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GENETICS OF INCIPIENT SPECIATION IN *DROSOPHILA MOJAVENSIS*. I. MALE COURTSHIP SONG, MATING SUCCESS, AND GENOTYPE X ENVIRONMENT INTERACTIONS

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Few studies have examined genotype by environment (GxE) effects on premating reproductive isolation and associated behaviors, even though such effects may be common when speciation is driven by adaptation to different environments. In this study, mating success and courtship song differences among diverging populations of *Drosophila mojavensis* were investigated in a two-environment quantitative trait locus (QTL) analysis. Baja California and mainland Mexico populations of *D. mojavensis* feed and breed on different host cacti, so these host plants were used to culture F_2 males to examine host-specific QTL effects and GxE interactions influencing mating success and courtship songs. Linear selection gradient analysis showed that mainland females mated with males that produced songs with significantly shorter L(long)-IPIs, burst durations, and interburst intervals. Twenty-one microsatellite loci distributed across all five major chromosomes were used to localize effects of mating success, time to copulation, and courtship song components. Male courtship success was influenced by a single detected QTL, the main effect of cactus, and four GxE interactions, whereas time to copulation was influenced by three different QTLs on the fourth chromosome. Multiple-locus restricted maximum likelihood (REML) analysis of courtship song revealed consistent effects linked with the same fourth chromosome markers that influenced time to copulation, a number of GxE interactions, and few possible cases of epistasis. GxE interactions for mate choice and song can maintain genetic variation in populations, but alter outcomes of sexual selection and isolation, so signal evolution and reproductive isolation may be slowed in diverging populations. Understanding the genetics of incipient speciation in *D. mojavensis* clearly depends on cactus-specific expression of traits associated with courtship behavior and sexual isolation.

KEY WORDS: **Cactus, courtship song, desert, *Drosophila mojavensis*, GxE interaction, QTL, sexual isolation, speciation.**

The emergence of behavioral isolation between diverging populations has long been recognized as a first step in the development of reproductive isolation. Dobzhansky (1937) and Muller (1939, 1942) suggested that divergence among populations in allopatry could lead to sexual isolation arising as a pleiotropic by-product of genetic drift or local adaptation. If behavioral divergence results in reduced gene flow, increasing genetic differentiation among populations may help to preserve incipient reproductive isolation, and if selection is strong enough, reproductive isolation can persist in sympatry despite low levels of interpopulational gene flow (Bush 1969; Feder 1998; Gomulkiewicz et al. 1999; Berlocher and Feder 2002). Evidence for directional selection causing evolutionary divergence among populations and species is widespread (Rieseberg et al. 2002), making the pleiotropy hypothesis an attractive mechanism for the evolution of new species.

The kinds of signaling systems that are expected to diverge under these conditions are likely to be lineage dependent, but also may depend on prior ecological and/or morphological adaptations (Streelman and Danley 2003). Although the pleiotropic consequences of adaptive evolution may be sufficient to influence courtship signal differentiation, and thus formation of premating isolating mechanisms, the genetic basis of these courtship signal differences in diverging populations is poorly understood (Ritchie and Phillips 1998; Hall and Kirkpatrick 2006). Within newly isolated demes, local environmental conditions (Endler 1992) and sexual selection can increase rates of signal evolution causing further interdemic sexual isolation (Boughman 2001).

When multiple signaling systems are involved with mate choice, ranking the importance of different types of mating signals may not always be straightforward (Etges 2002; Coyne and Orr 2004; Chenoweth and Blows 2006), but identifying the roles of different signals and partitioning sources of reproductive isolation can be very informative (Coyne and Orr 1989, 1997; Gerhardt 1992; Ramsey et al. 2003). In animals, behavioral isolating mechanisms among diverging populations often include acoustic signaling systems that are thought to evolve rapidly, are often species specific, and are good candidates for genetic analysis. Acoustic signals are common components of species recognition systems (Gerhardt and Huber 2002) and are often influenced by sexual selection (Ryan and Rand 1993; Jia and Greenfield 1997; Hoikkala et al. 1998; Welch et al. 1998; Rantala and Kortet 2003).

Uncovering the genetic architecture of naturally occurring variation within and among species for components of courtship songs involved in sexual selection and species recognition should lead to a clearer understanding of species formation (Shaw and Parsons 2002). Genetic analysis of courtship songs has revealed significant genetic variation within and among species, descriptions of candidate genes, and quantitative trait loci (QTL) influencing different song components (Gleason and Ritchie 2004). Insect songs, particularly in *Drosophila*, evolve rapidly (Gleason and Ritchie 1998), can influence female receptivity, and are involved in species recognition (Ewing and Bennet-Clark 1968; Liimatainen et al. 1992; Tomaru and Oguma 1994; Hoikkala et al. 2005). Species recognition is often based on variation in the interpulse interval (IPI), or the pulse frequency (Ewing and Bennet-Clark 1968; Bennet-Clark and Ewing 1969; Ritchie et al. 1999; Williams et al. 2001). Derived from mutation studies, a number of candidate genes that alter circadian rhythms, ion channels, sex determination pathways, auditory function, flight, locomotion, and body coloration influence expression of courtship songs (Gleason and Ritchie 2004; Gleason 2005). Quantitative genetic analysis has revealed numbers and locations of chromosomal regions influencing song variation within and between species, as well as the nature of genetic variability, gene additivity, dominance and epistasis (Shaw 1996; Gray and Cade 1999; Ritchie 2000; Henry et al. 2002). Locations of QTLs and candidate genes influencing song variation in mutational studies show limited overlap in the few species where genomic information is available (Gleason et al. 2002; Gleason and Ritchie 2004) suggesting that there may be many segregating genes that can affect *Drosophila* courtship song. A few of the genes affecting courtship song, such as *doublesex* and *fruitless*, are known to also influence sex determination and male behavior (Billeter et al. 2006), and are excellent candidates for future analysis of courtship songs in different species. Nonetheless, all QTL studies of courtship song in *Drosophila* have yet to address the importance of relevant, natural ecological variation during development and adult stages on expression of courtship songs, although genotype by environment (GxE) interactions is likely to be important for behavioral traits (Cotton et al. 2006).

Here, we take advantage of diverging populations of cactophilic *Drosophila mojavensis* to estimate the numbers and kinds of QTL influencing male courtship songs and mating success in flies reared under natural conditions. Populations of *D. mojavensis* use different host cacti across their range, so we can also directly assess the ecological determinants of courtship song variation and presence and magnitude of host-cactus–influenced GxE interactions. If courtship signals are condition dependent, or are sensitive to the environments in which they are expressed, they may be unreliable indicators of male quality (Greenfield and Rodriguez 2004). Such signal plasticity can be caused by GxE interactions if signaling genotypes are sensitive to significant environmental heterogeneity, and can help to preserve genetic variation in signaling traits in the face of strong sexual section (Danielson-Francois et al. 2006). Evidence for such GxE interactions influencing signal variation is not abundant, but has been shown in crickets (Olvido and Mousseau 1995), calling treefrogs (Welch 2003), waxmoth songs (Jia and Greenfield 1997; Jia et al. 2000; Danielson-Francois et al. 2006), and grain beetle pheromones (Rantala et al. 2003).

