NOTES AND COMMENTS

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PREMATING ISOLATION IS DETERMINED BY LARVAL SUBSTRATES IN CACTOPHILIC *DROSOPHILA MOJAVENSIS*

WILLIAM J. ETGES
Department of Biological Sciences, University of Arkansas, Fayetteville, AR 72701 USA

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Premating isolation among geographically isolated populations of *Drosophila mojavensis* is an often cited example of incipient speciation (Wasserman and Koepfer, 1977; Zouros and D'Entremont, 1980; Markow, 1981, 1991; Markow et al., 1983; Markow and Toolson, 1990; Koepfer, 1987*a*, 1987*b*; Krebs, 1990; Krebs and Markow, 1989; Heed, 1978, 1982; Heed and Mangan, 1986; Wasserman, 1982; Ehrman and Wasserman, 1987; Kaneshiro and Giddings, 1987). In laboratory tests, when mainland females from Sonora, Mexico, or Arizona are allowed to choose between males originating from the mainland or Baja California during courtship, mainland females and Baja males do not mate as frequently as all other male-female combinations causing low but significant one-way behavioral isolation. Since *D. mojavensis* have relatively simple courtship rituals that do not vary geographically (Markow, 1981; Krebs and Markow, 1989), determining the mechanism for the origin of this reproduction isolation is of great interest. At least three hypotheses have been advanced: 1) reproductive character displacement (Wasserman and Koepfer, 1977); 2) use of different host plants for feeding and oviposition that have caused physiological shifts in some traits such as cuticular hydrocarbons, contact pheromones in this species (Markow et al., 1983; Markow and Toolson, 1990); and 3) correlated responses in behavior resulting from adaptation to different host cacti due to genetic correlations between premating behavior and some component of fitness associated with differential host plant use (Etges, 1990). The latter two hypotheses may not be mutually exclusive, but suggest an important role of host cactus use on patterns of adult mate choice. Almost all *D. mojavensis* in Baja California use agria cactus, *Stenocereus gummosus*, and most mainland Sonora and Arizona populations use organ pipe cactus, *S. thurberi* (Heed and Mangan, 1986).

Variation in the profiles of adult cuticular hydrocarbons (CHCs) has been shown to influence mate choice in *D. mojavensis* (Markow and Toolson, 1990) and other drosophilid species (Antony and Jallon, 1982; Jallon and David, 1987; Cobb and Jallon, 1990). The preadult environment may influence adult CHC profiles because some adult cuticle precursors are assimilated during larval stages in holometabolous insects such as *D. pseudoobscura* (Toolson and Kuper-Simbron, 1989). The type of culture medium used for rearing larvae also can alter patterns of male mate choice in *D. pegasa* (Wasserman and Zweig, 1991).

All measurements of premating isolation in *D. mojavensis* have heretofore been made under laboratory conditions with flies reared on artificial media. Thus, artificial environmental conditions might influence adult mating behavior or physiological responses that are important in mate choice. CHC profiles differ between lab and wild adults (Toolson et al., 1990). The purpose of this study was to test the influences of rearing *D. mojavensis* in agria and organ pipe cultures on premating isolation between populations.

MATERIAL AND METHODS

All flies used in this experiment were collected in March, 1991 from naturally occurring cactus rots. The mainland stock was derived from 502 adults aspirated from agria rots in Punta Onah, Sonora. The Baja stock originated from Punta Prieta, Baja California Norte from 275 aspirated adults and 1,913 adults that emerged from eight agria rots collected in the field and returned to the lab. Both stocks were cultured with banana-yeast-malted barley-Karo-agar food in shell vials for four to five generations before the mating tests were performed. Several thousand adults from each population were then introduced into separate 12,720 cm^3 population cages with screw-on food cups. After two weeks, eggs were collected on softened food, transferred to lab food in ½ pint milk bottles, and grown at moderate densities in an incubator with a 14:10 LD photoperiod that cycled from 27° to 17°C. This generation in bottle culture was included to minimize any environmental variation/maternal effects caused by mass rearing in vials. Virgin adults from at least 12 bottles/population were separated by sex, aged for two weeks, and 200 adults of each sex were combined into individual oviposition chambers. Twelve replicate chambers were established for each population. Six chambers were randomly assigned to agria and the other six to organ pipe to yield 12 lines per population.

