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SPECIES HYBRIDS IN THE LABORATORY BUT NOT IN NATURE: A REANALYSIS OF PREMATING ISOLATION BETWEEN *DROSOPHILA ARIZONAE* AND *D. MOJAVENSIS*

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Understanding speciation relies critically on the identification of mechanisms responsible for maintaining species integrity (i.e., reproductive isolation) especially when closely related species are sympatric in nature. Studies of reproductive isolation in *Drosophila* often involve laboratory mating experiments that assume that patterns of mate choice in the laboratory are similar to those in the wild. Two sibling species, *Drosophila arizonae* and *D. mojavensis*, known to exhibit low levels of interspecific hybridization in the laboratory, but not in nature, were used in multiple-choice mating trials using various mating chamber designs as well as synthetic and natural media for developing larvae and courting adults. Sympatric populations were more sexually isolated than allopatric ones, consistent with past studies, and all experimental variables tested (chamber size, host plant presence and rearing substrates) had significant effects on levels of premating isolation between these species. Flies reared on cactus showed increased premating isolation versus those reared on synthetic laboratory food as did providing fermenting host plant tissue during mating trials. Also, surprisingly, smaller mating chambers led to an increase in premating isolation versus larger containers. The design of these types of mating trials is thus critical to understanding how mating behaviors in the laboratory are related to those in natural populations.

KEY WORDS: **Experimental design, mate choice, sexual isolation, speciation.**

The allopatric, or geographic, model of speciation (Mayr 1963) is the most basic and well-understood model concerning the origin of species. Genetic divergence between geographically separated populations occurs through adaptation to different environmental factors (i.e., by natural selection) and through the process of genetic drift. By diminishing gene flow, genetic divergence can lead to increased pre- and/or postzygotic isolation until speciation is complete. A reduction of gene flow via behavioral isolation is likely one of the most important causes for reproductive isolation in animals (Coyne and Orr 2004), particularly when closely related species exist in sympatry. Consistent with the biological species concept (Dobzhansky 1935; Mayr 1942) the extent to which hybridization occurs between populations is an important factor in determining species status (Cracraft 1992) and thus for understanding the speciation process in general and the origin of biological diversity.

Determining the causes for reproductive isolation between closely related species or diverging intraspecific populations in nature remains a challenge in speciation research. In many *Drosophila* species, this is partly due to a lack of knowledge of the

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natural biology and ecology of the species or species pairs under investigation, knowledge which has been labeled "depauperate" by Coyne et al. (2005). *Drosophila mojavensis* and *D. arizonae*, two desert-adapted sibling species, have been studied extensively and their host use and geographic distributions in nature are well documented (Fellows and Heed 1972; Heed 1978), making them a suitable system for understanding the forces that influence sexual isolation between closely related species. *Drosophila mojavensis* is considered to be in the initial stages of divergence, as there is significant reproductive isolation between populations (Zouros and D'Entremont 1980; Etges 1992).

In *Drosophila* spp., the degree to which species or intraspecific populations are reproductively isolated from one another is often investigated by carrying out mate choice experiments in the laboratory. However, as Spieth and Ringo (1983) have noted, the "normal rearing techniques and protocols used [in the laboratory] perturb the normal ontogeny of the flies." They state that "in the absence of prior knowledge about the effects of experimental design on mating behavior, the best design is the one that imitates nature most closely" (Spieth and Ringo 1983). Understanding how laboratory conditions affect mating behavior may help to elucidate mechanisms responsible for maintaining reproductive isolation between nascent species in nature (Noor and Ortiz-Barrientos 2006). For example, rearing techniques and mating chamber designs may cause changes in fly mating behavior that could affect measurements of sexual isolation, sexual selection, and mating propensity. If realistic estimates of these parameters are to be obtained, the effects of such conditions need to be disentangled.

Drosophila mojavensis and *D. arizonae* are two cactophilic members of the *mulleri* complex of the *D. repleta* group and show strong, yet incomplete, pre- and postzygotic isolation in the laboratory (Wasserman and Koepfer 1977; Reed and Markow 2004). Hybrids have not been observed in nature (Ruiz et al. 1990; Etges et al. 1999; Counterman and Noor 2006; Machado et al. 2007) suggesting that particular conditions in the laboratory cause interspecific hybridization. Both species complete their life cycle in the necrotic tissues of various cactus species and are endemic to the arid lands of the southwestern United States and Mexico. *Drosophila arizonae* is widespread with a range that extends from southern New Mexico and Arizona to Guatemala. Its range overlaps with that of *D. mojavensis* in Sonora and northern Sinaloa on the Mexican mainland and parts of southern Arizona. It has also been collected occasionally in low numbers in Baja California. *Drosophila mojavensis* is found in southern Arizona, Baja California, northwestern mainland Mexico, and southern California, including a population on Santa Catalina Island near Los Angeles. Cytological evidence suggests that *D. mojavensis* originated in Baja California and was derived from an ancestral population of a *D. arizonae*-like ancestor on the mainland (Ruiz et al. 1990). These derived mainland populations of *D. mojavensis*, therefore, subsequently colonized southern California, northwestern Mexico, and Arizona from Baja California by switching host plants. The estimated genetic distance ($D \pm 1$ SD) between species based on allozyme variation was 0.212 ± 0.121 (Zouros 1973) with estimated dates of divergence ranging from 0.15 million years ago (mya) based on mtDNA data (Park 1989) to 6 mya based on a combination of ADH and mtDNA sequence data (Pitnick et al. 1995). More recent estimates put the date of divergence at 2.4 ± 0.7 mya (Matzkin and Eanes 2003).

