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DIVERGENT ENVIRONMENTS AND POPULATION BOTTLENECKS FAIL TO GENERATE PREMATING ISOLATION IN *DROSOPHILA PSEUDOOBSCURA*

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Abstract.—While the feasibility of bottleneck-induced speciation is in doubt, population bottlenecks may still affect the speciation process by interacting with divergent selection. To explore this possibility, I conducted a laboratory speciation experiment using *Drosophila pseudoobscura* involving 78 replicate populations assigned in a two-way factorial design to both bottleneck (present vs. absent) and environment (ancestral vs. novel) treatments. Populations independently evolved under these treatments and were then tested for assortative mating and male mating success against their common ancestor. Bottlenecks alone did not generate any premating isolation, despite an experimental design that was conducive to bottleneck-induced speciation. Premating isolation also did not evolve in the novel environment treatment, neither in the presence nor absence of bottlenecks. However, male mating success was significantly reduced in the novel environment treatment, both as a plastic response to this environment and as a result of environment-dependent inbreeding effects in the bottlenecked populations. Reduced mating success of derived males will hamper speciation by enhancing the mating success of immigrant, ancestral males. Novel environments are generally thought to promote ecological speciation by generating divergent natural selection. In the current experiment, however, the novel environment did not cause the evolution of any premating isolation and it reduced the likelihood of speciation through its effects on male mating success.

Key words.—Assortative mating, divergent selection, founder event, novel environment, reproductive isolation, speciation.

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Laboratory evolution experiments have a long history in evolutionary biology and have made important contributions to our understanding of various fundamental processes including the origin of new species. Speciation experiments have generally focused on premating (behavioral) reproductive isolation and have provided important insight into the feasibility of various models for its evolution (e.g., see Rice and Hostert 1993). Two areas that have received much attention are the roles of divergent natural selection and genetic drift, as caused by population bottlenecks.

Many experiments have applied divergent selection to allopatric populations and asked whether premating isolation has evolved as a by-product. Few, however, have controlled for the effects of genetic drift in allopatry, meaning the relative roles of drift and selection could not be distinguished (Rice and Hostert 1993). To date, only two experiments (Kilias et al. 1980; Dodd 1989) have tested divergent selection with proper controls, asking whether reproductive isolation tends to accumulate between replicate populations adapted to different environments as compared to replicate populations adapted to the same environment. The result from both these experiments was clear: premating isolation evolved as a correlated response to natural selection adapting populations to their different environments.

The role of population bottlenecks in generating reproductive isolation has been more contentious. Some experiments suggest the plausibility of bottleneck-induced speciation (e.g., Dodd and Powell 1985; Ringo et al. 1985; Meffert and Bryant 1991; Galiana et al. 1993) and much attention has been given to drift-based models of speciation, both verbal (e.g., Carson and Templeton 1984) and mathematical (e.g., Gavrilets and Hastings 1996; Gavrilets and Boake 1998; Gavrilets and Vose 1998). Nevertheless, current opinion appears to be shifting away from the view that bottlenecks alone are an important cause of speciation. Past experiments have been criticized for poor replication and unnatural and/or inappropriate designs (Charlesworth et al. 1982; Rice and Hostert 1993; Rundle et al. 1998, 1999; but see Templeton 1996, 1999), and some of the isolation detected in earlier tests vanished in later trials (e.g., Moya et al. 1995; see Rundle et al. 1998). Recently, better replicated laboratory studies have found little evidence to support a role for population bottlenecks (Rundle et al. 1998; Mooers et al. 1999) and theoretical analyses suggest they are unlikely to cause significant reproductive isolation (Turelli et al. 2001). Evidence that bottlenecks have been important in nature is also weak (e.g., Vincek et al. 1997; Koskinen et al. 2002)

While bottlenecks alone may not generate significant premating isolation, it is possible that they still contribute to the speciation process. Founder events may often be associated with a change in environmental conditions and such co-occurrences of bottlenecks and divergent selection might have important consequences for the evolution of reproductive isolation (Rundle et al. 1998, 1999; Templeton 1999). For example, laboratory *Drosophila* experiments have demonstrated that population bottlenecks have highly variable and persistent effects on many of the fundamental quantitative genetic properties of a population, including the amount of additive genetic variation (V_A; Meffert 1995; Whitlock and Fowler 1999), phenotypic variation (V_P; Fowler and Whitlock 1999), environmental variation (V_E; Whitlock and Fowler 1999), and the shape of the genetic covariance matrix (**G** matrix; Phillips et al. 2001; Whitlock et al. 2002). In addition, the bottleneck-induced variability of these genetic properties of a population can increase if the environment also changes

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(Fowler and Whitlock 2002). Thus, bottlenecks, by altering the underlying genetic architecture of populations, may result in more variable evolutionary responses to novel environments, increasing the likelihood that reproductive isolation may evolve in some populations as a side effect.

Alternatively, population bottlenecks may have an inhibitory effect on the speciation process. For example, bottlenecks in *D. melanogaster* cause substantial and prolonged inbreeding depression of male mating success. Such inbreeding depression increases the likelihood of gene flow from an ancestor into a founder population because founder males will compete poorly for mates against immigrant males (Mooers et al. 1999; Saccheri and Brakefield 2002). In addition, while V_A is highly variable among bottlenecked populations, it decreases on average as predicted by population genetic theory (Whitlock and Fowler 1999). By reducing V_A, bottlenecks will, on average, decrease the overall response to selection and thus may reduce the likelihood of reproductive isolation evolving. Experimental studies of the interaction between divergent selection and bottlenecks during the evolution of reproductive isolation are needed to distinguish these alternatives. However, the topic has received little direct attention (but see Mooers et al. 1999).

