

# Pre-mating isolation is determined by larval rearing substrates in cactophilic *Drosophila mojavensis*.

## II. Effects of larval substrates on time to copulation, mate choice and mating propensity<sup>‡</sup>

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### Summary

It has been hypothesized that reproductive character displacement has evolved in mainland Sonora, Mexico populations of cactophilic *D. mojavensis* due to the presence of a sympatric sibling species *D. arizonae*. In laboratory tests using ancestral Baja California populations and derived, sympatric mainland populations, asymmetrical sexual isolation has been observed among populations of *D. mojavensis* where mainland females discriminate against Baja males. Effects of different pre-adult rearing environments on adult mating behaviour were assessed by comparing fermenting cactus tissues like those used in nature for breeding with laboratory media because previous studies have employed synthetic growth media for fly growth and development. Significant behavioural isolation was evident in all cases when larvae were reared on laboratory food, but was non-significant when flies were reared on fermenting cactus, except for the cactus used by most mainland populations, consistent with previous studies. Time to copulation of Baja females was greater than mainland females over all substrates, but male time to copulation did not differ between populations. Time to copulation for both sexes was significantly greater when flies were reared on laboratory food with one exception. The degree of behavioural isolation was weakly correlated with time to copulation across food types (Spearman rank correlation = 0.58,  $p = 0.099$ ). Therefore, use of laboratory media in this and previous studies exaggerated adult pre-mating isolation and time to copulation in comparison to natural breeding substrates. These experiments suggest that a change in host substrates by saprophagous insects (where chemical differences exist between hosts) may have subtle effects on mating behaviour in a manner which promotes low levels of sexual isolation as a by-product of their utilization of a particular substrate during larval development. For *D. mojavensis*, these results suggest that over evolutionary time, radiation into a new environment (from Baja to the mainland) allowed utilization of new host plants that may have incidentally promoted the sexual isolation patterns that have been observed within this species.

**Keywords:** cactus; pre-mating isolation; *Drosophila mojavensis*; host plant; character displacement; speciation

### Introduction

One of the most important classes of reproductive isolating mechanisms in animals is ethological or sexual isolation. Recent studies with a variety of *Drosophila* species have revealed that asymmetries in the patterns of sexual isolation between species or populations may be a common phenomenon (Kaneshiro, 1976, 1980; Wasserman and Koepfer, 1977, 1980; Ohta, 1978; Watanabe and Kawanishi, 1979; Zouros and d'Entremont, 1980; Markow, 1981, 1991; Markow

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*et al.*, 1983; Ehrman and Wasserman, 1987; Kaneshiro and Giddings, 1987; Koepfer, 1987a, b; Krebs and Markow, 1989; Markow and Toolson, 1990). Several models have been proposed that provide possible explanations for the origin of this kind of asymmetry (Kaneshiro, 1976; Wasserman and Koepfer, 1977; Watanabe and Kawanishi, 1979). Although different conclusions were reached (for reviews see, Wasserman and Koepfer, 1980; Giddings and Templeton, 1983; Heed, 1989), each of the models suggests that the existence of non-reciprocal isolation may reflect the mode of divergence by the populations studied.

In laboratory studies of *Drosophila mojavensis*, Wasserman and Koepfer (1977, 1980) and Zouros and d'Entremont (1980) suggested that the observed asymmetrical pre-mating isolation between populations of this species may have evolved as a response to the presence of its sibling species, *Drosophila arizonae*, in areas of sympatry, a hypothesis of reproductive character displacement. They suggested that selection against hybridization between these sibling species was responsible due to the expected loss of fitness in hybrids and to male sterility in  $F_1$  hybrids from crosses of *D. arizonae* females and *D. mojavensis* males (Crow, 1942; Baker, 1947). Wasserman and Koepfer (1977) clearly showed that in laboratory stocks originating from areas of sympatry, behavioural isolation was much stronger between *D. mojavensis* and *D. arizonae* than in stocks collected from allopatric populations.

Zouros and d'Entremont (1974) first observed a low but statistically significant level of sexual isolation between populations of *D. mojavensis* that were allopatric and sympatric with *D. arizonae*. Wasserman and Koepfer (1977) concluded that discrimination by sympatric females from mainland Sonora against allopatric males from the Baja peninsula arose as a by-product of selection against hybridization with *D. arizonae* and was responsible for the asymmetric nature of the isolation. Zouros and D'Entremont (1980) suggested that character displacement had resulted in a shift of the range of courtship behaviours acceptable to mainland females of *D. mojavensis*. By invoking the character displacement hypothesis, questions surrounding the origin of asymmetrical mating patterns and sexual isolation in *D. mojavensis* seemed resolved.

Although the character displacement hypothesis appears consistent with observations made in the laboratory, it has not been subjected to experimental testing. This is in part due to the fact that *D. mojavensis* and *D. arizonae* readily hybridize under laboratory conditions (Mettler, 1957), so far precluding rigorous laboratory experiments designed to isolate the effects of *D. arizonae* on shifts in mating behaviour of *D. mojavensis*. Field experiments may also be possible, but have not been tried. Differences in courtship behaviour between mainland and Baja populations of *D. mojavensis* are insignificant (Krebs and Markow, 1989) as they are between *D. mojavensis* and *D. arizonae* (Markow, personal communication) and mating songs do not differ between Baja and mainland populations (Ewing and Miyan, 1986). Other hypotheses have been suggested that explain the observed patterns of sexual isolation, such as effects of different host cacti on adult behaviour (Markow *et al.*, 1983). The pattern of host plant used by *D. mojavensis* underlies this alternative.

Changing artificial rearing substrates has long been known to alter patterns of mating behaviour in *Drosophila*, in addition to genotype, temperature and adult age (Kaul and Parsons, 1965; Spiess and Spiess, 1967; Spiess, 1987). More recently, the influence of natural substrates on mate choice has been demonstrated in cactophilic *Drosophila pegasa* (Wasserman and Zweig, 1991) and on behavioural isolation in *D. mojavensis* (Etges, 1992). The results of experiments presented in this paper provide further evidence suggesting an alternative to the character displacement hypothesis: pre-adult environmental conditions determine the intensity of adult pre-mating isolation, mating propensity and time to copulation. This alternate hypothesis was motivated by an understanding of the patterns of host plant used in natural populations of *D. mojavensis*.

### Natural history of *D. mojavensis*

The range of *D. mojavensis* covers most of the Sonoran Desert except the Mojave Desert in southern California and in northern Baja California (Heed, 1978, 1982; Heed and Mangan, 1986).

The Gulf of California is the geographical barrier that separates populations in Baja California from those in mainland Sonora and Sinaloa, Mexico (Fig. 1). *D. mojavensis* and *D. arizonae* co-occur on the mainland and in southern Baja California (Heed, 1982; Ruiz and Heed, 1988).

*D. mojavensis* feeds and breeds on the necrotic tissues, rots, of several cacti endemic to the Sonoran Desert. In Baja California, pitaya agria, *Stenocereus gummosus*, is used almost exclusively even though organ pipe cactus, *Stenocereus thurberi*, is sympatric throughout central and southern portions of the peninsula (Fellows and Heed, 1972; Heed, 1978, 1982; Etges and Heed, 1987). In mainland populations of *D. mojavensis*, the principle host plant is organ pipe except for a short strip along the Desemboque coastal region and the islands in the Gulf of California where agria grows. In southern Sonora and Sinaloa, Mexico, cina cactus, *Stenocereus alamosensis*, is used providing the ecological opportunity for *D. mojavensis* and *D. arizonae* to interact: both species have been reared from single arms of fermenting cina (Ruiz and Heed, 1988; W. B. Heed, personal communication). Populations of *D. mojavensis* in the Mojave Desert of southern California use barrel cactus, *Ferocactus acanthodes*, and they have been discovered on Santa Catalina Island, California where they use prickly pear rots, *Opuntia demissa* (Heed and Mangan, 1986). Thus, *D. mojavensis* is polyphagic, using different host cacti within its geographical range.

