The morphological terminology and the definitions of measurements and indices mostly follow McAlpine (1981), Zhang and Toda (1992), and Hu and Toda (2001). A total of 3090 flies belonging to different genera of the family Drosophilidae were collected during the period of present survey. (Table 1).

Altogether 26 species representing 8 genera of the family Drosophilidae were collected during preliminary surveys started from 2008 to 2010. Out of 26 species, 5 species are supposed to be new species. Among genus *Drosophila* altogether 14 species, in which nine species of subgenus *Sophophora*, one species of subgenus *Dorsilopha* and four species of subgenus *Drosophila*, were collected. Out of them one species of subgenus *Sophophora (Sophophora* sp. K1) was unidentified and are supposed to be new species. Genus *Leucophenga* (*Leucophenga* sp. A1, K1 and Z1) was represented by five species, out of which three species were unidentified and are supposed to be new species. Genus *Scaptodrosophila* was represented by only one species. K1 was unidentified and is supposed to be new species. Genus *Dettopsomyia, Scaptomyza, Gitona,* and *Zaprionus* were represented by one species each. The two Indian species of *Paraleucophenga, P. todai and P. neojavanai*, were also present in this area. The identification of yet unidentified species will be completed very soon.

The above table indicates the prominence of *D. melanogaster, D. immigrans, D. busckii, D. nepalensis, D. takahashi*, and *D. punjabiensis* in both the sampling sites of Srinagar, Pauri and Agastyamuni, Rudraprayag accompanied by *Scaptomyza himalayana* and *Zaprionus indianus.*

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Correlated changes in seasonal acquisition of desiccation resistance and cold-tolerance in *Drosophila simulans* along an altitudinal gradient in western Himalayas.

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Abstract

Correlated changes in seasonal increases in desiccation resistance and cold-tolerance were examined in field-collected adult females of *D. simulans*. Low temperature and desiccation stress are

thought to be mechanistically similar in insects, and several studies indicate that there is a degree of cross-tolerance between them, such that increased cold tolerance results in greater desiccation tolerance and vice versa. From autumn to winter, *D. simulans* flies exhibited an increase in cold-tolerance and desiccation resistance along an altitudinal gradient. The increase in cold-tolerance was probably due to a concomitant increase in trehalose level that acts as cryoprotectant in insects. In contrast to the gradual increase in cold-tolerance, flies exhibited reduced rates of water loss. The reductions in rates of water loss during seasonal changes were probably the result of reduced cuticular water loss as flies increased the amount of their cuticular hydrocarbons. The correlation between seasonal increases in trehalose content and reduction in rates of water loss may represent a link between desiccation resistance and cold-tolerance in this species. These findings provide little support for cross-tolerance between survival to cold stress and desiccation stress in *D. simulans*.
Keywords: *Drosophila simulans*, desiccation resistance, cold tolerance, altitude

Introduction

Many insects that overwinter in temperate and polar regions must tolerate not only extreme cold but desiccation stress. Recently, several reviews suggested that certain behavioral and physiological adaptations promoting cold-tolerance may also influence, or were originally adaptations for, desiccation resistance (Ring and Danks, 1994; Block, 1996; Danks, 2000). There are a remarkable number of overlaps in the tolerances of insects to differing environmental stresses. For example, mild desiccation elicits increased cold tolerance in the springtail *Folsomia candida* (Bayley et *al.*, 2001), and a period of anoxia induces increased cold tolerance in the house fly *Musca domestica* (Coulson and Bale, 1991). Cross-tolerance is thought to be a useful way to approach complex traits, and selection experiments in *Drosophila* have been frequently used to examine the relationships between environmental stressors, including high and low temperatures, desiccation and starvation (Nghiem *et al.*, 2000; Hoffmann *et al.*, 2003; Bubliy and Loeschcke, 2005). Freeze-tolerant insects use glycerol and other low-molecular-mass polyols and sugars, termed cryoprotectants, to decrease the amount of body water that freezes at a given temperature, thereby preventing excessive cellular dehydration (Baust and Lee, 1981; Storey and Storey, 1992; Zachariassen, 1991). Increased cryoprotectant concentrations may also lower water loss rates by collectively reducing the vapor pressure deficit between the insect's hemolymph and environmental water vapor (Ring and Danks, 1994; Bayley and Holmstrup, 1999; Sjursen *et al.*, 2001). However, most studies have focused on how adaptations promote low temperature survival with little attention to the possible effects these adaptations may have on water conservation.

