Premating Isolation is Determined by Larval Rearing Substrates in Cactophilic *Drosophila mojavensis*. VII. Effects of Larval Dietary Fatty Acids on Adult Epicuticular Hydrocarbons

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Abstract Low concentrations of dietary triacylglycerols including tristearin, triolein, and tripalmitolein, were assessed to determine their effects during egg to pupal stages on adult epicuticular hydrocarbon (EHC) variation in cactophilic *Drosophila mojavensis*. Triacylglycerols were added singly and in combination at concentrations of 1%, 3%, and 9% to a lipid-free culture medium. Control diets included Carolina Drosophila and lipid-free media. Egg to adult viability was reduced at triacylglycerol concentrations greater than 1%, except for tristearin. Both triolein and tripalmitolein increased EHC amounts to levels similar to those in combination and control diets. Tristearin caused significantly lower quantities of EHCs in adult flies than triolein or tripalmitolein, consistent with previous studies on reduced tristearin assimilation into adult EHCs. We rejected the hypothesis that unsaturated and saturated triacylglycerols were assimilated into unsaturated and saturated adult EHCs, respectively. Since these triacylglycerols comprise a fraction of known lipids in the columnar cacti used for breeding in nature, and EHCs serve as contact pheromones in *D. mojavensis*, these and other naturally occurring triacylglycerols may provide a direct causal link between host plant use and patterns of chemically mediated mate choice.

Keywords Cactus · Cuticular hydrocarbons · Desert · *Drosophila* · Fatty acids · Lipids · Mating behavior · Sexual isolation · Triacylglycerols

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Introduction

Acoustic and chemical signals can play important roles during mate choice in many insects, including *Drosophila* species (Gerhardt and Huber, 2002). Pheromonal signaling systems can influence both reproductive isolation and speciation, in addition to influencing proximate decisions during courtship (Greenfield, 2002). It is of considerable interest to identify particular mate–mate signals that influence mating success in local populations and those that may determine isolation between populations or species. In some cases, mate recognition systems can influence both sexual selection and sexual isolation (Boake et al., 1997; Carson, 2000). Understanding both the genetic and environmental influences on expression of these signaling systems should help to reveal the nature of genetic differences in mate recognition systems and their evolution (Etges, 2002).

In many *Drosophila* species, epicuticular hydrocarbons (EHC) are thought to serve as contact pheromones as well as to enhance desiccation resistance (Blomquist et al., 1987). Although the genetic basis of some hydrocarbon components is partially understood in some drosophilids (reviewed in Gleason et al., 2005), genetic and environmental influences on production and deposition (Wigglesworth, 1988) are poorly known for most species. Once revealed, the structure and function of individual hydrocarbon genes alone will be insufficient without knowledge of how EHCs are expressed in natural or experimental populations subject to potential environmental modification. We seek to gain a further understanding of the role of lifetime environmental influences on genetic variation in EHC production in insects and how this EHC variation influences behavior, mate choice, sexual selection, and sexual isolation in nature (e.g., Toolson and Kuper-Simbron, 1989; Howard et al., 1995; Howard, 1998; Liang and Silverman, 2000; Woodrow et al., 2000; Savarit and Ferveur, 2002; Buczkowski et al., 2005).

Here, we investigate the role of dietary triacylglycerols in larval *Drosophila mojavensis* on subsequent adult EHC profiles. Differences in natural larval feeding substrates vs. synthetic laboratory media influence EHC abundance in this species (Stennett and Etges, 1997). These differences also influence adult mating preferences (Etges, 1992, 1998; Brazner and Etges, 1993). In *D. mojavensis*, the function of cuticular lipids seems unrelated to desiccation resistance by reducing water loss (Gibbs et al., 1998), underscoring their role, and probable maintenance, as contact pheromones (Etges and Ahrens, 2001). We hypothesize that identifying substrate-specific precursors that influence adult EHC profiles will help to resolve host plant influences on mating behavior, as well as identify ecological sources of variation on patterns of reproductive isolation in nature.

Fatty acid metabolism and EHC biosynthesis have been well studied in *D. melanogaster* and should help in understanding lipid metabolism in *D. mojavensis.* Adult EHC production involves modification of fatty acid precursors, but little is known about the influences of fatty acids in larval diets on adult EHCs. Production of adult *D. melanogaster* sex pheromone (*Z,Z*)-7,11-heptacosadiene starts from either acetate or from medium-sized fatty acids (Chan Yong and Jallon, 1986; Jallon et al., 1986). From preformed fatty acids produced by fatty acid synthetase (de Renobales and Blomquist, 1984), palmitate is a better substrate than stearate and is desaturated into palmitoleate (ω-7) (Ferveur et al., 1989) by the *desat1* gene product (Wicker-Thomas et al., 1997; Dallerac et al., 2000; Labeur et al., 2002). From palmitoleate there is elongation to *cis*-vaccenate and subsequently several elongations, a second desaturation (the substrate of which has not yet been characterized) by another desaturase (Wicker-Thomas and Jallon, 2001; Jallon and Wicker-Thomas, 2003), and a subsequent decarboxylation (Jallon, 1984; Pennanec'h et al., 1997). Fatty acid

analysis of sexually mature *D. melanogaster* indicates high percentages of palmitoleate, but lesser quantities of oleate and *cis*-vaccenate, with oleate and linoleate decreasing as (*Z*,*Z*)-7,11-heptacosadiene synthesis occurs (Pennanec'h et al., 1997). Very long chain (C_{20}, C_{22}, C_{24}, and C_{26}) fatty acids with apparent ω-7 unsaturation and female dienoic acids with 22 and 24 carbons have also been detected (Pennanec'h et al., 1997). Together, these experiments and those of other groups (Keith, 1967a,b; Pennanec'h et al., 1991) suggest that fatty acids, either from biosynthesis or the diet, impact the biosynthesis of pheromones and other dipteran EHCs.

We hypothesize that different fatty acids in the cactus diets of *D. mojavensis* can change pheromone and other adult EHC profiles. A number of different hosts are used by *D. mojavensis*, including agria cactus, *Stenocereus gummosus*, in Baja California and organ pipe cactus, *S. thurberi*, in mainland Mexico and Arizona (Heed and Mangan, 1986). Fatty acid profiles of both agria and organ pipe cactus are mainly characterized by caproic (C_{10}) and lauric (C_{12}) acids in concentrations of 34–45%, with much lower concentrations of caprylic acid (C_8) and others in sizes up to C_{18} (Fogleman and Kircher, 1986). There are no overall significant differences between cactus species with respect to fatty acid composition (Fogleman and Kircher, 1986). However, cactus fatty acid analysis shows greater quantities of unsaturated fatty acids (12:1, 12:2, and 18:1) in agria vs. organ pipe cactus, but the location of double bonds has not been determined. It may be that minor quantities of unsaturated fatty acids in the diet of *D. mojavensis* influence biosynthesis of unsaturated EHCs, so we employed both saturated and unsaturated triacylglycerols to determine whether they influence EHC profiles of adult *D. mojavensis*.