In the northern part of its range, *D. arizonae* uses various columnar and *Opuntia* cactus species (Fellows and Heed 1972; Heed 1978). It has also been reared from rotting citrus, indicating its tendency to be a commensal with humans (Reed et al. 2007). *Drosophila. mojavensis* is more of a specialist than *D. arizonae* and host plant shifts are well documented (Fellows and Heed 1972; Heed and Mangan 1986; Ruiz and Heed 1988). For instance, *D. mojavensis* populations in Southern California and northwestern Arizona use barrel cactus, *Ferocactus cylindraceous*, whereas populations in southern Arizona, Sonora, and Sinaloa use organ pipe cactus, *Stenocereus thurberi*, as a principle host (Fig. 1). However, in Baja California, where organ pipe is also present, agria, *S. gummosus*, is used, as is the case in the Desemboque and Punta Onah regions of coastal Sonora. The ability to shift host plants is thought to have allowed *D. mojavensis* to expand its range across the deserts in this region. Populations of *D. mojavensis* from Baja California, mainland Mexico, and southern Arizona show low, but significant levels of sexual isolation; thus, *D. mojavensis* is thought to be diverging into at least two incipient species (Zouros and D'Entremont 1980; Etges 1992; Etges and Ahrens 2001). The degree to which *D. arizonae* and *D. mojavensis* are sympatric has been inferred from field collections from Sonora and Sinaloa in which both species were reared from the same agria and cina, *S. alamosensis*, cactus rots (Markow et al. 1983; Ruiz and Heed 1988; W. J. Etges, unpubl. data).

Wasserman and Koepfer (1977) provided the first evidence for reproductive character displacement in this system. Sexual isolation was estimated using the Joint I index and its standard error (Stalker 1942; Malagolowkin-Cohen et al. 1965) where $I = 0.926 \pm 0.019$ in sympatric $\times$ sympatric crosses and $I = 0.497 \pm 0.040$ in allopatric $\times$ allopatric crosses. Here, $I = 0$ represents random mating and $I = 1$ represents complete isolation. Thus, sympatric populations were more sexually isolated than allopatric ones, an observation supported by subsequent studies (Zouros and D'Entremont 1980; Massie and Markow 2005). Zouros and D'Entremont's (1980) study revealed a higher degree of sexual isolation in mating trials between strains of mainland and peninsular *D. mojavensis* than in crosses between strains from either within-mainland or within-peninsular populations and implicated the presence of *D. arizonae* on the mainland as the

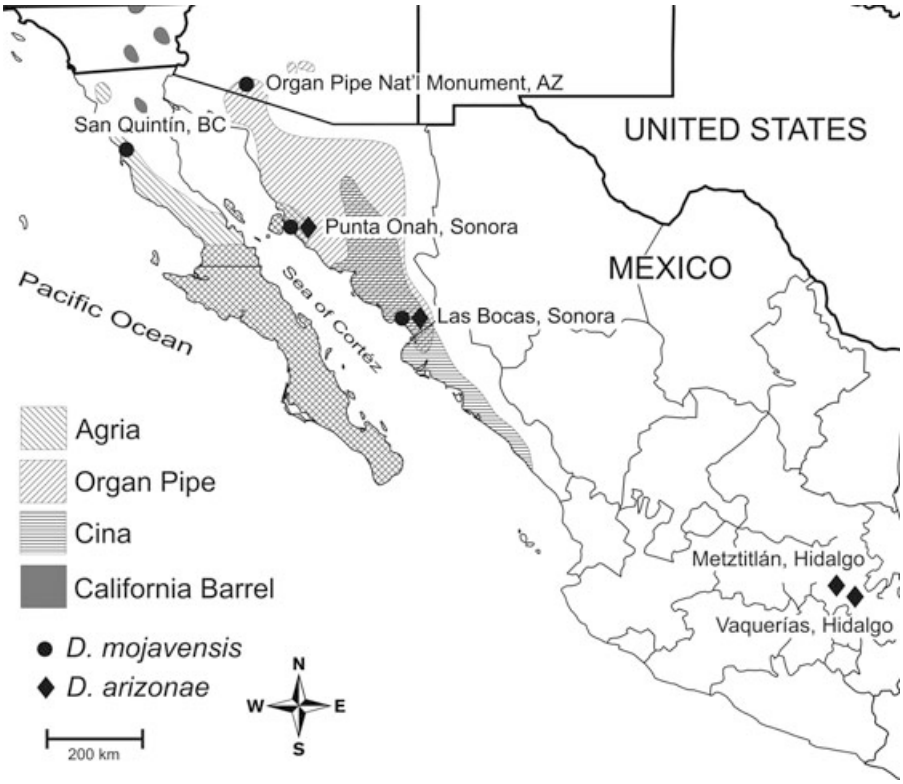

Figure 1. **Locations of the populations used in this study and the geographical distributions of four associated host cactus species.**

cause for reproductive character displacement that had caused increased levels of mate discrimination in mainland populations of *D. mojavensis*.

Although no evidence for hybridization between *D. mojavensis* and *D. arizonae* has been found in nature (Etges et al. 1999; Counterman and Noor 2006; Machado et al. 2007), hybrids can be formed in the laboratory, with incomplete postzygotic isolation. Population cage studies of hybrid swarms using *D. arizonae* and Mojave Desert populations of *D. mojavensis* revealed insights into chromosome replacement and heterosis (Mettler 1957; Nagle and Mettler 1969). However, when sympatric populations of *D. mojavensis* from mainland Sonora were used in population cage experiments, very little hybridization was observed and *D. mojavensis* usually replaced *D. arizonae* (Nagle 1965; Mettler and Nagle 1966). Furthermore, hybrid males with *D. arizonae* mothers are sterile whereas those with *D. mojavensis* mothers differ in sterility depending on the origin of the *D. mojavensis* population used in the cross (Ruiz et al. 1990; Reed and Markow 2004). Although we cannot rule out the possibility of complete extrinsic postzygotic isolation where both species are sympatric (e.g., hybrids may not survive to reproduce in cactus rots in high desert temperatures), the focus of the present study is on premating isolation.

