



Developmental adaptive strategies for water balance mechanisms mediates range shift in *Drosophila* species of the *takahashii* subgroup from the western Himalayas.

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Abstract

Physiological limits determine susceptibility to environmental changes and can be assessed at the individual, population, or species/lineage levels. *Drosophila nepalensis* could serve as an indicator species for analyzing range changes under changing climatic conditions. Ectothermic Drosophilids are profoundly affected by thermal selection (*i.e.*, genetic effects) or through induced effects on phenotypes (*i.e.*, plastic effects). Climatic data for the last fifty years involves a significant change in average temperature (T_{ave}) of Western Himalayas, which has affected the distribution and boundaries of various Drosophilids in this region. There is a significant decline in the number of *D. nepalensis* from lower ranges. *D. nepalensis* is more abundant under colder and drier montane habitats in the western Himalayas, but the mechanistic basis of such a climatic adaptation is largely unknown. Here we discuss the physiological levels in *D. nepalensis* and consider implications for determining species susceptibility to climate change. Thus, temperature-specific divergence in water-balance-related traits in this species is consistent with its adaptations to cold and dry habitats of Western Himalayas. Our results suggest that *D. nepalensis* from lowland localities seems vulnerable due to acclimation potential in the context of global climate change in the Western Himalaya. Finally, this is the first report on higher desiccation resistance of *D. nepalensis* due to developmental plasticity of cuticular melanisation when grown at 15°C, which is consistent with its abundance in temperate regions. Abbreviations: 'D', Desiccation resistant strains; I.F., Isofemale; J/mg, Joules/mg; RWL, Rate of water loss.

Introduction

Physiological limits occur when the abiotic conditions of an organism's environment become stressful, reducing fitness to survive, potentially leading to the extinction of populations and even species (Hoffmann and Parsons, 1991). Physiological limits can influence the susceptibility of organisms to climate change. Due to climate warming, many insect species have shifted their ranges to higher latitudes and altitudes (Hill *et al.*, 2011; Parmesan *et al.*, 1999; Parmesan and Yohe, 2003). Insects, especially those species that have narrow thermal tolerances (Addo-Bediako *et al.*, 2000; Deutsch *et al.*, 2008) are particularly sensitive to temperature changes. There is a significant decline in the number of *Drosophila nepalensis* from lower ranges, and climate warming mediates its range shift in the Western Himalayas (Parkash *et al.*, 2013; Singh, 2012).

D. nepalensis was first described from collections made from Nepal by Okada (Okada, 1955). Subsequently, Parshad and Paika (1964) reported that abundance of *D. nepalensis* (70–80%) was associated with lower temperature (T_{ave} =18–20°C) as well as humidity (~43–45% relative humidity) at Manali (2050 m). Thus, we expect that this species will differ in its desiccation-related traits for

different quantitative traits conferring adaptation to harsh climatic conditions in the Himalayas. Therefore; we compared *D. nepalensis* for evolved physiological mechanisms that may affect its adaptations to drier climatic conditions. *D. nepalensis* might employ phenotypic plasticity as a strategy to cope with colder and drier conditions in temperate regions, but this aspect has not been tested so far.

Water conservation is crucial to the ecological success of diverse insect taxa as well as terrestrial arthropods (Edney, 1977; Hadley, 1994). The ability to maintain water balance is associated with species distribution patterns (Willmer *et al.*, 2000). Several studies have shown substantial variation in desiccation resistance of *Drosophila* species living in different habitats (Gibbs and Matzkin, 2001; Gibbs *et al.*, 2003). Insects can increase their desiccation resistance through three different avenues of water balance: (1) higher bulk water, (2) reduced rate of water loss, and (3) greater dehydration tolerance (Hadley, 1994; Gibbs *et al.*, 1997). Insects with higher initial body water content can survive longer under arid conditions, *e.g.* laboratory-selected desiccation-resistant strains of *D. melanogaster* have shown a 300% increase in hemolymph water content compared with control strains (Folk *et al.*, 2001). If laboratory selection responses result in increases in the bulk water content, then it would be interesting to compare the water budget of *Drosophila* species differing in their desiccation resistance levels. Further, water balance related traits have been investigated in Indian populations of *D. nepalensis* reared at 21°C, but not at 15 and 25°C (Parkash *et al.*, 2012). Thus, the plasticity for evolved physiological mechanisms for water balance in *D. nepalensis* is largely unknown.

For ectothermic insects, more than 80% of body water loss occurs through the cuticle. Reduction in cuticular permeability has been associated with changes in the amount or composition of surface lipids in several large sized insect taxa such as scorpions and tenebrionid beetles (Hadley, 1977; Toolsons and Hadley, 1979; Hadley, 1994). In contrast, cuticular lipid amount did not vary between xeric and mesic *Drosophila* species; and therefore showed no correlation with habitats or water loss rate (Gibbs *et al.*, 2003). Further, similar relationships are evident between laboratory selected desiccation resistance and control strains of *D. melanogaster* (Gibbs *et al.*, 1997). However, it is not clear whether *D. nepalensis* grown at different growth temperatures have evolved changes in the amount of cuticular lipids to confer greater desiccation resistance. Further, association between cuticular permeability and quantity of cuticular lipids can be demonstrated through treatment of cuticular surfaces in over-etherised or dead insects with organic solvents such as hexane or chloroform: methanol (Hadley, 1989; Hadley and Quinlan, 1989; Hadley, 1994). This approach can be helpful in distinguishing cuticular lipids as water proofing barrier or not. There is evidence of acquisition of carbohydrates as energy reserves to alleviate the consequences of desiccation stress in laboratory selected desiccation resistant strains of *D. melanogaster* (Graves *et al.*, 1992; Gibbs *et al.*, 1997; Chippindale *et al.*, 1998; Djawdan *et al.*, 1998; Folk *et al.*, 2001; Folk and Bradley, 2005). In contrast, higher percentage of body lipid content has conferred greater survival under desiccation stress in a new set of laboratory selected desiccation resistant lines (Telonis-Scott *et al.*, 2006). It is likely that *Drosophila* species of the subgenus *Sophophora* might store and utilize similar or varying levels of energy metabolites to cope with desiccation stress.

