The Journal of Experimental Biology 216, 3301-3313

doi:10.1242/jeb.087650

RESEARCH ARTICLE

Sex-specific divergence for adaptations to dehydration stress in *Drosophila kikkawai*

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SUMMARY

Several studies on diverse *Drosophila* species have reported higher desiccation resistance of females, but the physiological basis of such sex-specific differences has received less attention. We tested whether sex-specific differences in cuticular traits (melanic females and non-melanic males) of *Drosophila kikkawai* correspond with divergence in their water balance mechanisms. Our results are interesting in several respects. First, positive clinal variation in desiccation resistance was correlated with cuticular melanisation in females but with changes in cuticular lipid mass in males, despite a lack of differences between the sexes for the rate of water loss. Second, a comparative analysis of water budget showed that females of the northern population stored more body water as well as hemolymph content and exhibited greater dehydration tolerance than flies from the southern tropics. In contrast, we found no geographical variation in the males for water content and dehydration tolerance. Third, an ~10-fold increase in the rate of water loss after organic solvent treatment of male *D. kikkawai* suggested a role of cuticular lipids in cuticular transpiration, but had no effect in the females. Fourth, geographical differences in the storage of carbohydrate content (metabolic fuel) were observed in females but not in males. Interestingly, in females, the rate of utilization of carbohydrates did not vary geographically, but males from drier localities showed a 50% reduction compared with wetter localities. Thus, body melanisation, increased body water, hemolymph, carbohydrate content and greater dehydration tolerance confer greater desiccation resistance in females, but a reduced rate of water loss is the only possible mechanism to cope with drought stress in males. Finally, acclimated females showed a significant increase in drought resistance associated with higher trehalose content as well as dehydration tolerance, while males showed no acclimation response. Thus, sex-specific differences in desiccation resistance of *D. kikkawai* are associated with divergence in some water balance strategies, despite a lack of differences in the rate of water loss between the two sexes.

Key words: drought resistance, sex-specific divergence, cuticular traits, energy metabolites, acclimation, *D. kikkawai*.

Received 1 March 2013; Accepted 7 May 2013

INTRODUCTION

Insects occupy a wide range of ecologically diverse niches and have evolved different physiological adaptations to ensure survival. There are three main ways in which insects can increase their resistance to desiccation: (1) increasing their total body water content, (2) reducing the rate of body water loss and (3) tolerating a larger proportion of overall water loss from the body (Hadley, 1994). Several studies on *Drosophila* species from temperate and tropical regions have shown a higher desiccation resistance of females than males, but the physiological basis of such sex-specific differences have received less attention (Gibbs and Matzkin, 2001; Gibbs et al., 2003; Matzkin et al., 2009). Further, it is likely that sexes may differ in body water content, rate of water loss and dehydration tolerance, but such possibilities have not been tested for geographical populations of diverse *Drosophila* species. However, at the interspecific level, analysis of water balance of 29 *Drosophila* species from xeric and mesic environments showed a negative correlation between water content and desiccation resistance in the males but a non-significant correlation for females (Gibbs and Matzkin, 2001). These observations on wild *Drosophila* species are in marked contrast to differences in water content between laboratory-selected desiccation-resistant and control lines of *D. melanogaster* (Gibbs et al., 1997). The laboratory-selected desiccation-resistant lines showed sexual dimorphism for desiccation-related traits, i.e. desiccation-resistant males showed no changes in dry mass but 15% more wet mass and 2.7-fold higher desiccation resistance as compared with control lines. In contrast, females of desiccation-selected lines exhibited significant changes in dry mass (11%) and wet mass (25%) and an approximately fourfold increase in desiccation resistance (Gibbs et al., 1997). Thus, sexes differ for water-balance-related traits when *D. melanogaster* populations are subjected to laboratory selection. However, most studies on interspecific and/or intraspecific variation for water balance traits have not considered both sexes (Hadley, 1994; Folk et al., 2001; Parkash et al., 2011; Parkash et al., 2012a).

In diverse insect taxa, body water loss occurs through three main routes: cuticular transpiration, respiration and excretion. Cuticular transpiration accounts for >80% of total body water loss in most insects, while losses due to respiration or excretion are quite low. The composition and/or amount of surface lipids can vary significantly at population and species levels and are associated with reduction in water loss, e.g. in the tenebrionid beetle *Eleodes armatus* (Hadley, 1977), the scorpion *Centruroides sculpturatus* (Toolson and Hadley, 1979), tiger beetles (Hadley and Schultz, 1987) and the grasshopper *Melanoplus sanguinipes* (Rourke, 2000), but only in a few drosophilids – *Zaprionus indianus* (Parkash et al., 2008a), *Drosophila busckii* (Parkash et al., 2011) and *D. nasuta* (Parkash et al., 2012a). For integumentary water loss, the role of epicuticular

lipids in water conservation has also been made apparent by the fact that removal of surface lipids with organic solvents can result in increased water loss rates from 10- to 100-fold (Hadley, 1994), e.g. in the black widow spider *Latrodectus hesperus* (Hadley and Quinlan, 1989), the cricket *Acheta domesticus* (Hadley, 1989) and the larvae of the gall fly *Eurosta solidaginis* (Ramløv and Lee, 2000). In contrast, in laboratory-selected desiccation-resistant lines, there was ~50% less water loss than control lines, despite a lack of changes in the amount of surface lipids (Gibbs et al., 1997). Further, some studies on wild *Drosophila* species found no association between water loss and surface lipid amount at intra- as well as inter-population levels (Parkash et al., 2008b; Parkash et al., 2012a). However, reduction in the rate of water loss was significantly correlated with increasing cuticular melanisation in some melanic *Drosophila* species (Parkash et al., 2008b; Parkash et al., 2012a). Such observations suggest the likelihood of different mechanisms of water balance in various *Drosophila* species from diverse types of habitats.

Desiccation resistance may vary because of differences in the rate of metabolite utilization under drier conditions, but this aspect has received less attention. A single study has investigated utilization of energy metabolites (carbohydrates, lipids and proteins) as a function of different durations of desiccation stress for three mesic and two desert *Drosophila* species (Marron et al., 2003). The mass-specific stored levels of carbohydrate or lipid contents did not vary between cactophilic and mesic *Drosophila* species, but there was a sevenfold higher utilization of carbohydrates under desiccation stress (Marron et al., 2003). Further, less attention has been paid to find sex-specific differences in the storage as well as utilization of energy metabolites under desiccation stress in different *Drosophila* species.

The effect of climatic stresses may not be the same in different life stages of drosophilids because adults are mobile and can behaviorally avoid the stressful conditions by seeking out favorable microhabitats (Kimura and Beppu, 1993; Feder et al., 2000; Dillon et al., 2009). As larvae are less mobile than adults, they are more vulnerable to desiccation stress and thus their analysis is ecologically relevant. However, a single study has shown that laboratory selection did not alter resistance to desiccation in the larvae of *D. melanogaster*, despite significant changes in the adult flies (Hoffmann and Parsons, 1993). For wild *Drosophila* species, it is not known whether water-balance-related traits are correlated between larval and adult stages. Because most previous studies have focused on adult desiccation tolerance, it would be interesting to examine the possible divergence of a mechanistic basis of desiccation resistance at the larval stage.

Insects can alter their desiccation resistance through acclimation, which may affect survival potential in drier habitats (Hoffmann, 1990; Hadley, 1994). Beneficial effects of drought acclimation are evident in the arctic collembolan *Onychiurus arcticus* (Holmstrup and Sømme, 1998), the soil-dwelling springtail (Sjursen et al., 2001), *Folsomia candida* (Holmstrup et al., 2002), *Belgica antarctica* (Benoit et al., 2007) and *Cryptopygus antarcticus* (Elnitsky et al., 2008). For four *Drosophila* species, dehydration acclimation also improved desiccation resistance in females but not in males, and showed no geographical differences in acclimatory response to desiccation stress (Hoffmann, 1991). Further, the physiological basis of acclimation responses to desiccation stress for both sexes of diverse *Drosophila* species has not received much attention so far.

Drosophila kikkawai Burla 1954 belongs to the subgenus *Sophophora* and has an oriental origin because of its abundance in Southeast Asia. This species has colonized the Indian subcontinent as well as Brazil, from where it was first described (Burla, 1954). The color dimorphism between sexes was described by Ohnishi and Watanabe (Ohnishi and Watanabe, 1985). The males of *D. kikkawai* lack body pigmentation on abdominal segments, while females show abdominal melanisation. However, the ecological significance of such sex-specific differences in body color remains unknown. The two sexes are likely to involve divergent strategies to cope with climatic stresses. Our previous work on *D. kikkawai* showed opposite latitudinal clines for starvation resistance and desiccation resistance (Karan and Parkash, 1998). However, *D. kikkawai* could be a model species for testing sex-specific differences in water-balance-related traits because sexes differ in their cuticular traits. In a previous study, we did not consider differences in total water budget as well as energy metabolites of male and female larvae and adults of latitudinally varying populations of *D. kikkawai* (Parkash et al., 2010). The two sexes of *D. kikkawai* might differ in their rate of water loss, consistent with differences in their desiccation resistance, and acclimation potential to dehydration stress, but there are no data to support such contentions. Further, water balance changes have not been investigated in seasonally varying populations of *D. kikkawai*. Thus, detailed analysis of sex-specific water conservation strategies in *D. kikkawai* is relatively unstudied despite the significant divergence of cuticular traits between sexes.