Heed (1978, 1982) and Johnson (1980) proposed Baja California as the most likely origin of *D. mojavensis* in the Sonoran Desert because of the isolation from the mainland afforded by changing sea levels and tectonic drift of the Baja peninsula northward from mainland Mexico. Agria cactus, the preferred host (Fellows and Heed, 1972; Downing, 1985), is widespread in Baja and nowhere else and abundant chromosome inversion polymorphism is present in Baja with strict monomorphism in mainland populations suggesting that Baja populations are ancestral to mainland populations (Heed, 1982; Etges, 1990). Therefore, it is likely that a host plant shift from agria to organ pipe and other host cacti occurred when *D. mojavensis* radiated into the Sonoran mainland and southern California from Baja.

Thus, experiments were designed to determine whether adult mating behaviour and sexual isolation between populations of mainland and Baja *D. mojavensis*, are expressed when reared on fermenting cactus tissues. The results of these experiments suggest that rearing environments, particularly laboratory culture conditions used in previous studies, effect changes in time to copulation, mating propensity and sexual isolation in *D. mojavensis*.

### Materials and methods

Two similar sets of experiments were carried out at different times in different laboratories by the authors. Each study is therefore described separately.

#### *Syracuse study*

Two strains of *D. mojavensis* collected in December 1979 were used for all experiments performed in 1982 and 1983 (Brazner, 1983). One strain was originally collected west of Cataviña (West Cataviña, A761), Baja California Norte, Mexico and the other was collected from the Santa Rosa Mountains (Santa Rosa, A742) west of Tucson, Arizona (Fig. 1). These strains were

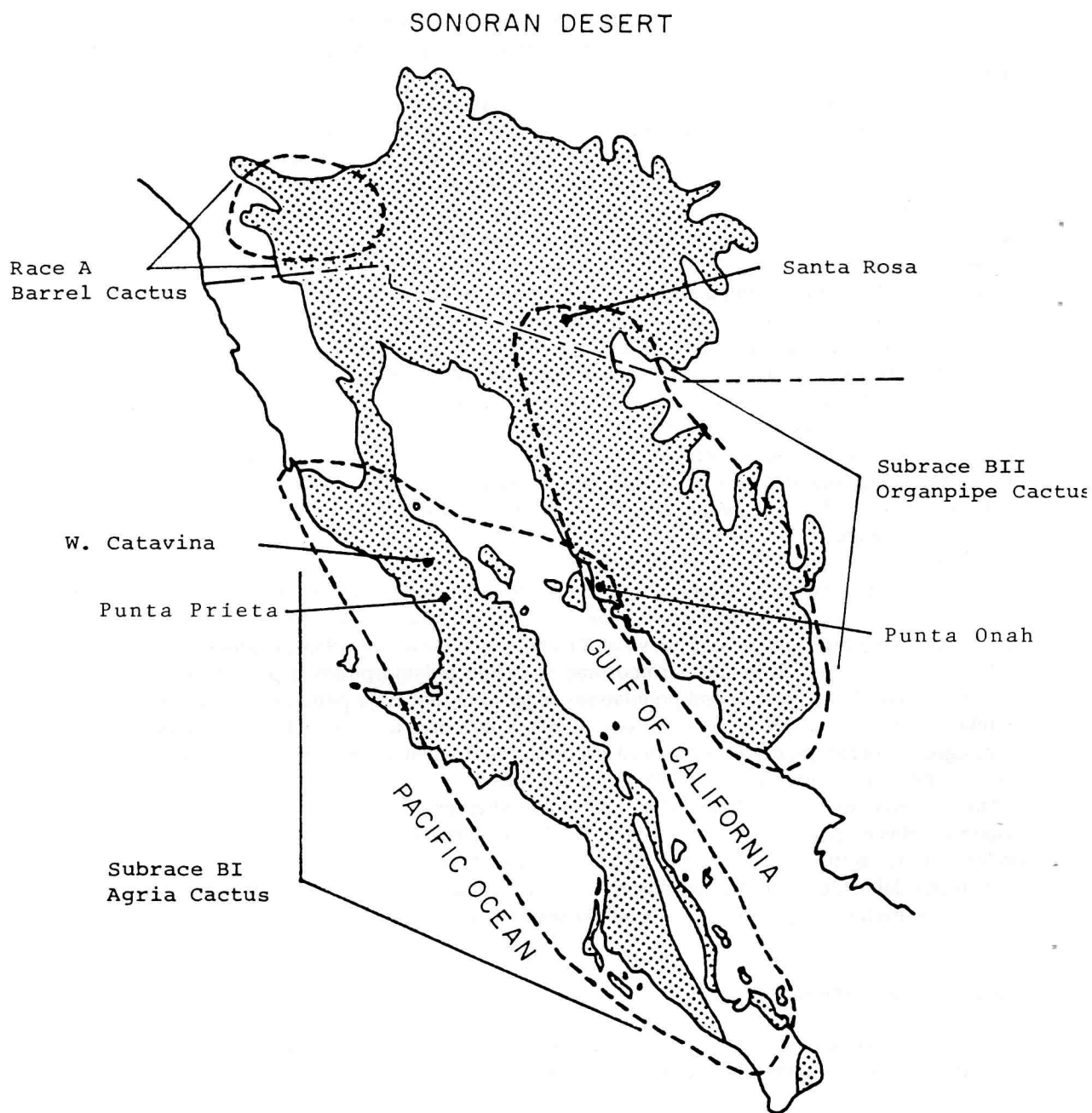


Figure 1. Map showing the distribution of *D. mojavensis* in the Sonoran Desert and the localities where flies in this study were collected. Race and subrace designations refer to the various geographically differentiated populations described in this study.



made axenic by dechoriation of eggs with chlorox (Starmer and Gilbert, 1982) and were maintained axenically on sterile laboratory food until adults were needed. Axenic cultures of each strain were reared from three substrate types: (1) bacteria fermented organ pipe tissue, (2) bacteria fermented agria tissue and (3) an artificial laboratory food (see Appendix).

At the start of each experiment, all substrates were autoclaved to ensure sterility. Approximately 10 g per vial of each cactus was then inoculated with a pectolytic bacterium to start fermentation. After a 48 h incubation period (for cactus only) approximately 10 male and 10 female adults were placed into each food vial. The adults were allowed to mate and lay eggs until moderate larval densities were apparent and then adults were removed from each vial. One to two days past eclosion, emerging adults were removed from each vial, sexed and placed onto fresh, sterile laboratory food. These adults were maintained as virgins for 10–14 days prior to being used in the mating experiments because male *D. mojavensis* reach sexual maturity after 8–10 days at 25°C (Markow, 1982).

#### *Time to copulation experiments*

Fifty male and 50 female, virgin adults reared from one of the three substrates were placed into a closed mating chamber (1000 ml Pyrex Ehrlenmeyer flask) and observed. The first 25 mated pairs were scored for time to copulation and removed. Several replicates of each strain from each substrate were completed.

#### *Sexual isolation experiments*

The effects of larval rearing environments on sexual isolation between populations were evaluated in both within-strain and between-strain trials. For the within-strain trials, only West Cataviña adults reared from banana and organ pipe were used. Rearing origin was ascertained by marking groups of males and females with fluorescent dust (US Radium Corp.) approximately 24 h prior to the start of each trial. Reciprocal marking revealed that dusting had no detectable effect on mating outcomes. Twenty-five males and 25 females from each rearing substrate were placed in a mating chamber to start each trial. The first 25 mated pairs were scored for time to copulation, larval rearing substrate and removed from the chamber. Four replicates of this design were completed.