From each oviposition chamber, eggs were collected over 10 hr intervals each day in petri dishes containing 2% agar-fermented cactus juice. The following day, eggs from each chamber were washed in deionized water, 70% ethanol, and again in sterile deionized water. Eggs were counted out in groups of 250, transferred to a 1 cm^2 square of filter paper and placed on fermenting

cactus. Cactus cultures were set up in ½ pint bottles with 75 g of aquarium gravel at the bottom. Bottles were autoclaved with just gravel, and again after 50 g of either agria or organ pipe tissue were in place. After cooling to room temperature, each culture was 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, P. mexicana, P. amethionina* var. *amethionina, Cryptococcus cereanus, Candida valida, C. ingens,* and *Torulopsis sonorensis.* Two replicate cactus cultures were started from each chamber.

All emerging adults from each bottle were collected daily and separated by sex. Egg to adult development time was measured in days, and viability was estimated after correcting for the number of unhatched eggs. These adults were aged for at least 12 to 14 days at room temperature (20 to 24°C) prior to the mating tests because male *D. mojavensis* reach sexual maturity after 8 to 10 d at 25°C (Markow, 1981). After the mating tests were completed, thorax sizes of 10 adults of each sex were measured to the nearest 0.0001 mm at 25× using the JAVA® video image analysis system (Jandel Scientific, Corte Madera, CA).

Behavioral 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 fermenting cactus juice. Two such mating chambers were observed simultaneously for 1 hr in a darkened room. Adults from each population were lightly dusted with fluorescent powder (Radiant Color, Richmond, CA) of different colors 24 hr prior to observation allowing identification of copulating pairs. Dust color was alternated between tests. All copulating pairs were observed for several minutes to avoid including any pseudo-copulations in the data set (Markow et al., 1983).

Three series of mating tests were performed. First, adults from both populations that had been cultivated on lab food in ½ pint bottles were combined in mating chambers. Fourteen replicates were observed. Second, adults from both populations that were cultured on the same cactus species were tested. Twelve replicates were observed for each cactus, two from each of the initial lines. These tests allowed for measurement of the effect of cactus substrate on premating isolation between mainland and Baja populations. Third, within population premating isolation was recorded by observing copulations between adults from the same population but cultured on different cacti. The same proportions of adults were used as described above in each of 12 pairs of observations. The latter series of mate choice tests were performed to determine whether cactus type could induce within-population premating isolation.

Premating isolation was estimated by calculating Stalker's (1942) joint isolation index,

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

where n_{11} and n_{22} are the number of homospecific matings, n_{12} and n_{21} are the number of heterospecific matings between females of the first type and males of the second type and vice versa, and N is the total number of observed copulations. For significance testing, Malagolowkin-Cohen et al. (1965) provided expressions for the standard error of I, where

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

and

$$\mathrm{t}(I) = I/\sqrt{\mathrm{var}(I)}.$$

Comparing statistical power, bias, and Type I error of several isolation statistics, Gilbert and Starmer (1985) found that this joint isolation index has relatively poor properties when mating propensity differs and sampling without replacement exists, as in the present study. They concluded that Yule's V index (Yule, 1912 discussed in Pielou, 1977) provides better unbiased estimation and hypothesis testing. This index, its variance, and t value are given by

$$V = [(n_{11}\cdot n_{22}) - (n_{12}\cdot n_{21})] \div \sqrt{(F1\cdot F2\cdot M1\cdot M2)}$$