In the present work, we investigated geographical variation in desiccation resistance, cuticular traits and energy metabolites in both sexes of *D. kikkawai*. We made detailed analysis of water-balance-related traits in both sexes for larvae and adults of one northern and one southern population. Changes in cuticular permeability under desiccation stress were investigated in organic-solvent-treated males and females as well as in control flies. We also examined storage and utilization of energy metabolites under desiccation stress in both sexes of both geographical populations. We assessed seasonal changes in desiccation-related traits in males and females of this species. Finally, we analysed physiological basis of drought acclimation in both sexes of *D. kikkawai*.

MATERIALS AND METHODS

Collections and cultures

Wild individuals of *D. kikkawai* (*N*=150–200 flies from each site) were collected in September 2010 using a net-sweeping method in a single trip for each of eight localities varying in latitude (latitudinal range: 8°06′–32°40′N): Trivendrum [8°06′N – southernmost location; average temperature (T_{ave})=27–29°C; average relative humidity (RH_{ave})=77–79%], Mysore (12°18′N), Mahabubnagar (16°45′N), Hirakud (21°32′N), Udaipur (24°37′N), Agra (27°11′N), Bhatinda (30°10′N) and Pathankot (32°40′N – northernmost location; T_{ave}=20–22°C; RH_{ave}=44–46%). Wild-caught females were used to initiate isofemale (IF) lines (20 lines per population). All cultures were maintained at low density (60–70 eggs per 37×100 mm vial) on cornmeal-yeast-agar medium at 21°C. Further, in order to check whether all the geographical populations belong to *D. kikkawai*, we made reciprocal crosses between virgin male and female flies of isofemale lines of northern *versus* southern populations. The genitalia of G_1 males of each isofemale line were examined to confirm species identification (Bock and Wheeler, 1972). All experiments were performed with G_6 and G_7 generations on 7-day-old virgin female and male flies. Further, wild flies (*N*=350–400 per season) were also collected in rainy (T_{ave}=25–27°C; RH_{ave}=73–76%) and winter seasons (T_{ave}=14–17°C; RH_{ave}=40–50%) from Pathankot. Climatic data for thermal variables of origin of populations were obtained from the Indian Institute of Tropical Meteorology (www.tropmet.res.in), but data on relative

humidity were obtained from climatological tables (Indian Meteorological Department, 2010).

Analysis of body melanisation

Body melanisation of individual flies from each isofemale line (N=20 IF lines per population × 10 replicates of each sex) was visually scored with an Olympus stereo-zoom microscope SZ-61 (www.olympus.com). Body melanisation was estimated from dorsal as well as lateral views of the fly abdomen, giving values ranging from 0 (no melanisation) to 10 (complete melanisation) for each of the six abdominal segments (second to seventh). Further, the relative size of each abdominal segment was calculated in proportion to the largest fourth abdominal segment, which was assigned the value of 1.0. Because the abdominal segments differ in size (i.e. 0.86, 0.94, 1.0, 0.88, 0.67 and 0.38 for the second to seventh segments, respectively), these relative sizes were multiplied by 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., 2008b). Clear differences in body melanisation patterns between subspecies helped us to distinguish *D. kikkawai* isofemale lines from other related subspecies (Bock, 1980).

Isolation of third instar larvae

Females mated once were allowed to lay eggs in culture vials (37×100 mm) for 2 h on cornmeal-yeast-agar medium at 21°C. Third instar larvae were separated on the basis of morphology of mouthparts and anterior spiracles (Ashburner, 1989). The larvae were sexed according to gonad morphology, i.e. testes being significantly larger than ovaries (Demerec and Kaufman, 1996). All assays were performed in late third instar larvae (after 144±1.03 h of egg laying) of both sexes.

Assessment of cuticular lipid mass

For estimation of cuticular lipid mass, third instar larvae and 7-day-old flies were used (N=20 IF lines per population × 10 replicates of each sex). Larvae or flies were dried overnight at 60°C to obtain dry mass, i.e. devoid of body water. Each dried fly or larva was kept in HPLC-grade hexane for 1 h and thereafter it was removed from the solvent and again dried at room temperature and finally reweighed on a Sartorius microbalance (model CPA26P, with precision 0.001 mg; www.sartorious.com). Cuticular lipid mass per centimeter squared was calculated as the difference in mass following solute extraction divided by surface area (cm^2).

Desiccation resistance

Desiccation resistance was measured as the time to lethal dehydration effect under dry air. Individual larvae or adults (N=20 IF lines per population × 10 replicates of each sex) were isolated in a dry plastic vial with 2 g of silica gel at the bottom and covered with a foam disc. Finally, the vials were placed in a desiccator chamber (Secador electronic desiccator cabinet; www.tarson.com) maintained at 0–5% relative humidity. The number of immobile individuals was counted at 1 h intervals and time period to lethal desiccation effect (LT_{100}) was recorded.

Effect of organic solvent on cuticular water loss

Changes in cuticular permeability due to organic solvents were tested on larvae as well as adult flies (N=20 IF lines per population × 10 replicates of each sex). The assays were conducted by treating over-etherized (dead) larvae or flies, either in 2 ml of hexane or chloroform:methanol (2:1), and gently vortexing them five times each for 30 s. Individuals were then blotted dry on tissue paper, weighed and placed in a desiccator chamber (Secador electronic desiccator cabinet; www.tarson.com) maintained at 0–5% relative humidity. Organic solvents vary in terms of the solubility properties of surface lipids, i.e. slower with hexane but faster with chloroform:methanol. Accordingly, the effects of hexane on water loss were monitored hourly but at 30 min intervals for chloroform:methanol. For control groups, no solvent treatment was given and cuticular water loss was determined at 2 h intervals until no further loss in body mass occurred for both sexes.

Basic measures of water balance

To estimate wet and dry body mass as well as total body water content, individual larvae of each isofemale line (N=20 IF lines per population × 10 replicates of each sex) were rinsed with distilled water and blotted dry on absorbent tissue and then weighed on a Sartorius microbalance (model CPA26P, precision 0.001 mg). Thereafter, each larva or adult was dried at 60°C for 8 and 24 h, respectively. These were reweighed after drying. Total body water content of larvae or adults is the difference in mass before and after drying at 60°C. Dehydration tolerance was estimated as the percentage of total body water lost due to desiccation (until death) 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 males and females of *D. kikkawai*, we followed Wharton's method (Wharton, 1985), modified by Benoit et al. (Benoit et al., 2005) and Yoder et al. (Yoder et al., 2009). Total body water content (m) was calculated as the difference between wet or fresh (M_f) and dry mass (M_d), i.e. $m=M_f-M_d$. Individual flies were weighed and placed at $0.00a_v$ (a_v=water vapor activity=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 the regression line from a plot of $\ln(m_t/m_0)$ against time according to Wharton's exponential equation: $m_t=m_0e^{-kt}$, 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 regression line and is expressed as percent per hour (Wharton, 1985).

Assessment of extractable hemolymph content in larvae and adults

Hemolymph content was estimated as the reduction in mass following hemolymph blotting (Cohen et al., 1986; Hadley, 1994; Folk et al., 2001). 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.

Estimation of energy metabolites

Body lipid content, proteins, trehalose and glycogen content were estimated in larvae as well as adults (N=20 IF lines per population × 10 replicates of each sex) of both sexes of *D. kikkawai* following the method of Parkash and co-workers (Parkash et al., 2012b).

Utilization of energy metabolites

We measured each metabolite in multiple replicate sets of isofemale lines (N=20 IF lines per population × 10 replicates of each sex) before and after its utilization under desiccation stress until death. Flies were subjected to different durations of desiccation stress (at 4 h intervals). Further, the rate of utilization of each metabolite was calculated as the regression slope value as a function of desiccation stress duration (Marron et al., 2003).

Assessment of desiccation acclimation responses

To measure acclimation pre-treatment time duration, 10 individuals of each sex (20 IF lines per population) were subjected to desiccation stress at ~0–5% RH. The initial body water content in replicate groups was recorded. The time period in which flies lost ~15–17% body water was assessed as the pre-treatment time duration. Further, for the recovery period, individuals were placed on food medium and tested at hourly intervals for an increase in body water until the lost body mass 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.

Statistical analyses

For each trait, population means (20 IF lines × 10 replicates of each sex) along with standard error (s.e.m.) were used for illustrations and tables. Inter- and intra-population trait variability for desiccation-related traits in eight latitudinal populations of *D. kikkawai* was examined by ANCOVA (body mass as a covariate) for both sexes. Regression analyses were used to determine clinal variation in desiccation resistance, cuticular traits and energy metabolites. Further, results of ANOVA (*F*-values) were used to compare significant differences in desiccation-related traits, basic measures of water balance or dehydration tolerance and energy metabolites in one northern and one southern population of *D. kikkawai*. Proportional data were arcsine transformed for ANOVA and ANCOVA. Kruskal–Wallis values of Dunn's multiple comparisons test (one-way ANOVA) were used to compare water loss between dead (control) and organic-solvent-treated (hexane or chloroform:methanol) larvae or adult flies of each sex. Statistica (Release 5.0, StatSoft, Tulsa, OK, USA) was used for calculations as well as illustrations.