Acclimation to desiccation stress has received lesser attention (Hoffmann, 1990, 1991; Bazinet *et al.*, 2010). It would be interesting to assess acclimation potential of *D. nepalensis* from the Western Himalayas. In the present study, we examined relative abundance of *D. nepalensis* as a function of changes in relative humidity along an altitudinal gradient in the Western Himalayas. We analyzed *D. nepalensis* for desiccation-related traits and investigated effects of developmental phenotypic plasticity (15° versus 25°C growth temperatures) on desiccation-related traits as well as energy metabolites. We tested three different routes of water balance that may result in differences of desiccation resistance. We assessed whether higher desiccation potential is associated with greater

storage of energy metabolites. Finally, we examined the desiccation acclimation potential for evolved physiological mechanisms that may affect its adaptations to drier climatic conditions.

Materials and Methods

Collections and cultures

D. nepalensis (n = 150–300) were collected in a single trip during autumn in October, 2008 from five altitudinal localities of the western Himalayas (Figure. 1). Wild-caught individuals of a midland locality (Solan, 1440 m; 30.55°N) were used to initiate 20 isofemale lines (for all analyses, 20 lines with 10 replicates each were used unless otherwise indicated). All cultures were maintained at low density (60–70 eggs per vial; egg vials were 40 × 100 mm in size) on cornmeal-yeast-agar medium at 15°, 21°, and 25°C and 65 ± 1% relative humidity in a temperature- and humidity-controlled incubator for five generations before experimental analysis. All assays were performed on 7-day-old female 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.*, 2008a). Climatic data for thermal variables and relative humidity were obtained from the Indian Meteorological Department, Government of India, New Delhi. Percent abundance was estimated as the number of individuals of a particular *Drosophila* species divided by the total number of individuals of all the different *Drosophila* species in the samples collected from a given locality.

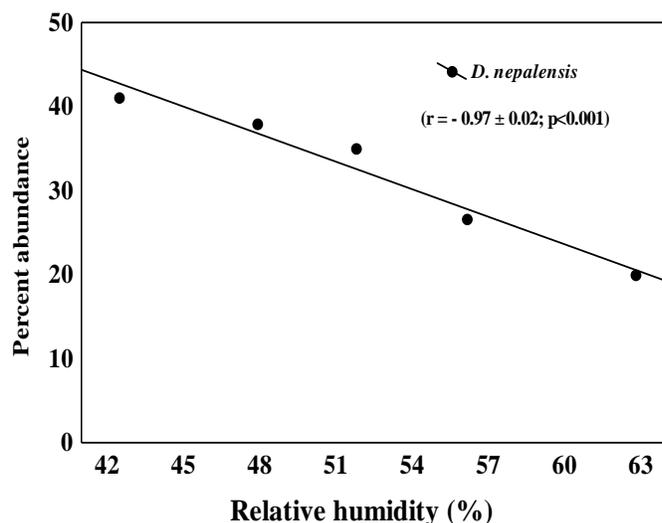


Figure 1. Regression analysis of percent abundance as a function of relative humidity (RH%) of the origin of five altitudinal populations of *D. nepalensis*. Populations include (altitude; RH%): (1) Parwanoo (512m; 62.8%); (2) Bhuntar: (1096m, 56.2%); (3) Solan (1440m, 51.8%); (4) Barog: (1680m, 47.9%); (5) Kasuali (1951m, 42.5 %).

Trait analysis

We used 10 individuals of each replicate (10 replicates × 20 isofemale lines each) to quantify body melanisation, epicuticular lipid mass, desiccation resistance, multiple measures of water balance, and levels of energy metabolites. For flies grown at 15°, 21°, and 25°C, we tested desiccation-related traits at their respective growth temperature, *i.e.*, at 15°, 21°, and 25°C, respectively. Therefore, growth temperature and experimental temperature was same in our experimental setup.

Analysis of body melanisation

The progeny of each isofemale line was examined for differences in body melanisation patterns on the abdominal segments. Body melanisation of individual female flies (n = 20 I.F. lines × 10 replicates per isofemale line) was visually scored with Olympus stereo zoom microscope SZ-61 (www.olympus.com). It was estimated from dorsal as well as lateral views of the female abdomen giving values ranging from 0 (no melanisation) to 10 (complete melanisation) for each of the six

abdominal segments (2nd to 7th). Further, the relative size of each abdominal segment was calculated in proportion of the largest 4th abdominal segment, which was assigned the value of 1.0. Since the abdominal segments differ in size, these relative sizes (*i.e.*, 0.86, 0.94, 1.0, 0.88, 0.67, and 0.38 for 2nd to 7th segments, respectively) were multiplied with segment-wise melanisation scores. Data on percent melanisation were calculated as $(\Sigma \text{ observed weighted melanisation scores of abdominal segments per fly} / \Sigma \text{ relative size of each abdominal segment} \times 10 \text{ per fly}) \times 100$ (Parkash *et al.*, 2008).

