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IMPACT OF EXPERIMENTAL DESIGN ON *DROSOPHILA* SEXUAL ISOLATION STUDIES: DIRECT EFFECTS AND COMPARISON TO FIELD HYBRIDIZATION DATA

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Abstract.—Many studies of speciation rely critically on estimates of sexual isolation obtained in the laboratory. Here we examine the sensitivity of sexual isolation to alterations in experimental design and mating environment in two sister species of *Drosophila*, *D. santomea* and *D. yakuba*. We use a newly devised measure of mating frequencies that is able to disentangle sexual isolation from species differences in mating propensity. Variation in fly density, presence or absence of a quasi-natural environment, degree of starvation, and relative frequency of species had little or no effect on sexual isolation, but one factor did have a significant effect: the possibility of choice. Designs that allowed flies to choose between conspecific and heterospecific mates showed significantly more sexual isolation than other designs that did not allow choice. These experiments suggest that sexual isolation between these species (whose ranges overlap on the island of São Tomé) is due largely to discrimination against *D. yakuba* males by *D. santomea* females. This suggestion was confirmed by direct observations of mating behavior. *Drosophila santomea* males also court *D. yakuba* females less ardently than conspecific females, whereas neither males nor females of *D. yakuba* show strong mate discrimination. Thus, sexual isolation appears to be a result of evolutionary changes in the derived island endemic *D. santomea*. Surprisingly, as reported in a companion paper (Llopart et al. 2005), the genotypes of hybrids found in nature do not accord with expectations from these laboratory studies: all F_1 hybrids in nature come from matings between *D. santomea* females and *D. yakuba* males, matings that occur only rarely in the laboratory.

Key words.—Mate choice, mate discrimination, mating propensity, reproductive isolation, sexual isolation.

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Behavioral isolation involving mate discrimination is probably one of the most important causes of speciation in animals (Coyne and Orr 2004). Such discrimination has been studied most extensively in the genus *Drosophila*, in which measurements of sexual isolation exist for nearly 150 pairs of closely related species (Coyne and Orr 1997). These measurements have been used in a variety of evolutionary studies, including those determining whether reinforcement increases mate discrimination in sympatry (Ehrman 1965; Noor 1995), comparing the evolutionary rate of sexual isolation with that of other isolating barriers (Patterson and Stone 1952; Coyne and Orr 1989, 1997), studying the genetics of sexual isolation (Tan 1946; Wu et al. 1995), and discovering cryptic species that are morphologically similar but behaviorally isolated (Ehrman 1965; Malagolowkin-Cohen et al. 1965). Because accurate measurements of sexual isolation are important in resolving many evolutionary questions—including studies of reinforcement, sympatric speciation, and founder-effect speciation—it is important to obtain measurements that realistically estimate the degree of mate discrimination in nature. Here we examine the effect of varying the conditions under which mating occurs on the degree of sexual isolation between two species of *Drosophila*, and we compare our results to hybridization seen in nature (detailed in a companion paper, Llopart et al. 2005).

As is true for many species, laboratory studies of sexual isolation in *Drosophila* usually involve one of four designs: (1) no-choice, in which single pairs of the opposite sex are confined together and the frequency of conspecific matings compared with that of heterospecific matings; (2) male-choice, in which a single male is confined with one conspecific and one heterospecific female; (3) female-choice, in which one female is confined with one conspecific and one heterospecific male; and (4) multiple-choice, in which males and females of two species (usually many of each) are placed in a mating chamber and copulating pairs identified (for a review of the methodology and results of many earlier studies, see Spieth and Ringo 1983). In all cases, the relative numbers of heterospecific and homospecific matings are used to construct an index of sexual isolation. The many suggested indices and their relative merits were discussed by Gilbert and Starmer (1985), Rolán-Alvarez and Caballero (2000), and Pérez-Figueroa et al. (2005).

Several problems arise with measuring sexual isolation in the laboratory. First, different test designs using the same pair of species may yield different indices of sexual isolation. In their comparative study, for example, Coyne and Orr (1989, 1997) implicitly assumed that different mating designs all provide similar estimates of sexual isolation. But there has been no systematic study of this possibility, and there are only three comparisons of the effects of experimental design on sexual isolation. Malagolowkin-Cohen et al. (1965) found that male-choice and multiple-choice tests in races of *D. paulistorum* gave similar estimates of sexual isolation. In contrast, Merrell (1954) found that in *D. pseudoobscura* and *D. persimilis*, male-choice experiments showed lower sexual isolation than did female-choice experiments, which themselves gave results similar to multiple-choice experiments. This implies that female discrimination is a primary cause of sexual isolation in these species, a conclusion later substantiated by Noor (1996). Comparing no-choice with male-

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choice experiments in *D. melanogaster* and *D. simulans*, Wood and Ringo (1980) found the counterintuitive result that *D. melanogaster* males courted *D. simulans* females more intensely when presented with both conspecific and heterospecific females than when presented only with *D. simulans* females. From the meager results at hand, then, it appears that experimental design may affect estimates of sexual isolation but not in a consistent way among diverse species. One expects such inconsistency if sexual isolation depends on female preference and male ardor to different degrees in different species. Yet, there has been no systematic exploration of the four different designs in a single pair of species.

Second, it is not clear that male- or female-choice experiments really test whether the designated sex exercises choice. Suppose, for example, that sexual isolation between species A and B occurs solely because females of species B refuse the courtship of males from species A, while both A and B males court females of both species indiscriminately. In a male-choice test, A males presented with both A and B females will mate only with A females. Yet, this does not reflect choice by A males, but rather the refusal of B females to mate with them. (Similarly, only one sex might be choosy in multiple-choice experiments.) Such discrimination must be assessed not by counting matings themselves but by observing and measuring courtship behavior.

Third, for most species, at least in *Drosophila*, it is not clear which experimental design most realistically mimics mate choice in nature, the implicit goal of laboratory experiments on sexual isolation. While there have been studies of intraspecific mating behaviors in nature, most notably in cactophilic species and lekking species from Hawaii (Shelly 1989; Krebs and Bean 1991; Pitnick 1993), as well as some work on the effects of changes in the laboratory environments on sexual isolation (see Spieth and Ringo 1983), there has been no systematic laboratory work investigating which factors influence sexual isolation in nature. Our ability to extrapolate laboratory tests of sexual isolation to nature thus depends on understanding how flies of different species encounter and evaluate each other as potential mates in the wild. Do females encounter males singly, so that a no-choice situation is more natural; do females encounter lekking males, so that a female-choice situation might exist; or do male and females encounter each other multiply on food resources, so that multiple-choice designs are most realistic?

Spieth and Ringo (1983, p. 247) asserted, ''In the absence of prior knowledge about the effects of experimental design on mating behavior, the best design is the one that imitates nature most closely. Therefore, the multiple-choice design would usually be preferable to the other three.'' But this conclusion does not follow. Given our depauperate knowledge of *Drosophila* ecology, we simply do not know whether a multiple-choice situation is natural. For example, even in cases where many flies of two species congregate on a resource, females could nevertheless evaluate males individually and sequentially and quickly reject unfavored ones.

