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Desiccation resistance and mating behaviour in laboratory populations of *Drosophila simulans* originating from the opposing slopes of Lower Nahal Oren (Israel)

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Abstract

Lower Nahal Oren in Northern Israel, often referred to as 'Evolution Canyon', has been proposed as a microscale model site for ecological evolution. However, conflicting stress resistance and mating assay results contribute to controversy over the Nahal Oren model. In this study, we further tested the Nahal Oren model, while extending its focus from *Drosophila melanogaster* to its sister species, *Drosophila simulans*. Using fly populations derived from the opposing canyon slopes and acclimated to laboratory conditions for 11–22 generations, we did not find a significant slope effect on desiccation resistance ($P = 0.96$) or body metabolic fuel content ($P > 0.43$), which would indicate a genetic basis for adaptation to local resource limitation. Multiple-choice mating assays (47–48% homotypic couples in two replicate populations) did not indicate divergence from a random mating pattern between north- and south-facing slope flies. In conclusion, our findings do not support divergence of *D. simulans* populations across Lower Nahal Oren.

Introduction

Water availability and temperature are the two main abiotic factors determining the distribution of terrestrial organisms, whose fitness largely depends on their ability to conserve body water in a generally dry environment. The small body size of insects compounds this challenge because of limited water storage ability and a relatively large surface area through which water is lost to the environment (Chown & Nicolson, 2004).

Ecologically based natural selection is currently considered central to population divergence and speciation (Rundle & Nosil, 2005). The Lower Nahal Oren, Mount Carmel, Israel, has been proposed as a model site for microscale ecological evolution (Nevo, 1997). The geology and microclimate of the north- and south-facing slopes of the canyon (NFS and SFS, respectively) are similar, but the orientation of the slopes dictates that SFS is exposed to considerably higher levels of solar radiation, and thus is warmer and drier than NFS. Interslope differences in genes and genomes as well as differences in species richness and resistance to environmental stressors have been reported for a wide range of taxa, including several *Drosophila* species (reviewed in Nevo, 2006). Korol *et al.* (2006) suggested that interslope divergence in adaptive genes, stress tolerance and mating behaviour in *Drosophila* support ecologically based incipient sympatric divergence, given the relatively close proximity of the NFS and SFS (100 m at the bottom and 400 m at the top) compared with reported dispersal distances in *Drosophila* (Coyne & Milstead, 1987).

The ability of drosophilids to withstand environmental stress in the form of resource limitation has been extensively studied. Higher stress-tolerance levels are associated with both increased resource storage and reduced loss rates (Gibbs & Gefen, 2009). A large-scale interspecific comparison showed that tropical species are significantly more susceptible to desiccation when compared with species from temperate regions, with resistance levels positively correlated with body lipid content (van Herrewege & David, 1997). A strong response to

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laboratory selection highlights the ample genetic variation for desiccation resistance in *Drosophila*. Desiccation-selected *Drosophila* have often been shown to exhibit lower metabolic rates under stressful conditions, lower rates of water loss and higher content of carbohydrates which may allow increased water storage capabilities (reviewed by Hoffmann & Harshman, 1999). Furthermore, when subjected to desiccation stress, fruit flies preferentially oxidize carbohydrates, whereas under starvation stress, a combination of metabolic fuels is utilized (Marron *et al.*, 2003). Laboratory selection for desiccation resistance in *Drosophila melanogaster* also results in increased fraction of longer-chain cuticular hydrocarbons, which correlates with increased resistance to water loss (Gibbs *et al.*, 1997). The dual role of cuticular hydrocarbons for both waterproofing and as mate-recognition agents (Antony & Jallon, 1982) suggests a possible avenue for linking divergent selection with reproductive isolation.

Recent studies indicated higher desiccation resistance for SFS populations of *D. melanogaster* compared with NFS flies (Korol *et al.*, 2006; A. Korol, personal communication), although a counter-intuitive pattern of higher resistance for NFS flies was reported initially (Nevo *et al.*, 1998). Likewise, a number of laboratory mate-choice assays of NFS- and SFS-derived flies resulted in nonrandom mating patterns, where flies showed preference for mates originating in the same slope (Korol *et al.*, 2000; Singh *et al.*, 2005), but other studies failed to replicate these results (Schlötterer & Agis, 2002; Panhuis *et al.*, 2003; see also Drake *et al.*, 2005).