Here I present the result of a long-term laboratory speciation experiment designed to test the separate effects of divergent environments and population bottlenecks, as well as their interaction, on the evolution of two aspects of between-population matings: premating isolation and male mating success. The experiment involves a simple two-way factorial design in which 80 replicate populations were assigned to one of four treatment combinations. The treatment factors were bottleneck (present/absent) and environment (ancestral/novel), yielding 20 replicate populations under each combination. Populations independently evolved under these conditions and were then tested for both assortative mating and male mating success against the ancestor from which they were all derived. The lines that did not undergo a bottleneck in the ancestral environment provide a control for the effects of allopatry; the contribution of bottlenecks and the novel environment and their interaction are evaluated by comparison with this baseline.

My study builds on previous experimental work on this topic (Mooers et al. 1999) that used a similar design yet failed to find any significant reproductive isolation evolving under any treatment combination. The current experiment differs in a number of important aspects that maximize the probability of speciation. First, the experiment was replicated at a larger scale and run for a longer period of time, thus giving populations a greater opportunity to adapt to the novel environment. In bottleneck-induced speciation, while the founder event acts as a trigger, it is the genetic changes that occur following the bottleneck that are hypothesized to generate reproductive isolation (Carson and Templeton 1984; Templeton 1989); it may take time for such genetic changes to accrue. Second, the novel and ancestral environments differed in several aspects (i.e., food, temperature, light), likely generating divergent selection on numerous phenotypic traits. Past experiments suggest that strong, multifarious divergent selection is more conducive to speciation than is selection on a single trait (Rice and Hostert 1993). Third, bottlenecked populations experienced two single-pair bottlenecks, allowing the populations to flush to large sizes in the interim. Multiple, serial bottlenecks should increase the likelihood of bottleneck-induced speciation (Carson 1968; Templeton 1999). Fourth, to maximize my chances of detecting any premating isolation that may have evolved, I measured assortative mating at two different times using two different techniques (see Materials and Methods). In addition, the second measurement tested each experimental line within its treatment environment, thus allowing me to detect subtle, environment-dependent forms of assortative mating. Finally, unlike Mooers et al. (1999), who used *D. melanogaster*, I used *D. pseudoobscura*. *Drosophila pseudoobscura* has been predicted to be more conducive to bottleneck-induced speciation than *D. melanogaster* (Templeton 1980, 1996, 1999; see Rundle et al. 1998, 1999) because it has a higher genomic recombination rate (total map length of *D. pseudoobscura* is 1.6 times that of *D. melanogaster*; True et al. 1996), its mating system does not respond to inbreeding with disassortative mating (Powell and Morton 1979) as does the mating system of *D. melanogaster* (Averhoff and Richardson 1974, 1976), and it is not a ''weedy,'' colonizing species with a ''general purpose genotype'' that may be resistant to bottleneck-induced speciation (Carson and Templeton 1984). *Drosophila pseudoobscura* has also been shown to evolve premating isolation in numerous past laboratory experiments (see Rice and Hostert 1993).

Materials and Methods

Stock Population

A population of *D. pseudoobscura* was obtained from J. Graves at Arizona State University. This stock was derived from a large sample of flies (> 200 mated females) collected in 1991 from Orange County, California, and was created to have high frequencies (> 95%) of the *Standard* (ST) inversion (J. Graves, pers. comm.). This stock was maintained in the laboratory under constant conditions (21°C; 24L:0D photoperiod; banana, corn syrup, and yeast medium) at a large population size (2000–3000 adults) with discrete, nonoverlapping generations. In June 1998, a large sample (> 400 adults) of this stock was moved to the University of British Columbia, where it was maintained in three population cages (22 × 26 × 32 cm) at 21°C and 12L:12D photoperiod, on the same medium. Generations were overlapping, with three new bottles of food added to each cage every week and the oldest three removed. Individual bottles remained in a cage for five weeks. Cages were cleaned every three months by transferring a large sample of adults and larvae to new cages, with flies mixed among the three cages at this time to ensure a single, large, panmictic population. This stock population (hereafter ''base'') was maintained under these conditions for 16 months prior to the start of the experiment and continued under these conditions throughout its duration.

Derivation and Maintenance of Experimental Lines

In October 1999, 80 separate lines were derived from the base population, each housed in its own single population cage. These lines were assigned in a two-way factorial design

to one of four experimental treatments, yielding 20 replicate lines within each treatment. Each line was independently established from the base population by random selection of either a large sample of adults (approximately 1000 flies), or a single male-female adult virgin pair (''unbottlenecked'' and ''bottlenecked'' lines, respectively). These lines were assigned to one of two environments, an ancestral or a novel environment, yielding the four following treatment combinations: unbottlenecked ancestral (UA), bottlenecked ancestral (BA), unbottlenecked novel (UN), and bottlenecked novel (BN). The ancestral environment maintained the same conditions under which the base population had been kept since its arrival at the University of British Columbia, including temperature (21°C), photoperiod (12L:12D), food (banana, corn syrup, and yeast medium), and feeding routine. The novel environment differed in temperature (25°C), photoperiod (0L:24D, with the exception of approximately 1.5 h every week during feeding), food (cornmeal, dextrose/sucrose, and yeast medium, with 5 g table salt/L food added), and feeding routine (same replacement schedule of three bottles/week, but individual bottles spent only four weeks in a cage). Two otherwise healthy lines went extinct early in the experiment due to a mite infestation (one UN and one BN), leaving 19 replicate lines within each of these treatments.