Fourth, the simple, sterile conditions of the laboratory may yield indices of sexual isolation very different from those obtained from individuals encountering each other in the complexity of nature. We completely agree with Spieth and Ringo's advice (1983, p. 247) that ''It may be worthwhile to measure sexual behavior under a variety of naturalistic conditions. For example, the complex environment of an insectarium (Jacobs 1978) might be used instead of the standard dry, plastic container.''

Finally, indices designed to measure sexual isolation often do not really do so. This is because some indices conflate sexual isolation with mating propensity: that is, species that differ only in their general willingness to mate, but show no discrimination against heterospecific members of the opposite sex, can nevertheless yield positive values of sexual isolation. Moreover, species differences in mating propensity can not only artifactually create sexual isolation, but can also cause significant biases in indices of sexual isolation (Gilbert and Starmer 1985; Rolán-Alvarez and Caballero 2000). Such biases have been demonstrated experimentally (Casares et al. 1998).

Here we study the effect of experimental design and mating environment on sexual isolation between two sister species, *Drosophila yakuba* and *D. santomea*, previously subject to other studies of sexual isolation in our laboratory (Coyne et al. 2002). *Drosophila yakuba* is widespread across sub-Saharan Africa and on islands near the continent. *Drosophila santomea*, discovered in 1998, is endemic to São Tomé, an 860-km^2 volcanic island 255 km from the coast of Gabon (Lachaise et al. 2000). *Drosophila yakuba* also inhabits São Tomé. On the mountain of Pico de São Tomé, *D. yakuba* lives at elevations below 1450 m, *D. santomea* at elevations above 1100 m, and between these elevations the species ranges' overlap, forming a hybrid zone in which one finds a low frequency ($\sim$ 0.3%) of hybrids (Lachaise et al. 2000; Llopart et al. 2005).

These species show substantial sexual isolation when tested in the laboratory (Coyne et al. 2002), with both interspecific matings occurring less often than intraspecific matings, and the mating between *D. santomea* females and *D. yakuba* males occurring very rarely. The pair fails to show any reinforcement, that is, there is no increase in sexual isolation between species in hybrid zone (Coyne et al. 2002). Molecular evidence puts the divergence between *D. yakuba* and *D. santomea* at about 400,000 years ago (Llopart et al. 2002). The ecology of these species is, as with most species in the genus, nearly unknown. In the African mainland, *D. yakuba* is widespread across sub-Saharan Africa from Senegal to South Africa, living in a diversity of open or semi-open habitats including semi-arid areas, lowland savannas, and montane grassland. In São Tomé, *D. yakuba* is found largely in disturbed, cutover areas, open plantations, edges of the rainforest, and towns, whereas *D. santomea* lives only in virgin montane rain and mist forest (Llopart et al. 2005). We suspect that *D. santomea* breeds largely, if not exclusively, in figs of the endemic subspecies *Ficus chlamydocarpa fernandesiana* (Llopart et al. 2005).

Our purpose in studying the effects of experimental design on sexual isolation is fourfold. First, we wished to determine whether the four major types of mating test designs give different estimates of sexual isolation. Such a finding would suggest that any estimate of isolation based on a single test is an unreliable general estimate of isolation. Second, we wished to determine whether varying environmental factors such as food availability, mating chamber volume, the pres-

ence of a more natural environment, and the relative density of species affects sexual isolation. To compare sexual isolation in these studies, we use a recently introduced statistic that is uncontaminated by interspecific differences in mating propensity. Third, we wished to see if direct observations of mating behavior confirm the implications of sexual-isolation tests about which sex is most responsible for sexual isolation. This tells us whether classical mate-discrimination experiments can help identify the dynamics of mating behavior. Finally, because we have now determined the genotypes of hybrids produced in nature (see Llopart et al. 2005), we wished to see whether those interspecific crosses that proceed most easily in the laboratory are also those yielding most hybrids found in the field. That is, does the asymmetrical sexual isolation seen in laboratory tests of these species also operate in nature, so that most F_1 hybrids have *D. yakuba* mothers?

Materials and Methods

We used one strain of each species. The *D. santomea* STO.4 stock was derived from the offspring of a single female collected on March 1998 in the Obo Natural Reserve on São Tomé Island, in the zone of sympatry with *D. yakuba*. The *D. yakuba* stock, Taï 18, was derived from the offspring of a female collected by D. Lachaise in 1983 in the Taï rainforest on the border between Liberia and the Ivory Coast. We used these stocks extensively in previous studies (Coyne et al. 2002; Llopart et al. 2002).

These species are morphologically distinguishable, as both sexes of *D. yakuba* have abdomens much more heavily pigmented than individuals of *D. santomea* (Lachaise et al. 2000). Thus, we did not need to artificially mark flies with dyes or wing-clipping to identify mating pairs.

Effects of Environmental Factors on Sexual Isolation

To compare sexual isolation under different conditions, we conducted five experiments. All flies used in these experiments were 4-day-old virgins reared in uncrowded half-pint bottles in incubators at 24°C under a 12:12 light:dark cycle. Flies were originally collected under CO_2 anesthesia, stored in groups of 10–15, and introduced into mating chambers by aspiration or (in cages) by opening the vials inside the cage. Mating experiments began at 0900 h (when incubator lights went on) and took place at room temperature, between 21°C and 23°C. Within each experiment, different experimental designs were used on the same days, often at the same time, using flies collected from the same bottles.

Experiment A: effects of choice

This experiment investigated the four classic designs of mating experiments to determine if they gave different measures of sexual isolation. The four designs were used simultaneously.

No-choice design.—A single male and female are placed in an 8-dram vial containing cornmeal-agar medium and observed for 1 h. Forty pairs were watched simultaneously: 10 of each of the four pairings (S × S, Y × Y, S × Y, and Y × Y, where S indicates *D. santomea*, Y *D. yakuba*, and the species of female is given first). Fifteen replicates of this experiment were made (150 possible matings for each pairing), and for each pair we recorded whether a copulation took place and how long after the start of the experiment it occurred (copulation latency).

Female-choice design.—This study was conducted as described above, except that each vial contained one female, one conspecific male, and one heterospecific male. Two groups of 15 vials each were watched simultaneously, with one group having *D. santomea* females (S × [S, Y]) and the other *D. yakuba* females (Y × [S, Y]). For each vial, we recorded the male copulating with the female (if any) and the copulation latency. Fifteen replicates were conducted, giving a total of 225 possible matings for each combination of flies.

Male-choice design.—This study was conducted as above, except that each vial contained one male, one conspecific female, and one heterospecific female. Two groups of 15 vials each were watched simultaneously, with one group having *D. santomea* males ([S, Y] × S) and the other *D. yakuba* males ([S, Y] × Y). For each vial, we recorded the female copulating with the male (if any) and the copulation latency. Fifteen replicates were conducted, giving a total of 225 possible matings for each combination of flies.

