

Adaptive differentiation of thermotolerance in *Drosophila* along a microclimatic gradient

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We examined whether a remarkable occurrence – the physiological evolution of two *Drosophila melanogaster* populations, despite a spatial separation of only 100–400 m, was idiosyncratic and temporary, or persisted over multiple years. We ascertained the high-temperature tolerance of *Drosophila* descended from populations on the north-facing slope (NFS) and south-facing slope (SFS) of ‘Evolution Canyon’ (Lower Nahal Oren, Mt Carmel, Israel), which were collected in 1997, 1999, and 2000. Results for these *Drosophila* uniformly resembled other studies in many respects: an inverse relationship between survival and

heat-shock temperature, male–female differences in thermotolerance, and inducible thermotolerance. Importantly, for all years of collection, SFS flies consistently exceeded NFS flies in basal and inducible thermotolerance after diverse heat shocks, with and without thermal pretreatment, and whether isofemale lines, synthetic populations, or inbred lines were compared. Inbred lines, however, had lower thermotolerance than outbred lines. Several nonexclusive processes may explain the evolution of such physiological differentiation. *Heredity* advance online publication, 22 March 2006; doi:10.1038/sj.hdy.6800784

Keywords: *Drosophila*; basal thermotolerance; inducible thermotolerance; heat shock; differentiation

Introduction

Selection can be a potent mechanism of phenotypic change in complex eukaryotes, effectively coupling trait values to environmental variables. One superb example, at least in laboratory populations, is the speed and magnitude of phenotypic evolution in *Drosophila melanogaster*. Indeed, diverse traits are highly responsive to selection by extreme temperature, desiccation, toxic substances, gravitrophism, starvation, spontaneous mortality, and reproductive effort, among others (Hoffmann and Parsons, 1993; Ricker and Hirsch, 1998; Djawdan *et al*, 1998; Promislov *et al*, 1998; Bryant and Reed, 1999; Singh and Singh, 2001; Hoffmann *et al*, 2003a). For at least some of these agents, moreover, relaxation of selection causes the resultant phenotypes to revert to their pre-existing levels, suggesting that ongoing selection may be necessary to maintain the values of such traits (Teotonio and Rose, 2001; Teotonio *et al*, 2002). Thus, these findings are consistent with all of the classical conditions for selection (ie, genetic encoding of relevant phenotypes, variation in the encoding genes, differential fitness under selection) persisting at sufficiently high levels in laboratory populations of *D. melanogaster*.

In nature, by contrast, the complex, dynamic, and unstable interplay of diverse environmental, demographic, and genetic variables can undermine the directionality, if not the potency of natural selection. That despite this dynamism selection can create monumental phenotypic diversification in nature is self-evident. But are such instances of diversification extremely unlikely, with reversals in selection pressure or demographic interference (eg, swamping via migration) typically eroding nascent differences before they have an opportunity to accumulate and fix? *D. melanogaster* is both small and highly mobile, which prospectively exposes it to diverse, if not contrasting, selection pressures on a micro scale and enhances gene flow among local populations. Thus, sustained unidirectional selection in *D. melanogaster* may be unlikely. Most conspicuous exceptions to this expectation involve large-scale clines (Alonso-Moraga *et al*, 1988; David *et al*, 1989; James *et al*, 1997; Robinson *et al*, 2000; Huey *et al*, 2001; Verrelli and Eanes, 2001; Duvernell *et al*, 2003; Hoffmann *et al*, 2003b; Gilchrist *et al*, 2004), which may override local heterogeneity in selection pressures, or peculiar instances of partial isolation (eg, wine cellar populations).