Previous studies examined several parameters of cold-tolerance and their possible link to desiccation resistance in cold-hardy insects collected in mid-winter (Williams *et al*., 2002). Of the many environmental stresses that insects face regularly, desiccation and cold are thought to be particularly closely related (Ring and Danks, 1994). Many overwintering insects consequently show reduced water content, and many of the biochemical adaptations to cold also serve to protect against desiccation (Ring and Danks, 1994). This relationship is well-supported in insects that are highly permeable (Holmstrup *et al.*, 2002), exposed to very cold, desiccating conditions (Ramløv and Lee, 2000), or are freeze tolerant (Sinclair and Wharton, 1997). However, in *Drosophila simulans*, the link between cold and desiccation is less well-explored. To survive the low temperatures and desiccating conditions of winter, *D. simulans* increase their cold-tolerance during the autumn and have extremely low rates of water loss. The seasonal increase in cold-tolerance is correlated with the accumulation of the cryoprotectant trehalose. However, it is unknown if seasonal changes in the rate of water loss exist, are these reductions in rate of water loss linked to physiological process of

increasing cold-tolerance? The purpose of the present study was to characterize seasonal changes in cold-tolerance and resistance to water loss in adult females of *D. simulans* to determine if the acquisition of desiccation resistance is linked to increases in cold-tolerance. To investigate this question we measured survival after exposure to low temperatures, trehalose levels as a measure of cryoprotectant, and rate to water loss of field collected flies from autumn to winter. In conjunction with the field study, we also examined the effect of mild desiccation stress on rates of water loss and cold-tolerance.

Materials and Methods

Collection and cultures

Wild living *D. simulans* individuals (n = 320–400 flies from each collection site) were collected by net sweeping and banana bait-trap methods in autumn as well as winter season of 2010 from six altitudinal sites (600–2226 m). For both the seasons, multiple collections over a week were made from each of the six localities. In the laboratory, after mild anesthesia with solvent ether, the wild caught females were sorted out to initiate isofemale lines (20 lines per population). All cultures were initiated with 6–8 h egg laying period and were maintained at low density (60–70 eggs per vial of 37 mm × 100 mm size) on cornmeal-agar medium at 21ºC. For seasonally varying as well as altitudinal populations, the laboratory progeny of wild-caught individuals were analyzed for all the traits. Analyses were made for each season at different times of the year, *i.e*. there was a time gap of 3 months corresponding to two different seasons. However, the experimental conditions were made uniform with the use of a temperature controlled room set at 21°C. Further, for each season, G_1 and G_2 (Generations 1 and 2) of the wild caught flies were analyzed in order to avoid possible effects of laboratory adaptation (which may result due to laboratory cultures maintained for months and years before analysis). However, there were no changes in cold tolerance and desiccation resistance in *D. simulans* cultures maintained for 10–12 generations for all the populations. Climatic data for the sites of origin of populations were obtained from Indian Institute of Tropical Meteorology (IITM; www.tropmet.res.in) and the data are shown in Table 1.

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

Populations/Alt. (m)	Lat. (°N)	Autumn			Winter		
		T_{min} (°C)	T_{max} (°C)	RH (%)	T_{min} (°C)	T_{max} (°C)	RH (%)
Kalka (600)	30.51	21.7	31.3	68.6	12.2	22.0	64.0
Bhuntur (1096)	30.20	20.5	29.5	63.2	10.0	18.0	60.0
D.shala (1211)	32.00	17.8	28.6	59.3	7.3	17.1	55.2
Solan (1440)	32.58	15.4	23.6	56.4	7.1	16.2	50.0
Kasauli (1951)	30.17	13.2	20.8	51.1	6.2	15.4	45.0
Shimla (2202)	31.06	10.2	18.6	50.2	5.1	13.2	42.0