Multiple-choice design.—This design used flies of both sexes and both species in a large mating chamber. Flies were introduced into a Plexiglas cage measuring 18 × 24 × 25 cm and having a 14-cm diameter circular hole on the side. A mesh sleeve covered the hole and allowed introduction of flies and access to the mating pairs. Each cage contained a hexagonal plastic weigh boat holding a 0.25-in. layer of fresh cornmeal-agar medium. Each trial used 30 males and 30 females of each species, totaling 120 flies. As each copulation occurred, the mating pair was aspirated from the cage, the time of copulation recorded, and the pair placed in a numbered vial for later identification. The experiment lasted until 30 mating pairs were collected (only half the possible matings, since the element of choice can diminish as flies are removed from the cage), or, if 30 pairs were not collected, until 1 h had elapsed. This study was replicated 15 times, giving a maximum number of 450 matings for each type (this would occur only if all matings were of the same type).

Experiment B: effects of space

This experiment was designed to test the effect of space on sexual isolation by allowing flies (presumably females) more space to escape from unwanted mates, possibly increasing the degree of sexual isolation. The experiment used a no-choice design, but was conducted in cages on the one hand and vials on the other. Each cage was set up as described in the multiple-choice design of experiment A, but contained 60 males of a single species and either 60 females of the same species or of the other species. Cages were watched for 1 h, with all mating pairs removed by aspiration. We made five replicates of each of the four combinations of flies, allowing a maximum number of 300 matings per combination. The vial experiment was conducted identically to the no-choice design used in experiment A, but we made 18 repli-

cates, allowing a maximum number of 180 matings per pairing.

The vial and cage experiments were conducted simultaneously. The cage dimensions allowed for a volume of 90.0 cm^3/fly, and a surface area of 24.7 cm^2/fly, while the vial dimensions allowed for a volume of only 6.5 cm^3/fly but a surface area of 22.9 cm^2/fly. Because flies usually rest on side of cage during these experiments, but can escape unwanted matings by flying, flies in the vial and cage experiments have about the same probability of encountering another fly but a substantial difference in their ability to escape unwanted mates.

Experiment C: effects of a plant

This experiment was intended to show whether a slight increase in environmental complexity, caused by the introduction of a plant, would affect sexual isolation. In this study we simply placed a potted plant (a flowering African violet, *Saintpaulia ionantha* in a 4-in. pot) in the Plexiglas mating chamber as well as the hexagonal weigh boat containing fly medium. (The African violet is an East African tropical plant and does not grow on São Tomé.) The control (no-plant) cage had the weigh boat but lacked the potted plant. The added environmental complexity (and additional surface area) could affect sexual isolation by allowing flies additional space to escape unwanted matings. Indeed, flies were often observed on the plant and its soil during the mating trials.

We used the multiple-choice design with 30 flies of each sex and both species (120 flies total). The flies were treated identically to flies in the multiple-choice design of experiment A: each cage was watched (with mating pairs removed) for 1 h or until 30 pairs had mated, whichever came first. For each cage we used 12 replicates, giving a maximum number of 360 matings of a given type. The control and plant cages were run in successive pairs daily, with the order alternated each day.

Experiment D: effects of environment

In this experiment we tried to simulate to some extent the natural habitat of these flies, using, as suggested by Spieth and Ringo (1983, p. 247), ''the complex environment of an insectarium instead of the standard dry, plastic container.'' We thus constructed an environmental cage by covering the bottom of the large Plexiglas mating cage with 1.5 cm of potting soil and then with a layer of dead leaves. We then placed two African violets in 4-in. pots in opposite corners of the cage. Food was provided as three dried figs soaked in water for 20 min, split in half (to simulate semifresh figs on rain forest floor), and randomly placed on the leaf litter. The Plexiglas control cage (food) contained only a weigh boat of fly medium. As with other multiple-choice cages, we observed the flies for 1 h or until 30 mating pairs were collected, whichever came first. There were 15 replicates of each cage (with one of each done daily), giving a maximum number of 450 matings of a given type.

Experiment E: effects of starvation

Flies in nature are not likely to be as well fed as the flies used in our studies. Matings are thus likely to involve hungry flies meeting on a food substrate. Under these circumstances females might be less discriminating. To determine if there was any effect of starvation on sexual isolation, we conducted multiple-choice mating tests on two groups of flies, normal and starved. For this experiment we collected virgin flies 4 days before mating. Twenty-four hours before the mating experiments (day 3 of the holding time), we transferred starvation flies to empty bottles containing a piece of filter paper soaked in distilled water. Control flies are placed in food-containing bottles at the same time. We then conducted 1 h multiple-choice experiments as described above, with six halves of soaked, dried figs placed in the bottom of each mating chamber. Eight replicates were conducted, giving a maximum of 240 matings for each combination of flies.

Effects of species composition.—This experiment was conducted some time after the other studies described in this paper; its intent was to see whether the high frequency of natural hybrids having *D. santomea* mothers and *D. yakuba* fathers (Llopart et al. 2005) could be explained by a relatively high proportion of *D. yakuba* in areas where the species mate. Although in the laboratory the cross between *D. santomea* females and *D. yakuba* males occurs much less frequently than the reciprocal cross (Coyne et al. 2002; this study), it is possible that this more difficult mating would occur more frequently in areas where *D. santomea* was outnumbered by *D. yakuba*. Where there is a higher frequency of the latter species than the former, *D. santomea* females will encounter *D. yakuba* males more frequently than conspecific males, and, even though these females are reluctant to mate heterospecifically, they may do so if courted incessantly by *D. yakuba* males. This dependence of apparent sexual isolation on species frequency is called the Wirtz effect (Wirtz 1999; Chan and Levin 2005).

We tested the effect of species frequency on sexual isolation by using two ratios of *D. yakuba*:*D. santomea*, 1:1 and 3:1. Multiple-choice tests were conducted in cages using a protocol identical to that of the multiple-choice design of experiment A. In the 1:1 cages, 30 virgin males and 30 virgin females from each of the two species were watched for 1 h or until 30 matings had occurred, whichever came first. In the 3:1 cages, we placed 45 males and 45 females of *D. yakuba* and 15 males and 15 females of *D. santomea*, with cages again watched until either 30 matings had occurred or 1 h had passed. Eleven replicates were performed at each ratio, with pairs of cages having different ratios performed on each day in alternating order.

Statistical Analysis of Sexual Isolation

The different mating designs (male-, female-, multiple-, and no-choice) differ also from a statistical point of view: in the male-, female-, and no-choice designs, the different pairs can be considered true statistical replicates, but in the multiple-choice design they should be considered as pseudoreplicates. This is because in the multiple-choice design many specimens of the same type and sex are placed in the same container, and one cannot exclude the possibility that the behavior of one pair is indirectly affected by the others. In fact, it has been demonstrated that mate choice can change in multiple-choice experiments as the trial is lengthened to

obtain the maximum number of mates (Casares et al. 1998). Casares et al. (1998) suggested that multiple-choice experiments should be terminated (as we did in our work) when half of the possible matings are obtained. In summary, we assume here that mating pairs obtained under multiple-choice experiments are true replicates (i.e., they are not basically affected by the other pairs). We have corroborated this assumption a posteriori by showing that statistical conclusions about multiple-choice experiments based on bootstrapping (and thus assuming true replication) were identical to those obtained using parametric tests on experimental replicates, a procedure not affected by pseudoreplication problems (see Results).