$$\begin{aligned}\mathrm{var}(V) = V^2\{&(-4/N) \\ &+ [n_{11}\cdot n_{22}(n_{11} + n_{22}) \\ &\quad + n_{12}\cdot n_{21}(n_{12} + n_{21})] \\ &\div [(n_{11}\cdot n_{22}) - (n_{12}\cdot n_{21})]^2 \\ &- 0.75\{[(F1 - F2)^2/(N\cdot F1\cdot F2)] \\ &\qquad + [(M1 - M2)^2/(N\cdot M1\cdot M2)]\} \\ &+ 0.50\{[(n_{11}\cdot n_{22}) - (n_{12}\cdot n_{21})] \\ &\qquad \cdot (F1 - F2)(M1 - M2)\} \\ &\div (N\cdot F1\cdot F2\cdot M1\cdot M2)\}\end{aligned}$$

where $F1$, $F2$, $M1$, and $M2$ are total numbers of females and males of the first and second populations, respectively, and

$$\mathrm{t}(V) = V/\sqrt{\mathrm{var}(V)}.$$

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 one and I_2 for strain two. The standard error of I_i (i = 1, 2) is

$$S_i = [(1 - I_i^2)/(n_{11} + n_{12})]^{1/2}.$$

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

TABLE 1. Results of mate choice tests: A) between Baja and mainland adults reared on lab food, and two host cacti, and B) within populations where males and females were cultured on different host cacti. Explanations for the isolation statistics are given in the text.

A. Between populations

	Number of matings				Isolation statistics				
	M × M	B × B	M♀ × B♂	B♀ × M♂	$I \pm$ SE	$V \pm$ SE	$I_1 \pm$ SE	$I_2 \pm$ SE	$\hat{k} \pm$ SE
Lab food	72	37	12	52	0.260*** ±0.073	0.303*** ±0.071	0.714*** ±0.076	−0.169 ±0.105	0.395** ±0.067
Organ pipe	51	79	39	46	0.209** ±0.067	0.197** ±0.068	0.133 ±0.105	0.264* ±0.086	1.216* ±0.167
Agria	47	83	56	46	0.121 ±0.065	0.101 ±0.066	−0.087 ±0.098	0.287* ±0.084	1.495* ±0.200

B. Within populations

	AG × AG	OP × OP	AG♀ × OP♂	OP♀ × AG♂	$I \pm$ SE	$V \pm$ SE	$I_1 \pm$ SE	$I_2 \pm$ SE	$\hat{k} \pm$ Se
Baja	83	51	68	67	−0.004 ±0.061	−0.018 ±0.061	0.099 ±0.081	−0.136 ±0.091	0.793* ±0.097
Mainland	66	53	69	51	−0.004 ±0.065	−0.002 ±0.065	−0.022 ±0.086	0.019 ±0.098	1.043 ±0.135

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

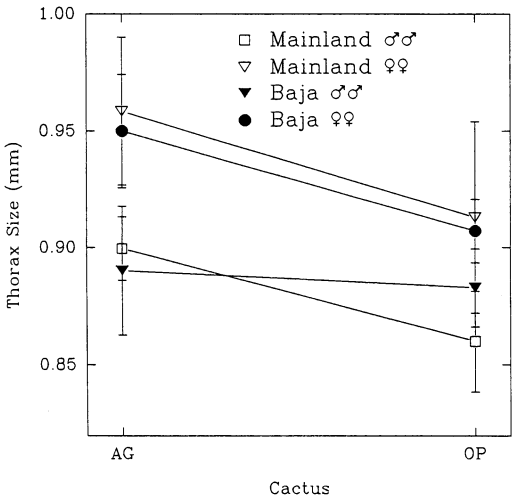

FIG. 1. Mean (±1 SD) thorax sizes of adult *D. mojavensis* used for premating isolation tests in this study. Flies grown in agria cultures (AG) were larger than those grown in organ pipe cultures (OP).

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

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

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

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

Both estimates of premating isolation, I and V, were compared with a Chi-squared test of homogeneity across lab food, organ pipe, and agria rearing environments using a planned comparisons test for independent sample means (Sauer and Williams, 1989). The algorithm produces a priori simultaneous contrasts among several sample means with standard errors.