Although the ecology and distribution of these species are well characterized, the degree to which they interact in nature has not been well studied. Krebs and Bean (1991) observed the behavior of *D. mojavensis* on natural cactus rots in mainland Sonora, characterizing the spatial distribution of flies in and around individual cactus rots, and concluded that "courtship structure of *D. mojavensis* is similar in the field and in the laboratory." However, the specific barriers to gene flow responsible for maintaining complete reproductive isolation between sympatric *D. mojavensis* and *D. arizonae* in nature, where both species feed on and carry out their life cycles in the same individual cactus rots, are still unknown.

In the present study, conditions in the laboratory were varied by experimentally manipulating mating chamber size, larval rearing substrate, and the physical environment within the mating chamber (i.e., presenting courting flies with a simulated host plant and fermenting cactus tissue) to identify whether and how these factors influence premating isolation in laboratory mating trials. Identifying which factors contribute to increased sexual isolation in the laboratory may help to elucidate the reasons why laboratory conditions promote hybridization not observed in the wild.

Materials and Methods

FLIES

Four populations – two sympatric (SYM) and two allopatric (ALLO) – of each species were used (Fig. 1, Table 1), combined in

Table 1. Origins of populations used in this study, dates of collection, numbers of adult flies collected that were used to establish the laboratory stocks, and whether populations are sympatric or allopatric with respect to known species distributions in nature.

Stock number	Locality	Date	Number of founders[1]	Allopatric/ Sympatric
D. mojavensis				
A990	Las Bocas, Sonora	1996	2559[B,C]	Sym
PO03	Punta Onah, Sonora	2003	2006[A,B]	Sym
SQ03	San Quintín, Baja California	2003	544[B]	Allo
OPNM02	Organ Pipe Nat'l Monument, AZ	2002	30[B]	Alllo
D. Arizonae				
A990	Las Bocas, Sonora	1996	7[B]	Sym
PO03	Punta Onah, Sonora	2003	198[A,B]	Sym
A1015	Metztitlán, Hidalgo	1999	44[C]	Allo
A1016	Vaquerías, Hidalgo	1999	41[B]	Allo

[1]Flies were collected by aspirating adults from cactus rots in the field (A), by baiting (B), or counting emerged adults from cactus rots returned to the laboratory (C).

four independent, interspecific types of mating trials (two SYM × SYM and two ALLO × ALLO). Since their collection, all stocks were mass cultured on banana agar food (Brazner and Etges 1993) in 8-dram shell vials at room temperature. Prior to the mating trials, stocks were maintained under moderate larval densities in an incubator under a 14:10 light:dark cycle and 27°C:17°C day:night temperature regime for at least two generations. All flies used in mating experiments were aged until sexually mature under these same light and temperature conditions.

MATING EXPERIMENTS

Mating trials were multiple-choice in design and took place in the afternoon hours (6–12 h after lights on) at approximately 26°C. Within three days of eclosion, flies were separated by sex under CO_2 anesthesia and stored in groups of 35 on laboratory food in shell vials for 10–14 days to ensure sexual maturity. For each trial, 120 sexually mature virgin flies (30 males and 30 females of each species) were lightly CO_2 anesthetized and gently introduced onto the floor of the mating chamber. Observations from hundreds of such multiple-choice trials (e.g., Etges 1992, 1998; Etges and Tripodi 2008) and this study (see Discussion) have shown that light CO_2 anesthetization has little detectable effects on courtship behavior. Cactus-reared flies typically begin courtship immediately after becoming active, sometimes several seconds after being introduced into the mating chamber. The mating chamber was then placed into a 50 × 53 × 57 cm cardboard enclosure, heated and illuminated from above by a single 250 W reflector heat lamp approximately 60 cm from the floor of the stage to reduce the effects of day-to-day variation in ambient temperature and lighting on fly behavior. Temperature was monitored continuously with a digital thermometer and the mating chamber was washed thoroughly after each trial.

Copulating pairs were aspirated out of the mating chamber as they occurred and stored in individual vials for subsequent sex and species identification. To avoid including pseudocopulations in the dataset, copulating pairs were removed only if both flies remained in copulo for at least 10 sec; based on previous observations, pseudocopulations were often accompanied by movement, either by the male attempting to engage in full intromission, or by the female in an attempt to reject the male by kicking with her hind legs. Experiments proceeded until half of all possible matings (30) had occurred because species-specific differences in vigor can lead to increased type I error rates if a more complete sampling of mating pairs is taken (Gilbert and Starmer 1985), or until one hour had elapsed.

Adults of both species were placed on laboratory food colored with one drop of either red or blue food coloring 12–24 h before each trial began (as in Wu et al. 1995) so that species identification could be verified. In rare cases in which color was not visible through the abdomen, the gut was dissected to ensure a proper identification. If color was still not visible after dissection, or if a paired fly escaped before identification, the pair was excluded from the data. Colors for each species were alternated between trials.

Four experimental treatments were used to test for the effects of mating chamber size, larval rearing substrate, and the presence of a simulated host plant on sexual isolation and other mating statistics using multiple-choice mating trials. The effects of geographic origin (allopatry vs. sympatry) were also examined for evidence of character displacement in these species by answering the question—do sympatric strains always exhibit stronger sexual isolation than allopatric ones under varying environmental conditions? Mating trials were carried out five to eight times for each of the four population combinations in each treatment (see

Table 2. Numbers and percentages (in parentheses) of each type of pair mating and corresponding I_{PSI} values for different each population cross and treatment. I_{PSI}, SD, and significance of sexual isolation were obtained by bootstrapping, resampling 10,000 times in JMATING. S or A indicates whether each cross was SYM×SYM (S) or ALLO×ALLO (A). For pair matings, M refers to *D. mojavensis* and A refers to *D. arizonae* with females listed first. *P<0.0001, indicates values significantly different from 0 (i.e., significant sexual isolation).