EHCs of adult *D. mojavensis* are composed of C_{28} to C_{40} alkanes, 2-methylalkanes, alkenes, methyl-branched alkenes, and multimethylene interrupted alkadienes, with the largest fraction of adult hydrocarbons composed of C_{35} alkadienes (Toolson et al., 1990; Stennett and Etges, 1997; Etges and Jackson, 2001). We hypothesize that the EHCs of *D. mojavensis* are derived from three fatty acid pools: ω-9, ω-7, and saturated fatty acids. The ω-9 fatty acids are precursors of the 9-alkenes and the 8, *X*-alkadienes (e.g., $C_8=C_{16}=C_9$, $C_8=C_{18}=C_9$, $C_8=C_{20}=C_9$, etc.) since they are really 9, *X*-alkadienes counting from the other end. The ω-7 fatty acids are precursors to the 7-alkenes and the 7,*X*-alkadienes (e.g., $C_7=C_{16}=C_{10}$, $C_7=C_{18}=C_{10}$, $C_7 = C_{20}=C_{10}$, etc.). The saturated fatty acids are precursors to the alkanes, and the 10-, 12-, and 8-alkenes also result from saturated fatty acids when desaturation occurs late in the biosynthetic pathway.

Given the previously described fatty acid profiles in agria and organ pipe cactus, and the substrates known to be involved in EHC biosynthesis, we used tripalmitolein (C16:1, ω-7), tristearin (C18), and triolein (C18:1, ω-9) in differing concentrations in larval diets to assess their influences on egg to adult viability and on adult EHC composition in two populations of *D. mojavensis*.

Methods and Materials

Fly Stocks

Populations of *D. mojavensis* were collected in 1996 from south of Santa Rosalia, Baja California Sur (stock no. A975, 15 adults), and El Fuerte, Sinaloa (stock no. A991, 185 adults), over baits or by collecting adults emerging from fermenting cactus tissues, "rots," returned to the laboratory. All flies were maintained on banana–malt–yeast–agar food at room temperature in mass cultures (Brazner and Etges, 1993). Several hundred adults from

each population were placed in separate oviposition chambers and kept in an incubator programmed for a 14:10 L/D photoperiod and a temperature cycle of 27:17°C. Eggs were collected daily on oviposition media from 8:00 AM to 6:00 PM in removable Petri dishes attached to each chamber. Eggs were washed with deionized water, 70% ethanol, and surface sterilized for 10 min in 70% ethanol. Eggs were then counted in sterile, deionized water in groups of 100 onto 1-cm^2 pieces of sterile filter paper and placed into vials containing Drosophila medium (see below). Filter paper was removed after 24–48 hr, and numbers of unhatched eggs were counted in order to calculate egg to adult viability.

Food vials were prepared with standard Instant Drosophila Medium® formula 4–24 (Carolina Biological Supply Co., Burlington, NC, USA); or with "defatted" medium (both controls), as well as by adding different concentrations of triacylglycerols (TAGS) to the dry, homogeneous defatted diet. For the defatted medium, lipids were removed from the standard medium by Soxhlet extraction with hexane. We prepared 9% stock solutions of each triacylglycerol. Triolein (ω-9), tripalmitolein (ω-7), and tristearin in pentane were added to 2 g of dry defatted diet in final concentrations of 1%, 3%, and 9%. Each 2-g aliquot of food was completely dried under a fume hood, added to a sterile 8 dram shell vial, and rehydrated with an equal volume of sterile, deionized water. Combinations of equal amounts of all three TAGs were also prepared so that the final concentrations of the three combined would be 1%, 3,% and 9%. These latter treatments were prepared to test the hypothesis that all three TAGS should produce EHC profiles more like the standard or defatted diets, but at comparable concentrations to the single TAG treatments. Two replicate cultures of each TAG concentration were created for each population of *D. mojavensis*, and six replicates were included for both the standard and defatted diets. Live baker's yeast was added to each vial *ad libidum* to insure larval survivorship.

Epicuticular Hydrocarbon Analyses

All eclosed adults were aspirated from each culture vial daily, sexed under light CO_2, and placed into separate vials containing banana–yeast–malt–agar food for at least 14 d and then frozen in hexane-rinsed glass vials at −20°C. EHCs were extracted from groups of males and females in Biosil minicolumns. Each column consisted of a Pasteur pipette containing packed glass wool and Biosil (silica gel, Sigma™ S-4133, Sigma-Aldrich, St. Louis, MO, USA) flushed several times with HPLC-grade hexane. Flies were added to the top of the column, washed in 8 ml of hexane, and the hydrocarbons were collected in hexane-rinsed vials. After the hexane was evaporated with nitrogen, each sample was sealed and stored at −20°C. When only one or two flies were available from some replicates, we used hydrocarbon extraction procedures described by Savarit et al. (1999). Flies were placed in conical glass microvials and immersed in 50 μl hexane per fly. The vials were agitated, and after 20 min, flies were removed, the hexane was evaporated off, and each vial was frozen.

Each hydrocarbon sample was redissolved in hexane (2 μl/fly) containing 382 ng of docosane (C_{22}) per μl as an internal standard with the exception of samples containing only one or two flies, which were redissolved in 5 μl hexane with the same concentration of internal standard. One μl of each sample was analyzed by capillary gas–liquid chromatography using a Shimadzu GC14 (Shimadzu Scientific Instruments, Columbia, MD, USA) fitted with a 30 m DB-1 fused silica column (Agilent Technologies, Palo Alto, CA, USA). Injector and detector temperatures were set at 345°C with the injector port in split mode (split ratio=100:1) and the column was heated from 200°C to 345°C at 10°C/min holding at 345°C for 5 min (Stennett and Etges, 1997).

Statistical Analyses

Egg to adult viability was calculated as the number of eclosed adults divided by the number of hatched eggs. Variation in viability due to substrate and TAG concentration was assessed with analysis of variance (ANOVA; SAS Institute, 2004). Viability data were arcsin transformed prior to analysis.

