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Premating isolation is determined by larval rearing substrates in cactophilic *Drosophila mojavensis*. VIII. Mating success mediated by epicuticular hydrocarbons within and between isolated populations

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Abstract

We tested the hypothesis that intrademic sexual selection has caused sexual isolation between populations of geographically isolated populations of cactophilic *Drosophila mojavensis*, and was mediated by epicuticular hydrocarbons (EHCs), contact pheromones in this system. Sexual selection and sexual isolation were estimated using a Baja California and mainland population by comparing the number of mated and unmated males and females in each of four pairwise population mating trials. EHC profiles were significantly different in mated and unmated males in the interdemic (Baja♀ × Mainland♂ and Mainland♀ × Baja♂), but not the intrademic mating trials. A small number of EHCs was identified that best discriminated among mated and unmated males, mostly alkadienes with 34 and 37 carbons. Females showed population-specific preferences for male EHC profiles. However, EHC profiles between mated and unmated males in the intrademic mating trials were not significantly different, consistent with undetectable sexual selection estimated directly from numbers of copulating pairs vs. unmated adults. Thus, sexual isolation among populations was much stronger than sexual selection within these populations of *D. mojavensis*.

Introduction

The formation of premating isolating barriers among recently diverged populations is often an early step in the process of speciation (Dobzhansky, 1937; Mayr, 1963; Coyne & Orr, 2004). Establishing the causes for different patterns of mate choice among populations usually requires analysis of multiple causes including sexual selection (Boughman, 2001; Boul *et al.*, 2007), mate preference for conspecifics or species recognition systems (Paterson, 1993; Gerhardt & Huber, 2002), natural selection via reinforcement (Howard, 1993; Noor, 1999; Servedio & Noor, 2003) and sensory bias (Basolo, 1995; Ryan, 1998; Boughman, 2002). Without phylogenetic information, hypotheses involving sensory bias will be difficult to test. Because of historical reliance on the Biological Species Concept (Mayr, 1942; Coyne & Orr, 2004), a preliminary step in the investigation of the degree of reproductive isolation between populations has often involved estimating sexual isolation. This can provide a general indication of the strength of premating isolation among populations, and perhaps inferences about species status, but does not necessarily inform us of how premating isolation evolved. Untangling sexual selection from sexual isolation (Carson, 1978; West-Eberhard, 1983; Boake *et al.*, 1997) requires experimentation to separate the causal roles of sexual selection within populations from the influences of sexual isolation between populations (Ewing & Miyan, 1986; Ryan & Rand, 1993; Sætre *et al.*, 1997; Panhuis *et al.*, 2001; Blows & Higgie, 2002). Although several exemplary cases of sexual selection-driven reproductive isolation have been described, speciation via sexual selection is thought

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to be more likely when accompanied by ecologically mediated natural selection (see Ritchie, 2007 for a review).

Here, we assess sexual isolation among geographically isolated populations of cactophilic *Drosophila mojavensis* by demonstrating how a key component of mate recognition, contact pheromones composed of epicuticular hydrocarbons (EHCs), influence mating success within and between populations. Low, but consistent, levels of premating isolation between Baja California and mainland Mexico and Arizona populations of *D. mojavensis* have been studied for over 30 years (Zouros & d'Entremont, 1974, 1980; Markow *et al.*, 1983; Markow, 1991; Etges, 1992; Etges *et al.*, 1999) and significant post-mating-prezygotic isolation (Knowles & Markow, 2001) suggest ongoing incipient speciation in allopatry, yet there is no evidence for post-zygotic isolation (Etges, 1990; Ruiz *et al.*, 1990). Male courtship songs differ consistently between isolated populations, are genetically differentiated (Etges *et al.*, 2006), and influence courtship success (Etges *et al.*, 2007). EHCs serve as mate recognition signals during the final stages of courtship when males and females are in close physical contact (Ferveur, 2007). By experimentally transferring EHCs from one group of males to another in 'perfuming experiments' (see Coyne *et al.*, 1994), *D. mojavensis* mating success was significantly increased compared with controls altering levels of sexual isolation between populations (Etges & Ahrens, 2001), demonstrating a pheromonal role of EHCs. However, sexual selection involving EHCs in *D. mojavensis* has not been studied, and how sexual selection may influence sexual isolation between allopatric populations considered to be incipient species is unknown.

Epicuticular hydrocarbons of *D. mojavensis*

The EHCs of *D. mojavensis* and its closest relatives are composed of approximately 30 different-branched alkanes, alkenes, branched alkenes and alkadienes, with the most abundant components having odd numbered carbon chains, ranging from C_{29} to C_{37} (Toolson *et al.*, 1990; Etges & Jackson, 2001). In a survey of 11 populations of *D. mojavensis* from Baja California and mainland Mexico, 13 different EHCs differed in amounts between populations grouped by region, with 16 EHCs showing significant sexual dimorphism, suggesting that EHC-related mate recognition in *D. mojavensis* involves multiple EHC components. Further, multiple EHC components showed significant sex × region interactions, indicating that sexual dimorphism in EHC amounts was not consistent across populations from Baja California and mainland Mexico (Etges & Ahrens, 2001; and see Discussion). Such sex × region interactions in EHCs were hypothesized to represent a significant shift in the mate recognition system of Baja California and mainland *D. mojavensis* populations.

Ecology and biogeography of *D. mojavensis*

A full understanding of the nature of reproductive isolation in *D. mojavensis* requires knowledge of its evolutionary history and ecology (Heed, 1982; Ruiz *et al.*, 1990). Populations of *D. mojavensis* are distributed throughout the Sonoran and Mojave Deserts, the Sinaloan thornscrub on the mainland, the Cape Region of southern Baja California, the Coastal Sage floristic province in northern Baja California and Santa Catalina Island near Los Angeles, California (Etges *et al.*, 1999). Populations in Baja California are considered ancestral to others in the species range (Ruiz *et al.*, 1990; Machado *et al.*, 2007) and carry out their life cycle in fermenting tissues of agria cactus, *Stenocereus gummosus*, while derived mainland populations primarily use organ pipe cactus, *S. thurberi*, with occasional use of *S. gummosus* in coastal Sonora and *S. alamosensis* with which it shares with its sibling species, *D. arizonae* (Fellows & Heed, 1972; Markow *et al.*, 1983; Ruiz & Heed, 1988). Mojave Desert populations use California barrel cactus, *Ferocactus cylindraceous*, and those on Santa Catalina Island, near Los Angeles, use *Opuntia demissa* (Heed, 1982). Thus, *D. mojavensis* is considered oligophagic, using different host plants in different parts of its species range.