This study aims to further test the incipient sympatric speciation model presented by Korol *et al.* (2006), which predicts that adaptive divergence of *Drosophila* populations from the opposing Nahal Oren slopes would elicit behavioural changes leading to interslope reproductive isolation. Using *Drosophila simulans* populations originating from opposing EC slopes and acclimated for laboratory conditions, we tested a possible genetic basis for interslope differences in desiccation resistance and in metabolic fuel contents which correlate with enhanced stress-resistance performance. This study extends available mate-choice data for another species, as with the exception of a 'pilot test' on *D. simulans* (Singh *et al.*, 2005); all available mating preference data from EC flies are limited to *D. melanogaster*.

Materials and methods

Flies

The flies were collected from the north- and south-facing slopes of Lower Nahal Oren, Mount Carmel, Israel (32°42′N, 34°58′E). Two replicate populations were created in the laboratory from flies collected on each slope on September 17th and 23rd (population 1) and October 12th, 2009 (population 2). Transparent plastic bottles (1.5 L) containing apple slices were hung on trees in midslope elevations on both the slopes. The traps were collected the following morning and brought to the lab, where individual females were placed in vials containing 10 mL standard cornmeal medium (Markow & O'Grady, 2006) at room temperature (23–25 °C) and constant light. Experimental populations were established from 10 male and 10 female progeny of each of 35 (population 1) and 25 (population 2) females from each slope (a total of 700 and 500 flies, respectively).

The flies were kept in Plexiglas population cages (25 × 20 × 15 cm) for at least 11 generations prior to initiation of experiments to minimize the possibility of artefacts resulting from adaptation to a new environment (Chippindale, 2006). Preadult stages were reared at densities of ~75 larvae in vials containing 10 mL of cornmeal medium. After 2 weeks, adult flies (approximately 4 days posteclosion; PE) were transferred to population cages having two petri dishes containing food. A cloth sleeve covered one end and allowed access to the cage. The medium was changed every 2 days. At 5–6 days PE yeast paste was added to stimulate egg production. Approximately 1500 eggs were collected at 6–7 days PE to found the next generation.

Flies from additional vials of similar egg densities were transferred to 175-mL plastic bottles containing 50 mL cornmeal medium. Flies (~150) were transferred at 3–4 days PE, and yeast paste was added to stimulate egg production. For egg collection, adults were transferred to empty 175-mL bottles covered with a 35 × 10 mm plate containing grape agar as a substrate for egg laying and placed upside down. Sets of 70–80 eggs were then counted and placed in vials containing approximately 10 mL of cornmeal food. The vials were incubated at room temperature and constant light. Eclosing flies were used for experimentation as detailed below.

Body mass

The vials were checked every 2 h for eclosing flies. The flies were sexed, and eight sets of 10 individuals (from each of two sexes × two replicate populations × two slopes) were weighed to the nearest 0.1 mg (CPA224S, Sartorius, Goettingen, Germany).

Desiccation resistance

After 21–22 generations of laboratory maintenance (population 1 and 2, respectively), adult males and females at 4 days PE from each of the four experimental populations (n = 27 to 34 for each sample) were transferred to empty vials individually after brief CO_2 anaesthesia and restricted to the lower half of the vials by a foam stopper. Silica gel was then added above the stopper to maintain low humidity, and the vial was sealed with parafilm. The vials were placed in an incubator (25.0 ± 0.1 °C; Friocell 222, MMM, Munich,

Germany), and mortality was recorded at hourly intervals. Calculation of the initial oxygen content of the sealed vial and metabolic rates of the sibling species *D. melanogaster* (Gefen & Gibbs, 2009) indicate an oxygen depletion of not more than 1.5% during the duration of the experiment.