Assessment of cuticular lipid mass

We assessed cuticular lipid mass in individual adult (20 I.F lines \times 10 replicates each) reared at 15°, 21°, and 25°C. Each individual was dried overnight at 60°C to get dry mass, *i.e.*, devoid of body water. Each dried individual was kept in HPLC-grade hexane in 2 ml eppendorf tube (www.tarsons.in) for 3 minutes and, thereafter, it was removed from the solvent and was again dried at room temperature and finally reweighed on a Sartorius microbalance (Model-CPA26P; 0.001 mg precision; www.sartorius.com). Cuticular lipid mass per cm² was calculated as the difference in mass following solute extraction divided by surface area (cm²).

Desiccation resistance

Desiccation resistance was measured as the time to lethal dehydration (LT₁₀₀) effect under dry air. Seven day old flies were separated out and placed individually in dry plastic vials (40 \times 100 mm) in which the open end was covered with muslin cloth. These vials were kept on top of another vial containing 2 g of silica gel at the bottom. Finally, this apparatus was made airtight with Parafilm and kept in the desiccator chamber (Secador electronic desiccator cabinet; www.tarsons.in), which maintained 0–5% relative humidity. Number of immobile flies was counted after every one hour interval, and LT₁₀₀ values in dry air were recorded.

Basic measures of water balance

To estimate total body water content and dehydration tolerance (%), 10 flies of each isofemale line (20 I.F lines \times 10 replicates each) were used. First, individual flies were weighed on Sartorius microbalance (Model-CPA26P; 0.001 mg precision) and then reweighed after drying overnight at 60°C. Total body water content was estimated as the difference between mass before and after drying at 60°C. Further, after mild anesthesia (one minute) 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 formula $(\text{wet body mass} - \text{body mass at death}) / (\text{wet body mass} - \text{dry body mass}) \times 100$ (Gibbs *et al.*, 1997). For calculation of the rate of water loss, we followed Wharton's method (1985). Total body water content (m) was calculated as the difference between wet (f) and dry mass (d), *i.e.*, $m = f - d$. Individual flies were weighed and placed at 0-5% relative humidity for a specified time at one hour 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 (Wharton, 1985) $m_t = m_0 e^{-k_t}$, where m_t is the water mass at time t , and m_0 is the initial water content. Rate (k_t) is the slope of the regression line and was expressed as % per hour.

Assessment of extractable hemolymph content

An individual adult fly was placed on a paper towel and cleaned with distilled water followed by air drying for 2 minutes. The dry individual was 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 observing through a stereo-zoom microscope (SZ-61; www.olympus.com). The leaking extractable hemolymph was absorbed with an absorbent tissue

moistened with an isotonic saline solution (Folk *et al.*, 2001). Hemolymph content was estimated as reduction in mass following hemolymph blotting (Cohen *et al.*, 1986; Hadley, 1994). 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.

Assessment of desiccation acclimation responses

To measure pretreatment duration, 10 adult individuals of each replicate (20 I.F lines \times 10 replicates each) were subjected to desiccation stress at ~0-5% relative humidity. The initial body water content in each replicate group was recorded. The time period in which flies lost ~15–17% body water was considered as the pre-treatment time duration. Further, for the recovery period, individuals were placed on laboratory food till the original mass was regained. Such individuals were subjected to desiccation stress until death in order to test the increased desiccation resistance due to acclimation. Thus, absolute acclimation capacity (increased desiccation survival hours) was calculated by subtracting the desiccation resistance (h) of non-acclimated (control) from desiccation resistance (h) of acclimated individuals. Control and treatment experiments were run simultaneously under identical experimental conditions.

Analysis of body lipid content

Individual adult flies were dried in 2 ml eppendorf tubes (www.tarsons.in) at 60°C for 48 h and then weighed on Sartorius microbalance (Model-CPA26P; 0.001 mg precision; www.sartorius.com). Thereafter, 1.5 ml di-ethyl ether was added in each eppendorf tube and kept for 24 h under continuous shaking (200 rpm) at 37°C. Finally, the solvent was removed and individuals were again dried at 60°C for 24 h and reweighed. Lipid content was calculated per individual by subtracting the lipid free dry mass from initial dry mass per fly.

Estimation of trehalose and glycogen

For trehalose and glycogen content estimation, 10 adult flies of each isofemale line were homogenized in a homogenizer (Labsonic@ M; www.sartorius.com) with 300 μ l Na₂CO₃ and incubated at 95°C for 2 hours to denature proteins. An aqueous solution of 150 μ l acetic acid (1M) and 600 μ l sodium acetate (0.2M) was mixed with the homogenate.

Thereafter, the homogenate was centrifuged (Fresco 21, Thermo-Fisher Scientific, Pittsburgh, USA) at 12000 rpm (9660 \times g) for 10 minutes. This homogenate was used for independent estimations of trehalose and glycogen as given below. For trehalose estimation, aliquots (200 μ l) 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, Shimadzu Scientific Instruments, Columbia, 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 50 μ l aliquot was incubated with 500 μ l *Aspergillus niger* glucoamylase solution (8.7 U/ml in 200 mM of acetate buffer) for 2 hours at 40°C with constant agitation, and the suspension was centrifuged at 4000 rpm (1073 \times g) for 5 minutes. 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 μ l of supernatant from the suspension and added with 170 μ l of a mixture of G6-DPH (0.9 U/ml); ATP (1.6mM); and NADP (1.25mM) in triethanolamine hydrochloride buffer (380mM TEA-HCl and 5.5mM of MgSO₄) and 10 μ l of

Hexokinase solution (32.5 U/ml in 3.2M ammonium sulphate buffer), and absorbance was measured at 340nm.