By contrast, in Lower Nahal Oren, Mt Carmel, Israel (‘Evolution canyon’), populations living in distinctive microclimates have diverged in multiple traits despite minimal spatial separation. The opposite slopes of this canyon show strong abiotic contrasts that are consequential for species composition and population genetic structure in diverse organisms, including several *Drosophila* species (Nevo, 1997, 2001; Nevo *et al*, 1998; Harry *et al*, 1999; Pavlicek *et al*, 2003). The *D. melanogaster*

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Received 27 April 2005; accepted 14 November 2005

populations on the slopes, separated by 100 and 400 m at the bottom and top, respectively, experience markedly different environments due to the higher illumination on the south-facing slope (SFS) than on the north-facing slope (NFS) (Pavlicek *et al*, 2003). The slopes also differ in temperature and aridity: NFS has comparatively lush vegetation of European origin, whereas the SFS is an open Park Forest or Xeric Savanna, primarily of African and Asian origin. The *Drosophila* populations in the canyon differ in habitat choice, thermotolerance and desiccation resistance, and life-history traits (Nevo *et al*, 1998; Rashkovetsky *et al*, 2000; Iliadi *et al*, 2001; Lupu *et al*, 2004), all corresponding to the prevailing microclimate. These populations also differ in sexual behavior, including mate choice (Korol *et al*, 2000; Iliadi *et al*, 2001; Drake *et al*, 2005). This remarkable divergence has evolved despite an interslope distance much smaller than the daily dispersal capability of *Drosophila* (Coyne and Milstead, 1987).

Here we ask: Were these first reports of interpopulation differences in 'Evolution Canyon' *Drosophila* an unrepresentative snapshot of a temporary deviation from homogeneity, or are these differences stable despite year-to-year and seasonal variation in environmental conditions and potential interslope migration? Our results support the latter.

Materials and methods

During July–October 1997, 1999, and 2000, we collected wild female *D. melanogaster* from yeasted banana bait at the two mid-stations (90 m above sea level) on the NFS and SFS of Lower Nahal Oren canyon (Mount Carmel, Israel). Isfemale lines were established from each female inseminated in nature and maintained under standard conditions (25°C; approximately 40% mean relative humidity; standard cornmeal–sugar–agar medium). Synthetic populations were established for each slope and year by combining 10 flies of each sex from 25 isfemale lines in a population cage, and maintained under random mating for 72 (1997 collection), 24 (1999), and 12 (2000) nonoverlapping generations. To examine the impact of inbreeding, lines established from the 1997 synthetic population were sib-mated for eight generations.

Thermotolerance measurements

Adult flies were transferred to fresh bottles and allowed to oviposit, and then cleared from the bottles. We collected the first brood of flies eclosing during an 18-h window beginning at 1800 h. After CO₂ anesthesia, these flies were sorted by sex into groups of 20, and each group transferred to 22 × 95 mm glass vials containing 8 ml of medium. After two additional days at 25°C, these vials were stoppered with cotton plugs, inverted, and fastened to plastic racks, which were submerged in circulating water baths (GFL-1083, Gesellschaft für Labor-technik mbH, Burgwedel) regulated within ±0.3°C of the temperatures indicated below. Survival was scored 24 h after heat treatment as the proportion of flies in a vial exhibiting any response to touching with a fine paint brush. This scheme was based on previous *Drosophila* thermotolerance studies (Loeschcke and Krebs, 1997;

Krebs and Feder, 1998; Bettencourt *et al*, 1999). Heat treatments were:

- Heat shock only (HS)*: 38.5 (for 1997 and 1999 populations) or 39 (for 2000 populations) ±0.3°C for 50, 60, or 70 min;
- Heat pretreatment (PT)* at 36°C for 1 h and 25°C for 1 h preceding heat shock as described above (PT + HS).

Comparisons and preliminary experimentation

Often, eggs deposited on the same day will yield adults that eclose over several days; we term adults eclosing on each day a brood. Preliminary studies revealed that brood affected thermotolerance (see also Sorensen and Loeschcke, 2004). Hence, except where noted, experiments used only the first brood (excepting rare rapidly eclosing adults) eclosing from any day's egg deposition.

We compared thermotolerance of synthetic populations, isfemale lines, and inbred lines from each slope in a factorial design with replicated tests. The factors included were: 'year' (1997, 1999, and 2000), 'population' (SFS and NFS), and 'treatment' (PT + HS and HS only).

Data analysis

Statistical tools including ANOVA, and log-linear analysis were employed for data analysis using Statistica software package (StatSoft, 1996).