In the between-strain trials, flies of both strains, reared from one of the three substrates, were used in separate banana and cactus isolation trials. The banana trials were performed exactly as in the within-strain isolation trials except that fluorescent dust was used to determine strain identity rather than larval rearing substrate. The isolation trials for cactus-reared flies were conducted in the same manner as for the banana-reared flies except that two cactus substrates were used in these trials and, therefore, twice the number of adults. Both the cactus and banana isolation trials were replicated several times.

#### *Rearing density – nutritional stress experiments*

Effects of larval nutritional stress on time to copulation and sexual isolation between populations of *D. mojavensis* were suspected, so body size differences caused by larval rearing density were manipulated in several banana food test vials. Emerging adults were grouped into two nutritional classes – stressed and unstressed. The stressed group was obtained by rearing larvae at high densities (approximately 200 larvae per vial) while an unstressed group was obtained by rearing larvae at low to moderate densities (approximately 50 larvae per vial). Mating isolation trials were performed using the same methods described above. Two replicates of this experiment were performed with flies from the Santa Rosa strain.

### Arkansas study

Wild *D. mojavensis* were collected in Punta Prieta, Baja California Norte (120 km southeast of Cataviña) in March 1991 by aspirating 275 wild adults from agria rots in the field and collecting 1913 adults that emerged from eight agria rots returned to the laboratory. Mainland flies originated from Punta Onah, Sonora north of Kino in the Desemboque region in May 1992. A total of 393 adults aspirated from 12 agria rots were combined with 4210 adults that emerged from four agria rots returned to the laboratory. Although collected from agria, Punta Onah flies are characteristically 'mainland' for chromosome and allozyme frequencies, life history traits and behaviour (Etges, 1990). Both stocks were reared at room temperature (21–24°C) on banana food (see Appendix) in eight dram shell vials prior to testing.

Both stocks were then cultured in 10 half-pint milk bottles on banana food at 24°C to increase population size. All adults emerging from the bottles were placed into 12 720 cm<sup>3</sup> Plexiglas population cages for 2 weeks and allowed to mate at room temperature. Eggs were collected over 24 h intervals in removable food cups and approximately 1000 eggs were then transplanted to each of 20 half-pint bottles containing four types of synthetic growth media used in all the previous studies: recipes used were provided by M. Wasserman, E. Zouros and S. Pitnick (Appendix). Cactus cultures were established by introducing approximately 300 eggs onto 50 g of sterilized agria or organ pipe tissue that had been inoculated with 0.1 cc of a pectolytic bacterium and 0.1 cc of a mixture of seven species of yeasts common in natural agria and organ pipe rots (Starmer, 1982; Fogleman and Starmer, 1985); *Pichia cactophila*, *Pichia mexicana*, *Pichia amethionina* var. *amethionina*, *Cryptococcus cereanus*, *Candida valida*, *Candida ingens* and *Torulopsis sonorensis*. Eight replicate cultures of each cactus type were started for each population. All cultures were grown at 24°C.

All emerging adults from these bottle cultures were separated by sex and aged for 2–3 weeks at room temperature prior to the mating tests because male *D. mojavensis* reach sexual maturity after 8–10 days at 25°C (Markow, 1982). Behavioural isolation between mainland and Baja adults was measured by recording the number of copulations in groups of 15 pairs of Baja males and females combined with 15 pairs of mainland males and females in a plastic Petri dish containing filter paper moistened with fermented cactus juice. Time to copulation was recorded for each pair from the time when the first male in a trial was observed courting. During each trial, two mating chambers were observed simultaneously for 1 h in a darkened room. Female *D. mojavensis* remate after approximately 24 h (Markow, 1982) so all matings recorded were of different females. Four trials were performed for each of the six food types. Adults from each population were lightly dusted with fluorescent powder (Radiant Color, Richmond, CA) of different colours 24 h prior to observation allowing identification of copulating pairs. Dust colour was alternated between trials. All copulating pairs were observed for several minutes, thus eliminating pseudo-copulations in the data set (Markow *et al.*, 1983).

### Statistical analysis

Pre-mating isolation was estimated using the joint behavioural isolation index, *I*, from Stalker (1942),

$$I = (n_{11} + n_{22}) - (n_{12} + n_{21}) / N$$

where *N* is the total number of observed copulations, *n*<sub>11</sub> is the number of copulations between females and males from strain 1, *n*<sub>12</sub> is the number of copulations between females of strain 1 and males of strain 2 and so on. The standard error and significance of *I* were calculated with the following formulae (Malagolowkin-Cohen *et al.*, 1965),

$$SE(I) = [(1-I^2)/N]^{1/2}$$

$$t(I) = I / [\text{var}(I)]^{1/2}$$

Calculation of the joint isolation index was included for comparison with previous studies. Gilbert and Starmer (1985) found that this index has relatively poor properties when mating propensity differs and sampling without replacement exists, as in this study. They concluded that Yule's  $V$  index (Yule, 1912; discussed in Pielou, 1977) provides a better unbiased estimation, hypothesis testing, statistical power and reduced type I error in comparison. Yule's  $V$ , its variance and  $t$  value are given by

$$V = [(n_{11} \times n_{22}) - (n_{12} \times n_{21})] / (F_1 \times F_2 \times M_1 \times M_2)^{1/2}$$

$$\text{var}(V) = V^2 \{ (-4/N) + [n_{11} \times n_{22}(n_{11} + n_{22}) + n_{12} \times n_{21}(n_{12} + n_{21})] / [(n_{11} \times n_{22}) - (n_{12} \times n_{21}) - 0.75 \{ [(F_1 - F_2)^2 / (N \times F_1 \times F_2)] + [(M_1 - M_2)^2 / (N \times M_1 \times M_2)] \}] + 0.50 \{ [(n_{11} \times n_{22}) - (n_{12} \times n_{21})][F_1 - F_2] / [M_1 - M_2] \} / (N \times F_1 \times F_2 \times M_1 \times M_2) \}$$

where  $F_1$ ,  $F_2$ ,  $M_1$  and  $M_2$  are total numbers of females and males of the first and second populations, respectively and

$$t(V) = V / [\text{var}(V)]^{1/2}$$

Indices of female-based assortative mating were calculated following Zouros and D'Entremont (1980) and Malagolowkin-Cohen *et al.* (1965) where

$$I_1 = (n_{11} - n_{12}) / (n_{11} + n_{12})$$

and

$$I_2 = (n_{22} - n_{21}) / (n_{21} + n_{22})$$

$I_1$  estimates the degree of female-based assortative mating for females of strain 1 and  $I_2$  for strain 2. The standard error of  $I_i$  ( $i = 1, 2$ ) is

$$SE(I_i) = [(1 - I_i^2) / (n_{i1} + n_{i2})]^{1/2}$$

Because the number of homospecific matings among members of each population differed between the laboratory food and cactus trials, differences in food-induced mating propensity were suspected. Zouros and D'Entremont (1980) provided an index of mating propensity where

$$\hat{k} = (n_{12} + n_{22}) / (n_{11} + n_{21})$$

and the variance of  $\hat{k}$ ,

$$\hat{V}(k) = \hat{k}(1 + \hat{k})^2 / N$$

If male mating propensity is equivalent between populations,  $\hat{k} = 1$ .

All isolation statistics were tested for heterogeneity across food types with a  $\chi^2$ -test for planned comparisons among independent sample means (Sauer and Williams, 1989). The algorithm produces *a priori* simultaneous contrasts among several sample means with standard errors.