Protein assay

Protein levels were determined by using the bicinchoninic acid method as followed by Gibbs and coworkers (Marron *et al.*, 2003). For protein assay, 10 female flies per isofemale line ($n = 10$ replicates \times 20 I.F. lines of each species) were homogenized in 3 ml distilled water and centrifuged at 10000 rpm for 5 minutes. Further, 50 μ l of aliquot was taken from supernatant and treated with 2 ml of Sigma BCA reagent and incubated at 25°C for 12 hours. Absorbance was recorded at 562 nm, and protein concentration was determined by comparing with standard curve.

Energy metabolites and energy budget

We measured each energy metabolite (carbohydrates, body lipids, or proteins) in multiple replicate sets of isofemale lines (20 I.F. lines \times 10 replicates each) in *D. nepalensis* reared at 15°, 21°, and 25°C. Total energy budget was calculated using standard conversion factors following Schmidt-Nielsen (1990).

Statistical analyses

For each trait, mean values (\pm S.E.; 20 isofemale lines, 10 replicate each) were used for illustrations and tables. Effects of developmental temperatures (15°, 21°, and 25°C) on desiccation-related traits, energy metabolites, body weight, basic measures of water balance, and dehydration tolerance were compared with ANOVA. Pearson's correlation coefficients were calculated on the basis of isofemale line data (10 I.F. lines \times 10 replicates each). For multiple comparisons, alpha value was adjusted with Bonferoni corrections. Energy contents due to carbohydrates, lipids, and proteins of adults were calculated using standard conversion factors (Schmidt-Nielsen, 1990; Marron *et al.*, 2003). Statistica (Statsoft Inc., Release 5.0, Tulsa, OK, USA) was used for calculations as well as illustrations.

Results

Data on percent abundance of wild-caught flies of *D. nepalensis* from five altitudinal localities (512–1951 m) as a function of relative humidity of origin of populations are shown (Figure 1). *D. nepalensis* is more abundant (~42.5%) in highland localities but occurs less frequently in lowland localities (~21%). The highland localities are moderately colder and drier ($T_{ave} = 15.2^\circ\text{C}$; RH = 42.5%), while lowland localities are warm and less desiccating ($T_{ave} = 27.6^\circ\text{C}$; RH = 62.8%). Therefore, significant reduction in T_{ave} (~2°C per 200 m) as well as relative humidity (~3.9% per 200 m) along an elevational gradient may act as selection factors for affecting its relative abundance. Thus, *D. nepalensis* is better adapted under colder and drier conditions in highland localities.

Comparison of plastic effects for desiccation-related traits data on desiccation resistance and energy metabolites due to growth temperatures, *i.e.*, (15°, 21°, and 25°C) in *D. nepalensis* are shown (Table 1). Body melanisation increased ~1.5-fold at 15°C and ~15-fold at 25°C from mid thermal range temperature (Figure 2a), but no change in epicuticular mass due to thermal plastic effects (melanisation: $p < 0.001$; epicuticular lipids: 15°C = $22.10 \pm 0.37 \mu\text{g cm}^{-2}$; 25°C = $22.00 \pm 0.27 \mu\text{g cm}^{-2}$) in *D. nepalensis*. We observed ~1.5-fold higher desiccation resistance at 15°C and ~3-fold lower desiccation resistance at 25°C from mid thermal range temperature, *i.e.*, 21°C (Table 1 and Figure 2b). Further, trehalose content was significantly higher (1.45-fold) at 15°C (0.131 ± 0.004 mg) than 21°C (0.090 ± 0.002 mg) and significantly lower (1.42-fold) at 25°C (0.063 ± 0.004 mg)

than 21°C (0.090 ± 0.002 mg) ($F_{2, 18} = 195.43$; $p < 0.001$). In contrast, our results did not evidence significant changes in the levels of proteins but in glycogen content (Table 2). Thus, plastic responses for desiccation related traits differ in *D. nepalensis*.

Table 1. Data (mean \pm S.E.) on cuticular components (cuticular melanisation and cuticular lipids), desiccation resistance hours, water balance related traits, and dehydration tolerance in adult flies ($n = 20$ I.F lines \times 10 replicates) of *D. nepalensis* grown at 15°C, 21°C, and 25°C (plastic effects). Trait values for each species grown at 15°C, 21°C, and 25°C were compared as ratio (fold-differences) and with ANOVA (F -values).

Traits	15°C	21°C	25°C	Ratio of 15°C vs. 21°C	Ratio of 21°C vs. 25°C	$F_{2, 18}$
1. Melanisation (%)	90.00 \pm 2.01	60.00 \pm 1.08	4.02 \pm 0.51	1.50	14.93	789.89***
2. Epicuticular lipids ($\mu\text{g cm}^{-2}$)	22.10 \pm 0.37	22.05 \pm 0.30	22.00 \pm 0.27	1.00	1.00	0.32ns
3. Desiccation hours	55.11 \pm 1.78	40.07 \pm 1.58	14.01 \pm 0.29	1.37	2.86	392.55***
4. Wet weight (mg fly^{-1})	2.265 \pm 0.03	1.516 \pm 0.05	1.075 \pm 0.02	1.494	1.410	254.63***
5. Dry weight (mg fly^{-1})	0.679 \pm 0.006	0.455 \pm 0.004	0.322 \pm 0.003	1.492	1.413	199.50***
6. Total water content (mg fly^{-1})	1.586 \pm 0.01	1.061 \pm 0.005	0.753 \pm 0.008	1.494	1.409	230.47***
7. Hemolymph content (mg fly^{-1})	0.747 \pm 0.006	0.501 \pm 0.008	0.355 \pm 0.003	1.491	1.411	165.98***
8. Hemolymph water content (mg fly^{-1})	0.523 \pm 0.004	0.350 \pm 0.001	0.249 \pm 0.002	1.494	1.405	286.33***
9. Tissue water content (mg fly^{-1})	1.063 \pm 0.003	0.711 \pm 0.003	0.504 \pm 0.003	1.495	1.410	268.94***
10. Dehydration tolerance (%)	82.09 \pm 0.05	82.18 \pm 0.02	82.07 \pm 0.04	1.001	1.001	1.86ns

