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ADAPTATION TO DESICCATION FAILS TO GENERATE PRE- AND POSTMATING ISOLATION IN REPLICATE *DROSOPHILA MELANOGASTER* LABORATORY POPULATIONS

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Many laboratory speciation experiments have raised allopatric populations in different environments to determine whether reproductive isolation evolves as a byproduct of adaptation (a form of ecological speciation). Few, however, have addressed the evolution of both pre- and postmating isolation or investigated the conditions affecting the process. We present results of an evolution experiment in which 12 lines of *Drosophila melanogaster* were derived from a common population and then independently evolved for more than 57 generations under alternative selection treatments. Six "desiccation" populations had access to food and water removed during a period of their adult lives generating strong desiccation selection, and six "starvation" populations had access to food but not water removed for the same period, generating a mild starvation stress. Substantial divergence of cuticular hydrocarbons occurred between the desiccation and starvation populations, key traits that have been implicated in sexual isolation in *Drosophila*. Despite this divergence, there was no detectable premating isolation between desiccation and starvation populations and postmating isolation was asymmetrical: the fitness of F1 hybrids was reduced in the desiccation but not the starvation environment. This asymmetry was likely caused by the absence of divergent selection: adaptation to desiccation appears to have come at no cost to performance in the starvation environment. Novel environments are thought to promote the evolution of reproductive isolation. Understanding the conditions that favor or hamper this remains a key challenge for speciation research.

KEY WORDS: **Cuticular hydrocarbons, ecological speciation, experimental evolution, reproductive isolation, stress resistance.**

Ecological speciation occurs when reproductive isolation evolves as a consequence of divergent natural selection acting on populations occupying different environments or niches (Mayr 1942; Dobzhansky 1951; Schluter 2000, 2001; Rundle and Nosil 2005). Pre- and/or postmating isolation accrue as natural selection drives populations up separate adaptive peaks in the fitness landscape. When populations are allopatric, reproductive isolation evolves indirectly, as a byproduct of the adaptive divergence of phenotypic traits; this is our focus herein. Laboratory experiments have confirmed the feasibility of this process (e.g., Kilias et al. 1980; Dodd 1989; Dettman et al. 2007, 2008), and there is strong evidence that it occurs in nature (reviewed in Schluter 2000; Coyne and Orr 2004). This includes cases in which reproductive isolation has evolved in parallel in independent populations inhabiting similar environments (i.e., "parallel speciation": Funk 1998; Rundle et al. 2000; Nosil et al. 2002; McKinnon et al. 2004).

Our understanding of the allopatric model of ecological speciation is nevertheless incomplete (Rundle and Nosil 2005; Hendry 2009; Nosil et al. 2009). Laboratory experiments have focused on the evolution of premating isolation, with much less

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attention being given to postmating isolation (but see Dettman et al. 2007, 2008), and we lack a comprehensive understanding of the relative rates at which different forms of reproductive isolation evolve. In addition, past laboratory experiments often failed to control for the effects of genetic drift in allopatry, which requires showing that reproductive isolation evolves to a greater extent between populations adapted to different environments than between replicate populations adapted to the same environment (Rice and Hostert 1993). Of those that did, reproductive isolation failed to evolve in some cases (e.g., Mooers et al. 1999; Rundle 2003), yet we have limited insight into why. Exploring these issues is an important task for future laboratory speciation experiments.

Here, we take advantage of a long-term evolution experiment involving 12 replicate populations of *Drosophila melanogaster* that have independently evolved under alternative desiccation treatments: six desiccation (D) populations in which access to both food and water was removed during a period of their adult lives every generation, which was sufficient to generate approximately 75% mortality, and six starvation (S) populations in which access to food but not water was removed for the same period of their adult lives every generation, generating a mild starvation stress that caused little to no mortality. In *Drosophila*, the evolution of desiccation resistance has been shown to involve extensive phenotypic remodeling (e.g., Hoffmann and Parsons 1989; Gibbs et al. 1997; Chippindale et al. 1998). Using these populations, Kwan et al. (2008) have shown that after 46 generations of experimental evolution, the D populations exhibit extended development and faster growth as larvae, contributing to a 20% increase on average in adult body weight relative to the S populations. These differences contribute to a 68% increase in survival time under severe desiccation stress in the D relative to the S populations. Here, we investigate the extent to which reproductive isolation has evolved as a byproduct of this adaptive divergence between environments, providing insight into the early stages of ecological speciation.

We explore two aspects of reproductive isolation. First, we further characterize the evolutionary divergence of these populations with respect to a suite of traits, cuticular hydrocarbons (CHCs), with links to both desiccation resistance and premating isolation. CHCs are long chain-length carbon compounds found on insect epicuticles. They have waterproofing capabilities (Nelson 1993) and their variation in *Drosophila* and other insects has been associated with desiccation resistance (Toolson and Kuper-Simbron 1989; Gibbs 1998; Howard and Blomquist 2005), including a pattern in *D. melanogaster* of increasing chain-length with latitude (Rouault et al. 2001) and in response to desiccation adaptation during a laboratory evolution experiment (Gibbs et al. 1997). Longer chain-length CHCs are thought to be more effective at preventing water loss because they have higher melting temperatures (Gibbs 1998). CHCs also function as a pheromonal system that is involved in sexual communication in *Drosophila* (Jallon 1984; Markow and Toolson 1990; Ferveur et al. 1997; Blows and Allan 1998; Etges and Ahrens 2001; Petfield et al. 2005; Grillet et al. 2006). Their expression is plastic, and individuals can rapidly alter CHC composition in relation to their social context in *D. melanogaster* (Kent et al. 2008; Krupp et al. 2008). CHCs have been shown to mediate sexual isolation among species in the *melanogaster* subgroup (e.g., Coyne 1996) and between races of *D. melanogaster* (e.g., Fang et al. 2002). All else being equal, the evolutionary divergence of CHCs in response to desiccation-selection should generate premating isolation eventually.

Second, in addition to investigating the divergence of a suite of traits implicated in desiccation resistance and mate choice, we directly test for the evolution of both pre- and postmating isolation between populations from these two environments. Our test for premating isolation uses multiple-choice mating trails to determine whether populations adapted to different environments mate assortatively. To test for postmating isolation, we employ a reciprocal transplant experiment in which the survival and fecundity of D and S individuals, as well as F1 hybrids between them, is estimated in both the desiccation and starvation environments. Postmating isolation would be indicated by a reduction in F1 hybrid fitness relative to the population adapted to that environment and could be caused by genetic incompatibilities that have arisen between the D and S genomes (i.e., "intrinsic genetic" postmating isolation), or by a mismatch between hybrid phenotype and environment (i.e., "ecologically dependent" postmating isolation) (Rice and Hostert 1993; Schluter 2000; Rundle and Whitlock 2001). Including individuals from both the D and S populations in these reciprocal transplants permits an additional test of a specific prediction of ecologically dependent postmating isolation: in the absence of genetic incompatibilities, the rank order of relative fitness in the desiccation environment should be D > F1 > S, and the reverse in the starvation environment. The inclusion of both D and S individuals also allows a test of adaptive divergence between environments, the expected outcome of divergent selection. Such divergence is a fundamental requirement of ecological speciation that has been linked to the success or failure of past laboratory experiments (Florin and Ödeen 2002).

Materials and Methods

DERIVATION AND MAINTENANCE OF EXPERIMENTAL POPULATIONS

A detailed description of the stock population and the evolution experiment is available in Kwan et al. (2008). In brief, the experiment was originally designed to isolate the effects of desiccation selection, the application of which necessitates the removal of any food as a source of water. Six replicate lines were therefore created in which access to food and water was removed during a period of their adult lives (desiccation or 'D' populations) and six

matched control lines that had access to water but not food during this same period. Removal of food in the control lines creates an environment that differs from the stock population, potentially causing selection for starvation resistance, and we therefore designate these as starvation (S) populations. Populations $D_{1\text{-}3}$ and $S_{1\text{-}3}$ were derived from the laboratory-adapted LH_M population (Chippindale and Rice 2001) that has wild-type (red) eyes; populations $D_{4\text{-}6}$ and $S_{4\text{-}6}$ were derived from a stock carrying the recessive brown-eye (*bw*) mutation in the LH_M genetic background (LH_M-*bw*). (In Kwan et al. (2008), populations $D_{1\text{-}3}$ and $S_{1\text{-}3}$ are labeled $DR_{1\text{-}3}$ and $CR_{1\text{-}3}$, respectively, and populations $D_{4\text{-}6}$ and $S_{4\text{-}6}$ are labeled $DB_{1\text{-}3}$ and $CB_{1\text{-}3}$, respectively.)

