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RESEARCH ARTICLE

Divergent strategies for adaptations to stress resistance in two tropical *Drosophila* species: effects of developmental acclimation in *D. bipectinata* and the invasive species *D. malerkotliana*

Ravi Parkash, Divya Singh* and Chanderkala Lambhod

ABSTRACT

Previous studies on two tropical *Drosophila* species (*D. malerkotliana* and *D. bipectinata*) have shown lower resistance to stress-related traits but the rapid colonization of *D. malerkotliana* in the past few decades is not consistent with its sensitivity to desiccation and cold stress. We tested the hypothesis that developmental acclimation at two growth temperatures (17 and 25°C) can confer adaptations to desiccation and thermal stresses. We found divergence in developmental plastic effects on cuticular traits, i.e. a significant increase of body melanisation (~2-fold) and of cuticular lipid mass (~3-fold) in *D. malerkotliana* but only 1.5-fold higher cuticular lipid mass in *D. bipectinata* when grown at 17°C compared with 25°C. A comparison of the water budget of these two species showed significantly higher effects of developmental acclimation on body water content, rate of water loss and dehydration tolerance resulting in higher desiccation resistance in *D. malerkotliana* than in *D. bipectinata*. When grown in cooler conditions (17°C), *D. malerkotliana* had greater resistance to cold as well as desiccation stress. In contrast, heat resistance of *D. bipectinata* was higher when grown at 25°C. These laboratory observations are supported by data on seasonally varying populations. Furthermore, adult *D. malerkotliana* acclimated to different stresses showed greater resistance to those stresses than *D. bipectinata* adults. Thus, significant increase in stress resistance of *D. malerkotliana* through developmental acclimation may be responsible for its invasion and ecological success on different continents compared with *D. bipectinata*.

KEY WORDS: Developmental acclimation, Stress resistance, *Drosophila bipectinata*, Invasion by *Drosophila malerkotliana*

INTRODUCTION

Water conservation and thermotolerance are crucial to the ecological success of different insect taxa from diverse types of habitats (Hadley, 1994; Willmer et al., 2000; Angilletta, 2009). Several studies have compared desiccation as well as cold and heat resistance levels of *Drosophila* species of temperate and tropical origin but few studies have investigated genetic variation for stress-related traits (Parsons, 1983; Hoffmann, 2010; Kellermann et al., 2012a; Kellermann et al., 2012b). Kellermann and co-workers have shown low genetic variation for desiccation and cold resistance (estimated as additive genetic variance and narrow sense heritability) in *D. bipectinata* and four other tropical species, whereas five widespread *Drosophila* species have been shown to have higher genetic variation (Kellermann et al., 2009). For the two tropical species (*D. malerkotliana* and *D. bipectinata*), there are no studies that have investigated the overall levels of genetic variation. However, *D. malerkotliana* has extended its range across tropical and subtropical regions on different continents despite its lower resistance to desiccation and thermal stress (Birdsley, 2003; Garcia et al., 2005). If *D. malerkotliana* and *D. bipectinata* have low levels of stress resistance for desiccation and cold, it is likely that these species have adopted other strategies, such as developmental acclimation effects, but this possibility has not been investigated so far. In order to cope with desiccation stress, phenotypic plasticity of one or both cuticular traits (body melanisation and/or cuticular lipid amount) are likely to affect water conservation mechanisms because both these cuticular traits are known to confer water proofing in *Drosophila* species (Hadley, 1994; Gibbs et al., 2003; Parkash et al., 2008, Parkash et al., 2013). Rearing temperatures can also affect thermotolerance of *Drosophila* species (Huey et al., 1999; Bubliy and Loeschcke, 2005). It is not clear whether these two tropical *Drosophila* species can improve their desiccation resistance through adult acclimation. Adult *Drosophila* flies can encounter short-term bouts of low humidity conditions in the field. A single study on adult acclimation to desiccation stress in two sibling rainforest *Drosophila* species of the montium subgroup has shown acclimation effects for *D. serratta* but not for *D. birchii* (Hoffmann, 1991). Furthermore, this study showed association between adult acclimation ability and range of species distribution on the Australian continent (Hoffmann, 1991). Thus, it would be interesting to compare the effects of developmental as well as adult acclimation to dehydration and thermal stresses in *D. malerkotliana* and *D. bipectinata*. These two tropical *Drosophila* species might use common or divergent mechanisms of water conservation and thermotolerance consistent with differences in their relative abundance levels on the Indian subcontinent.

Drosophilids can survive desiccating conditions by one or more of three different water conservation mechanisms: storing more water; dehydration tolerance (a greater tolerance of body water loss before succumbing to death); and by reduction in the rate of cuticular water loss (Hadley, 1977; Hadley, 1994; Benoit et al., 2005; Gibbs et al., 1997). For insects, more than 80% of body water loss occurs through the cuticle. Reduction in the cuticular permeability has been associated with changes in the amount or composition of surface lipids in several taxa of large insects, such as scorpions and tenebrionid beetles (Hadley, 1977; Toolson and Hadley, 1979; Hadley, 1994). In contrast, cuticular lipid amount did not vary between xeric and mesic *Drosophila* species and showed no correlation with rate of water loss (Gibbs et al., 2003). Similar relationships are evident between laboratory-selected desiccation resistant and control strains of *D. melanogaster* (Gibbs et al., 1997). However, it is not clear whether tropical *Drosophila* species of the

Department of Genetics, Maharshi Dayanand University, Rohtak-124001, India.

*Author for correspondence (singh.genetics@gmail.com)

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subgenus *Sophophora* have evolved changes in the amount of cuticular lipids to confer greater desiccation resistance in the subtropics, even though no such changes in the cuticular lipids were found in the related species, *Drosophila ananassae* (Parkash et al., 2010). Furthermore, association between cuticular permeability and quantity of cuticular lipids can be demonstrated through treatment of cuticular surfaces with organic solvents such as hexane or chloroform:methanol (Hadley, 1989; Hadley, 1994; Hadley and Quinlan, 1989). This approach can be helpful in distinguishing *Drosophila* species that use cuticular lipids as water proofing barrier and those that do not.

Recent invasion into new territories by some *Drosophila* species offer opportunities to examine climatic adaptations (Huey et al., 2000; Birdsley, 2003; Garcia et al., 2005). For example, European *D. subobscura* has invaded Southern and Northern America (Huey et al., 2000; Rezende et al., 2010). *D. malerkotliana* has also extended its range to warm temperate climates of southern states of North America [Florida: latitude 30°25′N; average temperature (T_{ave}) ~22°C; relative humidity (RH) ~70%; and Athens, Georgia: latitude 30°57′N; T_{ave} ~24.5°C; RH ~53.7%)] during the months of September and October (Birdsley, 2003; Garcia et al., 2005). Growing urbanization and climate warming are likely to provide warmer refuges during the spring season for *D. malerkotliana* in the southern states of USA (Birdsley, 2003; Garcia et al., 2005). Thus, it is probable that the invasion of *D. malerkotliana* into these localities is associated with global climate warming. Therefore, it will be interesting to compare changes in stress resistance traits of *D. malerkotliana* and its related species *D. bipectinata*, and the association of these traits with species-specific relative abundance in the subtropical localities on the Indian subcontinent.

The *ananassae* species subgroup of the subgenus *Sophophora* is represented by about a dozen rainforest *Drosophila* species, which are endemic to equatorial humid environments, but two species *D. ananassae* and *D. malerkotliana* have extended their range of distribution on different continents in the past few decades (Birdsley, 2003). In the present work, we investigated species-specific differences in the water conservation mechanisms resulting from developmental acclimation, i.e. changes in cuticular traits (body melanisation and cuticular lipids), body water content, rate of water loss, dehydration tolerance in both the species (*D. malerkotliana* and *D. bipectinata*) grown at 17 and 25°C. We assessed effects of organic solvents on the surface lipids to determine their waterproofing role. We compared storage of energy metabolites at two growth temperatures. Furthermore, we compared adult acclimation effects to low humidity for both the species. We also examined seasonal changes (rainy versus autumn) in stress-resistance traits in both the species. Finally, we assessed whether in the subtropics, greater abundance of *D. malerkotliana* than *D. bipectinata* matches its greater stress resistance.