Results

Thermotolerance: comparing synthetic populations

Slope, sex, year of collection, duration of heat shock, and pretreatment all affected the survival of heat shock (see Figure 1). Not unexpectedly, survival was inversely related to the duration of heat shock. Consistent with numerous prior reports, pretreatment increased the

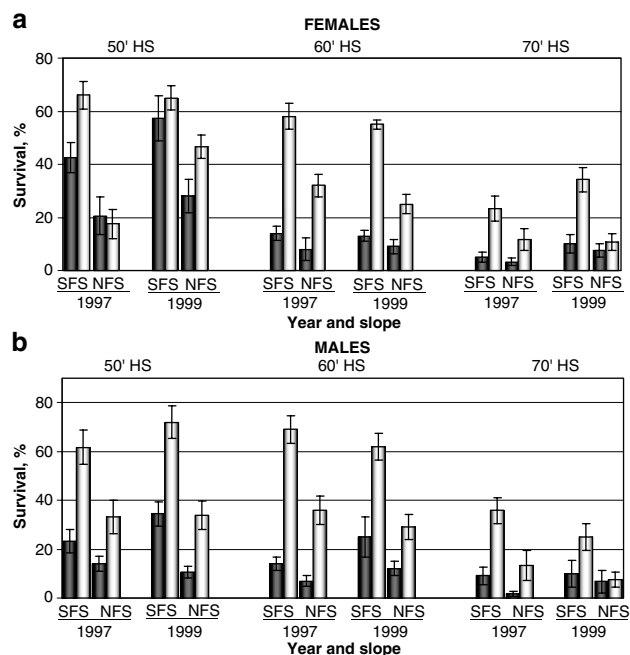


Figure 1 Basal (gray columns) and inducible (white columns) thermotolerance of *Drosophila* from the opposite slopes of Nahal Oren canyon. Heat shock was at 38.5°C and pretreatment was at 36°C for 60 min.

mean thermotolerance in every comparison (2 years × 3 temperatures × 2 sexes × 2 slopes), significantly so in 17 of 24 cases (Table 1).

Our major interest is in the interslope differences in thermotolerance. In each of the 24 SFS vs NFS comparisons (2 years × 3 temperatures × 2 sexes × ±pretreatment), the mean thermotolerance for the SFS sample exceeds the mean thermotolerance for the NFS sample (see Table 2).

These differences in means, moreover, were statistically significant in 11 of the 12 comparisons for acquired thermotolerance. The differences were less frequently significant for basal thermotolerance, where the mean thermotolerance was small relative to the sampling error. The mean change in thermotolerance between pretreated and unpretreated flies was greater for SFS than for NFS flies in 11 of 12 comparisons; overall, this difference was significant (Table 3). Repetition of the experiment with single heat shock duration, pretreated flies only, and flies collected in 2000 yielded similar outcomes (Tables 3, 4).

Thermotolerance: comparing isofemale lines

Repetition of the above studies with separate isofemale lines founded from flies collected in 2000 (Figure 2)

revealed patterns of variation similar to those evident in synthetic populations. Log-linear analysis revealed that the founders' slope of origin was the most significant factor affecting survivorship after pretreatment. As before, SFS flies were more tolerant than NFS flies (Figure 2). Sex had a lesser impact than for synthetic populations, which was significant only for SFS lines (Table 4). In addition, separate lines founded from parents from the same slope varied significantly in thermotolerance, and more so in the lines from the SFS. The greater variation in the SFS lines is evident from the ratio of two χ^2 statistics, which is distributed asymptotically as Fisher's *F*-statistics with corresponding degrees of freedom ($F_{7,5} = 154.71/24.33 = 6.36, P = 0.029$). Between-slope differences were principally due to the large differences among female flies.

