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Sexual and reproductive behaviour of *Drosophila melanogaster* from a microclimatically interslope differentiated population of 'Evolution Canyon' (Mount Carmel, Israel)

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The strong microscale interslope environmental differences in 'Evolution Canyon' provide an excellent natural model for sympatric speciation. Our previous studies revealed significant slope-specific differences for a fitness complex of *Drosophila*. This complex involved either adaptation traits (tolerance to high temperature, different viability and longevity pattern) or behavioural differentiation, manifested in habitat choice and non-random mating. This remarkable differentiation has evolved despite a very small interslope distance (a few hundred metres only). Our hypothesis is that strong interslope microclimatic contrast caused differential selection for fitness-related traits accompanied by behavioural differentiation and reinforced by some sexual isolation, which started incipient speciation. Here we describe the results of a systematic analysis of sexual behaviour in a non-choice situation and several reproductive parameters of *D. melanogaster* populations from the opposite slopes of 'Evolution Canyon'. The evidence indicates that: (i) mate choice derives from differences in mating propensity and discrimination; (ii) females from the milder north-facing slope discriminate strongly against males of the opposite slope; (iii) both sexes of the south-facing slope display distinct reproductive and behavioural patterns with females showing increased fecundity, shorter time before remating and relatively higher receptivity, and males showing higher mating propensity. These patterns represent adaptive life strategies contributing to higher fitness.

Keywords: *Drosophila*; microsite; sexual behaviour; reproductive behaviour; adaptive life strategy

1. INTRODUCTION

Behaviour can serve as an effective tool for the analysis of the evolution of animals, particularly in related groups of species (Spieth 1968). Significant behavioural reorganization may even arise as a consequence of a very small number of gene changes (Tauber & Tauber 1977; Tauber *et al.* 1977; Stratton & Uetz 1986). According to Mayr (1976), 'A shift into a new niche or adaptive zone requires, almost without exception, a change in behavior'. Sexual behaviour plays an important role in the establishment of reproductive isolation between populations. In *Drosophila*, mating behaviour includes a sequence of elements of male courtship and female responsive reactions that depend on the duration and quality of her stimulation. During courtship, the sexes exchange stimuli of different modalities: auditory (Bennet-Clark & Ewing 1969; Kyriacou & Hall 1982), visual (Willmund & Ewing 1982) and chemosensory (Jallon 1984; Ferveur *et al.* 1989). Usually, the sequence of stimuli produced by the male is typical and is regarded as elements of courtship ritual. Even a slight deviation from the specific sexual behaviour leads to a decrease of reproductive chances and fitness (Bastock & Manning 1955; Bastock 1956; Dow 1977; Jamart *et al.* 1993; Gleason & Ritchie 1998; reviewed by Spieth (1968) and Ewing (1983)). The general strategy for a population in improving its fitness may include: (i) increased male courtship activity, (ii) high female receptivity and an ability to discriminate between males, and (iii) optimized rate of egg laying and time of remating.

In our previous studies we showed a strong differentiation of *D. melanogaster* subpopulations from the opposite slopes of 'Evolution Canyon' for a complex of adaptive traits (Nevo *et al.* 1998; Korol 1999). This differentiation included various aspects of induced changes in viability and longevity caused by short-term and lifetime temperature treatments, different rates of mutation and recombination and habitat choice.

The slopes of 'Evolution Canyon' display dramatic physical and biotic contrasts at a microscale (Nevo 1995, 1997, 2001). Higher solar radiation (more than 600%) on the south-facing slope (SFS) makes it warmer, drier and spatiotemporally more heterogeneous and fluctuating than the north-facing slope (NFS). These slopes differ in their biota (across phylogeny, from bacteria to higher plants and animals). The NFS has comparatively lush, mesic vegetation of Euro-Asian origin, whereas the SFS has xeric savannah, primarily of Afro-Asian origin. This remarkable differentiation has evolved despite a very small interslope distance (only 100 m at the bottom and 400 m at the top). Thus, the geography of the canyon permits mixing of flies by easy migration across the microsite and allows us to consider this situation as sympatric.

There are two hypotheses concerning the origin of reproductive isolation. Dobzhansky (1970) suggested that premating isolation is a product of natural selection that may arise as a consequence of lower fitness hybrids in

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Table 1. Summary of sexual behavioural scores of flies from the opposite slopes of 'Evolution Canyon' for four mating combinations.

(F ratio given for two-way ANOVA on log-transformed data; d.f. = 1, 67 for each parameter except latency period where d.f. = 1,76; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$)

	female origin						
	NFS[a]		SFS[b]				
	male origin		male origin		F ratio for the effect of		
behavioural measure	NFS	SFS	NFS	SFS	FO[c]	MO[d]	FO × MO[e]
Percentage of successful mating	95	75	90	95			
Courtship latency (s)	25.91 ± 5.51	11.90 ± 3.60	28.51 ± 5.64	6.55 ± 3.07	0.85	15.08^{***}	1.69
Courtship duration (s)	307.78 ± 52.13	278.04 ± 45.88	236.80 ± 49.24	119.32 ± 37.30	13.31^{***}	5.95^{*}	5.44^{*}
Time in sexual activity (%)	69.74 ± 5.30	77.17 ± 2.90	52.12 ± 5.17	70.93 ± 4.47	6.37^{*}	7.71^{**}	1.45
Mating speed (s)	333.84 ± 54.75	292.77 ± 47.56	263.83 ± 50.33	126.07 ± 37.60	13.02^{***}	8.20^{**}	5.77^{*}
Duration of copulation (min)	19.68 ± 0.64	17.06 ± 0.85	19.22 ± 0.77	17.57 ± 0.53	0.01	9.50^{**}	0.50