RESULTS

Clinal variation in desiccation-related traits

Between-population differences in desiccation stress related traits were assessed through a common garden experiment by growing eight latitudinal populations of *D. kikkawai* at 21°C, which helped minimize the environmental effects and show the genetic differences. For three traits (desiccation resistance, cuticular traits and energy metabolites), we found significant differences in clinal variation. The slope values of clines for desiccation resistance do not differ between sexes, but for each population, females showed higher desiccation resistance than males (Fig. 1A). Further, females showed significant clines for melanisation and trehalose, but there was no geographical variation in the males (Fig. 1B,D). However, a cline for surface lipid amount was evident only in males (Fig. 1C). For

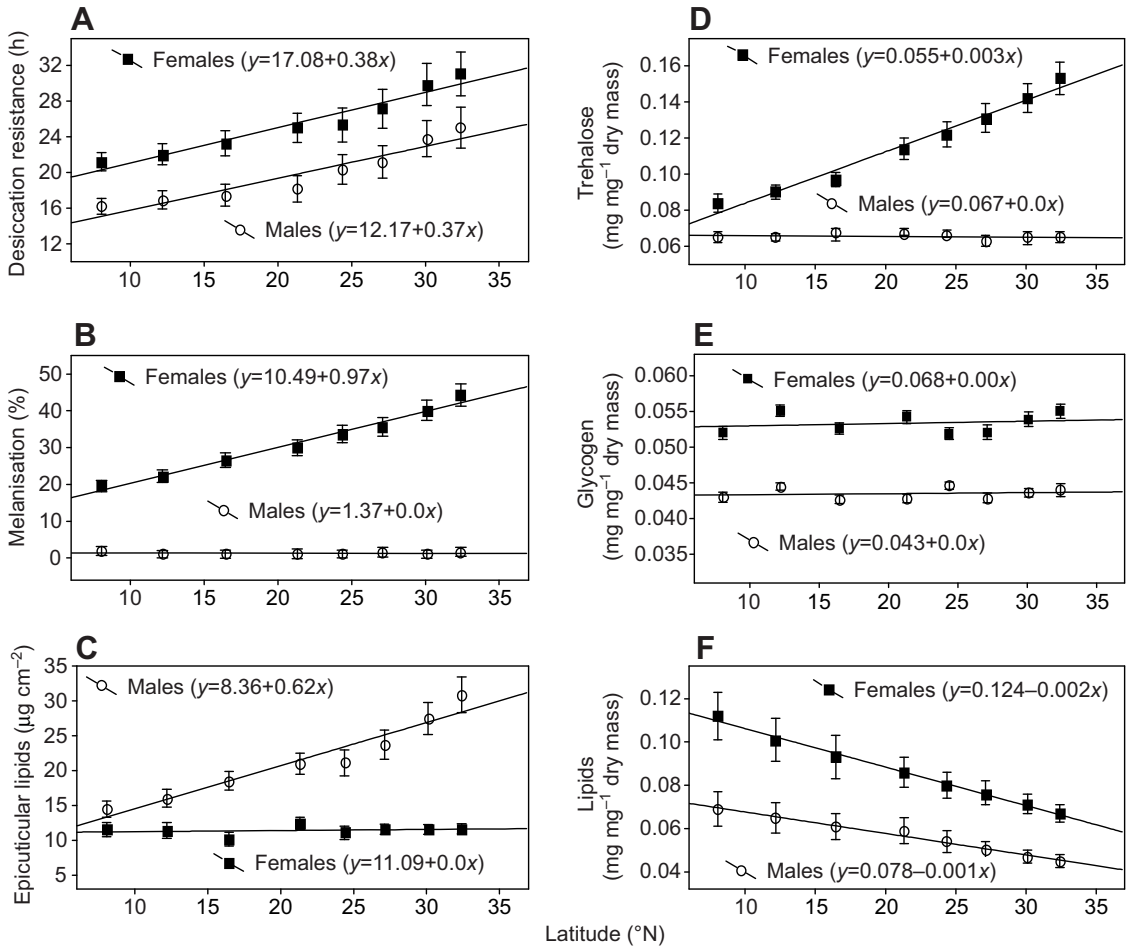

Fig. 1. Geographical variation in desiccation-related traits (A–C) and energy metabolites (D–F) in male and female *Drosophila kikkawai* (*N*=20 isofemale lines per population) collected from eight latitudinal localities of the Indian subcontinent. For each trait, the regression equation is also given. For clinal variation of all the variable traits, the level of significance is ***P<0.001. Data are means ± s.e.m.

Table 1. Results of ANCOVA for partitioning trait variability due to population (P), isofemale line (IF) and their interaction in males and females of eight latitudinal populations of *Drosophila kikkawai*

		Males				Females			
		Population	IF line	P×IF	Error	Population	IF line	P×IF	Error
Trait	d.f.	7	19	133	1439	7	19	133	1439
Desiccation hours	MS	25,084.21	4152	171.02	7.15	24,745.91	1794.80	85.46	3.89
	F	3256.41	513.29	21.12		6361.41	461.38	21.96	
	% variance	69.97***	25.39***	4.64*	3.21	77.29***	15.29***	7.42***	2.49
Melanisation (%)	MS	4.29	2.05	0.31	0.75	37,670.88	3248.37	231.38	6.15
	F	8.69	4.21	0.26		6125.34	528.19	37.68	
	% variance	2.89$^{n.s.}$	5.02$^{n.s.}$	1.56$^{n.s.}$	90.53	72.36***	19.08***	5.49*	3.07
Cuticular lipids (μg cm^{-2})	MS	23,256.02	3289.5	162.35	5.23	7.68	4.01	0.28	0.97
	F	2968.79	489.67	19.86		14.23	6.59	0.39	
	% variance	77.23***	18.69***	2.84***	1.24	3.69$^{n.s.}$	5.79$^{n.s.}$	1.23$^{n.s.}$	89.29
Carbohydrates	MS	8.90	4.81	0.30	0.86	27,817.57	2380.61	128.51	4.94
(mg mg^{-1} dry mass)	*F*	10.34	5.59	0.34		5631.08	481.91	26.03	
	% variance	4.27$^{n.s.}$	7.32$^{n.s.}$	2.81$^{n.s.}$	85.60	73.66***	18.49***	6.29*	1.56
Lipids (mg mg^{-1} dry mass)	MS	15,727.48	2458.36	90.27	3.24	19,136.77	1699.20	83.96	2.06
	F	4854.16	758.75	27.86		9289.69	824.85	40.75	
	% variance	62.79***	29.09**	5.63***	2.58	72.09***	20.27***	6.01***	1.63
Rate of water loss (mg h^{-1})	MS	22,365.47	3365.89	166.38	3.65	23,657.12	3698.20	174.26	6.47
	F	3594.26	369.8	13.89		3821.05	336.42	12.05	
	% variance	67.69***	19.66***	9.86***	2.79	69.32***	18.65***	9.02***	3.01
Body water	MS	3.26	1.32	0.22	0.47	13,526.02	1426.14	75.64	2.96
	F	5.78	3.66	0.19		2965.47	278.63	35.26	
	% variance	1.79$^{n.s.}$	3.44$^{n.s.}$	1.23$^{n.s.}$	93.54	68.76***	17.64***	11.23***	2.37
Dehydration tolerance (%)	MS	6.76	4.91	0.09	0.32	15,583.22	2102.35	75.39	3.43
	F	21.09	15.31	0.28		4257.95	606.26	23.78	
	% variance	8.27$^{n.s.}$	7.22$^{n.s.}$	2.11$^{n.s.}$	82.40	63.27***	27.16***	6.76***	2.81

For each trait, mean square (MS), *F*-value and percentage variance explained are shown. Percent data were arcsine transformed. *P<0.05; **P<0.01; ***P<0.001; n.s., not significant.

both sexes, body lipids showed a negative cline but there was no variation in the glycogen content (Fig. 1E,F). For two water-balance-related traits (body water and dehydration tolerance), females showed clinal variation but males did not. However, rate of water loss showed similar negative clines in males and females. Thus, desiccation-related traits showed divergence in geographical variation between the two sexes of *D. kikkawai*.

The results of ANCOVA with body size as a covariate for different quantitative traits are given in Table 1. For each trait, the mean square (MS), *F*-value and percent variance due to populations and isofemale lines are shown. In males, we found lack of within- as well as between-population variability for body melanisation, body water, dehydration tolerance and carbohydrates. However, males showed ~70% variability between populations and ~20% in isofemale lines for desiccation resistance, surface lipid amount and rate of water loss (Table 1). In contrast, females showed within- (20%) and between-population trait variability (70%) for all the desiccation-related traits except surface lipid amount (Table 1). Thus, we found significant trait variability for different traits consistent with clinal variation in males and females of *D. kikkawai*.