Measurement of cold-tolerance

Cold resistance was measured individually on ten males or females from each isofemale line (n = 20 per population). Adult flies were aspirated and placed individually in 42 ml glass vials which were placed in 10% glycol solution at 0ºC for 8 hours (Hoffmann, Anderson and Hallas, 2002). This was followed by transfer of flies to petri-plates (9 cm diameter) in a temperature controlled room at 21°C, and chill coma recovery period (in minutes) was recorded. Recovery to chill coma is inversely related to cold resistance, *i.e*. longer recovery period to cold stress means lesser resistance level. For each population, recovery time in minutes was subtracted out of 100 to denote relative resistance level to cold stress. This helped in comparing the data on resistance to heat and cold stress.

Measurement of desiccation resistance related traits

2.4.1. Desiccation resistance

To measure desiccation resistance, five wild caught or 10 laboratory reared individuals from each line (six replicates per isofemale line) were isolated in a dry plastic vial, which contained 2 g of silica gel at the bottom and was covered with a piece of foam. Such vials with muslin wraps were placed in a desiccation chamber (Secador electronic desiccator cabinet) which maintained 4–5% relative humidity at 21 ± 0.5ºC. The vials were inspected every hour and the numbers of dead flies (completely immobile) were recorded. The desiccation survival hours were based on LT_{50} values.

2.4.2. Cuticular water loss

For individual flies, total body water content was estimated from the difference between wet and dry weights (dried overnight at 60ºC) and divided by initial wet weight × 100. Cuticular water loss (under dehydration) was analyzed by giving short-term desiccation stress (8 h) in groups of five wild-caught or ten laboratory reared flies. After mild anesthesia with solvent ether, flies were weighed on a Sartorius microbalance (Model-CPA26P; with precision 0.001 mg) both before and after desiccation, and the cuticular water loss was calculated: (initial body weight – body weight after 8 h desiccation stress)/initial body weight × 8; and the values were given in mg h^{-1}.

2.4.3. Cuticular lipids

For cuticular lipid estimation, each individual fly was dried overnight at 60ºC to get constant dry mass, *i.e*. devoid of body water. Each dried fly was kept in HPLC-grade hexane for 1 h and thereafter it was removed from the solvent and was again dried at room temperature and finally reweighed. For each individual fly, cuticular lipid mass was estimated 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; and surface area scaled to 2/3 power of the wet body mass). Finally, the data on cuticular lipid mass per fly were shown as mg cm^{-2} fly^{-1}. According to scaling relationships (Schmidt-Nielsen, 1984; Brown *et al.*, 2000; Gibbs, 2002), changes in surface area due to increase in body mass were represented by the equation: $y = xb$; where y = dependent variable; x = independent variable (body mass), b = scaling exponent (*i.e*., ratio of surface in squares to volume in cubes = 2/3).