RESULTS AND DISCUSSION

Sexual isolation between mainland and Baja adults was low, but significantly different from zero in trials using flies reared on banana food (Table 1A) consistent with previous studies of premating isolation between a variety of Baja and mainland populations reared on artificial media (Wasserman and Koepfer, 1977; Zouros and D'Entremont, 1980; Brazner, 1983; Koepfer, 1987*a*; Markow, 1981, 1991; Markow et al., 1983). The magnitude of behavioral isolation, $I = 0.26$ (SE = 0.073), was caused by the paucity of heterogametic matings, M♀ × B♂, and the significance of the index of female based assortative mating for mainland adults, I_1 (Table 1). Mating propensity differences were also apparent as indicated by the significance of $\hat{k}$: Baja males were less likely to mate with females from either population when reared on lab food. Brazner (1983) observed banana food-reared Baja males had four-fold slower mating speeds than when reared on agria or organ pipe, but mating speeds of mainland males were much less sensitive to rearing environments. Therefore, these results are largely consistent with those of all previous studies employing artificial culture media.

However, when these populations were cultured on fermenting cactus tissues, patterns of mate choice and mating propensity changed (Table 1A). Both isolation indices, Joint I and Yule's V, decreased slightly for organ pipe-cultured adults and were not significantly different from zero for agria adults. Lab food and agria isolation indices differed by more than two standard errors suggesting that agria cactus effectively eliminates the pattern of premating isolation observed on banana food. However, values of Joint I were homogeneous across substrates ($\chi^2 = 2.125$, $P = 0.346$) as were values of Yule's V ($\chi^2 = 4.343$, $P = 0.114$; Sauer and Williams, 1989). Both cactus substrates increased female-based indices of assortative mating of Baja females, I_2, as opposed to mainland females on lab food. Lab food induced-male mating propensity differences were eliminated by both cacti, especially by increasing the number of Baja homospecific matings.

Cactus substrates had no influence on premating isolation or patterns of female-based assortative mating in the within-population tests. Comparisons with lab

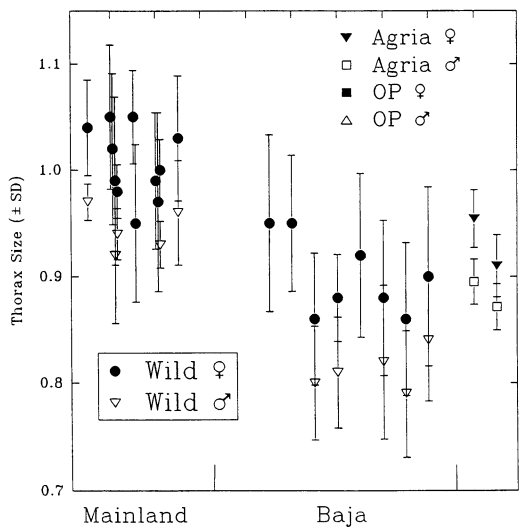

FIG. 2. Thorax sizes of wild-caught adult *D. mojavensis* from 13 natural populations compared to thorax sizes of the adults in this study. Data for flies in this study were grouped by sex and cactus because no differences between populations were observed (Table 2). The origin of wild flies and sample sizes by number are (Mainland, 1–5; Baja, 6–13; graphed in ascending order on the X axis): 1. Vallecito, California (25 ♀ and 17 ♂); 2. Sil Nakya, Arizona, four collections. a. 22 ♀ b. 21 ♀ c. 186 ♀ and 143 ♂ d. 31 ♀ and 21 ♂; 3. Tepopa, Sonora, two collections. a. 30 ♀ b. 40 ♀; 4. Punta Onah, Sonora, three collections. a. 30 ♀ b. 48 ♀ c. 131 ♀ and 141 ♂; 5. San Carlos, Sonora (29 ♀ and 39 ♂); 6. San Simon, Baja California Norte (17 ♀); 7. West Catavina, Baja California Norte (65 ♀); 8. Punta Prieta, Baja California Norte (103 ♀ and 130 ♂); 9. South Viscaino, Baja California Norte (5 ♀ and 33 ♂); 10. Punta Balandra, Baja California Sur (31 ♀); 11. A. V. Bonfil, Baja California Sur (149 ♀ and 53 ♂); 12. San Bartolo, Baja California Sur (71 ♀ and 35 ♂); 13. Virgin Maria, Baja California Sur (37 ♀ and 36 ♂).