RESULTS

Changes in species relative abundance

Data on relative abundance of wild caught flies, *D. malerkotliana* and *D. bipectinata*, from five altitudinal localities are shown in Fig. 1A. *Drosophila malerkotliana* is more abundant in midland localities whereas *D. bipectinata* is prevalent in the lowland localities. The midland localities are cooler and drier (T_{ave} 19.12°C; RH 50%) whereas lowland localities are warmer and humid (T_{ave} 24.9°C; RH 78%). Repeated patterns were evident in seasonal changes (rainy versus autumn season) in the relative abundance of these two *Drosophila* species collected during 2010 and 2011

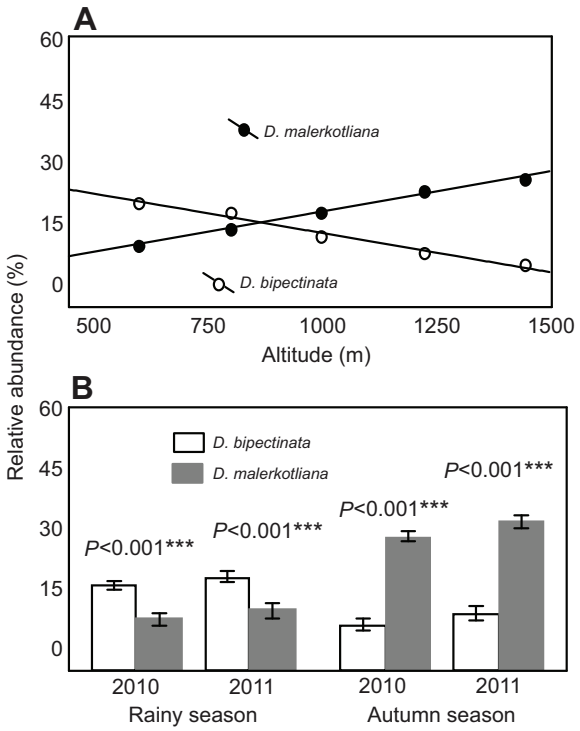

Fig. 1. Relative abundance in their natural habitats of the two species studied. (A) Regression analysis of relative abundance (with respect to other *Drosophila* species) as a function of altitude of the original location of five populations of *D. bipectinata* and *D. malerkotliana*. Flies were obtained from (altitude): (1) Parwanoo (600 m); (2) Mandi (800 m); (3) Chamba (1000 m); (4) Dharamshala (1220 m); (5) Solan (1440 m). (B) Seasonal changes (rainy versus autumn) in species-specific relative abundance in population samples collected from Chamba (1000 m) during 2010 and 2011.

(Fig. 1B). *D. malerkotliana* is abundant in autumn whereas *D. bipectinata* is more prevalent during the rainy season. Therefore, substantial reduction in T_{ave} and RH along an elevational gradient as well as across seasons could act as selection factors affecting species' relative abundance.

Species-specific differences in desiccation resistance

Data on desiccation resistance as a function of different durations of desiccation stress for *D. bipectinata* and *D. malerkotliana* females grown at 17 and 25°C are shown in Fig. 2A,B. For *D. bipectinata* there are significant differences in desiccation resistance of flies reared at 17°C [half-maximal lethal time (LT_{50})=11.81 h; maximal lethal time (LT_{100})=23.5 h] compared with 25°C (LT_{50}=7.23 h; LT_{100}=13.5 h). However, *D. malerkotliana* could survive desiccation much longer when reared at 17°C (LT_{50}=15.31 h and LT_{100}=30.62 h; Fig. 2). In order to determine whether these two different measures (LT_{50} versus LT_{100}) of desiccation resistance can be used to compare interspecific differences for flies grown at 17 and 25°C, we calculated correlations between LT_{50} and LT_{100} values of 20 isofemales lines of each of the two *Drosophila* species, and the results are illustrated in Fig. 2C,D. For *D. bipectinata* we found significant correlation values between LT_{50} and LT_{100} measures (17°C: 0.81±0.16, P<0.001; 25°C: 0.74±0.19, P<0.001; Fig. 2C). Likewise, we found significant correlations between LT_{50} and LT_{100} for desiccation resistance of *D. malerkotliana* (Fig. 2D). These results suggest that both LT_{50} and LT_{100} are valid measures to compare desiccation resistance between these two tropical *Drosophila* species.

The Journal of Experimental Biology (2014) doi:10.1242/jeb.096818

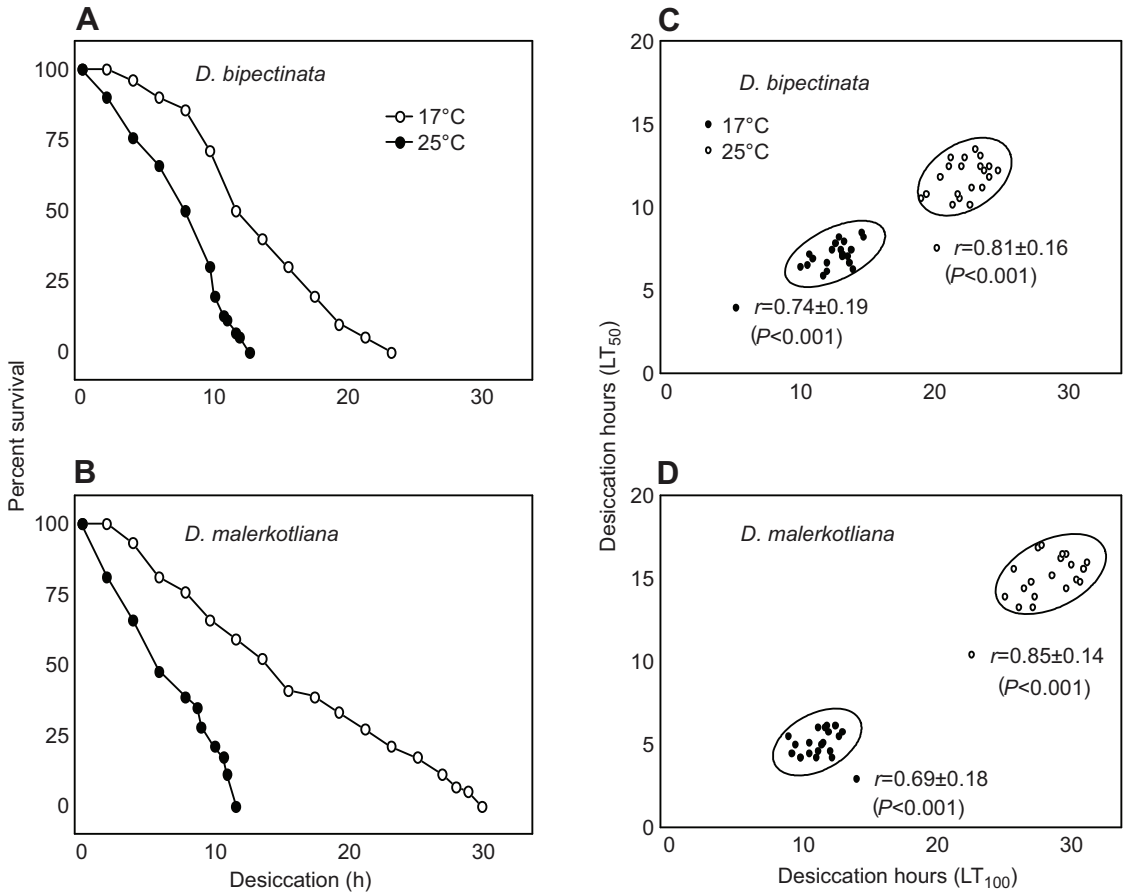

Fig. 2. Desiccation resistance. (A,B) Percentage survival as a function of different durations of desiccation stress at ~5% relative humidity (RH) in *D. bipectinata* (A) and *D. malerkotliana* (B) reared at 17 and 25°C. (C,D) Correlations between LT_{100} and LT_{50} values for desiccation resistance in 20 isofemale lines each of *D. bipectinata* (C) and *D. malerkotliana* (D) reared at 17 and 25°C.