## Results

### Sexual isolation experiments

In all cases, when *D. mojavensis* were reared on synthetic laboratory media, significant pre-mating isolation was evident (Table 1, Fig. 2). Estimates of the joint isolation index,  $I$  and Yule's

Table 1. (a) Results of mate choice tests between Baja (B) and mainland (M) adults reared on different laboratory media and two host cacti<sup>a</sup>. (b) Isolation statistics for Baja and mainland adults reared on their typical hosts and alternate hosts

| (a)                             | Number of matings |                  |                  |                  |  | Isolation statistics |                    |                     |                     |                    | $\chi^2$ |
|---------------------------------|-------------------|------------------|------------------|------------------|--|----------------------|--------------------|---------------------|---------------------|--------------------|----------|
|                                 | M × M             | B × B            | Mf × Bm          | Bf × mm          |  | I ± SE               | V ± SE             | I <sub>1</sub> ± SE | I <sub>2</sub> ± SE | k̂ ± SE            |          |
| Wasserman media                 | 72                | 59               | 17               | 44               |  | 0.365***<br>±0.067   | 0.389***<br>±0.065 | 0.618***<br>±0.083  | 0.146<br>±0.098     | 0.655**<br>±0.097  | 29.10*** |
| ASU media                       | 67                | 52               | 25               | 35               |  | 0.330***<br>±0.071   | 0.329**<br>±0.071  | 0.457***<br>±0.093  | 0.195<br>±0.105     | 0.755**<br>±0.114  | 19.38*** |
| Syracuse media                  | 60                | 6                | 4                | 14               |  | 0.571***<br>±0.090   | 0.312*<br>±0.147   | 0.875***<br>±0.061  | -0.400<br>±0.205    | 0.135**<br>±0.046  | 8.20**   |
| Zouros media                    | 65                | 47               | 22               | 47               |  | 0.238***<br>±0.072   | 0.254***<br>±0.071 | 0.494***<br>±0.093  | 0.0<br>±0.103       | 0.616***<br>±0.094 | 11.70*** |
| Banana media                    | 39                | 50               | 28               | 30               |  | 0.211**<br>±0.081    | 0.207**<br>±0.081  | 0.164<br>±0.121     | 0.250*<br>±0.108    | 1.130<br>±0.187    | 6.28*    |
| Organ pipe bacteria             | 23                | 31               | 11               | 28               |  | 0.161<br>±0.102      | 0.195<br>±0.100    | 0.353*<br>±0.161    | 0.051<br>±0.130     | 0.824<br>±0.172    | 3.55     |
| Organ pipe yeast                | 60                | 44               | 32               | 41               |  | 0.169*<br>±0.072     | 0.166*<br>±0.071   | 0.304**<br>±0.093   | 0.023<br>±0.103     | 0.745*<br>±0.094   | 5.20*    |
| Agria yeast                     | 56                | 55               | 37               | 48               |  | 0.133<br>±0.081      | 0.133<br>±0.081    | 0.204<br>±0.121     | 0.068<br>±0.108     | 0.885<br>±0.187    | 3.64     |
| Agria bacteria                  | 21                | 12               | 7                | 23               |  | 0.048<br>±0.126      | 0.101<br>±0.123    | 0.500**<br>±0.164   | -0.314*<br>±0.161   | 0.432**<br>±0.119  | 0.64     |
| SW test X <sup>2b</sup>         |                   |                  |                  |                  |  | 23.644**             | 11.517             | 57.296***           | 16.414*             | 94.806***          |          |
| 2 group SW test X <sup>2c</sup> |                   |                  |                  |                  |  | 13.046***            | 5.591*             | 5.092*              | 0.883               | 0.488              |          |
| (b)                             |                   |                  |                  |                  |  |                      |                    |                     |                     |                    |          |
| Natural host <sup>d</sup>       | M-OPf<br>× M-OPm  | B-AGf<br>× B-AGm | M-OPf<br>× B-AGm | B-AGf<br>× M-OPm |  | 0.094<br>±0.125      | 0.201<br>±0.117    | 0.704***<br>±0.137  | -0.351<br>±0.154    | 0.333*<br>±0.096   | 2.58     |
| 'Wrong' host                    | M-AGf<br>× M-AGm  | B-OPf<br>× B-OPm | M-AGf<br>× B-OPm | B-OPf<br>× M-AGm |  | 0.268*<br>±0.106     | 0.378***<br>±0.095 | 0.750***<br>±0.135  | 0.069<br>±0.131     | 0.708<br>±0.159    | 11.73*** |

\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

<sup>a</sup> Media recipes are given in the Appendix and the cactus media listed are those from the Syracuse study where only bacterial fermentation was used or from the Arkansas study where both bacteria and yeast were used. Details for these food differences and the isolation statistics are given in the text.

<sup>b</sup>  $\chi^2$  tests of homogeneity across all food types for each isolation statistic, all with eight df (Sauer and Williams, 1989).

<sup>c</sup>  $\chi^2$  tests of homogeneity between laboratory (five types) and cactus (four types) food types, all with one df (Sauer and Williams, 1989).

<sup>d</sup> Natural host refers to the cactus used by natural populations of *D. mojavensis*; agria for Baja California populations, organ pipe for mainland populations and the 'wrong' host refers to culturing flies on the hosts each population does not usually use in nature. The crosses denote which population was reared on which species of cactus, e.g. M-OP refers to mainland flies reared on organ pipe-bacteria food, B-AG refers to Baja flies reared on agria-bacteria food, and so on.