In the present study, we test the hypothesis that EHC-related sexual selection has directly influenced premating isolation between populations by employing separate intrademic and interdemic mating trials. Previous laboratory studies have shown that premating isolation is often influenced by increased female discrimination by mainland females in multiple choice trials (Zouros & d'Entremont, 1980; Markow, 1991), so we hypothesized that sexual selection may be stronger in trials involving mainland females. As levels of premating isolation depend on rearing substrates, mainly lab food vs. cactus (Etges, 1992; Stennett & Etges, 1997), all flies were reared on fermenting cactus tissues. We estimated sexual selection and sexual isolation in different mating trials and show that sexual selection in between population, but not within population trials, drives patterns of mate choice in *D. mojavensis*. Further, we identify contrasting patterns of sex-specific differences in EHCs that are associated with differences in mate choice within and between populations of *D. mojavensis*, consistent with their role as contact pheromones.

Materials and methods

Husbandry and cactus rearing

A population of *D. mojavensis* was derived from 544 adults collected over banana baits in an agria cactus patch east of San Quintin, Baja California in January 2003, and an outbred stock of *D. mojavensis* derived from ~30 wild females collected in 2002 from Organ Pipe Natl Monument, Arizona was provided by T. Markow. Upon return

to the laboratory, both outbred stocks were mass reared on banana food (Brazner & Etges, 1993) in 8 dr shell vials at room temperature. Each population was considered a random sample from each geographical area previously characterized for host plant use and EHC variation (Etges, 1990; Etges & Ahrens, 2001).

Both populations were reared for a week or two in large numbers in 12 720 cm^3 plexiglass population cages prior to the mating experiments in 2003 and 2004 to avoid the effects of inbreeding in vials and allow for random mating. Eggs collected from these large populations were reared on banana food at moderate larval densities in half-pint bottles. Twelve replicate cultures were established for each population in an incubator set to a 14 : 10 LD cycle and 27 °C during the day and 17 °C at night. Emerging adults were collected daily, separated by sex, and aged until sexually mature (10–12 days). Two hundred mature males and 200 females were placed in plastic freezer boxes fitted with oviposition media in removable 5 cm diameter petri dishes (Etges, 1992). Adults were allowed to mate and oviposit over an 8-h period each day. Eggs from each population were rinsed in sterilized water, soaked in 70% ethanol for 10 min, counted out in groups of 200 on to a 1-cm^2 piece of sterilized filter paper, and placed onto fermenting cactus tissues.

Cactus cultures were prepared using 60 g of thawed agria cactus placed in sterilized half-pint bottles containing 75 g of aquarium gravel. We used agria cactus because it reduces levels of premating isolation, like organ pipe, rather than lab food, and it produces similar effects on adult cuticular hydrocarbons as organ-pipe cactus (Etges & Ahrens, 2001). These cultures were then autoclaved for 8–10 min and at 20 lbs. of pressure, allowed to cool, and then inoculated with a 2 mL solution of seven species of cactophilic yeast including *Pichia mexicana*, *Sporopachyderma cereana*, *Dipodascus starmeri*, *P. cactophila*, *Starmera amethionina var amethionina*, *Candida valida* and *C. sonorensis*, and 2 mL of a pectolytic bacterium, *Pectobacterium (=Erwinia) cacticida*, common in agria and organ-pipe rots in nature (Starmer, 1982; Alcorn *et al.*, 1991). Twelve replicate cactus cultures were established for each population. Eggs and filter paper were removed after several days and number of unhatched eggs counted to assess egg to adult viability. All enclosing adults were aspirated from each bottle daily, separated by sex and aged until sexually mature (10–12 days) on lab media prior to the mating trials.

Mating trials

Four types of mating trials were used to characterize adult mating success within and between populations. For each trial, 10 virgin males and 10 virgin females were observed for 30 min. (i) Baja females were paired with Baja males (B♀ × B♂); (ii) Baja females were paired with mainland males (B♀ × M♂); (iii) mainland females were paired with Baja males (M♀ × B♂); and (iv) mainland females were paired with mainland males (M♀ × M♂). All aged adults were randomly assigned to different trails. Trials were conducted in a darkened room during the active morning period in observation chambers consisting of a 50-mL Erlenmeyer flask turned on its side and plugged with cotton. Courtship was observed, copulating pairs were observed for at least a minute to insure that pseudocopulations were not included in the data (Markow *et al.*, 1983). All copulating pairs were aspirated out of the mating chamber, separated by sex, and frozen individually at −20 °C for EHC analysis.

Variation in courtship latency, or the time from the initiation of a trial to the beginning of observed courtship, can be quite large in *D. mojavensis*. Some males seem especially able to mate especially quickly while others do not (Brazner & Etges, 1993; Etges, 1998). In a recent QTL analysis of mating success, courtship songs and EHCs in F_2 male *D. mojavensis*, time to copulation in 60-min multiple choice trials averaged 23.4 min, SD = 13.6 min, $n = 902$ (Etges *et al.*, 2007). Thus, we hypothesized that the variance in time to copulation was because of variation in EHC profiles. To test this hypothesis, we separated mated pairs into groups that mated in the first 10 min of a trial from those that mated in the remaining 20 min, times based on our earlier observations of mating success. Matings observed in the first 10 min were recorded as mated first (F), and subsequent copulations were denoted as mated (M). At the end of the 30-min period, all unmated flies (U) were aspirated out and frozen individually. A total of 17–18 trials of each of the four types (1–4) were completed. Multiple trials of each type were performed each day in alternating order.