Effects of developmental acclimation on stress-related traits

Data on desiccation hours, cuticular lipid mass, heat knockdown time, chill coma recovery time and different energy metabolites in *D. bipectinata* and *D. malerkotliana* reared at 17 and 25°C are shown in Table 1. We found significant increase for different traits for both the species reared at 17 compared with 25°C. For each species, the effects due to developmental acclimation can be appreciated from fold ratio differences (Table 1). For example, there was an approximate threefold increase in desiccation resistance and epicuticular lipid mass but a fivefold increase in body melanisation and 60% increase in the carbohydrate level of *D. malerkotliana* reared at 17°C compared with 25°C. In contrast, corresponding developmental acclimation effects were much lower (~50% increase at 17°C compared with 25°C) in *D. bipectinata*. For each trait, results of ANOVA for trait variability in the isofemale lines are shown in Table 1. We found significantly higher developmental acclimation effects for stress resistance traits in *D. malerkotliana* compared with *D. bipectinata*. Furthermore, nested ANCOVA data on trait variability resulting from species, growth temperatures, isofemale lines and their interactions are given in Table 2. Results of ANCOVA showed that maximum trait variability (~50%) occurred from developmental acclimation at the two growth temperatures, whereas between-species variation was ~20–28% for different traits. For each stress-related trait, we observed significant trait variability (~10%) between isofemale lines (Table 2). Thus, both *D. malerkotliana* and *D. bipectinata* have within population trait variability for stress-related traits.

Species-specific divergence in water balance-related traits

Data on the effects of developmental acclimation (17 versus 25°C) on different measures of water balance-related traits of *D. bipectinata* and *D. malerkotliana* are given in Table 3. Both the species showed higher trait values for total body water, dry mass, haemolymph and tissue water when grown at 17°C compared with 25°C. The fold difference for different traits is ~1.6 in case of *D. malerkotliana* but ~1.4 for *D. bipectinata* (Table 3). Interestingly, the effect of developmental acclimation on dehydration tolerance is significantly higher for *D. malerkotliana*, i.e. we observed ~65% dehydration tolerance in flies grown at 17°C but the corresponding value is 55% at 25°C. In the case of *D. bipectinata*, dehydration tolerance is ~55% at 17°C and ~50% at 25°C. The effects of developmental acclimation are significantly higher for most of the water balance-related traits of *D. malerkotliana* compared with *D. bipectinata*.

We used Wharton's method to compare the rate of water loss of *D. bipectinata* and *D. malerkotliana* grown at 17°C and 25°C, and the results are illustrated in Fig. 3. For flies grown at 17°C, the slope value of *D. malerkotliana* is lower (b=–0.007±0.0002) than *D. bipectinata* (b=–0.011±0.0004) consistent with species-specific differences in desiccation resistance (Figs 2, 3). However, for species grown at 25°C, an interspecific comparison of slope values for rate of water loss showed no differences ($t_{1,19}$=0.45, n.s.) between *D.*

The Journal of Experimental Biology (2014) doi:10.1242/jeb.096818

Table 1. Data on stress-related traits in adult female flies [*N*=20 isofemale (IF) lines × 10 replicates] of *Drosophila bipectinata* and *D. malerkotliana* grown at 17 and 25°C

	D. bipectinata				*D. malerkotliana*			
Trait	17°C	25°C	Ratio	$F_{1,398}$	17°C	25°C	Ratio	$F_{1,398}$
Desiccation (LT_{100}; h)	22.53±0.36	13.05±0.42	1.73	3101.55***	30.62±0.55	11.55±0.43	2.65	2420.63***
Desiccation (LT_{50}; h)	11.81±0.33	07.23±0.21	1.63	1877.16***	15.31±0.38	05.67±0.23	2.70	4129.67***
Melanisation (%)	02.06±0.13	02.00±0.51	1.03	1.58$^{n.s.}$	38.01±2.01	07.60±0.56	5.00	2956.17***
Epicuticular lipids (μg cm^{-2})	18.90±0.37	12.45±0.27	1.51	2608.59***	30.69±0.23	10.22±0.29	3.00	3529.56***
Heat knockdown (min)	12.24±0.42	19.18±0.29	1.56	3435.44***	07.30±0.37	12.10±0.42	1.63	4063.19***
Chill coma recovery (min)	35.85±0.07	65.04±0.11	1.81	4121.56***	12.49±0.49	40.32±0.51	3.22	5049.23***
Dry-mass-specific energy metabolites								
Trehalose (mg mg^{-1} dry mass)	0.221±0.004	0.147±0.002	1.50	37,421.11***	0.532±0.004	0.266±0.003	2.00	38,956.56***
Glycogen (mg mg^{-1} dry mass)	0.144±0.003	0.096±0.005	1.50	29,750.89***	0.247±0.002	0.128±0.004	1.93	30,017.08***
Lipids (mg mg^{-1} dry mass)	0.263±0.003	0.172±0.005	1.52	20,901.66***	0.186±0.003	0.133±0.003	1.39	10,697.89***
Proteins (mg mg^{-1} dry mass)	0.128±0.002	0.125±0.003	1.02	2.45$^{n.s.}$	0.126±0.002	0.125±0.004	1.00	0.96$^{n.s.}$

Values are means ± s.e.m.
Percentage data were arcsin transformed for ANOVA.
For each species, trait values were compared as ratios (fold-differences) and also with ANOVA (*F*-values).
***P<0.001; n.s., not significant.

malerkotliana (b=–0.034±0.0011) and *D. bipectinata* (b=–0.030±0.0001). We found species-specific differences in the rate of water loss of *D. malerkotliana* and *D. bipectinata* when reared at 17°C but not at 25°C (Fig. 3).

Assessment of water proofing role of cuticular traits

We tested whether gentle washing of flies with hexane can affect cuticular water loss in dead flies and the data on time series changes in the loss of body water as a function of different durations of desiccation stress are illustrated in Fig. 4. A comparison of control and hexane-treated flies showed significant increase in water loss after treatment with organic solvent, i.e. in 3–4 h in *D. bipectinata* whereas control flies took significantly more time (~22 h for flies grown at 17°C) but our results for *D. malerkotliana* were quite different (Fig. 4B, Table 4). The slope values for rate of water loss in control and hexane-treated flies are shown in Table 4. A comparison of slope values of control and treated flies showed significantly higher effects in *D. bipectinata* compared with *D. malerkotliana*. The much reduced effect of hexane on water loss rate of *D. malerkotliana* is due to increased body melanisation at 17°C compared with 25°C (Fig. 4B; Table 4).

Correlation between energy budget and desiccation resistance

We examined whether species-specific storage levels of carbohydrates (metabolic fuels for desiccation resistance) are

Table 2. Results of nested ANCOVA for trait variability due to species, growth temperature, isofemale line (nested in species) and their interactions for *D. bipectinata* and *D. malerkotliana* (*N*=20 IF lines × 10 replicates each) grown at 17 and 25°C

		Species	Temperature	IF line	S×T	IF×T	Error
Trait	d.f.	1	1	38	1	38	720
Desiccation resistance	MS	20,135.87	113,068.18	242.33	8653.37	101.41	0.42
	F	48.27***	1032.59***	1163.53***	85.33***	316.91***	
	% Variation	26.86	48.90	7.92	13.85	2.16	0.31
% Melanisation	MS	30,345.32	45,786.32	212.65	4123.52	95.23	0.54
	F	131.45***	1152.32***	1123.71***	32.62***	112.13***	
	% Variation	67.50	18.83	5.49	4.64	2.32	1.22
Cuticular lipid mass	MS	48,718.30	111,573.32	587.73	7583.82	137.77	1.89
	F	55.67***	659.04***	292.40***	55.05***	68.54***	
	% Variation	19.81	61.11	6.89	8.36	3.01	0.82
Heat knockdown	MS	34,325.33	225,232.41	210.59	4562.12	112.22	1.33
	F	121.56***	433.48***	263.21***	34.75***	60.23***	
	% Variation	26.32	55.26	9.85	4.42	4.00	0.15
Chill coma recovery	MS	48,245.22	356,487.25	328.46	5123.20	78.26	0.87
	F	142.52***	526.32***	216.52***	6785.58***	165.22***	
	% Variation	28.23	50.46	11.21	5.82	3.41	0.87
Carbohydrate content	MS	25,698.26	34,353.56	170.01	2952.01	65.73	0.63
	F	134.69***	574.37***	178.96***	44.91***	69.19***	
	% Variation	39.43	37.54	14.82	5.06	2.41	0.74
Lipid content	MS	32,718.30	104,573.32	587.73	7583.82	137.77	2.01
	F	55.67	759.04	292.40	55.05	68.54	
	% Variation	30.12***	28.81***	20.89***	14.36***	5.01***	0.87
Protein content	MS	24.79	16.38	7.74	15.16	9.64	0.95
	F	9.14	6.04	2.85	8.33	1.94	
	% Variation	0.03$^{n.s.}$	0.01$^{n.s.}$	0.02$^{n.s.}$	0.02$^{n.s.}$	0.01$^{n.s.}$	0.94

S, species; T, temperature; IF, isofemale.
***P<0.001; n.s., not significant.