[a] North-facing slope.
[b] South-facing slope.
[c] Female origin.
[d] Male origin.
[e] Sex interaction.

comparison with the conspecific groups. Consequently, an increasing mating discrimination to avoid gamete wasting is thus expected between sympatric populations, leading to further reproductive isolation (the reinforcement hypothesis) (see, for example, the review by Noor (1999)). The other hypothesis, advanced by Darwin, Muller, Mayr and Carson, is that isolation arises as a by-product of gene pool divergence for environmental adaptations in a geographically isolated population (Mayr 1963).

Recently, we found non-random assortative mating manifested by flies from both slopes of 'Evolution Canyon' (Korol *et al.* 2000), with a preference for sexual partners originating from their own slope. We hypothesized that strong interslope microclimatic contrast caused differential selection for stress-related gene complexes, accompanied by behavioural differentiation and reinforced by incipient sexual isolation. Clearly, these findings call for in-depth analysis of the revealed differentiation (see the commentary of Schneider (2000) on Korol *et al.* (2000)). In particular, mating non-randomness could result from such specific courtship behaviours as discrimination and mating propensity. Therefore, for correct interpretation of observed positive assortative mating it is mandatory to unravel the underlying behavioural components (Markow 1981; Dodd 1989; Casares *et al.* 1998). Here, we describe the results of a systematic analysis of sexual behaviour in a non-choice situation, and several reproductive parameters of the sympatric populations of *D. melanogaster* from the opposite slopes of 'Evolution Canyon'.

2. MATERIALS AND METHODS

(a) *Stocks and culture conditions*

Flies used in this study were collected during August–September 1997 from the opposite slopes of 'Evolution Canyon' (Lower Nahal Oren, Mt Carmel, Israel; see details in Nevo 2001). Earlier, established isofemale lines from the opposite slopes had been used to construct two synthetic populations, for NFS and SFS. Each slope-population was constructed by the crossing of 25 isofemale lines (10 females and 10 males of each isofemale line were combined in a population cage), five generations after the capture of flies from nature. The populations were maintained as a mass culture under random mating during 25 generations, before the start of the experiments. The flies were reared on standard cornmeal–sugar–yeast–agar medium in half-pint milk bottles at a temperature of 24 ± 1°C and on a 12 L:12 D cycle.

(b) *Behavioural procedures*

(i) *Experimental design*

For behavioural observations, experimental virgin females and males were collected separately without anaesthesia 4–6 h after eclosion and were placed into culture vials with 5 ml food medium. Females were placed with density not higher than 10–12 flies per vial. To exclude any influence of previous sexual experience, each male was placed individually in a vial. All experiments were performed at 24 ± 1°C; the humidity was not controlled. Males and females were 5 days old at testing. For registration of sexual behaviour we used four mating combinations: (i) female and male of NFS (abbreviated as NN, where the first letter stands for origin of females and the second for origin of males); (ii) female and male of SFS (SS); (iii) female of NFS and male of SFS (NS); (iv) female of SFS and male of NFS (SN). There were 20 replicates per combination. All handling of flies was by aspiration. The behaviour of a single pair was observed in a Perspex experimental chamber (20 mm diameter, 5 mm deep) supplied with a movable transparent Perspex cover, a sliding opaque partition that divided the chamber into two halves and two lateral entries (3 mm diameter) with stoppers. Before testing, the chamber was cleaned with 50% ethanol and dried. For testing, a naive male (with no experience of sexual contact) was placed in the

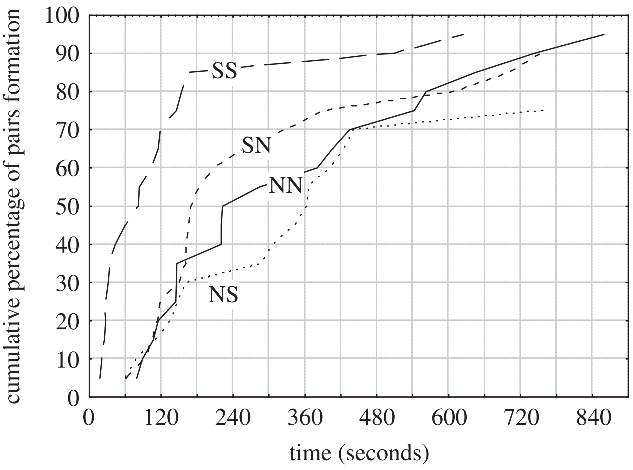

Figure 1. Mating speed for single pairs of flies from 'Evolution Canyon' expressed as cumulative percentage of copulating pairs during 900 s observations. Abbreviations (the first letter stands for the origin of females, the second for the origin of males: female and male of NFS (NN); female and male of SFS (SS); female of NFS and male of SFS (NS); female of SFS and male of NFS (SN)).

experimental chamber together with a virgin female. The flies were introduced into the chamber through separate holes. After 45 s, the divider was carefully withdrawn and the flies were left together until copulation, or for 15 min.