Strongly inbred lines

Strongly inbred lines exhibited essentially the same patterns of variation in thermotolerance that synthetic populations and isofemale lines displayed, except that the average thermotolerances were dramatically lower after comparable treatments in the strongly inbred lines

Table 1 Effect of pretreatment on the percentage of *Drosophila* from NFS and SFS surviving heat shock of 38.5°C

Sex	Year	Treatment duration	% Survival SFS		P	% Survival NFS		P	Δ for SFS	Δ for NFS	Pairwise t-test for av. means	
			Inducible	Basal		Inducible	Basal					
Females	1997	50	66.0±5.3	vs	42.5±5.7	0.007	17.5±5.6	vs	20.5±7.1	0.374	23.5	-3
		60	58.0±4.9	vs	14.0±2.7	0.00001	32.0±4.2	vs	8.0±4.2	0.002	44.0	24
		70	23.3±4.7	vs	5.0±2.0	0.005	11.7±4.2	vs	3.3±1.4	0.087	18.3	8.4
	1999	50	65±4.6	vs	57.2±8.5	0.439	46.5±4.4	vs	28.0±6.4	0.039	7.8	18.5
		60	55.0±1.7	vs	13.0±2.1	0.00001	25.0±3.7	vs	9.0±2.8	0.006	42	16
		70	34.2±4.5	vs	10.0±3.5	0.0017	10.8±3.1	vs	7.5±2.5	0.427	29.7	8.3
Males	1997	50	61.7±7.1	vs	23.3±4.9	0.0012	33.2±7.0	vs	14.1±3.1	0.032	38.4	19.1
		60	69.0±5.7	vs	14.0±2.7	0.00001	36.0±5.8	vs	7.0±2.1	0.001	55	29
		70	35.8±5.4	vs	9.2±3.6	0.0022	13.3±6.1	vs	1.7±1.1	0.0908	26.6	11.6
	1999	50	71.9±6.7	vs	34.4±4.9	0.0011	34.0±5.8	vs	10.6±2.4	0.0039	37.5	23.4
		60	62.0±5.5	vs	25.0±8.2	0.0038	29.0±5.1	vs	12.0±2.9	0.0159	37	17
		70	25.0±5.6	vs	10.0±5.4	0.0827	7.5±3.1	vs	6.7±4.5	0.8865	15	0.8
Average means (value)									31.2±3.9	14.4±2.7	$t = 5.74; P < 0.001$	

Basal thermotolerance refers to survival without pretreatment; inducible thermotolerance refers to survival after pretreatment at 36°C for 60 min. *P* was calculated in accordance to nonparametric Wilcoxon test.

Table 2 Effect of slope of origin on the percentage of *Drosophila* from the NFS and SFS surviving heat shock of 38.5°C

Sex	Year	Treatment duration	% Survival with pretreatment		P	% Survival without pretreatment		P		
			SFS	NFS		SFS	NFS			
Females	1997	50	66.0±5.3	vs	17.5±5.6	0.0001	42.5±5.7	vs	20.5±7.1	0.0363
		60	58.0±4.9	vs	32.0±4.2	0.0024	14.0±2.7	vs	8.0±4.2	0.2573
		70	23.3±4.7	vs	11.7±4.2	0.0924	5.0±2.0	vs	3.3±1.4	0.5022
	1999	50	65±4.6	vs	46.5±4.4	0.0157	57.2±8.5	vs	28.0±6.4	0.0207
		60	55.0±1.7	vs	25.0±3.7	0.00001	13.0±2.1	vs	9.0±2.8	0.2798
		70	34.2±4.5	vs	10.8±3.1	0.0016	10.0±3.5	vs	7.5±2.5	0.5740
Males	1997	50	61.7±7.1	vs	33.2±7.0	0.0170	23.3±4.9	vs	14.1±3.1	0.1438
		60	69.0±5.7	vs	36.0±5.8	0.0023	14.0±2.7	vs	7.0±2.1	0.0121
		70	35.8±5.4	vs	13.3±6.1	0.0201	9.2±3.6	vs	1.7±1.1	0.0744
	1999	50	71.9±6.7	vs	34.0±5.8	0.0016	34.4±4.9	vs	10.6±2.4	0.0014
		60	62.0±5.5	vs	29.0±5.1	0.0013	25.0±8.2	vs	12.0±2.9	0.1160
		70	25.0±5.6	vs	7.5±3.1	0.0211	10.0±5.4	vs	6.7±4.5	0.6489

Pretreatment was at 36°C for 60 min.