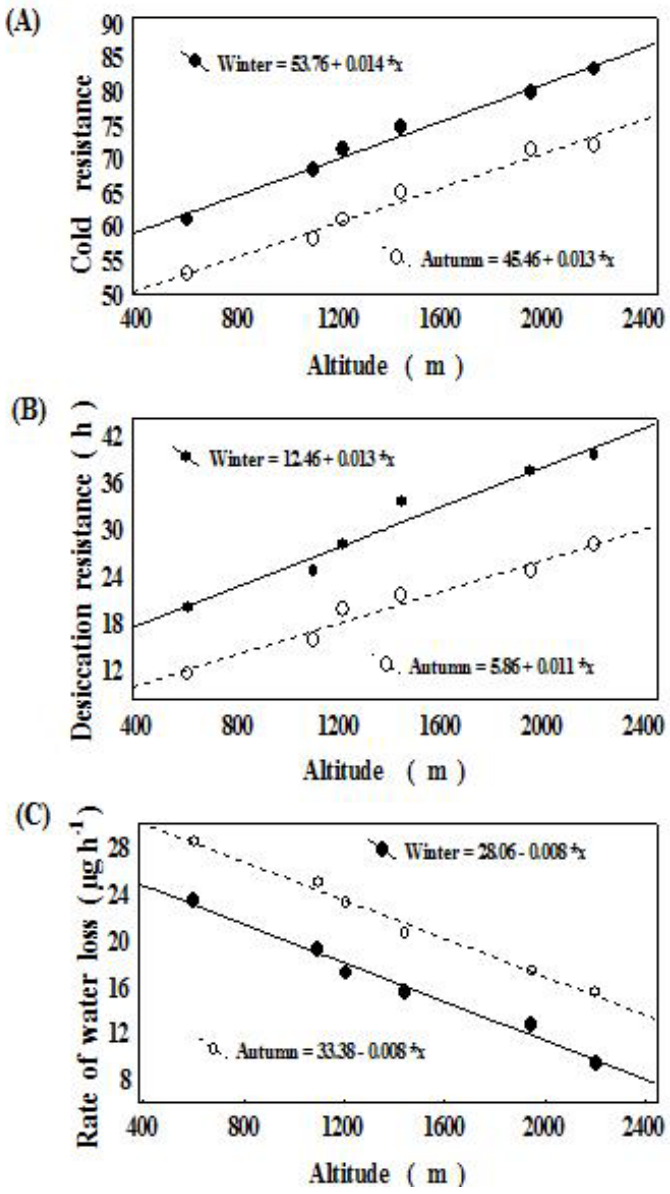
Trehalose estimation

For trehalose estimation, aliquots (200 µl) were placed in two different tubes; one was taken as a blank while the other was digested with trehalase at 37ºC (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 340nm (UV-2450-VIS; USA). Pre-existing glucose level in the sample was determined in a control reaction lacking trehalase and subtracted from total glucose concentration.

Effect of moderate desiccation stress on cold-tolerance and desiccation resistance

Flies collected in late autumn were used to determine the effects of mild water stress on cold-tolerance and desiccation resistance. To measure pretreatment time duration, 10 female individuals of each replicate (20 I.F lines × 10 replicates each) were subjected to desiccation stress at ~0 - 5%

relative humidity. The initial body water content in replicate groups was recorded. The time period in which flies lost ~ 15-17% body water was assessed as pre-treatment time duration. Further, for recovery period, individuals were placed on non-nutritive agar and tested at hourly intervals for increase in body water till the lost body weight was regained. Such flies were subjected to desiccation stress until death in order to test the increased desiccation resistance due to acclimation. Increased desiccation survival hours were calculated after subtracting the desiccation resistance hours of non-acclimated (control) from acclimated individuals. Control and treatment experiments were run simultaneously under identical experimental conditions.



Statistical analyses

Seasonal data were analyzed using a one-way analysis of variance (ANOVA). Variance (%) due to populations, seasons, and their interactions were calculated as proportion of MS × degree of freedom out of the total sum of such values. Correlation values (r) were calculated for flies from each season. For climatic data analysis, we used multiple regressions of trait values as a simultaneous function of Taverage ($T_{ave.}$) and relative humidity (RH) of the sites of origin of populations. Statistica (Statsoft Inc., Release 5.0, Tulsa, OK, USA) was used for calculations as well as illustrations.

Figure 1. Comparison of altitudinal slope values for cold resistance (A); desiccation resistance (B); and rate of water loss (C) flies collected during autumn and winter seasons from six altitudinal populations of *D. simulans*. Values for linear regression analysis (y = a + bx) are shown where 'a' = intercept and 'b' = slope value.