Courtship songs from geographically isolated populations of *D. mojavensis* show divergence among populations that use different host cacti (Byrne 1999; Etges et al. 2006). *Drosophila mojavensis* is thought to have originated in isolation in what is now Baja California and then diverged into mainland Sonora, Sinaloa, Arizona, and southern California. During this transition, *D. mojavensis* switched from its ancestral host pitaya agria, *Stenocereus gummosus*, to organ pipe, *S. thurberi*; sina cactus, *S. alamosensis*, in Sonora and Sinaloa; and California barrel cactus, *Ferocactus cylindraceous*, in southern California (Heed and Mangan 1986). Courtship songs of ancestral Baja California populations of *D. mojavensis* are characterized by longer IPIs with shorter, but more variable, burst durations than those of mainland populations. In a quantitative genetic analysis, genes influencing IPI differences were found to be largely additive in effect, with dominance toward shorter IPIs in a mainland population. Burst duration was influenced by autosomal genes and both X and Y chromosomes (Etges et al. 2006) in an antagonistic fashion: additive autosomal effects significantly decreased burst duration while both X and Y chromosomes increased it. A suite of other characters have diverged genetically between Baja and mainland populations, including host-plant–specific shifts in egg to adult viability, development time, and thorax size (Etges and Heed 1987; Etges 1989, 1990, 1993; Etges et al. 1999), host-related physiologies (Starmer et al. 1977; Etges and Klassen 1989), as well as allozyme and inversion frequencies (Zouros 1974; Etges et al. 1999). Thus, adaptive divergence of *D. mojavensis* has been accompanied by evolution of altered courtship songs, suggesting that these traits may not be independent (see Endler 1992).

We used a recently developed genetic map for *D. mojavensis* (Staten et al. 2004) and the assembled genome sequence (Gilbert 2005) to further assess the genetic basis of courtship song differences between Baja California and mainland populations using recombinant F_2 males reared on two host cacti. Multiple components of song were analyzed and used in a selection gradient analysis to determine how strongly different song components were correlated with mating success. We developed single and multilocus restricted maximum likelihood (REML) models to analyze QTL effects and GxE interactions due to host cactus on courtship behavior and song variation. We show that rearing substrates caused significant differences in mating success associated with different aspects of courtship song, and GxE interactions underlying these song traits were common.

Materials and Methods

STRAINS AND CROSSES

A population of *D. mojavensis* was derived from 544 adults collected over banana baits in a hillside population of agria cactus near San Quintin, Baja California Norte, in January 2003, returned to the laboratory, and mass reared on banana food (Brazner and Etges 1993) in 8-dr shell vials at room temperature. Multiple pair-mated lines were established and repeatedly inbred to establish homokaryotypic lines for gene arrangement LP (q^5) on chromosome II and ST on chromosome III. A multi-female stock of *D. mojavensis* from Organ Pipe National Monument, Arizona, collected in 2002 was obtained from T. Markow. This population was chosen because previous surveys revealed that these flies are homozygous LP/LP for chromosome II, and ST/ST for chromosome III (Etges et al. 1999). Multiple homokaryotypic lines were established from each population and cytologically verified: no inversions were segregating. One homokaryotypic line from each population was inbred for another five generations to reduce microsatellite heterozygosity.

A series of mass reciprocal crosses using these lines were then performed over the course of the experiment, and all F_2 flies from each cross were reared on fermenting agria or organ pipe cactus. Cactus cultures were set up in plugged half pint bottles with 75 g of aquarium gravel at the bottom covered with a 5.5-cm diameter piece of filter paper. Bottles were then autoclaved, and after 60 g of either agria or organ pipe tissues were in place, they were autoclaved again for 10 min. After cooling to room temperature, each culture was inoculated with 0.5 mL of a pectolytic bacterium, *Erwinia cacticida* (Alcorn et al. 1991) and 1.0 mL of a mixture of seven yeast species common in natural agria and organ pipe rots (Starmer 1982): *Dipodascus starmeri*, *Candida sonorensis*, *Starmera amethionina*, *Candida valida*, *Pichia cactophila*, *Pichia mexicana*, and *Sporopachydermia cereana*. Eggs were collected from replicate sets of aged F_1 adults for 10 h and washed in deionized water, 70% ethanol, and again in sterile deionized water. Eggs were counted in groups of 200, transferred to a 1 cm^2 piece of sterilized filter paper, and placed on fermenting cactus in an incubator programmed at 27°C during the day and 17°C at night on a 14:10 LD cycle. All unhatched eggs were counted to allow calculation of egg to adult viability. Eclosed adults from each replicate culture were counted daily allowing determination of egg-to-adult development time, separated by sex, and aged until sexually mature (8–10 days at 25°C) on banana food in vials at room temperature.

BEHAVIORAL ANALYSIS

Male mating success was measured in 1-h trials by placing 10 mature F_2 males in a 50-mL conical flask with 10 mature, virgin Organ Pipe National Monument (mainland) females, and each copulating pair was aspirated out. Mainland females reared on lab food were used in these trials because they tend to be more choosy than Baja California females in laboratory mating tests (Zouros and d'Entremont 1980; Brazner and Etges 1993). Time to copulation (min) from the start of each trial to successful intromission was recorded for each pair. In these trials, we attempted to

randomize males across different development times. After these mating tests, each male was placed in a Plexiglas mating chamber ($1.5 \times 1 \times 0.5$ cm) with two virgin mainland females whose wings had been removed so that any female wing vibration would not complicate analysis of courtship songs. Male songs were recorded using an INSECTAVOX electric microphone (Gorczyca and Hall 1987) onto cassette tape. Temperature inside the recording chamber was monitored continuously with a digital thermometer. Additional cactus-reared males not used in the mating tests were recorded to increase sample sizes.