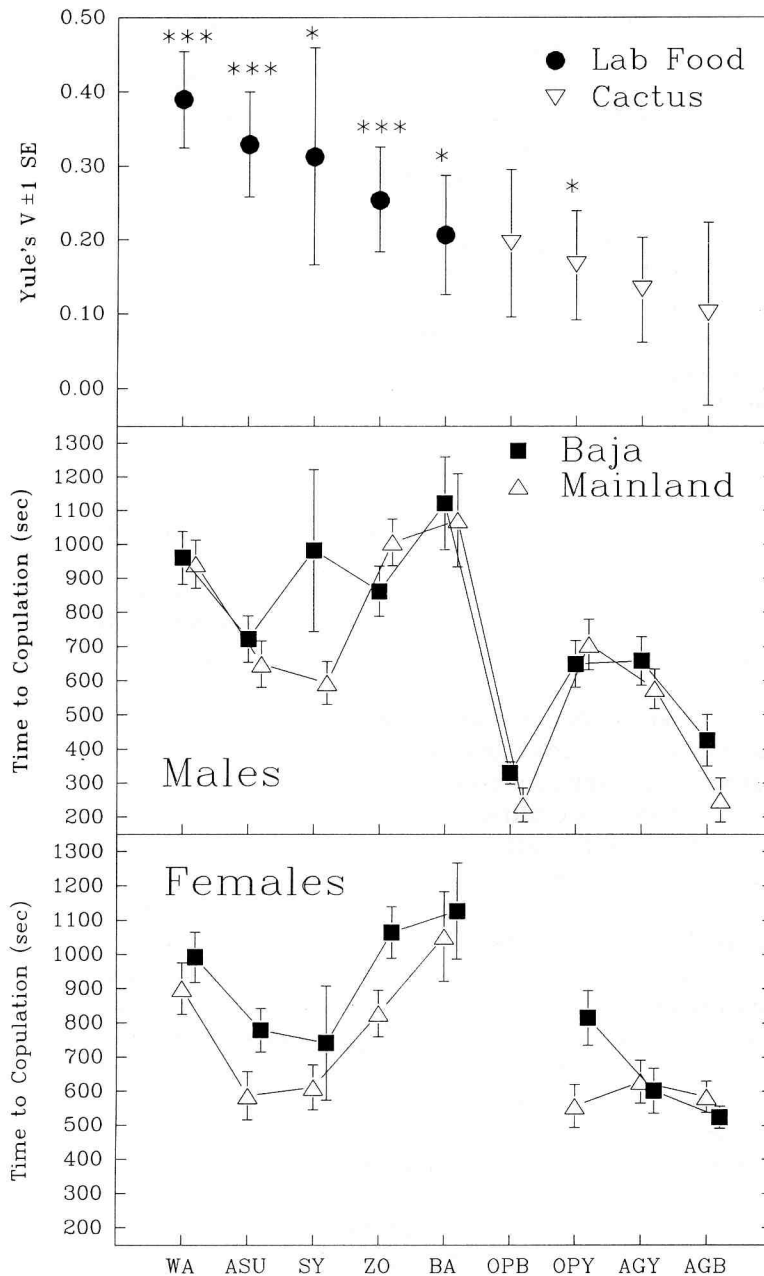


Figure 2. Pre-mating isolation estimated by Yule's  $V \pm 1$  SE and mean time to copulation ( $\pm 1$  SE) for male and female *D. mojavensis* as influenced by larval rearing substrate and geographic origin. Food types arrayed along the x axis are WA, Wasserman; ASU, Arizona State University; SY, Syracuse; ZO, Zouros; BA, banana (Heed); OPB, organ pipe-bacteria; OPY, organ pipe-yeast; AGY, agria yeast; AGB, agria bacteria. Times to copulation for AGB females were actually for all pooled cactus-reared females in the Syracuse study. Details of the media used are given in the text and in the Appendix.

$V$  were similar in all trials except for the Syracuse study where mating propensity of Baja males was low which probably biased the estimate of  $I$  (Gilbert and Starmer, 1985). The degree of isolation for banana-reared flies was uncharacteristically lower than in previous studies (Etges, 1992) and the level of female-based assortative mating,  $I_1$ , was non-significant. In all other cases on laboratory food,  $I_1 > 0$ , showing that mainland females mated more often with mainland males versus Baja males. Under these conditions, Baja females did not discriminate between Baja and mainland males as shown by the lack of statistical significance of  $I_2$  in all cases except for the banana food trials (Table 1). Results of  $2 \times 2$  Chi-squared contingency table analyses testing the hypothesis of equal numbers of matings were also included. In all cases for flies reared on laboratory media, unequal numbers of matings were observed with a clear pattern of assortative mating.

For flies reared on Syracuse and banana media, the pattern was asymmetrical but not in the form expected if character displacement was responsible. The preponderance of mainland male by mainland female matings produced significant isolation and asymmetry but the number of Baja male by Baja female matings was less than that predicted by the character displacement hypothesis, i.e. approximately equal numbers of homospecific matings. This hypothesis predicts that the number of mainland female by Baja male matings (Mf  $\times$  Bm) should consistently be less than the number of Baja female by mainland male matings (Bf  $\times$  Mm). Across all media used, the number of Mf  $\times$  Bm matings was lower than the number of Bf  $\times$  Mm matings (two sample  $t$ -test,  $t = 2.598$ ,  $df = 16$ ,  $p < 0.02$ ). However, for the flies reared on laboratory food only, pre-mating isolation was not always observed to be 'one-way' but the number of Mf  $\times$  Bm matings was marginally lower than the number of Bf  $\times$  Mm matings (two sample  $t$ -test,  $t = 2.052$ ,  $df = 8$ ,  $0.1 < p < 0.05$ ).

For cactus-reared flies, pre-mating isolation was not significantly different from 0 except for the organ pipe cactus-yeast-reared flies, consistent with previous results (Etges, 1992). Like laboratory food, organ pipe cactus induced pre-mating isolation between Baja and mainland *D. mojavensis*, as well as female-based assortative mating,  $I_1$  and mating propensity of mainland males,  $\hat{k}$ . The type of tissue fermentation also seemed to have an effect on patterns of female-based assortative mating and mating propensity. In the Syracuse study, the artificial 'rots' were initiated with a naturally occurring pectolytic bacterium only, whereas this bacterium plus cactophilic yeasts were used in the Arkansas study. These treatments may represent real differences in the type of fermenting tissues encountered in nature because bacterial degradation and cell lysis precede yeast fermentation during microbe community succession (Fogleman and Starmer, 1985). Except for the organ pipe-yeast food, the  $\chi^2$  analyses failed to show differences in the types of matings observed for cactus-reared flies. The pattern of one-way pre-mating isolation was suggested across all cactus media, but reduced from that observed in the laboratory food-reared flies (two sample  $t$ -test,  $t = 1.404$ ,  $df = 6$ ,  $p > 0.2$ ).

Differences in all the isolation statistics caused by growth media were assessed by testing the homogeneity of means using a  $\chi^2$  analysis devised for survivorship data (Sauer and Williams, 1989). Significant heterogeneity was detected across all food types for each statistic except for Yule's  $V$  (Table 1). The disparity between the values of joint  $I$  and Yule's  $V$  was limited to the Syracuse study where sample sizes were smaller and mating propensity differences were greater than in the Arkansas study. To further resolve the differences between artificial and cactus media effects on these statistics, a two-group comparisons test was carried out, laboratory food versus cactus food (Table 1). Pre-mating isolation and mainland female-based assortative mating was greater for flies reared on artificial media than cactus media.

Behavioural isolation of mainland and Baja adults reared on the host typically used in nature was negligible compared to the isolation expressed when they were reared on alternate host



tissues (Table 1B). These data were part of the Syracuse study involving cactus-bacteria food. These results emphasize the degree to which rearing substrates other than those typically used in nature can alter patterns of mate choice. However, laboratory food- and organ pipe-reared groups of the West Cataviña population did not exhibit pre-mating isolation ( $V = 0.079$ ,  $SE = 0.111$ , Table 2), but more organ pipe-reared males and females were involved in matings than laboratory food-reared males and females ( $\chi^2 = 17.28$ , 1 df,  $p < 0.001$ ). The largest number of matings took place between organ pipe-reared males and females (nearly three times more than any other cross) producing significant organ pipe-reared, female-based assortative mating and increased male mating propensity. Thus, laboratory food severely reduced the mating activity of West Cataviña adults.

#### Rearing density – nutritional stress experiments

The results of isolation experiments performed with flies reared under stressed and non-stressed conditions indicated that differences in larval nutrition may lead to sexual isolation between groups of flies reared in different nutritional regimes within a substrate type, even though both groups originated from the same strain (Table 3). This result was due to the differences in time to copulation exhibited by the nutritionally stressed flies versus the nutritionally unstressed flies.

Table 2. The number of matings observed between Syracuse laboratory food- and organ pipe-reared groups of the West Cataviña strain of *D. mojavensis*

| Females    | Males      |        |       |
|------------|------------|--------|-------|
|            | Organ pipe | Banana | Total |
| Organ pipe | 47         | 16     | 63    |
| Banana     | 16         | 8      | 24    |
| Total      | 63         | 24     | 87    |

The level of pre-mating isolation from these data was  $V = 0.079$ ,  $SE = 0.113$ ,  $p > 0.4$ , laboratory food-reared female-based assortative mating,  $I_1 = 0.492$ ,  $SE = 0.110$ ,  $p < 0.05$  and male mating propensity,  $k = 0.381$ ,  $SE = 0.091$ ,  $p < 0.001$ .