Gas chromatography

Individual flies were immersed in 50 μL of high-performance liquid chromatography-grade hexane in a 300-μL vial insert for 20 min with 1 min of agitation. Flies were removed and the extracts were dried at 40 °C in a heating block for 1 h. These extracts were then frozen at −20 °C until GC analysis by capillary gas–liquid chromatography. Hydrocarbon extracts were redissolved with 5 μL of heptane containing 385 ng docosane (C_{22}) μL^{-1} as an internal standard. One microlitre of each sample was analysed using an automated Shimadzu GC-17H High Speed FID/GC fitted with an AOC-20i autosampler tower (Shimadzu Scientific, Columbia, MD, USA) fitted with a 15 m (ID = 0.22 mm) Rtx-5 fused-silica column. An initial oven temperature of 200 °C was increased to 345 °C at a rate of 15 °C min^{-1}, and held at 345 °C for 2 min (Etges & Ahrens, 2001). Injector temperature was 290 °C and detector temperature was 345 °C with the injector port in split mode (split ratio = 3 : 1). Amounts of 30 EHC components (Stennett & Etges, 1997; Etges & Ahrens, 2001; Etges & Jackson, 2001) were quantified in

all flies by analysis of peak integrations using CLASS VP 4.2 software provided by Shimadzu. Amounts of each EHC were estimated using the amount of C_{22} as an internal standard and expressed as nanograms/fly.

Data analysis

Patterns of mating success in the four different trials were assessed by comparing the numbers of copulating pairs and unmated adults. Sexual selection was estimated directly as the relative mating success of Baja and mainland males and females. Statistical analyses were performed using JMating (Carvajal-Rodríguez & Rolán-Alvarez, 2006) allowing estimation of sexual selection (*PSS*) and sexual isolation (*PSI*) in each pair-wise combination of matings, as well as overall sexual isolation (I_{PSI}) among these mainland and Baja California populations (Rolan-Alvarez & Caballero, 2000). Indices of mating asymmetry (IA_{PSI}) were calculated to assess deviations from unity in numbers of copulations within populations and between populations, often observed in *D. mojavensis*. Significance of all JMating statistics was obtained by 10 000 bootstrap iterations.

Male and female EHC data were $\log_{10}$ transformed to improve normality. All analyses were performed using SAS procedures (SAS-Institute, 2004). ANOVA and MANOVA with trial, sex and population as main effects were evaluated for EHC components and compared with earlier surveys of broad-scale geographic differentiation in EHC composition of Baja California and mainland populations *D. mojavensis* (Etges & Ahrens, 2001). MANOVA also revealed differences in the 30 EHC components between flies in the first (F), mated (M) and unmated (U) groups across the four different mating trials. *Post hoc* multiple comparisons were evaluated after MANOVA to identify significant differences among F, M and U groups. Canonical discriminant function (CDF) analysis was performed for each mating trial separately and with pooled data. Stepwise discriminant function analysis (DFA) was performed for each type of mating trial to reduce the total number of EHCs to those that best discriminated among the three groups (F, M and U) in each trial. We compared stepwise and backward elimination methods using the significance of the squared partial correlation for each EHC that predicted the discriminatory power of the model, controlling for the effects of the variables already selected for the model in PROC STEPDISC (SAS-Institute, 2004). Initial significance was set at $P = 0.15$ (Costanza & Afifi, 1979) and only EHCs with partial correlations significant at $P = 0.05$ were retained. We analysed EHC variation in each of the four types of mating trials separately because we predicted that flies from these geographically isolated populations should have different mate preferences given the number of region × sex interactions in EHC amounts previously reported (Etges & Ahrens, 2001).

Results

Egg to adult viability did not differ among populations ($P = 0.08$) and averaged 84.7% (SD = 13.90), consistent with past studies of cactus-reared flies (Etges, 1990, 1998). A total of 71 mating trials were performed including the four types of matings. Overall, 401 copulations were observed (Table 1), and mating success varied from 71.6% (126/176) in M♀ × M♂ trials to 41.1% (72/175) in the B♀ × M♂ trials, similar to observed rates of copulation success (mean ± SD = 0.77 ± 0.10, $n = 17\,280$) in past multiple choice experiments with cactus-reared flies (Etges, 1998).

Sexual selection and sexual isolation based on observed copulations

Based on the number of observed copulations and the number of unmated flies, overall sexual isolation between these populations was low, but significant ($I_{PSI} = 0.12$, $t = 2.36$, $P = 0.02$) consistent with previous studies using agria-reared flies (Etges, 1992, 1998; Brazner & Etges, 1993). The properties of the *PSS* and *PSI* statistics allowed closer inspection of the underlying mating patterns responsible for significant sexual isolation between populations (Rolan-Alvarez & Caballero, 2000). Decomposing I_{PSI} into pairwise sexual selection and isolation statistics, *PSS* and *PSI*, revealed that pair sexual selection, *PSS*, in the M♀ × B♂ and B♀ × M♂ trials was statistically significant, but in opposing directions (Table 2). Here, Baja males achieved more copulations with mainland females than expected from random mating ($PSS = 1.196$, $P < 0.0001$) while mainland males achieved significantly fewer matings with Baja females ($PSS = 0.817$, $P = 0.019$) than expected (Table 2), but there was no difference in overall mating success between Baja and mainland males revealed by the cross-product estimator of sexual selection ($W_1 = 0.925$, SD = 0.093, $P = 0.199$; Rolan-Alvarez & Caballero, 2000). Notably, this pattern is the opposite of that described for flies reared on lab

Table 1 Numbers of observed copulations and unmated adults (percent of total in parentheses) in the four different mating trials with mainland (M) and Baja California (B) populations of *Drosophila mojavensis*.

	M♀ × M♂	B♀ × B♂	M♀ × B♂	B♀ × M♂
Mated first (F)	88 (0.50)	62 (0.38)	65 (0.39)	51 (0.29)
Mated (M)	38 (0.22)	35 (0.22)	41 (0.24)	21 (0.12)
Total mated (F + M)	126 (0.72)	97 (0.60)	106 (0.63)	72 (0.41)
Unmated (U)	50 (0.28)	66 (0.40)	62 (0.37)	103 (0.59)
Total	176	163	168	175
No. trials	18	17	18	18

Each trial lasted 30 min, with copulations occurring in the first 10 min designated 'mated first', and all other copulations as 'mated'. Deviations from total numbers of individuals in each trial (170, 180) were because of escaped flies.