Sex-specific differences in cuticular traits and energy metabolites

Comparisons of geographical variation in cuticular traits and energy metabolites in both sexes of *D. kikkawai* are given in Table 2. The surface lipid amount varied in male larvae as well as adults, but no change was observed in females. Another cuticular trait (body melanisation) showed significant increase in females but no change in males of *D. kikkawai* (Table 2). For all traits, female larvae as well as adults showed higher trait values than males. In both the larvae and adults of males, carbohydrate content did not vary between geographical populations, whereas northern females showed higher storage of carbohydrates compared with the southern population (Table 2).

Sex-specific differences in water balance traits

Results on the analysis of different parameters of water budget of one southern and one northern population of *D. kikkawai* are shown in Table 3. For male larvae as well as adults, except for rate of water loss, the two geographical populations showed no significant differences in water-balance-related traits (i.e. for total body water, hemolymph content, tissue water or dehydration tolerance; Table 3, Fig. 2). However, for males, the northern population showed a significantly lower rate of water loss than the southern population, which is consistent with the twofold increase in surface lipids in northern males of *D. kikkawai*. In contrast, females showed significant differences in all the water-balance-related traits, i.e. females of *D. kikkawai* from northern drier localities have evolved changes in all the three avenues of water conservation (increased body water, reduced rate of water loss and increase in dehydration tolerance; Table 3, Fig. 2).

Lack of sex-specific differences in rate of water loss

In order to test whether sex-specific differences in desiccation resistance correspond with changes in rate of water loss, we followed Wharton's method to compare rate of water loss between sexes as well as between two geographical populations of *D. kikkawai* (Fig. 3). We found no difference in the rate of water loss between the two sexes for a given population; however, rate of water loss was 50% higher in the southern population as compared with the northern population (Fig. 3). Thus, sex-specific differences in desiccation resistance cannot be explained on the basis of changes in the rate of water loss.

Table 2. Mean (±s.e.m.; *N*=20 isofemale lines × 10 replicates) data on ecophysiological traits – dry mass-specific desiccation resistance hours, cuticular lipid mass and energy metabolites (trehalose, glycogen, lipid and protein content) in male and female third instar larvae and adult flies of one southern (Trivendrum: 8°06′N) and one northern population (Pathankot: 32°40′N) of *D. kikkawai*

	Males				Females			
Trait	Southern	Northern	Ratio	$F_{1,398}$	Southern	Northern	Ratio	$F_{1,398}$
Third instar larvae								
Desiccation hours	7.53±019	12.37±0.33	1.64	21,463.14***	8.14±0.22	14.17±0.41	1.74	29,361.25***
Epicuticular lipids (μg cm^{-2})	12.71±0.04	27.35±0.07	2.15	36,894.22***	8.23±0.03	9.15±0.03	1.0	5.61$^{n.s.}$
Trehalose (mg mg^{-1} dry mass)	0.056±0.002	0.055±0.002	1.0	0.44$^{n.s.}$	0.076±0.004	0.141±0.007	1.85	32,012.35***
Glycogen (mg mg^{-1} dry mass)	0.038±0.001	0.039±0.001	1.0	0.39$^{n.s.}$	0.049±0.001	0.048±0.001	1.0	0.44$^{n.s.}$
Lipid (mg mg^{-1} dry mass)	0.066±0.003	0.041±0.002	0.62	39,586.47***	0.105±0.004	0.063±0.002	0.60	56,231.98***
Protein (mg mg^{-1} dry mass)	0.050±0.002	0.052±0.002	1.0	0.54$^{n.s.}$	0.047±0.002	0.049±0.002	1.0	0.52$^{n.s.}$
Adults								
Desiccation hours	16.18±0.38	25.03±0.48	1.54	30,249.22***	21.19±0.43	34.03±0.57	1.60	69,365.28***
Epicuticular lipids (μg cm^{-2})	14.48±0.06	30.89±0.09	2.13	33,246.28***	11.53±0.06	11.63±0.06	1.0	5.23$^{n.s.}$
Trehalose (mg mg^{-1} dry mass)	0.062±0.003	0.065±0.003	1.0	0.69$^{n.s.}$	0.084±0.004	0.153±0.006	1.82	44,586.52***
Glycogen (mg mg^{-1} dry mass)	0.043±0.001	0.044±0.001	1.0	0.46$^{n.s.}$	0.052±0.002	0.055±0.002	1.0	0.51$^{n.s.}$
Lipid (mg mg^{-1} dry mass)	0.069±0.004	0.045±0.002	0.65	33,564.25***	0.112±0.005	0.068±0.003	0.61	54,631.27***
Protein (mg mg^{-1} dry mass)	0.054±0.002	0.056±0.002	1.0	0.49$^{n.s.}$	0.053±0.002	0.055±0.002	1.0	0.53$^{n.s.}$
Melanisation	1.08±0.41	1.07±0.38	1.0	2.56$^{n.s.}$	19.58±2.15	44.25±2.89	2.26	49,415.44***

ANOVAs (*F*-values) were used for between-population comparisons. Differences in trait values between the two geographical populations are shown as ratios. ***P<0.001; n.s., not significant.

Effects of organic solvents on cuticular permeability

We assessed changes in body water loss of male and female larvae as well as adults before and after washing the cuticle with organic solvents (hexane or chloroform:methanol), and changes in cuticular permeability were analysed with Dunn's multiple comparison test (Table 4). Lack of changes in body water loss after hexane treatment of females with organic solvents suggested no role of surface lipids in cuticular permeability. In contrast, in male larvae as well as adults, we found a significant increase in body water loss after hexane (larvae: 2.5-fold; adults: 4.46-fold) and chloroform:methanol (larvae: 6.2-fold; adults: 10.19-fold) treatment (Table 4). Further, effects of organic solvents on surface lipids (cuticular permeability) were higher in adults than in larvae. For water loss changes, results from Dunn's multiple comparison test were significant for males but not for females (Table 4). Further, we compared loss of total body water as a function of desiccation stress durations in control and solvent-treated males and females of *D. kikkawai* (Fig. 4). Maximum body water loss was observed after 12 h in control male larvae, but after 5 h for hexane-treated larvae and after 2 h in the case of chloroform:methanol-treated male larvae (Fig. 4A); the corresponding values for adult males were 23 h for control and 5 and 2 h for hexane and chloroform:methanol treatments, respectively (Fig. 4B). In contrast, females showed no difference in the loss of total body water after treatment with organic solvents as compared with controls (Fig. 4C,D). Thus, our results suggest effect of organic solvents on cuticular permeability of males but not on females of *D. kikkawai*.

Table 3. Mean (±s.e.m.; *N*=20 isofemale lines × 10 replicates) data on different measures of water balance, rate of water loss and dehydration tolerance in male and female third instar larvae and adults of one southern and one northern population of *D. kikkawai*

	Males				Females			
Trait	Southern	Northern	Ratio	$F_{1,398}$	Southern	Northern	Ratio	$F_{1,398}$
Third instar larvae								
Wet mass (mg fly^{-1})	1.452±0.023	1.459±0.024	1.0	1.35$^{n.s.}$	1.589±0.027	1.772±0.035	1.11	15,623.21**
Dry mass (mg fly–^{1})	0.450±0.012	0.454±0.013	1.0	0.54$^{n.s.}$	0.492±0.016	0.519±0.021	1.05	8564.52***
Total water content (mg fly^{-1})	1.002±0.019	1.005±0.019	1.0	0.81$^{n.s.}$	1.097±0.023	1.258±0.029	1.15	17,231.47***
Hemolymph content (mg fly^{-1})	0.494±0.013	0.496±0.012	1.0	2.26$^{n.s.}$	0.539±0.017	0.631±0.023	1.17	18,695.62***
Hemolymph water content (mg fly^{-1})	0.392±0.009	0.394±0.009	1.0	0.17$^{n.s.}$	0.432±0.013	0.513±0.018	1.19	18,854.55***
Tissue water content (mg fly^{-1})	0.610±0.019	0.611±0.020	1.0	0.08$^{n.s.}$	0.665±0.021	0.745±0.025	1.12	15,726.35***
Rate of water loss (mg h^{-1})	0.065±0.004	0.039±0.002	0.60	23,251.25***	0.067±0.005	0.040±0.003	0.60	27,121.14***
Dehydration tolerance (%)	48.12	49.04	1.0	1.01$^{n.s.}$	50.12	60.05	1.20	20,100.17***
Adults								
Wet mass (mg fly–^{1})	0.931±0.018	0.935±0.017	1.0	1.42$^{n.s.}$	1.694±0.024	1.897±0.032	1.12	16,852.34***
Dry mass (mg fly^{-1})	0.284±0.009	0.283±0.010	1.0	0.97$^{n.s.}$	0.510±0.018	0.580±0.023	1.14	16,999.81***
Total water content (mg fly^{-1})	0.651±0.016	0.652±0.017	1.0	0.19$^{n.s.}$	1.184±0.020	1.317±0.028	1.11	14,976.25***
Hemolymph content (mg fly^{-1})	0.312±0.010	0.309±0.009	1.0	0.57$^{n.s.}$	0.587±0.017	0.676±0.025	1.15	17,025.36***
Hemolymph water content (mg fly^{-1})	0.249±0.007	0.247±0.008	1.0	0.45$^{n.s.}$	0.466±0.014	0.565±0.019	1.21	21,154.32***
Tissue water content (mg fly^{-1})	0.402±0.013	0.405±0.013	1.0	0.83$^{n.s.}$	0.718±0.018	0.752±0.022	1.05	8985.51***
Rate of water loss (mg h^{-1})	0.050±0.003	0.036±0.001	0.70	29,156.14***	0.042±0.002	0.029±0.001	0.69	28,697.25***
Dehydration tolerance (%)	51.79	52.12	1.0	1.15$^{n.s.}$	55.96	68.18	1.22	21,052.14***

ANOVAs (*F*-values) were used for between-population comparisons. Differences in trait values between two geographical populations are shown as ratios. Percent data were arcsine transformed for ANOVA. **P<0.01; ***P<0.001; n.s., not significant.