Cross (S/A)	Treatment	n	MM	MA	AM	AA	I_{PSI} (SD)
A990×A990 (S)	Small LF	8	145 (60.7)	7 (2.9)	1 (0.4)	86 (36.0)	0.936 (0.022)*
	Large LF	8	118 (49.2)	21 (8.8)	5 (2.1)	96 (40.0)	0.798 (0.036)*
	Large CS	8	106 (44.2)	7 (2.9)	2 (0.8)	125 (52.1)	0.927 (0.024)*
	Large LF HP	5	91 (60.7)	4 (2.7)	3 (2.0)	52 (34.7)	0.908 (0.035)*
PO03×PO03 (S)	Small LF	8	133 (55.9)	1 (0.4)	1 (0.4)	103 (43.3)	0.983 (0.012)*
	Large LF	8	151 (62.9)	5 (2.1)	9 (3.8)	75 (31.3)	0.883 (0.031)*
	Large CS	8	146 (60.8)	9 (3.8)	2 (0.8)	83 (34.6)	0.912 (0.026)*
	Large LF HP	5	100 (67.1)	4 (2.7)	3 (2.0)	42 (28.2)	0.906 (0.035)*
OPNM202×A1016 (A)	Small LF	8	130 (54.2)	11 (4.6)	4 (1.7)	95 (39.6)	0.879 (0.030)*
	Large LF	8	138 (57.5)	29 (12.1)	13 (5.4)	60 (25.0)	0.651 (0.051)*
	Large CS	8	138 (57.5)	6 (2.5)	5 (2.1)	91 (37.9)	0.909 (0.027)*
	Large LF HP	5	88 (58.7)	2 (1.3)	3 (2.0)	57 (38.0)	0.934 (0.029)*
SQ03×A1015 (A)	Small LF	8	127 (53.4)	18 (7.6)	14 (5.9)	79 (33.2)	0.730 (0.045)*
	Large LF	8	109 (45.2)	25 (10.4)	37 (15.4)	70 (29.0)	0.485 (0.057)*
	Large CS	8	134 (56.3)	13 (5.5)	20 (8.4)	71 (29.8)	0.721 (0.046)*
	Large LF HP	5	81 (55.5)	14 (9.6)	6 (4.1)	45 (30.8)	0.734 (0.056)*

Table 2), resulting in a total of 116 replicates. The experiments took place from September 2007 to March 2008, with 1–3 trials per day. Below, each treatment is described separately, along with the statistical analyses used to identify treatment effects.

SMALL MATING CHAMBER, LABORATORY FOOD-REARED FLIES, NO HOST PLANT (Small LF)

This treatment was designed to reduce the physical space available to individual flies during courtship, thus increasing the number of interactions with potential mates. Flies were reared on laboratory food and mating trials were carried out in a 20 mL cylindrical glass specimen jar fitted with a perforated latex lid. A small slit was made in the latex to allow for the removal of copulating pairs. The container lay on its side during trials so that flies began each trial on the concave "floor" of the cylinder, after which they were free to move about the chamber. Mating trials were replicated eight times for each independent cross, resulting in 32 total trials for this treatment.

LARGE MATING CHAMBER, LABORATORY FOOD-REARED FLIES, NO HOST PLANT (Large LF)

To greatly increase the amount of physical space available to flies during trials, a clear Plexiglas box (30.5 × 30.5 × 30.5 cm, or 28.4 L) with a fine mesh ceiling and cardboard floor was used. A small opening in the middle of the mesh ceiling allowed for fly removal. Flies were reared on laboratory food and eight mating trials were carried out for each population cross.

LARGE MATING CHAMBER, CACTUS-REARED FLIES, NO HOST PLANT (Large CS)

In this treatment, the large (28.4 L) container was used as described above, however, all flies were reared either on agria or organ pipe cactus tissue instead of laboratory food. Cactus cultures were established using standard techniques (Etges 1992). Seventy-five grams of aquarium gravel was covered with a circular piece of filter paper in a half-pint milk bottle and then autoclaved. Sixty-five grams of cactus tissue, including a small piece of midrib, was then placed on top of the filter paper and sterilized by autoclaving bottles for 10–12 min at approximately 20 psi. After cooling to room temperature, tissues were immediately inoculated using a sterile syringe containing 3 mL of an aqueous solution of cactophilic yeasts and bacteria. Seven species of yeasts, *Pichia mexicana*, *P. cactophila*, *Sporopachyderma cereana, Dipodascus starmeri, Starmera amethionina var. amethionina, Candida valida, and C. sonorensis*, and one species of bacteria, *Pectobacterium cacticida*, were used (Starmer 1982; Fogleman and Starmer 1985). Yeast solutions were prepared by mixing one inoculating loop full of each species into 50 mL of sterile deionized water. Each yeast species was cultured in a Petri dish on YM agar for three days at 37°C so that all cultures were in or near log-phase growth when used.

Eggs were obtained by placing 150 males and 150 females of each population in a 10 × 9 × 9 cm plastic box where females oviposited on sugar/cactus juice agar in a 6 cm diameter Petri dish for 24 h. Eggs were then removed from the agar, rinsed with

sterile deionized water, soaked in 70% ethanol for 10 min, and then rinsed again with sterile deionized water. Eggs were counted and placed in groups of 250 on sterile 1 × 1 cm pieces of filter paper, which were in turn placed on the cactus tissue in the bottles. Four replicates were carried out for each population and cactus type, resulting in 32 total replicates for this treatment.

LARGE MATING CHAMBER, LABORATORY FOOD-REARED FLIES, HOST PLANT PRESENT (Large LF HP)

A cactus model consisting of a 1000 mL pale green plastic bottle, sealed and tapered distally, and laden with fermenting agria tissue, was fixed to the floor of the mating chamber at a 45° angle in an attempt to simulate a natural cactus rot. Cactus tissue was prepared by placing a 12 g disc of thawed agria cactus, approximately 1 cm thick, on top of 75 g of aquarium gravel in a cylindrical 5-cm-diameter plastic cup and inoculated with yeasts and bacteria as described above. Each cup was sealed tightly with a lid and incubated for three days at 37°C. Prior to each trial, a single cup of fermenting cactus tissue was fitted into a hole made near the top of the plastic model so that flies were allowed access not only to the volatiles associated with tissue breakdown, but also to the tissue itself. Five mating trials were carried out for each population cross, resulting in 20 total replicates for this treatment.