TABLE 2. ANOVA results for thorax size data presented in Figure 1 for the two populations of *D. mojavensis* cultured on agria and organ pipe cactus in this study.

Source of variation	*df*	Mean square	*F*	*P*
Model	9	0.0053	8.54	0.0001
Population	1	0.0000	0.00	0.9729
Cactus	1	0.0138	22.03	0.0001
Sex	1	0.0289	46.15	0.0001
Line	2	0.0010	1.58	0.2191
Pop × Cact	1	0.0009	1.46	0.2342
Pop × Sex	1	0.0006	0.94	0.3383
Cact × Sex	1	0.0013	2.04	0.1615
Pop × Cact × Sex	1	0.0007	1.06	0.3107
Error	38	0.0006		

food-cultured flies were not performed. Matings between flies from the same population, but reared on alternate cacti were random (Table 1B). These results suggest that the effects of rearing environment per se do not explain the reduction of isolation in the between-population tests of cactus-reared flies. Since adult size can influence premating isolation (Brazner, 1983), analysis of thorax size variation of the flies used in the mating tests was performed because thorax sizes of mainland adults are genetically larger than Baja adults when cultured on cactus (Etges, 1989, 1990) and adult size is influenced by larval nutrition in *D. mojavensis* (Heed and Mangan, 1986; Etges and Heed, 1987).

Agria cactus caused larger adult thorax sizes than organ pipe (Fig. 1, Table 2), but overall body size did not differ among populations. Therefore, size-related mate choice or vigor cannot explain the reduction of premating isolation in flies reared on either cactus in the first set of mating trials (Table 1A). Thorax sizes of these cactus-reared flies were grossly comparable to wild-caught adults from a number of Baja and mainland localities (Fig. 2), suggesting that these lab rots produced flies similar in size to those collected in nature. However, the larger size of mainland adults was eliminated here due to the high culture densities used. Mainland females are genetically less homeostatic for thorax size than Baja females as culture densities increase, particularly on organ pipe (Etges and Heed, 1987). So, at higher larval densities, mainland females, but not males, tend to emerge smaller than Baja females, even though mainland adults are genetically larger (Etges, 1989). If cactus-induced size variation determines patterns of mate choice, then some premating isolation should have been evident in the last series of within-population mating tests because flies from either population grown on agria were larger than those grown on organ pipe (Fig. 1, Table 2). No cactus-induced premating isolation was evident (Table 1B). Brazner (1983) did not measure thorax sizes of flies he used to study the effects of body size on premating isolation so comparisons with the present study are not possible.

Thus, some other influence of the variation among cacti and lab food during preadult stages affected patterns of mate choice. Since all adults used were aged in vials containing banana food for up to two weeks, it is unlikely that adults could detect the scent of the type of substrate an individual of the opposite sex emerged from. Since mating behavior in the lab is essentially the same as that of wild flies (Krebs and Bean, 1991), variation among rearing substrates is likely to have influenced the composition of CHCs. Wild flies reared from organ pipe cactus contain less total epicuticular hydrocarbon, including differences in the kinds of alkenes and alkadienes as compared to laboratory reared flies (Toolson et al., 1990). Males and females exhibit dimorphism for the ratio of C_{35} and C_{37} alkadienes, and Sonora males differ from Sonora females and Baja adults in diene profiles that influence mate choice (Markow and Toolson, 1990).

If the type of cactus used for breeding determines CHC mediated patterns of mate choice and mating speed, then the results of this study suggest that premating isolation between Baja and mainland populations *in nature* may be far different than that revealed by past studies with laboratory medium-reared flies. Further investigation of the role of cactus substrates on adult epicuticular hydrocarbons as contact pheromones will clearly provide increased resolution of the causes of incipient speciation in this species.

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Corresponding Editor: J. Ringo