The Journal of Experimental Biology (2014) doi:10.1242/jeb.096818

Table 3. Data on different measures of water balance and dehydration tolerance of *D. bipectinata* and *D. malerkotliana* isofemale lines (*N*=20 IF lines × 10 replicates each) grown at 17 and 25°C

	D. bipectinata				*D. malerkotliana*			
Trait	17°C	25°C	Ratio	$F_{1,398}$	17°C	25°C	Ratio	$F_{1,398}$
Basic measures of hemolymph and tissue water								
Wet mass (mg fly^{-1})	1.513±0.03	1.065±0.02	1.42	254.63***	1.203±0.04	0.762±0.03	1.58	196.21***
Dry mass (mg fly^{-1})	0.451±0.006	0.315±0.03	1.43	199.50***	0.361±0.008	0.221±0.005	1.63	230.37***
Total water content (mg fly^{-1})	1.062±0.01	0.750±0.08	1.41	230.47***	0.842±0.03	0.541±0.01	1.56	183.96***
Hemolymph content (mg fly^{-1})	0.500±0.006	0.352±0.03	1.42	165.98***	0.396±0.007	0.246±0.003	1.61	214.66***
Hemolymph water content (mg fly^{-1})	0.353±0.004	0.251±0.02	1.40	286.33***	0.277±0.005	0.184±0.002	1.50	260.41***
Tissue water content (mg fly^{-1})	0.709±0.003	0.499±0.03	1.42	186.66***	0.565±0.008	0.357±0.007	1.58	131.89***
Different measures of dehydration tolerance								
Water remaining at death after desiccation (mg fly^{-1})	0.482±0.07	0.380±0.03	1.26	212.17***	0.290±0.005	0.240±0.001	1.20	176.56***
Water lost under desiccation stress (mg fly^{-1})	0.582±0.01	0.370±0.09	1.57	177.42***	0.550±0.009	0.300±0.003	1.83	118.81***
Dehydration tolerance (%)	54.80	49.33	1.11	109.56**	65.32	55.45	1.17	121.42**

F-values of ANOVA were used for comparison of trait values at 17°C and 25°C.
Percent data were arcsine transformed for ANOVA; **P<0.01; ***P<0.001.
Values are means ± s.e.m.

correlated with their corresponding level of desiccation resistance, and the data are given in Fig. 5 and Table 5. For both the species, the energy budget from stored carbohydrates was higher at 17°C than at 25°C growth temperature (Table 5). In addition, the energy budget (carbohydrates) of *D. malerkotliana* was significantly higher compared with that of *D. bipectinata* (Fig. 5A). A simultaneous analysis of carbohydrate content and desiccation resistance in 10 replicates of each of 10 isofemale lines showed significant correlation between these traits (Fig. 5B,C). It may be noted that storage levels of carbohydrates in *D. bipectinata* were significantly lower (~50% less) compared with *D. malerkotliana*. However, we found correlated changes in the level of stored carbohydrates as well as desiccation resistance in both the species.

Trait correlations

In order to check whether there are correlated changes in the resistance to desiccation and cold stress in both the species, we simultaneously analyzed these two traits in individual flies (N=50) of each of 20 isofemale lines of each species, and the data are illustrated in Fig. 6. Note that a faster chill coma recovery reflects

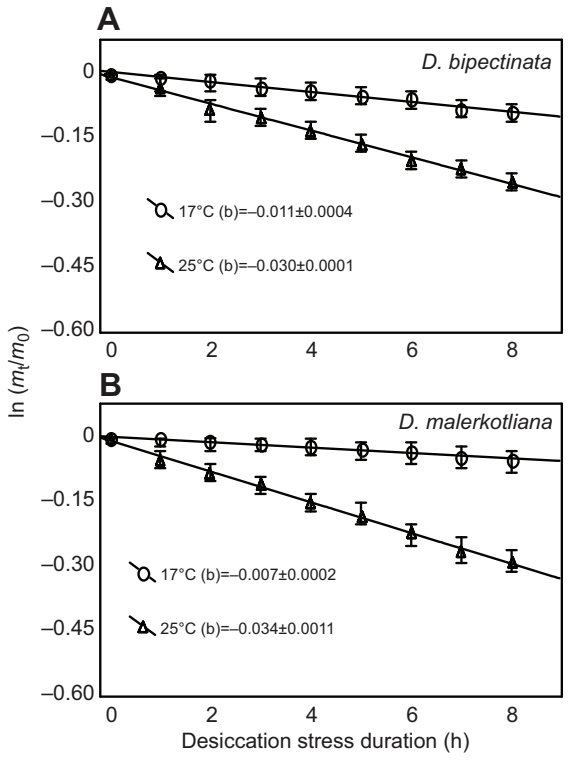

Fig. 3. Water loss. (A,B) Water loss rate (according to Wharton's method) in adult flies of *D. bipectinata* (A) and *D. malerkotliana* (B) grown at 17°C and 25°C. The water loss rate was derived from the slope of $\ln(m_t/m_0)$ as a function of different durations of desiccation stress at ~5% relative humidity. Slope values (means ± s.e.m.) vary significantly between species reared at 17°C ($t_{1,19}$=–3.92; P<0.01) but not at 25°C ($t_{1,19}$=0.45; not significant).

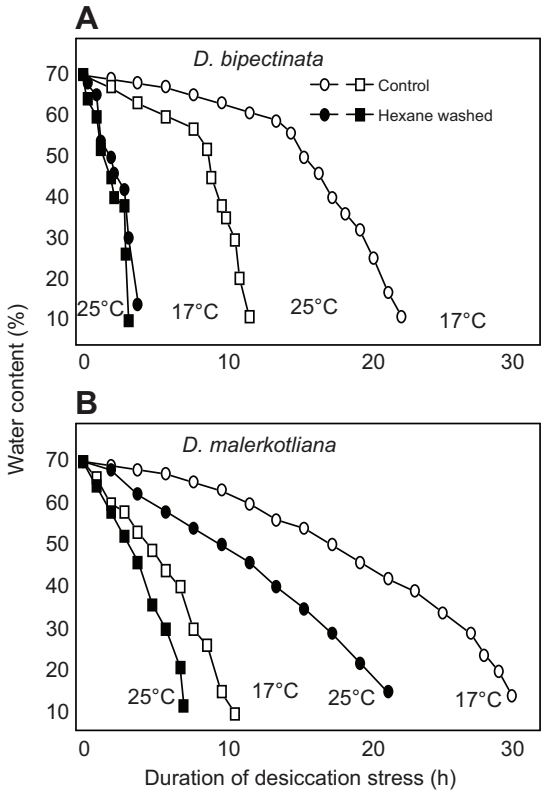

Fig. 4. The effect of cuticular lipids on water loss. (A,B) Changes in the body water loss in control (untreated) and organic solvent (hexane)-treated flies as a function of desiccation stress duration for *D. bipectinata* (A) and *D. malerkotliana* (B) grown at 17°C and 25°C. Significant differences due to hexane treatment are evident for both species.

The Journal of Experimental Biology (2014) doi:10.1242/jeb.096818

Table 4. Slope values for rate of water loss according to Wharton's method in control (dead over-etherized flies) and hexane-treated flies of *D. bipectinata* and *D. malerkotliana*

	17°C	25°C
D. bipectinata		
Control	0.011±0.0004	0.030±0.0001
Hexane treated	0.002±0.0001	0.001±0.0001
$t_{1,19}$	16.66 (P<0.001)	46.51 (P<0.001)
D. malerkotliana		
Control	0.007±0.0002	0.034±0.0011
Hexane treated	0.003±0.0001	0.010±0.0004
$t_{1,19}$	36.41 (P<0.001)	73.40 (P<0.001)

Rate of water loss in control versus hexane treated flies was compared with a *t*-test.
Values are means ± s.e.m.

cold tolerance whereas a longer chill coma recovery represents cold sensitivity. For both the species, the two traits were significantly correlated despite species-specific differences, i.e. *D. malerkotliana* is more tolerant to cold and desiccation stress than *D. bipectinata* (Fig. 6).