Metabolic fuel assays

Sixteen males and females from each of the four experimental populations were collected within 2 h PE, sexed, weighed and frozen at −20 °C. After thawing, individual flies were homogenized in microfuge tubes containing 200 μL 0.05% Tween-20 (in water) using a hand-held grinder. The samples were then centrifuged for 1 min at 16 000 r.p.m., and the supernatant was transferred to new tubes for the determination of the following metabolites:

Carbohydrates
Triplicates (10 μL) from each sample were loaded on 96-well microplates, and 10 μL of *Aspergillus niger* amyloglucosidase (0.1 mg mL^{-1}, 10115; Sigma-Aldrich, St. Louis, MO, USA) was added to each well to catalyze the conversion of glycogen and trehalose into glucose. The plates were then left overnight at room temperature. The following day, 100 μL of Liquid Glucose Reagent (Pointe Scientific Inc., Canton, MI, USA) was added to each sample, and absorbance at 340 nm was read using a PowerWave XS2 microplate reader (BioTek Instruments, Winooski, VT, USA). Carbohydrate concentrations were determined using standards of known glycogen concentration.

Triglycerides
Triglyceride content was measured using Serum Triglyceride Determination kits (TR 0100; Sigma-Aldrich). Samples were placed in microplates in 30 μL triplicates, and 100 μL free glycerol reagent was added before absorbance was read at 540 nm. Then, 25 μL of triglyceride reagent was added, and the plates were allowed to sit in room temperature for 15 min, before absorbance was read again at 540 nm. The amount of triglycerides was calculated as the difference between free glycerol levels before and after the use of the triglyceride reagent, using standards of known glycerol concentration.

Protein
Protein content was determined using the bicinchoninic acid kit for protein determination (BCA1; Sigma-Aldrich). Supernatants were diluted with water at 1 : 1 and 1 : 2 ratios for males and females, respectively. For measuring protein content, 8 μL triplicates from each sample were loaded on a microplate, and 200 μL of protein assay reagent (50 parts bicinchoninic acid solution to one part 4% $CuSO_4$) was added. The plates were then incubated overnight at room temperature, and absorbance at 562 nm was measured the following day. Protein concentrations were determined using standards of bovine serum albumin.

Mating assays

Flies from the four populations were sexed within 2 h PE and placed in fresh food vials in sets of 10. At 2 days PE, the flies were transferred to vials with fresh dyed (red or blue food colouring) food for 2 days for later identification. Food colour treatment was alternated even though possible effects on mating preferences in *Drosophila* have been ruled out previously (e.g. Korol *et al.*, 2000; Gefen & Gibbs, 2009).

Multiple-choice mating assays were conducted at room temperature with adult flies at 4 days PE, when vials with 10 NFS females, 10 NFS males, 10 SFS females and 10 SFS males were opened in the 25 × 20 × 15 cm Plexiglas assay cages at a random order. Mating couples were aspirated out and placed in empty vials for identification based on their gut content colour, which was usually visible through their abdomen. When identification was not possible through the abdomen, the gut was dissected out. The assays lasted 1 h during which the number of copulations never exceeded 10 (50% of possible copulations). A total of 42 assays were conducted for each replicate population.

Statistics

Statistical analyses of body mass, desiccation resistance and metabolite contents were carried out using Statistica 8.0 (Statsoft, Tulsa,OK, USA). For ANOVA, sex and slope were treated as fixed effects and replicate populations as random effects. For ANCOVA on carbohydrate and triglyceride content, we used protein content as a covariate, as it was a good predictor of body size (see Results).