Results

Seasonal acquisition of cold-tolerance and desiccation resistance

For wild-caught flies, there is significant population divergence for all the traits as a function of altitude of origin (Figure 1A– D). The clinal trends were compared as a ratio of trait values of lowest *vs*. highest altitudinal populations. Based on this criterion, cold resistance and desiccation

related traits showed a 1.40-fold divergence (Figure 1). Although trait values differed across seasons, the magnitude of clinal increase was identical across seasons. By contrast, there were no changes in the total body water content (69.4 ± 0.13%) across seasons as well as along altitude. For assessing the effects due to seasons, we analyzed different quantitative traits in wild-caught individuals of *D. simulans* and the data are given in Table 2. For the quantitative traits (cold resistance, desiccation resistance, cuticular water loss, and trehalose content), there were similar magnitudes of % variance due to populations; seasons, and their interactions (Table 2). Thus, there were significant parallel effects of seasonal changes for desiccation related traits.

Table 2. ANOVA for wild caught individuals trait variability due to population (P), seasons (S) and their interactions for six altitudinal populations of *D. simulans*.

Traits	df	Populations (5)	Seasons (1)	P x S (5)	Error (1188)
1. Cold survival	*MS*	91.23	1126.25	72.32	0.12
	F	12.32**	9445.14***	8.43*	
2. Desiccation resistance	*MS*	84.01	996.85	23.36	0.03
	F	36.23***	1236.02***	11.23**	
3. Rate of water loss	*MS*	6.23	112.69	3.23	0.0001
	F	11.23**	6532.21***	9.36**	

Trait correlations

Associations of desiccation resistance with cuticular lipids as well as cuticular water loss are illustrated in Figure 2 (for within population level). Analyses of seasonal wild caught samples clearly indicated positive associations between desiccation resistance and cuticular lipids ($r = 0.90 \pm 0.010$, $p < 0.001$) but negative with cuticular water loss ($r = -0.97 \pm 0.007$, $p < 0.001$; Figure 2A–B). Correlation analysis evidenced positive association of trehalose content with cold resistance and of cold resistance with desiccation resistance ($r = 0.90 \pm 0.012$, $p < 0.001$; Fig. 2C - D). Thus, for a given population, cold resistant flies during winter were more desiccation resistant and showed reduced cuticular water loss. By contrast, flies with lower cold resistance during autumn showed lower desiccation resistance but higher cuticular water loss. Further, the gradual increase in cold-tolerance of *D. simulans* was mirrored by increases in trehalose content (Figure 2C). Values for trehalose content increased significantly ($P < 0.05$) at successive testing season. In contrast to the increase in cold-tolerance, water loss rates decreased during the winter.

Cold-tolerance and desiccation resistance after moderate desiccation stress

To determine whether desiccation stress could induce changes in rates of water loss and cold-tolerance, autumn collected flies from one highland locality were subjected to desiccating conditions in the laboratory. Flies were subjected to either 0-5% RH at 21°C for 2 days prior to assessing their cold-tolerance, desiccation resistance, rate of water loss and changes in trehalose level, if any. There was an apparent trend toward increased resistance at 0°C after prior treatment to desiccation stress. Moderate desiccation stress enhanced desiccation resistance as rates of water loss were significantly lower ($P < 0.05$) for flies subjected to lower humidity conditions, than the field group (Figure 3A-B). Further, desiccation pre- treated flies had higher levels of trehalose content as compared to control groups. Cuticular lipid amount did not change in control and desiccation treated flies. These data suggest that mild desiccation stress induced an enhanced cold by increasing levels of cryoprotectant

(trehalose) and desiccation resistance by decreasing rate of water loss in *D. simulans*. Further, results of ANOVA explaining the trait variability are shown in Table 3. We observed significant *F* values for increase in desiccation resistance as well as cold resistance (desiccation resistance: $F = 3923.23$; $P < 0.001$; cold resistance: $F = 2196.47$; $P < 0.001$) due to prior exposure to stress in *D. simulans.*