EHC amounts were estimated by analysis of peak integrations using EZCHROM software (ver. 2.1) provided by Shimadzu. Each sample amount was normalized by the amount of internal standard. Replicate groups of flies were analyzed together. All data were expressed as ng/fly of EHCs and were analyzed by ANOVA and multivariate analysis of variance (MANOVA) using PROC GLM in SAS (SAS Institute, 2004) with population, TAG, TAG concentration, and sex as main effects and all interactions between main effects. Population and all interactions with population were considered random effects.

Post-hoc multiple comparisons were evaluated, and canonical correlation analysis in PROC GLM and CANDISC was employed to reveal EHC responses to different TAGs. Contrasts among diet treatments were also carried out using EHCs grouped by chemical structure according to Etges and Jackson (2001).

Results

Effects of Triacyglycerols on Egg to Adult Viability

Type of TAG, concentration of TAG, and their interactions had significant effects on egg to adult viability in these two populations of *D. mojavensis* (Fig. 1, Table 1). Overall, flies reared on standard medium, defatted medium, or defatted medium with tristearin had equivalent viabilities that were significantly higher than those from flies reared on defatted medium with triolein, tripalmitolein, or combinations of all three TAGs (Tukey's multiple range test, $P<0.05$). Except for tristearin, concentrations of TAGs exceeding 1% drastically reduced viability: egg to adult viability of controls (mean±1 SD on standard diet=0.47± 0.21; on defatted diet=0.57 ± 0.30) and of defatted diet with all three TAGs at 1% (0.24± 0.10) were higher than viabilities for diets with TAGs at 3% and 9% (both 0.18; Tukey's multiple range test, $P<0.05$). Egg to adult viability of laboratory populations of *D. mojavensis* reared on fermenting cactus or banana food tend to be much higher (>0.80, see Etges and Ahrens, 2001) than on standard diet, suggesting that the latter medium reduced preadult fitness in these experiments. Similar viabilities of flies reared on standard and defatted diets were surprising given the removal of lipids in the latter (Fig. 1), but may have been mediated by the addition of live baker's yeast.

At triolein and tripalmitolein concentrations of 3% and 9%, very few flies survived to eclosion (Fig. 1), with many never making it to the pupal stage. At these higher concentrations, flies that did eclose seemed to be unable to initiate cuticle hardening and died before wing expansion.

Effects of Triacylglycerols on Adult Epicuticular Hydrocarbons

Differences in EHC amounts due to TAG type were apparent for all but six of the 25 EHC components assayed (Table 2 for quantities and Appendix for ANOVA of the 20 most abundant EHCs). The ANOVA results were based on all TAGs employed, plus combinations of them at each concentration for which flies eclosed. The two control media were not included in these ANOVAs so we could focus on TAG differences. In most cases,
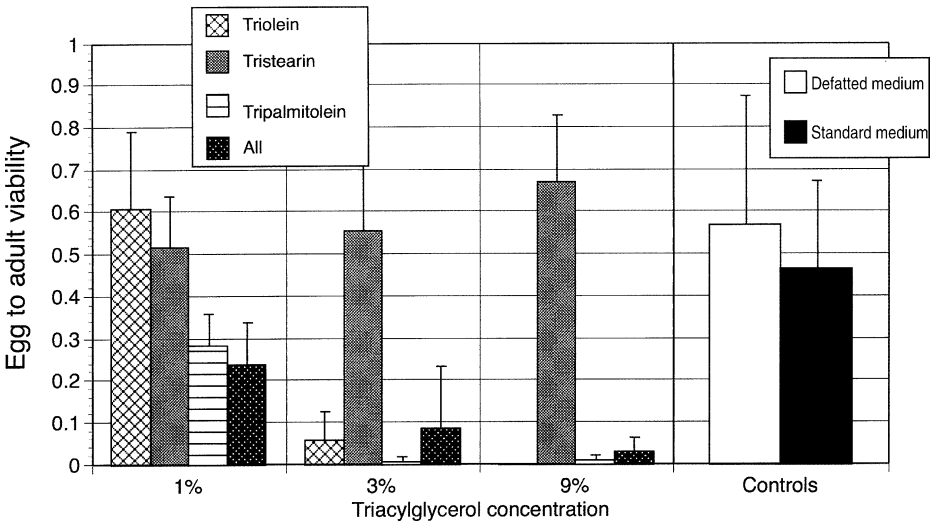
Fig. 1 Egg to adult viability (±1 SD) of two populations of *Drosophila mojavensis* cultured on defatted media with 1%, 3%, and 9% triacyglycerols alone and in combination. Live Baker's yeast was added to all cultures when initiated. See the text for details

EHC amounts were lowest for flies reared on defatted media, but similar among flies reared on standard medium and defatted media supplemented with TAGs (Table 2). Missing data due to high or total egg to adult mortality, particularly in the 3% and 9% tripalmitolein-containing cultures, meant that TAG type and concentration were somewhat confounded in the ANOVAs. This may partially explain the lack of a concentration effect in these analyses, except in a few cases.

EHC differences due to TAG type were overlaid on the known population differences that in some cases resulted in Population × TAG interactions (Appendix). Consistent with most previous studies, 13 of the EHC components differed between the Baja California and Sinaloa (mainland) populations, especially 2-methyloctacosane ($C_{28.65}$), 8,24-tricontadiene ($C_{33.63}$), 9,25-pentatricontadiene ($C_{34.59}$), and 9,27-heptatricontadiene ($C_{36.5}$). The latter three alkadienes showed characteristic regional differentiation in amounts, where mainland populations all expressed much greater amounts of these EHCs than Baja California populations (Stennett and Etges, 1997; Etges and Ahrens, 2001). Two of these EHCs, $C_{33.63}$ and $C_{34.59}$, exhibited Population × TAG interactions suggesting that even though amounts

Table 1 ANOVA results for the effects of triacylglycerol type and concentration on egg-to-adult viability in two populations of *Drosophila mojavensis*[a]

Source	df	Type III SS	F	P
Population	1	0.118	2.28	0.140
Triacylglycerol (TAG)	4	2.375	11.42	<0.001
Concentration	2	0.641	6.19	0.005
Population × TAG	5	0.247	0.95	0.461
TAG × Concentration	6	0.866	2.78	0.025
Population × TAG × Concentration	8	0.084	0.20	0.989
Error	36	1.871		

[a] Both control substrates were included in this analysis and all data were arcsin transformed prior to analysis.