Mating frequencies, propensities and possible deviations from random mating were analyzed using JMATING software (version 1.0.8; available at http://www.uvigo.es/webs/c03/webc03/XENETICA/XB2/JMsoft.htm) for the analyses of mating frequency data (Carvajal-Rodriguez & Rolan-Alvarez, 2006). Calculated coefficients were pair sexual selection (PSS), pair sexual isolation (PSI) and pair total isolation (PTI). Briefly, PSS and PSI estimate the contribution of sexual selection and sexual isolation for each pair type, whereas PTI (a ratio of observed mating frequencies to those expected if mating between types was random) is an estimate of their combined effect (Rolan-Alvarez & Caballero, 2000). JMATING also calculates an index of sexual isolation (I_{PSI}) for the entire experiment with the values ranging from −1 (representing negative assortative mating) to 1 (representing positive assortative mating) and a value of 0 representing random mating (Coyne *et al.*, 2005). Coefficient estimates and their significance were determined by bootstrapping 10 000 times using JMATING software.

Unless stated otherwise, values throughout the paper are means ± SE.

Results

Body mass

No significant effect of either slope (3-way ANOVA; $F_{1,24}$ = 0.47, P = 0.50) or replicate population ($F_{1,24}$ = 0.003, P = 0.95) on body mass was detected. Significant sexual dimorphism in body mass ($F_{1,24}$ = 66.97, $P << 0.001$) mirrored the ~20% difference between female (0.97 ± 0.01 mg) and male (0.79 ± 0.01 mg) body mass (values are means ± SE of n = 10 flies; 16 groups of 10 females and males were weighed).

Desiccation resistance

Figure 1 shows cumulative death ratios of male and female flies from the four experimental populations. Female survival under desiccating conditions at 25 °C (17.3 ± 0.4 h; n = 120) was more than two-fold longer compared with males (7.9 ± 0.2 h; n = 120) (3-way ANOVA; $F_{1,232}$ = 364.8, $P << 0.001$). However, no significant effects of slope ($F_{1,232}$ = 0.003, P = 0.96) or replicate population ($F_{1,232}$ = 0.27, P = 0.61) were observed.

Metabolic fuel contents

Table 1 presents metabolic fuel contents in newly eclosed male and female *D. simulans* from each of the four experimental populations. All assayed metabolic fuels varied significantly between males and females (Tables 2–4). As with survival under desiccation conditions, no significant slope effect was evident on protein, triglyceride or carbohydrate levels ($P > 0.4$ in all comparisons). A highly significant effect of replicate population on carbohydrate content was observed, with lower levels measured for population 2. *Post hoc* comparisons did not yield significant differences between females of the two replicate populations, but population 1 males had significantly higher carbohydrate levels than the males of population 2 (Tukey HSD test, α = 0.05; Table 1). A significant sex x replicate population interaction on triglyceride levels was also observed, with the higher levels in females of population 1 compared with population 2 but no difference between males of the two replicate populations (Tukey HSD test, α = 0.05).

Mean metabolic fuel contents for the eight experimental groups (males and females of the four populations) were regressed on mean body mass measured for the respective samples. Body protein content was significantly regressed on body mass ($P < 0.001$; r^2 = 0.94). In comparison, variation in body size accounted for 57% and 83% of variation in carbohydrate and triglyceride content, respectively. As body mass of individual flies could not be determined, we used protein content as a covariate to assess carbohydrate and triglyceride content while accounting for body size. Protein content (hence body size) was a good predictor of triglyceride content in both males and females (Table 5), whereas carbohydrate content is largely independent of body mass (Table 6), suggesting preferential storage rather than the body size effect on elevated carbohydrate content in population 1 males.