Six months after beginning the experiment, a second bottleneck was performed on each of the bottlenecked lines (BA and BN). For each line, five vials were created with each vial containing a single, randomly chosen, virgin male-female pair. Pairs that failed to produce offspring were subsequently discarded and then one of the remaining vials was randomly chosen to propagate the line. With the exception of these two bottlenecking events, population sizes were not regulated in any population throughout the experiment. Rather, all experimental lines and the base population were allowed to reach their self-imposed carrying capacities in their respective environments. However, for approximately three months prior to the final mating trails, all BN lines were temporally removed from their cages and put through a series of transfers in bottles to create sufficient numbers of flies for the trails. At the end of the experiment, a complete census of all adult individuals was conducted with the following results (mean number of adults per line ± SE): UA (1000 ± 53); BA (555 ± 81); UN (1066 ± 85); BN (471 ± 36); base (2124; total for all three cages).

Tests for Premating Isolation

Premating isolation was measured between each experimental line and the base population by performing female-choice mating trials at two different times using two different techniques (vials and cages; see below). All mating trials involved virgin flies collected at eclosion using CO_2 anesthesia. Adult virgin flies were subsequently held separately by sex in bottles (containing 50 mL of their respective medium) of approximately 100 individuals prior to testing, with live yeast added to bottles containing females but not to those containing males (to facilitate marking of males; see below). Adult flies ranged in age from 3 to 8 days posteclosion at the time of testing. To permit their identification during mating trials, line and base males were marked using food coloring, as described in Mooers et al. (1999). Replicate lines were reciprocally marked to balance any color effects. As in past studies (Rundle et al. 1998; Mooers et al. 1999), no such effects were seen; of the 16,298 observed matings, 49.8% involved red males, which does not differ from random expectation (χ_c^2-test with $\hat{p} = 0.5$, $P = 0.63$).

Thirteen to 14 months (approximately 15 generations) after the start of the experiment, mating trials were performed in vials using a standard female-choice design. Basically, a single female and two males (one line male and one base male) were placed together in a vial containing 10 mL medium (for details see Mooers et al. 1999). Trials were performed under conditions similar to the ancestral environment and pairs were given 180 min in which to mate, with vials being tapped lightly after 120 and 150 min to disturb the flies (a procedure that initiates active courtship by males; pers. obs.). For each line, 100 replicate vials were performed, half using base females and half using line females. Mating occurred in approximately 78% of the trials, with both line and base females mating at similar frequencies (50.2% of the matings were by base females; χ_c^2-test with $\hat{p} = 0.5$, $P = 0.79$).

The second round of mating trials were conduced 33–34 months (approximately 37 generations) after beginning the experiment. These trails were performed using a female-choice design analogous to that in vials above, except they were performed in cages using multiple individuals of each type. Female-choice was used instead of a multiple-choice design (e.g., Hollocher et al. 1997) because differences in mating rates among sexes and populations can generate assortative mating in multiple-choice experiments in the absence of any biologically significant behavioral isolation. Unlike the previous trials in vials, each line was tested under conditions designed to replicate its treatment environment. Thus, lines from the ancestral environment (UA and BA) were tested at 21°C and ambient light levels (''light'' cages), whereas lines from the novel environment (UN and BN) were tested at 25°C in a dark room (''dark'' cages). There was no food present in any of the cages, and flies were introduced without anesthesia, having been aspirated into vials of the correct number the day prior to the experiment. To permit observation, light from a Intralux 5001-1 cold light source (20 V/150 W tungsten halogen bulb; Volpi Manufacturing, Auburn, NY) was passed through a 2-m flexible fiber-optic cable (10-mm fiber diameter; Volpi Manufacturing) with a 670 nm Scott and Hoya long pass filter (Thermo Oriel Ltd., Stratford, CT) attached to the end. This filter only transmits wavelengths longer than 670 nm (far red). *Drosophila* are less sensitive to long wavelengths than humans and are thought to be insensitive to light above approximately 650 nm (Ashburner 1989; Carulli et al. 1994; Salcedo et al. 1999). Unlike their behavior under normal light, when illuminated at these wavelengths, flies failed react to the close presence of an object such as an aspirator (pers. obs.).

Six replicate cages were used when testing most lines (average number of cages/line ± SE: 5.8 ± 0.08), with each containing 40 base males and 40 line males. Base females were present in three of the cages and line females in the other three, with 40 females/cage for ancestral environment lines (i.e., light cages; UA and BA) and 60 females/cage for novel environment lines (i.e., dark cages; UN and BN); pre-

liminary work established that mating rates were lower in the dark. Mating pairs were aspirated out of the cage for identification, with individual cages being run until 25 matings occurred or 45 min had passed. Twenty-five matings occurred rapidly in all light cages (< 20 min in all cases), but fewer than 25 were common in the dark cages (average number of matings/dark cage in 45 min: UN = 20.2, BN = 19.0). Base and line females did not differ significantly in their propensity to mate in the dark (51.2% of matings were by base females; χ_c^2-test with $\hat{p} = 0.5$, $P = 0.14$).

Analysis

Assortative mating was evaluated, for vials and cages separately, using the index *Y* (Bishop et al. 1975). *Y* varies from −1 (perfect negative assortative mating) to +1 (perfect assortative mating), with zero indicating nonassortative mating (for details see Rundle et al. 1988; Mooers et al. 1999). Because I am primarily interested in average effects of the treatments, I treated *Y* as simply a measure of premating isolation and calculated it, for both vial and cage mating trials, using all matings between a line and the base population. I evaluated the effects of environment and bottlenecking on *Y* using a general linear model that included both main effects and their interaction.

While my focus was on treatment effects, the presence of significant assortative mating in individual lines is also of interest. In this case, the significance of *Y* can be evaluated using the statistic $X^2(Y)$, which is χ^2 distributed with 1 df (Fienberg 1977; Spieth and Ringo 1983), provided that the matings contributing to the calculation of *Y* are independent replicates. While this may be true for mating data from vials, it is not true for cage data. In cages, multiple matings are not independent of one another because the relative frequencies of the different types of flies change as the trial proceeds and flies are mated. For this reason, when evaluating the significance of assortative mating for any one line, separate cages are the only true replicate. Significance values of assortative mating for individual lines (reported here as *Y*), when tested in cages, are thus calculated using a *z*-test (with $\hat{p} = 0.5$) of the proportion of homotypic matings (arcsine square-root transformed) within a cage, treating separate cages as replicates.