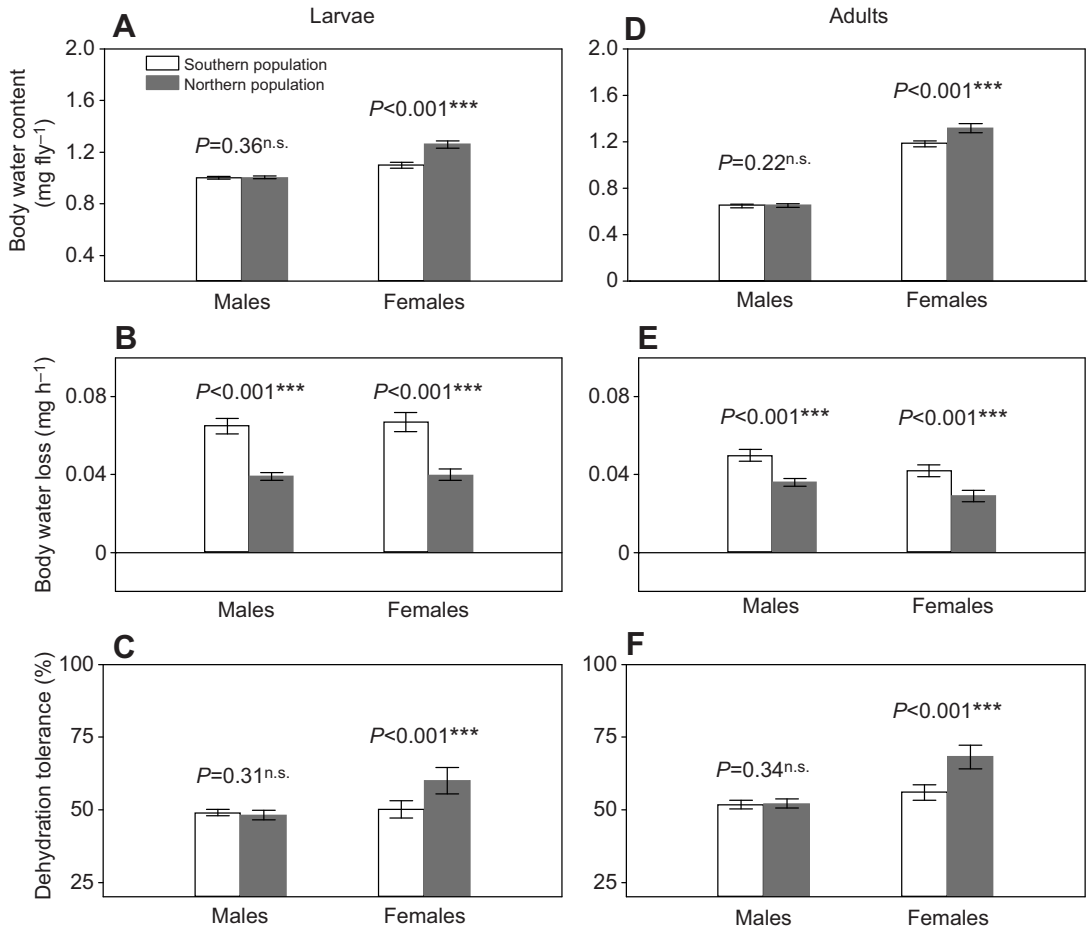

Fig. 2. Differences in total body water content (A,D), body water loss (B,E) and dehydration tolerance (C,F) in larvae (A–C) and adult flies (D–F) of both sexes from one southern (Trivendrum, 8.06°N) and one northern (Pathankot, 32.40°N) population of *D. kikkawai*. Asterisks denote a significant difference between populations based on (ANOVA; ****P*<0.001; n.s., not significant). Data are means ± s.e.m.

Utilization of metabolic fuels

Data on energy budget of male and female individuals of *D. kikkawai* due to storage of metabolic fuels (carbohydrates, lipids and proteins) are shown in Table 5. For proteins, the energy budget is similar across sexes as well as populations, but these are not utilized under desiccation stress. Interestingly, the southern population exhibited 54% more energy due to lipids in males but 65% more in females despite a lack of utilization of lipids under desiccation stress. In contrast, both sexes of *D. kikkawai* showed contrasting patterns for storage as well as rate of utilization of carbohydrates under desiccation stress. The energy budget of males in terms of stored carbohydrates did not show geographical variation whereas females stored 50% more carbohydrate in the northern population than in the southern population (Table 5). We observed a higher rate of utilization of carbohydrates under desiccation stress in males of the southern population when compared with the northern population, but the rate of utilization did not vary in females of geographical populations of *D. kikkawai*. Thus, in *D. kikkawai*, there is sexual dimorphism for storage as well as utilization of carbohydrates under desiccation stress.

Trait associations differ between sexes

We used data on isofemale line variability to assess correlations of desiccation resistance with different traits (surface lipid amount, hemolymph content and trehalose content) in males and females of *D. kikkawai* (Fig. 5). Between-line variation in surface lipid amount exhibited a significant correlation with desiccation resistance in males (Fig. 5A) but not in females (Fig. 5D). In contrast, isofemale line variation in hemolymph and trehalose content in females showed a significant correlation with desiccation resistance (r>0.89±0.07 for hemolymph; r>0.93±0.06 for trehalose; Fig. 5E,F). However, in males, we did not find line variation in hemolymph or trehalose content and neither was there any correlation with desiccation resistance (Fig. 5B,C). Finally, there was a significant correlation between line variation for cuticular melanisation and desiccation resistance in females (r>0.92, P<0.001) but not in males. Thus, trait associations differ significantly between the two sexes of *D. kikkawai*.

Seasonal changes in desiccation-related traits

Males and females of *D. kikkawai* showed approximately twofold higher desiccation resistance during winter as compared with the rainy season (Table 6). These results are consistent with variable selection pressures during wet *versus* dry environments during the two seasons. For cuticular traits, seasonal changes showed approximately threefold increases in the amount of surface lipids in males while a threefold increase in melanisation was evident in females (Table 6). Three water balance traits (an increase of 15% for total body water, 64% for carbohydrates content and 36% for dehydration tolerance) showed significant changes across seasons

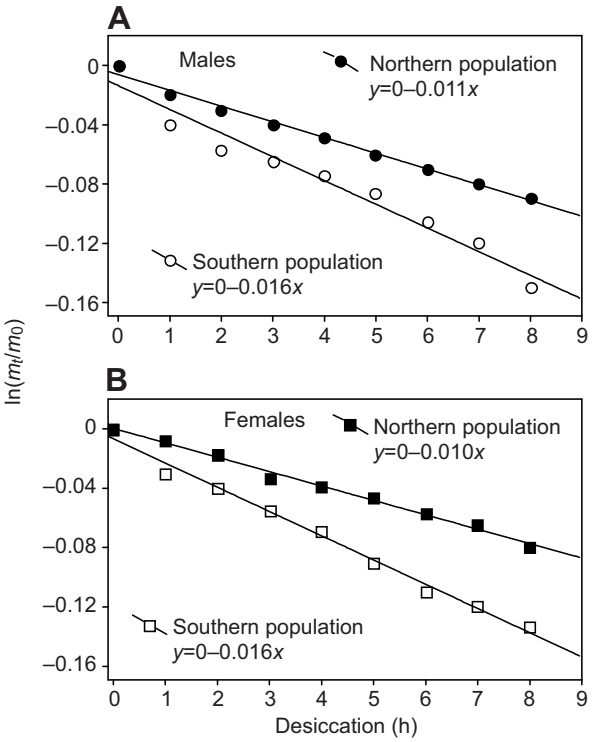

Fig. 3. Comparison of the rate of water loss according to Wharton's method in males (A) and females (B) of one northern and one southern population of *D. kikkawai*. The water loss rate was derived from slope (*b*) of $\ln(m_t/m_0)$ as a function of different desiccation stress durations, where m_t is the water lost at time *t*, and m_0 is the initial water content. Rate of water loss varied between populations but not for the two sexes of a given population.

in the females, but no seasonal effects on these water balance traits were seen in males (Table 6). Thus, seasonal effects on desiccation-related traits vary significantly across sexes of *D. kikkawai*.