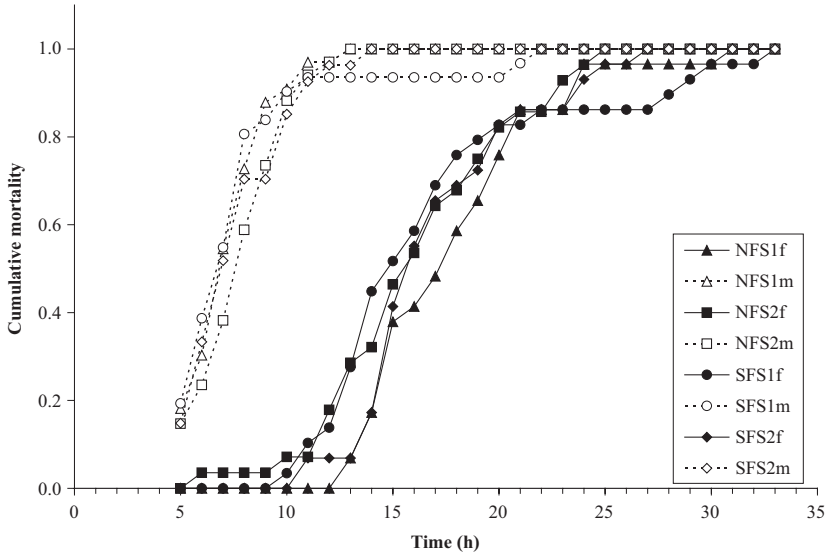

Fig. 1 Cumulative mortality fractions among male (m) and female (f) flies from two replicate populations (1 and 2) of flies originating in the Nahal Oren north- and south-facing slopes (NFS and SFS, respectively), as a function of exposure time to desiccation stress at 25 °C (see Materials and Methods).

Table 1 Metabolic fuel contents (mean ± SE; $n = 16$) in female (F) and male (M) *Drosophila simulans* from the two replicate populations originating from the north- and south-facing slopes (NFS and SFS, respectively) of the Lower Nahal Oren.

Sex	Slope	Replicate population	Protein (μg fly^{-1})	Carbohydrates (μg fly^{-1})	Triglycerides (μg fly^{-1})
F	NFS	1	89.7 ± 3.4	16.2 ± 0.8	28.6 ± 1.9
F	NFS	2	91.3 ± 1.2	15.9 ± 0.6	24.4 ± 1.7
F	SFS	1	95.8 ± 2.3	17.5 ± 0.7	31.1 ± 1.1
F	SFS	2	91.9 ± 1.3	14.7 ± 0.7	25.3 ± 1.0
M	NFS	1	70.0 ± 2.1	13.3 ± 0.9	18.2 ± 2.0
M	NFS	2	72.4 ± 1.2	10.5 ± 0.6	24.1 ± 1.6
M	SFS	1	71.5 ± 1.8	14.7 ± 0.8	19.8 ± 1.5
M	SFS	2	67.7 ± 2.0	10.7 ± 0.5	20.6 ± 1.4

Table 2 ANOVA for fly protein content.

	SS	d.f.	MS	*F*	*P*
Population	27.5	1	27.5	0.40	0.527
Sex	15188.7	1	15188.7	222.92	0.000*
Slope	25.0	1	25.0	0.37	0.546
Population × sex	2.0	1	2.0	0.03	0.865
Population × slope	274.0	1	274.0	4.02	0.047*
Sex × slope	193.5	1	193.5	2.84	0.095
Population × sex × slope	0.9	1	0.9	0.01	0.909
Error	8176.1	120	68.1		

* indicates significant effect ($P < 0.05$).

Table 3 ANOVA for fly carbohydrate content.

	SS	d.f.	MS	*F*	*P*
Population	199.04	1	199.04	23.449	0.000*
Sex	458.97	1	458.97	54.071	0.000*
Slope	5.42	1	5.42	0.639	0.426
Population × sex	27.18	1	27.18	3.202	0.076
Population × slope	27.57	1	27.57	3.248	0.074
Sex × slope	5.87	1	5.87	0.691	0.407
Population × sex × slope	3.10	1	3.10	0.365	0.547
Error	1018.60	120	8.49		

* indicates significant effect ($P < 0.05$).

Mating assays

A total of 192 (population 1) and 188 (population 2) copulations were observed, ~45% of possible matings in the 84 trials. The four possible mating combinations ranged from 23% to 27% of total observed copulations in either replicate populations, with both indices for sexual isolation (PSI) and sexual selection (PSS) not significantly different from 1. Therefore, Table 7 gives the total number of possible homotypic and heterotypic mating pairs pooled for both the replicate populations. The near 25% fraction for all mating pair types indicates a random mating pattern between *D. simulans* flies from the opposing Nahal Oren slopes. Estimated PSI and PSS not statistically different from 1 highlight the lack of mate discrimination or differences in mating propensities of flies from the opposing slopes. The calculated I_{PSI} statistic for both replicate populations was not significantly different from 0. Values for population 1 ($I_{PSI} = 0.04$, SD = 0.07, $P = 0.55$) and population 2 ($I_{PSI} = 0.05$, SD = 0.07, $P = 0.46$) confirm the apparent pattern of random mating between NFS and SFS flies.