STATISTICAL ANALYSIS

For each individual mating experiment, we estimated sexual isolation, sexual selection, and other aspects of mate choice based on the relative numbers of each type of mating pair (MM, MA, AM, or AA; M = *mojavensis*, A = *arizonae*, females always listed first) using JMating software (Rolán-Alvarez and Caballero 2000; Carvajal-Rodriguez and Rolan-Alvarez 2006). The index of sexual isolation, I_{PSI}, ranges from −1 to 1, where 1 is complete sexual isolation, −1 is complete disassortative mating (i.e., all matings are interspecific), and 0 represents random mating. Other indices of sexual selection and isolation were *PSS* (pair sexual selection), *PSI* (pair sexual isolation), and *PTI* (pair total isolation) coefficients for each type of mating pair. Briefly, *PSS* coefficients estimate the contribution of sexual selection for each pair type, *PSI* coefficients are estimates of sexual isolation effects for each pair type, and *PTI* coefficients represent the combined effects of sexual isolation and sexual selection for each pair type ($PTI = PSS \times PSI$). Mating asymmetry, that is, differences in *PSI* for homo- or heterospecific pair matings, was estimated by IA_{PSI}, where $AA/MM = PSI_{11}/PSI_{22}$ and $AM/MA = PSI_{12}/PSI_{21}$. Also, we compared differences in mating success of each species and sex (i.e., M♀, M♂, A♀, and A♂) using the cross-product estimator of sexual selection (W), which estimates mating fitness relative to one species (Rolán Alvarez and Caballero 2000).

Sexual isolation and other response variables were initially compared across treatments and by geography using ANOVA in PROC GLM (SAS Institute 2004) as recommended by Coyne et al. (2005) and Rolán-Alvarez (personal comm.). We used these results to glean patterns of variation in our data, and then performed nonparametric Kruskal–Wallis sign-rank tests using PROC NPAR1WAY (SAS Institute 2004) to assess differences due to mating chamber type, rearing substrates, and allopatric versus sympatric populations for the various estimators of sexual isolation and sexual selection. In all cases, when multiple comparisons were made using single datasets, probability levels were adjusted using sequential step-down Bonferroni correction (Rice 1989).

Results

MATING STATISTICS AND TREATMENT EFFECTS

One hundred and sixteen mating trials (29 per population cross) were carried out in total using the four different treatment designs. Of these trials, 22 (≈19%) yielded complete sexual isolation estimates, i.e., $I_{PSI} = 1$. None of the mating trials in the large container with flies reared on laboratory food (*Large LF*) resulted in complete sexual isolation, thus increasing chamber volume decreased levels of assortative mating in these two species.

Total numbers and percentages of each type of pair mating and corresponding I_{PSI} values for each population cross and treatment are presented in Table 2. Sexual isolation was significant ($P < 0.05$; 10,000 bootstraps) in all mating trials except two, both of which were ALLO × ALLO crosses in the *Large LF* treatment (Table S1). Sexual isolation (I_{PSI}) was significantly affected by container size, rearing substrate, and the presence of the simulated host plant; the effects of each treatment are described in the following sections. All results from individual mating trials are available in Table S1.

Overall sexual isolation (I_{PSI}), mating asymmetries for homo- and heterospecific mating (IA_{PSI}), and relative sexual fitness estimators (W) for each treatment are summarized in Table 3. IA_{PSI} *AA/MM*, or the relative mating success of *D. arizonae* pairs versus *D. mojavensis* pairs was significantly less than 1 (bootstrapped $P < 0.0001$) in all mating chamber designs due to the greater number of homospecific *D. mojavensis* matings throughout the experiment. For heterospecific pairings, IA_{PSI} *MA/AM* was significantly greater than unity only in the small container with flies reared on laboratory food (bootstrapped $P = 0.0238$) even though all IA_{PSI} *MA/AM* estimates were greater than 1 suggesting that the low numbers of heterospecific pairings across treatments decreased power of this test. Neither asymmetry index differed across treatments. The cross-product fitness estimators, W♂ and W♀, were significantly higher for *D. mojavensis* than for *D. arizonae* males and females (bootstrapped $P < 0.0001$) reinforcing

Table 3. Mating statistics for each treatment based on total numbers of pair matings pooled across populations and bootstrapped 10,000 times in JMATING (SD in parentheses). Significance of I_{PSI} indicates that values are significantly different than 0. Significance of asymmetry (IA_{PSI}) and cross-product estimators (W) indicates that values are significantly different than 1.

Treatment	I_{PSI}	IA_{PSI} AA/MM	IA_{PSI} MA/AM	*Wmoj*♀	*Warz*♀	*Wmoj*♂	*Warz*♂
Small LF	0.88 (0.015)*	0.71 (0.043)*	1.82 (0.496)*	1	0.67*	1	0.72*
Large LF	0.70 (0.024)*	0.69 (0.035)*	1.18 (0.140)	1	0.61*	1	0.66*
Large CS	0.87 (0.016)*	0.74 (0.044)*	1.21 (0.282)	1	0.72*	1	0.73*
Large LF HP	0.87 (0.020)*	0.59 (0.045)*	1.59 (0.531)	1	0.55*	1	0.59*

*$P<0.05$.

the differences in IA_{PSI} *AA/MM* (Table 3). However, W♂ and W♀ estimates did not differ across treatments. Thus, mating success of *D. mojavensis* populations was higher overall than that of *D. arizonae* in this study. *PSS, PSI,* and *PTI* coefficients for each pair mating in each treatment are presented in Table 4.