Around five minutes of each song recording was digitized for analysis using a Cambridge Electronic Design (Cambridge, England,); C.E.D. 1401 A/D converter (at 2 kHz after bandpass filtering at around 100 Hz to 1 kHz). All analyses used custom-written scripts in the "Spike2" language (© C.E.D.). Individual pulses were identified and the mean IPIs measured (Fig. 1). The IPIs are bimodal in *D. mojavensis* (Ewing and Miyan 1986; Byrne 1999; Etges et al. 2006) so "long" (L) and "short" (S) IPIs are distinguished here (the mean IPIs were not highly correlated, r^2 between mean L-IPI and S-IPI = 6.7%). L-IPIs were more common than S-IPIs (mean per recording = 918 vs. 205). L-IPIs were arranged into bursts with a clearer burst structure than is typically found in fly song (Fig. 1) and the burst durations and IPIs were also analyzed. Song frequency was not measured as the extremely short pulse length precludes accurate measurement by Fourier techniques, and suggests that this trait is unimportant to females in this species (Byrne 1999). We also analyzed the total number of bursts of song produced during courtship, which is likely related to male vigor, and potentially influenced by host cactus type. Prior to analysis, all parameters were regressed against temperature. L- and S-IPIs significantly covaried with temperature, as expected for fly song (e.g. Shorey 1962; Ritchie et al. 2001), so both were adjusted to an average temperature of 21°C using their regression coefficients, –0.380 and –0.053, respectively. Interburst intervals (IBIs) were log transformed and all song data were standardized (mean = 0, SD = 1) prior to analysis to eliminate any scale effects.

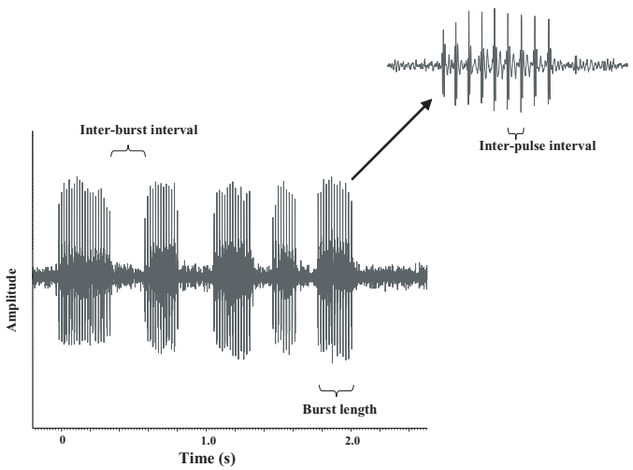

Figure 1. Typical waveform of the courtship song of *Drosophila mojavensis*.

We carried out selection gradient analysis (Lande and Arnold 1983; Brodie et al. 1995) on song traits using copulation success as a measure of fitness to examine how different song components were related to mating success with mainland females. We only considered linear components of selection and calculated univariate (as a measure of total selection on each trait) and multivariate regression coefficients (as a measure of the partial selection pressure on each trait, controlling for other traits). The magnitudes and standard errors of selection coefficients were derived from conventional linear models. Because mating success is binomial, probability values were calculated from binomial logistic generalized linear models (following Fairbairn and Preziosi 1996, appendix 1). For univariate analysis, we first fitted a general linear model with mating success, rearing cactus, and the interaction term as model parameters to test whether the pattern of selection was dependent on host rearing effects.

MARKER SCORING

After song recording, DNA was extracted from each male using a Puregene DNA isolation kit (Gentra Systems, Inc., Minneapolis, MN) and frozen at –80°C. DNA samples were gridded into 96-well format, and genotyped for 21 microsatellite markers. Many additional markers were attempted but were not used because of lack of variability between the two lines crossed. Unexpectedly, even after the intense inbreeding, we still observed extensive allelic variation within the lines, and sometimes there were alleles shared between the two lines for some of the markers used. As a result, we only scored those individuals that had alleles unambiguously derived from a particular parental line. Of the 21 markers used, 16 were described previously (Staten et al. 2004), one was designed on the fifth chromosome (Dmoj5200b) near a previously described microsatellite (Dmoj5200; Staten et al. 2004), and four new markers were designed to be near candidate genes (all < 15 kb away) affecting cuticular hydrocarbon profile or courtship song (Dmoj2_2868a is near *Slowpoke*, Dmoj2_6540c is near *fruitless*, Dmoj2_1603a is near *desat2*, and Dmoj5_1232a is near *croaker*: see Fig. 2).

Polymerase chain reaction (PCR) was performed in 10-μL reactions containing 0.5–1.0 μL of fly DNA preparation, using the following touchdown cycling protocol: 1 min 95°C, 3× (95°C, 30 sec; 56°C, 30 sec; 72°C, 30 sec), 3× (95°C, 30 sec; 53°C, 30 sec; 72°C, 30 sec), 30× (95°C, 30 sec; 50°C, 30 sec; 72°C, 30 sec) (Palumbi 1996). Products were visualized on a polyacrylamide gel using a LiCor DNA analyzer (Li-Cor Biosciences, Lincoln, NE). Genotypes were scored manually and entered into Microsoft Excel.

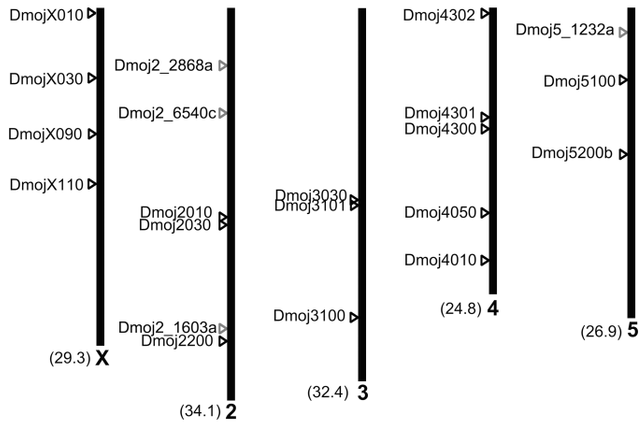

Figure 2. Locations of microsatellite markers in this study using the *D. mojavensis* genome assembly (Gilbert 2005). Physical distances for each chromosome are indicated in parentheses. Gray triangles indicate markers < 15 kb from candidate genes: Dmoj2_2868a is near *slowpoke*, Dmoj2_6540c is near *fruitless*, Dmoj5˙1232a is near *croaker*, and Dmoj2_1603a is near *desat2*. As a definitive transcript for *croaker* is not yet known, we developed a marker within the *D. mojavensis* region bearing similarity to the *D. melanogaster croaker* region (M. Noor, unpubl. results).