Treatments were implemented as follows. On day 12 of every generation (designating day 1 as when the eggs were laid), desiccation-selection occurred in each D population by introducing approximately 4000 adult flies from their rearing vials into a sealed plastic cage lacking food or water and containing a packet of Drierite desiccant (W.A. Hammond Drierite Company Ltd., Xenia, OH). Selection was terminated when approximately 75% mortality had occurred in a population. Each S population was maintained contemporaneously with its matched D population (e.g., S_1 with D_1) by introducing approximately 1000 individuals into a separate cage containing only a nonnutritional agar plate as a source of water. Starvation stress in a particular S population was terminated when desiccation-selection in its matched D population was ended. Once selection was terminated, survivors were supplied with fresh food and live yeast for two days while in their cage, after which they were then allowed to oviposit on fresh medium for 14–16 h. On day 16, 100–125 of the eggs were introduced into rearing vials (40 for the D; 10 for the S) to create the next generation. Except during the selection treatments, larvae and adults were reared at 25°C, 50% relative humidity, and 12L:12D cycle.

The selection protocol paired the D and S populations such that those bearing the same subscript (e.g., D_1 and S_1) were handled at the same time (e.g., collection and transferring of individuals, introduction into their cages, etc.) and spent the same amount of time in their respective selection treatments. Because little to no mortality occurred in the S populations during starvation stress, census population sizes of adults after selection were similar in the two treatments (i.e., 75% mortality in a D population left approximately 1000 individuals alive). Effective population sizes of the two treatments may have differed for a number of reasons, however, including a strongly female-biased sex ratio in the D populations after selection (females survive desiccation stress much better than males; Kwan et al. 2008), the opportunity of the D females to have mated with a greater diversity of males prior to selection, and stronger selection in the desiccation treatment. Prior to all assays, approximately 1000 individuals per population were reared for two generations in a common environment under relaxed selection, with access to both food and water, to minimize environmental effects.

RESPONSE TO SELECTION OF CHCs

After 69 generations of experimental evolution, CHCs were extracted from individuals from all 12 experimental populations. Extractions were performed under two stress treatments: nonstressed (benign conditions) and stressed (desiccation conditions). This factorial design allows us to determine whether there are evolved differences in CHC expressions between the D and S populations, whether individuals alter their CHC expressions in a direct response to desiccation stress (i.e., phenotypic plasticity), and whether there is an interaction between these factors (e.g., evolutionary divergence between the D and S populations in their plastic response to desiccation stress).

On day 11, individuals were lightly anesthetized with CO_2, separated by sex, and housed in holding vials containing food. For the nonstressed treatment, CHCs were extracted on day 12 from individuals taken directly from these holding vials. For the stressed treatment, prior to CHC extraction on day 12, individuals were desiccated for 4–5 h in vials containing approximately 6 g of Drierite (separated from the flies by a thin foam stopper) and sealed with Parafilm (Pechiney Plastic Packaging, Menasha, WI).

CHCs were extracted from 30 males and 30 females from each combination of population and stress treatment. Extractions were performed by washing individual flies in 100 μl of hexane, containing 10 ng/μl of octadecane (C_{18}) as an internal standard, for approximately 3 min and then vortexing for 1 min. Individuals were then removed, dried for 36 h at 65°C, and weighed to the nearest 10^{-4} g.

CHC samples were analyzed using a dual-channel Agilent Technologies 6890N gas chromatograph fitted with HP5 columns of 50 m $\times$ 0.32 mm internal diameter, pulsed splitless inlets, and flame ionization detectors. The temperature program began by holding 57°C for 1.10 min, increasing to 190°C at a rate of 100°C/min, holding at 190°C for 1.20 min, increasing to 270°C at a rate of 5°C/min, and finally increasing to 300°C at a rate 120°C/min and then holding at 300°C for 5 min. Individual CHC profiles were determined by integration of the area under 31 peaks in females and 28 peaks in males, representing all those that could be reliably identified. Although the pattern of peaks was broadly consistent with those chemically identified by Foley et al. (2007) in a different population of *D. melanogaster*, the precise correspondence of some peaks was unclear. We therefore refer to the individual CHCs by their sequential number within the profiles of each sex (Figs. S1 and S2). Because sexual dimorphism in *D. melanogaster* includes qualitative differences in the identity of some hydrocarbons, with females having a greater number of CHCs than males, peaks identified by the same number in the two sexes do not therefore necessarily indicate homologous traits.

The total quantity of CHCs present on an individual was calculated by summing the abundance of all their CHCs and then normalizing this value by dividing by the abundance of the internal C_{18} standard (to control for sample evaporation and technical error during gas chromatograph). Variation in total CHC quantity was then analyzed using these values. This analysis was also repeated after further normalizing these values by dividing by each individual's dry weight. Total CHC quantities were square-root transformed and body weights were cube-root transformed prior to this normalization because CHC expression reflects a surface area of the cuticle whereas body weight is a volumetric measurement (Lande 1977; Lande and Arnold 1983).

Nonbiological variation among samples in overall CHC concentration can be large, even with the use of an internal standard (Blows and Allan 1998). To test for variation in the expression of individual CHCs, we therefore expressed the abundance of each CHC as a proportion of the total abundance of hydrocarbons extracted from that individual to remove this technical error. Log-contrasts were then generated to break the unit-sum constraint associated with proportional data (Aitchison 1986; Blows and Allan 1998) and were calculated for each of the n CHCs for every individual using logcontrast(CHC_n) = $\log_{10}$(proportion[CHC_n]/proportion[CHC_x]), where CHC_x is an arbitrarily chosen common divisor (here, CHC_{11} in females and CHC_5 in males). Note that this transformation reduces the number of traits by one, resulting in 30 log-contrast CHCs in females and 27 log-contrast CHCs in males. Prior to subsequent analyses, seven (six) multivariate outliers were identified and removed in females (males) using the Mahalanobis distance technique implemented in the software package JMP version 7.0.2 (SAS Institute Cary, NC).

Statistical analyses of both absolute and relative (i.e., log-contrast) CHC data employed a nonadditive mixed linear model for factorial randomized complete block designs (Newman et al. 1997; Quinn and Keough 2002) in which evolution treatment (D vs. S) and stress treatment (stressed vs. nonstressed immediately prior to CHC extraction) were fixed effects, and block (1–6) was a random effect representing the six D–S pairs of populations. Significance tests for the main effects of evolution treatment and stress treatment employed the respective interaction with block as the F ratio denominator, thereby recognizing that each D–S pair represents a single, evolutionary replicate. There is no test of the three-way interaction because it cannot be estimated separately from the residual error variance (Quinn and Keough 2002).

For the relative concentrations of individual CHCs, the ideal analysis would have been a multivariate version of the above model that included all log-contrast CHCs as response variables. However, given the large number of traits relative to the modest number of replicate populations that can be accommodated in a long-term evolution experiment, this model could not be fitted. We therefore employed a univariate approach that analyzed each log-contrast CHC separately. All models were fit using maximum-likelihood implemented by the mixed procedure in SAS version 9.1 (SAS Institute Cary, NC).

TEST FOR PREMATING ISOLATION

Assortative mating between the D and S populations was tested after 57 generations of experimental evolution. Replicate multiple-choice assays were used in which 50 virgin males and 50 virgin females from a single population of each treatment were placed together in a cage. Choice tests like this have been shown to be more sensitive at detecting assortative mating than no-choice tests (Coyne et al. 2005), and multiple-choice tests permit both male and female choice to contribute to assortative mating. Such a design is also biologically appropriate because it is representative of the context under which mating normally occurs in these populations. Six separate combinations of the D and S populations were tested (Table 1), all involving unique populations. These

Table 1. Results of multiple-choice mating trials between six unique combinations of the desiccation (D) and starvation (S) populations. For each combination, the assortative mating score (Y) and the relative mating success of D versus S individuals of each sex (p) were evaluated using a one-sample t-test (t) treating cages as replicates (df=7 in all cases).