Table 2 Quantities of the major epicuticular hydrocarbons in *Drosophila mojavensis* (ng/fly)[a]

Hydrocarbon (ECL)[c]	Group[d]	Diet[b]											
		Standard	N	Defatted	N	Triolein	N	Tristearin	N	Tripalmitolein	N	Combination	N
n-Octacosane (C$_{28}$)	M fem	6.93	6	9.37	2	10.33	3	5.35	6	11.90	4	0.61	1
	M mal	3.62	5	9.88	2	9.30	3	3.41	4	0.0	2	16.91	2
	B fem	2.24	5	2.80	2	3.36	3	6.38	6	0.89	2	2.84	6
	B mal	2.44	6	4.24	2	2.97	3	4.54	6	1.04	2	0.58	5
2-Methyloctacosane (C$_{28.65}$)	M fem	65.73	6	40.64	2	81.53	3	64.79	6	102.87	4	89.96	4
	M mal	84.10	5	57.92	2	92.22	3	58.73	4	107.93	2	109.96	2
	B fem	52.22	5	59.56	2	81.63	3	46.96	6	57.54	2	74.29	6
	B mal	37.69	6	28.81	2	42.03	3	26.08	6	59.81	2	47.66	5
2-Methyltricontane (C$_{30.65}$)	M fem	74.05	6	55.49	2	83.09	3	77.55	6	136.06	4	79.76	1
	M mal	104.75	5	83.68	2	116.44	3	82.35	4	135.11	2	117.03	2
	B fem	114.65	5	107.78	2	147.18	3	98.59	6	116.39	2	116.54	6
	B mal	104.48	6	69.45	2	77.57	3	58.10	6	148.58	2	89.83	5
7- and 9-Hentricontene (C$_{30.78}$)	M fem	11.17	6	9.81	2	12.19	3	8.72	6	10.21	6	14.45	4
	M mal	12.21	5	5.72	2	11.27	3	7.07	4	14.81	4	19.45	2
	B fem	8.50	5	11.81	2	20.00	3	11.77	6	8.54	6	12.91	2
	B mal	7.55	6	6.00	2	10.46	3	5.67	6	8.24	6	8.10	2
Unknown (C$_{33br1}$)	M fem	11.89	6	9.75	2	10.38	3	8.71	6	15.07	6	15.42	4
	M mal	11.72	5	12.02	2	11.87	3	7.90	4	15.96	4	12.29	2
	B fem	5.99	5	6.18	2	5.92	3	6.29	6	3.77	6	6.74	2
	B mal	4.24	6	4.71	2	5.58	3	3.96	6	4.96	6	5.79	2
11-and 13-Methyldotricontane (C$_{33br2}$)	M fem	13.62	6	10.35	2	13.32	3	15.04	6	15.12	6	17.4	4
	M mal	13.72	5	11.21	2	13.12	3	7.82	4	21.19	4	14.27	2
	B fem	6.26	5	8.18	2	6.50	3	6.82	6	5.29	6	7.86	2
	B mal	4.13	6	4.37	2	5.98	3	4.00	6	5.79	6	5.42	2
31-Methyldotricont-8-ene (C$_{32.47}$)	M fem	51.93	6	33.66	2	47.94	3	56.32	6	63.64	6	62.59	
	M mal	66.81	5	51.19	2	58.92	3	37.01	4	74.78	4	73.43	2
	B fem	42.53	5	52.67	2	67.37	3	44.86	6	43.79	6	51.55	6
	B mal	34.13	6	26.61	2	43.63	3	27.34	6	50.93	6	40.83	5

Table 2 (continued)

Hydrocarbon (ECL)[c]	Group[d]	Diet[b]											
		Standard	N	Defatted	N	Triolein	N	Tristearin	N	Tripalmitolein	N	Combination	N
8,24-Tricontadiene (C$_{32.63}$)	M fem	23.89	6	14.89	2	26.93	3	23.13	6	29.67	4	50.56	1
	M mal	45.91	5	30.05	2	32.95	3	27.95	4	38.87	2	63.86	2
	B fem	9.29	5	13.80	2	17.39	3	10.17	6	9.78	2	14.34	6
	B mal	8.57	6	6.89	2	13.41	3	6.84	6	13.25	2	11.60	5
7,25-Tricontadiene (C$_{32.70}$)	M fem	44.30	6	29.83	2	38.01	3	44.91	6	65.43	4	60.78	1
	M mal	67.41	5	44.83	2	40.96	3	37.42	4	74.63	2	87.85	2
	B fem	36.35	5	46.33	2	56.33	3	38.48	6	42.40	2	44.89	6
	B mal	29.68	6	23.62	2	26.69	3	22.85	6	51.06	2	29.23	5
10-, 12-, and 14-Tritricontene (C$_{32.79}$)	M fem	28.95	6	18.23	2	24.28	3	25.30	6	25.24	4	37.39	1
	M mal	26.59	5	12.24	2	19.11	3	12.45	4	32.05	2	45.68	2
	B fem	12.27	5	17.53	2	23.57	3	12.66	6	12.40	2	17.44	6
	B mal	7.79	6	6.95	2	9.17	3	6.22	6	14.09	2	9.43	5
8,26-Tetracontadiene (C$_{34diene1}$)	M fem	4.29	6	1.71	2	4.16	2	3.49	6	6.55	3	5.35	1
	M mal	7.91	5	8.35	2	7.72	2	4.71	4	6.48	2	12.32	2
	B fem	5.78	5	2.16	2	5.58	2	2.46	6	3.77	2	2.58	6
	B mal	5.02	6	3.66	2	4.92	2	3.29	6	4.19	2	3.55	5
6,26- and 6,24-Tetracontadiene (C$_{34diene2}$)	M fem	16.52	6	7.40	2	11.50	3	11.24	6	18.96	3	16.87	1
	M mal	26.97	5	18.32	2	18.89	3	16.78	4	28.57	2	43.75	2
	B fem	10.23	5	12.57	2	19.36	3	8.37	6	9.69	2	12.93	6
	B mal	14.45	6	11.35	2	20.54	2	10.55	2	21.65	2	17.45	5
10-, 12-, and 14-Tetratricontene (C$_{34ene}$)	M fem	5.02	6	3.71	2	3.66	3	3.22	3	8.43	3	4.69	1
	M mal	15.08	5	6.41	2	8.72	3	5.59	3	7.89	2	12.99	2
	B fem	6.47	5	12.22	2	23.19	3	8.68	3	8.04	2	11.64	6
	B mal	21.14	6	7.11	2	12.72	2	8.43	2	20.64	2	13.95	5
33-Methyltetratricont-10-ene (C$_{35ene1}$)	M fem	13.94	6	6.66	2	11.44	3	11.18	6	15.08	4	14.08	1
	M mal	14.99	5	10.26	2	12.28	3	8.34	3	21.23	2	13.00	2
	B fem	12.49	5	8.42	2	12.15	3	8.24	6	9.28	2	10.15	5