(ii) *Recording the observations*

The ethograms were recorded by an original computer program. The duration of the male's courtship elements (orientation and pursuit, vibration, licking, attempted copulation) was fixed by pressing corresponding keys on the computer keyboard. Registration began when the male performed his first bout of vibration. However, if during the first 10 min a male did not pay attention to the female (because of exploratory behaviour and preening, probably induced by the procedure of his replacement), he was excluded from the calculation. Spoken commentaries by the investigator concerning female rejection behaviour (fending off, kicking and decamping) were written down by another investigator. For a detailed description of *Drosophila* courtship behaviour see Cobb *et al.* (1985). The time sequence for the specified behavioural elements was recorded as a file for each experimental male. Another program was used for decoding the ethograms, calculating various behavioural parameters, and organizing the resulting data.

(iii) *Locomotor activity*

Locomotor activity was measured (individually for each fly) by a count of the number of times a fly crossed a line drawn across the middle of the chamber bottom. Testing time was 600 s following 180 s allowed for adaptation.

(c) *Analysis of components of reproductive behaviour*

(i) *Fecundity*

Fecundity was determined by a count of the numbers of eggs laid by a female per day. One day after copulation each female was introduced into a single vial with food and a separable bottom. Counts of the eggs laid and the transfer of flies into a fresh medium were made each day for two weeks. To assess the average time of egg laying, the following index was employed: $\sum in_i/\sum n_i$, where n_i is number of eggs laid on the ith day.

(ii) *Remating*

Once mated, females were given the opportunity to mate once more, and the period between the two matings was scored individually. For this, during each of the following 21 days, each female was tested individually with a single 3–5 day old virgin male for 3 h, with inspections at least every 10 min. Remated females were excluded from further scoring. Twenty replicates were made for each mating combination.

(d) *Statistical analysis*

All data were tested for normality by the Kolmogorov–Smirnov test. Non-normally distributed data had been log-transformed before calculation of means and errors. Student's t test was applied when variances were homogeneous. In cases of non-equal variances, an approximate t test was used. The Model I of two-way ANOVA (considering the origin slope of males and females as fixed effects) was used for analysis of the effects of male and female origin. All experiments were performed with double blind control.

3. RESULTS

(a) *Basic features of mating behaviour*

Starting from the moment of introduction of a male and a female into the mating chamber, we can define several basic features of mating behaviour. Time of pair formation, or mating speed, is determined as the period from removal of the divider between the sexes until copulation. This interval may be separated into two components: courtship latency and courtship duration. Courtship latency is the time spent by the male until his first bout of vibration. This parameter reflects threshold in male sexual response (Connolly *et al.* 1974). Courtship duration includes male sexual activity (time spent by male only in courting) and non-courtship behaviour (locomotion, preening, resting).

The scoring results of the foregoing mating parameters for different mating combinations are presented in table 1. A lower (although not significantly lower) level of mating success (defined as the percentage of flies achieving copulation within 15 min) was observed for the combination NS than with the other mating combinations. Two-way ANOVA revealed a shorter courtship latency period ($F_{1,76} = 15.08$, $p < 0.001$) and duration of copulation ($F_{1,76} = 9.50$, $p < 0.05$) of SFS males than NFS males, regardless of different female origin (table 1). Under appropriate circumstances, differences in latency period may be a reflection of variation in sexual response threshold among males. An interesting pattern was observed for courtship duration: conspecific pairs (of the same canyon slope) showed more than twofold differences in courtship duration, whereas heterospecific pairs (from the opposite slopes) were intermediate between these extremes and closer to the NN combination. ANOVA revealed a highly significant effect of female origin (FO) ($p < 0.001$) and a much less, although still significant ($p < 0.05$) effect of male origin (MO) and of interaction FO × MO. Regarding only the part of time spent by the male in sexual activity, the difference between the contributions of MO and FO becomes less essential (table 1).