Combination	Females	Males	Assortative mating			Relative mating success					
						D females			D males		
			Y (SE)	t	P	p (SE)	t	P	p (SE)	t	P
1	D_1, S_1	D_2, S_2	0.15 (0.10)	1.61	0.152	0.51 (0.05)	0.25	0.807	0.42 (0.04)	−2.00	0.085
2	D_2, S_2	D_3, S_3	−0.11 (0.05)	−2.28	0.056	0.59 (0.03)	3.21	0.015*	0.39 (0.04)	−2.93	0.022*
3	D_3, S_3	D_1, S_1	0.10 (0.10)	1.09	0.313	0.55 (0.03)	1.49	0.180	0.40 (0.02)	−5.09	0.001*
4	D_4, S_4	D_5, S_5	0.00 (0.08)	−0.05	0.961	0.54 (0.03)	1.77	0.120	0.24 (0.03)	−8.40	<0.001*
5	D_5, S_5	D_6, S_6	−0.08 (0.12)	−0.68	0.518	0.62 (0.03)	4.33	0.003*	0.33 (0.04)	−4.63	0.002*
6	D_6, S_6	D_4, S_4	0.07 (0.06)	1.14	0.292	0.56 (0.03)	2.08	0.076	0.33 (0.05)	−3.53	0.010*

*Significant at P<0.05.

combinations controlled for both population of origin, preventing individuals of either sex from having a choice between individuals from their own population versus a different population, and eye-color (previous assays indicated reduced mating success of LH_M-*bw* relative to LH_M males; L. Kwan, unpubl. data). Eight replicate cages were performed for each of the six combinations using unique individuals in all cases.

Flies for use in the mating trials were collected as virgins on day 10 and housed in temporary holding vials containing food. Mating trials were performed on day 14. The eight replicate cages for each mating combination were run simultaneously and the different mating combinations were run sequentially within a single day. Cages were checked approximately every 11 min and mating pairs were removed and identified. Cages were terminated after the first 25 mating pairs were identified (to minimize changes in the frequency of receptive individuals as matings occur, reducing any nonindependence among these matings) or 3 h had elapsed (an average of 24.5 ± 0.3 matings occurred in each cage). To permit their identification, prior to the assay all individuals were marked by feeding them overnight (days 13–14) in vials with abundant yeast saturated with red or blue commercial food coloring. Replicate cages within a mating combination were reciprocally marked to balance any color effects (two replicate cages using each of the four possible color combinations involving two populations and two sexes). Consistent with past studies (Mooers et al. 1999; Rundle 2003), such effects were weak or absent (Tables S1 and S2).

Assortative mating was evaluated using the index *Y* (Bishop et al. 1975) based on the cross-product ratio (α) of a 2×2 contingency table containing the number of matings of each combination. *Y* is calculated as $(\sqrt{\alpha} - 1)/(\sqrt{\alpha} + 1)$, where α is the product of the number of S × S and D × D matings, divided by the product of the number of S × D and D × S matings. *Y* varies from -1 for perfect negative assortative mating to $+1$ for perfect positive assortative mating, with zero indicating random mating. *Y* is a margin-free index and is therefore not biased by varying propensities to mate (Bishop et al. 1975). *Y* was treated as a simple measure of assortative mating and was calculated for each replicate mating cage (because matings within a cage are not independent of one another). A single overall test for assortative mating between the D and S treatments was performed using a one-sample *t*-test that treated the six mating combinations as replicates. In this test, *Y* for each mating combination was the mean value from the eight replicate cages. Within each specific mating combination, assortative mating between that particular pair of populations was evaluated using a one-sample *t*-test that treated cages as replicates. Results of all of the above tests remain essentially unchanged if I_{PSI} (Pérez-Figueroa et al. 2005) is used in place of *Y* as an index of sexual isolation (L. Kwan, unpubl. data).

For each sex, the relative mating success of D individuals was calculated as the proportion (*p*) of total matings in a cage that involved D individuals. As for *Y* above, we are primarily interested in overall treatment effects, so a single one-sample *t*-test was performed to determine whether mean *p* differed significantly from 0.5 (indicating equal mating success of D and S individuals of that sex), treating the six mating combinations as replicates. Within each specific mating combination, deviations from random mating for a particular pair of populations were tested using a one-sample *t*-test that treated cages as replicates. Proportions were arcsine-square root transformed prior to the analysis.

Female mating rates

To determine whether D females mate faster than S females, a separate assay was conducted after 67 generations of experimental evolution to estimate the time of mating of both types of females when individually presented with LH_M virgin males. Individuals were collected as virgins on day 10 and housed separately by sex in holding vials containing food. On day 14, we determined the time in seconds it took for a single female to copulate with a single male transferred into her vial. Females from a single D population and their matched S population were tested simultaneously (e.g., pair 1: D_1 females and S_1 females). Within each pair, there were 50 trials using D females and 50 trials using S females. Trials were terminated after copulation or 1 h had elapsed (copulation occurred in 577 of the 600 trials).

A single overall test for a difference in time to mating between D and S females was performed using a paired *t*-test, treating the six D–S pairs as replicates. Within each specific pair, a two-sample *t*-test was used to determine whether D females mated significantly faster than S females. Times were log transformed prior to the analysis. *P*-values are one-tailed given an a priori expectation from the results of the premating isolation assay of faster mating in D than S females.

Male inbreeding depression

After 69 generations of experimental evolution, the potential effect of inbreeding depression on male mating success and assortative mating was explored. We created matched pairs of F1 "hybrids" between the D_1 and D_3 populations ($F1_{D1\text{-}3}$) and the S_1 and S_3 populations ($F1_{S1\text{-}3}$), and between the D_4 and D_6 populations ($F1_{D4\text{-}6}$) and the S_4 and S_6 populations ($F1_{S4\text{-}6}$; Table 2). These F1s were created from an equal number of crosses in both directions (i.e., D_1 females × D_3 males and D_3 females × D_1 males) and were pooled prior to the mating assay below. From these F1 crosses, 50 males were then used in multiple-choice mating trials using the same protocol as before. Eight replicate cages were tested for each of the two mating combinations (Table 2), controlling for population of origin and eye-color. Food coloring

Table 2. Results of multiple-choice mating trials involving outbred F1 males created by matings between the desiccation (D) and starvation (S) populations. For each combination, the assortative mating score (*Y*) and the relative mating success of D versus S females and $F1_D$ versus $F1_S$ males (*p*) were evaluated using a one-sample *t*-test (*t*) treating cages as replicates (df=7 in all cases). *Significant at *P*<0.05.

Combination	Females	Males	Assortative mating			Relative mating success					
						D females			$F1_D$ males		
			Y (SE)	*t*	*P*	*p* (SE)	*t*	*P*	*p* (SE)	*t*	*P*
1	D_2, S_2	$F1_{D1\text{-}D3}$, $F1_{S1\text{-}S3}$	−0.00 (0.05)	−0.04	0.966	0.56 (0.04)	1.59	0.156	0.43 (0.02)	−3.86	0.006*
2	D_5, S_5	$F1_{D4\text{-}D6}$, $F1_{S4\text{-}S6}$	0.14 (0.07)	1.90	0.100	0.62 (0.02)	5.05	0.002*	0.43 (0.03)	−2.43	0.045*

was again applied in a balanced design and no effects were detected (Tables S1 and S2).

TEST FOR POSTMATING ISOLATION

We tested for postmating isolation between the D and S populations using a reciprocal transplant assay in which survival and female fecundity were evaluated for D and S individuals, as well as F1 "hybrids" between them, in both the desiccation and starvation environments. F1s were created by matings between the matched D and S populations, thereby creating six F1 populations corresponding to the original six D–S pairs. For each D–S pair, crosses were performed using equal number of individuals in both directions (e.g., D_1 females × S_1 males and S_1 females × D_1 males) and the resulting F1 offspring were pooled.

The assay was performed over three generations, with two D–S pairs and their resulting F1 hybrids tested in both environments during each generation (gen. 72: $D_1/S_1/F1_{D1\text{-}S1}$ and $D_4/S_4/F1_{D4\text{-}S4}$; gen. 75: $D_2/S_2/F1_{D2\text{-}S2}$ and $D_5/S_5/F1_{D5\text{-}S5}$; gen. 76: $D_3/S_3/F1_{D3\text{-}S3}$ and $D_6/S_6/F1_{D6\text{-}S6}$). The environments mirrored the selection treatments used during experimental evolution, involving flies of the same age (day 12) and held under the same conditions. For each of the three phenotypes (D, S, and F1), exactly 1000 males and 1000 females were desiccated and approximately 250 males and 250 females were mildly starved. When 75% mortality had occurred in the F1 population in the desiccation environment, desiccation/starvation stress was removed for all flies in each environment. All survivors were then supplied with food and live yeast for two days in their cages, again matching the normal maintenance during experimental evolution. Any additional deaths occurring during this time were recorded. Surviving females (to a maximum of 100) were collected using light CO_2 anesthesia and then transferred to standard food vials and allowed to oviposit for 14–16 h. Adult progeny were counted 12 days later.