GENETIC ANALYSIS

We first assessed linkage among the 21 microsatellite loci (Fig. 2) using MapMaker (Lander et al. 1987). Only two loci were linked (Dmoj2010 and Dmoj2030), separated by 29.1 cM. Thus, interval mapping with QTL Cartographer (Basten et al. 2002) was not possible, so we resorted to single-marker regression and REML analysis (Genstat5-Committee 1993; SAS Institute 2004). Sequential single-marker regressions were performed separately for all traits. Any significant results suggested linkage between the microsatellite marker and an adjacent QTL. It is possible that we could overestimate the number of QTLs due to linkage between markers, but the fact that the markers segregated independently suggests this was not a major problem. Furthermore, multiple regression models were also applied, which would counter this potential problem. Additive models included locus as a covariate, cactus type, and their interaction. Because a series of reciprocal crosses were cultured to produce adequate numbers of adult flies, we also added "reciprocal cross" as a fixed effect in the model: this had no effect on significance levels of locus, cactus, or their interaction for any marker and trait. Because recombination rates in *D. mojavensis* are 2–3× that in *D. melanogaster* (Ortiz-Barrientos et al. 2006) and only two of our microsatellite loci showed evidence of linkage, QTLs revealed here by regression are likely to be independent from one another.

A five-song trait × 21 locus matrix with three significance terms per cell was produced. Strict Bonferroni corrections were not applied to the entire table, but sequential Bonferroni corrections ($P < 0.05$) were used across all loci for traits of interest. Multilocus analyses were performed for each trait and markers were chosen based on results of the single-locus analyses. Three markers (Dmoj4300, Dmoj4302, and Dmoj2_2868) were implicated in multiple traits, so these were always included in the multilocus models. Additional loci were included if either the main locus or GxE interaction terms from the single-marker regressions produced a P value < 0.01. The final fitted model was a REML model with main terms for loci (as covariates), cactus, and locus × cactus and locus × locus interaction terms. From the full model, significance of each coefficient (b) was assessed using a Wald test, based on a Z statistic formed as $Z = \hat{\beta}/SE$. This Z value is then squared, yielding Wald statistics distributed as chi-squared.

There were several missing values for genotypic data in our final data set. If each fly with any missing values was omitted from the analysis, sample sizes were often fairly small (average $n = 165$). We therefore replaced missing genotypic values with the mean for each locus. This is unbiased for the main effects and yielded identical results as the single-marker regression. This was also independent of cactus, and is therefore conservative for finding GxE interaction effects. An additive genetic model for multiple unlinked loci with interaction terms for locus × cactus and between main locus terms resulted. We could not fit a full model with all loci—attempts to do so failed due to computer memory constraints with too many parameters for estimation.

Results

MATING SUCCESS, TIME TO COPULATION, AND SONG VARIATION

A total of 902 F_2 males (average ± SD = 16.9 ± 3.4 days old) were scored for mating success and subsequently genotyped. A total of 332 of 902 (36.8%) successfully mated in these multiple-choice tests with mature, unmated mainland females. Time to copulation among successful males varied greatly (average ± SD = 23.4 ± 13.6 min) and there was no overall effect of host cactus (one-way ANOVA, $F = 2.17$, $P = 0.14$). Univariate selection coefficients associated with each component of courtship song ($n = 443$) incorporated all selection (direct and indirect) on each trait. All except mean S-IPI were subject to selection (Table 1). All selection coefficients were negative except for number of bursts, indicating that mainland female *D. mojavensis* prefer males that produce more songs per unit time. Number of bursts was included as a general indicator of male willingness to sing, and was not significant in the full multivariate model (Table 1). These linear selection gradients were not influenced by cactus rearing substrate. All partial regression coefficients in the multivariate model were also negative indicating that males producing songs with shorter L-IPIs, shorter burst durations, and shorter IBIs experienced significantly greater mating success (Fig. 3).

Table 1. Results of single and multiple regression analyses with mating success (0, 1) as the dependent variable, showing the relationship of courtship song components on mating success in male *Drosophila mojavensis*. See text for details.

Trait	Univariate β (SE)	*P*	Substrate effect*	*P*	Multivariate β (SE)	*P*
L-IPI	−0.064	0.006	0.011	ns	−0.059	0.017
	(0.024)				(0.025)	
S-IPI	−0.014	ns	−0.04	ns	−0.009	ns
	(0.024)				(0.024)	
Burst duration	−0.062	0.003	−0.021	ns	−0.052	0.037
	(0.024)				(0.025)	
Interburst interval	−0.079	<0.001	0.008	ns	−0.088	0.028
	(0.023)				(0.041)	
Number bursts	−0.057	0.015	−0.013	ns	0.002	ns
	(0.024)				(0.064)	
Total						<0.001

*Substrate effect is the difference in the regression coefficients when fitting separate lines for flies from each cactus, that is, between flies reared on organ pipe (arbitrarily first slope) versus agria cactus (second slope). For L-IPI, adding agria (the second cactus in this analysis) increased the regression coefficient by 0.011 over that for OP. For burst duration, it was decreased by 0.021, etc.

QUANTITATIVE TRAIT LOCI

We first examined which loci influenced male mating success and whether females tended to prefer to mate with males reared on the host plant they use in nature. Male mating success was marginally associated with a single second chromosome marker, Dmoj2_1603a, and with four significant GxE interactions (Table 2). For 16 of the 21 marker loci, males reared on organ pipe cactus had higher mating success than males reared on agria in these single-locus tests, but these cactus effects were not significant after sequential Bonferroni correction (although this difference was significant in the multiple-locus REML analysis, see below). Thus, most of the marker regions were weakly influenced by rearing substrates, showing that mainland females tended to mate more often with males reared on the host plant they use in nature—organ pipe cactus. Furthermore, of the four GxE interactions (all significant after Bonferroni correction, Table 2), three resulted from mainland genotypes with higher mating success when reared on organ pipe cactus, and to a lesser extent Baja genotypes with higher mating success when reared on agria (Fig. 4).

Overall, there was little overlap in the genomic regions influencing mating success and each of the song components (Table 2) as only Dmoj2_1603a influenced IBI, and L-IPI variation through a GxE interaction. However, in terms of common QTLs, time to copulation was influenced by two of the same QTLs as courtship song, associated with Dmoj4300 and Dmoj4302, and additionally by Dmoj4050. Main effects of locus for several other QTLs for time to copulation associated with Dmoj2010 and Dmoj2030 were not significant after Bonferroni correction. MM and MB genotypes (M = mainland allele, B = Baja allele) at QTL near Dmoj2_2868, Dmoj4300, and Dmoj4302 were associated with significantly shorter times to copulation than Baja genotypes, but in slightly different ways. For Dmoj4300, MB heterozygotes averaged the shortest times to copulation suggesting some heterozygote advantage (MB < MM < BB; least square means, $P < 0.0002$), and for both Dmoj2_2868 and Dmoj4302, MM homozygotes had the shortest times to copulation (MM < MB, BB; least square means, $P < 0.0001$). Conversely, mainland genotypes for Dmoj4050 were associated with significantly longer times to copulation with mainland females (MM ≫ MB, BB; least square means, $P < 0.0001$). Thus, genotypic differences for four unlinked QTL on the fourth chromosome influenced both time to copulation and different components of courtship songs.