Male mating success of a line was measured as the proportion (p) of total matings for that line achieved by line males. Because there was no difference in the propensity to mate between base and line females, females were pooled within lines prior to calculating male mating success. As for *Y* above, I am primarily interested in treatment effects on male mating success. Thus, lines were treated as replicates, and the effects of environment and bottlenecking on male mating success (arcsine square-root transformed p) were tested using a general linear model that included both main effects and their interaction. In addition, within treatment combinations I used a one-sample *t*-test, treating lines as replicates, to determine whether mating success of line males deviated significantly from one-half (i.e., $\hat{p} = 0.5$; equal number of mates obtained by line and base males). Finally, to determine the immediate effect of the novel environment on male mating success (in quantitative genetic terms, the environmental effect of the novel environment on male mating success), I retested 10 randomly selected UN lines against base males that had been raised for two generations in the novel environment. I used three replicate dark cages for each line, using only base females that had been raised in their normal, ancestral environment.

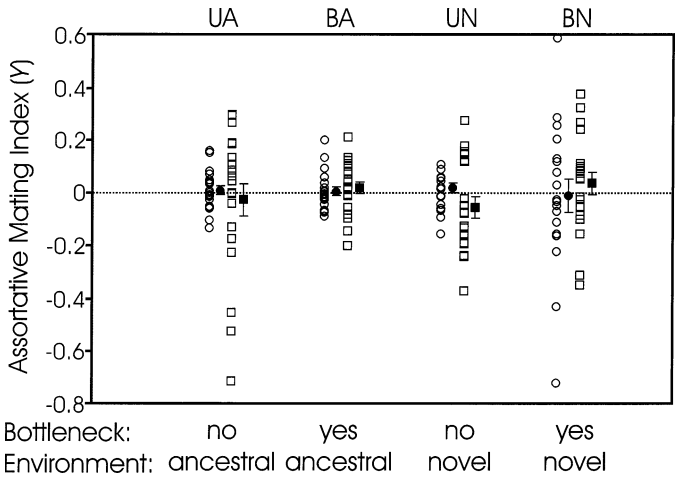

FIG. 1. Assortative mating scores (*Y*) of individual lines by treatment combination for mating trials performed in cages (circles) and vials (squares). *Y* can vary from −1 (perfect negative assortative mating) to +1 (perfect assortative mating), with zero indicating nonassortative mating (dashed line). None of the treatment means (closed symbols ± SE) differ significantly from nonassortative mating (*t*-tests with H_0: $Y = 0$, $P > 0.19$ in all cases).

RESULTS

Premating Isolation

For mating trials in both vials and cages, assortative mating (*Y*) scores were approximately normally distributed within treatments and varied little among treatments (Fig. 1). Environment, bottleneck, or their interaction had no significant overall effect on mean *Y* (Table 1). In addition, mean *Y* did not differ significantly from zero (nonassortative mating) for any of the four treatment combinations when measured in vials or cages (*t*-tests with H_0: $Y = 0$, $P > 0.19$ in all cases).

Assortative mating scores of individual lines were variable within treatments. For mating trials in vials, 14 lines showed significant assortative mating (eight positive and six negative), which is more than would be expected due to Type I error under the null hypothesis of random mating ($\hat{p} = 0.05$; $\chi_c^2 = 24.9$, $P < 0.001$). However, none of these 14 lines showed significant assortative mating when retested using larger sample sizes in cages (Table 2). Rather, in cages only two lines showed significant assortative mating (one BA and one UA), no more than expected due to Type I error ($\chi_c^2 = 1.55$, $P = 0.212$). In the vial trials, the excess of significant tests (indicating both positive and negative assortative mating) over what would be expected from Type I error was likely the result of nonindependence of separate replicate vials within a line. Row effects, caused by different groups of flies spending varying amounts of time under cold anesthesia when setting up rows of vials for the mating trials,

TABLE 1. ANOVAs of the effects of environment, bottleneck, and their interaction on assortative mating (*Y*) and the proportion of line males mating (p; arcsine square-root transformed) during mating trials performed in vials and cages. Each term had a single degree of freedom.

Method	Source	SS	*F*	*P*
Assortative mating (*Y*)				
Vials	environment	0.647×10^{-3}	0.017	0.897
	bottleneck	93.2×10^{-3}	2.41	0.124
	environment × bottleneck	8.95×10^{-3}	0.232	0.631
Cages	environment	2.63×10^{-4}	0.001	0.915
	bottleneck	52.6×10^{-4}	0.227	0.635
	environment × bottleneck	35.3×10^{-4}	0.153	0.697
Proportion line males mating (p)				
Vials	environment	0.7436	45.88	<0.0001
	bottleneck	0.1040	6.42	0.0134
	environment × bottleneck	0.0506	3.12	0.0815
Cages	environment	2.3947	393.53	<0.0001
	bottleneck	0.0201	3.31	0.0730
	environment × bottleneck	0.0771	12.68	0.0007

could create such nonindependence of separate vials. Such nonindependence will inflate the degrees of freedom in statistical tests, causing an excess of significant results.