We calculated the mean fitness of each phenotype (D, S, and F1) in each environment as the product of the average fecundity of surviving females of that phenotype in that environment and their observed probability of surviving in that environment. As with CHCs, results were analyzed using a nonadditive mixed linear model for factorial randomized complete block designs (Newman et al. 1997; Quinn and Keough 2002) in which phenotype (D vs. S vs. F1) and environment (desiccation vs. starvation) were fixed effects and D–F1–S pair was a random block effect. This analysis recognizes that each D–S pair, along with the F1 hybrids between them, represents a single evolutionary replicate. The phenotype × environment interaction is of particular interest because it tests whether the fitness of the phenotypes varies between the two environments. Given a significant interaction, the effect of phenotype was subsequently tested separately within each environment (removing all environment terms from the model), followed by post-hoc pairwise comparisons. All models were fit using maximum likelihood implemented by the mixed procedure in SAS version 9.1 (SAS Institute Cary, NC).

Results

RESPONSE TO SELECTION OF CHCs

After 69 generations of experimental evolution, females from the D populations produced significantly more CHCs overall than females from the S populations (Table 3; Fig. 1A). D individuals were larger on average than S individuals (L. Kwan, unpubl. data), however, consistent with previous results (Kwan et al. 2008). When total CHC content was normalized for body weight to account for this, differences between the D and S populations vanished (Fig. 1A), providing no evidence that D females had increased total CHC content per unit body weight. In females, total CHC content also tended to be higher in stressed than nonstressed individuals for both the D and S populations (Fig. 1A), although this was only significant when normalized for body weight (Table 3). There was little evidence of any consistent evolved or stress-induced effects in males (Fig. 1B) and all evolution treatment × stress treatment interactions were nonsignificant (Table 3), indicating that the plastic response to desiccation stress did not diverge between the D and S populations.

In addition to total CHC content, variation also existed in the relative concentrations of individual CHCs. In females, the

Table 3. Results of mixed linear models testing for evolved differences in the total CHC quantity between the desiccation (D) and starvation (S) populations (evolution treatment), plastic changes in response to immediate desiccation-stress (stress treatment), and their interaction, indicating evolved differences in the plastic response to stress of D versus S populations. *Significant at $P<0.05$.

CHCs	Females						Males					
	Evolution		Stress		Interaction		Evolution		Stress		Interaction	
	F	P^a	F	P^a	F	P^a	F	P^b	F	P^b	F	P^b
Total	18.88	0.012*	3.32	0.142	0.08	0.790	1.27	0.310	1.07	0.349	0.65	0.458
Total (weight corrected)	1.18	0.338	13.42	0.022*	0.06	0.814	0.47	0.524	0.02	0.890	2.40	0.182

[a]df=1,4.
[b]df=1,5.

eight longest chain-length CHCs had significantly higher relative concentrations in the D than S populations, along with one short chain-length CHC (Table 4; Fig. 2A). Significant differences in nine of 30 traits is more than would be expected by chance alone (binomial probability of nine or more successes from 30 trials with $\hat{p} = 0.05$; $P < 0.001$), and this does not account for the clustering of significant effects in the long chain-length CHCs nor the consistency in effect direction (D > S for all these CHCs). In contrast, in males the relative concentration of seven CHCs was significantly lower in the D as compared to the S populations and they tended to be concentrated among the shorter chain-length CHCs (Table 4; Fig. 2B). Seven of 27 traits showing significant effects is more than would be expected by chance alone (binomial probability with $\hat{p} = 0.05$; $P < 0.001$), and this again does not account for the clustering or the consistency in effect direction (S > D for all these CHCs).

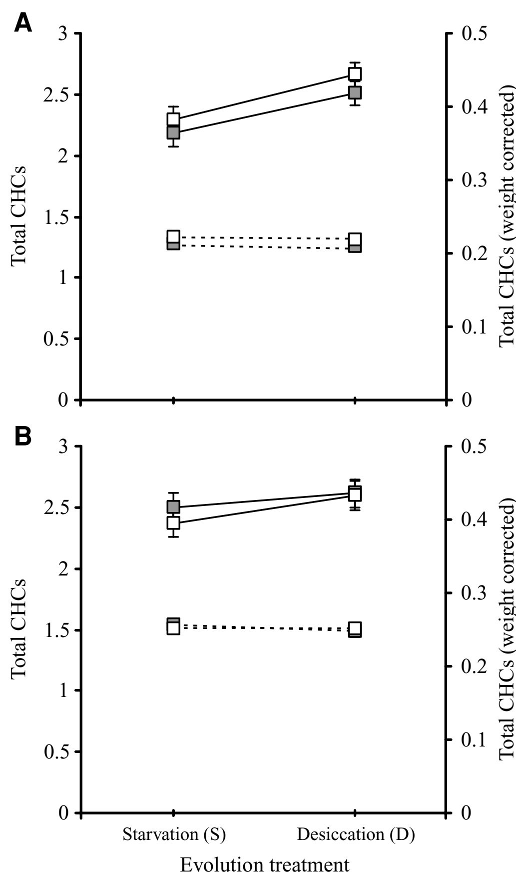

Figure 1. Total CHC quantity in females (A) and males (B) that have evolved in the starvation (S) and desiccation (D) treatments and that experienced nonstressed (filled squares) or stressed (open squares) environments immediately prior to CHC extraction. Total quantity is normalized to the internal standard (solid lines, left axis) and body weight as well (dashed lines, right axis). Error bars are ±1 SE.

CHC plasticity in response to desiccation stress was less pronounced, with significant effects being detected in four CHCs in females and only one in males (Table 4). None of these remained significant after correction for multiple comparisons. In females, there was no clear pattern that chain-length and effect sizes (difference in mean log-contrast CHC values, D–S ± SE; $CHC_6 = -0.05 \pm 0.03$; $CHC_{17} = -0.11 \pm 0.03$; $CHC_{18} = -0.08 \pm 0.05$; $CHC_{27} = -0.05 \pm 0.02$) tended to be smaller than that of the evolved differences in this sex (Fig. 2A). Nonstressed individuals, however, expressed higher relative concentrations as compared to stressed individuals in all cases for both males and females. The evolution treatment × stress treatment interaction was significant for a single CHC in one sex (i.e., CHC_9 in females; Table 4) and this was lost after correcting for multiple comparisons, providing little indication that the plastic response to desiccation stress diverged between the D and S populations.

TEST FOR PREMATING ISOLATION: MATING TRIALS

After 57 generations of experimental evolution, assortative mating scores (Y) between the D and S populations did not differ significantly from zero overall ($t_5 = 0.53$, $P = 0.618$; Fig. 3), indicating the absence of any premating isolation. Mean Y also did not differ significantly from zero within any of the six specific mating combinations (Table 1). Although combination 2

Table 4. Results of mixed linear models testing for evolved differences in the relative concentrations of individual CHC between the desiccation (D) and starvation (S) populations (evolution treatment), plastic changes in response to immediate desiccation-stress (stress treatment), and their interaction, indicating evolved differences in the plastic response to stress of the D versus S populations (df=1,5 in all cases). In females, 31 traits were identified with CHC_{11} used as the common divisor in calculating log-contrasts. In males, 28 traits were identified with CHC_5 used as the common divisor in calculating log-contrasts. CHCs are designated numerically within each sex and the chemical identities of compounds sharing a label do not necessarily correspond between the sexes (see Figs. S1 and S2). *Significant at P<0.05.