Differences in time to copulation were influenced by rearing substrates at seven different QTL (Table 2, all significant after

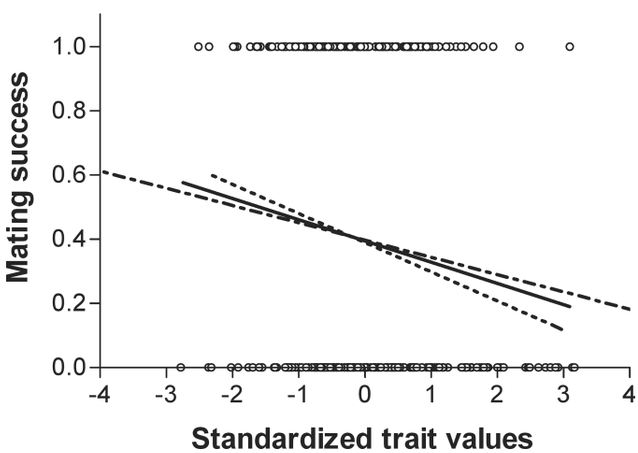

Figure 3. Linear selection gradients associated with male mating success (0, 1) determined by variation in L-IPI (——), IBI (·····), and burst duration (— · —). See Table 1 for significance values.

Table 2. Single-locus ANOVA results for mating success, time to copulation, and courtship song components showing the significance of the main effects of locus (as a covariate), cactus, and locus by cactus (GxE) interactions. Strongly significant effects ($P \leq 0.01$) are indicated in bold.

Trait	Effect	X chromosome				Second chromosome					
		X010	X030	X090	X110	2_2868a	2_6540c	2010	2030	2_1603a	2200
Mating success	Locus-									0.047	
	cactus	**0.010**			0.022	0.023	0.044	0.041	0.003	**0.010**	
	interaction										0.035
Time to copulation	Locus-					0.024		0.011	0.042		
	cactus	0.002	0.024		0.011						
	interaction			0.053			0.03		0.036		0.015
L-IPI	Locus-					0.026					
	cactus			0.039					0.018		**0.001**
	interaction										
S-IPI	Locus-										
	cactus										
	interaction										
Burst duration	Locus-				0.050						
	cactus										
	interaction										
Interburst interval	Locus-	0.014			0.056	**0.003**				0.042	
	cactus										
	interaction			0.050		0.031		**0.010**			
Number of bursts	Locus-	0.035				**0.009**					
	cactus	0.021		0.047	0.029			0.043	0.043		0.064
	interaction			0.020		0.021					

Trait	Effect	Third chromosome				Fourth chromosome				Fifth chromosome		
		3030	3101	3100	4010	4050	4300	4301	4302	5_1232a	5100	5200b
Mating success	Locus-											
	cactus	**0.007**	0.031	0.036	0.021		0.023	**0.005**		0.026	0.044	0.030
	interaction	0.032	0.016						**0.008**			
Time to copulation	Locus-					0.02	**<0.001**		**0.003**			
	cactus		**<0.001**						**0.000**		0.007	
	interaction											0.022
L-IPI	Locus-			0.015							0.027	
	cactus	0.030		0.049			0.030		**0.009**		**0.004**	**0.001**
	interaction	0.032	–									
S-IPI	Locus-							**0.008**				
	cactus											
	interaction		–									
Burst duration	Locus-						0.025				0.027	
	cactus						0.056	0.027				
	interaction		–	0.031								
Interburst interval	Locus-								0.026	0.030		
	cactus											
	interaction		–				0.030					
Number of bursts	Locus-						**0.009**		**0.001**	0.017		0.052
	cactus	0.024		**0.001**			**0.009**			0.054	0.036	
	interaction		–		**0.009**		**0.001**					

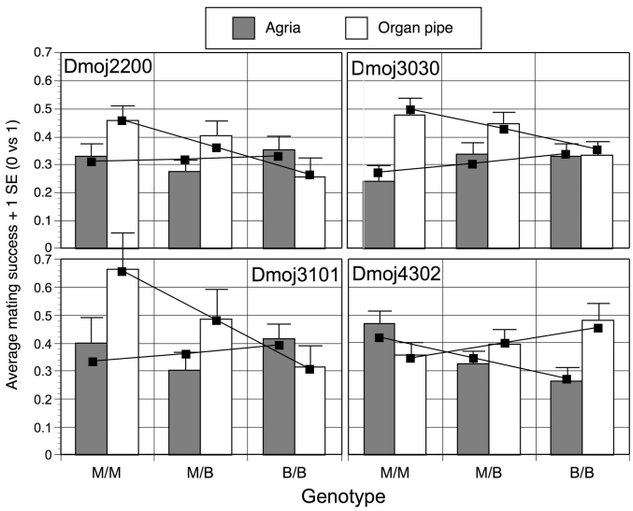

Figure 4. Significant GxE interactions influencing male mating success of F_2 recombinant *D. mojavensis* males with mainland females. Lines connect the best fitted mean additive effects for the three genotypes for both cactus substrates. Sample sizes and significance values are presented in Table 3.

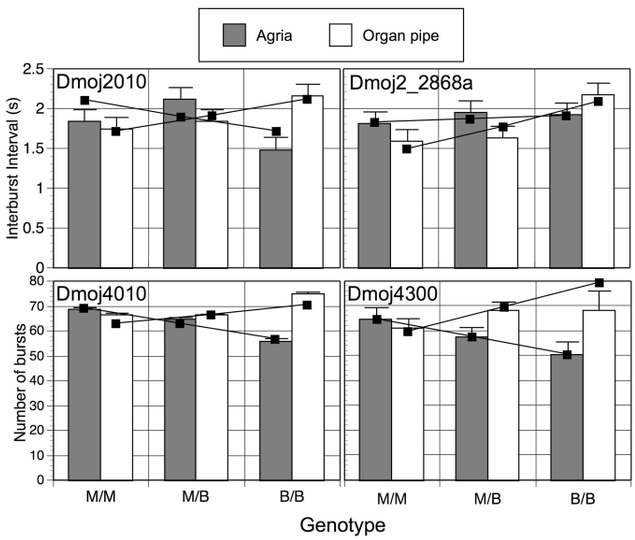

Figure 5. GxE interactions for song traits at four QTL for F_2 male *D. mojavensis* in this study. Lines connect the best fitted mean additive effects for the three genotypes for both cactus substrates.

Bonferroni correction), but only three of these also showed cactus differences in mating success. For all seven QTL, organ-pipe-cactus–reared males took significantly longer to achieve copulations than agria-reared males (results not shown). Therefore, although organ-pipe–reared males took longer to successfully mate with mainland females, their courtship success was ultimately greater than males reared on agria cactus. Thus, for agria-reared *D. mojavensis* males, these seven QTL were associated with shorter times to copulation, and reduced mating success with mainland females.