Male Mating Success

Mating success of line males (p) varied by treatment for trials conducted in both vials and cages (Fig. 2), with environment having a consistent and highly significant effect overall (Table 1). For lines from the novel environment treatment, line males competed poorly with base males, obtaining significantly fewer matings on average in both unbottlenecked (vials: $p_{UN} = 0.411$, cages: $p_{UN} = 0.210$) and bottlenecked (vials: $p_{BN} = 0.298$, cages $p_{BN} = 0.142$) lines (*t*-tests with $\hat{p} = 0.5$, $P < 0.001$ for all cases). However, when base males were reared for two generations in the novel environment prior to the mating trials, their mating advantage was lost (Fig. 2; mean male mating success of line males when tested against novel-environment-reared base males = 0.494, $P = 0.832$). In contrast to the results from the novel environment treatment, the mating success of line males from the ancestral environment treatment was not reduced. Line and base males had similar mating success in bottlenecked (vials: $p_{BA} = 0.530$, cages: $p_{BA} = 0.505$; $P > 0.34$ for both) and unbottlenecked populations when measured in cages ($p_{UA} = 0.475$; $P = 0.107$); when measured in vials, ancestral environment line males actually obtained significantly more matings than base males ($p_{UA} = 0.553$, $P = 0.029$), although the difference was small.

The effects of bottlenecking on line male mating success

TABLE 2. Assortative mating scores (*Y*) for lines showing significant assortative mating (positive or negative) for trials performed in either vials or cages. For vials, the significance of *Y* is evaluated using the statistic $X^2(Y)$, which is χ^2 distributed with 1 df (Fienberg 1977; Spieth and Ringo 1983). For cages, significance is determined from a z-test of the proportion females mating with a homotypic male for each cage, under the null hypothesis of $\hat{p} = 0.5$ (nonassortative mating).

	Vials		Cages	
Line	*Y*	*P*	*Y*	*P*
Significant in vials only				
BA13	0.212	0.041	0.020	0.325
BN3	−0.314	0.013	−0.432	0.617
BN5	0.377	0.014	−0.110	0.689
BN8	0.323	0.015	0.078	0.396
BN9	0.286	0.036	−0.157	0.701
UA2	0.293	0.014	−0.026	0.757
UA5	0.298	0.007	0.082	0.146
UA11	−0.713	0.001	0.033	0.376
UA14	−0.524	<0.001	0.154	0.206
UA15	−0.454	<0.001	−0.007	0.539
UA16	0.269	0.009	−0.050	0.674
UN1	−0.371	<0.001	0.109	0.394
UN8	−0.238	0.037	0.062	0.504
UN16	0.276	0.019	0.055	0.473
Significant in cages only				
BA9	0.123	0.263	0.203	0.044
UA12	−0.173	0.126	0.160	0.008

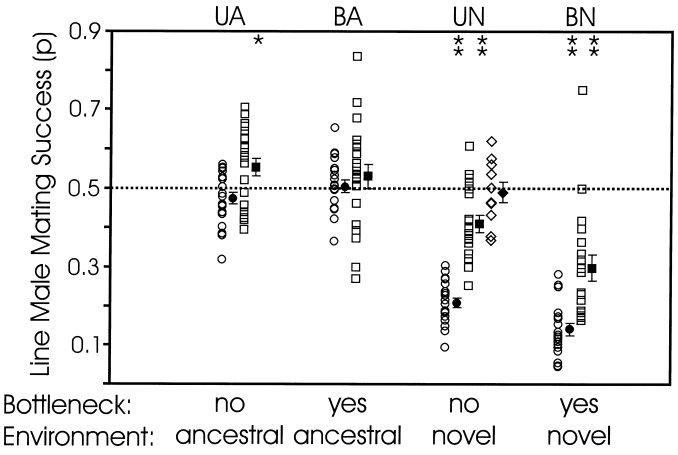

FIG. 2. Proportion of matings obtained by line males (p) by treatment combination for mating trials performed in cages (circles) and vials (squares). Treatment means (closed symbols ± SE) that are significantly different from $\hat{p} = 0.5$ (dashed line; equal mating success of base and line males) are indicated by the asterisks along the top of the panel (*$P < 0.03$, **$P < 0.001$). Results of the test to estimate the environmental effect of the novel environment on male mating success are also shown (far right of UN column, diamonds; see text for details).

overall were smaller and differed between environments for both vial and cage trials (Fig. 2). When measured in vials, bottlenecking significantly reduced male mating success (Table 1). However, this effect was primarily the result of differences in the performance of males from the novel environment treatment; in the novel environment lines, bottlenecked males competed poorly with base males for mates ($p_{BN} = 0.298$; $P < 0.0001$) and were less successful at obtaining mates than were unbottlenecked males ($p_{BN} = 0.298$ vs. $p_{UN} = 0.411$; $t_{36} = -3.38$, $P = 0.002$). In contrast, in the ancestral environment treatment, bottlenecked line males competed roughly equally with base males for mates ($p_{BA} = 0.530$, $P = 0.34$) and their success at obtaining mates did not differ significantly from that of unbottlenecked line males ($p_{BA} = 0.530$ vs. $p_{UA} = 0.553$; $t_{38} = -0.59$, $P = 0.58$). Results were similar when measured in cages, where the effect of bottlenecking again varied by environment (see interaction term in Table 1). As in vials, bottlenecking affected male mating success primarily in the novel environment treatment; bottlenecked males from these lines competed poorly with base males for mates ($p_{BN} = 0.142$, $P < 0.0001$) and were less successful than unbottlenecked line males at obtaining mates ($p_{BN} = 0.142$ vs. $p_{UN} = 0.210$; $t_{36} = -3.35$, $P = 0.002$). Also as in vials, bottlenecking effects were minimal in the ancestral environment treatment, with bottlenecked males from these lines suffering no mating disadvantage compared to base males ($p_{BA} = 0.505$, $P = 0.75$). Contrary to vial results, however, bottlenecked males from these lines were actually slightly more successful than unbottlenecked line males at competing for mates ($p_{BA} = 0.505$ vs. $p_{UA} = 0.475$), thus generating the overall environment $\times$ bottlenecking interaction. However, this difference was not significant ($t_{38} = 1.38$, $P = 0.17$).