Table 2 Estimates of sexual isolation (*PSI*), sexual selection (*PSS*) and total isolation (*PTI*) for each pairwise combination of males and females in the four mating trials in this study.

	Mainland females (344)			Baja females (338)		
	PTI	*PSI*	*PSS*	*PTI*	*PSI*	*PSS*
Mainland males (351)						
Copulations		126			72	
Estimate	1.210	1.100	1.100	0.704	0.863	0.816
Bootstrap mean	1.212	1.108	1.100	0.705	0.870	0.817
SD	1.005	0.120	0.086	0.076	0.127	0.079
P-value	0.019	0.352	0.263	0.000	0.325	0.019
Baja males (331)						
Copulations		106			97	
Estimate	1.080	0.903	1.196	1.006	1.134	0.887
Bootstrap mean	1.078	0.907	1.196	1.005	1.144	0.887
SD	0.089	0.104	0.035	0.089	0.151	0.085
P-value	0.401	0.371	0.000	0.962	0.314	0.182

The total number of adults used of each kind are shown in parentheses, and standard deviations (SD) and significance values from 10 000 bootstrap resamplings for each statistic are indicated.

food (Zouros & d'Entremont, 1980; Markow, 1991; Etges, 1992), where M♀ × B♂ matings are typically lowest in frequency because of the lowering of mating propensity of lab food-reared Baja males. Thus agria cactus significantly increased the mating success of Baja California male *D. mojavensis*, consistent with previous studies of cactus-reared flies (Brazner & Etges, 1993).

These contrasting pair sexual selection estimates, *PSS*, also suggested a significant mating disadvantage of Baja females relative to mainland females ($W_2 = 0.745$, SD = 0.076, $P = 0.001$) even though *PSS* was not significant in the intrademic trails (Table 2). Therefore, Baja females mated less often with males from either population either because they were more discriminating than mainland females or less inclined to mate. These results parallel the patterns of male EHC differences (see below; Fig. 1) where there were no significant differences in the intrademic trails, but mated and unmated males differed significantly in EHC profiles in the interdemic trials.

We evaluated the asymmetry in the numbers of heterogametic matings by assessing pair isolation of B♀ × M♂ matings where $PSI_{BM} = 0.863$, significantly lower than that of M♀ × B♂ matings, $PSI_{MB} = 0.903$, ($PSI_{MB/BM}$ ratio = 1.048, SD = 0.032, $P = 0.029$). Notably, this 'direction' of asymmetry was in the opposite direction to the 'one-way' premating isolation for lab food-reared *D. mojavensis* in multiple choice trials described above. No pair isolation indices (*PSI*) deviated from random (null hypothesis = 1), but total pair isolation indicated more M♀ × M♂ copulations than expected (*PTI* = 1.212, $P = 0.019$), consistent with greater mating propensity of mainland females, or because they were less choosy. These results suggest that overall sexual isolation between these Baja California and mainland populations was driven by nonrandom mating in the B♀ × M♂ and M♀ × B♂ trials, consistent with the differences in EHC amounts between mated and unmated adults (see below; Fig. 1).

Epicuticular hydrocarbon variation

Epicuticular hydrocarbon extracts of 615 individuals, 485 males and 130 females, were used in the analysis of hydrocarbon-associated differences in mating success. Over all trials, there were significant differences in EHC amounts between mated and unmated flies (Wilks' $\lambda = 0.8567$, $F = 1.54$, d.f. = 60,1148, $P = 0.0061$). Sex (Wilks' $\lambda = 0.1723$, $F = 91.9$, d.f. = 30,574, $P < 0.0001$) and population (Wilks' $\lambda = 0.1633$, $F = 98.0$, d.f. = 30,574, $P < 0.0001$) differences in EHC amounts between the mainland and Baja California populations were also significant, as was a population × sex interaction (Wilks' $\lambda = 0.6252$, $F = 11.47$, d.f. = 30,574, $P < 0.0001$), consistent with regional Baja vs. mainland EHC differences (Etges & Ahrens, 2001). Differences in EHC amounts between the four types of mating trials were also significant (results not shown), but were confounded by sex and population differences of the flies used in each trial.

Male EHCs and mating success

MANOVA revealed significant EHC differences among mated and unsuccessful males across all trials for the 30 measured hydrocarbon components (Wilk's $\lambda = 0.8374$, $F = 1.40$, 60,906 d.f., $P = 0.027$). Planned multivariate contrasts between groups with all four trials pooled revealed that EHC profiles differed significantly between mated (M) and unmated (U) males (Wilk's $\lambda = 0.8830$, $F = 2.00$, 30,453 d.f. $P = 0.002$), but not between F vs. M or F vs. U males (both $P > 0.1$). We also carried out separate planned contrasts in each of the four independent mating trials to investigate EHC differences that may have been obscured by pooling data across trials and to focus on intrademic vs. interdemic trials. There were significant differences in EHC profiles between F and U males in the M♀ × B♂ (Wilk's $\lambda = 0.5831$, $F = 1.83$, 30,77 d.f., $P = 0.018$) and B♀ × M♂ trials (Wilk's $\lambda = 0.6731$, $F = 1.68$, 30,104 d.f., $P = 0.028$), but differences in EHC profiles between M and U males in the M♀ × B♂ trial were not significant ($P = 0.088$). Thus, the EHC profiles of 'first mated' Baja males were associated with more rapid copulation ability with mainland females than males that mated after 10 min supporting our hypothesis that very short times to copulation were EHC based. We then pooled F and M males because there were no significant differences in EHCs among these groups (Table 3). Differences in EHCs between mated (F + M) vs. Unmated groups for M♀ × B♂ and B♀ × M♂ trials were significantly different, but not so in the B♀ × B♂ trials ($P = 0.085$). Thus, mating success among