Data were arcsin transformed for ANOVA.
ns – nonsignificant, *** $P < 0.001$.

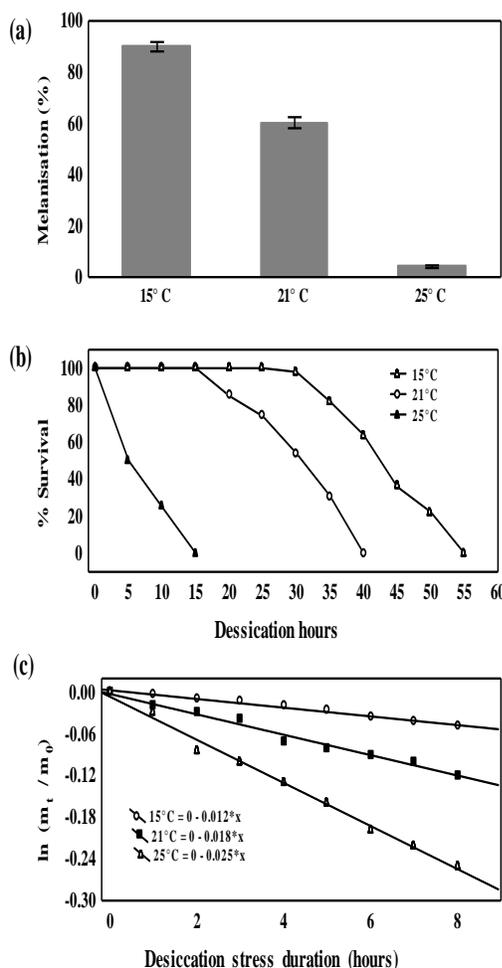


Figure 2. A comparison of melanisation, desiccation hours, and water loss rate (according to Wharton's method) in adult flies of *D. nepalensis* grown at 15, 21, and 25°C. The water loss rate was derived from the slope (b) of $\ln(m_t / m_0)$ as a function of different durations of desiccation stress at $< 5\%$ relative humidity. Slope values for rate of water loss vary significantly between species when grown at 15, 21, and 25°C.

Analysis of trait variability

We used ANOVA for partitioning % variance in three desiccation-related traits (desiccation resistance, cuticular lipid mass, and total carbohydrate content) in 20 isofemale lines (20 I.F lines \times 10 replicates) in *D. nepalensis* grown at 15°, 21°, and 25°C (Table 3). Interestingly, the results of ANOVA for all the three desiccation-related traits showed similar levels of variability in 7 day old adult flies. The percent variance for desiccation resistance and carbohydrate content were 72.90% and 60.12% due to growth temperatures, isofemale lines, and interaction effects, respectively. However, for cuticular lipid mass, adult flies have shown 0.01% and 0.02% non significant variability due to growth

temperatures, isofemale lines, and their interactions, respectively. Thus, we found major differences in desiccation-related traits due to growth temperatures (Table 3).

Table 2. Comparison of energy budget at 15, 21, and 25°C in *D. nepalensis*. Data are from 20 isofemale lines, 10 replicates each.

Metabolites	15°C	21°C	25°C	Fold difference due to 15°C vs. 21°C	Fold difference due to 2°C vs. 25°C
1. Carbohydrates	3.537	2.411	1.689	1.47	1.43
2. Lipids	3.615	2.397	1.689	1.51	1.42
3. Proteins	0.925	0.925	0.925	1.00	1.00
4. Total	8.077	5.733	4.303	1.41	1.33

Conversion factors: 17.6 Jmg⁻¹ for carbohydrates, 39.3 Jmg⁻¹ for lipids and 17.8 Jmg⁻¹ for proteins. (Schmidt and Nielsen, 1990; Marron et al., 2003).

Table 3. Analysis of variance (n = 20 I.F × 10 replicates each) for explaining trait variability due to growth temperatures (T), isofemale lines and their interactions in *D. nepalensis*.

	df	Temperature (T) 2	IF lines 19	IF × T 38	Error 540
1. Desiccation resistance	MS	130068.18	372.33	101.41	0.32
	F	1282.59	1163.53	316.91	
	% Var	72.90***	7.93***	2.16***	0.13
2. Cuticular lipid mass	MS	16.38	7.74	9.64	0.95
	F	6.04	2.85	1.94	
	% Var	0.01ns	0.02ns	0.01ns	0.94
3. Carbohydrate content	MS	104573.32	587.73	137.77	2.01
	F	759.04	292.40	68.54	
	% Var	60.12***	12.89***	3.01***	0.87

ns = nonsignificant; ****P* < 0.001.