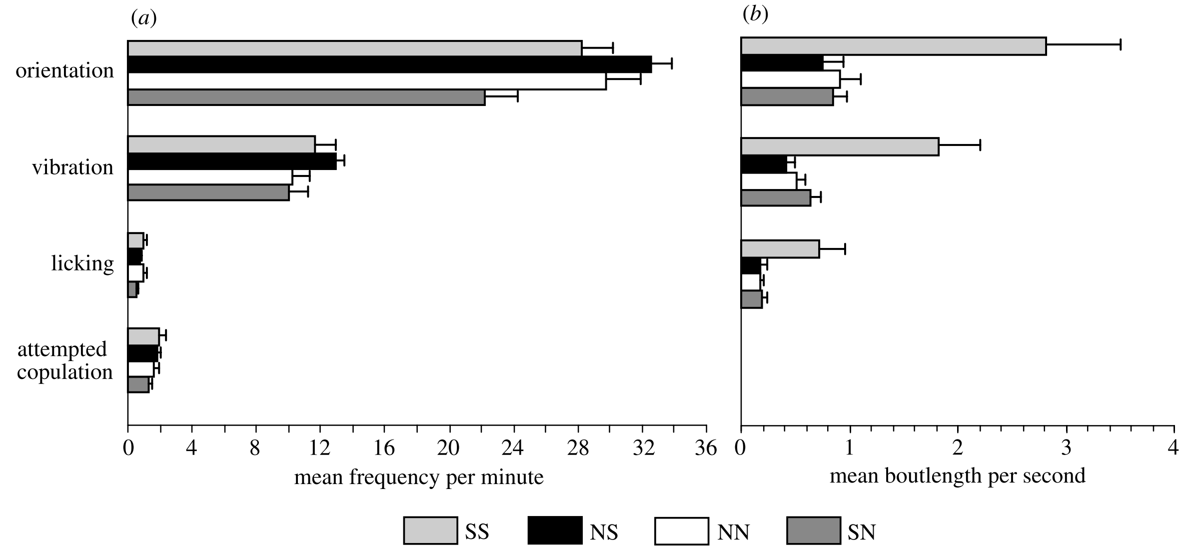

Figure 2. Mean frequency of different male courtship elements per minute (*a*) and corresponding mean bout length of these elements per second (*b*). For abbreviations see figure 1.

Thus, these data clearly show that duration of courtship and sexual activity *per se* reflect different mating attributes. The dynamics of pair formation (mating speed) are shown in figure 1.

An interesting fact is that about 70% of flies of the conspecific SS combination mated during the first 2 min, much faster than the corresponding proportions observed for this period in other combinations (21% in NN, 25% in SN and 17% in NS). This result, together with a relatively faster mating speed manifested by flies of the SN combination, seemingly reflects the effect of female origin (i.e. a higher receptivity of SFS females) and the dependence of SFS female reaction on the origin of the mate partner (see also table 1).

The origin of males had a significant effect on duration of copulation (table 1), with SFS males having a shorter copulation period regardless of female origin.

(b) *Male courtship behaviour*

Four basic male courtship elements (orientation, vibration, licking and attempted copulation) were registered for each pair. Each of these scores reflects simultaneously the mean frequency of initiation of each behavioural element (per time unit) and the duration (bout length) (figure 2).

For the mean frequency parameters, the only significant difference between conspecific and heterospecific pairs was found for orientation: NFS males initiated this element more frequently with NFS females than with SFS females (figure 2*a*). For the duration of three elements, SFS males displayed a much more pronounced effect, with striking bias towards SFS females (figure 2*b*), whereas NFS males did not differentiate their partners by origin. The longer bout length for all courtship elements in SS mating pairs may have resulted from a difference in rejection or acceptance behaviour of females.

(c) *Locomotor activity*

Twenty flies of each slope and sex were tested individually for 600 s (table 2). ANOVA revealed no significant differences either between sexes ($F_{1,76} = 0.113$, $p = 0.737$) and slopes ($F_{1,76} = 0.617$, $p = 0.435$) or their interaction ($F_{1,76} = 0.719$, $p = 0.399$).

(d) *Female courtship behaviour*

The repertoires of rejection behaviour for virgin and fertilized females are different (Connolly & Cook 1973; Connolly *et al.* 1974; Cook 1975). The rejection response of a fertilized female is mainly extruding. The female turns her abdomen towards the courting male and extends her ovipositor, thereby impeding most copulation attempts. The fertilized female perhaps reinforces this rejection response by releasing aversive pheromones (Jallon 1984). In our studies we used virgin females, which blocked courtship by kicking, fending off (the female extends legs on the side of the body nearest to the male and 'wards him off') and decamping. The scoring of female rejection behaviour is presented in figure 3.

No significant differences were found in kicking and fending off between mating combinations, although the mean frequency of kicking displayed by the NFS females to NFS males (1.88 bouts per min) and SFS males (0.80 bouts per min) differed more than twofold. For the frequency of decamping we found the only significant effect for NFS females: these females rejected courtship of SFS males by decamping significantly ($p < 0.01$) more than they did with respect to NFS males. During courtship a female may remain in the same place or move around the mating chamber. Obviously, this should influence either the frequency or bout length of the male's courtship elements (Burnet & Connolly 1974). The percentage of time spent standing or moving by females courted by conspecific and heterospecific males is illustrated in figure 4.

Two important conclusions can be derived from this diagram. First, in conspecific pairs, females of both slopes spent significantly more time standing than moving ($p < 0.001$ and $p < 0.05$, for SFS and NFS pairs, respectively). Second, in heterospecific pairs, NFS females spent much less time standing than moving ($p < 0.001$), whereas SFS females showed no difference in these two activities. Here, as in the case of female's rejection

Table 2. Locomotor activity of flies from the opposite slopes of 'Evolution Canyon'.

testing flies	line crossing
NFS[a] females	71.20 ± 3.71
NFS males	69.15 ± 4.50
SFS[b] females	70.95 ± 3.65
SFS males	75.70 ± 4.12

[a] North-facing slope.

[b] South-facing slope.