Table 4 ANOVA for fly triglyceride content.

	SS	d.f.	MS	*F*	*P*
Population	21.46	1	21.46	0.556	0.457
Sex	1430.34	1	1430.34	37.057	0.000*
Slope	4.65	1	4.65	0.120	0.729
Population × sex	559.95	1	559.95	14.507	0.000*
Population × slope	86.85	1	86.85	2.250	0.136
Sex × slope	52.28	1	52.28	1.354	0.247
Population × sex × slope	24.89	1	24.89	0.645	0.424
Error	4631.79	120	38.60		

* indicates significant effect ($P < 0.05$).

Table 5 ANCOVA for male and female *Drosophila simulans* triglyceride content (protein content as covariate).

	SS	d.f.	MS	*F*	*P*
Male					
Protein	244.11	1	244.11	6.166	0.016*
Population	201.23	1	201.23	5.083	0.028*
Slope	3.40	1	3.40	0.086	0.770
Population × slope	43.06	1	43.06	1.088	0.301
Error	2335.69	59	39.59		
Female					
Protein	423.72	1	423.72	15.353	0.000*
Population	345.61	1	345.61	12.523	0.001*
Slope	7.15	1	7.15	0.259	0.613
Population × slope	0.03	1	0.03	0.001	0.975
Error	1628.27	59	27.60		

* indicates significant effect ($P < 0.05$).

Table 6 ANCOVA for male and female *Drosophila simulans* carbohydrate content (protein content as covariate).

	SS	d.f.	MS	*F*	*P*
Male					
Protein	1.74	1	1.74	0.195	0.660
Population	187.95	1	187.95	21.103	0.000*
Slope	10.20	1	10.20	1.145	0.289
Population × slope	7.24	1	7.24	0.813	0.371
Error	525.48	59	8.91		
Female					
Protein	7.17	1	7.17	0.874	0.354
Population	37.18	1	37.18	4.530	0.037*
Slope	0.32	1	0.32	0.039	0.844
Population × slope	20.10	1	20.10	2.449	0.123
Error	484.21	59	8.21		

* indicates significant effect ($P < 0.05$).

Table 7 PSI, PSS and PTI estimates, standard deviations (SD) and *P* values based on pooled numbers of mating pairs (*n*; % of total matings in parentheses) in both replicate populations.

	NFS ♂			SFS ♂		
	PSI	PSS	PTI	PSI	PSS	PTI
NFS ♀						
n (%)	93 (24.5)			103 (27.1)		
Estimate	0.96	1.03	0.98	1.05	1.04	1.08
SD	0.12	0.09	0.09	0.13	0.09	0.09
P	0.74	0.74	0.85	0.70	0.65	0.33
SFS ♀						
n (%)	96 (25.3)			88 (23.1)		
Estimate	1.06	0.96	1.01	0.96	0.97	0.93
SD	0.14	0.09	0.09	0.13	0.09	0.09
P	0.69	0.72	0.86	0.74	0.80	0.43

NFS, north-facing slopes, SFS, south-facing slopes, PSS, pair sexual selection; PSI, pair sexual isolation; PTI, pair total isolation.