The mating behavior in multiple-choice experiments can be decomposed into sexual selection and sexual isolation effects for each mating type using the *PTI*, *PSI*, and *PSS* coefficients of Rolán-Alvarez and Caballero (2000). No-choice experiments can be analyzed using the same approach when the experiment includes all possible combinations of mating pairs (see Nosil et al. 2002). We used a similar strategy for male-choice and female-choice experiments to compare the different experimental designs, although in these latter designs the mechanisms causing sexual isolation and sexual selection (mate choice and propensity) are confounded.

The *PTI*, *PSI*, and *PSS* coefficients have been formally described elsewhere (Rolán-Alvarez and Caballero 2000). Briefly, *PSI* coefficients estimate the strength of sexual isolation and are thus most directly related to processes of speciation. *PSI* coefficients reflect a choice between different types of mates when mating occurs. *PSS* coefficients, on the other hand, describe the effects of sexual selection, that is, selection typically caused by different mating propensities of different species or by asymmetric mating discrimination between species (see Lewontin et al. 1968). The *PTI* coefficients combine both processes ($PTI = PSI \times PSS$) and are simply the ratios of the observed frequencies of matings to those expected given random mating among the experimental frequencies of sexes and species (in our case, S × S, S × Y, Y × S, and Y × Y). Thus, *PTI* statistics estimate the deviation from random mating using population frequencies, whereas *PSI* statistics measure the deviations from random mating using exclusively data taken from mating pairs. All of these coefficients vary between zero and infinity, with values of one indicating that species do not show sexual isolation or do not differ in mating propensity and larger values indicating that species differ either in mate propensity, mate choice, or both.

For example, in the first table from experiment A (see Table 2) the *PTI*-values for the S × S pair is 1.35, which is the ratio between the observed number of mating (120) and the expected number of matings in the experiment (356/4, or 89). These and similar coefficients describe the consequences of mating behavior, but, as noted above deviations from random mating ($PTI = 1$) can be produced by distinct causes: mate choice (preferences) and mating propensity (Merrell 1954; Lewontin et al. 1968; Spieth and Ringo 1983). There is no simple way to know a priori which is the best strategy to study mating preferences, although in these species we have some expectation from previous work that female-choice discrimination among the species of males is plays a major role in nonrandom mating (Coyne et al. 2002). If mate choice is operating exclusively (i.e., if there is no detectable difference in mating propensity between species), the *PTI* coefficients are the best estimates of the true mating preferences (Rolán-Alvarez and Caballero 2000).

The significance of *PTI* estimates was determined by resampling 10,000 times using our compiled *PTI* software (available at http://webs.uvigo.es/c03/webc03/XENETICA/XB2/pti.zip). When a 0-value was observed in a particular mate pair combination, it was replaced by 1 to allow resampling under bootstrap techniques. The significance of the *PTI* statistics can be also verified indirectly by using the overall observed mating frequencies in the table, because under our experimental designs (at least those having equal frequencies of sexes and species) *PTI*-values are exclusively influenced by changes in the number of observed mating pairs. Thus, overall differences in *PTI*-values can be tested by the *G*-test for goodness of fit for single-classification frequency distributions to check if the observed distribution of mating frequencies follows the expected distribution based on the population (experimental) frequencies (Sokal and Rohlf 1995). The *G*-value is compared to the critical value of a χ^2 for ($n - 1$) degrees of freedom. The *G*-test can also be used to compare the overall *PTI* values across treatments to detect heterogeneity. This test can be decomposed additively into different components, with *G*-statistics calculated for each treatment separately as well for the pooled data (G_{pooled}). Thus, we expect that G_{pooled} would be equal to G_{total} (the sum of *G*-values for each treatment separately) if different treatments were completely homogeneous. The heterogeneity *G*-value ($G_h = G_{total} - G_{pooled}$; Sokal and Rohlf 1995, pp. 697–715) can be used to statistically check the existence of differences in *PTI* between treatments. However, differences between treatments in *PTI* are not equivalent to differences in sexual isolation, because different *PTI*-values can yield the same overall values of sexual isolation. The best alternative for estimating sexual isolation caused by mating preferences under biologically realistic sample sizes is the I_{PSI} statistic (Pérez-Figueroa et al. 2005). This is similar to the joint isolation index *I* first used by Malagolowkin-Cohen et al. (1965), but uses *PSI* coefficients instead of observed numbers of matings. Thus,

$$I_{PSI} = \frac{(PSI_{SS} + PSI_{YY}) - (PSI_{SY} + PSI_{YS})}{(PSI_{SS} + PSI_{YS} + PSI_{SY} + PSI_{YY})}, \qquad (1)$$

with PSI_{SS}, PSI_{SY}, PSI_{YS}, and PSI_{YY} being the *PSI* coefficients for the respective mating pair combinations in the table (see Rolán-Alvarez and Caballero 2000). We used this estimator to describe overall sexual isolation in the experiments. I_{PSI} varies from −1 to 1, with −1 representing maximum disassortative mating, 0 representing random mating, and 1 representing the maximum possible degree of assortative mating (complete sexual isolation). The sexual isolation index can also be calculated using the *PTI* indexes as well (this is adequate when *PTI* is the most relevant estimate of mate choice), although in such circumstances we would expect that I_{PTI} would be identical to I_{PSI} (i.e., sexual isolation is caused only by mate choice).

The significance of I_{PSI} estimates was determined by re-

TABLE 1. Five experiments (A–E) showing effect of choice versus no-choice tests and experimental conditions on sexual isolation. See Materials and Methods for details. In pairings, species of female is given first; S, *Drosophila santomea*; Y, *D. yakuba*.