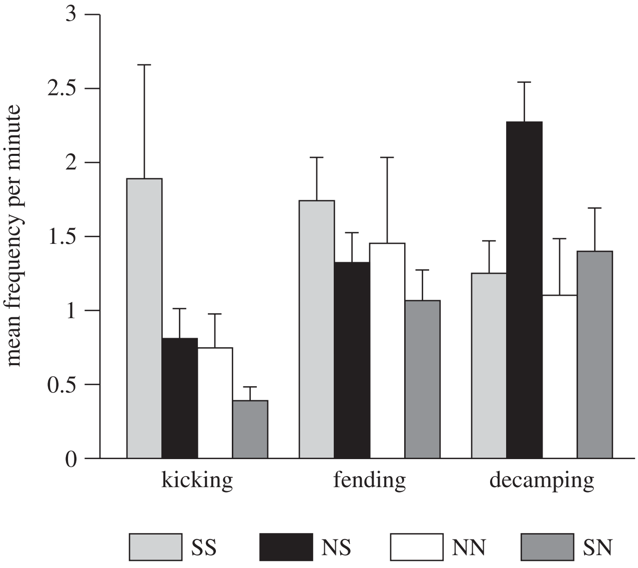

Figure 3. Mean frequency of different elements of female rejection behaviour. For abbreviations see figure 1.

behaviour, we may also consider standing as acceptance behaviour and moving as avoidance behaviour. Thus, summarizing the results on female courtship behaviour and the data on time of pair formation, we can hypothesize that SFS females are more receptive and less discriminating than NFS females.

(e) *Parameters of reproductive behaviour*

We were interested in elucidating the relationships among fecundity, dynamics of egg laying, and repeated mating. Table 3 displays the results on fecundity, average times of egg laying and remating, for females inseminated by conspecific or heterospecific males.

Neither female nor male origin, nor their interaction had any effect on fecundity. Nevertheless, our comparison shows that females from conspecific pairs (SS and NN) manifested the most divergent scores (significant difference at $p < 0.05$). Females from opposite slopes showed clear differences in dynamics of egg laying (figure 5, table 3). Maximum fecundity of SFS females inseminated by conspecific males was observed on the second day of egg laying and then dropped rapidly (figure 5*a*). NFS females, also from conspecific crosses, showed a gradual increase character in the egg laying until the maximal level, which was reached on the sixth day, and then a smooth decrease. In the heterospecific combinations (figure 5*b*), females showed a similar pattern of egg laying during the first 4 days; then SFS females again dropped more rapidly compared with NFS females. Because of the non-significant effect of the male's origin ($p = 0.26$; table 3) on average time of egg laying during the whole period of observation (two weeks), we merged together fecundity data for females with con- and heterospecific males, as shown in figure 5*c*. ANOVA revealed highly significant differences ($F_{1,56} = 14.74$, $p < 0.001$) between NFS and SFS females for this parameter (table 3).

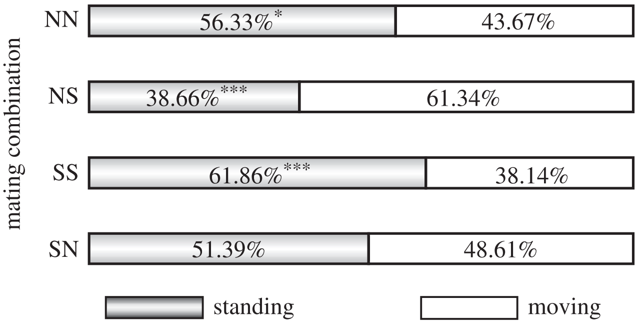

Figure 4. Mean proportion of time spent in standing and moving by females courted by conspecific and heterospecific males. Differences between standing and moving behaviour are significant at: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. For abbreviations see figure 1.

Females from the SFS were much more likely to repeat mating than females from the NFS ($F_{1,76} = 42.22$, $p < 0.001$). The average time of remating for NFS females was twice that of SFS females. Here, as in oviposition dynamics, the origin of males was unimportant (figure 6, table 3).

The relationship between the dynamics of egg laying and repeated mating is illustrated in figure 7. Clearly, the mating combinations are couple-grouped according to the females' origin. SFS females characterized by increased egg laying speed also displayed the shortest remating time. Thus, faster remating appears to be associated with earlier timing of egg laying.

4. DISCUSSION

Conclusions about sexual isolation and incipient speciation are usually derived from results of mate choice experiments. Clearly, a deviation from random mating might be caused by the fly's discriminating ability or its mating propensity (differences in male's sexual activity and female's receptivity). Unfortunately, these alternatives are not easy to distinguish, and often these are considered in discussion and not in real tests. As pointed out by Spieth & Ringo (1983), a significant non-random mating may be revealed if males and females have different rates of mating propensity, even if the two types mate indiscriminately. It is known from many studies in *Drosophila* (Manning 1963; Kessler 1969, Connolly *et al.* 1974; Singh & Chatterjee 1987; Casares *et al.* 1993; Singh & Sisodia 1999) that variation in parameters of mating propensity may appear on the level of populations and strains. Recently, it was clearly shown that differences in mating propensity can lead to non-random mating in mate choice situations and be erroneously interpreted as discrimination (Casares *et al.* 1998).

Table 3. Reproductive characteristics of flies from the opposite slopes of 'Evolution Canyon' for four mating combinations.