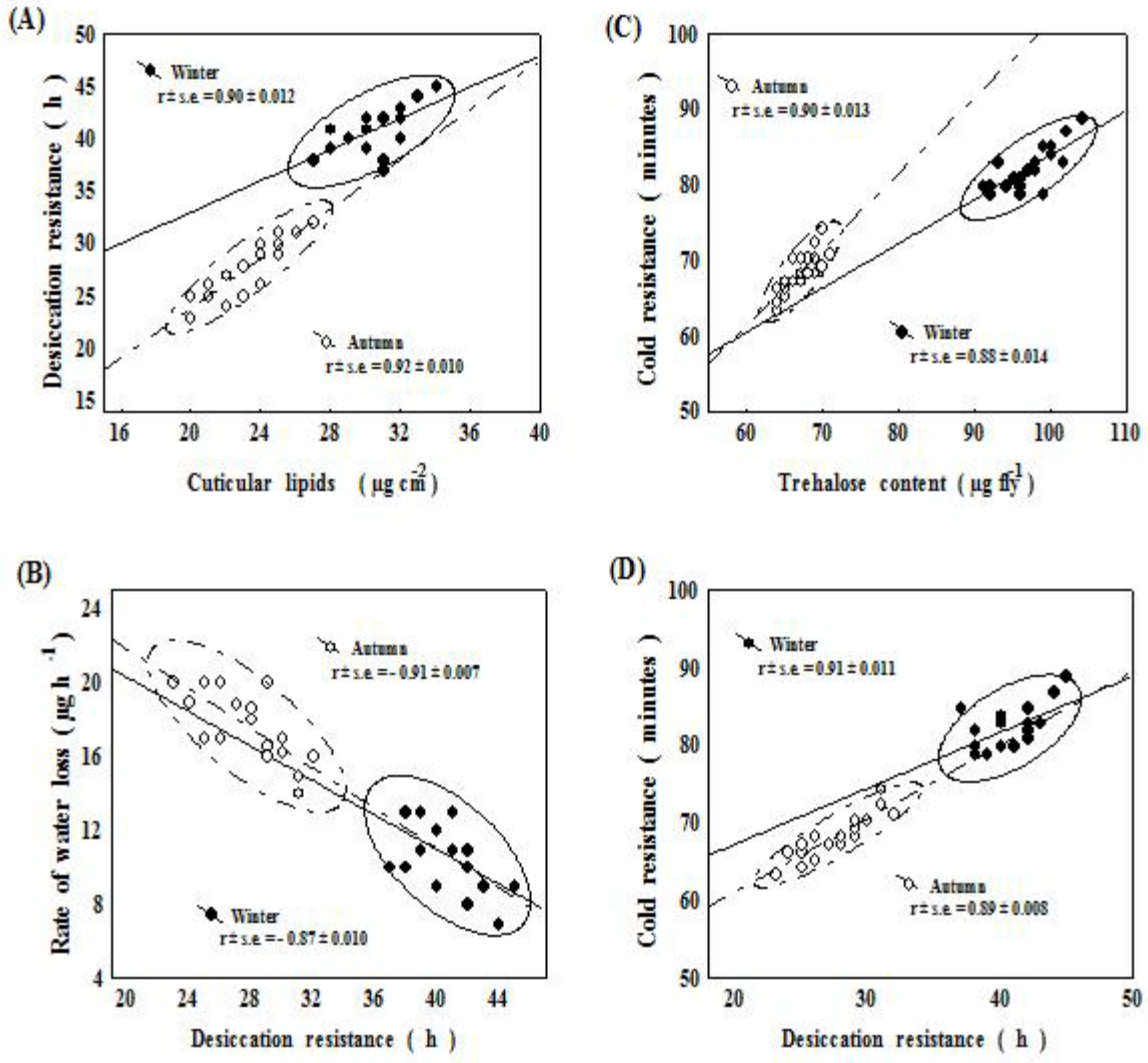


Figure 2. Trait correlations based on within population analysis. (A) Correlations between desiccation resistance with cuticular lipids; (B) negative correlation of desiccation resistance with cuticular water loss mg h^{-1}; (C) significant positive correlation of trehalose content per fly with cold resistance; and (D) positive correlation of desiccation resistance with cold resistance in individuals from autumn *vs*. winter populations of *D. simulans* from a highland locality. All the correlation values are highly significant ($p < 0.001$), but the signs are different.