Discussion

Divergent selection on fly populations from the opposing Nahal Oren slopes has been suggested as the driving force for incipient sympatric speciation (Korol *et al.*, 2006). However, contradicting experimental evidence has raised doubts over the model site often termed 'Evolution Canyon' (Coyne & Orr, 2004). Water availability is one of the two most important abiotic factors determining species distribution, and therefore, if adaptive divergence was the driving force for interslope reproductive isolation, variation in desiccation resistance could be expected. Furthermore, evolved desiccation resistance by means of reduced cuticular permeability may involve changing levels of cuticular hydrocarbons which also affect the mating behaviour (Antony & Jallon, 1982; Gibbs *et al.*, 1997). Data on desiccation resistance performance of SFS and NFS flies have so far been available for *D. melanogaster* only. Reported increased desiccation resistance in populations originating in the drier SFS (Korol *et al.*, 2006) contradicts the results of an earlier study (Nevo *et al.*, 1998). Results in this study clearly indicate a lack of genetic basis for interslope divergence in desiccation resistance (Fig. 1). A two-fold difference in survival time under desiccating conditions between males and females was coupled with a highly insignificant slope effect ($P = 0.96$). Resistance of natural populations to resource limitation was reported to be compromised during multi-generation acclimation to laboratory conditions and maintenance protocols (Hoffmann *et al.*, 2001). However, the authors reported that a significant decrease in desiccation resistance was observed only after 3 years of laboratory maintenance, whereas desiccation resistance assays in this study were conducted after 21–22 generations of laboratory rearing. Furthermore, preliminary tests at generation 14–15 (data not shown) revealed no significant interslope variation or higher resistance levels compared with those reported here. It is therefore unlikely that similar resistance levels to desiccation for NFS and SFS populations reported here result from compromised originally higher resistance of either population during laboratory acclimation.

There is ample experimental evidence, from both laboratory-selected and natural populations, indicating that the physiological basis for resisting environmental resource limitation stress involves metabolic fuel accumulation, storage and allocation. Selection for desiccation resistance results in significantly higher carbohydrate content in *D. melanogaster* (Graves *et al.*, 1992; Chippindale *et al.*, 1998; Gefen *et al.*, 2006), whereas higher body lipid content is associated with increased starvation resistance (Graves *et al.*, 1992; Djawdan *et al.*, 1998). In natural populations, higher lipid contents in temperate compared with tropical *Drosophila* species were correlated with increased desiccation and starvation resistance (van Herrewege & David, 1997). However, xeric *Drosophila* species did not exhibit higher body glycogen content in comparison with mesic species (Marron *et al.*, 2003). The authors also report that all studied species preferentially catabolize carbohydrates during desiccation, whereas a mixture of energy sources was oxidized during starvation. Results in this study show no significant slope effect on triglyceride, carbohydrate or protein content in *D. simulans* populations from Lower Nahal Oren and are thus not supportive of adaptive divergence resulting from possible resource limitation affecting SFS populations. Metabolic fuel contents were determined within 2 h PE and therefore, do not represent possible effects of adult feeding. Nevertheless, previous studies have shown that selection for stress resistance results in increased resource acquisition during larval stages (Chippindale *et al.*, 1998; Gefen *et al.*, 2006). Interestingly, there was a significant effect of replicate population on carbohydrate (but not triglyceride or protein) content (Tables 1–4). This was not a result of variation in body size (as indicated by measured protein content), suggesting preferential storage of carbohydrates. The first field collection took place following a long dry season and only a minor (< 4 mm) bout of rain in mid-September (Israel Meteorological Service: http://www.ims.gov.il/IMSENG/All_Tahazit/homepage.htm). In contrast, the flies used for founding population 2 were caught in October following two events of substantial rainfall in the late September and early October. No significant effect of replicate population on desiccation resistance was observed, but metabolic fuel data coupled with somewhat slower cumulative mortality curves for population 1 (Fig. 1) may indicate higher prevalence of 'resistant' genotypes following the months long dry season. Interestingly, a study of Nahal Oren *D. melanogaster* populations revealed higher temporal than interslope genetic differentiation (Schlötterer & Agis, 2002; but see Colson, 2002; Korol *et al.*, 2006).