(F ratio given for two-way ANOVA on log-transformed data; d.f. = 1, 56. $^{***}p < 0.001$.)

	female origin						
	NFS		SFS				
	male origin		male origin		F ratio for the effect of		
parameter of reproductive behaviour	NFS	SFS	NFS	SFS	FO[a]	MO[b]	FO × MO[c]
Fecundity	11.18 ± 1.42	12.60 ± 3.41	3.70 ± 2.62	16.91 ± 2.35	1.93	0.89	0.13
Average time of egg laying (day)[d]	4.76 ± 0.39	4.30 ± 0.49	2.82 ± 0.82	3.63 ± 0.21	14.73^{***}	0.26	3.48
Remating (day)	1.10 ± 1.19	13.40 ± 1.27	5.50 ± 0.57	6.30 ± 0.68	42.22^{***}	2.52	0.59

[a] Female origin.
[b] Male origin.
[c] Sex interaction.
[d] For this trait, average scores for individual females were obtained before ANOVA, as described in § 2.

(a) *Sexual behaviour*

(i) *Mating propensity*

Usually, male mating propensity is measured in non-choice tests as time elapsed from introduction of mating partners into the mating chamber until copulation (Casares *et al.* 1998) or the number of females mated (Ringo *et al.* 1986). These definitions are probably not absolutely correct for all cases. Indeed, the foregoing parameters result from interaction between sexual partners, male sexual activity and discrimination on the one hand and female receptivity and discrimination on the other. We believe that measuring male courtship latency and duration of copulation may provide a more reliable criterion of male sexual activity. In our ANOVA tests, only these two parameters showed dependence on male origin, SFS or NFS (table 1). Thus, based on the obtained scores of courtship latency and duration of copulation, we can conclude that SFS males display higher sexual activity than NFS males and consequently have a higher mating propensity.

Detailed characteristics of sexual behaviour and elements of females' courtship response in our data corroborate the common assumption that females are more discriminating than males in choosing the mating partner (Bastock 1967; Spiess 1970; Trivers 1972; Schilcher & Dow 1977). The results for female sexual behaviour and several parameters of reproductive behaviour presented in table 4 call for explanatory comments concerning 'acceptance' and 'rejection' behaviour in females.

We submit that the difference among these female behavioural categories includes a complex of traits reflecting a higher or lower degree in the females' readiness to mate.

Female receptivity might depend upon male sexual activity, but in contrast to the latter, it can be more easily measured. In order to determine a female's receptivity, we have to compare scores of mating speed and courtship duration of different females with males of equal mating propensity, i.e. of the same origin. In our experiments, asymmetric receptivity of SFS females proved obvious (table 1). In particular, with conspecific males these females manifested a significantly higher receptivity than with heterospecific ones. This asymmetric character of receptivity probably reflected the difference in the amount of stimulation that SFS females received from SFS and NFS males. Indeed, time spent by SFS males courting SFS females was significantly higher than that spent by NFS ones (see the scores for 'time in sexual activity' in table 1). This suggests that for some reason SFS females are less attractive to NFS males. In the same tests, the NFS females showed no differences in receptivity to males of a different origin.

The locomotor activity of females during courtship also might reflect the degree of acceptance or rejection. When a female is motionless, she receives more complete courtship from the male, and in such cases more typical courtship patterns of interaction between the sexes are displayed. Our quantitative scoring of time spent by females standing and moving showed significant differences between conspecific (more standing) and heterospecific (more moving) pairs. Besides, NFS females spent markedly more time in standing being courted by NFS than SFS males. By contrast, SFS females showed no significant difference in standing among pairs with SFS and NFS males (figure 4). Additional single-fly tests of locomotor activity (table 2) did not reveal any significant differences between SFS and NFS flies either for males or females. This, in turn, reflects the specificity of the female's activity during her interaction with the sexual partner described above.

(ii) *Discrimination*

The female's rejection response to the male's courtship is also of great interest in interslope divergence. These data, together with the results on mating pair formation, may be informative with respect to the degree of discrimination. We measured three rejection elements of sexual female behaviour: kicking, fending off, and decamping. Perhaps the most interesting and the only significant effect was found for decamping (figure 3). The female displayed kicking and fending off mainly when standing, i.e. this cannot be considered as active