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. Results of ANOVA (isofemale lines were nested in treatment group) for explaining trait variability due to desiccation acclimation on cold resistance and trehalose content in *D. simulans.*

Traits	df	MS	*F*
1. Desiccation resistance			
Treatment	1	47912.21	3923.23***
IF lines	38	203.25	14.27***
Error	360	10.23	
2. Cold resistance			
Treatment	1	62306.32	2196.47***
IF lines	38	458.45	22.36***
Error	360	17.36	

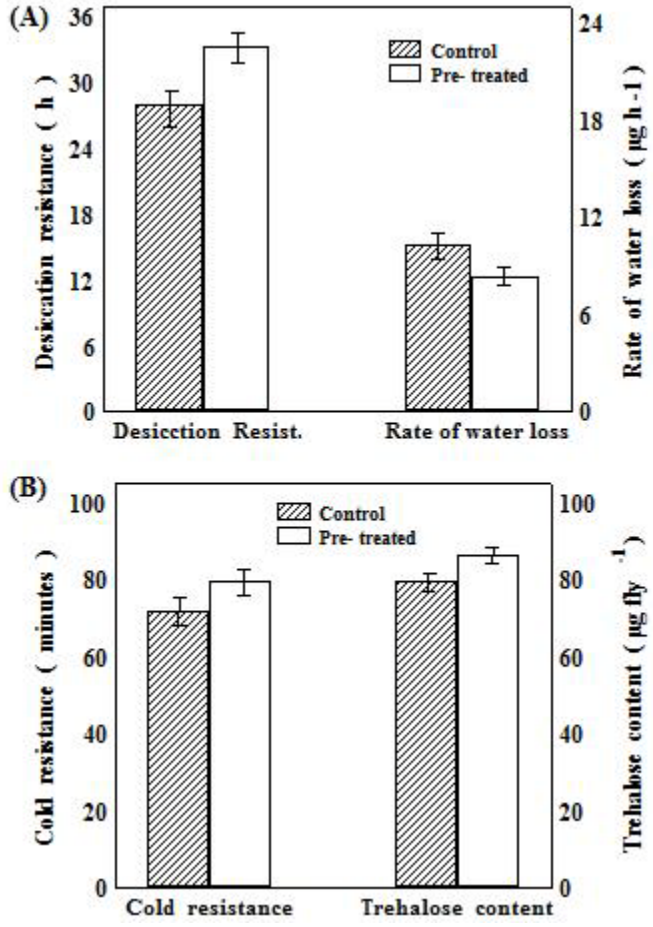


Figure 3. Bars represent changes in desiccation resistance and rate of water loss (A) and cold resistance and trehalose content (B) in control and desiccation pre-treated flies of *D. simulans* from a highland locality (20 I.F lines × 10 replicates each).

Weeks, 2007). In temperate regions, several studies have shown huge variations in body size for different *Drosophila* species. However, a large part of such heterogeneity results due to thermal and nutritional stress under wild conditions and accordingly the heritability in the field is much reduced (Coyne and Beecham, 1987; Imasheva *et al.*, 1994). 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 desiccation and cold stress related traits across two seasons (autumn and winter). However, seasonal plastic effects for body size are not correlated with desiccation resistance. Further, there are correlated changes in cuticular lipids, which account for variations in desiccation resistance across seasons. To the best of our knowledge, adaptive significance of cuticular lipid plasticity to cope with seasonally varying desiccation stress has not been previously analyzed.

The mechanistic basis of desiccation resistance involves reduction of cuticular water loss due to changes in cuticular lipids (Zachariassen *et al.*, 1987; Hadley, 1994; Gibbs, 1998; Addo- Bediako

et al., 2001). Several studies on thermal acclimation for cuticular lipids have shown correlated changes in cuticular water loss (Hadley, 1977; Toolson and Kuper-Simbron, 1989). However, there are reports which contradict such associations, *e.g*., in *D. mojavensis*, there are no effects of thermal acclimation on the rate of cuticular water loss (Gibbs *et al.*, 1998). In *D. melanogaster*, analysis of latitudinal populations has previously shown lack of geographical variations in the amount of cuticular lipids per fly (Parkash *et al.*, 2008). Laboratory selection for desiccation resistance has also shown a lack of difference in the amount of cuticular lipids per fly between resistant and sensitive strains (Gibbs *et al.*, 1997). By contrast, in the present study, we observed plastic effects in the amount of cuticular lipids per fly across seasons as well as in altitudinal populations. Thus, cuticular lipids do confer desiccation resistance in seasonally varying population of *D. simulans*.