Most song traits were influenced by about six QTL with significant genotypic effects (Table 2). Main terms of loci were more common (average = 3.7 per trait) than interaction terms, but interaction terms were common (average = 2.6; Fig. 5). A single fourth chromosome QTL near Dmoj4301 influenced S-IPI variation suggesting that the genetic basis of this trait may be influenced by a smaller portion of the genome than other traits. Unlike mating success and time to copulation, the main effect of cactus influenced only L-IPI and number of bursts, but none of the other song traits for these 21 marker regions. Burst duration was influenced by two QTL and one GxE interaction. Inspection of the GxE interactions for IBI revealed consistent effects of cactus that mirrored the relationship of these song traits and mating success (Fig. 5). GxE interactions for Dmoj2010 and Dmoj2_2868a were caused by mainland genotypes that decreased IBI when reared on organ pipe cactus, but agria either had little effect on trait expression (Dmoj2_2868a) or seemed to cause heterotic effects (Dmoj2010). For a QTL associated with fourth chromosome marker Dmoj4010, numbers of song bursts were influenced by a GxE interaction (Table 2). Baja genotypes increased burst numbers in organ-pipe–reared flies and mainland genotypes increased burst numbers in agria-reared flies, but the largest effect was due to organ pipe cactus (Fig. 5). Because more song bursts increased male mating success with mainland females (Fig. 3), cactus-specific Dmoj4010 genotype effects were reversed from those influencing IBI.

Results of the multiple-locus models showed significant effects for all traits, although these were weak for L-IPI and burst duration. Genotype × cactus effects were relatively common, and as likely to be seen as main effects. The main effect of rearing substrate was significant for mating success and for L-IPI (Table 3). Organ-pipe–reared males had higher mating success and shorter L-IPIs, as in the single-locus analyses. Of the four QTL showing significant GxE for mating success, only Dmoj4302 was included here in the multiple-locus models because we included those loci that seemed to influence multiple traits. Although the additive effects of Dmoj4302 genotypes on mating success were cactus-specific (Fig. 4, Table 3), Baja alleles at this locus significantly decreased the number of song bursts (MM > MB, BB, $P < 0.05$; additive effect size = – 5.34, SE = 2.66), as did the Dmoj2_2868a GxE interaction, and an epistatic interaction between QTLs associated with Dmoj4300 and Dmoj4302, but these effects were much weaker.

The genotypic effects of Dmoj4300, Dmoj4302, and Dmoj2_2868a on time to copulation were similar (Table 3): in all cases Baja homozygotes caused longer times to copulation, and for Dmoj4300, heterozygotes had significantly shorter times to copulation than either homozygote (least square means, $P < 0.03$).

Table 3. Results of multilocus REML models for mating success, time to copulation, and courtship song traits (mean ±1 SD). *P* values derived from Wald tests with 1 df following sequential addition to a REML model are indicated if $P \approx 0.05$, and in bold if $P \approx 0.01$ or less. Fitted effects (±1 SE) are shown.

Mating success (0, 1) (0.368±0.483, *n*=902)				Time to copulation (min) (23.44±13.58, *n*=322)			L-IPI (msec) (18.85±1.01, *n*=443)		
Fixed term	Wald statistic	*P*	Effect size (SE)	Wald statistic	*P*	Effect size (SE)	Wald statistic	*P*	Effect size (SE)
Cactus	5.99	**0.014**	0.07 (0.03)	1.8	ns		4.73	0.03	−0.21 (0.10)
Dmoj4300	0.74	ns		14.73	**<0.001**	3.67 (1.59)	3.52	0.061	
Cactus×Dmoj4300	3.55	ns		1.7	ns		0.21	ns	
Dmoj4302	0.57	ns		7.04	**0.008**	3.42 (1.53)	0.21	ns	
Cactus×Dmoj4302	6.79	**0.009**	0.14 (0.05)	0.04	ns		0	ns	
Dmoj2_2868a	1.65	ns		6.68	**0.01**	3.60 (1.35)	3.44	ns	
Cactus×Dmoj2_2868a	0.49	ns		1.73	ns		0.01	ns	
Dmoj4300×Dmoj4302	0	ns		1.87	ns		0.06	ns	
Dmoj4300×Dmoj2_2868a	0.01	ns		0.3	ns		0.39	ns	
Dmoj4302×Dmoj2_2868a	2.31	ns		3.58	ns		0	ns	

Burst duration (msec) (423±84.5, *n*=443)				Interburst interval (sec) (1.89±1.06, *n*=441)		
Fixed term	Wald statistic	*P*	Effect size (SE)	Wald statistic	*P*	Effect size (SE)
Cactus	2.06	ns		0.18	ns	
Dmoj4300	4.98	0.026	11.26 (10.17)	0.15	ns	
Cactus×Dmoj4300	0.08	ns		5.76	**0.015**	−0.13 (0.09)
Dmoj4302	0.29	ns		3.75	0.053	
Cactus×Dmoj4302	1.49	ns		0.14	ns	
Dmoj2_2868a	1.44	ns		8.31	**0.004**	0.012 (0.05)
Cactus×Dmoj2_2868a	0.8	ns		4.44	0.036	0.152 (0.07)
Dmoj4300×Dmoj4302	0.75	ns		2.23	ns	
Dmoj4300×Dmoj2_2868a	0.02	ns		1.35	ns	
Dmoj4302×Dmoj2_2868a	0.58	ns		0	ns	

S-IPI (msec) (11.25±0.54, *n*=411)				Number of bursts (66.38±29.16, *n*=443)			
Fixed term	Wald statistic	*P*	Effect size (SE)	Fixed term	Wald statistic	*P*	Effect size (SE)
Cactus	0.71	ns		Cactus	3.92	0.048	
Dmoj4300	0.02	ns		Dmoj4300	0.05	ns	
Cactus×Dmoj4300	0.01	ns		Cactus×Dmoj4300	12.19	**<0.001**	10.34 (5.35)
Dmoj4301	7.27	**0.007**	−0.14 (0.06)	Dmoj4010	0.82	ns	
Cactus×Dmoj4301	0.1	ns		Cactus×Dmoj4010	7.42	**0.006**	6.78 (3.84)
Dmoj4302	1.61	ns		Dmoj4302	8.36	**0.004**	−5.34 (2.66)
Cactus×Dmoj4302	0	ns		Cactus×Dmoj4302	0.39	ns	
Dmoj2_2868a	0.29	ns		Dmoj2_2868a	5.37	0.021	1.15 (2.70)
Cactus×Dmoj2_2868a	0.53	ns		Cactus×Dmoj2_2868a	4.27	0.039	−9.33 (3.72)
Dmoj4300×Dmoj4302	0.47	ns		Dmoj4300×Dmoj4302	4.83	0.028	−8.58 (4.01)
Dmoj4300×Dmoj2_2868a	2.34	ns		Dmoj4300×Dmoj2_2868a	2.61	ns	
Dmoj4302×Dmoj2_2868a	3.36	0.067		Dmoj4302×Dmoj2_2868a	1.94	ns	
Dmoj4300×Dmoj4301	0.22	ns		Dmoj4010×Dmoj4300	0.04	ns	
Dmoj4301×Dmoj4302	4.12	0.042	−0.15 (0.07)	Dmoj4010×Dmoj4302	2.31	ns	
Dmoj4301×Dmoj2_2868a	0.26	ns		Dmoj4010×Dmoj2_2868a	1.02	ns	