	Matings	Average latency (min)	Standard error, latency	Replicates	Matings possible
Experiment A					
Female-choice					
S × S	157	12.66	0.86	15	225
S × Y	13	16.65	3.92	15	225
Y × S	28	21.78	2.44	15	225
Y × Y	158	20.85	1.11	15	225
Male-choice					
S × S	175	12.67	0.783	15	225
S × Y	25	24.13	2.66	15	225
Y × S	25	15.32	1.31	15	225
Y × Y	133	20.90	1.20	15	225
Multiple-choice					
S × S	120	18.16	1.35	15	450
S × Y	8	23.46	6.99	15	450
Y × S	28	17.94	2.72	15	450
Y × Y	200	23.68	1.04	15	450
No-choice					
S × S	101	17.75	1.34	15	150
S × Y	15	30.63	3.02	15	150
Y × S	61	24.59	1.59	15	150
Y × Y	119	20.70	1.11	15	150
Experiment B					
No-choice vial					
S × S	110	17.76	1.30	18	180
S × Y	19	31.95	3.03	18	180
Y × S	73	24.78	1.49	18	180
Y × Y	144	20.21	.98	18	180
No-choice cage					
S × S	86	21.11	1.73	5	300
S × Y	33	35.84	2.54	5	300
Y × S	67	21.13	1.86	5	300
Y × Y	195	22.82	1.07	5	300
Experiment C					
No plant					
S × S	83	16.96	1.48	12	360
S × Y	3	24.01	3.89	12	360
Y × S	22	20.87	3.05	12	360
Y × Y	171	20.60	1.03	12	360
Plant					
S × S	83	18.43	1.72	12	360
S × Y	1	16.40	N/A	12	360
Y × S	25	18.57	3.24	12	360
Y × Y	191	20.16	.96	12	360
Experiment D					
Environment					
S × S	7	16.375	4.568	15	450
S × Y	0	—	—	15	450
Y × S	5	36.850	8.078	15	450
Y × Y	62	27.070	2.102	15	450
Food					
S × S	103	27.51	1.66	15	450
S × Y	0	—	—	15	450
Y × S	19	24.76	2.83	15	450
Y × Y	170	27.23	1.20	15	450
Experiment E					
Food					
S × S	64	25.95	1.95	8	240
S × Y	2	39.67	—	8	240
Y × S	7	21.50	3.82	8	240
Y × Y	81	28.50	1.77	8	240
Starved					
S × S	56	20.30	1.97	8	240
S × Y	0	0	—	8	240
Y × S	7	31.05	5.65	8	240
Y × Y	85	29.17	1.63	8	240

sampling 10,000 times using the *PTI* software described above. To compare the levels of sexual isolation between treatments, we used ANOVA comparing the mean values (and Duncan test for the a posteriori differences between treatments) of isolation across treatments using the different experimental replicates (see Table 1; Sokal and Rohlf 1995). A few replicates were pooled a priori with the subsequent ones if the sample size was too small to calculate the statistics.

When multiple statistical tests were performed on a single dataset, significance was assessed after applying the sequential Bonferroni test (Rice 1989) to the multiple probability values.

Direct Observations of Mating Behavior

We observed courtship and copulatory behaviors of the *D. yakuba* and *D. santomea* strains to determine how sexual isolation operated and which aspects of behavior were affected. We wished to understand from this study the degree to which sexual isolation between these species is due to discrimination by females as opposed to reduced courtship by males and to check, through direct observation, conclusions derived from the mating tests described above. We again used the *D. yakuba* Taï 18 and *D. santomea* STO.4 lines. Each day, an equal number of the four possible pairings between these species were observed in an 8-dram food-containing vial (with the stopper pushed down to about 2 cm from the food surface to reduce the volume) for 30 min at room temperature. The following statistics were recorded or calculated (see also Coyne et al. 1994): courtship latency (for males): the time elapsing from when a male was introduced into a vial to when he began courtship (defined as orienting toward a female and vibrating a wing); copulation latency: the time elapsing from when a fly was introduced into a vial or cage to when it achieved successful copulation (i.e., a copulation lasting more than 30 sec); courtship duration (no-choice tests): within an observation period, the amount of time during which a male courts a female (defined as orienting toward a female, following her, or vibrating his wings near her); copulation attempts: each attempt constitutes one episode in which the male curls his abdomen beneath him and makes genital contact with the female, contact that does not result in copulation; and proportion of time courting: pro-

TABLE 2. Estimates of sexual isolation and mate propensity in the different experimental designs of mate choice (experiment A). Four treatments (mating designs) were compared: multiple choice (MuC), no choice (NC), female choice (FC), and male choice (MC). The *PTI* coefficients (estimates of mating preferences) and their standard deviation (in parentheses) for each mating pair combination are shown. The I_{PSI} coefficient (estimating sexual isolation) and its standard deviation is presented in bold. The coefficients, their standard deviations, and their significance (probability of rejecting the null hypothesis) were calculated by resampling the observed values 10,000 times using the software PTI (see Materials and Methods).

		Male					Male		
	Female	S		Y		Female	S		Y
MuC	S	1.35*** (0.100)	**0.81***** (**0.030**)	0.09*** (0.031)	FC	S	1.76*** (0.106)	**0.77***** (**0.032**)	0.15*** (0.040)
	Y	0.31*** (0.057)		2.25*** (0.104)		Y	0.31*** (0.057)		1.77*** (0.105)
NC	S	1.36*** (0.110)	**0.54***** (**0.046**)	0.20*** (0.051)	MC	S	1.95*** (0.104)	**0.72***** (**0.037**)	0.28*** (0.054)
	Y	0.82 ns (0.094)		1.61*** (0.063)		Y	0.28*** (0.054)		1.49*** (0.101)

***$P < 0.001$ (all significant using Bonferroni correction); ns, not significantly different from null hypothesis.

portion of time occupied by male courtship behavior during the period between the onset of courtship and either until copulation occurred or, if copulation did not occur, until the end of the observation period; in former case, the proportion is calculated as (copulation latency − courtship latency)/ courtship duration, in the latter as (30 − courtship latency)/ courtship duration; these proportions are bounded by zero and one.

We emphasize that this study was conducted with only one isofemale line from each species, and there is thus the possibility that our results would differ with other strains. However, the study of Coyne et al. (2002) found no systematic difference in sexual isolation between these species whether the strains used were isofemale lines or synthetic strains made by combining several isofemale lines. An additional caveat is that all of our tests were done using virgin flies, while flies in nature may often mate several times, storing sperm from multiple males (e.g., Harshman and Clark 1998). (Clearly, however, a large number of matings in nature must involve virgin females because all mated females are virgins before their first mating.) Given that mated females are often reluctant to remate, experiments such as ours would be difficult using nonvirgin females. Nevertheless, such experiments would be an important extension of the work reported here.

RESULTS

Effects of Environmental Factors on Sexual Isolation

Comparing the *PTI*, *PSI*, and *PSS* coefficients for the different treatments and experiments (results not shown), we found that *PTI* and *PSI* were significantly correlated ($r = 0.50$, df = 46, $P < 0.001$). In addition, both heterospecific and homospecific ratios of *PSI* coefficients (PSI_{SS}/PSI_{YY} and PSI_{SY}/PSI_{YS}, which estimate the relative strength of mate choice coefficients) were significantly and highly correlated with the sexual fitness of *D. santomea* related to *D. yakuba* females (sexual fitness ratio = $[PSS_{SS} + PSS_{SY}]/ [PSS_{YS} + PSS_{YY}]$; this itself estimates the relative strength of sexual selection; ($r = -0.80$, df = 10, $P = 0.002$; $r = 0.60$, df = 10, $P = 0.039$, respectively), suggesting that in our dataset, sexual selection and sexual isolation are caused by the same mechanism—asymmetric mating preferences due to male or female-choice ($PTI_{SY} < PTI_{YS}$; see below). We therefore used *PTI* coefficients in estimating and dissecting the causes of mate choice, as these are the best estimators of sexual isolation when there are no detectable species differences in mating propensities (see Materials and Methods). Although ideally the I_{PTI} statistic is the best way estimate sexual isolation, we used I_{PSI} statistics for several reasons. First, the I_{PTI} coefficients were nearly identical to and almost perfectly correlated with I_{PSI} coefficients ($r = 0.99$, df = 10, $P < 0.001$); this result implies that mate choice is the only factor contributing to sexual isolation). Second, the statistical properties of the I_{PSI} statistic are well known and are more favorable than properties of alternative statistics (see Pérez-Figueroa et al. 2005). Thus, we have taken the I_{PSI} statistics as our best estimates of sexual isolation.