CHC	Females						Males					
	Evolution		Stress		Interaction		Evolution		Stress		Interaction	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
1	0.08	0.794	0.02	0.897	1.92	0.224	7.31	0.043*	0.21	0.668	2.91	0.149
2	0.93	0.379	0.02	0.900	2.77	0.157	5.16	0.072	0.14	0.724	3.37	0.126
3	11.01	0.021*	1.82	0.236	2.11	0.206	4.33	0.092	2.89	0.150	0.09	0.773
4	0.16	0.708	0.05	0.828	1.17	0.328	25.51	0.004*	0.06	0.821	2.65	0.165
5	5.28	0.070	0.11	0.755	1.56	0.267						
6	1.74	0.245	7.56	0.040*	4.60	0.085	1.53	0.271	1.61	0.260	<0.01	0.967
7	2.62	0.166	0.06	0.822	1.39	0.292	2.98	0.145	1.15	0.333	3.25	0.131
8	0.78	0.418	0.13	0.733	1.93	0.223	7.43	0.042*	0.17	0.695	0.31	0.604
9	5.52	0.066	0.17	0.701	8.66	0.032*	9.72	0.026*	2.72	0.160	3.29	0.130
10	1.26	0.313	0.19	0.680	0.05	0.838	27.47	0.003*	0.04	0.841	0.51	0.508
11							1.40	0.290	2.39	0.183	2.25	0.194
12	0.71	0.438	1.41	0.289	0.92	0.381	17.52	0.009*	0.22	0.661	0.90	0.386
13	2.08	0.209	<0.01	0.996	3.50	0.120	4.51	0.087	1.67	0.253	1.74	0.244
14	2.86	0.152	0.63	0.464	4.16	0.097	10.47	0.023*	8.27	0.035*	4.87	0.078
15	1.35	0.298	0.67	0.451	0.22	0.659	3.21	0.133	1.42	0.286	4.89	0.078
16	0.88	0.392	4.39	0.090	1.71	0.248	0.02	0.897	1.69	0.250	1.59	0.263
17	0.80	0.412	9.59	0.027*	1.52	0.272	0.08	0.788	3.52	0.120	1.60	0.261
18	4.84	0.079	8.39	0.034*	1.18	0.326	0.39	0.559	1.58	0.264	1.70	0.249
19	4.08	0.100	3.74	0.111	1.55	0.268	1.39	0.291	2.36	0.185	1.94	0.222
20	1.41	0.288	2.11	0.206	1.47	0.279	0.98	0.367	4.54	0.087	2.34	0.187
21	3.66	0.114	0.57	0.484	0.25	0.636	0.15	0.713	1.15	0.332	1.25	0.315
22	2.87	0.151	0.19	0.678	0.13	0.731	0.34	0.585	1.26	0.313	1.24	0.316
23	2.86	0.152	2.56	0.171	0.02	0.900	0.25	0.636	1.47	0.280	1.58	0.264
24	9.53	0.027*	5.31	0.070	1.51	0.274	0.05	0.829	2.23	0.195	2.04	0.212
25	46.39	0.001*	4.07	0.100	0.01	0.909	2.53	0.173	2.16	0.201	2.11	0.206
26	7.29	0.043*	1.60	0.261	0.05	0.829	4.23	0.095	1.58	0.265	1.69	0.250
27	42.62	0.001*	7.33	0.042*	0.03	0.863	1.47	0.280	1.65	0.256	1.45	0.282
28	54.22	0.001*	1.81	0.236	0.20	0.673	6.06	0.057	1.86	0.231	1.39	0.291
29	69.48	<0.001*	4.53	0.087	0.05	0.829						
30	35.62	0.002*	3.25	0.131	1.81	0.236						
31	39.40	0.002*	1.69	0.250	0.20	0.674						

approached significance, mean Y was less than zero in this case, indicating a weak tendency toward negative assortative mating.

Treatment effects were evident, however, in the relative mating success of both females and males. D females achieved more matings than S females in all six mating combinations (Fig. 4), a difference that is significant overall ($t_5 = 4.01$, $P = 0.010$). This higher mating success of D than S females was significant in two of the six specific mating combinations (Table 1) and was irrespective of the type of male ($P = 0.57$ and 0.56 overall with D and S males, respectively). In males, the opposite pattern was evident in that S males achieved significantly more matings than D males in all six mating combinations (Fig. 4), a difference that is also significant overall ($t_5 = -5.63$, $P = 0.003$). This increased mating success of S males was significant in five of the six specific mating combinations (Table 1) and was also irrespective of the type of female ($P = 0.64$ and 0.66 overall with D and S males, respectively).

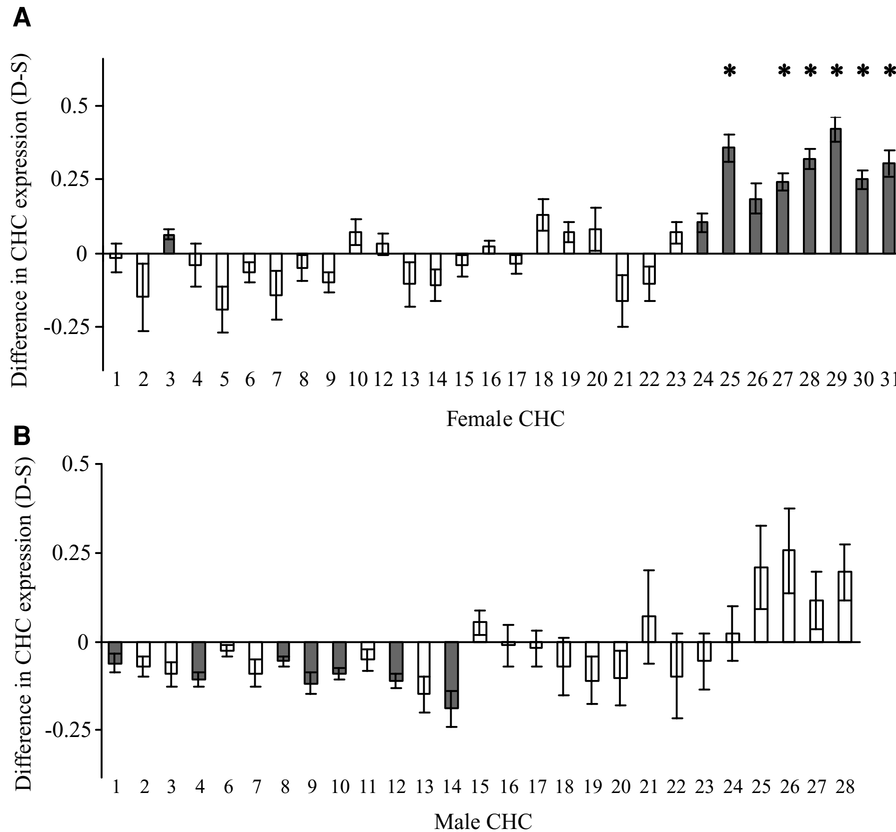

Figure 2. Differences in the individual logcontrast CHCs between females (A) and males (B) that have evolved in the desiccation (D) versus starvation (S) treatments. More positive values indicate higher mean relative concentrations of that CHC in the D than the S populations. Shaded bars indicate CHCs for which differences are significant when tested individually; asterisks indicate those that remain significant after correction for false discovery rate (Benjamini and Hochberg 1995). Error bars are ±1 SE. CHCs are designated numerically within each sex and the chemical identities of compounds sharing a label do not necessarily correspond between the sexes (Figs. S1 and S2).

Female mating rates

In five of the six D–S pairs, D females were quicker on average than S females to mate individually with LH_M males (Fig. 5), a difference that was significant overall ($t_5 = -2.14$, $P_{\text{one-tailed}} = 0.043$). The shorter time of mating for D compared to S females was significant for two of the specific population pairs (Fig. 5).

Male inbreeding depression

Mating trials in which the males were outbred, F1 crosses between populations within their respective evolution treatments produced similar results to the previous mating trials. Mean *Y* did not differ from zero in either specific combination (Table 2) and the point estimates fell within the previously observed range (Table 1), indicating that assortative mating between treatments remained absent when males were outbred. The mating success of D females also remained high relative to S females, with the difference being significant in one of the two mating combinations (Table 2). F1 males from the D treatment again achieved fewer matings than F1 males from the S treatment, with the difference being significant in both mating combinations (Table 2).

TEST FOR POSTMATING ISOLATION: RECIPROCAL TRANSPLANT

Mean fitness, estimated as the average fecundity of females multiplied by their observed probability of surviving, showed a significant phenotype × environment interaction ($F_{2,10} = 123.30$, $P < 0.001$), indicating that the fitness of the phenotypes (D, S, and F1) was environment-dependent. Main effects of environment ($F_{1,5} = 138.27$, $P < 0.001$) and phenotype ($F_{2,10} = 151.78$, $P < 0.001$) were also significant. The phenotype effect remained significant when the analyses were performed separately in each environment (starvation environment: $F_{2,10} = 21.90$, $P < 0.001$; desiccation environment: $F_{2,10} = 243.86$, $P < 0.001$).