Comparison of rate of water loss

We used nine sets of independent experiments to determine changes in the rate of body water loss in control *versus* flies exposed to different durations (1 to 8 h) of desiccation stress in *D. nepalensis* reared at 15°, 21°, and 25°C; and the data are shown in Figure 2b. Comparison of slope values (Wharton's method) has shown a significant increase in rate of water loss in adult flies (Figure 2c) grown at 25°C than 21° and 15°C. These observations on rate of water loss suggest greater desiccation resistance of *D. nepalensis* when grown at 15°C.

Effects of growth temperature on acclimation potential

We tested whether *D. nepalensis* show similar or different desiccation acclimation responses to prior treatment of desiccation stress when compared across three growth temperatures (15°, 21°, and 25°C). Interestingly, similar trends for acclimation effects at 15° and 21°C but varied in trait values. *D. nepalensis*, reared at 15°C showed significant increase in desiccation resistance (net

increase in desiccation resistance due to acclimation: 8.48 ± 0.38 h) and corresponding values at 21°C were significant (6.00 ± 0.05 h; Figure 3a), *i.e.*, *D. nepalensis* showed higher acclimation response at both 15°C and 21°C (Figure 3), and at 25°C it shows negligible response. However, we did not find any significant change in the cuticular lipid mass as a consequence of desiccation acclimation across three growth temperatures (Figure 3b). Interestingly, we observed a non-significant reduction in rate of water loss due to acclimation across the growth temperatures (Figure 3c). Thus, our results suggest that *D. nepalensis* responds to acclimation for desiccation stress at lower growth temperature.

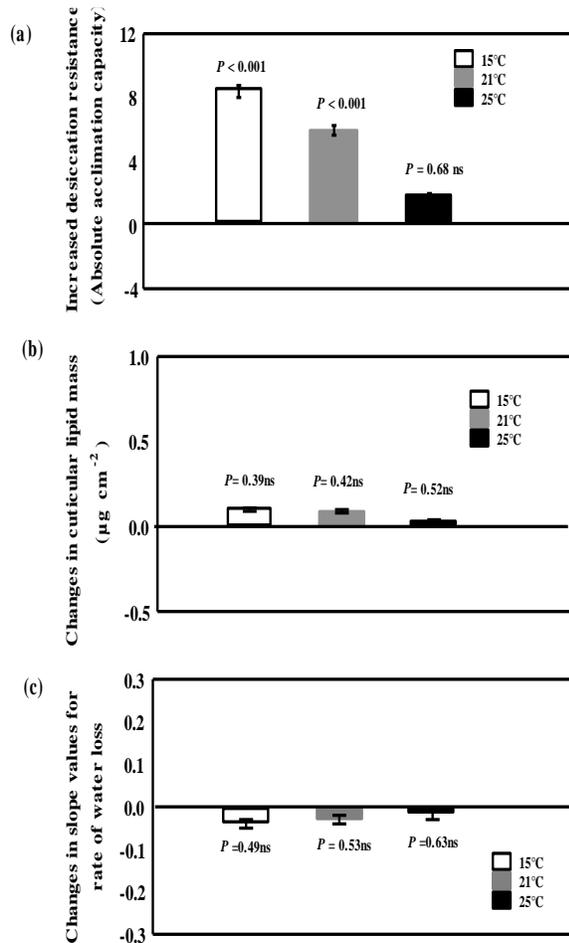


Figure 3. Changes in desiccation related traits due to acclimation of adult flies of *D. nepalensis* grown at 15, 21, and 25°C . Changes in trait values are shown for desiccation resistance (a), cuticular lipid mass (b), and rate of water loss (c). For (a), absolute acclimation capacity = desiccation hours of acclimated – non-acclimated adults.

Differences in basic measures of water balance and dehydration tolerance

A comparative analysis of body mass has shown consistent differences in multiple measures of body weight as well as body water content in *D. nepalensis* (wet mass: $F_{2, 18} = 254.63$; $p < 0.001$; dry mass: $F_{2, 18} = 199.50$; $p < 0.001$; body water content: $F_{2, 18} = 230.47$; $p < 0.001$) when grown at three different temperatures, *i.e.*, 15° , 21° , and 25°C . Hemolymph content and hemolymph water content have shown ~ 2 -fold increase and decrease at 15°C than 25°C , respectively, as compared to 21°C (Figure 4). We observed ~ 2 fold increase in tissue water due to variation in growth temperatures ($F_{2, 18} = 268.94$, $p < 0.001$). However, dehydration tolerance did not change significantly due to variation in growth temperatures at 15°C and 25°C as compared with 21°C ($F_{2, 18} = 1.86$, ns).

Correlation between desiccation resistance and energy budget

Data on differences in the storage of desiccation resistance and energy budget in *D. nepalensis* when reared at 15° , 21° , and 25°C are shown (Tables 1 and 2), respectively. We calculated energy budget due to carbohydrates (which are actually consumed under desiccation stress) by using standard conversion factors (Schmidt-Nielsen, 1990), and the data for adult flies are shown (Figure 5c). The energy budget due to stored carbohydrates is about 30-50% higher at 15°C than 21°C (Table 2). Further, storage levels of carbohydrates are about 30-50% lower at 25°C than 21°C . We found significant correlations between carbohydrates energy budget (J/mg) and desiccation resistance (Figure 5c) of *D. nepalensis* grown at 15° , 21° , and 25°C . Thus, there are significant correlations between carbohydrate energy budget and desiccation resistance.