Table 1 gives the numbers of matings and the mean copulation latencies for all five tests of the effect of mating environment on sexual isolation. As observed in our previous study (Coyne et al. 2002), sexual isolation is substantial, with heterospecific matings in all tests occurring less frequently than conspecific matings. The mating between *D. santomea* females and *D. yakuba* females (S × Y) is rarer than the reciprocal mating (Y × S), a difference seen in 10 of the 11 mating tests in experiments A–E as well as in the study of Coyne et al. (2002). Sexual isolation tends to be between 70% and 90% of its maximum possible value in choice tests, and 40–50% in no-choice tests.

Experiment A: effects of choice

Between 40% and 50% of possible matings occurred in the no-choice, female-choice, and male-choice experiments, but this proportion was nearly 80% in the multiple-choice experiment. Table 2 gives the estimates of mating preferences (*PTI* coefficients) for the different mating designs from experiment A. The expected value is one when observed matings equal those expected under a hypothesis of no preference, values less than one indicate a deficiency of observed matings, and values greater than one show an excess of observed matings.

The pattern of mate choice described by the *PTI* statistics is compatible with strong assortative mating in all four mat-

TABLE 3. Total, pooled, and heterogeneity *G*-tests studying the effects of different ''choice'' designs (experiment A), and those comparing different treatments that might affect mating preferences (experiments B–D).

Experiment	Comparison	Grouping	df	*G*
A	all	pooled	3	781.5***
		total	12	854.1***
		heterogeneity	9	72.6***
	multiple-choice vs. female-choice	pooled	3	526.0***
		total	6	537.1***
		heterogeneity	3	11.1
	multiple-choice vs. male-choice	pooled	3	4721.6***
		total	6	504.8***
		heterogeneity	3	33.2**
	multiple-choice vs. no-choice	pooled	3	365.1***
		total	6	396.7***
		heterogeneity	3	31.6**
B	vial vs. cage	pooled	3	249.0***
		total	6	262.0***
		heterogeneity	3	13.0*
C	plant vs. no plant	pooled	3	574.7***
		total	6	576.3***
		heterogeneity	3	1.6
D	environment vs. food	pooled	2	408.0***
		total	4	430.5***
		heterogeneity	2	22.5**
E	food vs. starved	pooled	2	311.1***
		total	4	314.4***
		heterogeneity	2	3.3
A–B	A: multiple-choice vs. B: cage	pooled	2	399.3***
		total	4	507.0***
		heterogeneity	2	107.7***

*$P < 0.05$; ***$P < 0.01$; ***$P < 0.001$ after the sequential Bonferroni multitest correction (Rice 1989).

ing designs, with all values significantly larger than one for conspecific matings (S × S and Y × Y) and significantly lower than one for heterospecific matings (S × Y and Y × S; Table 2). The sole exception is the Y × S pairing in the no-choice design, whose frequency does not differ significantly from that expected under random mating.

The differences in *PTI* among mating designs can be assessed using the heterogeneity *G*-test given in Table 3: treatments are significantly heterogeneous in *PTI* coefficients (Table 4). When we compare the mating frequencies (and *PTI* coefficients) in each test to that seen in the multiple-choice test, we find that the differences are significant for both male-choice and no-choice tests, but not for the female-choice test (Table 3). These results imply female-choice (i.e., female preference) is the principal cause of sexual isolation in the multiple-choice experiments, although one must remember the caveat that female-choice experiments could also incorporate male-choice effects. The contribution of males to mating preferences is seen when comparing the multiple-choice cage treatment of experiment A with the no-choice cage treatment from experiment B: if female discrimination were the only cause of mating preferences, one might expect a homogeneous distribution of mating pairs between no-choice and multiple-choice experiments (Table 3, bottom). However, mating preferences were substantially higher when there was choice, even in the larger volume of this cage. Thus, if our earlier interpretation is correct, and female choice is the main mechanism contributing to sexual isolation, then the mating preferences of the *D. yakuba* females are those most sensitive to whether heterospecific males are present, as these females lost their mating preferences under no-choice conditions (see PTI_{YS}-values in Table 2).

In addition, we can estimate the degree of sexual isolation using the index I_{PSI} and compare this index across mating designs. Sexual isolation was high and significant in all four mating designs, but the no-choice design showed the lowest degree of sexual isolation (Table 2). There was significant heterogeneity among mating designs in sexual isolation using empirical estimates of the variation among replicates ($F_{3,59} = 11.1$, $P < 0.001$). This heterogeneity was caused by the low sexual isolation observed in the no-choice design, because there was no significant difference between the other three designs (Duncan test at $P < 0.05$). Additionally, the empirical means and standard errors of the sexual isolation for the four mating designs were very similar to the bootstrap estimates presented in Table 2: multiple-choice ($I_{PSI} = 0.83$, SE = 0.037), female-choice ($I_{PSI} = 0.78$, SE = 0.041), no-choice ($I_{PSI} = 0.55$, SE = 0.040), and male-choice ($I_{PSI} = 0.74$, SE = 0.033). The same result was observed in the rest of our analyses, so we present only the bootstrap estimates (see Tables 4, 6).

The contribution of both sexes (but the greater importance of females) to sexual isolation is shown by the direct observations of courtship described below.

Effects of other environmental factors

Table 1 gives the mating results for the different environmental treatments (B–E), Table 3 analyzes their heterogeneity in *PTI*, and Table 4 describes the mating preferences extracted

TABLE 4. Estimates of sexual isolation and mate propensity in the different experimental designs of mate choice (experiments B–D; see details in Materials and Methods). Different treatments were compared: no choice in vial (NC vial) versus no choice in cage (NC cage); multiple choice with plants (MuC plant) versus multiple choice without plants (MuC no plant); multiple choice with special environment (MuC environ) versus multiple choice with food only (MuC food); multiple choice with food (MuC fed) versus multiple choice starved (MuC starved). *PTI* coefficients (estimates of mating preferences) and their standard deviation (in parentheses) for each mating pair combination are shown. The I_{PSI} coefficients (estimating sexual isolation) and their standard deviations are presented in bold. The coefficients, their standard deviations, and their significance (probability of rejecting the null hypothesis) were calculated by resampling the observed values 10,000 times using the software PTI (see Materials and Methods).

