., J.R. Killeen, D.B. Goldstein, and L. Partridge 2003, Evolution 57(4): 932–936; Kettlewell, H., 1955, Heredity 9: 323–342; Kettlewell, H., 1956, Heredity 10: 287–301; Kingsolver, J.G., and D.C. Wiernasz 1991, American Naturalist 137: 816–830; Lee, R.E., and D.L. Denlinger 1991, Chapman & Hall, New York; Llopart, A., S. Elwyn, D. Lachaise, and J.A. Coyne 2002, Evolution 56: 2262–2277; Loeschcke, V., R.A. Krebs, and J.S.F. Barker 1994, Biol. J. Linnean Soc. 52: 83–92; Majerus, M.E.N., 1990, Bull. Amateur Ento. Soc. 49: 123–131; Majerus, M.E.N., 1998, Oxford University Press, Oxford, UK; Mani, M.S., 1968, *Ecology and Biogeography of High Altitude Insects*; Marron, M.T., T.A. Markow, K.J. Kain, and A.G. Gibbs 2003, J. Insect Physiol. 49: 261–270; Mckenzie, J.A., and P.A. Parsons 1974, J. Biol. Sci. 27: 441–456; Ohnishi, S., and T.K. Watanabe 1985, Jap. J. Genet. 60: 355–358; Osawa, N., and T. Nishida 1992, Heredity 69: 297–307; Overgaard, J., and J.G. Sorensen 2008, Cryobiology 56 (2): 159–162; Parkash, R., D. Aggarwal, P. Ranga, and D. Singh 2012, J. Comp. Physiol., B.; Parkash, R., B. Kalra, and V. Sharma 2010, Evol. Ecol. 24: 207–225; Parkash, R., S. Rajpurohit, and S. Ramniwas 2008b, J. Ins. Physiol. 54: 1050–1056; Parkash, R., V. Sharma, and B. Kalra 2008a, Fly 2: 111–117; Parkash, R., and A.K. Munjal 1999, J. Zool. Syst. Evol. Res. 37: 195–202; Parkash, R., S. Rajpurohit, and S. Ramniwas 2008c, J. Insect Physiol. 54: 1050–1056; Parkash, R., S. Rajpurohit, and S. Ramniwas 2009, J. Insect Sci. 8: 97–103; Parkash, R., P.K. Tyagi, I. Sharma, and S. Rajpurohit 2005, Physiol. Ento. 530: 353–361; Parshad, R., and I.J. Paika 1964, Res. Bull. (N.S.) Punjab Uni. 15: 225–252; Pool, J.E., and C.F. Aquadro 2007, Mol. Ecol. 16 (14): 2844–2851; Rajpurohit, S., R. Parkash, and S. Ramniwas 2008, Ento. Res. 38: 49–60; Rapoport, E.H., 1969, Evolution 23: 622–626; Roskam, J.C., and P.M. Brakefield 1999, Biol. J. Linnean Soc. 66: 345–356; Schmidt-Nielsen, K., 1990, Cambridge University Press, Cambridge; Shapiro, A.M., 1976, Evol. Biol. 9: 259–333; Tantawy, A.O., 1965, Evolution 18: 560–570; Tauber, C.A., and M.J. Tauber 1981, Annual Review of Ecol. and Systematics 12: 281–308; Tauber, M.J., C.A. Tauber, J.P. Nyrop, and M.G. Villani 1998, Env. Ento. 27: 523–530; Torres, F.R., and L. Madi-Ravazzi 2006, Ser. Zool., Porto Alegre. 96: 437–444; True, J.R., 2003, Trends in Ecol. and Evol. 18: 640–647; Tutt, J., 1896, George Routledge, London; Watt, W.B., 1968, Evolution 22: 437–458; Wharton, G.W., 1985, Comprehensive Ins. Physiology, Biochem. Pharmaco. 4: 565–603; Wittkopp, P.J., S.B. Carroll, and A. Kopp 2003, Trends in Genetics 19: 495–504; Yoder, J.A., B.Z. Hedges, J.B. Benoit, and G.D. Keeney 2009, J. Comp. Physiol. B 179: 729–736; Zar, J.H., 1999, *Biostatistical Analysis*, 4th ed. Prentice-Hall, London.