In the desiccation environment, Ds outperformed F1 hybrids which outperformed Ss (Fig. 6). All three pairwise combinations of these phenotypes differed significantly in post-hoc multiple comparisons after a false discovery rate correction (Benjamini

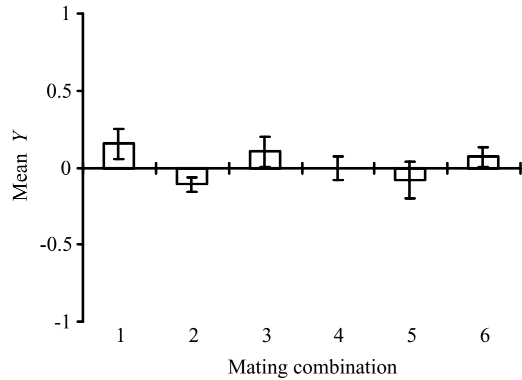

Figure 3. Assortative mating scores (*Y*) between populations that evolved in the desiccation (D) versus starvation (S) treatments. *Y* can vary between −1 (indicating perfect negative assortative mating) and +1 (indicating perfect positive assortative mating), with zero indicating random mating. Mating combinations are given in Table 1. Error bars are ±1 SE.

and Hochberg 1995), indicating that F1 hybrid fitness was reduced relative to the native D phenotype (i.e., the evolution of postmating isolation). The rank order of fitness (D > F1 > S) is also consistent with ecologically dependent postmating isolation, demonstrating that performance increased with phenotypic similarity to the D populations. Results differed in the starvation environment, with F1s performing best and little difference between Ss and Ds (Fig. 6), providing no indication of any postmating isolation. Evidence for divergent selection was also lacking, with Ds performing as well as Ss in the starvation environment, suggesting that adaptation to the desiccation environment came at no cost in terms of performance in the starvation environment.

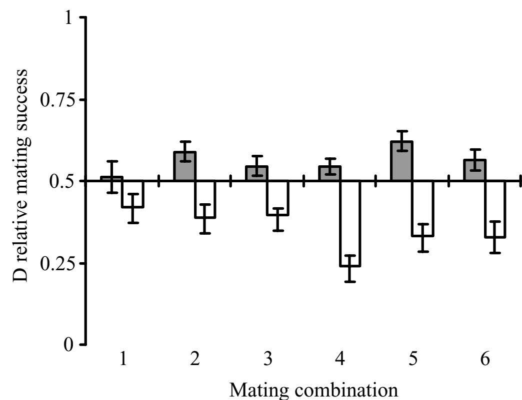

Figure 4. Mean proportion of total matings achieved by females (filled bars) and males (open bars) from the desiccation-evolved treatment (D). A proportion of 0.5 indicates equal mating success of D and S individuals of that sex. Mating combinations are given in Table 1. Error bars are ±1 SE.

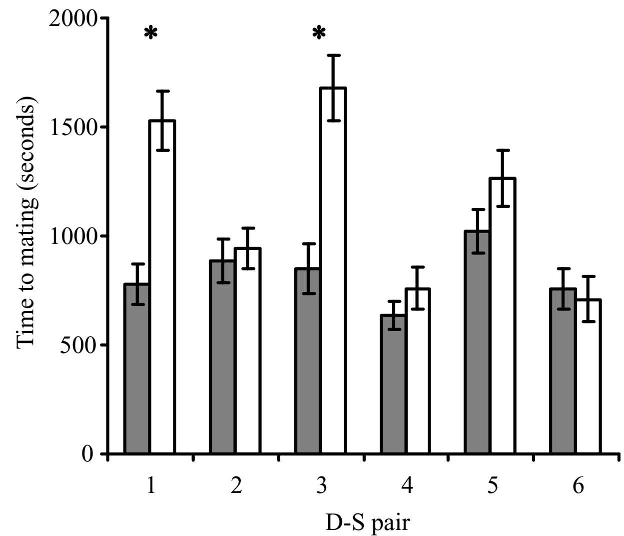

Figure 5. Time to mating of females that evolved in the desiccation (D; filled bars) and starvation (S; open bars) treatments when presented with ancestral LH_M males. Asterisks denote specific population pairs for which time to mating differs significantly in a two-sample *t*-test ($P < 0.05$, one-tailed). Error bars are ±1 SE.

Discussion

Despite empirical demonstrations from both nature and the laboratory that ecological speciation can occur, our understanding of the details of the process is incomplete. In particular, cases of "failed"

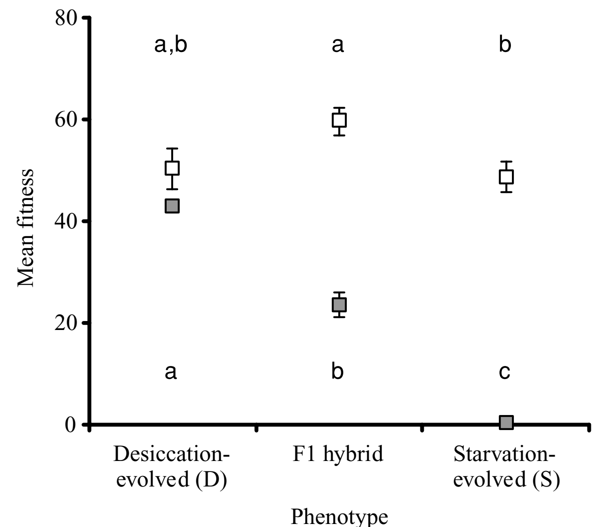

Figure 6. Mean fitness (probability of survival × fecundity) of various phenotypes derived from the desiccation (D) and starvation (S) evolution treatments, as well as F1 hybrids between them, when raised in desiccation (filled squares) and starvation (open squares) environments. Within each environment, phenotypes with different letters were significantly different in post hoc comparisons (starvation environment above, desiccation environment below). Error bars are ±1 SE.

ecological speciation in laboratory experiments are poorly understood. Here, we took advantage of a long-term desiccation-selection experiment in *D. melanogaster* to study the potential evolution of reproductive isolation in allopatry as a byproduct of adaptation. First, we demonstrated the evolutionary divergence of these populations in a number of CHCs, traits previously implicated in both desiccation resistance and mating behavior in *D. melanogaster*. Second, we directly tested for the evolution of both pre- and postmating isolation. These tests, however, provided little evidence of the early stages of ecological speciation: premating isolation was absent and postmating isolation was asymmetrical, present in one environment but not in the other. We discuss the implications of these results below.

EVOLUTIONARY DIVERGENCE OF CHCs

CHCs have been previously implicated in desiccation resistance in *Drosophila* and other insects (e.g., Toolson and Kuper-Simbron 1989; Rouault et al. 2001) and patterns of CHC variation in nature support this (e.g., Gibbs 1998; Rouault et al. 2001; Ferveur 2005). We are only aware of a single study, however, in which an evolutionary manipulation has been used to provide a direct test of the relationship between CHC expression and desiccation resistance. Gibbs et al. (1997) selected replicate populations of *D. melanogaster* for desiccation resistance for more than 100 generations using a protocol similar to the one adopted here. They found that selected flies had higher relative concentrations of longer chain-length CHCs compared to control flies, and that females had longer mean chain-lengths than males. Consistent with this, lipid melting temperatures were higher in selected than control flies, and in females than males. Resolution was limited, however, because CHCs were pooled by chain-length, yielding only six composite traits (CHCs with 21–31 carbon atoms, odd numbers only).

Here, we extracted CHCs from more than 1000 D and S flies that were exposed to either stressed or nonstressed conditions immediately prior to CHC extraction. As a plastic response to desiccation stress, females produced more CHCs per unit body weight (Table 3; Fig. 1A). This creates a thicker waterproofing layer and should thereby reduce rates of water loss (Gibbs et al. 1997; Gibbs 1998). No such pattern, however, was apparent in males (Table 3; Fig. 1B). Plastic changes in the relative concentrations of individual CHCs in response to desiccation stress were smaller in magnitude and involved fewer CHCs as compared to the evolved responses (Table 4). The lack of correspondence between evolved differences and plastic responses suggests these changes are nonadaptive, although the degree to which such changes may depend on the severity and duration of desiccation stress, and the timing of CHC extraction, is unknown.