Species-specific divergence in adult acclimation

Developmental acclimation effects on stress resistance traits of *D. malerkotliana* and *D. bipectinata* are shown in Table 1. However, data on adult acclimation effects are illustrated in Fig. 7. A comparison of changes in trait values due to adult acclimation effects shows species-specific as well as trait-specific differences, i.e. adult acclimation effects are higher for desiccation resistance, carbohydrate content and chill coma recovery in *D. malerkotliana* compared with *D. bipectinata* (Fig. 7A,B,D). The adult acclimation effects for heat knockdown are higher for *D. bipectinata* reared at 25°C but such effects are much lower for *D. malerkotliana* (Fig. 7C). Thus, for stress-related traits, we found significant differences, based on ANOVA (data not shown), in the absolute acclimation capacity (trait values of acclimated flies minus non-acclimated) of flies reared at 17 and 25°C (Fig. 7). Our results suggest that adult acclimation effects are constrained by their basal level of stress resistance.

Seasonal changes in stress related traits

Data on seasonal changes (rainy versus autumn of 2010 and 2011) in desiccation-related and thermotolerance traits of wild-caught *D. bipectinata* and *D. malerkotliana* are given in Table 6. For cuticular traits (body melanisation), *D. malerkotliana* exhibited approximately fivefold higher trait values in autumn compared with rainy season, despite lack of any seasonal change in the body melanisation of *D. bipectinata*. In contrast, there was a threefold increase in the cuticular lipid mass as well as in desiccation resistance of autumn *D. malerkotliana*, whereas there was a modest (50%) increase in these traits for *D. bipectinata*. The cold tolerance of autumn *D. malerkotliana* increased about threefold but there was only a 60% increase in the case of *D. bipectinata*. Finally, during the rainy season, heat resistance of *D. bipectinata* was about twofold higher but there was only slight increase (~50%) in the case of *D. malerkotliana*. Thus, desiccation and cold resistance of *D. malerkotliana* showed significant increase during the autumn season, and such changes are consistent with changes in cuticular traits and are associated with low humidity and cooler climatic conditions. However, heat resistance of *D. bipectinata* is associated with the warmer higher humidity conditions of the rainy season during 2010 as well as 2011. In the present work, we found repeated

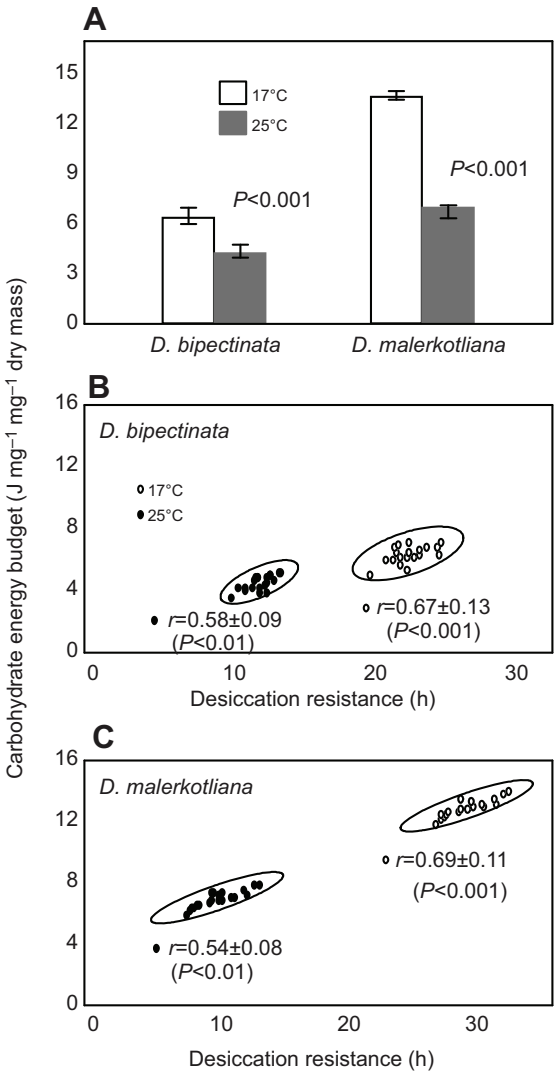

Fig. 5. Energy budgets and desiccation resistance. (A) Energy budgets (means ± s.e.m.) from stored carbohydrates in *D. bipectinata* and *D. malerkotliana* isofemale lines (N=20 IF lines) grown at 17 and 25°C. (B,C) Correlation between desiccation resistance and carbohydrate energy budget in *D. bipectinata* (B) and *D. malerkotliana* (C). For isofemale line variability, ellipses with 90% confidence limits are shown.

patterns of seasonal changes in the relative abundance as well as stress-related traits that are consistent with changes in the climatic conditions of rainy versus autumn seasons.

DISCUSSION

In the present work, we investigated the effects of developmental acclimation on stress resistance traits in 20 isofemale lines each of two tropical *Drosophila* species (*D. malerkotliana* and *D. bipectinata*) reared at two ecologically relevant growth temperatures (17 and 25°C). A previous study found that these species are sensitive to desiccation and cold stress and narrow in their distribution patterns (Kellermann et al., 2009). The invasion of *D. malerkotliana* into the southern states of North America during the last decade reflected a mismatch between its limited physiological tolerance levels and invasion potential. Our results have shown significant increase in resistance to cold and desiccation in *D. malerkotliana* reared at 17°C. The developmental acclimation of *D. malerkotliana* evidenced significant plastic effects in cuticular traits,

The Journal of Experimental Biology (2014) doi:10.1242/jeb.096818

Table 5. Comparison of energy budget from carbohydrates, lipids and proteins in *D. bipectinata* and *D. malerkotliana* isofemale lines reared at 17 and 25°C

	Energy budget (J mg^{-1} mg^{-1} dry mass)			
	17°C	25°C	Ratio	$t_{1,19}$
D. bipectinata				
Carbohydrates	06.42	04.28	1.50	4.74***
Lipids	10.33	06.76	1.53	19.05***
Proteins	02.28	02.23	1.02	0.63$^{n.s.}$
Total	19.03	13.27	1.43	12.13***
D. malerkotliana				
Carbohydrates	13.71	06.93	1.98	26.23***
Lipids	07.31	05.22	1.40	11.27***
Proteins	02.24	02.23	1.00	0.53$^{n.s.}$
Total	23.26	14.38	1.62	19.87***

Trait values (dry-mass adjusted level of each metabolite × conversion factor) in adult female flies of both species were compared with *t*-tests.
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).
***P<0.001; n.s., not significant.

which are associated with threefold increase in desiccation as well as cold resistance. The occurrence of plastic changes in both the cuticular traits seems a novel feature of this tropical species. For both species tested, we showed a role for cuticular lipids in waterproofing by treating flies with organic solvents that significantly increased cuticular efflux of body water. The effects of developmental acclimation at lower growth temperature are consistent with significant increase in desiccation resistance of *D. malerkotliana*. In contrast, stress-related traits increased by only 50% in *D. bipectinata* through developmental acclimation at 17°C. Thus, except heat resistance of *D. bipectinata*, physiological tolerance levels of *D. bipectinata* are lower compared with those of *D. malerkotliana*. Simultaneous analysis of chill coma recovery followed by desiccation resistance in species-specific isofemale lines evidenced significant correlation between these traits in both the species. For stress resistance traits, the effects due to developmental acclimation are significantly higher compared with adult acclimation. Therefore, *D. malerkotliana* has evolved acclimation strategies to elevate its physiological tolerance levels, which might be consistent with its invasion of new territories on the American continent. We have shown that these two related tropical *Drosophila* species living in the same environment differ in their relative abundance level across rainy versus autumn seasons of two different years.

Species-specific divergence of stress resistance traits

Several studies have considered desiccation resistance levels between generalist and tropical endemic *Drosophila* species reared at a common growth temperature of 25°C but there is paucity of data on adult as well as developmental acclimation effects (Gibbs and Matzkin, 2001; Gibbs et al., 2003; Kellermann et al., 2012b). In the present work, we found species-specific differences in water balance-related traits, i.e. we observed ~60% increase in body water content, haemolymph and tissue water in *D. malerkotliana* reared at 17°C compared with 25°C, whereas the corresponding differences in *D. bipectinata* were only 40%. The effect of developmental acclimation on dehydration tolerance was ~11% higher in the case of *D. malerkotliana* but only ~5% in *D. bipectinata*. Therefore, we suggest that developmental acclimation could be a potent mechanism to elevate desiccation resistance in these two *Drosophila* species.