Compound	Group[d]	mean	N	mean	N	mean	N	mean	N	mean	N	mean	N
	B mal	10.12	6	4.23	2	5.29	2	5.50	6	15.80	2	9.31	4
33-Methyltetracont-8-ene (C_{35ene2})	M fem	12.83	6	7.22	2	9.91	3	10.59	6	16.65	4	11.77	1
	M mal	16.17	5	10.88	2	15.25	3	7.97	4	19.59	2	13.67	2
	B fem	19.35	5	15.52	2	19.36	3	15.52	6	19.46	2	15.70	5
	B mal	23.41	6	10.85	2	12.05	2	11.42	6	30.64	2	19.63	4
9,25-Pentatriacontadiene ($C_{34.59}$)	M fem	119.17	6	53.13	2	128.50	3	118.84	6	196.25	4	192.53	1
	M mal	251.47	5	131.32	2	194.74	3	129.08	4	174.05	2	276.47	2
	B fem	48.79	5	62.75	2	104.00	3	35.65	6	39.46	2	65.30	6
	B mal	40.47	6	42.14	2	89.88	3	36.26	6	50.14	2	45.67	5
8,26- and 7,27-Pentatriacontadiene ($C_{34.66}$)	M fem	235.63	6	150.38	2	211.04	3	196.62	6	370.85	4	264.95	1
	M mal	360.07	5	273.21	2	325.89	3	195.64	4	335.62	2	410.35	2
	B fem	285.24	5	379.67	2	621.31	3	299.10	6	241.24	2	386.02	6
	B mal	312.52	6	245.37	2	409.28	3	227.41	6	448.76	2	287.34	5
9,27-Heptatriacontadiene ($C_{36.5}$)	M fem	27.11	6	9.45	2	28.94	3	18.84	6	27.96	4	25.21	1
	M mal	26.29	5	14.02	2	21.75	3	12.68	4	26.94	2	29.46	2
	B fem	7.38	5	11.40	2	18.67	3	8.70	6	6.88	2	11.45	6
	B mal	10.62	6	7.60	2	12.01	3	5.99	6	14.26	2	6.91	5
8,28-Heptatriacontadiene ($C_{36.6}$)	M fem	48.30	6	22.37	2	30.96	3	33.98	6	60.23	4	37.61	1
	M mal	31.00	5	21.48	2	27.59	3	16.08	4	42.04	2	28.71	2
	B fem	22.32	5	26.55	2	59.59	3	17.94	6	26.04	2	30.55	6
	B mal	24.05	6	11.33	2	34.33	3	11.91	6	32.25	2	21.01	5
14-, 16-, and 12-Hexatriacontene ($C_{36.7}$)	M fem	29.71	6	19.06	2	24.71	3	23.49	6	32.42	4	22.70	1
	M mal	22.76	5	15.78	2	21.48	3	11.41	4	31.78	2	26.89	2
	B fem	33.59	5	39.39	2	82.67	3	34.30	6	27.44	2	43.09	6
	B mal	31.30	6	20.69	2	34.66	3	18.82	6	33.85	2	20.56	5

[a] See Toolson et al. (1990) and Etges and Jackson (2001) for details of the GC-MS analyses and determination of the double bond locations.

[b] Diet refers to the two control diet treatments in this study and the three triacylglycerols (concentrations combined) added to the defatted diet. Combination refers to the combination of all three triacylglycerols. N=number of replicate cultures.

[c] ECL is the equivalent chain length of each hydrocarbon component, if known.

[d] Group definitions are M fem=mainland females; M mal=mainland males; B fem=Baja females; B mal=Baja males.

differed between populations, Sinaloa EHCs responded to the three TAGs far differently than Baja California EHCs (Fig. 2a). In both cases, combinations of the three TAGs increased EHC amounts in the Sinaloa population far more than in the Baja population. For the large $C_{34.59}$ diene component, 9% TAG concentrations vastly reduced the amounts of this EHC as compared to 1% and 3% concentrations (Tukey's multiple range test, $P<0.05$).

Multivariate ANOVA of EHC differences revealed significant variation due to all treatments except for the Population × Sex interaction (Table 3). Here, Roy's Greatest Root is the largest characteristic root or eigenvalue that explains the greatest proportion of variation (percent) in each factor of the MANOVA. The largest eigenvalues for population and sex accounted for 100% of the total variation in EHC abundance, with more complex sources of variation explaining TAG differences given that the first eigenvalue accounted for approximately half of the variation (Table 3). Males had more EHCs than females (likelihood ratio=0.3331, F=4.76, df=24/57, $P<0.001$). Because the data were unbalanced by the absence of flies in the 3% and 9% triolein and tripalmitolein treatments (Fig. 1), concentration effects were confounded with TAG type, and may have spuriously resulted in a significant TAG X concentration interaction term.

The overall pattern of EHC variation due to control and TAG treatments was assessed by calculating Mahalanobis distances between groups in PROC CANDISC (Table 4). Significance of these differences indicated that these dietary treatments almost always caused significantly different EHC profiles, except for a number of comparisons involving control diets (overall variation indicated by Roy's Greatest Root=3.33, F=7.91, df=24/57, $P<0.001$). EHC profiles were different between triolein and tripalmitolein treatments, and among these two diets and all others. Both control diets produced similar EHC profiles, but only the defatted control diet resulted in similar EHC profiles as the combination treatment (P=0.159, Table 4).

Loadings for each diet on the first three canonical variables, accounting for 89.8% of TAG variation, showed clearer similarities between tristearin and control diet treatments (Fig. 2b). The overall effects of tripalmitolein and triolein strongly influenced the effects of the combination diets, i.e., EHC differences due to the three TAGs in combination were more "similar" to the tripalmitolein and triolein centroids than to the tristearin centroid (Fig. 2b). Removing the control diets from this analysis made this tripalmitolein-combination comparison somewhat more apparent (Fig. 2a). Here, population differences were included, revealing some overlap between EHC differences from tristearin-treated Baja California files and those due to the combination diet. Qualitatively, these results suggest that there may have been a greater influence of this unsaturated TAG in the combination diets for this Baja California population than for the Sinaloa population. However, replicate numbers were limited for the Sinaloa population.