EFFECTS OF CONTAINER SIZE

Interestingly, decreasing the physical space available to flies resulted in significant increases in sexual isolation. More interspecific matings occurred in the large container ($n = 144$) than in the small container ($n = 57$) resulting in a significant difference in I_{PSI} between treatments ($\chi^2 = 15.34$, $P < 0.0001$, $N = 64$). Thus, the amount of physical space in the mating chamber influenced premating isolation in these species in multiple-choice situations, with increased fly density resulting in a decrease in interspecific copulations; mean $I_{PSI} \pm 1$ SD was 0.88 ± 0.12 in the small container and 0.71 ± 0.20 in the large container. Both types of heterospecific matings (MA and AM) increased for each population in the large container, leading to significant differences in *PSI* for three-fourths of the pair types, that is., PSI_{MM} ($\chi^2 = 10.27$, $P = 0.0014$, $N = 64$), PSI_{MA} ($\chi^2 = 12.24$, $P = 0.0005$, $N = 64$), and PSI_{AM} ($\chi^2 = 17.66$, $P < 0.0001$, $N = 64$); PSI_{AA} did not differ significantly between treatments. *PSS* did not differ between treatments in this or any other comparison, so the significant differences in PTI_{MA} ($\chi^2 = 11.34$, $P = 0.0008$, $N = 64$), PTI_{AM} ($\chi^2 = 13.15$, $P = 0.0003$, $N = 64$), and PTI_{AA} ($\chi^2 = 6.58$, $P = 0.0103$, $N = 64$) were influenced more by *PSI* than *PSS* as $PTI = PSS \times PSI$ (Rolán-Alvarez and Caballero 2000).

EFFECTS OF LARVAL REARING SUBSTRATES

Rearing flies on fermenting cactus also increased sexual isolation. More heterospecific matings were observed when flies were reared on laboratory food ($n = 144$) when compared to cactus-reared flies ($n = 64$), resulting in a significant difference in I_{PSI} between treatments ($\chi^2 = 13.01$, $P = 0.0003$, $N = 64$). Mean $I_{PSI} \pm$ SD was 0.87 ± 0.19 for cactus reared flies and 0.71 ± 0.20 for flies reared on laboratory food, indicating the sensitivity of mate-choice to differences in larval diet. For all estimates of sexual isolation and sexual selection, there were no differences between flies reared on agria and those reared on organ pipe cactus, so the data were pooled for analysis. Similar to the effects of container size, PSI_{MM} ($\chi^2 = 8.62$, $P = 0.0033$, $N = 64$), PSI_{MA} ($\chi^2 = 12.9$, $P = 0.0005$, $N = 64$), and PSI_{AM} ($\chi^2 = 11.45$, $P = 0.0007$, $N = 64$) differed significantly between laboratory food and cactus-reared flies, suggesting that numbers of mating pairs involving *D. mojavensis* were influenced by larval rearing substrates, but not *D. arizonae*, as PSI_{AA} did not differ between treatments. However, differences in PTI_{MA} ($\chi^2 = 11.36$, $P = 0.0008$, $N = 64$), PTI_{AM} ($\chi^2 = 9.71$, $P = 0.0018$, $N = 64$), and PTI_{AA} ($\chi^2 = 6.04$, $P = 0.0140$, $N = 64$) due to rearing substrates suggest some influence of sexual selection on PTI_{AA}, as PSI_{AA} did not differ across rearing substrates. Thus, increased sexual isolation between species due to cactus rearing substrates was influenced by the numbers of homospecific *D. arizonae* pairings and some small contribution from sexual selection.

EFFECTS OF SIMULATED HOST PLANT

Sexual isolation was significantly greater in mating trials in which flies were provided with a simulated host plant exposing them to fermenting agria tissue. Here, the percentage of heterospecific matings was lower than in the container in which the cactus model and tissue were absent (6.6% vs. 15.0%, respectively), resulting in a significant difference in I_{PSI} between treatments ($\chi^2 = 9.32$, $P = 0.0023$, $N = 52$); mean $I_{PSI} \pm 1$ SD was 0.71 ± 0.20 when the host plant was absent and 0.88 ± 0.10 when the host plant was present. This was again driven in part by differences in *PSI* rather than *PSS* (results not shown).

EFFECTS OF GEOGRAPHIC ORIGIN

In three of the four treatments, crosses between sympatric strains resulted in stronger sexual isolation than crosses between allopatric strains (Table 5). I_{PSI} differed significantly between sympatric and allopatric crosses in the *Small LF*, *Large LF,* and *Large CS* treatments, but not in the large container with the simulated host plant present (*Large LF HP*), perhaps due to a smaller sample size in this treatment. With the data pooled across all treatments, the difference between allopatric and sympatric crosses remained highly significant ($\chi^2 = 27.5$, $P < 0.0001$, $N = 116$). Thus, even

Table 4. ***PSS, PSI,*** **and** ***PTI*** **estimates, SD, and** ***P*****-values for each pair mating in each treatment based on total numbers, pooled across cross types, bootstrapped 10,000 times. Numbers in bold (*N*) represent total numbers of pair matings observed.**