In contrast to the plastic responses, there was no evidence that desiccation-adapted flies evolved a higher total CHC content per unit body weight (Table 3; Fig. 1). Evolved differences were detected in the relative concentrations of a number of individual CHCs, with little evidence of any interaction with plastic responses (Table 4). In females, adaptation to desiccation resulted in relatively greater amounts of long chain-length CHCs in Ds compared to Ss, whereas in males it involved relatively lesser amounts of shorter chain-length CHCs in Ds compared to Ss (Table 4; Fig. 2). The correspondence with the results of Gibbs et al. (1997) is remarkable: in their experiment, desiccation-adapted females had higher relative concentrations of the four longest chain-length groups (C25–C31), with two of these being significant (C25 and C29), whereas desiccation-adapted males had significantly lower relative concentrations of the two shortest chain-length groups (C21 and C23).

The evolution of higher relative concentrations of long chain-length CHCs in females is consistent with the proposed mechanistic link in which longer chain-length hydrocarbons provide a better barrier to water loss because they melt at higher temperatures (Gibbs et al. 1997; Gibbs 1998; Ferveur 2005). D females must survive the desiccation stress to ensure their fitness, generating strong natural selection for stress-resistant traits (e.g., body size; Kwan et al. 2008). The absence of significant increases in relative concentration of long chain-length CHCs in males may have occurred because males do not need to survive desiccation stress to ensure their fitness, and few actually do survive. Rather, the majority of males gain their fitness posthumously via matings that occur prior to desiccation (Kwan et al. 2008). The absence of significant increase in long chain-length CHCs in males also implies the genetic independence of these traits between the sexes, consistent with other work (e.g., Foley et al. 2007).

The evolutionary mechanism generating lower relative concentrations of short chain-length CHCs in D males is less clear. Shorter chain-length CHCs may serve an unknown but potentially important role in desiccation resistance, although this would be inconsistent with the proposed mechanistic link between CHC chain-length and water loss. Alternatively, differences in sexual selection between the desiccation and starvation environments could be responsible. As noted previously, females must mate prior to desiccation because few males survive (and mating does not occur during desiccation). This may have favored the evolution of less-choosy D females, weakening or otherwise altering sexual selection on D males and explaining the evolution of lower mating success as compared to S males (Fig. 4). We return to this possibility below when discussing the evolution of mating behaviors.

THE EVOLUTION OF REPRODUCTIVE ISOLATION

Reproductive isolation has been shown to evolve as a by-product of adaptation in a limited number of laboratory speciation experiments (Kilias et al. 1980; Dodd 1989; Dettman et al. 2007;

Dettman et al. 2008), demonstrating that the initial stages of ecological speciation can occur quite rapidly. In contrast, there was little evidence of any reproductive isolation between the D and S populations in the current experiment. Multiple-choice tests provided no indication of any premating isolation in the form of assortative mating (Table 1; Fig. 3), and reciprocal transplant experiments revealed asymmetrical postmating isolation in which F1 hybrid fitness was reduced relative to the D populations in the desiccation environment, but was higher than the S populations in the starvation environment (Fig. 6). Asymmetrical postmating isolation represents a weak barrier to gene flow because even low levels of migration from a D into S population would be unopposed by selection, preventing their evolutionary divergence.

Two general explanations exist for the absence of reproductive isolation in laboratory speciation experiments. First, populations may not have adapted to their different environments, preventing speciation from occurring as a side effect. Tests for adaptation are sometimes lacking in past experiments (e.g., Rundle 2003) and when conducted, have provided a likely explanation for the failure to observe any reproductive isolation (e.g., Mooers et al. 1999). The absence of such tests hampers interpretation of negative results because this possibility cannot be addressed. This is not the case, however, in the current experiment. Adaptation to the novel desiccation environment was extensive in the D populations, generating a 68% increase in survival time under arid conditions relative to the S populations, and involved divergence in life-history traits and body size (Kwan et al. 2008), as well as a number of CHCs (Table 4; Fig. 2).

The second explanation for the absence of reproductive isolation is that adaptation occurred in a manner that did not produce it as a byproduct. Whether reproductive isolation evolves likely depends on a range of factors, including the form and intensity of selection, duration of the experiment, genetic basis of the traits underlying selection, and effective population sizes (Ödeen and Florin 2000; Florin and Ödeen 2002; Hendry 2009; Nosil et al. 2009). Evaluating the relative roles of these factors is an important goal for laboratory speciation experiments. In our case, the experiment involved population sizes and a duration similar or greater than many past studies that have found reproductive isolation, and reproductive barriers appear to have evolved over such time scales in nature (Hendry et al. 2007). Adaptation to desiccation also involved changes in a diverse set of traits, increasing the chance that reproductive isolation would evolve as a byproduct (i.e., multifarious divergent selection; Rice and Hostert 1993; Nosil et al. 2009).

The form of selection, however, stands out here as a potentially key factor affecting the likelihood of speciation. Ecological speciation occurs when reproductive isolation evolves as a result of ecologically based divergent selection, or in other words, when selection acts in contrasting directions on populations inhabiting different environments or exploiting alternative niches (Schluter 2000, 2001; Rundle and Nosil 2005). The extensive adaptation to the desiccation environment in this experiment, however, appears to have come at no cost to performance in the starvation environment, revealing the presence of directional but not divergent selection. The absence of a cost to adaptation can arise if increases in fitness in one environment produce a correlated increase in fitness in another environment (Whitlock 1996; Bell 1997; Kassen 2002). In this experiment, the starvation stress, although nonlethal, may have generated selection via variation among individuals in their subsequent fecundity or mating success. (Experiments with the LH_M population have shown that female fecundity is strongly dependent on their consumption of live yeast [Morrow et al. 2005].) If the genetic basis of resistance to desiccation and starvation stress is largely shared, adaptation to desiccation may have increased fitness in the starvation environment as a correlated response. Consistent with this, after 37 generations of experimental evolution, the S populations were significantly better able to resist desiccation stress than their ancestors (L. Kwan, unpubl. data). The apparent higher fitness of F1 hybrids in the starvation environment may have been caused by heterosis. Whether any reproductive isolation evolved between the D and S populations and their LH_M ancestor as a byproduct of adaptation to these environmental stresses has not been explored.

Despite the absence of premating isolation, there is nevertheless evidence of divergence in mating behavior. Females from all six D populations achieved more matings than S females (Table 1; Fig. 4), suggesting the evolution of either faster choosing or less discriminating D females. A no-choice mating rate assay employing standard LH_M males directly confirmed this (Fig. 5). The evolution of faster mating females in the D populations is likely an adaptation to the selection protocol. Given that very few D males survive desiccation, D females must ensure that they acquire sufficient quantities of sperm prior to selection to maximize their postselection fecundity. This is not an issue for S females, however, because virtually all S males survive starvation stress. If D females mated faster by becoming less discriminating of males, this could have weakened sexual selection on male CHCs, potentially explaining the evolution of decreased expression of short chain-length CHCs observed in males.

Opposite to the pattern in females, males from all six D populations achieved fewer matings than S males (Table 1; Fig. 4). Inbreeding depression of male mating success, which has been previously reported in *D. melanogaster* (Rundle et al. 1998), was not responsible here because outbred D males remained less successful than outbred S males (Table 2). One possible explanation is the evolution of unattractive males due to sexual conflict. Sexual conflict arises from differences in the evolutionary interests of males and females (Parker 1979). Because few D males

survive selection and mating is not observed during selection (Kwan et al. 2008), the sexes may have dissimilar fitness interests before desiccation stress: early maturation and mating in males versus resource acquisition to maximize survival and later reproduction in females. Strong selection on stress-resistant traits in D females may have caused correlated responses in D males that lowered their mating success, in effect pulling males off their sexual selection fitness peak via intralocus sexual conflict (Rice and Chippindale 2001). The shared trait(s) lying at the centre of such a potential conflict are unknown, although body size is one candidate. Whatever the cause, the reduced mating success of D males will hamper speciation by increasing the mating success of immigrant S males.

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Supporting Information

The following supporting information is available for this article:

Figure S1. Gas chromatography profile of a typical female *D. melanogaster*.

Figure S2. Gas chromatography profile of a typical male *D. melanogaster*.

Table S1. Tests for overall color effects in multiple-choice mating trials between unique combinations of red and blue individuals in the premating isolation and male inbreeding depression assays.

Table S2. Tests for specific color effects in multiple-choice mating trials between unique combinations of red and blue individuals in the premating isolation and male inbreeding depression assays.

Supporting Information may be found in the online version of this article.

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Supplementary Information

Table S1. Tests for overall colour effects in multiple-choice mating trials between unique combinations of red and blue individuals in the premating isolation and male inbreeding depression assays. For each assay, the assortative mating score and the relative mating success of red versus blue individuals of each sex were evaluated using a single overall one-sample *t*-test (*t*), treating specific mating combinations as replicates.