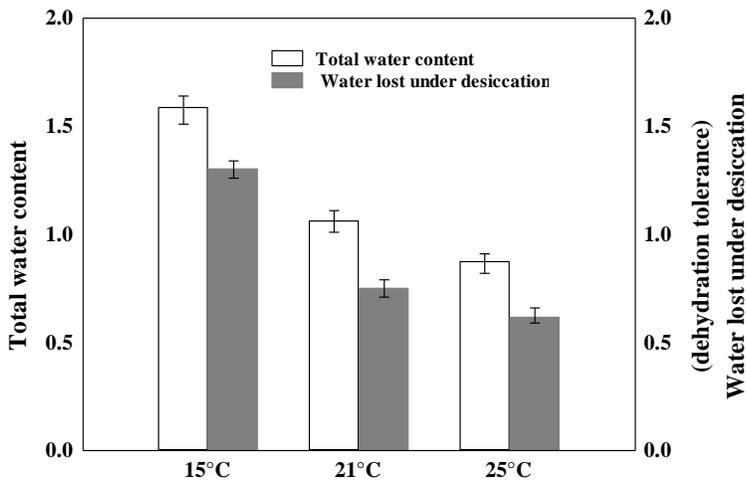


Figure 4. Bars (mean ± SE) represent differences in total body water content and dehydration tolerance in *D. nepalensis* reared at 15, 21, and 25°C. There is higher total water content as well as dehydration tolerance in *D. nepalensis* at colder growth temperature as compared to warmer growth temperature.

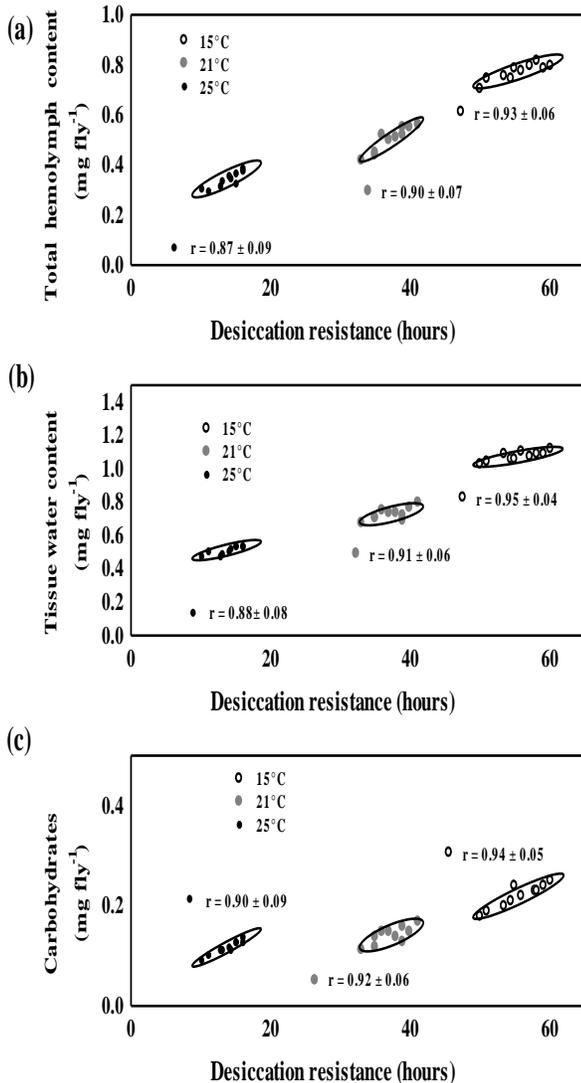


Figure 5. Trait correlations are based on 10 isofemales lines (10 replicates) of *D. nepalensis*. Desiccation resistance is positively correlated with Hemolymph (a), Tissue water content (b), and Carbohydrate (c). Correlation coefficients are shown ± s.e.m.

Discussion

In the present study, we found significant differences in desiccation-related traits in *D. nepalensis* across three growth temperatures that differ in their abundance under field conditions. Interestingly, there are developmental plastic effects for body melanisation. For *D. nepalensis*, plastic response for body melanisation is consistent with higher desiccation potential. Further, differences in desiccation resistance due to developmental plasticity match significant increase in the body mass (wet and dry mass), body water content, and hemolymph water when reared at 15°C as compared to 21°C. We also observed a significant effect of growth temperatures on the storage of carbohydrates. For example, there was a lower storage level of carbohydrates when grown at 25°C, whereas the reverse trend was evident at 15°C. Finally, we found that absolute desiccation

acclimation capacity was quite low in *D. nepalensis* reared at 25°C, and this might reflect its future vulnerability under global climate warming.

Role of cuticular lipids

Insect cuticle is a complex structure and its components might vary between species and populations (Willmer *et al.*, 2000). Several studies have shown variable cuticular permeability due to changes in the composition or amount of cuticular lipids in diverse insect taxa (Edney, 1977; Toolson, 1984; Hadley, 1994; Rourke, 2000). However, no previous study has examined developmental plastic effects (due to growth temperatures) on cuticular lipid mass. In the present work, we observed negligible increase in the cuticular lipid mass of adults reared at 15°, 21°, and 25°C. In contrast, developmental plastic effects for cuticular melanisation are evident. Thus, we found a decrease in cuticular permeability due to single component (cuticular melanisation). As evident (Figure 4b), we may argue that cuticular lipids are not contributing to the total desiccation survival hours.