Drought acclimation: effects due to sex and population

Data on the effects of drought acclimation on desiccation-related traits (desiccation resistance and water balance traits) in males and females of two geographical populations of *D. kikkawai* are shown in Fig. 6. Three ecophysiological traits (cuticular lipid amount, melanisation and rate of water loss) did not vary between control and acclimated flies. Results on the rate of water loss following Wharton's method were similar between control (Fig. 3) and acclimated flies (rate of water loss: control *versus* acclimated; F=1.13, P>0.87). Males of *D. kikkawai* showed no increase in desiccation resistance after acclimation for either geographical population (Fig. 6A). In contrast, females of the northern population showed a greater desiccation acclimation response (9.04 h) than southern females (5.3 h), despite a lack of difference in relative hardening capacity (26%) between the populations (Fig. 6D). The lack of acclimation response in males was consistent with a lack of changes in storage level of energy metabolites (carbohydrates) and dehydration tolerance for both geographical populations. However, significant geographical variation in the increase in carbohydrate content (northern population, 16.34%; southern population, 8.08%) and dehydration tolerance (northern population, 9.45%; southern population, 5.46%) was found in acclimated females of *D. kikkawai* (Fig. 6E,F). Thus, we found desiccation acclimation responses and geographical variation for desiccation acclimation only in female *D. kikkawai*.

DISCUSSION

In the present study, we found significant sex-specific differences in water-balance-related traits (water budget, energy metabolites and dehydration tolerance) of *D. kikkawai*. However, we did not find a difference in the rate of water loss between the two sexes consistent with differences in their desiccation resistance levels. We found divergence of cuticular traits, i.e. variable melanised cuticle in females, but this was due to changes in the amount of surface lipids in the males. In females originating from drier localities (northern population), we found increased levels of total body water, hemolymph and carbohydrate content associated with higher dehydration tolerance. In contrast, males from drier habitats showed no changes in water-balance-related traits except the reduced rate of water loss consistent with an increased amount of surface lipids. Thus, higher desiccation of females is likely due to higher carbohydrate content as well as dehydration tolerance when compared with males. Further, the two sexes vary in the levels of storage and utilization of carbohydrates, i.e. storage of carbohydrates did not vary in the males originating from southern wet *versus* northern drier habitats, whereas females from drier northern localities evidenced higher levels of stored carbohydrates (50% higher) compared with females from southern humid localities. Thus sexes differ in their storage level of carbohydrates as energy metabolites, which flies utilize under desiccation stress. Interestingly, the rate of utilization of carbohydrates did not vary between females from dry *versus* wet localities (northern

Table 4. Mean (±s.e.m.; 20 isofemale lines × 10 replicates) water loss (mg h^{-1}) in control (dead overetherised larvae or flies) and hexane- or chloroform:methanol-treated dead larvae/adults of both the sexes of the northernmost population (Pathankot) of *D. kikkawai*

Treatment	Males	Females
Third instar larvae		
Dead larvae (control)	0.072±0.003	0.066±0.002
Hexane	0.180±0.005***	0.065±0.003$^{n.s.}$
Chloroform:methanol (2:1)	0.452±0.006***	0.065±0.003$^{n.s.}$
K	18.24***	1.97$^{n.s.}$
Adults		
Dead flies (control)	0.026±0.004	0.039±0.003
Hexane	0.116±0.007***	0.040±0.005$^{n.s.}$
Chloroform:methanol (2:1)	0.265±0.009***	0.040±0.005$^{n.s.}$
K	26.37***	1.75$^{n.s.}$

Based on Dunn's multiple comparison test, Kruskal–Wallis values (*K*) showed no differences between control and treated female larvae (K=1.97, P=0.31) or adults (K=1.75, P=0.39). However, there were significant differences for male larvae (K=18.24, P<0.001) as well as adults (K=26.37, P<0.001). ***P<0.001; n.s., not significant.

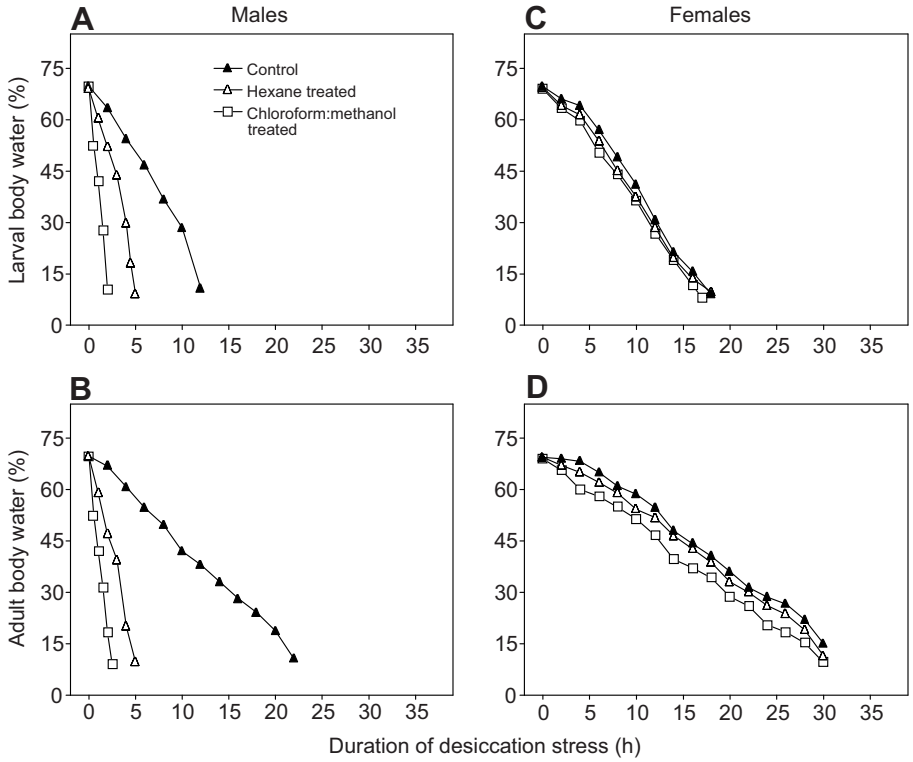

Fig. 4. Comparison of sex-specific changes in body water loss of third instar larvae (A,C) and adult flies (B,D) as a function of different durations of desiccation stress in control and organic-solvent-treated individuals of both sexes of one northern population (Pathankot) of *D. kikkawai*.

versus southern). In contrast, the rate of utilization of carbohydrates was 50% lower in males from northern drier habitats when compared with males from southern humid localities. Thus, we found divergent strategies for storage as well as utilization of carbohydrates under desiccation stress between the two sexes of *D. kikkawai*. Further, the two sexes showed divergence in their drought acclimation response; females increased desiccation resistance after prior treatment to non-lethal levels of desiccation stress, but such a response was not evident in the males. Finally, females showed geographical variation in desiccation acclimation but males did not. Thus, our results provide a physiological basis for sex-specific differences in desiccation resistance of *D. kikkawai*.

Sex-specific differences in clinal variation

Further, across different *Drosophila* species, the occurrence of clinal variation in desiccation-related traits is considered as an adaptive response due to natural selection (Endler, 1986). Several studies have shown geographical variation in morphological and ecophysiological traits in different *Drosophila* species, but not all studies have considered both sexes of a species (Hoffmann and Harshman, 1999; Parkash and Munjal, 1999; Robinson et al., 2000; Hoffmann and Weeks, 2007; Telonis-Scott et al., 2011). In the present work, for males of *D. kikkawai*, geographical variation is related to changes in surface lipid amount and rate of water loss, but other water balance traits showed no variation within or between populations. In contrast, in females, the effects of climatic selection can be argued for all the desiccation-related traits except changes in surface lipid amount. Both sexes of *D. kikkawai* encounter a similar gradient of relative humidity conditions with latitude in India, but different cuticular traits are selected in the two sexes of *D. kikkawai*. Further studies are needed to examine sex-specific differences in surface lipids and/or body melanisation in various *Drosophila* species as well as diverse insect taxa.

Table 5. Rate of metabolite utilization (regression slope values as a function of different durations of desiccation stress) and stored energy budget ($J\,mg^{-1}$) due to carbohydrates, lipids and proteins in adult flies of both sexes of one southern and one northern population (N=20 isofemale lines × 10 replicates each) of *D. kikkawai*

	Males			Females		
Metabolites	Southern	Northern	*t*-test	Southern	Northern	*t*-test
Rate of metabolite utilization						
Carbohydrates	–6.19±0.018***	–4.05±0.013***	**	–6.42±0.019***	–6.70±0.020***	n.s.
Lipids	–0.019±0.031$^{n.s.}$	–0.018±0.032$^{n.s.}$	n.s.	–0.023±0.042$^{n.s.}$	–0.026±0.044$^{n.s.}$	n.s.
Proteins	–0.010±0.029$^{n.s.}$	–0.011±0.026$^{n.s.}$	n.s.	–0.012±0.041$^{n.s.}$	–0.013±0.42$^{n.s.}$	n.s.
Stored energy budget						
Carbohydrates	1.89	1.92	n.s.	2.40	3.67	***
Lipids	2.71	1.76	***	4.40	2.67	***
Proteins	0.96	0.99	n.s.	0.94	0.97	n.s.