Discussion

Population Bottlenecks and Speciation

The idea that population bottlenecks alone may be an important mechanism in the origin of new species seems to endure, despite accumulating evidence to the contrary (Turelli et al. 2001). In the current experiment, two successive and extreme bottlenecks failed to generate significant premating isolation overall in 20 replicate populations inhabiting their ancestral environment (Fig. 1). Results similar to the current study, following single population bottlenecks, have been found in two past studies using *D. melanogaster* (Rundle et al. 1998; Mooers et al. 1999). The current study strengthens the conclusion that population bottlenecks alone do not cause the evolution of reproductive isolation, especially because, as outlined earlier, it was performed under conditions hypothesized to be highly conducive to bottleneck-induced speciation.

Population bottlenecks not only failed to generate any reproductive isolation, but they hampered the speciation process by reducing the mating success of bottlenecked males, relative to unbottlenecked males, in the novel environment (7–11% decrease; Fig. 2). The most likely explanation is inbreeding depression. Such persistent (the effects were detected over two years after the second bottleneck) inbreeding depression will enhance the mating success of immigrant, ancestral males, thus increasing gene flow into a population (Mooers et al. 1999). Inbreeding depression of a similar magnitude has been found in studies measuring male mating success in *D. melanogaster* (Sharp 1984; Mooers et al. 1999) and has also been shown to persist for 20 generations after the bottleneck, despite relatively large population sizes during that time (Mooers et al. 1999). This suggests that substantial and persistent inbreeding depression of male mating success is not a rare phenomenon, at least in *Drosophila*. Understanding how often population bottlenecks occur in natural populations, and when they do, if they produce similar effects is an important goal for future speciation research.

In contrast to the reduced male mating success of bottlenecked lines from the novel environment, bottlenecked lines from the ancestral environment (BA lines) suffered no such effect (Fig. 2). The absence of inbreeding depression in these lines is surprising and the cause is not clear, although it is likely that these lines were less inbred than the novel environment (BN) lines (mean census size $\pm$ SE: BA = 555 $\pm$ 81; BN = 471 $\pm$ 36). The effective population sizes of the BN lines was likely further reduced both by smaller census sizes in the generations immediately following the bottlenecks and also by strong selection in the novel environment (Santiago and Caballero 1995). Nevertheless, the ancestral environment lines experienced two successive, extreme bottlenecks yet showed little effect in terms of male mating success. Inbreeding effects have been shown to vary among environments for other traits (Keller and Waller 2002), so it is possible that inbreeding depression of male mating success varied when measured in the different environments during cage mating trails. However, no such environmental differences existed when male mating success was measured in vials. A better understanding of the interaction between bottlenecks and novel environments, especially as it affects male mating success, requires further study.

Novel Environments and Speciation

In past experiments, premating isolation has evolved in multiple populations adapted to different environments (Kilias et al. 1980; Dodd 1989; Rice and Hostert 1993), demonstrating the feasibility of ecological speciation (Rundle and Schluter 2003) and suggesting that premating isolation can evolve rapidly and with relative ease. In contrast to these results, I detected no significant premating isolation between any of 19 replicate unbottlenecked populations inhabiting a novel environment and their ancestor (Fig. 1). In addition, the novel environment lines showed a substantial reduction in male mating success as compared to the ancestral environment lines (14–36% decrease; Fig. 2). While reduced male mating success could arise as a pleiotropic effect of adaptation to a novel environment, in the current experiment it was a nonadaptive result of environmentally induced phenotypic plasticity (as demonstrated by the mating success of males from 10 UN lines when retested against base males raised in the novel environment for two generations; Fig. 2). Although novel environments are commonly thought to promote speciation by generating divergent selection (Schluter 2000; Rundle and Schluter 2003), the novel environment in the current study failed to generate any premating isolation

and it reduced male mating success in such a way as to decrease the likelihood of speciation. The important question is how often novel environments produce such an effect in nature.

There are two possible explanations for the lack of premating isolation: either the populations did not adapt to their novel environment, or they adapted in a manner that did not produce premating isolation as a by-product. Which is responsible for the current result is not clear. There is no evidence that the populations adapted to their novel environment, and it is possible that divergent selection was sufficiently strong to have reduced genetic variation and hampered their response (although census sizes at the end of the experiment were similar in both environments). Well-replicated fitness measures could help distinguish these alternatives, although they are not necessarily mutually exclusive. Both alternatives are certainly plausible and likely occur in natural populations under various circumstances. While their underlying causes may differ and they thus warrant further investigation, the ultimate effect of both is to reduce the likelihood of speciation.

Whether premating isolation evolves when populations colonize novel environments likely depends on many factors. These include, among others, the form and intensity of selection, the genetic architecture of the traits under selection, details of the mating systems, and the effective sizes of the populations. Understanding how these various factors interact to affect the likelihood of speciation is an important goal for future laboratory speciation experiments (Ödeen and Florin 2000). To this end, the publication of negative results is fundamental; one wonders how many unsuccessful divergent selection experiments have never made it out of our collective filing cabinets. Although not discussed earlier because they failed to control for the effects of genetic drift, there are 12 additional divergent selection laboratory experiments summarized in Rice and Hostert (1993). Of these 12 studies, four failed to find any significant premating isolation. If a significant publication bias also exists, then novel environments may not often generate reproductive isolation, at least as rapidly as uggested by some studies.