Plastic changes for hemolymph content and dehydration tolerance

In insects, hemolymph is a major source for changes in higher level of body water to support longer survival under dehydration stress (Hadley, 1994; Chown and Nicolson, 2004; Folk *et al.*, 2001; Folk and Bradley, 2005). In contrast, several studies on wild populations of various *Drosophila* species have not considered changes in hemolymph content to enhance survival under desiccation stress (Gibbs and Matzkin, 2001; Gibbs *et al.*, 2003; Parkash *et al.*, 2010). In the present study, we found changes in hemolymph content as a consequence of developmental plastic effects (*i.e.*, 15°, 21°, and 25°C). *D. nepalensis* has shown changes in hemolymph content consistent with their different levels of desiccation resistance potential. Further, most arthropods can tolerate ~30-50% loss of body water, but some taxa adapted to drier habitats have evidenced higher dehydration tolerance (Hadley, 1994; Willmer *et al.*, 2000; Benoit *et al.*, 2005). In the present study, we found increased dehydration tolerance at 15°C as compared with 21°C, which is consistent with differences in desiccation resistance. Therefore, dehydration tolerance has evolved as a common physiological mechanism to support survival under desiccation stress in *D. nepalensis*.

Differences in the storage of energy metabolites

The acquisition of greater energy reserves has been associated with increased survival under dehydration stress (Gibbs 2002; Chown and Nicolson 2004). Laboratory selected desiccation resistant lines (D) have shown higher storage of carbohydrates as compared with control (Graves *et al.*, 1992; Gibbs *et al.*, 1997; Djawdan *et al.*, 1998; Chippindale *et al.*, 1998; Folk *et al.*, 2001; Folk and Bradley, 2005). In contrast, a new set of laboratory selected desiccation resistant lines has shown increased lipid content in selected (D) lines when compared with control (Telonis-Scott *et al.*, 2006). Therefore, results of laboratory selection experiments are not consistent whether carbohydrates or lipids support survival under desiccation stress. Further, wild *Drosophila* species from xeric and mesic habitats vary in desiccation resistance, despite lack of differences in the storage of energy metabolites (Marron *et al.*, 2003). Further, no previous study has examined changes in the storage levels of energy metabolites due to thermal plastic effects. In the present study, we observed higher levels of carbohydrates when *D. nepalensis* reared at 15° than 21°C and lower levels of carbohydrates when *D. nepalensis* reared at 25° than 21°C, which is in agreement with differences in their desiccation resistance at different growth temperatures. Thus, *D. nepalensis* have stored higher levels of carbohydrates (15°C) to alleviate the effects of desiccation stress. In contrast, low storage

level of carbohydrates at 25°C is consistent with significantly lower desiccation resistance. Our results suggest that storage of energy metabolites is constrained by growth temperatures.

Acclimation potential

Ectothermic organisms are capable of increasing their stress resistance level due to prior exposure of few or more bouts of thermal stresses (Bale, 2002; Hoffmann *et al.*, 2003). In *Drosophila* species, there are a few studies which have shown increase in desiccation resistance due to prior treatment of non-lethal level of desiccation stress in two Australian populations of *D. melanogaster* and *D. simulans* (Hoffmann, 1991) and in one Canadian population of *D. melanogaster* (Bazinet *et al.*, 2010). Two pairs of sibling species grown at 25°C (*D. serrata* versus *D. birchii*; and *D. melanogaster* versus *D. simulans* from Australia) have shown species-specific differences in the acclimation to desiccation stress (Hoffmann, 1991). In that study, both *D. melanogaster* and *D. simulans* from Cairns (Australia) showed increased desiccation resistance due to acclimation to desiccation stress. Thus, higher acclimation capacity was evident in adults of *D. simulans* as compared with *D. melanogaster* (Hoffmann, 1991). However, the effects of acclimation at ecologically relevant growth temperatures have not been considered in any *Drosophila* species so far. In the present study, we found higher acclimation capacity in *D. nepalensis* (~8.4 h) and (~6 h) when grown at 15° and 21°C, respectively. In contrast, at 25°C, it showed negligible acclimation response. Our results suggest that contrasting levels of acclimation capacity are constrained by their basal levels of desiccation resistance at different growth temperatures. *D. nepalensis* at warmer growth temperature have shown lower desiccation potential as well as lower acclimation response as compared with colder growth temperatures. Thus, *D. nepalensis* can be vulnerable under global climate warming. Therefore, acclimation to drought conditions is adaptive for *D. nepalensis* only with its potential varies according to growth temperatures. *D. nepalensis* is a stenothermal species and physiologically adapted to a narrow range of developmental temperatures. Thermal sensitivity has the potential to influence ecology and fitness of a species. Several investigations have shown the role of thermal plastic effects on quantitative traits for adaptations to temporal and spatial changes in climatic conditions (Willott and Hassall, 1998; Mousseau *et al.*, 2000). In the present study, *D. nepalensis* has shown significant plastic effects for traits. The evolutionary changes in these traits can be explained on the basis of climatic selection of trait variability. In *D. nepalensis*, trait values are significantly reduced at 25°C, which can limit its occurrence at higher temperatures. Thus, due to global warming, the cold adapted species *D. nepalensis* has retracted from low to midland localities as a consequence of plasticity. In conclusion, the observed range changes of *D. nepalensis* have resulted as a consequence of plasticity in traits under global warming effects at the northern border limits.

Temperature differences are sufficiently pronounced in various geographical regions. Hence, we might predict a positive correlation between various traits and adaptation to different geographical regions. Phenotypic plasticity in Drosophilids is still insufficiently investigated but is relevant to understanding natural population ecology. Temperate and tropical species differ significantly in the thermal range at which they can develop under laboratory conditions. The associations of various traits with the environments suggest that such traits may present the adaptive characteristics underlying the diversification and distribution of *Drosophila* species. The present study suggests that *Drosophila* species respond to climate change by shifting their distribution range, changing in abundance and physiology.

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