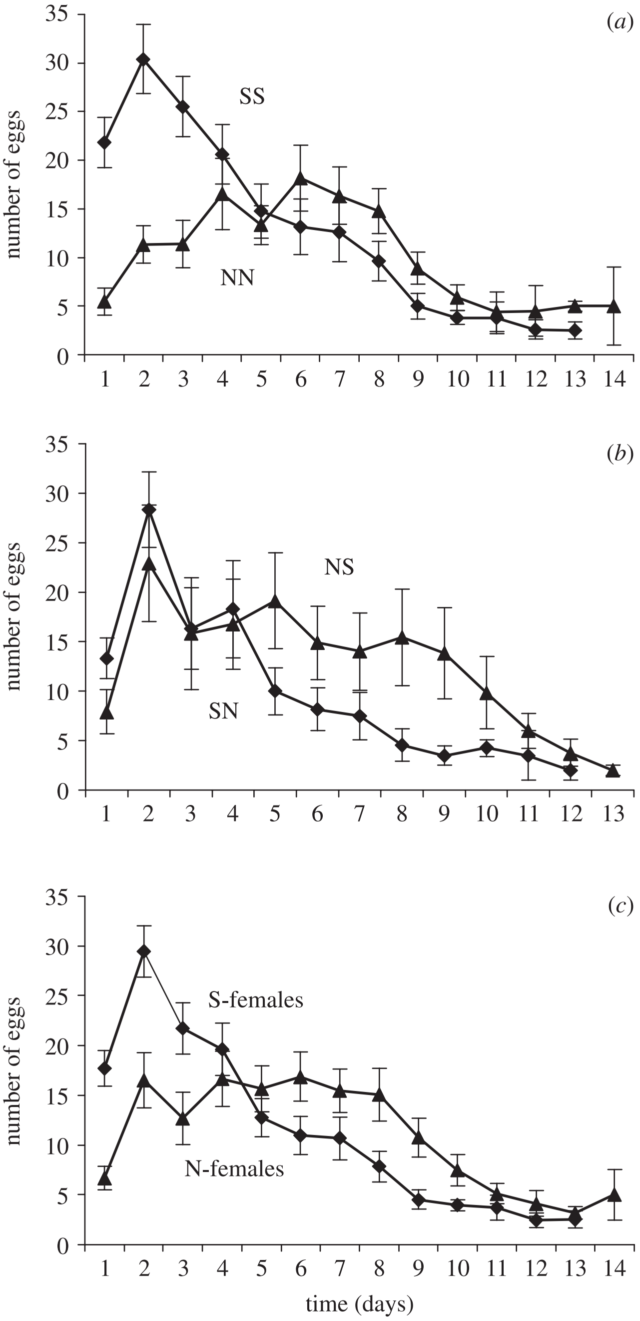

Figure 5. Dynamics of egg laying measured in females mated by (*a*) conspecific males, (*b*) heterospecific males and (*c*) in respect of the female's origin. For abbreviations see figure 1.

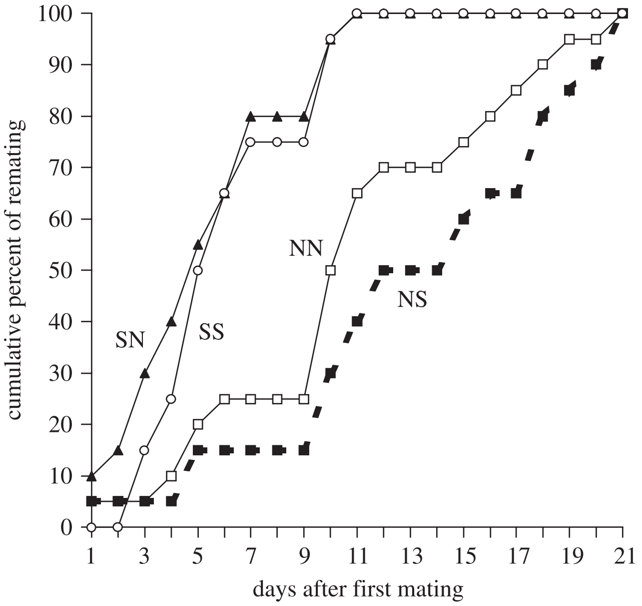

Figure 6. Cumulative percentage of remating in females that had first been mated by conspecific and heterospecific males. For abbreviations see figure 1.

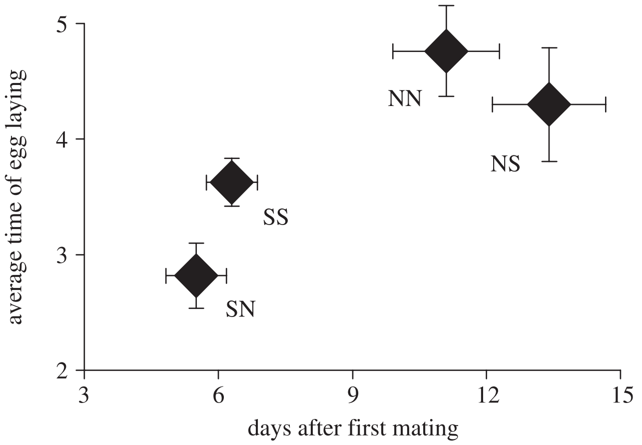

Figure 7. Relationship between time of egg laying and remating in females mated by conspecific and heterospecific males. For abbreviations see figure 1.

escape. Moreover, this female behaviour may provoke even more prolonged courtship. By contrast, a female's decamping behaviour (running away, jumping, and flying away from the courting male) can be regarded as an active avoidance. It is especially important to take this element into account for situations in nature, because decamping of a female may lead to full loss of contact with the courting male. With regard to percentage of formed mating pairs, here again the NS combination differed (although non-significantly, probably because of an insufficient sample size) from the others. All these results (table 1; figures 3, 4) imply that females derived from the opposite slopes of the canyon are able to discriminate between conspecific and heterospecific males, and the result of this discrimination is the accepted or rejected male's courtship. Our tests clearly demonstrated that mate choice in *D. melanogaster* derived from nature might depend on differences in mating propensities and on discrimination.

Thus, the evidence obtained on sexual behaviour of flies from the opposite slopes of 'Evolution Canyon' was as follows. (i) SFS males exhibited a higher mating propensity than NFS males. (ii) Females from the SFS displayed asymmetric receptivity, with preference for conspecific males, whereas NFS females were approximately equally receptive to both SFS and NFS males. (iii) NFS females showed strong discrimination against heterospecific males. We hypothesize that this pattern of sexual behaviour is closely associated with different strategies of adaptation of flies from the opposite slopes of 'Evolution Canyon'. The following discussion of the experimental evidence obtained for reproductive behaviour supports this point of view.