	Assortative mating		Relative mating success			
			Red females		Red males	
Assay	*t*	*P*[a]	*t*	*P*[a]	*t*	*P*[a]
Premating isolation	-1.11	0.316	-2.15	0.084	-1.26	0.262
Male inbreeding depression	-0.23	0.856	0.31	0.810	-1.67	0.344

[a]*df* = 5 in premating isolation; *df* = 1 in male inbreeding depression

Table S2. Tests for specific colour effects in multiple-choice mating trials between unique combinations of red and blue individuals in the premating isolation and male inbreeding depression assays. For each mating combination, the assortative mating score (*Y*) and the relative mating success of red versus blue individuals of each sex (*p*) were evaluated using a one-sample *t*-test (*t*), treating cages as replicates (*df* = 7 in all cases).

		Assortative mating			Relative mating success					
					Red females			Red males		
Assay	Combination	*Y* (SE)	*T*	*P*	*p* (SE)	*t*	*P*	*p* (SE)	*t*	*P*
Premating isolation	1	-0.16 (0.10)	-1.61	0.151	0.39 (0.03)	-3.80	0.007*	0.53 (0.05)	0.56	0.593
	2	-0.02 (0.06)	-0.33	0.748	0.47 (0.04)	-0.70	0.504	0.51 (0.06)	0.18	0.863
	3	0.16 (0.08)	1.88	0.102	0.48 (0.03)	-0.75	0.478	0.47 (0.04)	-0.82	0.440
	4	-0.15 (0.06)	-2.70	0.031*	0.46 (0.03)	-1.31	0.233	0.45 (0.10)	-0.48	0.647
	5	-0.07 (0.12)	-0.59	0.573	0.49 (0.05)	-0.22	0.831	0.50 (0.07)	0.05	0.959
	6	-0.07 (0.06)	-1.22	0.263	0.51 (0.04)	0.14	0.891	0.41 (0.07)	-1.25	0.252
Male inbreeding depression	1	0.07 (0.04)	1.93	0.094	0.56 (0.04)	1.59	0.156	0.49 (0.03)	-0.31	0.763
	2	-0.11 (0.08)	-1.39	0.206	0.47 (0.05)	-0.65	0.537	0.50 (0.04)	-0.07	0.949

*Significant at $P < 0.05$

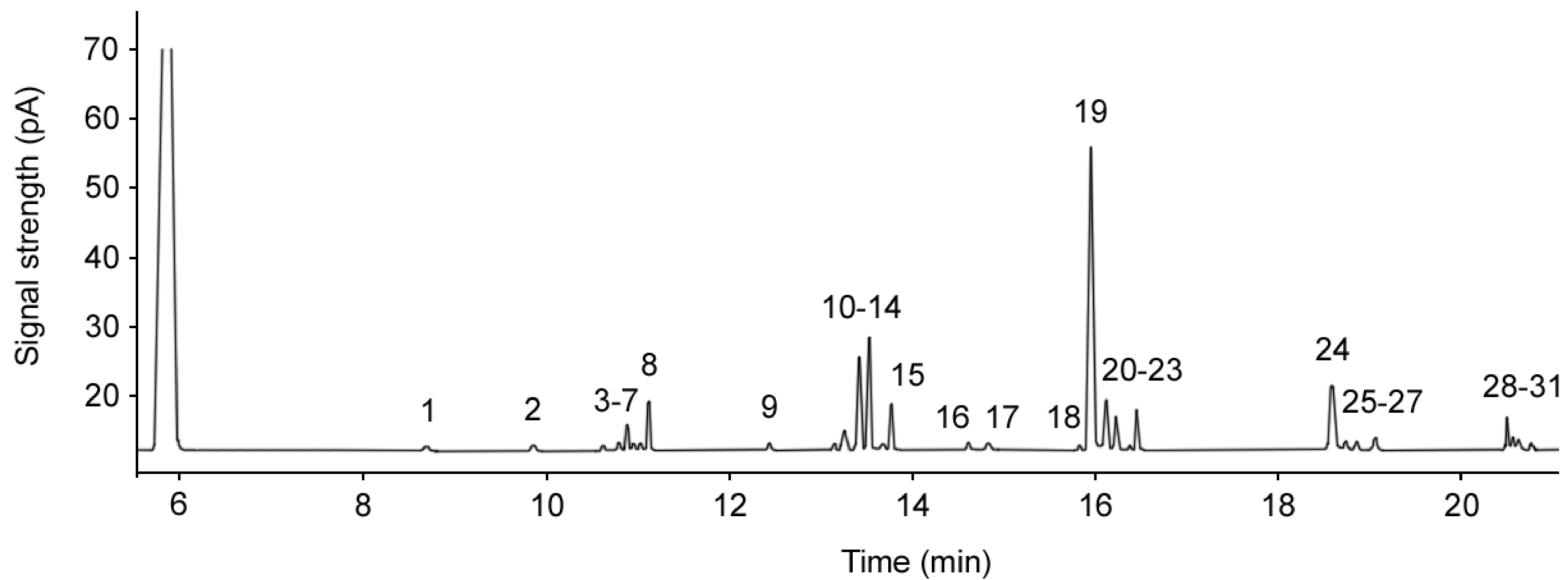

Figure S1. Gas chromatography profile of a typical female *D. melanogaster*. CHCs integrated in the current study are identified numerically, with the internal standard appearing just prior to 6 min and unlabeled.

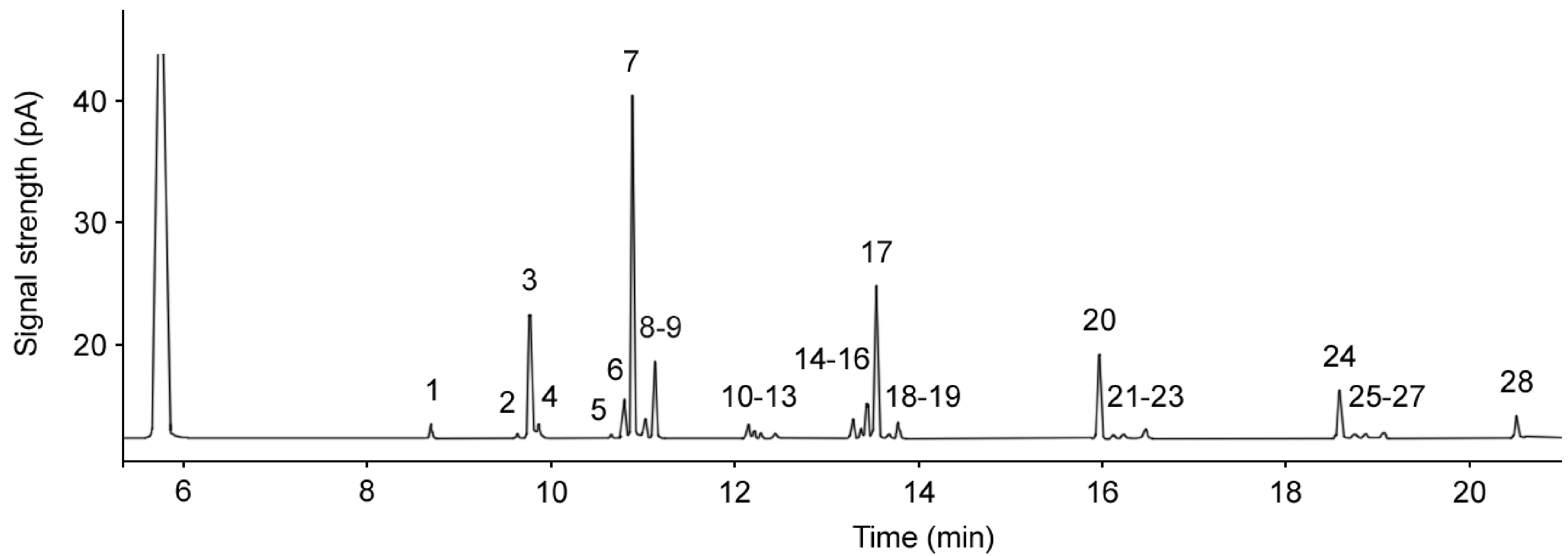

Figure S2. Gas chromatography profile of a typical male *D. melanogaster*. CHCs integrated in the current study are identified numerically, with the internal standard appearing just prior to 6 min and unlabeled.