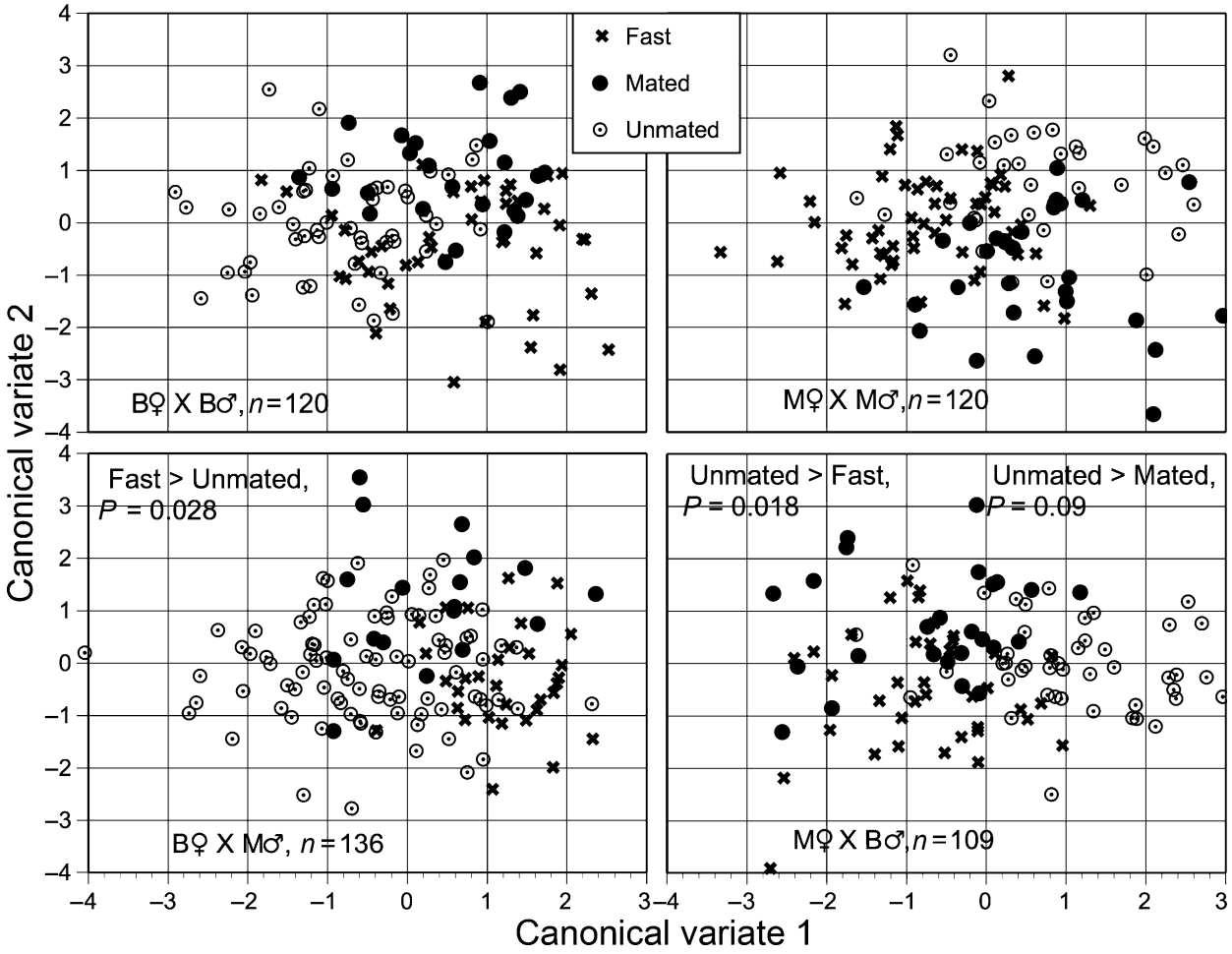

Fig. 1 Plots of male *Drosophila mojavensis* epicuticular hydrocarbon scores along the first two canonical variates in the four different mating trials according to whether males mated (M), mated in the first 10 min of the trial (F), or were unmated (U).

Table 3 Pairwise, *post hoc* multivariate contrasts between male EHCs in the three mating categories for each of the four mating trials; mated first (F), mated (M) and unmated (U), based on 30 epicuticular hydrocarbons in mainland (M) and Baja California (B) populations of *Drosophila mojavensis*.

		Contrast											
		First vs. mated			First vs. unmated			Mated vs. unmated			F + M vs. unmated		
Mating trial	d.f.	Wilk's λ	*F*-value	$P > F$	Wilk's λ	*F*-value	$P > F$	Wilk's λ	*F*-value	$P > F$	Wilk's λ	*F*-value	$P > F$
M♀ × M♂	30,89	0.6963	1.28	0.188	0.6899	1.32	0.161	0.7326	1.07	0.391	0.7109	1.21	0.247
B♀ × B♂	30,88	0.7906	0.78	0.780	0.6862	1.34	0.147	0.7203	1.14	0.313	0.6687	1.47	0.085
M♀ × B♂	30,77	0.8226	0.55	0.964	0.5831	1.83	0.018	0.6349	1.48	0.088	0.5461	2.16	0.004
B♀ × M♂	30,104	0.7921	0.91	0.604	0.6731	1.68	0.028	0.7912	0.91	0.597	0.6893	1.58	0.048

these Baja California and mainland males was largely associated with differences in EHC profiles in interdemic, but not intrademic mating trials.

Canonical DFA revealed the magnitude of EHC differences (+/−) for mated and unmated flies. Despite insignificant EHC differences among males in the intrademic trials, we were interested in seeing whether the variation in EHC amounts was similar between intrademic and interdemic trials, i.e. were the same trends apparent? Plotting the first two canonical variates for each CDF analysis revealed a startling difference in mating preferences of Baja California and mainland

females: Baja females tended to mate more often with males with more (+) of the EHCs correlated with CV1 (Fig. 1) while mainland females preferred males with less (−) EHCs correlated with CV1. These differences were largest in the interdemic trials, but not significant in the intrademic trials, complicating our attempts to link intrademic and interdemic patterns of mate choice. However, the array of mated vs. unmated males along CV1 was strikingly similar for Baja vs. mainland females respectively (Fig. 1). Inspection of the EHC-based Euclidean distances between groups showed that mated (M) and unmated (U) males were more different ($P = 0.002$) than first mated (F) and unmated (U) males ($P = 0.113$). This pattern was obscured in the M♀ × M♂ trials because of the lack of significant difference in EHCs between mated and unmated males (Table 3). This may have been caused by the low number of unmated M♀ × M♂ males (34/120) relative to the other trials and a corresponding lack of power to detect differences in EHC amounts.