		MOJ♂			ARZ♂		
		PSS	PSI	PTI	PSS	PSI	PTI
Treatment: Small LF							
MOJ♀	N		**535**			**37**	
	EST	1.611	1.393	2.24	0.155	1.003	0.155
	SD	0.085	0.061	0.065	0.026	0.061	0.025
	P	0.000	0.000	0.000	0.000	0.95	0.000
ARZ♀	N		**20**			**363**	
	EST	0.09	0.932	0.084	2.277	0.672	1.521
	SD	0.021	0.055	0.019	0.192	0.048	0.063
	P	0.000	0.22	0.000	0.000	0.000	0.000
Treatment: Large LF							
MOJ♀	N		**516**			**80**	
	EST	1.436	1.499	2.148	0.340	0.983	0.333
	SD	0.075	0.063	0.065	0.042	0.056	0.036
	P	0.000	0.000	0.000	0.000	0.745	0.000
ARZ♀	N		**64**			**301**	
	EST	0.291	0.916	0.266	2.094	0.602	1.253
	SD	0.04	0.054	0.032	0.194	0.047	0.06
	P	0.000	0.126	0.000	0.000	0.000	0.000
Treatment: Large CS							
MOJ♀	N		**524**			**35**	
	EST	1.628	1.347	2.188	0.149	0.987	0.146
	SD	0.088	0.061	0.064	0.026	0.056	0.024
	P	0.000	0.000	0.000	0.000	0.812	0.000
ARZ♀	N		**29**			**370**	
	EST	0.127	0.961	0.121	2.203	0.705	1.545
	SD	0.024	0.056	0.022	0.184	0.05	0.063
	P	0.000	0.48	0.000	0.000	0.000	0.000
Treatment: Large LF HP							
MOJ♀	**N**		**360**			**24**	
	EST	1.49	1.628	2.419	0.171	0.955	0.162
	SD	0.09	0.081	0.081	0.037	0.07	0.032
	P	0.000	0.000	0.000	0.000	0.519	0.000
ARZ♀	**N**		**15**			**196**	
	EST	0.114	0.893	0.101	2.541	0.525	1.318
	SD	0.03	0.069	0.026	0.312	0.055	0.077
	P	0.000	0.132	0.000	0.000	0.000	0.000

though some of these stocks had been maintained in the laboratory for several years, greater sexual isolation was still evident among sympatric populations of these species, consistent with the reproductive character displacement hypothesis (Wasserman and Koepfer 1977). Estimates of *PSI* and *PTI* coefficients differed significantly by geography in various treatments, whereas *PSS* coefficients did not (Table 6), reinforcing the view that the contribution of sexual selection to differences in sexual isolation between sympatric and allopatric populations of *D. mojavensis* and *D. arizonae* was weak to nonsignificant.

Table 5. Mean I_{PSI}, SD (in parentheses), and results of Kruskal–Wallis comparisons between allopatric × allopatric (ALLO) crosses and sympatric × sympatric (SYM) crosses in each treatment. See text for treatment definitions.

Treatment	SYM	ALLO	χ^2	*N*	*P*
Small LF	0.96 (0.05)	0.81 (0.11)	16.60	32	<0.0001*
Large LF	0.85 (0.09)	0.57 (0.18)	14.85	32	<0.0001*
Large CS	0.92 (0.07)	0.82 (0.14)	4.32	32	0.0376*
Large LF HP	0.91 (0.07)	0.84 (0.12)	2.22	20	0.136
Pooled	0.91 (0.08)	0.75 (0.18)	27.50	116	<0.0001*

*Significant after Bonferonni correction.

Discussion

Laboratory estimates of sexual isolation between *D. arizonae* and *D. mojavensis* were sensitive to larval rearing substrates, mating chamber size, and the presence of a simulated host plant during mating trials. Decreasing chamber size, rearing flies from cactus instead of synthetic laboratory food, and providing a simulated host plant all increased premating isolation between species, but none of these treatments completely eliminated interspecific mating despite that 22 of 116 total mating trials resulted in complete sexual isolation, that is, $I_{PSI} = 1$. Also, sympatric populations exhibited stronger sexual isolation than allopatric ones, consistent with the reproductive character displacement hypothesis (Wasserman and Koepfer 1977).

Reproductive isolation between *D. arizonae* and *D. mojavensis* has been investigated numerous times (Baker 1947; Patterson 1947; Markow 1981; Markow and Hocutt 1998). Increased sexual isolation between sympatric populations has been observed repeatedly using flies reared on synthetic laboratory food and has been interpreted as a result of reproductive character displacement (Wasserman and Koepfer 1977; Zouros and D'Entremont 1980; Massie and Markow 2005). In this study, although rearing flies on fermenting cactus tissue increased sexual isolation between species, the differences in sexual isolation between sympatric and allopatric populations were reduced where fermenting cactus tissue was used, either as larval rearing substrate or as a simulated host during mating trials (Table 5). Sexual isolation was higher between sympatric populations of *D. arizonae* and *D. mojavensis* than between allopatric populations when experiments were carried out in an empty mating chamber with flies reared on laboratory food (*Small LF* and *Large LF*, both $P < 0.0001$) but this difference decreased when cactus tissues were used, particularly in the *Large LF HP* treatment in which a simulated host plant

Table 6. Probabilities associated with Kruskal–Wallis tests for differences in pair sexual isolation, *PSI,* and pair total isolation, *PTI,* between sympatric and allopatric crosses in each of the mating chamber designs. M refers to *D. mojavensis* and A refers to *D. arizonae* with females listed first. See text for details.

Mating chamber design	Pair combination							
	MM		MA		AM		AA	
	PSI	PTI	PSI	PTI	PSI	PTI	PSI	PTI
Small LF	ns	ns	0.0003	0.007	0.0008	0.0006	ns	ns
Large LF	0.005	ns	0.0005	0.0059	0.0001	0.0001	0.0224	0.0047
Large CS	0.0157	ns	ns	ns	ns	ns	ns	ns
Large LF HP	ns	ns	ns	ns	0.0026	0.0014	ns	0.0136

with fermenting tissues was present. Here, premating isolation was not significantly different between allopatric and sympatric populations ($\chi^2 = 2.22$, $P = 0.136$, $N = 20$; Table 5).

Reduction in sexual isolation and time to copulation due to cactus rearing substrates was first discovered by Brazner (1983) in crosses between populations of *Drosophila mojavensis*. Flies reared on either agria or organ pipe cactus tissue had a fourfold decrease in copulation latency (or time to copulation) when compared to flies reared on synthetic laboratory media. Subsequent investigations showed that premating isolation between populations was increased to significant levels when flies were reared on laboratory food versus cactus (Etges 1992, and see Table 1 in Brazner and Etges 1993) and that agria cactus reduced premating isolation between *D. mojavensis* and *D. arizonae* (W. J. Etges, unpubl. manuscript). Substrate type has also been shown to affect the composition of epicuticular hydrocarbons in *D. mojavensis* and *D. arizonae* (Stennett and Etges 1997) that serve as contact pheromones, mediating sexual isolation between populations (Etges and Ahrens 2001; Etges and Tripodi 2008; Etges et al. 2009).