Table 4. Slope-specific sexual and reproductive female's behaviour in flies derived from 'Evolution Canyon'.

female origin	sexual behaviour		reproductive behaviour		
	acceptance	rejection	fecundity	time of egg laying	time of remating
NFS	toward SFS males—very weakened	toward SFS males—strong	low	long	slow
SFS	toward NFS males—slightly weakened	toward SFS and NFS males—equal	high	short	fast

(b) *Reproductive behaviour*

Significant differences were revealed when we compared the fecundity of females mated by conspecific males only. The non-significant effect found by ANOVA for all four mating combinations may have reflected some peculiarities of a male's reproductive status. Thus, SFS males slightly increased the fecundity of NFS females, but NFS males tended to decrease this parameter in SFS females. The shorter time of egg laying and fast repeated mating in SFS females (table 3; figures 5, 6, 7) are probably also connected with the strategy of adaptation to the climatically more stressful environment of the SFS. Our preliminary laboratory tests (T. Pavlicek, K. Iliadi and E. Rashkovetsky, unpublished data) suggest that *Rubia tenuifolia*, *Tamus communis* and *Opuntia* sp. are the putative host plant species for *D. melanogaster* in 'Evolution Canyon'. The population density of these plant species on the SFS is very low because the SFS is an open park forest and the individual plants grow separately from each other. The warmer and drier microclimatic conditions of the SFS result in a faster withering of the falling fruits. Consequently, the probability of finding suitable fruits there for successful oviposition seems to be rather low. Besides, this situation might create competition among females for oviposition sites. Therefore, increased egg laying speed is far more important for SFS than for NFS females. In turn, significant shortening of remating time in SFS females may be a consequence of egg-laying behaviour. Indeed, our data corroborate the results of Pyle & Gromko (1978) who demonstrated that remating is not time-dependent, but is correlated with the rate of progeny production and sperm utilization. Females remate when their productivity, fertility, and fecundity begin to decrease as a result of the depletion of stored sperm. For SFS flies this behaviour should be evolutionarily advantageous because it increases the expected progeny size.

(c) *Adaptive life strategies*

We can propose several rather different scenarios to explain slope-specific adaptive strategies of *D. melanogaster* in 'Evolution Canyon', including non-random mating. The first scenario is based on the assumption that *Drosophila* occupies these places seasonally. The flies settle on both slopes randomly and soon selection for adaptation (e.g. for thermotolerance) begins. In this context, the data of Kilias & Alahiotis (1985) are of interest. They found that even short-term (10 generations) indirect selection in *Drosophila* for heat sensitivity or resistance could induce significant adaptive and evolutionary changes. Selected strains showed a slight tendency to non-random mating at lower temperatures. This tendency was not due to conspecific preferences and reflected asymmetrical sexual isolation. The authors considered this pattern of reproductive success as a first stage of reproductive isolation. Other such examples are found (del Solar 1966; Markow 1981; Dodd 1989).

An alternative and perhaps more appropriate explanation assumes that flies can live on these slopes permanently and have evolved genetic adaptations to different ecological conditions on the slopes, including physiological (stress tolerance) and behavioural (e.g. habitat preferences) adaptations (Nevo *et al.* 1998). Such adaptive differentiation over a very short distance must be maintained by strong permanent selection and reinforced by non-random mating (Korol *et al.* 2000). A similar natural situation was found in populations of *Drosophila persimilis* at the Sierra Nevada Mountains, USA (Taylor & Powell 1977). Flies differed in their frequencies of inversion and allozymic variants. The observed differentiation was also explained in terms of the habitat choice mechanism.

The contrasting microclimatic conditions on the opposite slopes of 'Evolution Canyon' probably are the reasons for the development of several life strategies in *Drosophila*. The genotype's fitness means its relative ability to contribute to future generations (Ehrman & Parsons 1981), and that depends on the environments to which the genotype is exposed. In accordance with this concept, we propose that increased fecundity, shorter time before repeated mating and relatively higher receptivity in SFS females, and higher mating propensity in males from the same niche, reflect their adaptive strategy contributing to higher fitness. NFS females, living in milder, less stressful conditions, display strong discrimination against heterospecific males. We hope that future studies will reveal the real mechanisms underlying this discrimination. It could be due to a qualitatively different pattern of the female's cuticular hydrocarbons, which act as contact pheromones inducing male courtship (Cobb & Jallon 1990). Another cause of discrimination may be connected with different patterns of wing vibration generating species-specific sounds (pulse song and sine song), which are important for mating success and species isolation (Bennet-Clark & Ewing 1969; Ewing 1983; Ritchie *et al.* 1999).

The observed tendency of incipient premating isolation in the *D. melanogaster* population at 'Evolution Canyon' (see also Korol *et al.* 2000) could have evolved as an incidental by-product of gene pool divergence caused by microclimatic interslope differentiation (in accordance with the concepts of Darwin, Muller, Mayr, and Carson concerning reproductive isolation). Alternatively, this tendency may have resulted from selection, direct or indirect, owing to its contribution to higher progeny fitness of the carriers of this mating strategy (Dobzhansky's concept). The very fact that the divergences in adaptive and behavioural traits between the populations at the microsites of the study are accompanied by non-random mating, makes this natural system a promising model to link theory and evidence in an in-depth analysis of adaptation and incipient speciation under heterogeneous stressful conditions.

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