The postulation that ecological adaptation leads to fly population divergence in Nahal Oren is based on adaptive changes overriding gene flow between SFS and NFS populations. With the distance between the opposing slopes well within the dispersal range of fruit flies, it has been suggested that interslope gene flow is limited by assortative mating (see Korol *et al.*, 2006). A significant assortative mating pattern has been reported for NFS and SFS *D. melanogaster* populations reared under laboratory conditions for at least 12 and 48 generations (Korol *et al.*, 2000; see also Singh *et al.*, 2005). Results of multiple-choice assays were complemented by single-choice tests that indicate intraslope mate preferences in both males and females. However, other studies using Nahal Oren flies failed to replicate these results (Panhuis *et al.*, 2003). Intriguingly, a study conducted in two locations yielded conflicting multiple-choice assay results indicating assortative mating (Haifa, Israel) and random mating (Burnaby, Canada) (Drake *et al.*, 2005). Although conflicting mating assay results for *D. melanogaster* contribute to the Nahal Oren controversy (Coyne & Orr, 2004), very little data is currently available for testing the generality of the interslope ecologically driven mate preference model. Singh *et al.* (2005) provided results of a pilot test using *D. simulans* from the opposing Nahal Oren slopes in which the level of assortative mating (70% homotypic pairs) was higher than that previously reported for *D. melanogaster*. Results in this study fail to replicate these findings, with 48% and 47% of mating couples being homotypic in replicate populations 1 and 2, respectively. Lack of divergence from random mating results from similar mating propensities of NFS and SFS male and female *D. simulans* (PSS values not significantly different from 1) and slope-independent mate preference (PSI values not different from 1) (see Coyne *et al.*, 2005). The nearly identical indices for the two replicate populations in this study, founded from a total of 60 isofemale lines, suggests that deviation from earlier Nahal Oren studies based on similar population sizes (e.g. Korol *et al.*, 2000) is unlikely to be a result of genetic drift. Moreover, laboratory maintenance in this study was shorter than previously reported for Nahal Oren *Drosophila* populations exhibiting assortative mating patterns. Possible reasons for conflicting results among *D. melanogaster* mating behaviour studies have been discussed and include differences in maintenance and assay protocols and other 'unknown environmental factors' (see Drake *et al.*, 2005). These could also play a part in the discrepancies between our results for *D. simulans* and those reported by Singh *et al.* (2005), who used half-pint bottles for each 40-fly multiple-choice assay compared with the much larger (~7.5 L) mating chambers used in this study. Interestingly, a recent study showed that increased fly density in the mating chamber resulted in lower frequencies of interspecific copulations between *D. arizonae* and *D. mojavensis* (Jennings & Etges, 2010). Nevertheless, even though results in this study do not support divergence of NFS and SFS *D. simulans* populations, they could still reconcile with earlier reports for *D. melanogaster* if reproductive isolation in this species across Nahal Oren is indeed driven by adaptive divergence to the slopes differing in the desiccation stress levels. *D. simulans* exhibits lower genetic variance for desiccation resistance in comparison with *D. melanogaster* (Hoffmann & Parsons, 1993), and thus, the response to selective pressures that characterize the Nahal Oren SFS may result in a milder response in the former. If reproductive isolation of populations from the two opposing slopes is a consequence of adaptive divergence to local conditions, then (given similar rates of interslope gene flow) a more pronounced divergence from random mating would be expected in *D. melanogaster*.

A possible mechanism for reproductive isolation of *Drosophila* populations as a by-product of ecological divergence involves the dual role of cuticular hydrocarbons. Laboratory *D. melanogaster* selected for desiccation resistance exhibit significant changes in the fraction of CH containing 27 and 29 carbon atoms in females and 23 and 25 carbon atoms in males, consistent with the established role of CHs in the cuticle's waterproofing abilities (Gibbs *et al.*, 1997). These adaptive changes involve cuticular hydrocarbons that have been shown to elicit courtship behaviour (Antony & Jallon, 1982) and sexual isolation between closely related species (Coyne *et al.*, 1994). Intriguingly, *D. simulans* are monomorphic, whereas *D. melanogaster* are sexually dimorphic in relation to their cuticular hydrocarbon profile (see Ferveur, 2005). Therefore, adaptive changes to desiccation stress reflected in alteration to their cuticular hydrocarbon profile could result in contrasting effects on courting behaviour in the two species.

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