Table 3. (a) Number of matings between nutritionally stressed and unstressed populations of the Santa Rosa population of *D. mojavensis* including mean time to copulation (TTC in s) and standard errors (SE) for each marginal category<sup>a</sup>. (b) Timing of matings classified as early (mated in first half of trial) versus late (mated in second half of trial) for the stressed and unstressed flies used in the crosses from (a)<sup>b</sup>

| (a)<br>Females | Males          |                |       |                | (b)<br>Time | Level of nutrition |          |       |
|----------------|----------------|----------------|-------|----------------|-------------|--------------------|----------|-------|
|                | Unstressed     | Stressed       | Total | TTC            |             | Unstressed         | Stressed | Total |
| Unstressed     | 28             | 11             | 39    | 196<br>(SE=22) | Early       | 29                 | 9        | 38    |
|                |                |                |       |                | Late        | 17                 | 23       | 40    |
| Stressed       | 13             | 13             | 26    | 388<br>(SE=58) | Total       | 46                 | 32       | 78    |
| Total          | 41             | 24             | 65    |                |             |                    |          |       |
| TTC            | 196<br>(SE=19) | 389<br>(SE=62) |       |                |             |                    |          |       |

<sup>a</sup> The index of sexual isolation based on these data was  $V = 0.221 \pm 0.125$ , ( $0.1 < p < 0.05$ ).

<sup>b</sup> See text for details. The contingency chi-squared value for these data is 9.21 ( $p < 0.01$ ).

The unstressed flies mated faster (in the first half of the trial) than the stressed flies, if they mated at all ( $\chi^2 = 9.21$ , 1 df,  $p < 0.01$ , Table 3B). Therefore, variation in larval nutrition can influence time to copulation and has a marginal effect on sexual isolation, whether through body size-mediated differences in courtship success or other more subtle effects on mating behaviour.

### Time to copulation

Larval rearing substrates had significant effects on time to copulation for the four possible mating combinations by altering male and female time to copulation (Table 4, Figs 2 and 3). Laboratory food extended male and female time to copulation in comparison to cactus substrates with the exception of the ASU recipe. Data from the Syracuse study were not included in the ANOVA

Table 4. ANOVA and Scheffe's multiple comparisons test results for analysis of rearing substrates (food) on time to copulation (TTC) of the (a) four mating combinations (mate), (b) population differences in male time to copulation and (c) female time to copulation, as shown in Fig. 2

| (a) All pairwise mating combinations |        |              |       |             |       |         |     |
|--------------------------------------|--------|--------------|-------|-------------|-------|---------|-----|
| Source                               | df     | Type IV SS   |       | Mean square | F     | Pr > F  |     |
| Model                                | 23     | 37931001.16  |       | 1649173.96  | 3.49  | 0.0001  |     |
| Food                                 | 5      | 21130183.41  |       | 4226036.68  | 8.94  | 0.0001  |     |
| Mate                                 | 3      | 4504455.54   |       | 1501485.18  | 3.18  | 0.0234  |     |
| Food × mate                          | 15     | 6198140.11   |       | 413209.34   | 0.87  | 0.5934  |     |
| Error                                | 974    | 460281578.48 |       | 472568.36   |       |         |     |
| Food types <sup>a</sup>              |        | BA           | WA    | ZO          | OPY   | ASU     | AGY |
| n                                    | 72     | 181          | 192   | 178         | 179   | 196     |     |
| TTC (s)                              | 1094.4 | 950.3        | 948.8 | 681.08      | 679.8 | 614.2   |     |
| Mating combination <sup>b</sup>      |        | Bf × Mm      | B × B | M × M       |       | Mf × Bm |     |
| n                                    | 234    | 280          | 340   | 144         |       |         |     |
| TTC (s)                              | 890.9  | 855.8        | 741.1 | 668.1       |       |         |     |
| (b) Male time to copulation          |        |              |       |             |       |         |     |
| Source                               | df     | Type IV SS   |       | Mean square | F     | Pr > F  |     |
| Model                                | 11     | 28098661.97  |       | 2554423.82  | 5.36  | 0.0001  |     |
| Food                                 | 5      | 24974610.94  |       | 4994922.19  | 10.48 | 0.0001  |     |
| Population                           | 1      | 3056.95      |       | 3056.95     | 0.01  | 0.9362  |     |
| Food × population                    | 5      | 1651951.16   |       | 330390.23   | 0.69  | 0.6289  |     |
| Error                                | 986    | 470113917.64 |       | 476788.96   |       |         |     |
| (c) Female time to copulation        |        |              |       |             |       |         |     |
| Source                               | df     | Type IV SS   |       | Mean square | F     | Pr > F  |     |
| Model                                | 11     | 34135077.87  |       | 3103188.90  | 6.59  | 0.0001  |     |
| Food                                 | 5      | 25280471.92  |       | 5056094.38  | 10.74 | 0.0001  |     |
| Population                           | 1      | 4142799.38   |       | 4142799.38  | 8.80  | 0.0031  |     |
| Food × population                    | 5      | 2712228.49   |       | 542445.70   | 1.15  | 0.3308  |     |
| Error                                | 986    | 464077501.77 |       | 470666.84   |       |         |     |

<sup>a</sup> Food types are defined in Fig. 2. Means joined by a line are not significantly different by Scheffe's multiple comparisons test.

<sup>b</sup> See Table 1.

The Syracuse data were not included in this analysis. See text for details.

because the raw data from Brazner (1983) were not available. Multiple comparison tests across food types for all pairwise mating combinations (Table 4) produced similar results for male and female times to copulation, so only the former result is shown. Baja females took longer to mate than mainland females no matter which type of male was courting, except for flies reared on agria-yeast media and the Syracuse cactus media (data for females reared on agria-bacteria and organ pipe-bacteria media were pooled in Brazner (1983)). Mainland females accepted mates faster, particularly Baja males (Table 4). Male time to copulation differed over all substrates (Table 4), but Baja and mainland males exhibited similar times to copulation except in the Syracuse study (Fig. 2).

In order to clarify the rankings of the four possible mating types for time to copulation, the data were separated into laboratory food and cactus food groups and reanalysed by ANOVA. This analysis permitted a closer inspection of the effects of cactus versus laboratory food on time to copulation: only the data from the Arkansas study were included in this comparison because the raw data from Brazner (1983) were unavailable. No significant differences in time to copulation across mating combinations or between agria and organ pipe substrates were found (Table 5). However, the interaction between cactus type (agria versus organ pipe) and mating combination was marginally significant ( $p = 0.058$ ) indicating that these two cactus substrates altered the rankings of mating combinations for time to copulation. Organ pipe substrates caused lower times to copulation than agria for matings involving mainland females and Baja males (Fig. 3). This combination is expected to be the rarest under the model of 'one-way' pre-mating isolation yet was consistently the group showing the lowest times to copulation on laboratory food (Tables 4 and 5) and organ pipe tissue in the Arkansas study. Furthermore, this combination