		Male					Male	
	Female	S		Y		Female	S	Y
Experiment B								
	S	1.27*** (0.101)		0.22*** (0.049)		S	0.89 ns (0.085)	0.34*** (0.057)
NC vial			**0.52*** (0.045)**		NC cage			**0.47*** (0.048)**
	Y	0.84 ns (0.043)		1.66*** (0.107)		Y	0.69*** (0.079)	2.07*** (0.102)
Experiment C								
	S	1.13 ns (0.109)		0.04*** (0.025)		S	0.99 ns (0.102)	0.01*** (0.014)
MuC plant			**0.84*** (0.029)**		MuC no plant			**0.86*** (0.025)**
	Y	0.30*** (0.063)		2.52*** (0.115)		Y	0.30*** (0.062)	2.69*** (0.110)
Experiment D								
	S	0.35*** (0.133)		0.00*** (0.057)		S	1.40*** (0.112)	0.00*** (0.014)
MuC environ			**0.81*** (0.102)**		MuC food			**0.88*** (0.025)**
	Y	0.29*** (0.123)		3.36*** (0.180)		Y	0.26*** (0.058)	2.34*** (0.119)
Experiment E								
	S	1.70*** (0.165)		0.03*** (0.029)		S	1.51*** (0.153)	0.00*** (0.025)
MuC fed			**0.92*** (0.032)**		MuC starved			**0.90*** (0.032)**
	Y	0.14*** (0.062)		2.13*** (0.168)		Y	0.20*** (0.069)	2.29*** (0.156)

***P

TABLE 5. Effect of density on mating frequency. Two species ratios were used in replicate cages of 120 individuals: 1:1 *Drosophila yakuba*:*D. santomea* and 3:1 *D. yakuba*:*D. santomea*. Eleven replicate cages were made for each species ratio; total matings and average copulation latencies are shown, as well as total possible matings of each type.

Species ratio and mating type	*N*	Average latency (min)	Standard error, latency	Matings possible
1:1 (Y:S)				
S × S	89	17.47	1.28	330
S × Y	0	—	—	330
Y × S	18	13.14	2.28	330
Y × Y	191	18.07	0.97	330
3:1 (Y:S)				
S × S	22	11.86	2.71	165
S × Y	2	21.27	10.60	165
Y × S	12	17.10	3.18	165
Y × Y	275	14.52	0.74	495

Effect of space on multiple-choice versus no-choice designs.—As it is not possible to do multiple-choice experiments in small vials (Table 1, bottom), it is possible that the disparity between the high sexual isolation of the multiple-choice design and the lower sexual isolation of the no-choice design in experiment A was caused at least partly by the confinement of flies in small vials. We can test this hypothesis by comparing the sexual isolation seen in the multiple-choice cage design in experiment A with the sexual isolation seen in the no-choice cage design of experiment B. The difference remains highly significant ($F_{1,19} = 29.7$, $P < 0.001$). This is not surprising, given the lack of significant difference in sexual isolation between no-choice experiments conducted in vials or in cages (Table 1, experiment B). Space itself thus appears to be an unimportant factor in sexual isolation.

Effects of species composition.—We investigated whether the proportion of species in the mating pool affected the pattern of sexual isolation, using two relative frequencies of *D. santomea* (25% and 50%). The mating data are shown in Table 5, and the analysis of sexual isolation is shown in Table 6. The number of flies mating was very similar in the 1:1 (298 matings) and 3:1 (309 matings) experiments. However, there was significant heterogeneity in *PTI* statistics between the two treatments ($G_h = 76.9$, df = 3, $P < 0.001$; Table 6). This was caused by the larger number of *D. yakuba* conspecific matings and the slightly smaller number of matings between *D. santomea* females and *D. yakuba* males in the 1:1 experiment compared with the 3:1 experiment (Table 6).

Statistical comparison of the two treatments (Table 6) showed no significant differences in sexual isolation ($F_{1,24} = 0.1$, $P = 0.896$). Thus, while the change in species frequency might slightly affect mate choice, it does not significantly affect sexual isolation under these laboratory conditions. Levene and Dobzhansky (1945) similarly showed that changes in the proportion of the sister species *D. pseudoobscura* and *D. persimilis* did not affect the degree of sexual isolation.

Copulation latency

In all experiments (except for the two treatments in experiment C, the two treatments in experiment D, the food treatment of experiment E, and the two species-proportion treatments), copulation latency was significantly heterogeneous among the four mating types (all probabilities below 0.008). In general, the mating between *D. santomea* females and *D. yakuba* males took longest to occur, reflecting the paucity of these crosses due to female discrimination (see below). The reciprocal interspecific mating was not noticeably slower than intraspecific matings.

Summary

In these species only one environmental factor appeared to have a significant effect on sexual isolation: the possibility of choice, that is, the ability to choose a mate when presented with individuals of both species. This aspect of experimental design had a great impact on sexual isolation, at least in these species: no-choice experiments always yielded substantially less sexual isolation than did male-choice, female-choice, or multiple-choice experiments. In addition, statistics imply that both sexes contribute to sexual isolation, but that female discrimination plays the largest role. This conclusion is buttressed by direct observations of mating behavior described in next section.

We also found, however that mate preferences in *D. santomea* females are influenced by some environmental factors (space in the no-choice design, complexity of the environment and relative frequency of species in the multiple-choice experiments; Tables 3, 4, 6), although this difference in pref-

TABLE 6. Estimates of sexual isolation and mate propensity in the experiment testing the effect of density (Y, *Drosophila yakuba*; S, *D. santomea*) on sexual isolation. Two densities were used: equal species ratios (1Y:1S), and a ratio of three *D. yakuba* to one *D. santomea* (3Y:1S). The *PTI* coefficients (estimates of mating preferences) and their standard deviation (in parentheses) for each mating pair combination are shown. The I_{PSI} coefficients (estimating sexual isolation) and their standard deviations are presented in bold. The coefficients, their standard deviations and their significance (probability of rejecting the null hypothesis) were calculated by resampling the observed values 10,000 times using the software PTI (see Materials and Methods).

		S		Y			S		Y
	S	1.34*** (0.109)		0.00*** (0.013)		S	1.14 ns (0.234)		0.04*** (0.024)
1Y:1S			**0.89*** (0.023)**		3Y:1S			**0.90*** (0.028)**	
	Y	0.24*** (0.055)		2.42*** (0.112)		Y	0.21*** (0.024)		1.57*** (0.032)

***$P < 0.001$ (all significant after the Bonferroni correction); ns, not significantly different than one.

TABLE 7. Measurements of courtship and mating statistics in the four types of pairings. Twenty-five replicates were observed for each type. Female given first in the mating type. All times are given in minutes. Analysis of variance (ANOVA) were used for comparing mean values in behavioral traits between mating types; courtship proportions were arcsine transformed before testing.