Table 3 Log-linear analysis of thermotolerance in *D. melanogaster* populations derived from opposite slopes of 'Evolution Canyon'

Factor	d.f.	Basal thermotolerance		Inducible thermotolerance		Joint analysis	
		χ^2	P	χ^2	P	χ^2	P
P	1	111.8	<10 ⁻⁶	363.3	<10 ⁻⁶	460.8	<10 ⁻⁶
P × Y	1	0.7	0.42	0.1	0.71	0.05	0.82
P × D	2	3.9	0.27	10.2	0.017	10.83	0.01
P × T	1	—	—	—	—	10.03	0.0015
P × S	1	0.5	0.46	0.01	0.94	0.16	0.69
P × Y × D	2	6.0	0.11	13.7	0.003	2.54	0.47
P × Y × T	1	—	—	—	—	1.06	0.30
P × Y × S	1	0.8	0.37	4.2	0.04	4.00	0.05
P × Y × D × T	2	—	—	—	—	18.77	0.0003
P × Y × D × S	2	4.0	0.27	13.9	0.003	13.17	0.004

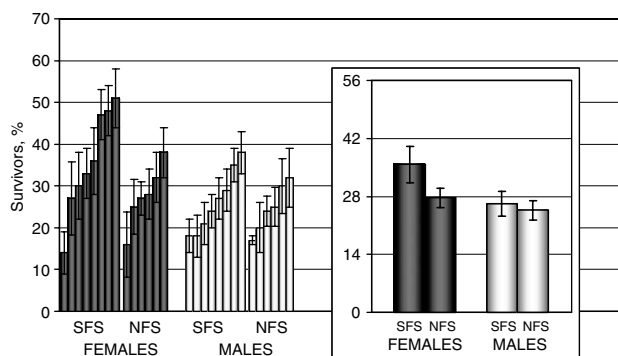
Heat shock was at 38.5°C. Pretreatment was at 36°C for 60 min.

P – Population origin (SFS vs NFS), Y – sampling year (1997 vs 1999), D – treatment duration, T – type of treatment (HS vs PT+HS), S – sex.

Table 4 Analysis of the effects of lines, flies' origin and sex on inducible thermotolerance for *Drosophila* collected in 2000

Factor	d.f.	χ^2	P
<i>NFS vs SFS lines</i>			
Population	1	15.9	<7 × 10 ⁻⁵
Sex	1	4.9	0.027
<i>NFS lines</i>			
Line	5	24.3	0.0002
Sex	1	0.3	0.57
<i>SFS lines</i>			
Line	7	154.7	<10 ⁻⁶
Sex	1	8.6	0.0034

Heat shock was at 39°C for 60 min. Pretreatment was at 36°C for 60 min.

**Figure 2** Thermotolerance of isofemale lines from the opposite slopes. Inset: Grand means by slope and sex for the isofemale lines. Means are plotted ± 1 SE. Heat shock was at 39°C.

(Table 5, Figure 3). Importantly, thermotolerances of inbred flies were more variable for lines founded from the SFS than from the NFS.

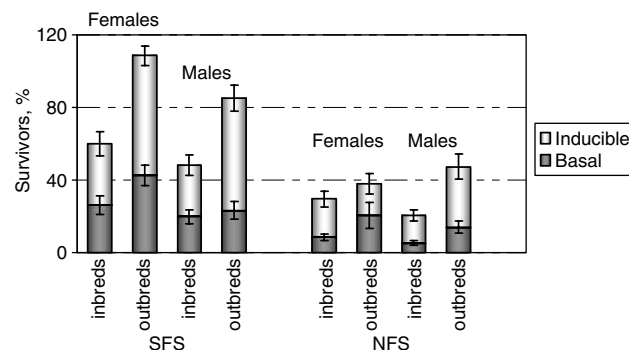
Discussion

The initial reports of inter-slope differentiation in 'Evolution Canyon' have elicited considerable controversy. *Drosophila* adults are able to disperse long