Conversion factors: 17.6 $J\,mg^{-1}$ for carbohydrates, 39.3 $J\,mg^{-1}$ for lipids and 17.8 $J\,mg^{-1}$ for proteins (Schmidt-Nielsen, 1990; Marron et al., 2003). Slope values represent rate of metabolite utilization as a function of time ($\mu g\,h^{-1}$; a negative sign indicates that metabolite level decreased with time under desiccation stress). ***P<0.001; n.s., not significant.

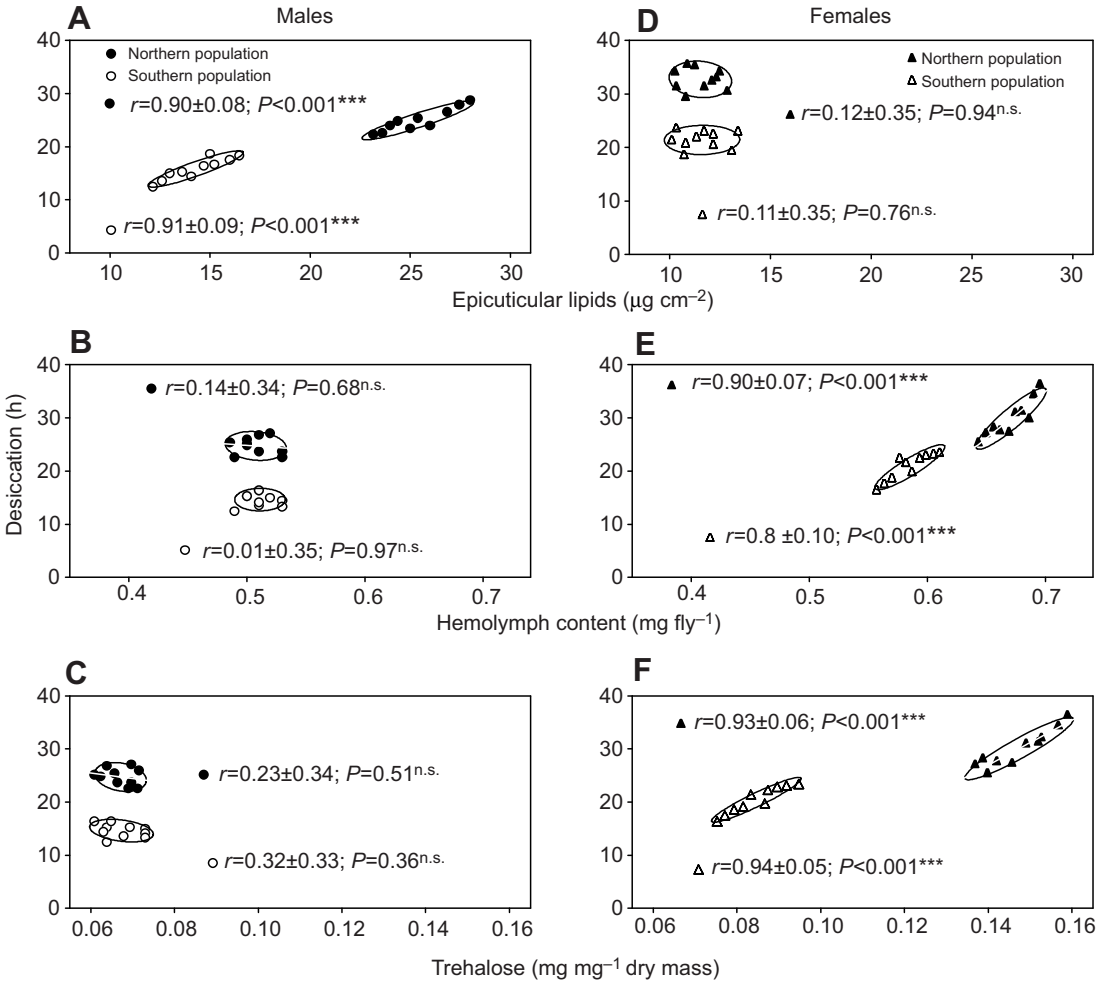

Fig. 5. Trait correlations between desiccation resistance and epicuticular lipid mass (A,D), hemolymph content (B,E) or trehalose content (C,F) in males (A–C) and females (D–F) of one northern and one southern population of *D. kikkawai*. Correlation values (±s.e.m.) between traits are also shown (***P<0.001; n.s., not significant).

Several studies have shown quantitative changes in surface lipids associated with desiccation resistance in a wide variety of insects (Armold and Regnier, 1975; Yoder et al., 1995; Kaneko and Katagiri, 2004; Benoit and Denlinger, 2007; Urbanski et al., 2010). In the case of the Asian tiger mosquito, *Aedes albopictus*, diapause involves quantitative but not compositional changes in surface lipids (Urbanski et al., 2010). This study showed an ~33% increase in surface hydrocarbons in diapause eggs of a temperate population of *A. albopictus* exposed to short day photoperiodic conditions. It was suggested that in temperate regions, changes in surface lipid amount of the eggs are associated with increased desiccation resistance of *A. albopictus*, which is likely to contribute to the rapid global spread of this invasive mosquito (Urbanski et al., 2010). However, similar studies have not been carried out with drosophilids showing quantitative changes in surface lipids under variable temporal and/or spatial climatic conditions. Further, it would be interesting to make genetic crosses between isofemale lines of *D. kikkawai* with a high or low amount of surface lipids and to

Table 6. Mean (±s.e.m.) seasonal changes in desiccation resistance, cuticular traits (melanisation and epicuticular lipid mass), carbohydrate content and three avenues of water balance (rate of water loss, total body water and dehydration tolerance) in male and female *D. kikkawai* collected from Pathankot (32.14°N)

	Males				Females			
Trait	Rainy season	Winter season	Ratio	*t*-test	Rainy season	Winter season	Ratio	*t*-test
Desiccation hours	14.45±0.36	28.25±0.57	1.95	***	18.60±0.41	35.86±0.69	1.93	***
Melanisation (%)	1.02±0.03	1.05±0.03	1.0	n.s.	16.59±1.36	48.23±2.77	2.90	***
Epicuticular lipids (µg cm^{-2})	11.30±0.04	32.66±0.10	2.89	***	11.02±0.02	11.23±0.03	1.0	n.s.
Carbohydrates (mg mg^{-1} dry mass)	0.101±0.005	0.103±0.005	1.0	n.s.	0.121±0.007	0.199±0.011	1.64	***
Rate of water loss (mg h^{-1})	0.056±0.002	0.034±0.001	0.60	***	0.054±0.004	0.032±0.002	0.59	***
Body water (mg fly^{-1})	0.639±0.004	0.643±0.005	1.0	n.s.	1.071±0.011	1.235±0.016	1.15	***
Dehydration tolerance (%)	49.25	49.65	1.0	n.s.	51.63	70.00	1.36	***

Differences in trait values between two seasons are shown as ratios. ***P<0.001; n.s., not significant.