The lack of premating isolation in the current experiment is nevertheless surprising for several reasons. First, the novel environment differed from the ancestral environment in a number of aspects (e.g., food, temperature, light) that are expect to have generated divergent selection on numerous traits, maximizing the chance that premating isolation would evolve as a by-product (Rice and Hostert 1993). Second, effective population sizes were likely relatively large in comparison with past studies (Ödeen and Florin 2000), given the census sizes of the populations at the end of the experiment (average census size $\pm$ SE of UN lines: 1066 $\pm$ 85). Effective population size has been suggested to be a key parameter in speciation experiments (Ödeen and Florin 2000), and it seems unlikely that a lack of variation would have prevented a response to selection in the current study. Third, past successful experiments used environments that differed in similar ways; Dodd (1989) varied food (starch vs. maltose based) in *D. pseudoobscura*, and Kilias et al. (1980), working with *D. melanogaster*, varied photoperiod (0L:24D vs. 12L:12D), temperature (14–18°C vs. 25°C), and relative humidity (43% vs. 90%; humidity was not controlled in the current study). In both past studies, however, neither environment was ancestral for any of the populations, but rather each likely generated strong, novel selection. Thus, tests for assortative mating were conducted between two divergently selected lines, as opposed to tests between lines under novel selection and their ancestor. Reproductive isolation may be more likely in the former situation because it maximizes divergence between lines (Florin and Ödeen 2002) or because other factors that may otherwise generate negative assortative mating between populations (e.g., differences in inbreeding and/or environmental effects on mating success) are reduced (Ödeen and Florin 2002). Exploring these factors is another important goal for future laboratory speciation experiments.

Experimental Power

The statistical power of an experiment is of concern whenever the outcome includes nonsignificant treatment effects. In the current experiment, it is unlikely that the lack of significant treatment effects (both bottleneck and environment) on the evolution of assortative mating were caused by weak statistical power. My experiment used 120–150 replicate matings when calculating assortative mating scores (in cages) for every line, and 19 or 20 replicate lines within each treatment combination. Such replication is greater than that used in past studies that have found assortative mating between populations adapted to alternate environments (e.g., Kilias et al. 1980; Dodd 1989) and similar or greater than that used in studies that have found assortative mating following population bottlenecks (see Rundle et al. 1998). With 19 or 20 replicate lines within each treatment combination, the minimum detectable treatment effect (Zar 1996) in the current experiment was small; a difference in assortative mating scores of $Y = 0.07$ between bottleneck or environment treatments had a 90% chance of detection. In past experiments (Kilias et al. 1980; Dodd 1989), assortative mating scores (Y) between lines adapted to different environments have been much higher, ranging from 0.16 to 0.49; assortative mating scores from past bottlenecking experiments are highly variable and tend to suffer from experimental and statistical concerns that complicate their interpretation (see Rundle et al. 1998, 1999).

Finally, results from the current experiment provide no indication of any interaction between population bottlenecks and divergent selection in the evolution of reproductive isolation; no significant premating isolation evolved between any of the 19 replicate bottlenecked lines in the novel environment and their ancestor (Fig. 1). While this is consistent with previous results (Mooers et al. 1999) and suggests that bottlenecks do not interact with divergent selection to increase the likelihood of speciation, the lack of an effect of the novel environment alone hampers this interpretation by limiting the power to detect such an interaction. Evaluation of the combined role of these factors in the evolution of reproductive isolation deserves further study.

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Literature Cited

Ashburner, M. 1989. *Drosophila*: a laboratory handbook. Cold Spring Harbor Laboratory Press, New York.

Averhoff, W. W., and R. H. Richardson. 1974. Pheromonal control of mating patterns in *Drosophila melanogaster*. Behav. Genet. 4:207–225.

———. 1976. Multiple pheromone system controlling mating in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA 73: 591–593.

Bishop, Y. M. M., S. E. Fienberg, and P. W. Holland. 1975. Discrete multivariate analysis: theory and practice. MIT Press, Cambridge, MA.

Carson, H. L. 1968. The population flush and its genetic consequences. Pp. 123–137 *in* R. C. Lewontin, ed. Population biology and evolution. Syracuse Univ. Press, Syracuse, NY.

Carson, H. L., and A. R. Templeton. 1984. Genetic revolutions in relation to speciation phenomena: the founding of new populations. Annu. Rev. Ecol. Syst. 15:97–131.

Carulli, J. P., D. M. Chen, W. S. Stark, and D. L. Hartl. 1994. Phylogeny and physiology of *Drosophila* opsins. J. Mol. Evol. 38:250–262.

Charlesworth, B., R. Lande, and M. Slatkin. 1982. A neo-Darwinian commentary on macroevolution. Evolution 36:474–498.

Dodd, D. M. B. 1989. Reproductive isolation as a consequence of adaptive divergence in *Drosophila pseudoobscura*. Evolution 43: 1308–1311.

Dodd, D. M. B., and J. R. Powell. 1985. Founder-flush speciation: an update of experimental results with *Drosophila*. Evolution 39:1388–1392.

Fienberg, S. E. 1977. The analysis of cross-classified categorical data. MIT Press, Cambridge, MA.

Florin, A.-B., and A. Ödeen. 2002. Laboratory environments are not conducive for allopatric speciation. J. Evol. Biol. 15:10–19.

Fowler, K., and M. C. Whitlock. 1999. The distribution of phenotypic variance with inbreeding. Evolution 53:83–96.

———. 2002. Environmental stress, inbreeding, and the nature of phenotypic and genetic variance in *Drosophila melanogaster*. Proc. R. Soc. Lond. B 269:677–683.

Galiana, A., A. Moya, and F. J. Ayala. 1993. Founder-flush speciation in *Drosophila pseudoobscura*: a large-scale experiment. Evolution 47:432–444.

Gavrilets, S., and C. R. B. Boake. 1998. On the evolution of premating isolation after a founder event. Am. Nat. 152:706–716.

Gavrilets, S., and A. Hastings. 1996. Founder effect speciation: a theoretical reassessment. Am. Nat. 147:466–491.

Gavrilets, S., H. Li, and M. D. Vose. 1998. Rapid speciation on holey adaptive landscapes. Proc. R. Soc. Lond. B 265: 1483–1489.