Burst duration was also influenced by a QTL associated with Dmoj4300 in this multiple-locus model (additive effect size = 11.26, SE = 10.17), but Dmoj5100 was not included here (Table 2). Allelic effects at both loci were similar in effect—Baja alleles significantly increased burst duration (BB, MB > MM), consistent with mainland female preference for songs with shorter bursts (Table 1). There was only one case of epistasis detected between Dmoj4300 and Dmoj4302 QTL for number of bursts (Table 3). A marginal epistatic interaction between Dmoj4301 and Dmoj4302 QTL was also detected for S-IPI, revealing how the genomic region near Dmoj4302 influenced mating success and song in multiple ways.

Discussion

Mainland *D. mojavensis* females copulated more often with males that produce courtship songs with shorter L-IPI, IBI, and burst duration (Fig. 3). In a previous study, it was found that mainland *D. mojavensis* males have indeed evolved shorter L-IPIs (Etges et al. 2006). However, mainland males also have longer, but less variable burst durations than Baja California males (Byrne 1999; Etges et al. 2006). There was no indication of selection associated with S-IPI, but these short IPI songs were produced less frequently than L-IPI songs. The paucity of S-IPIs seems to be related to geography: males from southern California produce more S-IPI songs than males from populations in northwestern Mexico and Baja California (Byrne 1999). Observation of mainland female preference for shorter burst duration (Table 1, Fig. 3) was at odds with the genetically longer burst durations typical of mainland males. Baja alleles at both QTL influencing burst duration, near Dmoj4300 and Dmoj5100, significantly increased burst duration, the reverse of the population level difference, and there were no GxE interactions (Tables 2 and 3). Thus, there may be other loci of small effect that contribute to shorter burst duration in mainland males that were not accounted for in our genetic model that contribute to the population differences in burst duration. For example, in a genetic analysis of an interpopulational cross comparing parentals, F_1s and F_2s, both X and Y chromosomes significantly increased burst duration (Etges et al. 2006), suggesting a complex epistatic basis of this song trait. Mainland females may have retained an ancestral preference for shorter burst durations, or they reacted to the courtship of these F_2 males differently than mainland males. Unfortunately, no Y-linked markers have yet been developed for *D. mojavensis*.

Consistent differences between populations in IBIs were not detected in previous studies. Byrne (1999) surveyed a number of populations of both *D. mojavensis* and *D. arizonae*, but reported no variation in IBI. Variation in IBI was found among Baja California and mainland populations of *D. mojavensis*, but there was little evidence for consistent differences between geographic regions (Etges et al. 2006), and in some cases, there was more variation between populations in Baja California than between regions (M. G. Ritchie, unpubl. results). Thus, female preference for short IBIs may be specific to particular mainland populations, such as the one used in our QTL analysis or might reflect female preference for males that produce more intense courtship stimuli, that is, more song. If IBI varies geographically, it will be necessary to replicate QTL analyses to multiple population crosses to investigate the generality of these female preferences (Table 1), and the genetic basis of this trait.

Genetic differentiation in courtship songs has evolved with divergence of *D. mojavensis* populations using alternate host plants in different parts of its range. Direction of song evolution is largely interregional, with most differences observed between ancestral Baja California populations and derived populations in mainland Mexico and Arizona (Etges et al. 2006). The direction of song evolution also mirrors the low, but usually significant premating isolation observed in assortative mating tests between populations from these regions (Zouros and d'Entremont 1974, 1980; Markow et al. 1983; Markow 1991; Etges 1992). Most of these premating isolation studies have pointed to the increased choosiness of mainland females as the source of sexual isolation between populations in Baja California and the mainland, but this is not always consistent: often female discrimination is equivalent among populations (Brazner and Etges 1993; Etges 1998). Thus, the degree of genetic differentiation in Baja California and mainland courtship songs did not precisely match patterns of female preference revealed in the selection gradient analyses (Table 1).

Courtship success, song variation, and the QTLs that influence them were often influenced by rearing substrates, and showed GxE interactions, revealing a pervasive role of the use of different host plants in the evolution of mating signals between populations of *D. mojavensis*. Specifically, courtship success was greater when males were reared on the same host, organ pipe cactus, used in nature by the mainland females in this study, even though the females used in our mating tests were reared on lab food. The extent of GxE interaction for mating success, IBI, number of bursts, and to a lesser extent L-IPI (Tables 2, 3) directly implicates rearing substrates as determinants of courtship song-related mating success. Because mainland females were used in the mating trials, the observations that organ-pipe–reared males mated more often (Table 3), but took longer to locate and court females, point to female preference specifically for males reared on organ pipe cactus. Furthermore, three QTL showed GxE interactions where mainland alleles were associated with increased mating success when males were reared on organ pipe cactus (Fig. 4). Previous observations are consistent with these results: organ pipe cactus increased female discrimination over that of agria cactus for mainland males, and caused somewhat longer times to copulation of mainland males in multiple choice tests (Brazner and Etges 1993). Thus, the kinds of mating signals known to be important

in sexual selection and species recognition in other *Drosophila* species, and involved in sexual isolation among populations of *D. mojavensis* in the initial stages of species formation, are often influenced by rearing substrates and GxE interactions.