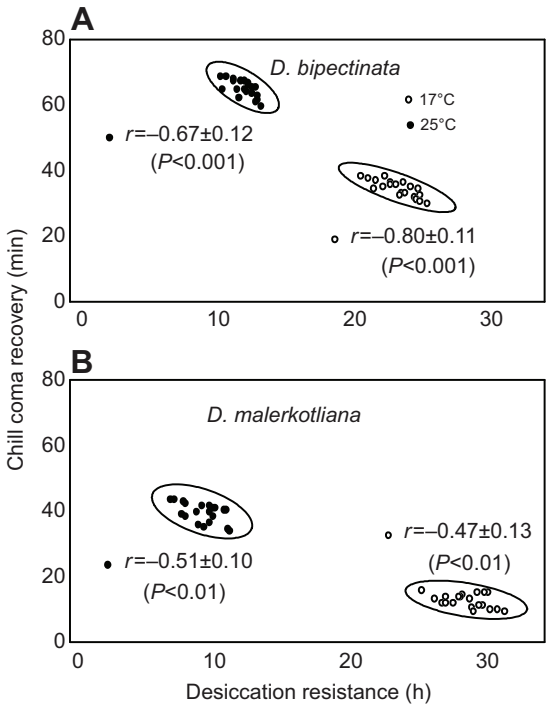

Fig. 6. Desiccation resistance and cold stress. (A,B) Correlations between desiccation resistance and chill coma recovery of 20 isofemale lines of *D. bipectinata* (A) and *D. malerkotliana* (B) reared at 17 and 25°C. For isofemale line variability, ellipses with 90% confidence limits are shown.

Species-specific divergence in cuticular traits

We assessed changes in the amount of cuticular lipids in *D. malerkotliana* and *D. bipectinata* resulting from developmental acclimation despite lack of such evidence in *D. melanogaster* or *D. ananassae* (Gibbs et al., 1997; Parkash et al., 2010). Interestingly, there was a threefold increase in the amount of cuticular lipid of *D. malerkotliana* reared at 17°C compared with 25°C, whereas there was a modest increase of 50% in *D. bipectinata*. Treatment with organic solvents resulted in a faster loss of body water in *D. bipectinata* compared with *D. malerkotliana*. This difference could be due to the effect of developmental acclimation on body melanisation of *D. malerkotliana* (a fivefold increase) but there was no effect on body melanisation of *D. bipectinata* (Fig. 4). If *D. malerkotliana* had only cuticular lipids as barriers to water loss, hexane treatment would have had a similar effect to that in *D. bipectinata*, but this was not so. This is because *D. malerkotliana* has developed body melanisation, which is also a cuticular barrier to water loss. Our results suggest that the two cuticular traits (body melanisation and epicuticular lipids) might serve to waterproof *D. malerkotliana*, consistent with its significantly higher level of desiccation resistance. Thus, these two *Drosophila* species are able to increase their desiccation resistance through plastic mechanisms. However, these species were considered as desiccation sensitive in the past. To the best of our knowledge, changes in the amount of cuticular lipids as well as body melanisation through developmental acclimation have not been reported for any tropical *Drosophila* species so far.

Species-specific divergence in thermotolerance

Several studies have shown that rearing temperatures can affect thermal stress tolerance in diverse insect taxa (Hoffmann and Watson, 1993; Huey et al., 1999; Bubliy and Loeschcke, 2005; Ragland and Kingsolver, 2008). In the temperate mosquito

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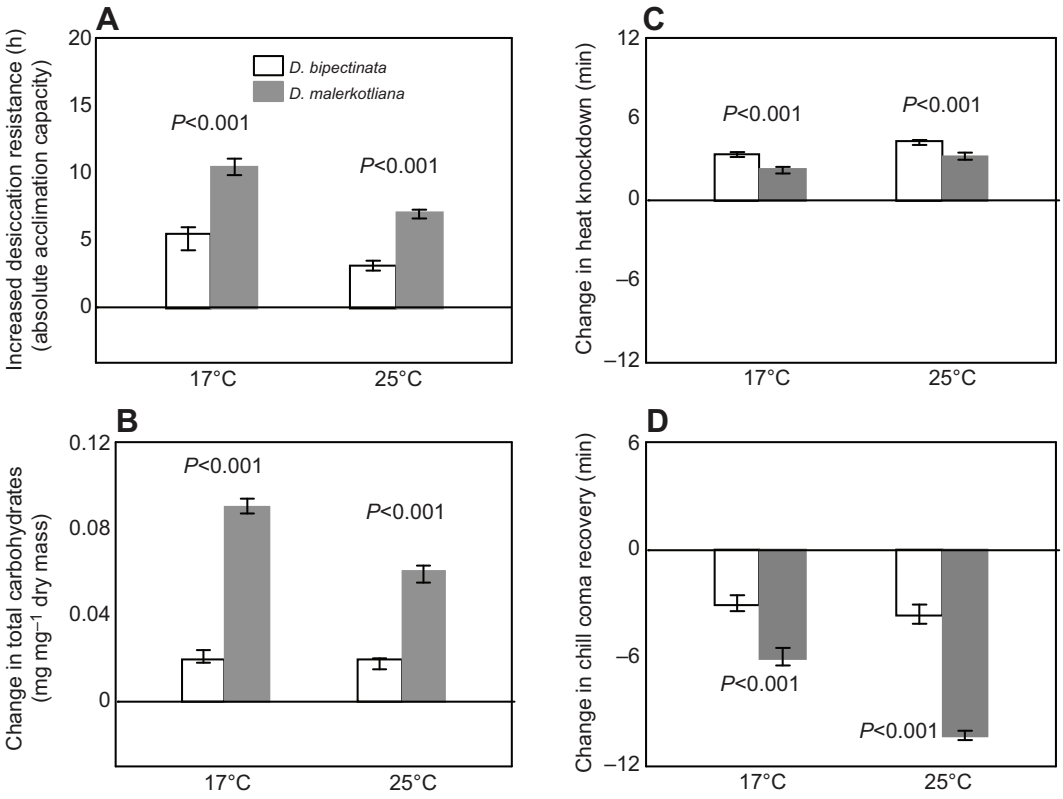

Fig. 7. Species-specific differences in adult acclimation. (A-D) Species-specific changes (means ± s.e.m.) in the absolute adult acclimation capacity (trait values of acclimated minus non-acclimated flies) for desiccation resistance (A), carbohydrate content (B), heat knockdown time (C) and chill coma recovery time (D) in *D. bipectinata* and *D. malerkotliana* isofemale lines (*N*=20 IF lines) grown at 17 and 25°C. For each species, changes in trait values due to adult acclimation of flies reared at 17 and 25°C were tested with ANOVA and the *P*-values are shown.

Wyeomyia smithii, there was significant acclimation effects for cold tolerance but not for heat tolerance (Ragland and Kingsolver, 2008). In the present work, heat tolerance of *D. bipectinata* was higher than of *D. malerkotliana*. In contrast, for cold tolerance, developmental acclimation effects were threefold higher for *D. malerkotliana* grown at 17°C compared with 25°C. *D. bipectinata* is also a cold-sensitive species because flies grown at 25°C took ~1 h to recover. These laboratory observations are consistent with similar trends of seasonal changes in thermotolerance of both these species. Thus, our results support the view that developmental acclimation can significantly affect thermotolerance of these two tropical *Drosophila* species. It is probable that increased cold tolerance of *D. malerkotliana* due to developmental acclimation could enable this species to colonize cooler localities on different continents.