Previous studies have created discrepancy on the association between desiccation resistance and basal cold tolerance. Selection for desiccation (and the starvation control) does not affect the basal tolerance of adult *D. melanogaster* to acute cold exposure, nor does selection affect the ability of *D. melanogaster* to exhibit the RCH response (Sinclair *et al*., 2007). The present study appears to be the first to examine the correlated changes of desiccation resistance on another stress resistance trait (cold resistance). The correlated change in cold tolerance observed here matches with the results of a study by Bubliy and Loeschcke (2005), who show that desiccation selection confers increased tolerance to a long exposure at 0°C; with Telonis-Scott *et al.* (2006), who show a decrease in cold tolerance after selection for desiccation resistance in one of their two selected lines; and with Hoffmann *et al.* (2005), who show that *D. melanogaster* selected for starvation tolerance have decreased survival of an acute cold shock. Telonis-Scott *et al.* (2006) employ an acute cold exposure (2.5 h at – 2°C) as their assay, so their metric is likely directly comparable with that used in the present study. Moreover, these three studies combined with the present contrasts with a laboratory-evolved desiccation resistance and basal cold tolerance study in *D. melanogaster* (Sinclair *et al.*, 2007). It is unclear whether this divergence in relationship is due to chance variation in trajectories of selection lines, or due to genetic differences between the founding populations, other studies perform cold tolerance experiments on mated females (Bubliy and Loeschcke, 2005; Hoffmann *et al.*, 2005; Telonis-Scott *et al.*, 2006). However, Telonis-Scott *et al.* (2006) perform many of their stress assays (but not cold tolerance) on both males and females and demonstrate that the direction of response is consistent between genders for other traits.

In the present study increased desiccation resistance as a result of prior treatment to desiccating conditions was correlated closely with increases in trehalose levels and suggests a link between desiccation resistance and cold-tolerance in *D. simulans* (Figure 3). Prior desiccation stress increased cold resistance in *D. simulans*. Several studies have shown that dehydration pretreatment followed by rehydration can equip insects or terrestrial arthropods to tolerate a subsequent exposure to dehydration (Holmstrup and Somme, 1998; Sjursen *et al.*, 2001; Benoit *et al.*, 2007). However, a single study has analyzed acclimation to desiccation stress in four *Drosophila* species from Australia (Hoffmann, 1991). This study has shown lack of acclimatory response in tropical rainforest *D. birchii* but improved desiccation resistance due to acclimation in *D. serrata*, *D. simulans*, and *D. melanogaster*. Further, *D. birchii* and *D. serrata* are sibling species of montium species subgroup and have shown divergence in acclimation response to dehydration stress, and such observations are in agreement with divergence in their desiccation resistance (Hoffmann, 1991). Subsequently, another study on a single population of *D. melanogaster* has provided indirect evidence that acclimation to desiccation resistance may be affected by reduction in cuticular water loss rates but not through changes in respiratory water loss (Bazinet *et al.*, 2010). Our results have shown an increase in desiccation resistance as well as cold tolerance in *D. simulans* due to desiccation pretreatment, and such changes are consistent with their stress resistance level under field conditions. In summary, desiccation resistance and cold-tolerance of *D. simulans* increased during the winter season as

compared with autumn. An initial rapid decrease in seasonal rates of water loss was correlated with decreased water loss as increased cuticular lipids. Later in the autumn, cryoprotectant accumulation may have affected cold tolerance in *D. simulans*.

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In-vivo* testing for genotoxicity of ethyl methanesulfonate (EMS) using *Drosophila melanogaster.

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Abstract

Drosophila melanogaster is one among the heavily exploited model organisms, used to study genetics and more commonly used in the field of genetic toxicology due to the primary factor of