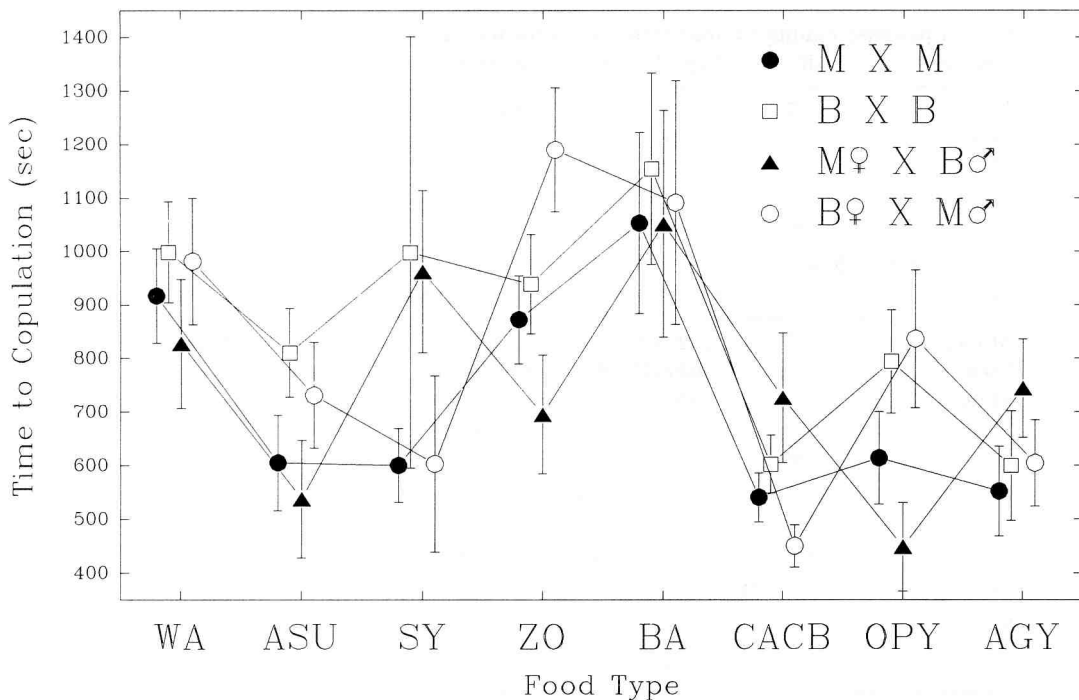


Figure 3. Time to copulation for all possible mating combinations of mainland and Baja adults as determined by pre-adult substrates. Food types are defined in Fig. 2 except for CACB which refers to all pooled cactus-reared flies in the Syracuse study.

showed the largest times to copulation on agria, as well as in the Syracuse study, but in both cases no pre-mating isolation was evident (Table 1). Therefore, these results emphasize that patterns of pre-mating isolation in *D. mojavensis* are not consistently related to differences in time to copulation.

Brazner (1983) concluded that time to copulation was affected by the combined influences of rearing substrate and strain identity ( $F= 25.42, p<0.0001$ ). A least significance difference test (LSD) on these data at the 0.05 level of significance revealed that:

- (1) West Cataviña adults reared from Syracuse (SY) media had significantly slower times to copulation than other groups (SY, OPB and AGB food only),
- (2) West Cataviña males reared from agria-bacteria food had significantly slower times to copulation than all other cactus-reared groups,
- (3) Santa Rosa males reared from banana food displayed significantly longer times to copulation than when reared on organ pipe-bacteria food and
- (4) Santa Rosa males reared from the two cactus substrates did not exhibit significant differences in time to copulation.

Thus, substrate-induced variation in time to copulation for males differed between the Syracuse and Arkansas studies, particularly in the magnitude of laboratory food versus cactus food differences (Fig. 2). Whether this can be attributed to the use of different mainland and Baja

Table 5. ANOVA and Scheffe's multiple comparisons test results for analysis of rearing substrates (food) on time to copulation (TTC) of the (a) four mating combinations on cactus food only (mate) and (b) four mating combinations on laboratory food only

| (a) All pairwise mating combinations on cactus food only |         |              |             |         |        |
|--|---------|--------------|-------------|---------|--------|
| Source   | df      | Type IV SS   | Mean square | F       | Pr > F |
| Model  | 7       | 4846762.17   | 692394.60   | 1.61    | 0.1318 |
| Food   | 1       | 209917.29    | 209917.29   | 0.49    | 0.4855 |
| Mate   | 3       | 1377272.20   | 459090.73   | 1.07    | 0.3634 |
| Food × mate  | 3       | 3256476.15   | 1085492.05  | 2.52    | 0.0577 |
| Error  | 366     | 157611396.51 | 430632.23   |         |        |
| (b) Laboratory food only                                 |         |              |             |         |        |
| Source   | df      | Type IV SS   | Mean square | F       | Pr > F |
| Model  | 15      | 19295002.2   | 1286333.5   | 2.58    | 0.0009 |
| Food   | 3       | 10299127.9   | 3433042.6   | 6.90    | 0.0001 |
| Mate   | 3       | 3209741.4    | 1069913.8   | 2.15    | 0.0929 |
| Food × mate  | 9       | 2361632.5    | 262403.6    | 0.53    | 0.8553 |
| Error  | 608     | 302670182.0  | 497812.8    |         |        |
| Food types <sup>a</sup>                                  | BA      | WA           | ZO          | ASU     |        |
| n  | 72      | 181          | 192         | 179     |        |
| TTC (s)  | 1094.4  | 950.3        | 948.8       | 679.8   |        |
| Mating combination <sup>b</sup>                          | Bf × Mm | B × B        | M × M       | Mf × Bm |        |
| n  | 144     | 181          | 224         | 75      |        |
| TTC (s)  | 1002.4  | 948.7        | 822.5       | 724.7   |        |

<sup>a</sup> Food types are defined in Fig. 2. Means joined by a line are not significantly different by Scheffe's multiple comparisons test.

<sup>b</sup> See Table 1.

The Syracuse data were not included in this analysis. See text for details.

populations, different culturing conditions (bacteria versus yeast fermentation) or uncontrolled environmental variation is unclear. Food-induced differences in pre-mating isolation and female time to copulation were similar in both studies.

The correlation between Yule's  $V$  and male time to copulation was weak (Spearman rank correlation,  $r = 0.583$ ,  $p = 0.099$ ) due to the variation in time to copulation across cactus food and the decreased times to copulation induced by the ASU and SY food types relative to the other artificial media. The observation that the mating combination of mainland females and Baja males showed the lowest times to copulation (Table 4) yet was the least abundant combination of all suggests that substrate-induced differences in male time to copulation do not sufficiently explain the one-way pre-mating isolation in this species when reared on artificial laboratory media. When reared on cactus, the relationship between time to copulation and sexual isolation was influenced more by the type of cactus, agria versus organ pipe and type of tissue fermentation, bacteria versus bacteria + yeast.

## Discussion

Artificial media used in all previous studies caused significant pre-mating isolation, increased mainland female-based assortative mating and increased mainland male mating propensity as compared to fermenting cactus tissues (Table 1). With one exception, laboratory media caused increased times to copulation for males and females from both mainland and Baja populations. Although behavioural isolation among mainland and Baja California populations has been cited as a case of incipient speciation (Wasserman and Koepfer, 1977; Heed, 1978, 1982; Zouros and D'Entremont, 1980; Markow, 1981; Wasserman, 1982; Markow *et al.*, 1983; Heed and Mangan, 1986; Ehrman and Wasserman, 1987; Kaneshiro and Giddings, 1987; Koepfer, 1987a,b; Krebs and Markow, 1989; Etges, 1989, 1992; Krebs, 1990; Markow and Toolson, 1990; Markow, 1991), it is clear from the present study that patterns of sexual behaviour leading to pre-mating isolation depend on pre-adult rearing conditions. Brazner (1983) first demonstrated a significant influence of rearing substrates on time to copulation and patterns of pre-mating isolation. His interpretation that substrate-induced differences in time to copulation, particularly laboratory food-induced increases in time to copulation of Baja males, explained the patterns of one-way pre-mating isolation as an alternate to the character displacement hypothesis is not general for all rearing substrates (Figs 2 and 3, Tables 4 and 5). Therefore, the expression of pre-mating isolation in this species is dependent on pre-adult rearing environments but not due totally to substrate-induced differences in time to copulation.