Pairwise *post-hoc* comparisons revealed the effects of dietary treatments on EHC profiles (Table 5). Missing data due to the absence of flies in the 3% and 9% triolein and tripalmitolein treatments did not permit the use of the CONTRAST option in the MANOVA for all EHCs together except for one case, so pairwise contrasts between treatments were performed, despite a loss of power (Scheiner, 1993). Only one *post-hoc* comparison was possible, tristearin vs. TAG combination, revealing the nature of this unsaturated TAG on EHC profiles (F=3.62, df=24/7, P=0.035). Least square means were greater for a majority of the EHCs assayed due to the TAG combination diets than tristearin, including $C_{28.65}$, $C_{30.78}$, $C_{32.63}$, $C_{32.70}$, $C_{32.79}$, all C_{34} components, $C_{34.59}$, $C_{34.66}$, and $C_{36.5}$. Thus, although not toxic even at higher concentrations (Fig. 1), tristearin is insufficient alone as a substrate for EHC biosynthesis vs. the combination of the unsaturated and saturated TAGs used here.
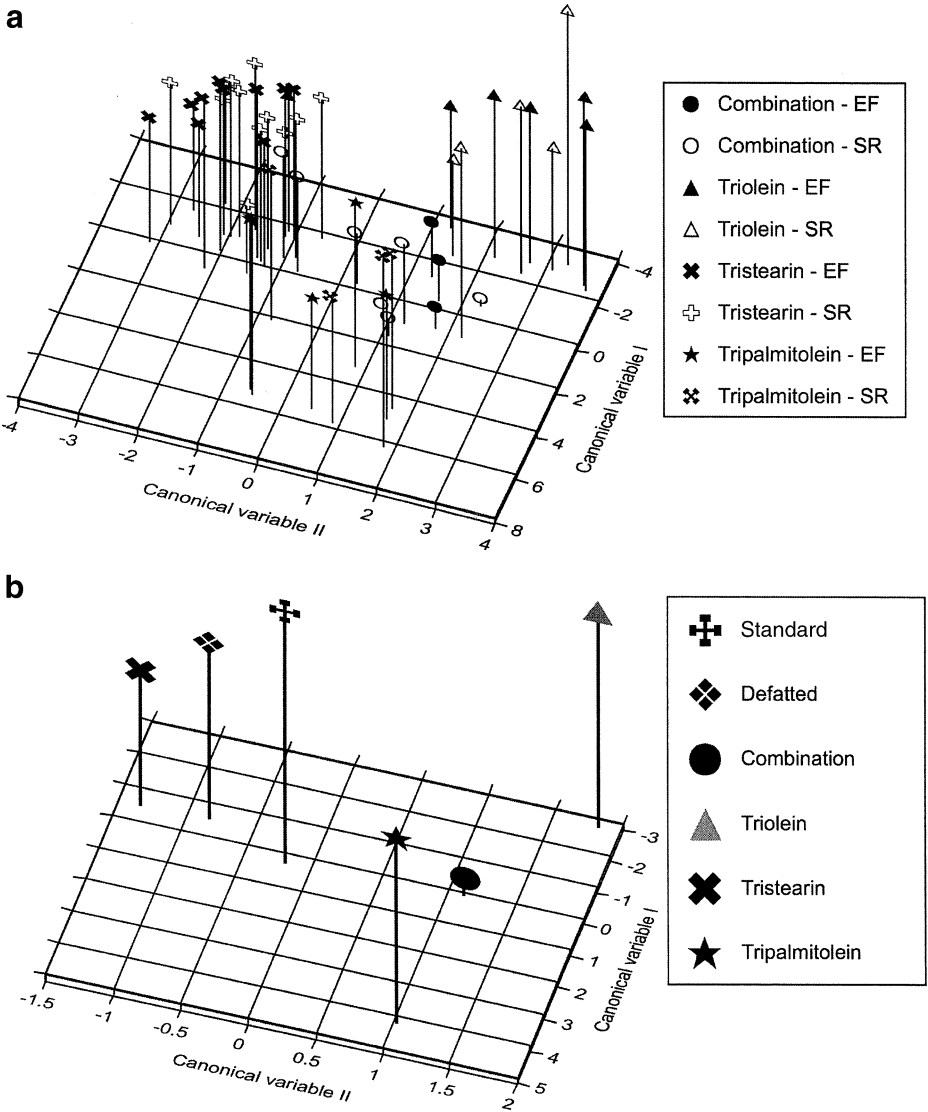
Fig. 2 Canonical discriminant function plot (a) showing the effects of triacylglycerols for each population (EF: El Fuerte, Sinaloa, mainland; SR: Santa Rosalia, Baja California) with the control diet data excluded to better illustrate the effects of the three triacylglycerols on epicuticular hydrocarbon variation. Canonical discriminant function plot (b) showing the effects of the three triacylglycerols alone and in combination, plus the two control diets on variation in epicuticular hydrocarbon profiles for all *Drosophila mojavensis* in this study grouped by population, sex, and triacylglycerol concentration

The remaining pairwise TAG comparisons for EHC profiles caused a reduction in the degrees of freedom for the error term required for significance testing, and this resulted in undefined eigenvalues. To increase degrees of freedom, EHC components were combined into hydrocarbon classes, i.e., alkanes, methylalkanes, alkenes, methylalkenes, and alkadienes (Etges and Jackson, 2001). Grouping the EHCs together by chemical structure

Table 3 MANOVA results for the effects of the experimental treatments on epicuticular hydrocarbon composition in two populations of *Drosophila mojavensis*[a]

Source	Roy's Greatest Root				
	Percent	Root	F	df	P
Population	100.0	47.81	13.94	24/7	<0.001
Triacylglycerol (TAG)	52.1	24.17	9.06	24/9	<0.001
Concentration	77.5	16.92	5.64	24/8	0.008
Sex	100.0	18.17	5.30	24/7	0.015
Population × Sex	100.0	10.42	3.04	24/7	0.067
Sex × TAG	73.1	16.87	6.33	24/9	0.004
Population × TAG	61.1	11.93	4.47	24/9	0.012
TAG × Concentration	43.98	16.86	7.02	24/10	0.001
Population × TAG × Sex	75.54	15.39	5.77	24/9	0.005

[a] Combinations of all three triacylglycerols (TAGs) were included, but control diets were not, in order to assess TAG effects in combination with the other factors.