Identity and relative amounts of the EHCs preferred by Baja and mainland females were revealed by evaluating the structure of the first canonical variate for each of the four trials (Table 4). A large proportion of EHCs was associated with differences in mating success, indicated here by the number of significant correlations between EHC amounts and CV1 for each trial. Other than in the B♀ × B♂ trial where only four hydrocarbons showed weak correlations, EHC amounts consistently covaried positively or negatively among successful vs. unsuccessful mainland and Baja males respectively (Table 4). Mainland females preferred Baja males with lower (−) amounts (Fig. 1) of 14 EHC components, yet Baja females preferred mainland males with increased (+) amounts of eight of these EHCs plus seven others.

Stepwise DFAs were used to identify the best discriminating EHCs in each trial. There were generally few EHCs that discriminated between F, M and U males, and even fewer that were common among trials (Table 5). Stepwise DFA was far more conservative and generally retained fewer EHCs in each discriminant function than backward elimination DFA. These results suggested a much smaller set of EHC components that functioned as pheromones. Further, since there were no overall EHC differences between successful and unsuccessful males in

Table 4 Correlations of epicuticular amounts with the first canonical variate.

Hydrocarbon	ECL†	M♀ × M♂	B♀ × B♂	M♀ × B♂	B♀ × M♂
2-Methyloctacosane	$C_{28.65}$	0.303***	0.158	−0.233*	0.290***
2-Methyltricontane	$C_{30.65}$	0.239**	0.107	−0.218*	0.218*
7- and 9-hentricontene	$C_{30.78}$	0.232**	0.129	−0.296**	0.237**
Unknown alkene	C_{33br1}	0.053	−0.150	0.116	0.110
11- and 13-methyldotricontane	C_{33br2}	−0.001	−0.094	−0.100	0.197*
Unknown alkene	C_{33br3}	0.184*	−0.016	−0.035	0.091
31-Methyldotricont-8-ene	$C_{32.47}$	0.230*	−0.015	−0.215*	0.288***
31-Methyldotricont-6-ene	$C_{32.56}$	0.098	−0.217*	−0.210*	0.344****
8,24-Tritricontadiene	$C_{32.63}$	0.331***	0.126	0.063	0.277**
7,25-Tritricontadiene	$C_{32.70}$	0.285**	0.030	−0.350***	0.022
10-, 12- and 14-tritricontene	$C_{32.79}$	0.231**	−0.028	−0.283**	0.226**
Unknown	$C_{32.86}$	0.205*	0.076	−0.211*	0.054
8,26-Tetratricontadiene	$C_{34diene}$	0.281**	0.210*	−0.519****	0.124
6,24- and 6,26-tetracontadiene	$C_{34diene}$	0.219*	0.091	−0.250**	0.112
10-, 12- and 4 tetretricontene	C_{34ene}	0.235**	0.074	−0.095	−0.006
33-Methlytetratricont-10-ene	C_{35alk1}	0.162	−0.040	−0.149	0.326***
33-Methlytetratricont-8-ene	C_{35alk2}	0.212*	0.002	−0.031	0.301***
Unknown alkene	C_{35alk3}	0.276**	0.054	−0.178	0.210*
9,25-Pentatricontadiene	$C_{34.59}$	0.174	0.060	−0.185	0.098
8,26- and 7,27-pentatricontadiene	$C_{34.66}$	0.228*	0.082	−0.253**	0.205*
Unknown	$C_{34.73}$	0.167	0.166	−0.124	0.169*
Unknown alkene	C_{36a}	0.265**	0.133	−0.309**	0.090
Unknown alkene	C_{36b}	0.293**	0.166	−0.052	−0.038
35-Methylhexatricont-10-ene	C_{37br}	0.270**	0.131	0.001	0.130
9,27-Heptatricontadiene	$C_{36.5}$	0.305***	0.229*	−0.214*	0.137
8,28-Heptatricontadiene	$C_{36.6}$	0.183*	0.123	−0.227*	0.210*
14-, 16- and 12-hexatricontene	$C_{36.7}$	0.336***	0.159	−0.215*	0.281***
Unknown alkene	C_{38}	0.182*	0.223*	−0.048	0.390****
Unknown alkene	C_{39}	0.216*	0.165	−0.268**	0.137
Unknown alkene	C_{40}	0.333***	−0.030	−0.287**	0.124

†Equivalent chain length as calculated in Stennett & Etges (1997) or other hydrocarbon name if component not yet identified (Etges & Jackson, 2001).

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

Table 5 The best discriminating epicuticular hydrocarbons for each mating trial based on mated first (F), mated (M) and unmated (U) groups of males.

ECL†	M♀ × M♂	B♀ × B♂	M♀ × B♂	B♀ × M♂
$C_{30.78}$			7- and 9-hentricontene***	7- and 9-hentricontene**
C_{33br2}	11- and 13-methyldotricontane*			
C_{33br3}			C_{33} branched alkane 3**	C_{33} branched alkane 3*
$C_{32.56}$		31-Methyldotricont-6-ene**		
$C_{32.63}$	8,24-Tritricontadiene**			
$C_{32.70}$	7,25-Tritricontadiene**			
$C_{32.86}$	*C_{33} branched alkene***			C_{33} branched alkene*
$C_{34diene}$		8,26-Tetratricontadiene*	*8,26-Tetratricontadiene****	
C_{34ene}			10-, 12- and 14-tetratricontene**	
C_{35alk1}				33-Methyltetratricont-10-ene*
$C_{34.59}$	9,25-Pentatricontadiene***	*9,25-Pentatricontadiene***		
$C_{34.66}$	8,26-Pentatricontadiene*			
$C_{36.5}$	9,27-Heptatricontadiene*	*9,27-Heptatricontadiene***		
$C_{36.6}$				8,28-Heptatricontadiene
$C_{36.7}$	14-, 16- and 12-hexatricontene*			*14-, 16- and 12-hexatricontene**
C_{38}				*C_{38} alkene*

Italicized names indicate best discriminating hydrocarbon components identified in both stepwise and backward selection models. See text for details.