Table 5 Analysis of the effects of flies' origin, lines, sex and type of treatment on inbred lines' thermotolerance

Factor	d.f.	χ^2	P
<i>SFS+NFS lines</i>			
Slope	1	121.6	<10 ⁻⁶
Sex	1	14.8	0.0001
Replica	5	148.7	<10 ⁻⁶
Treatment	1	54.5	<10 ⁻⁶
Slope × Sex	1	0.6	0.426
Slope × Treatment	1	11.3	8 × 10 ⁻⁴
<i>NFS lines</i>			
Line	4	17.2	0.0017
Sex	1	8.1	0.004
Replica	5	13.6	0.018
Treatment	1	52.1	<10 ⁻⁶
Line × Sex	4	2.2	0.709
Line × Treatment	4	9.6	0.047
<i>SFS lines</i>			
Line	4	47.0	<10 ⁻⁶
Sex	1	7.2	0.0072
Replica	5	190.4	<10 ⁻⁶
Treatment	1	15.5	8 × 10 ⁻⁵
Line × Sex	4	5.6	0.231
Line × Treatment	4	1.9	0.762

Heat shock was at 39°C for 60 min. Pretreatment was at 36°C for 60 min.

**Figure 3** Comparison of thermotolerance in inbred and outbred lines of *Drosophila* from the opposite slopes of Nahal Oren canyon.

distances (10–15 km) overnight (Coyne and Milstead, 1987; Coyne *et al*, 1987). Therefore, that slope-specific adaptive gene complexes could evolve at all and escape

recombinational collapse is perplexing, even to the authors, and has prompted numerous subsequent investigations, which themselves are controversial. For example, if the populations on the two slopes are indeed distinct on a sustained basis, their genes should diverge in sequence. Indeed, our recent estimates based on microsatellite markers (Michalak *et al.*, 2001) revealed a substantial interslope differentiation for microsatellites in *D. melanogaster* as great as between it and its sibling species, *D. simulans*, and indicated that gene flow should be rather restricted among the slopes. Schlotterer and Agis (2002) and Colson (2002), by contrast, examining many of the same microsatellites in flies collected at nearly identical times, found scant genetic differentiation. Additionally, NFS and SFS populations sampled in 1995 did not differ at specific loci for the *Acp* gene family (Panhuis *et al.*, 2003). Interslope genetic differentiation in *D. melanogaster* derived from 'Evolution Canyon' was revealed in our recent study of the *period* gene known to affect sexual behavior. Variants of the (Thr-Gly)_n repeat of the *period* gene, $n = 17$ and $n = 20$, which are abundant in natural populations of *D. melanogaster* in Africa and Europe (Kyriacou *et al.*, 1996; Sawyer *et al.*, 1997), were found to predominate in the Canyon. A noteworthy fact is that the less abundant 'European' allele ($n = 20$) occurred on the NFS about three-fold compared to the SFS (Zamorzaeva *et al.*, 2005). These reports could be reconciled if the 'Evolution Canyon' *Drosophila* populations were undergoing dynamic demographic and environmental change, which is certainly possible for small insects living in such a variable environment. Thus, the Introduction asked: Were the first reports of interpopulation differences in 'Evolution Canyon' *Drosophila* an unrepresentative snapshot of a temporary deviation from homogeneity, or are these differences stable despite year-to-year and seasonal variation in environmental conditions and potential interslope migration?

Our principal finding is that the difference in thermotolerance between flies from the two slopes of the canyon is ongoing and robust. Greater thermotolerance in SFS *Drosophila* than in NFS slope *Drosophila* is now evident for flies collected in 1997, 1999, and 2000. These differences, moreover, are in both basal thermotolerance and inducible thermotolerance, and appear in synthetic populations, isofemale lines, and inbred lines. *Drosophila* from the two slopes also differ in pre-adult viability and developmental time (Rashkovetsky *et al.*, 2000). Since the original study, interslope differences in habitat choice (Nevo *et al.*, 1998) and mating preference (Korol *et al.*, 2000; Iliadi *et al.*, 2001; Drake *et al.*, 2005) have also come to light. At least in phenotypes reported here, the NFS and SFS populations differ.