Stress resistance and invasive potential

Three cases of recent invasion of drosophilids on different continents have been documented. These species might have evolved similar or different mechanisms to adapt and extend their range, i.e. invasion of European *D. subobscura* in southern and

Table 6. Data on seasonal changes in desiccation resistance, body melanisation, epicuticular lipid mass, heat knockdown and chill coma recovery of *D. bipectinata* and *D. malerkotliana* collected during rainy and autumn seasons from one northern locality (Chamba; 1000 m) in the years 2010 and 2011

	D. bipectinata					*D. malerkotliana*			
Trait	Year	Rainy	Autumn	Ratio	$t_{1,149}$	Rainy	Autumn	Ratio	$t_{1,149}$
Desiccation (h)	2010	12.46±1.47	19.52±1.68	1.56	28.11***	9.21±1.47	29.37±1.83	3.18	76.21***
	2011	13.10±1.51	21.63±1.78	1.65	33.17***	10.17±1.67	31.42±1.96	3.08	83.41***
Melanisation (%)	2010	2.15±0.56	2.10±0.54	1.02	0.49$^{n.s.}$	8.20±1.37	39.53±2.29	4.82	82.56***
	2011	2.64±0.63	2.78±0.57	1.05	0.54$^{n.s.}$	9.50±1.51	43.55±3.24	4.58	88.32***
Epicuticular lipids (μg cm^{-2})	2010	13.20±1.29	19.15±1.57	1.45	20.42***	9.38±0.86	28.25±1.40	3.01	106.97**
	2011	13.86±1.33	19.70±1.61	1.42	29.82***	10.65±0.98	30.66±1.38	2.87	121.29***
Heat knockdown (min)	2010	21.34±2.40	11.47±1.80	1.86	42.56***	10.29±1.67	6.57±0.94	1.56	60.23***
	2011	24.46±2.52	13.56±1.83	1.80	49.02***	13.68±1.78	8.12±0.97	1.68	73.54***
Chill coma recovery (min)	2010	68.23±3.56	34.27±2.82	1.99	53.56***	38.51±2.46	14.49±1.82	2.65	107.01***
	2011	67.13±3.22	33.67±2.91	1.99	59.23***	41.63±2.86	16.54±1.97	2.51	129.38***

For each species and season, wild-caught female flies (*N*=150) were tested for stress-related traits.
Yearly data for each trait in each species as well as season showed non-significant differences as determined with *t*-tests (data not shown).
Values are means ± s.e.m.

northern America (Huey et al., 2000); *Zaprionus indianus* in Brazil (Tidon et al., 2003) and *D. malerkotliana* in South America and its extension into southern states of North America (Birdsley, 2003; Garcia et al., 2005). Both *D. malerkotliana* and *D. bipectinata* are of tropical origin and their sensitivities to cold and desiccation stress when grown at 25°C are consistent with their abundance in the warm and humid equatorial regions. However, recent invasion of *D. malerkotliana* into a few southern states of North America seems a mismatch because of its lower physiological tolerance levels to climatic stresses. It is not clear how *D. malerkotliana* has adapted to the cooler and drier habitats of sub-tropical regions on different continents and has achieved a sub-cosmopolitan status (Markow and O'Grady, 2006). This might be because no previous study has investigated stress resistance traits in these two tropical *Drosophila* species reared at lower temperatures. The comparison of stress resistance traits in *Drosophila* species reared at 25°C could bias their stress resistance level. In the present work, greater resistance to cold and desiccation in *D. malerkotliana* as a result of developmental acclimation at 17°C are consistent with its ability to cope with the cooler and drier habitats (midland locations) in the western Himalayas. Furthermore, in a southern Indian montane locality (Chamundi hills with a maximum altitude of ~1000 m), there is an approximate fivefold higher relative abundance of *D. malerkotliana* compared with *D. bipectinata* (Guru Prasad and Hegde, 2006). This study has shown greater abundance of *D. malerkotliana* associated with cooler and drier habitats of a montane locality. We also found similar trends of abundance of *D. malerkotliana* compared with *D. bipectinata* in midland localities of the western Himalayas. For *D. malerkotliana,* changes in the species distribution range and its invasion capacity could be associated with developmental acclimation. We also found significant increase in the cold and desiccation resistance of *D. malerkotliana* resulting from adult acclimation. It is possible that *D. malerkotliana* has been able to invade southern parts of North America because of developmental as well as adult acclimation effects. Therefore, this species may be a convenient indicator species to assess the effects of global warming on different continents. More field data are required on the relative abundance of these two tropical species on different continents, which could be helpful in assessing the impact of global warming on related species living under shared environments.

MATERIALS AND METHODS

Collections and culture

Wild individuals of *Drosophila malerkotliana* Parshad and Paika 1965 and *D. bipectinata* Duda 1923 (N=250–300 flies from each site) were collected in a single trip in October 2010 from five subtropical localities of the western Himalayas (Parwanoo, 600 m; Mandi, 800 m; Chamba, 1000 m; Dharamsala, 1220 m; Solan, 1440m). The identification of species were made on the basis of taxonomic characteristics (Parshad and Paika, 1964; Markow and O'Grady, 2006). These two species were identified on the basis of sex combs and abdominal pigmentation in the progeny of each of several isofemale lines for each species. The abdomens of male *D. malerkotliana* are shiny black, whereas they are pale in both male and female *D. bipectinata*. Female *D. malerkotliana* have narrower brown bands on the posterior margin of all the abdominal tergites (Bock, 1971). However, sex combs in the males of these two species are quite different. In *D. bipectinata*, sex combs comprise two oblique but adjacent rows of six to nine strong teeth on the metatarsus. In contrast, in *D. malerkotliana*, sex combs on the foreleg comprise two sets on the metatarsus with one and four teeth, respectively, and two sets on the next tarsal segment with one and three teeth (Parshad and Paika, 1964; Bock and Wheeler, 1972; Markow and O'Grady, 2006). Furthermore, we made crosses between all of the isofemale lines of both the species collected from a midland locality with their species-specific strains obtained from the *Drosophila* stock centre, Mysore (India). For each species, crosses produced fertile F1 and F2 flies of both the sexes which confirmed the identification of these two *Drosophila* species.

Percentage species 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. Wild-caught individuals from a midland locality (Dharamsala) were used to initiate 20 isofemale lines (geographical variables: altitude 1220 m, latitude 32°45′N, longitude 77°06′E; climatic variables: T_{min}=16.80°C, T_{max}=23.50°C, T_{ave}=19.12°C, RH=50%). Replicate cultures of each species were maintained at low density (60–70 eggs per 40×100 mm vial) on cornmeal-yeast-agar medium and reared at 17 and 25°C for four generations under the same thermal and humidity condition in order to remove the potential effects of environmental variation on wild-caught flies. The isofemale lines reared in the laboratory for four generations can minimize the effects of laboratory adaptations on the stress-resistance traits (Hoffmann, 2010). For each isofemale line, four out of eight batches of eggs (each ~200) were reared at 17°C while the remaining four were reared at 25°C in 250 ml wide-mouth culture bottles with cotton plugs. All assays were performed on adult females (6 days post eclosion) grown at 17 and 25°C. Climatic data for thermal and humidity variables were obtained from the Indian Meteorological Department (Indian Meteorological Department, 2010). The climatic conditions of Florida and Athens (Georgia; USA) were obtained from www.worldweatheronline.com.

Trait analysis

All assays for stress-related traits were performed on individual flies from the progeny of each of isofemale lines of both the species. Multiple replicates (~50) of each isofemale line were run simultaneously to estimate the effects of organic solvents on cuticular permeability as a function of different durations of desiccation stress. For stress-related traits, we used individual adult flies, but a group of 10 individuals were examined to analyze the storage levels of energy metabolites. For flies grown at 17 and 25°C, we tested desiccation-related traits at their respective growth temperatures. For mortality- or recovery-based assays for different stress-related traits, female flies were tested and scored individually for 20 isofemale lines of each *Drosophila* species. For data analysis, we used the means of 20 isofemale lines of *D. bipectinata* as well as *D. malerkotliana*.

Seasonal collections and trait analyses

For analysis of species-specific differences in stress-related traits, seasonal (rainy versus autumn) collections of *D. malerkotliana* and *D. bipectinata* (N=150 each) were collected from one locality (Chamba, 1000 m) during the years 2010 and 2011. For each species and seasonal collection, three groups of wild-caught flies (3×50 female flies) were directly tested for two or three stress-related traits. Flies of the first group (N=50) were scored for body melanisation followed by chill coma recovery of individual flies; and after recovery these flies were individually tested for desiccation resistance. For the second group (N=50), after scoring body melanisation and chill coma recovery, the epicuticular lipid mass was estimated for each fly. For the third group of wild-caught flies (N=50), we measured body melanisation and heat knockdown of individual female flies. Similar assays of stress-related traits were made for independent collections made during the rainy and autumn seasons of 2010 and 2011.