reduced the dimensionality and perhaps some of the variation in the data (Table 5). Several of the comparisons were not significantly different despite the significance of the Mahalanobis' distances (Table 4). Pairwise *post-hoc* comparisons again showed that most EHC differences were caused by tristearin vs. the unsaturated TAGs (Fig. 2b), suggesting that TAG substrate-induced shifts in adult EHCs may not have necessarily influenced specific classes of hydrocarbons, but caused overall shifts in most types of hydrocarbons (Table 5). Triolein caused greater amounts of all EHCs than tristearin, particularly in 2-methyloctacosane ($C_{28.65}$), 2-methyltricontane ($C_{30.65}$), 7- and 9-hentricontene ($C_{30.78}$), 8,24-tricontadiene ($C_{32.63}$), 9,25-pentatricontadiene ($C_{34.59}$), 8,26- and 7,27-pentatriconta-diene ($C_{34.66}$), and all three major C_{37} components (Table 2 and Tukey's multiple range tests, all $P<0.05$ based on ANOVAs in Appendix). Similarly, tripalmitolein caused greater amounts of all EHCs than tristearin in many of the same EHCs including 2-methyloctaco-sane ($C_{28.65}$), 2-methyltricontane ($C_{30.65}$), 7, 25-tricontadiene ($C_{32.70}$), 10-, 12-, and 14-tritricontene ($C_{32.79}$), 10-, 12-, and 14 tetratricontene (C_{34ene}), 9,25-pentatricontadiene ($C_{34.59}$), 8,26- and 7,27-pentatricontadiene ($C_{34.66}$), 9,27-heptatricontadiene ($C_{36.5}$), and 8,28-heptatricontadiene ($C_{36.6}$) (Table 2 and Appendix, Tukey's multiple range tests, $P<$

Table 4 Pairwise probabilities for squared mahalanobis distances among *Drosophila mojavensis* epicuticular hydrocarbon profiles due to standard and defatted control diets as well as to defatted diets supplemented with three triacylglycerols[a]

	Combination[b]	Defatted[c]	Standard[d]	Triolein	Tripalmitolein	Tristearin
Combination	–	0.159	0.028	0.004	0.001	0.023
Defatted		–	0.609	0.036	0.001	0.995
Carolina			–	0.001	<0.001	0.070
Triolein				–	<0.001	<0.001
Tripalmitolein					–	<0.001

[a] Overall, diets caused significant differences in hydrocarbon profiles, Roy's greatest root=3.33, $F=7.91$, $df=24/57$, $P<0.001$.

[b] Defatted media plus all 1%, 3%, or 9% total concentration of all three fatty acids.

[c] See text for details.

[d] Instant Drosophila Medium® formula 4–24 (Carolina Biological Supply Co.).

Table 5 Pairwise *post-hoc* comparisons among the three individual and combination tag treatments based on class of epicuticular hydrocarbon[a] of *Drosophila mojavensis*

	df	Alkane		Methylalkanes		Alkenes		Methylalkenes		Alkadienes	
		SS	F	SS	F	SS	F	SS	F	SS	F
All	3	19.65	0.75	72,263.6	5.46	12,939.3	2.85[+]	10,668.8	2.25	670,380.7	3.66*
TO vs. TS	1	11.64	0.38	35,263.6	9.86**	9702.4	5.99*	8644.3	5.23*	463,505.5	7.88**
TO vs. TP	1	43.90	1.23	10,111.2	2.27	18.3	0.01	91.4	0.07	68,134.7	0.85
TS vs. TP	1	7.73	0.26	52,260.4	12.85**	3942.5	4.37*	4895.7	2.86	268,444.1	5.91*
CB vs. TO	1	0.35	0.02	748.1	0.15	60.9	0.03	383.3	0.27	383.3	0.27
CB vs. TP	1	0.59	0.03	16,307.9	2.71	32.3	0.02	1064.1	0.74	6402.7	0.10
CB vs. TS	1	7.71	0.36	15,210.8	3.46[+]	6670.5	5.24*	2329.4	1.34	384,530.9	7.35*

[a] Pairwise comparisons for each class of molecule. TO: triolein, TS: tristearin, TP: tripalmitolein, CB: combination.

+:$0.10 < P < 0.05$; *: $P < 0.05$; **: $P < 0.01$.

0.05). Thus, we tentatively rejected the hypothesis that particular unsaturated or saturated TAGs in larval diets induced variation in specific saturated or unsaturated EHCs in the adults. Both unsaturated TAGs caused higher levels of all adult EHCs, suggesting they are more easily incorporated into EHC biosynthesis than tristearin despite the higher toxicity of the unsaturated TAGs during larval development (Fig. 1).

Discussion

Low larval diet concentrations of some triacylglycerols present in the fermenting cactus tissues used by *D. mojavensis* as breeding sites caused significant shifts in amounts of EHCs expressed in mature adults. The hypothesis that different fatty acid pools from larval diets would be preferentially used as substrates for different saturated and unsaturated adult EHCs was rejected. Unsaturated triacylglycerols caused increases in a wide range of adult EHCs in *D. mojavensis* (Tables 1, 2, and 5), and although not toxic at higher concentrations, tristearin diets were consistently associated with lower quantities of most adult EHCs. Thus, tristearin is not likely a suitable substrate for lipid biosynthesis in *D. mojavensis*, consistent with Pennanec'h et al. (1997), who found that stearic acid was not a preferred precursor for hydrocarbon production in adult *D. melanogaster*, yet palmitic acid was readily incorporated. The chemical cues that determine mate recognition and sexual isolation in *D. mojavensis* are therefore sensitive to some of the triacylglycerols present in natural rearing substrates.

The observed variation in TAG response shown by the Population × TAG interactions (Appendix) in EHC abundance suggests these genetically differentiated populations differ in ability to synthesize adult EHCs from fatty acid precursors. The large geographic differences in EHC profiles, particularly due to differences between Baja California and mainland Mexico and Arizona populations (Stennett and Etges, 1997; Etges and Ahrens, 2001) in amounts of 8,24-tricontadiene ($C_{32.63}$), 9,25-pentatricontadiene ($C_{34.59}$), and 9,27-heptatricontadiene ($C_{36.5}$) are thus sensitive to TAG variation. Overall, variation in amounts of EHCs was more influenced by population differences than by the types of TAGs used in this study (Fig. 2a, Table 1), but the mainland population here (Sinaloa) was more sensitive to TAG amounts than the Baja California population. Such geographical variation is consistent with previous observations that mainland populations of *D. mojavensis* are more

sensitive to available nutrients in rearing substrates than Baja populations (Etges and Heed, 1987). At higher larval densities in cactus cultures where competition for food is increased, mainland populations express reduced viability and longer development times than Baja California populations, suggesting lower genetic homeostasis in mainland populations for these components of fitness (Etges, 1989).