†Equivalent chain length as calculated in Stennett & Etges (1997) or other hydrocarbon name if component not yet identified (Etges & Jackson, 2001).

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

the M♀ × M♂ trials, and few differences in the B♀ × B♂ trials (Table 3), the backward elimination method probably included too many EHCs. Baja males with more 8,26-tetratricontadiene were more successful obtaining copulations in both the B♀ × B♂ and M♀ × B♂ trials (amounts of 8,26-tetratricontadiene were greater in M vs. U males in B♀ × B♂ trials, ANOVA, $F = 5.37$, $P = 0.022$). Except for the relatively minor 7- and 9-hentricontene and C_{33}-branched alkene components, there were no best discriminating EHCs common to both interdemic (M♀ × B♂ and B♀ × M♂) trials (Table 4). Baja males with more C_{34} EHCs mated more often with mainland females, while mainland males with more C_{37} and C_{38} EHCs obtained more copulations with Baja females. The C_{37} alkadienes, including 8,28-heptatricontadiene, have been previously associated with sexual isolation between populations (Markow & Toolson, 1990), but sexual dimorphism in these hydrocarbons varies from Baja California to mainland populations resulting in sex × region interactions (Etges & Ahrens, 2001).

Female EHCs and mating success

A reduced number of female *D. mojavensis* from both intrademic mating trials were assayed for EHC variation permitting analysis of variation among mated and unmated adults. No significant differences were observed between M + F females so these groups were pooled, and CDF analysis was performed based on all 30 EHCs. Unfortunately, small female sample sizes in the heterogametic trials caused singularity of covariance matrices precluding CDF analysis. Unmated females in B♀ × B♂ trials had significantly less EHCs than mated (M + F) females (Wilks' $\lambda = 0.0794$, $F = 3.09$, d.f. = 30,8, $P = 0.049$).

Stepwise DFA identified no best discriminating female EHCs in the B♀ × B♂ and M♀ × B♂ trials. Even with smaller sample sizes, stepwise DFA identified two EHCs in the B♀ × M♂ trials that discriminated among mate and unmated females; 10-, 12- and 14-tetretricontene and 8,28-heptatricontadiene. The latter C_{37} alkadiene was also implicated as a best discriminating EHC among males (Table 5), suggesting amounts of this EHC are associated with mating success in both sexes.

Discussion

The strength of sexual isolation driving premating isolation among geographically isolated populations of *D. mojavensis* is clearly related to the differences in EHC quantities between mated and unmated males, but intrademic sexual selection was so weak as to be generally undetectable in this study. In both kinds of interdemic mating trials (B♀ × M♂ and M♀ × B♂), EHCs were significantly different between mated and unmated males and mating success was nonrandom resulting in significant pair sexual selection, *PSS* (Table 1). Since mating success in these two trials was associated with differences in EHCs among mated and unmated males, but not in the within population trials (Fig. 1), we reject the hypothesis that sexual isolation among Baja California and mainland populations of *D. mojavensis* has resulted from within population sexual selection

(cf. Carson, 2000). There have been no previous attempts to measure sexual selection based on EHCs in *D. mojavensis* and previous studies of sexual isolation have used numbers of observed copulations only (cf. Zouros & d'Entremont, 1980; Markow, 1991; Brazner & Etges, 1993). Certainly, further study of EHC-based sexual selection should be broadened to include more populations of *D. mojavensis*, increased numbers of both sexes, and other cactus substrates. Sexual selection could also operate on male courtship song variation as several courtship song traits were significantly associated with mating success in male Baja-mainland F_2 hybrids (Etges *et al.*, 2007). Nevertheless, using the four types of mating trials revealed little evidence of EHC-related sexual selection, and a clear role for a small set of EHCs responsible for premating isolation between allopatric populations of *D. mojavensis* considered to be incipient species.

How many EHCs function as contact pheromones?

Most of the EHC components identified in *D. mojavensis* are sexually dimorphic and vary geographically between Baja California and mainland populations (Etges & Ahrens, 2001), yet only a handful of these EHCs seem to act as pheromones, primarily C_{34} and C_{37} alkadienes (Table 5). Several C_{33}, C_{35} and C_{37} alkadienes discriminated among mated vs. unmated males in the M♀ × M♂ trials despite there being no overall differences among groups. Differences in amounts of the C_{34} alkadiene, 8,26-tetratricontadiene, in both the B♀ × B♂ and M♀ × B♂ trials between malted and unmated males (Table 5) provided the strongest evidence for a single EHC that mediated mating success. Baja females mated with Baja and mainland males with nonoverlapping EHC profiles suggesting male recognition does not require the same EHCs. Therefore, sexual isolation between mainland and Baja populations of *D. mojavensis* involves multiple EHCs as in other *Drosophila* species (Oguma *et al.*, 1992; Nemoto *et al.*, 1994; Blows, 2002) and there was no general pattern to the intrademic vs. interdemic roles of individual EHCs.

Courtship latency and mating success

It was possible to identify contrasting patterns of EHC amounts in mated and unmated adults by considering each of the four mating trials as independent groups. Although fewer females were available for analysis (many samples were lost because of contamination), some EHCs like 8,28-heptatricontadiene differed among mated and unmated of both sexes. The *post hoc* multivariate contrasts among groups for each mating trial (Table 3) revealed significant differences in EHC profiles that pooling trials had obscured, particularly the largest sources of variation in the heterogametic trials. Further, EHC differences between the F and U groups of males were not initially apparent. In both the B♀ × M♂ and M♀ × B♂ trials, there were significant differences between EHC profiles of F vs. U males, but marginal or insignificant differences between M vs. U males (Table 3). Even though there were no significant EHC differences between F and M males, these F males were able to achieve more rapid, successful copulations, suggesting that these males were especially attractive to females, and that this attractiveness was EHC related.