Mating success and time to copulation were influenced largely by different QTL (Tables 2 and 3). Each "trait" encompasses well-recognized differences in the sequence of events leading to successful intromission and copulation (Spieth 1952). In the early stages of courtship, males orient toward and identify prospective mates, followed by repeated extensions of their proboscis contacting the female's genitalia, or "licking," accompanied by male wing vibration producing courtship song. Licking and repeated male foreleg "thumping" of the female's venter involve functionally different forms of signaling, including the phase when pheromonal recognition is thought to occur mediated by male and female epicuticular hydrocarbons. Females may then signal acceptance by wing spreading, followed by attempted male intromission. Thus time to copulation, including latency of courtship and courtship duration, and mating success are likely to be determined by different genes, and indeed are uncorrelated in lab food–reared *D. mojavensis* mate choice trials (Alonso-Pimentel and Tobin 1992). This is consistent with the high variation in times to copulation observed here, and the differences in QTLs influencing mating success, time to copulation, and courtship songs. Although the influence of Dmoj2_1603a on mating success was marginally significant (Table 2), with mainland alleles increasing mating success (least square means, MM, MB > BB, $P < 0.05$), this QTL also influenced a number of epicuticular hydrocarbons thought to be involved with mate choice among mainland and Baja California populations of *D. mojavensis* (Etges et al., unpubl. data). Thus, the genetic basis of mating success is somewhat independent of that influencing time to copulation, where mating success may also be determined by signals exchanged by physical contact prior to attempted copulation after courtship and song production, that is, epicuticular hydrocarbons (Etges and Ahrens 2001).

A single QTL associated with Dmoj4302 influenced both time to copulation and mating success. Mainland alleles at this locus tended to decrease times to copulation (MM, MB < BB) with mainland females, an a priori expectation given the population-specific assortative mating observed is past studies (Zouros and d'Entremont 1980; Markow 1991; Brazner and Etges 1993). A pleiotropic effect of this QTL on mating success was expressed as a GxE interaction, but not a main effect of genotype (Table 3, Fig. 4): here, mainland alleles increases mating success in males reared on agria cactus, and Baja alleles increased mating success when reared in organ pipe cactus. The "direction" of these cactus-specific effects on QTL alleles is notable because the effects are the reverse of the other three GxE interactions for mating success (Fig. 4).

GxE INTERACTIONS AND SIGNAL EVOLUTION

Even with the relatively small number of QTLs revealed here that influenced courtship songs and mating success, it is clear that QTLs, male signals, and female preference for them were influenced by cactus substrates and GxE interactions. These QTL effects suggest that the genetic architecture of these song traits may be determined by a small number of marker regions and GxE interactions, but the limited number of available marker loci surveyed and increased recombination rates in *D. mojavensis* (see Ortiz-Barrientos et al. 2006) currently limit resolution of our analysis.

In natural populations of *D. mojavensis* that use more than one host cactus, there is potential for phenotypic plasticity in male courtship songs and female preference. Models of sexual selection and signal evolution have largely overlooked GxE effects, even though context or condition dependence of signaling/sexually selected traits is widely recognized (Griffith et al. 1999; Lesna and Sabelis 1999; Welch 2003; Greenfield and Rodriguez 2004 for a review). "Good genes" models of sexual selection predict that females choose among males because some signals are heritable and reliable so that her offspring will share those males' high viability (Pomiankowski 1988; Kokko et al. 2002). When signal traits are plastic and influenced by GxE interactions, such as IBI and number of bursts (Tables 1 and 3), female choice may result in poorer fitness of her progeny should they experience an alternate cactus host during development. This can result in the maintenance of additive genetic variance in the population (Gillespie and Turelli 1989; Greenfield and Rodriguez 2004) allowing for continued selection on the trait by female choice, but interference with any immediate increases in fitness that might result from sexual selection.

Under Fisherian sexual selection (Lande 1981; Kirkpatrick 1987), females may mate with males whose song traits are perceived as attractive and heritable such that her sons might experience greater mating success because they will inherit these preferred traits. If song traits are plastic and influenced by GxE interactions, her sons may be less successful in attracting potential mates if they experience larval development on an alternate host. This suggests that females should evolve preference for the most reliable indicators of male fitness or least environmentally influenced signal traits, such as L-IPI that showed only one GxE interaction (Tables 1–3). However, mainland *D. mojavensis* females clearly discriminate among males based on IBI and number of songs bursts, traits that are influenced by multiple GxE interactions (Tables 2 and 3). For IBI, each of the two GxE terms had an opposite additive effect on IBI (Table 3). Thus, in diverging populations that have evolved low levels of sexual isolation, such host-induced signal plasticity and female choice should maintain genetic variation for these traits, but slow rates of differentiation in sexually selected traits (Danielson-Francois et al. 2006). Of course, knowledge of the effects of rearing

substrate on female preferences is required for a full understanding of the effects of these interactions on sexual selection (e.g., Lesna and Sabelis 1999). In premating isolation tests with mainland and Baja California adults, the degree of mainland female discrimination was higher and significant for organ-pipe-cactus–reared flies compared with agria-reared females (Brazner and Etges 1993), but the consequences to offspring fitness of these host-influenced behaviors is not known.

The natural history of *D. mojavensis* is the key to understanding the significance of GxE interactions on courtship song differences that have diverged in allopatric populations. Clearly, the specific effects on signal traits of these GxE interactions will depend on the ecological details that influence the expression of these traits. Signal plasticity determined by effects of different host cacti can only influence potential mate choice and sexual selection in which more than one host is used by local populations of *D. mojavensis*. Rearing records from nature (Fellows and Heed 1972; Ruiz and Heed 1988) and results of host preference tests (Newby and Etges 1998) clearly show that populations of *D. mojavensis* prefer and predominantly use agria cactus even when other hosts are present. Agria and organ pipe cactus are sympatric in the southern half of Baja California, the midriff islands in the Gulf of California, and a small area in coastal Sonora, but records of *D. mojavensis* emerging from organ pipe "rots" from these areas are infrequent (Heed 1978; Etges et al. 1999). Throughout most of northwestern Mexico and Arizona, organ pipe cactus is the sole host plant for *D. mojavensis*. However, there are alternate sympatric hosts, occasionally used by *D. mojavensis* throughout the species range (Heed and Mangan 1986). In central and southern Sonora, organ pipe is sympatric with sina cactus, *S. alamosensis*, which *D. mojavensis* sometimes shares with *D. arizonae* (Markow et al. 1983; Ruiz and Heed 1988). Saguaro cactus, *Carnegiea gigantea*, is also infrequently used by *D. mojavensis* on the mainland, and the large endemic columnar cactus, *Myrtillocactus cochal*, is used in Baja California. Thus, there are ample opportunities for song plasticity and GxE interactions to influence the evolution of female choice in natural populations.

Genetic analysis of population divergence in different environments has revealed abundant evidence of ecological specialization and adaptation by GxE interaction (Via 1990; Via et al. 1995; Pigliucci 2001), but components of behavioral isolation have received comparatively less attention. The expansion of *D. mojavensis*' range and adaptation to the use of different host cacti has been facilitated by GxE interactions for host-associated components of fitness and low, across-host genetic correlations (Etges 1993). Analysis of the genetic basis of traits directly associated with host use and fitness, and those determining mate choice should help resolve the causes driving divergence and reproductive isolation among these geographically isolated populations of *D. mojavensis*.

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