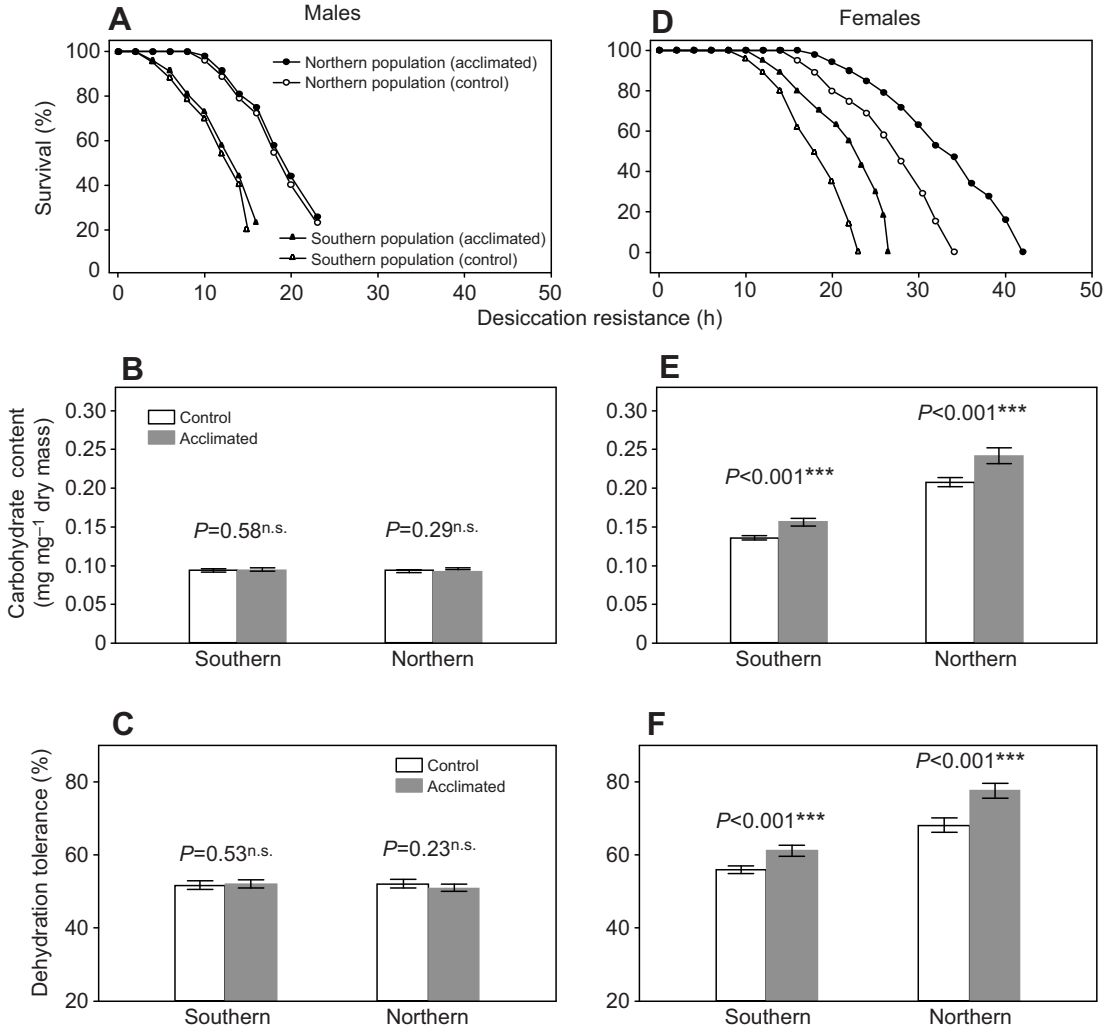

Fig. 6. Effects of drought acclimation on desiccation resistance (A,D), carbohydrate content (B,E) and dehydration tolerance (C,F) in males and females of one northern and one southern population of *D. kikkawai*. Geographical variation in acclimation responses was compared with ANOVA (***P<0.001; n.s., not significant).

investigate changes in water balance characteristics of the resulting male and female individuals. Future studies on *D. kikkawai* may also consider whether quantitative changes in surface lipids are associated with changes in photoperiod and/or relative humidity across seasons as well as along a geographical transect.

Sex-specific differences in hemolymph and storage of carbohydrates

Some studies on laboratory-selected desiccation resistance lines of *D. melanogaster* have shown a significant increase in carbohydrate content compared with control lines (Djawdan et al., 1998; Chippindale et al., 1998). Further, females of desiccation-selected lines showed correlated changes in body water content, hemolymph as well as carbohydrates, but males were not investigated in this study (Folk et al., 2001). In the present work, our results on females of *D. kikkawai* are similar for water balance traits when compared with laboratory-selected desiccation-resistant females of *D. melanogaster*, as reported by Folk and colleagues (Folk et al., 2001). However, in the males of *D. kikkawai* originating from drier habitats, there was no increase in body water, hemolymph or carbohydrates. Interestingly, we found a significant correlation between increased hemolymph and carbohydrates of females with greater desiccation resistance (r>0.80 for hemolymph; r>0.90 for trehalose; P<0.001) while males of *D. kikkawai* did not show such correlations. Thus, desiccation resistance is a complex trait that might have evolved in different ways in the two sexes of *D. kikkawai*.

Sexual dimorphism for utilization of carbohydrates as metabolic fuel

Previous studies on laboratory selection of desiccation-resistant as well as control lines did not consider the rate of utilization of an increased level of carbohydrates in desiccation-resistant lines (Gibbs et al., 1997; Chippindale et al., 1998; Djawdan et al., 1998). However, Marron and colleagues investigated the rate of utilization of metabolic fuels in both sexes of five *Drosophila* species (Marron et al., 2003). That study showed significant utilization of carbohydrates under desiccation stress, but males and females revealed similar patterns of energy consumption (Marron et al., 2003). However, in the present work, we found sexual dimorphism in the rate of utilization of carbohydrates under desiccation stress in *D. kikkawai*. Interestingly, for females, the rate of utilization of carbohydrates did not vary between geographical populations of *D. kikkawai*, despite higher storage of carbohydrates in the northern population, which encounters drier conditions in nature. Thus,

geographical variation in desiccation resistance of *D. kikkawai* females is associated with differences in storage level rather than rate of utilization of carbohydrates. In contrast, males of *D. kikkawai* showed quite different patterns for storage and utilization of carbohydrates, i.e. male individuals of populations from wet *versus* dry (southern *versus* northern) environments did not vary in the storage of carbohydrates, but there was a significant reduction (~50%) in the rate of utilization of carbohydrates in the males originating from northern (drier) habitats. The present study has shown that storage and utilization of metabolic fuel vary between the sexes of *D. kikkawai*. Further investigations are needed to examine sexual dimorphism for utilization of metabolic fuel under desiccation stress in different *Drosophila* species.

Sex-specific differences in water conservation of seasonally varying populations

In subtropical regions, seasonal changes in temperature and relative humidity cause desiccation stress during winter (colder and dry) as compared with the rainy season (warmer and wet). During winter, *D. kikkawai* males and females showed an approximately twofold increase in desiccation resistance consistent with a 60% reduction in the rate of body water loss compared with flies from the rainy season. For seasonally varying populations, sexual dimorphism was evident for cuticular traits (an approximately threefold increase in surface lipid amount in males, but an increase in melanisation in females); increased dehydration tolerance (36%) and higher carbohydrate content (64%) were also observed in females, but no such changes were seen in males of *D. kikkawai*. Seasonally varying males showed differences in the rate of water loss consistent with changes in the amount of surface lipids. Thus, seasonally varying climatic conditions have evidenced sex-specific differences in desiccation-related traits of *D. kikkawai*.

Sexual dimorphism for acclimation to drought conditions

In subtropical regions, *Drosophila* species are likely to encounter prior exposure to low-humidity conditions for few hours in nature. Such acclimation to drought-like conditions showed an increase in desiccation resistance of some widespread species (*D. melanogaster*, *D. simulans* and *D. serrata*) from the Australian continent, but an acclimation response was not evident in the rainforest *D. birchii* (Hoffmann, 1991). This study showed a lack of acclimation response in the males and also between two geographical populations of three *Drosophila* species (Hoffmann, 1991). Hoffmann anticipated likely changes in the rate of water loss, consistent with changes in surface lipids, and a possible reduction in metabolic rate in acclimated female flies and thereby increased desiccation resistance, but these aspects were not investigated in that study (Hoffmann, 1991). However, in the present work, we found a lack of drought acclimation in the males of *D. kikkawai*, which was consistent with a lack of changes in rate of water loss and surface lipid amount. In contrast, females of *D. kikkawai* revealed geographical variation in the desiccation acclimation response. However, increased desiccation of acclimated females is associated with an increase in carbohydrate content as well as dehydration tolerance, despite a lack of changes in rate of water loss as well as body melanisation. Thus, our results have shown sexual dimorphism in the acclimation response in *D. kikkawai*.

Conclusions

In the present work, we found significant sex-specific divergence in the mechanistic basis of water-balance-related traits consistent with differences in the desiccation resistance of the two sexes of *D. kikkawai*. Geographical as well as seasonal variations in desiccation resistance are associated with changes in surface lipids of males but body melanisation in females. For a given population, higher desiccation resistance of females is associated with increased levels of body water, hemolymph, carbohydrates and dehydration tolerance, while males showed no such changes in water balance traits. Similar results were evident between the sexes for desiccation-related traits in seasonally varying populations of *D. kikkawai*. The males showed clinal variation in epicuticular lipid mass but there were no changes in carbohydrate content. Thus, sex-specific differences in desiccation resistance of *D. kikkawai* correspond with divergence in their water balance traits despite a lack of changes in the rate of water loss. For energy metabolites (trehalose), we found a geographical cline in females but not in males. In contrast, the rate of utilization of carbohydrates under desiccation stress did not vary for females but there was an ~50% reduction in the rate of utilization in males from drier (northern) environments compared with those from humid (southern) localities. Thus, sexes differ in their mode of adaptation to drought conditions in *D. kikkawai*. For desiccation acclimation, females of *D. kikkawai* showed an increase in desiccation resistance associated with increased levels of carbohydrates as well as dehydration tolerance, but male flies showed no acclimation responses. Further investigations are needed to examine whether related *Drosophila* species of the *montium* species subgroup as well as other *Drosophila* species also show similar levels of sexual dimorphism in the physiological basis of desiccation resistance.

ACKNOWLEDGEMENTS

We are indebted to the reviewers for several helpful comments, which improved the manuscript.

AUTHOR CONTRIBUTIONS

P.R. carried out the experiments and analyzed the data, and was associated with R.P. who designed the experiments and wrote the manuscript.

COMPETING INTERESTS

No competing interests declared.

FUNDING

Financial assistance provided by the Council of Scientific and Industrial Research, New Delhi, to R.P. [Emeritus Scientist project no. 21(0847)/11 EMR-II] is gratefully acknowledged. P.R. is thankful to the University Grants Commission, New Delhi, for award of a Rajiv Gandhi National research fellowship (RGNF-SC-HAR-7423).

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