In the present study, flies reared on cactus tissue were noticeably more active than flies reared on laboratory food, consistent with decreased time to copulation in cactus-reared flies (Brazner and Etges 1993). When laboratory food-reared flies recovered from light CO_2 anesthesia, they sometimes remained motionless for several minutes whereas flies reared on cactus tissues immediately began walking, flying, and/or initiating courtship, remaining more active throughout the duration of the trial. When the simulated host plant was provided during mating trials in the large container (*Large LF HP*), flies were often observed directly on the fermenting cactus tissue. Females generally arrived first, where males would subsequently arrive and attempt to initiate courtship with a feeding female. Up to nine females were observed on the simulated rot before a single male arrived. When males did arrive, they rarely fed on the cactus tissue and courtship usually began immediately. There were no noticeable differences in activity, physical distribution, or mating behavior of flies in sympatric crosses versus those in allopatric ones. Clearly, carrying out mating trials in the small container, with cactus reared flies and a simulated host plant (i.e., "*Small CS HP*") would have likely provided interesting results, and a full-factorial design with all substrates and container sizes included should be performed.

Coyne et al. (2005) noted that "space itself. . .appears to be an unimportant factor in sexual isolation," yet the present study demonstrates its importance in the *D. mojavensis*/*D. arizonae* system. We initially predicted that providing flies with more physical space would increase premating isolation, and that confining large numbers of flies in close quarters would result in more interspecific mating, due perhaps to interference of male mating signals (e.g., courtship songs or epicuticular hydrocarbons), increased interaction of individuals with flies of a different species, or simply a lack of space for females to escape undesirable males. However, crowding flies in small chambers actually increased sexual isolation.

Our interpretation of this finding is that the small chamber increased the possibility for females to choose between con- and heterospecific males, and that this increase in the element of choice caused the observed increase in premating isolation. In the small (20 mL) container, most females were courted by multiple males simultaneously, if not sequentially, thus increasing the frequency of interaction among potential mates of both species. In the large container, however, flies explored the floor of the chamber at the beginning of each trial and were in fairly close proximity, but their spatial distribution changed with time. Females often walked up the container walls where they stopped and remained motionless, sometimes for the entire duration of the experiment. Males appeared to roam about the container until a lone female was encountered. Therefore, many of the females in the large container were courted by only a single male and many of the males courted only a single female, a situation more closely resembling a no-choice situation.

This result corroborates the mate choice results with *D. yakuba* and *D. santomea;* Coyne et al. (2005) found that multiple-choice mating experiments yielded significantly higher estimates

of sexual isolation (I_{PSI}) than no-choice, male-choice, or female-choice experiments. Hoikkala and Aspi (1993) provided similar evidence using a different experimental design. In their study, providing females with the ability to choose between two males of differential fitness, due to wing manipulation, significantly increased the mating success of the fittest male (thus reducing the mating success of males with decreased fitness). In the three species used in their study (*D. littoralis, D. montana,* and *D. ezoana*), discrimination between conspecific normal and wing manipulated males by females increased when both males were present, as opposed to no-choice situations, and was strongest when the females were courted by both types of male during the trial rather than just one of them (see Fig. 3 in Hoikkala and Aspi 1993).

Another possible explanation for our inability to completely eliminate interspecific copulations may be that all flies used in this study were virgins, separated by sex shortly after eclosion, and allowed to mature in vials without any interaction with the other sex or species. Early sexual experience has been shown to influence sexual isolation (Mayr and Dobzhansky 1945; Spieth 1958; LeMoli and Mainardi 1972; O'Hara et al. 1976) and more recently, Dukas (2008) provided evidence for the adaptive role of learning in the context of mating behavior. Male *D. persimilis* that were allowed to court and experience rejection by female *D. pseudoobscura* exhibited stronger sexual isolation in subsequent mating trials, mating less frequently with heterospecific females than inexperienced males (Dukas 2008). In nature, flies are exposed to both conspecific and heterospecific males and females of various ages, and males court females that are not yet sexually mature (Spieth and Ringo 1983). Theoretical models indicate that this type of learning could be adaptive and may play a role in increasing assortative mating between diverging populations (Lachlan and Servedio 2004; Beltman and Metz 2005; Verzijden et al. 2005), although it is unknown whether similar effects may play a role in the *D. arizonae*/*D. mojavensis* system.

Experimental design of mating experiments can clearly influence the intensity of sexual isolation within and between species. Thus, failure to take into account ecologically relevant aspects of the natural mating environment (rearing substrates, chemical cues, etc.) in the laboratory, may lead to biased measurements of sexual isolation. Determining which factors affect sexual isolation between *D. mojavensis* and *D. arizonae* has yielded valuable information about the possible mechanisms responsible for maintaining reproductive isolation in nature when sexual isolation breaks down under laboratory conditions. Attempts to create a more natural setting in the laboratory, with respect to certain biotic and abiotic factors, may yield more realistic estimates of sexual isolation in natural populations. The combination of these and other factors may render interspecific matings extremely infrequent or even absent in nature. Furthermore, it is still unknown whether hybrid larvae are present in nature and whether they can complete development in cactus tissues. This aspect of extrinsic postzygotic isolation in this system needs further attention. Along with fieldwork aimed at determining the frequency and nature of interspecific courtship and copulation in the field (e.g., Liimatainen and Hoikkala 1998) and hybrid larval viability in natural host plant tissues, more laboratory studies should be carried out to better characterize the effects of early sexual experience on reproductive isolation between these, and other, sibling species.

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Supporting Information

The following supporting information is available for this article:

Table S1. Output results from JMATING software (Carvajal-Rodriguez, and Rolan-Alvarez. 2006. BMC Evol. Biol. 6:40) used in the mating studies of Jennings and Etges, 2009.

Supporting Information may be found in the online version of this article.
(This link will take you to the article abstract).