Even though all larval cultures were supplemented with live baker's yeast and adults were aged to maturity on banana media, EHC profiles from triolein- and tripalmitolein-treated flies were significantly different among other treatment groups, with few differences due to the control diets (Table 4). Flies were aged at least 12–14 d on lab food in order to ensure that they were sexually mature (ca. 8–10 d for males at 25°C; Markow, 1982). This may have influenced adult EHC profiles despite the effects of these TAGs during larval stages, along with the effects of including live yeast to insure survivorship on the diet. Supplemental yeast provides larvae with nucleic acids, sterols, and essential vitamins and nutrients, including fatty acids. However, cultures from all treatment groups included the yeast, so if the additional yeast affected fatty acid metabolism in the flies, all treatment groups should have been influenced to the same degree. Because EHC amounts increase in abundance from eclosion to sexual maturity in *D. mojavensis* (Toolson et al., 1990), we wanted to be sure that our assays were performed with adults that had been reared in the same way as in previous experiments that had shown the consequences of preadult rearing environments on adult mating behavior.

EHC production has been intensively studied in adult insects (reviewed in Blomquist, 2003), but the influence of metabolism of lipids during larval stages on adult EHC profiles is not well understood. Biosynthesis of adult EHCs has been intensively studied, particularly in immature males and females in *D. melanogaster*, *D. simulans*, and *D. erecta*. EHC sexual dimorphism increases with age until sexual maturity is attained (Jallon and Wicker-Thomas, 2003). In *D. mojavensis*, rearing larvae on fermenting cactus lowers premating isolation compared to laboratory media (Etges, 1992; Brazner and Etges, 1993) despite a maturation period of up to 2 wk on banana food. We used this protocol to directly compare the effects of preadult rearing conditions without changing adult rearing procedures used in previous experiments. Thus, the rearing substrates experienced by preadult *D. mojavensis* cause a "carryover" effect that influences adult EHC composition and mating behavior past the age of sexual maturity.

The effects of tristearin in larval diets on adult *D. mojavensis* EHCs were consistent with earlier observations of its role as a lesser used substrate for adult EHC biosynthesis (Pennanec'h et al., 1997). Tristearin's effects on larval viability suggest it is either not metabolized effectively in larvae, or once assimilated, it has negligible effects even at higher concentrations (Fig. 1). Since the effects of tristearin on EHC profiles were not significantly different from either of the control diets, but very different from both triolein and tripalmitolein (Table 4), it may not be assimilated into adult hydrocarbons. More data will be needed to confirm this. Tripalmitolein, tristearin, and triolein are present in much lower concentrations (0–2.3%) in agria and organ pipe cacti than the most abundant saturated fatty acids, capric (C_{10}) and lauric (C_{12}) acid, which together account for 30–45% of total fatty acids (Fogleman and Kircher, 1986). Capric and lauric acids have been shown to have negligible effects on larval viability in *D. mojavensis* at 0.5% concentrations, but lauric acid significantly depressed viability at 1%. Thus, saturated fatty acids *per se* are not poor substrates for lipid and hydrocarbon metabolism: some are assimilated during larval growth and development and have variable effects depending on concentration.

Direct chemical links between host substrates and the resulting pheromonal cues used as both mate recognition signals and part of species recognition systems have broad

significance to studies on ecological speciation (Funk, 1998; Nosil et al., 2002; Sandoval and Nosil, 2005). In general, host plant adaptation typically involves host chemistry to some degree (Harborne, 1982; Bernays and Chapman, 1994; Thompson, 1994; Becerra, 1997; Funk et al., 2002), so host plant fatty acid or TAG determinants of EHC abundance in adult *D. mojavensis* provide direct evidence for how differential host use might determine reproductive isolation among insects. Although future studies will be necessary to show that TAG-induced shifts in adult EHC abundance directly influence patterns of mate choice, these diet-induced pheromonal shifts need to be considered in studies on reproductive isolation, particularly in *Drosophila* species. Enough is known about cactus stem chemistry that we can now design further experiments to examine the effects of these secondary compounds on the behavior and physiology of the insects that use these host plants.

Perhaps half of the species in the large *D. repleta* group (ca. 100 species) use cacti to carry out their life cycles. Along with a few other drosophilids that are cactus specialists, e.g., species in the *nannoptera* group (Heed, 1982), there are likely other species in which adult physiology and behavior has been shaped by the host plants used for feeding and breeding. In the Sonoran Desert, cactophilic *Drosophila* use different species of columnar cacti as breeding sites (Heed and Mangan, 1986). Host chemistry has been studied in detail in order to understand why certain host cacti are used preferentially by particular *Drosophila* species (Fogleman and Abril, 1990; Fogleman and Danielson, 2001). For *D. mojavensis*, crude lipid extracts from its major host plants, agria and organ pipe cactus, are not toxic because fatty acids and sterol diols are esterified to either neutral triterpenes, betulin and calenduladiol, or di-hydroxysterols (Fogleman et al., 1986). These neutral triterpenes are not toxic, but low concentrations of medium chain fatty acids and sterol diols can significantly depress larval viability. However, *D. mojavensis* is more tolerant of free fatty acids during larval growth and development than other desert drosophilids (Fogleman and Kircher, 1986). Agria and organ pipe cacti contain higher concentration of these fatty acids than other host cacti, so this may help to explain why *D. mojavensis*, but not other *Drosophila* species, tend to specialize on these cacti.

Throughout its evolutionary history, *D. mojavensis* has expanded its range by switching to different host cacti. Originating in Baja California, *D. mojavensis* colonized mainland Mexico, Arizona, and southern California by switching host plants. Use of agria cactus in Baja California is considered ancestral, with derived mainland populations using organ pipe cactus in Sonora and Sinaloa, Mexico, as well as southern Arizona (Etges et al., 1999). Sina cactus, *S. alamosensis*, is occasionally used in Sonora and northern Sinaloa, and in southern California, California barrel cactus, *Ferocactus cylindraceous*, is a major host. The colonization of mainland Mexico from Baja California by switching to organ pipe cactus has caused widespread genetic differences in life history traits, chromosome and genic frequency shifts, and physiological adaptation to these secondary hosts that has also resulted in the evolution of altered mating preferences in mainland populations (Etges, 1998; Etges and Ahrens, 2001, 2002). The evolutionary relationships between *D. mojavensis* and its host cacti suggest that this is an ideal system with which to pursue the molecular and chemical causes of ecological speciation, particularly if the mechanisms underlying these evolutionary shifts are related to host plant chemistry. These host plants have shaped the development and physiology of the insects that use them, causing not only adaptive responses in terms of host use and specialization, but also potential reproductive isolation among species that use different hosts.

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