These results imply that factors other than character displacement alone may have been important in the evolution of the sexual isolation patterns observed between populations of *D. mojavensis*. Reasons for substrate-mediated effects on time to copulation are very likely related to larval nutrition. Robertson (1960) and Royes and Robertson (1964) have shown that suboptimal larval diet (either due to missing nutrients or overcrowded conditions) slows larval maturation and leads to reduced size in adults in *D. melanogaster*. Neither Robertson nor Royes tested for associated effects on adult sexual maturation or mating activity, but preliminary results with *D. mojavensis* have provided suggestive results (Brazner, unpublished data). When adult *D. mojavensis* females were reared as larvae and adults on a low protein/high fructose diet and then used in mating experiments along with females which had been fed standard banana food, only females from the latter group were found to have mated in all but a few instances (39 out of a total 45 matings). Furthermore, when the ovaries of females from both groups were dissected, only females from the standard diet group were found to contain eggs. This implies that variation in larval nutrition, as well as adult nutrition, influences mating activity and egg maturation rates

(cf. Etges and Heed, 1992). However, cactus substrate-induced differences in body size for the culture conditions used in the present study do not influence patterns of pre-mating isolation (Etges, 1992). Only when larvae are severely stressed, producing abnormally small adults, is mating behaviour affected (Table 3).

The results of the isolation trials in both studies did not always follow the predictions of the character displacement model closely, i.e. equivalent numbers of mainland male by mainland female, Baja male by Baja female and mainland male by Baja female matings were observed along with a relatively low number of Baja male by mainland female matings (Table 1). Even though fewer numbers of Baja male by mainland female copulations were observed on laboratory food than mainland male by Baja female copulations, the differences were marginally significant (see Results) whereas with the across cactus cultures, no statistically significant differences were found.

Of the substrates used for these experiments, the most obvious differences in nutrition were apparent between artificial media and cactus substrates. These substrate specific differences were of great enough magnitude to promote sexual isolation. Evidence presented by Kircher (1982), Gibson and Horak (1978) and Starmer (1982) indicates that there are also significant nutritional differences between agria and organ pipe cacti including higher concentrations of triterpene glycosides in agria (Kircher, 1982). Such host plant differences may have effects on adult mating behaviour because of the type of chemical stimuli that are thought to influence patterns of mate choice in *D. mojavensis*. Long-chain hydrocarbons in the adult cuticle act as contact pheromones in this species (Markow and Toolson, 1990). Amounts of cuticular hydrocarbons (CHC) are lower and the kinds of alkenes and alkadienes differ in wild organ pipe-reared adults as compared to laboratory food-reared adults (Toolson *et al.*, 1990). Since lipid precursors of the adult cuticle in *Drosophila* are assimilated during larval development (Toolson and Kuper-Simbron, 1989; J. Fogleman, personal communication) as in most holometabolous insects, variation in CHC profiles may be directly influenced by diet and thereby determine patterns of mate choice. This is currently under investigation (Etges, unpublished data).

The populations used in all the experiments reported here were chosen because their behaviour patterns are characteristic of other populations from these areas. They exhibited asymmetrical mating and sexual isolation patterns similar to those previously observed between several different mainland and peninsular populations (i.e. Zouros and d'Entremont, 1980). They also exhibited mean times to copulation representative of 17 other populations collected from mainland and peninsular regions (D.G. Gilbert, personal communication). Age at first reproduction, as measured by the proportion of a specific age class that mated in a given trial, was found to be comparable in length and sexual dimorphism to previous studies (Markow, 1982). Based on this information, it is likely that the populations used for these experiments were representative of other populations from the mainland and peninsular regions, therefore, it seems reasonable to use the results obtained here to generalize to these other populations.

The pattern of behavioural isolation in *D. mojavensis* is therefore dependent upon larval substrates. While the effects of agria and organ pipe tissues on pre-mating isolation differed somewhat between the two studies described in this paper, it is nonetheless clear that use of artificial media alters several important features of mating behaviour. Organ pipe tissues fermented with bacteria and yeast were most similar to all of the artificial media used. In nature, pre-mating isolation, increased mainland female-based assortative mating and increased mainland male mating propensity would only be expressed when *D. mojavensis* breeds in organ pipe rots, but the frequency with which Baja and mainland adults interact in the wild, if ever, is unknown. Changes in the use of host plants by insects feeding and breeding on them may



therefore influence mate choice in a manner which promotes sexual isolation as a by-product of utilization of that substrate during pre-adult development.

The causes of sexual isolation among bisexually reproducing populations may be more related to the forces shaping mate choice behaviours in local demes promoting reproductive efficiency than selection reducing the production of less fit hybrid progeny (Paterson, 1978; Carson and Lande, 1984; Carson, 1987). Unfortunately, the relative importance of sexual selection in *D. mojavensis* in studies of pre-mating isolation has yet to be investigated. The search for the causes of behavioural divergence among Baja and mainland *D. mojavensis* certainly requires consideration of those local ecological conditions which might influence mating success. This view does not preclude the character displacement hypothesis but suggests that proximate ecological factors such as differential host plant use may influence sexual isolation. Obviously, without knowledge of the pattern of host plant use and range expansion in *D. mojavensis*, this alternative hypothesis could not be tested.

### Acknowledgements

W. T. Starmer and D. G. Gilbert assisted every phase of the Syracuse study. L. L. Wolf, W. M. Shields, S. L. McNaughton, E. Zouros and an anonymous reviewer provided helpful criticism of earlier versions of this paper, W. B. Heed and J. C. Fogleman collected flies for the Syracuse study and A. Gurevitch and E. Waltz provided statistical advice. B. Metcalf, J. Montague, C. Hodges, D. Droney and D. Buckley answered all our questions and V. Aberdeen and P. Brown provided excellent technical assistance. M. Wasserman, E. Zouros and S. Pitnick provided food recipes that D. Koroma prepared and used for culturing the flies in the Arkansas study. Finally, special thanks go to M. Benedict for both technical and personal support and W. Johnson for being there. Financial support came from NSF grants to W. T. Starmer and NIH BRSG 2 SO7 RRO7101-12 to W. J. Etges.

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#### Appendix: recipes for laboratory media

(1) Banana (Heed's) media. This media contained 1000 ml deionized water, 7.7 g agar, 46.15 g nutritional yeast, one banana, 48 ml Karo syrup, 48 ml malt extract and 8.5 ml propionic acid. The agar solution was heated to a rapid boil and the yeast added. The banana, Karo and malt extract were combined in a blender and then added to the agar-yeast mixture. This was then cooked for 10 min at a low boil, cooled, propionic acid added and then poured.

(2) Wasserman media. This recipe consisted of a cooked and refrigerated yeast mixture of 41.2 g live brewer's yeast and 78.4 ml deionized water, a mixture of 10.7 g agar, 392 ml deionized water and three-quarters of a tablespoon of Karo. The yeast mixture mentioned above was added to one-tenth of a mason jar of *Opuntia* cactus fruit, a half teaspoon of malt extract, two bananas, 294 ml deionized water and was blended for 4 min. An additional 235 ml deionized water was added and then this mixture was heated to boiling. After cooling for 20 min, 39.2 ml of 15% propionic acid was added.

(3) Arizona State (ASU) media. This medium contained 7.3 g agar, 11.56 g baker's yeast, 87 g cornmeal, 106.6 ml molasses, 1000 ml deionized water and 16 ml propionic acid.

(4) Zouros medium. This recipe consisted of three-quarters of a medium-size banana, one spoonful of molasses, three-quarters of a spoonful of malt extract, 10 g agar, 20 ml ethanol (added during the last few minutes of cooling to prevent evaporation), 30 g live baker's yeast, 20 ml of 10% Tegosept in 70% ethanol (substituted for the suggested 20 ml of 10% nipagin) and enough deionized water to bring the solution to 1000 ml.

(5) Syracuse media. This mixture contains 1 banana, 60 g brewer's yeast, 10 g malt extract, 10 ml light corn syrup and 15 g *Drosophila* agar per litre of distilled water.

All the recipes were made according to the instructions provided. After cooling, approximately 50 ml of media was poured into sterilized half-pint bottles yielding approximately 20 bottles per recipe. The bottles were then wrapped securely with sterilized cloth and left to stand overnight. They were then plugged with gauze covered cotton balls and refrigerated until needed.