Hollocher, H., C.-T. Ting, F. Pollack, and C.-I. Wu. 1997. Incipient speciation by sexual isolation in *Drosophila melanogaster*: variation in mating preference and correlation between sexes. Evolution 51:1175–1181.

Keller, L. F., and D. M. Waller. 2002. Inbreeding effects in wild populations. Trends Ecol. Evol. 17:230–241.

Kilias, G., S. N. Alahiotus, and M. Pelecanos. 1980. A multifactorial genetic investigation of speciation theory using *Drosophila melanogaster*. Evolution 34:730–737.

Koskinen, M. T., T. O. Haugen, and C. R. Primmer. 2002. Contemporary Fisherian life-history evolution in small salmonid populations. Nature 419:826–830.

Meffert, L. M. 1995. Bottleneck effects on genetic variance for courtship repertoire. Genetics 139:365–394.

Meffert, L. M., and E. H. Bryant. 1991. Mating propensity and courtship behavior in serially bottlenecked lines of the housefly. Evolution 45:293–306.

Moya, A., A. Galiana, and F. J. Ayala. 1995. Founder-effect speciation theory: failure of experimental corroboration. Proc. Natl. Acad. Sci. USA 92:3983–3986.

Mooers, A. Ø., H. D. Rundle, and M. C. Whitlock. 1999. The effects of selection and bottlenecks on male mating success in peripheral isolates. Am. Nat. 153:437–444.

Ödeen, A., and A.-B. Florin. 2000. Effective population size may limit the power of laboratory experiments to demonstrate sympatric and parapatric speciation. Proc. R. Soc. Lond. B 267: 601–606.

———. 2002. Sexual selection and peripatric speciation: the Kaneshiro model revisited. J. Evol. Biol. 15:301–306.

Phillips, P. C., M. C. Whitlock, and K. Fowler. 2001. Inbreeding changes the shape of the genetic covariance matrix in *Drosophila melanogaster*. Genetics 158:1137–1145.

Powell, J. R., and L. Morton. 1979. Inbreeding and mating patterns in *Drosophila pseudoobscura*. Behav. Genet. 9:425–429.

Rice, W. R., and E. E. Hostert. 1993. Laboratory experiments on speciation: What have we learned in 40 years? Evolution 47: 1637–1653.

Ringo, J., D. Wood, R. Rockwell, and H. Dowse. 1985. An experiment testing two hypotheses of speciation. Am. Nat. 126: 642–661.

Rundle, H. D., and D. Schluter. 2003. Natural selection and speciation in sticklebacks. Pp. 192–209 *in* U. Dieckmann, H. Metz, M. Doebeli, and D. Tautz, eds. Adaptive speciation. Cambridge Univ. Press, Cambridge, U.K.

Rundle, H. D., A. Ø. Mooers, and M. C. Whitlock. 1998. Single founder-flush events and the evolution of reproductive isolation. Evolution 52:1850–1855.

———. 1999. Experimental tests of founder-flush: a reply to Templeton. Evolution 53:1632–1633.

Saccheri, I. J., and P. M. Brakefield. 2002. Rapid spread of immigrant genomes into inbred populations. Proc. R. Soc. Lond. B 269:1073–1078.

Salcedo, E., A. Huber, S. Henrich, L. V. Chadwell, W. Chou, R. Paulsen, and S. G. Britt. 1999. Blue- and green-absorbing visual pigments of *Drosophila*: ectopic expression and physiological characterization of the R8 photoreceptor cell-specific Rh5 and Rh6 rhodopsins. J. Neurosci. 19:10716–10726.

Santiago, E., and A. Caballero. 1995. Effective size of populations under selection. Genetics 139:1013–1030.

Schluter, D. 2000. The ecology of adaptive radiation. Oxford Univ. Press, Oxford, U.K.

Sharp, P. M. 1984. The effect of inbreeding on competitive male-mating ability in *Drosophila melanogaster*. Genetics 106: 601–612.

Spieth, H. T., and J. M. Ringo. 1983. Mating behaviour and sexual isolation in *Drosophila*. Pp. 223–284 *in* M. Ashburner, H. L. Carson, and J. N. Thomson, eds. The genetics and biology of *Drosophila*. Vol. 3c. Academic Press, New York.

Templeton, A. R. 1980. The theory of speciation via the founder principle. Genetics 94:1011–1038.

———. 1989. Founder effects and the evolution of reproductive isolation. Pp. 329–344 *in* L. V. Giddings, K. Y. Kaneshiro, and W. W. Anderson, eds. Genetics, speciation and the founder principle. Oxford Univ. Press, New York.

———. 1996. Experimental evidence for the genetic-transilience model of speciation. Evolution 50:909–915.

———. 1999. Experimental tests of genetic transilience. Evolution 53:1628–1632.

True, J. R., J. M. Mercer, and C. C. Laurie. 1996. Differences in crossover frequency and distribution among three sibling species of *Drosophila*. Genetics 142:507–523.

Turelli, M., N. H. Barton, and J. Coyne. 2001. Theory and speciation. Trends Ecol. Evol. 16:330–343.

Vincek, V., C. O'Huigin, Y. Satta, N. Takahata, P. T. Boag, P. R. Grant, B. R. Grant, and J. Klein. 1997. How large was the founding population of Darwin's finches? Proc. R. Soc. Lond. B 264: 111–118.

Whitlock, M. C., and K. Fowler. 1999. The changes in genetic and environmental variance with inbreeding in *Drosophila melanogaster*. Genetics 152:345–353.

Whitlock, M. C., P. C. Phillips, and K. Fowler. 2002. Persistence of changes in the genetic covariance matrix after a bottleneck. Evolution 56:1968–1975.

Zar, J. H. 1996. Biostatistical analysis. 3rd ed. Prentice Hall, Englewood Cliffs, NJ.

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