These differences may be related to adaptation to the contrasting environmental regimes prevailing on the two slopes (Pavlicek *et al.*, 2003). The difference in inducible thermotolerance, moreover, has a candidate genetic basis. The inducible molecular chaperone Hsp70 is responsible for a substantial portion of inducible thermotolerance. In the 'Evolution Canyon' populations, naturally occurring P transposable elements disrupt the proximal promoters of at least two of the five Hsp70-encoding genes. Such disruptions can reduce *hsp70* mRNA transcription, Hsp70 protein levels, and thermotolerance (Lerman *et al.*, 2003; Lerman and Feder, 2004). In

the 'Evolution Canyon' *Drosophila*, *hsp70* alleles in which the P element is present or absent segregate in a balanced polymorphism, but at higher allelic frequencies in the NFS population than in the SFS population. This pattern is consistent with the lesser thermotolerance of the NFS population.

Above we show that strong inbreeding reduces thermotolerance. Thus, an alternative explanation is that the inter-slope differences in thermotolerance reflect differing magnitudes of inbreeding on the two slopes. A definitive portrait of the demographic and environmental dynamism that *Drosophila* undergo in 'Evolution Canyon' may need to await the development of truly miniaturized equipment that can report flies' environment, position, and with which other flies they interact.

An additional explanation of the interslope differentiation is that, although *Drosophila* can travel long distances (Coyne and Milstead, 1987; Coyne *et al.*, 1987), those in 'Evolution Canyon' do not. That is, either migration in Nahal Oren canyon is much lower than usually thought for such small distances or there is significant deviation from common simple population-genetic assumptions (ie, panmixia, random dispersal, and weak-to-moderate selection), or all these deviations work together (Korol *et al.*, 2000; Iliadi *et al.*, 2001). Laboratory comparisons of migratory activity between flies from the canyon and a population collected from an open forest park on the Golan Heights (Iliadi *et al.*, 2002) revealed no differences, suggesting that the 'Evolution Canyon' flies are not atypical migrators.

A final possible explanation of the discrepancy in the results could be that differential selection initiates the creation of alternative gene complexes (haplotypes) for loci affecting the selected traits on the two slopes, and that their maintenance in the face of gene flow/recombination is due to selection facilitated by certain habitat choice (Nevo *et al.*, 1998) and assortative mating (Korol *et al.*, 2000; Iliadi *et al.*, 2001; Singh *et al.*, 2005). In this scenario, adaptive differentiation can withstand the disruptive effects of migration and recombination. Such adaptive differentiation, however, would not necessarily be accompanied by differentiation of selectively neutral markers, unless the latter are in linkage disequilibrium with selected loci. This last condition can also persist despite migration, but only under tight linkage and strong selection. In a number of *Drosophila* genes, linkage disequilibrium decays within a few kilobases (kb), or even within 1 kb (Langley *et al.*, 2000). Therefore, differentiation of adaptive trait complexes seems to provide better evidence for interslope differential selection than that displayed by genetic distances estimated using molecular markers.

In conclusion, initial responses to reports of evolved differences between *Drosophila* populations on the two sides of 'Evolution Canyon' were appropriately skeptical. The present study clearly demonstrates that these differences were not a one-time occurrence, but either persist or re-evolve over multiple years. In principle, moreover, the microevolution of thermotolerance should not be confined to 'Evolution Canyon', but demonstrable wherever similar microclimatic gradients exist. Testing this prediction might well elucidate the specific evolutionary mechanisms that have given rise to the differentiation in 'Evolution Canyon'.

Acknowledgements

We acknowledge Kamal Ibrahim (Editor) and two reviewers for constructive comments that improved the paper. We thank T Pavlicek for field assistance and helpful discussions. This study was supported by the United States–Israel Binational Science Foundation (grant 9800443), the Israel Science Foundation (grant 601/03-17.3), Israeli Ministry of Absorption, and Ancell–Teicher Research Foundation for Genetics and Molecular Evolution for EN.

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