Analysis of body melanisation

The progeny of each isofemale line was examined for differences in body melanisation on the abdominal segments. For *D. malerkotliana*, body melanisation of individual female flies (N=20 IF lines ×10 replicates each) was visually scored with an Olympus SZ-61 stereo-zoom microscope (www.olympus.com). It was estimated from dorsal as well as lateral views of the female abdomen, and giving values ranging from 0 (no melanisation) to 10 (complete melanisation) for each of the six abdominal segments (second to seventh). The relative size of each abdominal segment was calculated in proportion of the largest fourth abdominal segment which was assigned the value of 1.0. Because 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 second to seventh segments, respectively) were multiplied with segment-wise melanisation scores. The percentage melanisation was 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., 2008). However, flies of *D. bipectinata* did not show abdominal bands and were paler in colour.

Assessment of epicuticular lipid mass

We assessed cuticular lipid mass in individual flies (20 IF lines ×10 replicates each) of both the species reared at 17 and 25°C. Each fly was dried for 24 h at 60°C to obtain the dry mass. Each dried fly was kept in 2 ml HPLC-grade hexane in Eppendorf tubes (Tarsons Products Pvt Ltd, New Delhi, India) for 3 min and then it was removed from the solvent, dried at room temperature and finally reweighed on a sartorius microbalance (Model-CPA26P; 0.001 mg precision; www.sartorious.com).

Desiccation resistance

Desiccation resistance was measured as the time to lethal dehydration effect under dry air. We placed individual flies of each isofemale line in dry plastic vials (40×100 mm) in which the open end was covered with a muslin cloth. These vials were kept on the 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 desiccation chamber (Secador electronic desiccator cabinet; Tarsons Products) which maintained ~5% RH. The number of immobile flies was counted every hour, and LT_{100} values in dry air were recorded. LT_{50} values were calculated with the help of Probit method using SPSS software for windows.

Basic measures of water balance

To estimate total body water content and dehydration tolerance, individual flies of each isofemale (IF) line (20 IF lines ×10 replicates each) were used. For each isofemale line, individual flies were weighed on a Sartorius microbalance (Model-CPA26P; 0.001 mg precision) and then reweighed after drying for 24 h at 60°C. Total body water content was estimated as the difference between the mass before and after drying at 60°C. After mild anaesthesia with ether (1 min), individual flies were weighed on a Sartorius microbalance both before and after desiccation stress until death. In individual flies, dehydration tolerance was estimated as the percentage of total body water lost until death due to desiccation; and was calculated using the formula (wet body mass−body mass at death)/(wet body mass−dry body mass)×100 (Gibbs et al., 1997). Thus, all the traits were measured in individual flies from the progeny of each isofemale line.

For calculation of the rate of water loss, we followed Wharton's method modified by Benoit and co-workers (Wharton, 1985; Benoit et al., 2005). Total body water content (m) was calculated as the difference between wet or fresh (f) and dry mass (d), i.e. $m=f–d$. Individual flies were weighed and placed at ~5% RH for a specified time at 1 h intervals (1–8 h), and reweighed. The rate of water loss was derived from the slope of the regression line on a plot of $1n(m_t/m_0)$ against time according to Wharton's exponential equation (Wharton, 1985) $m_t=m_0e^{-kt}$, 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.

Effect of organic solvent on the cuticular water loss

Changes in the cuticular permeability due to organic solvent were tested on individual adult flies (20 IF lines ×50 replicates). The assays were conducted by treating over-etherized (dead) flies immediately with 2 ml of hexane and shaking gently for 5×30 s. Soon thereafter, flies were blotted dry, weighed and placed in a desiccation chamber (Secador electronic desiccator cabinet) which was maintained at ~5% RH. The effect of hexane on rate of water loss was monitored at 30 min intervals for *D. bipectinata* and *D. malerkotliana* reared at 17 and 25°C. For control groups, no solvent treatment was given and cuticular water loss was determined at 1 h intervals.

Assessment of extractable haemolymph content

Individual flies were carefully pinned to a microdissection dish at its anterior and posterior ends with microdissection pins, and a narrow incision was made through the cuticle with a third pin while observing through a stereo-zoom microscope (SZ-61). The leaking extractable haemolymph was absorbed with an absorbent tissue moistened with an isotonic saline solution (Folk et al., 2001). Haemolymph content was estimated as reduction in mass following haemolymph blotting (Hadley, 1994). In order to achieve accuracy, several pilot experiments on haemolymph blotting were done to standardize the method. Independent observations by two people gave higher correlation values (r=0.87) ensuring repeatability of our results. Tissue water was estimated after subtracting exsanguinated mass before and after drying. From the same data, we also calculated haemolymph water content by subtracting tissue water from total body water content.

Assessment of adult desiccation acclimation responses

To measure adult acclimation duration, individual adults of each isofemale line (20 IF lines ×10 replicates each) of *D. bipectinata* and *D. malerkotliana* were subjected to desiccation stress at ~5% RH. Based on pilot experiments, the acclimation time at 17°C was 12 h and at 25°C was 4 h, i.e. the time period in which flies lost ~15–17% body water. Variation in water lost during adult acclimation in the replicates was insignificant under our assay conditions. For the recovery period, individuals were placed on non-nutritive agar and tested at hourly intervals for increase in body water until the lost body water was regained. The recovery time at 17°C was 12 h and at 25°C was 4 h. Such individuals were then subjected to desiccation stress until death in order to test the increased desiccation resistance due to acclimation. Thus, acclimation effects (increased desiccation survival) 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. Changes in the carbohydrate content were also determined after adults were acclimated to desiccation stress.

Analysis of heat knockdown and chill coma recovery

For heat resistance assays, individual adult females of *D. bipectinata* and *D. malerkotliana* grown at 17 and 25°C (20 IF lines ×10 replicates each) were placed in stoppered 10 ml glass vials submerged in a glass tank with water held at a constant temperature of 39°C. Resistance was scored as the time taken for flies to be knocked down. To evaluate chill coma recovery, 10 adult females of each species (20 IF lines ×10 replicates each) were placed in 10 ml glass vials, which were submerged in a 10% glycol solution cooled to 0°C. The vials were removed after 8 h and recovery time was scored. The flies were considered recovered when they were able to stand up on their legs. Flies were heat acclimated for 1 h at 35°C followed by 6 h recovery at 25°C before assessing heat knockdown resistance. For cold acclimation, adult flies were pretreated to 4°C for 8 h and allowed to recover for 12 h before assessing chill coma recovery. Thereafter, their thermotolerance changes (increase or decrease) due to thermal acclimation were recorded.

Estimation of energy metabolites

Trehalose, glycogen, body lipid content and proteins were estimated following the method of Parkash et al. (Parkash et al., 2012), and full details of homogenate preparation, use of the Megazyme Trehalose Assay Kit for trehalose estimation and use of the gluco-amylase assay for glycogen, estimation of proteins using the bicinchoninic assay, and estimation of lipids per fly by subtracting the lipid free dry mass from initial dry mass per fly, are given therein.

Statistical analyses

For each trait, means ± s.e.m. of 20 isofemale lines were used for illustrations and tables. For both the species reared at 17 and 25°C, we calculated correlation coefficients between LT_{50} and LT_{100} (h) measures of desiccation resistance. Effects of developmental temperatures (17 versus 25°C) on desiccation-related traits, energy metabolites, body mass, and basic measures of water balance, dehydration tolerance, heat knockdown and chill coma recovery were compared using ANOVA. For comparing slope values for rate of water loss (based on Wharton's method), we used t-tests as suggested by Zar (Zar, 1999). However, we used nested ANCOVA (isofemale lines nested into species) to assess the percentage variance due to species, growth temperatures, isofemale lines and their interactions effects for desiccation resistance, cuticular lipid mass, heat knockdown time, chill coma recovery time and energy metabolites. Pearson's correlation coefficients were calculated from data of 20 isofemale lines for desiccation resistance, carbohydrate content and chill coma recovery. Data on energy

metabolites were dry-mass adjusted and energy contents of 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. $P<0.05$ was considered statistically significant in all tests.

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Competing interests
The authors declare no competing financial interests.

Author contributions
R.P. helped in the preparation of the manuscript and interpretation of the results. D.S. was involved in execution of the experiments and analyzing the results. C.L. helped in the culture maintenance and execution of the experiments.

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