EHCs are part of the mate recognition system of male and female *D. mojavensis*

Previous evidence for the pheromonal role of EHCs came from 'perfuming' experiments, where EHCs were transferred from mainland males to Baja California males (Etges & Ahrens, 2001). By enclosing flies in a small space for several days, fly activity causes transfer of EHCs to other flies (Coyne *et al.*, 1994). Baja males 'perfumed' with mainland EHCs showed increased levels of transferred mainland EHCs and corresponding greater mating success with mainland females compared with controls. Together, these results suggest that EHCs are part of the mate recognition system of male and female *D. mojavensis* that mediate mating success in the terminal phases of courtship when there is physical contact between prospective mates (Spieth, 1952; Krebs & Markow, 1989; Alonso-Pimentel & Tobin, 1992) similar to other *Drosophila* species (Antony & Jallon, 1982; Oguma *et al.*, 1992; Tompkins *et al.*, 1993; Howard *et al.*, 2003).

The population specific preferences of Baja vs. mainland females for contrasting EHC amounts (Fig. 1) may also help to explain the contrasting sex-specific differences in EHC amounts across the species range. Across a broad range of cactus-reared Baja and mainland populations, almost all EHCs were sexually dimorphic, but total hydrocarbons per fly were equivalent (Etges & Ahrens, 2001). However, variation in eight of these EHCs was influenced by significant sex by region (Baja vs. mainland) interaction terms from ANOVA, indicating sexual dimorphism in EHCs varied geographically (Fig. 2). Such sex by region interactions can be generated by significant differences in EHC amounts in one or both regions. Closer inspection of these interactions shows that most of them were because of large decreases in EHC amounts in mainland males relative to females, with gender differences among Baja adults being much smaller (Fig. 2). Many of these EHCs in the present study were significantly correlated with the first canonical variate (Fig. 1, Table 4), including two of the best discriminating C_{37} EHCs, 14-, 16- and 12-hexatricontene and 8,28-heptatricontadiene (Table 5). Thus, EHC-related differences in mating success (Fig. 1) were consistent with the larger, overall pattern of EHC differences between Baja California and mainland Mexico populations where sexual dimorphism in

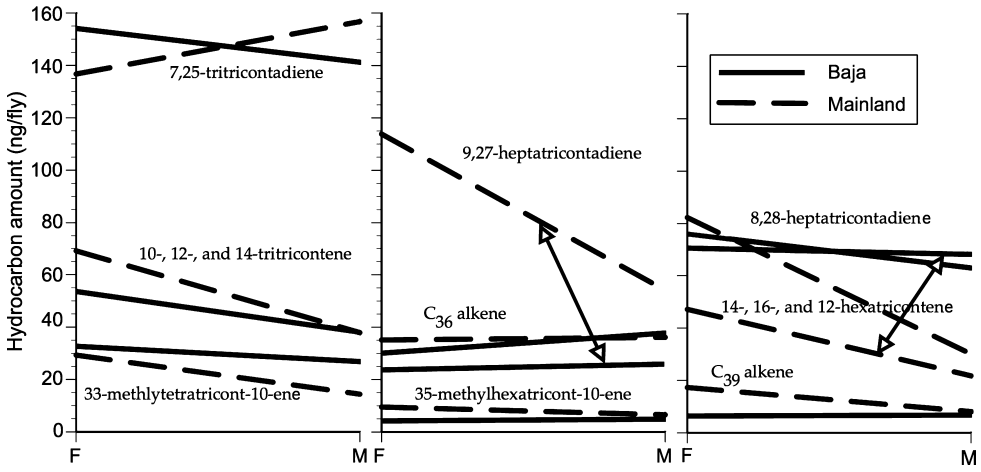

Fig. 2 Plots of the epicuticular hydrocarbons (EHCs) showing significant sex × region interactions in the population survey in Etges & Ahrens (2001). Female (F) and male (M) EHC amounts are plotted as pairs of Baja and mainland lines as reaction norm plots. Arrows connect line pairs that are separated by different EHCs. The plot of C_{39} was included showing similar trends, although this sex × region interaction was marginally significant ($P = 0.09$).

mainland adults is greater than in Baja California adults (Etges & Ahrens, 2001; Fig. 2).

The causes for these region-specific EHC differences remain unresolved. Determining whether EHC profiles diverged because of genetic drift in allopatry since *D. mojavensis* invaded the mainland, adaptation to different environments such as host plant shifts, or to the presence of *D. arizonae* requires further study. Experiments with *D. arizonae* have yet to be devised because these species hybridize in the lab (Wasserman & Koepfer, 1977; Markow, 1981) precluding experimental tests of character displacement (cf. Higgie *et al.*, 2000). Among natural populations, EHC differences in *D. mojavensis* and *D. arizonae* are accentuated in sympatry, but only one Baja California population of *D. mojavensis* was studied (Etges & Jackson, 2001). The sex × region interaction terms together with evidence for sexual selection in the between-population mating trials suggests that pheromone divergence probably did not arise from intrademic sexual selection (Carson, 2000; Blows, 2002) given the overall lack of EHC differences between mated and unmated males in the within-population trials (Table 3, Fig. 1), but more populations need to be studied.

The kinds of signalling systems that evolve early in the process of species divergence are likely to be lineage and environment dependent (see Etges *et al.*, 2007 for a review). Both contact pheromones, EHCs and courtship songs have diverged in geographically isolated populations of *D. mojavensis* and cause differences in mating success. Thus, early in the process of divergence, these signalling systems have evolved with no apparent postmating isolation as *D. mojavensis* expanded its range by switching to alternate host cacti (Heed, 1982), and the interdemic sexual isolation that has resulted is multimodal, including courtship songs and EHCs. While extensive sex-specific and geographic EHC diversification has occurred during this process, a small number EHCs seem to be associated with overall mating success mediated by interdemic (B♀ × M♂ and M♀ × B♂) sexual selection (Table 2). Mate recognition and mating success within local populations mediated by sexual selection seem to have had undetectable consequences for sexual isolation between demes. The relative strengths of courtship songs and EHCs in determining mating success still needs to be evaluated, as well as further genetic analysis of EHC differences, but the few EHCs responsible for mate recognition suggest that analysis of incipient speciation in *D. mojavensis* will be possible by focusing on a well-defined set of acoustic signals and contact pheromones that are driving divergence.

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