Copulation mating type	Matings	Courtship latency (SE)	Copulation Attempts (SE)	Copulation latency (SE)	Courtship duration (SE)	Courtship proportion (mated) (SE)	Courtship proportion (unmated) (SE)
S × S	22	2.35 (0.69)	1.36 (0.54)	7.71 (1.04)	5.75 (1.29)	0.88 (0.06)	0.57 (0.29)
S × Y	2	1.95 (0.37)	2.04 (0.74)	23.45 (5.05)	16.50 (2.42)	0.95 (0.05)	0.63 (0.09)
Y × S	10	2.22 (0.27)	1.08 (0.29)	11.04 (2.71)	3.82 (0.55)	0.48 (0.10)	0.17 (0.02)
Y × Y	16	2.74 (1.03)	0.16 (0.09)	7.86 (1.62)	9.90 (2.04)	0.91 (0.05)	0.82 (0.03)
Differences among types (ANOVA)	df	3, 92	3, 96	3, 46	3, 96	3, 41	3, 41
	F	0.32	2.59	4.31	10.50	5.85	7.02
	P	0.870	0.060	0.009	<0.001	0.002	0.001

erence was not sufficient to significantly affect sexual isolation between the species.

Direct Observations of Mating Behavior

Table 7 gives courtship and copulation statistics for the four types of matings, each watched in a no-choice situation for 30 min. As in the experiments described above, we found that sexual isolation rests largely on the paucity of matings between *D. santomea* females and *D. yakuba* males, although there are fewer of both types of interspecific matings than of either intraspecific mating. We found no significant difference among the four mating types for mean courtship latency and number of copulation attempts. Copulation latency, however, was significantly heterogeneous among mating types; this is due entirely to the two long-delayed matings between *D. santomea* females and *D. yakuba* males (under Fisher's PLSD test, all three comparisons involving S × Y matings are significant, while no other comparison is significant). As one can see from the statistics (Table 7) the marked deficiency of S × Y matings is due to discrimination by *D. santomea* females: *D. yakuba* males courted *D. santomea* females persistently, but were refused just as persistently. Males in the S × Y pairings showed significantly higher courtship duration than males in the other three matings ($P < 0.01$, Fisher's PLSD test) and showed a higher proportion of time devoted to courting, regardless of whether matings did or did not occur.

Courtships between *D. yakuba* females and *D. santomea* males, in contrast, appeared relatively unsuccessful for a different reason: *D. santomea* males showed reduced interest in heterospecific compared to conspecific females. Thus, the Y × S pairing had the lowest duration of courtship and the lowest proportion of courtship among all pairings, whether pairs mated or not. In these comparisons, the Y × S mating showed significantly lower courtship proportion in five of the six pairwise comparisons (all comparisons of arcsine-transformed data using Fisher's PLSD test; $P < 0.03$). Observation showed no obvious behavior of *D. yakuba* females (such as extrusion of the ovipositor) that would discourage *D. santomea* males; rather, many *D. santomea* males appeared to lose interest in courting females after a brief encounter. This loss of interest is probably not based on interspecific differences in cuticular hydrocarbons (CH), as males and females of both species show similar CH profiles consisting largely of 7-tricosene (Llopart et al. 2002). (There is, however, always the possibility that slight differences in minor components of the CH profile could produce sexual isolation.) Nevertheless, 10 of the 25 pairs still mated, a frequency five times higher than in the reciprocal pairing.

Thus, as implied by experiment A described above, sexual isolation of these species involves differences in both male and female behavior, with each sex acting in a different reciprocal cross. *Drosophila santomea* males are less interested in heterospecific than conspecific females, whereas *D. santomea* females refuse the persistent courtship of heterospecific males. However, the female refusal clearly causes more sexual isolation than the male loss of interest, as seen in the consistently lower proportion of S × Y than Y × S matings. This again accords with mating studies showing that sexual isolation is nearly as strong in female-choice as in multiple-choice tests.

DISCUSSION

Effects of Environmental Factors on Sexual Isolation

Despite our use of several mating designs and different environments in these studies of sexual isolation, the only factor that appeared to affect the degree of sexual isolation when both species were in equal frequency was the possibility of choice. The most striking result of our study is that interspecific mating is much less likely when flies are given the possibility of choosing between conspecific and heterospecific partners than when no choice is possible. This result is perhaps not surprising, but it has not previously been documented by systematic comparison of several mating designs. Thus, in these species, the willingness to mate heterospecifically is not a probability that is invariant under all conditions.

Moreover, in choice experiments, sexual isolation is highest when multiple-choice or female-choice designs are used and lower when male-choice is used. This implies that much of the sexual isolation between *D. yakuba* and *D. santomea* results from discrimination by females, although this conclusion rests on the possibly fallacious assumption that one-sex choice experiments in fact do reflect choice by only that sex. (In fact, much of the isolation in the male-choice experiments surely results not from male's preferential courting of conspecific females but from the rejection of courting *D.*

yakuba males by *D. santomea* females.) However, we found that different environmental factors actually affected mate choice coefficients, although they did not change the relative proportion of homotypic versus heterotypic pairs. These environmental effects on mate choice coefficients suggest that the environmental factors may have some minor role on sexual isolation in the wild.

Mating Behavior and Its Evolution

Our conclusion that female discrimination is important in sexual isolation is strongly supported by our direct observation of mating behaviors, as well as by previous work (Coyne et al. 2002) showing that *D. santomea* females refuse the persistent courtship of *D. yakuba* males. However, male-choice experiments also yield more sexual isolation than do no-choice experiments, implying that male discrimination among females also plays a role. This is again supported by the direct observation of mating behaviors: *D. santomea* males do not court *D. yakuba* females as ardently as they court conspecific females.

The observation that *D. santomea* males and females show discrimination against the sister species, but *D. yakuba* does not, implies that sexual isolation between the species results largely from evolution that occurred in the derived island species, *D. santomea*. Perhaps the changes in sexual behavior of *D. santomea* that underlie its isolation from *D. yakuba* are by-products of natural selection acting on the island species alone—selection possibly associated with colonization of São Tomé.

One possibility is that *D. santomea* has shifted its system of sexual signaling via sensory-drive evolution (Endler 1992; Boughman 2002) after invasion of a novel habitat. According to this hypothesis, an adaptive shift in the island species can produce a divergence in sexual signaling from that present in the ancestral species, and thus indirectly increase sexual isolation. The greater sensitivity of the *D. santomea* pairing (S × S) to some environmental conditions in the laboratory accords with this view. It is not completely clear, however, how adaptation to a new habitat would lead both males and females of the new species to discriminate more strongly against heterospecifics when mating.

It should be noted that sexual isolation resulting from increased discrimination of island-endemic females is found in one other species in the *D. melanogaster* subgroup, *D. mauritiana* (found on Mauritius). This species was presumably the result of colonization of Mauritius by *D. simulans*–like flies from the mainland about 250,000 years ago (Kliman et al. 2000). The sexual isolation between *D. simulans* and *D. mauritiana* is due largely to *D. mauritiana* females rejecting persistently courting *D. simulans* males (Coyne 1996). (We note in passing that this rejection of mainland species by island females violates the Kaneshiro hypothesis that, because low-density populations will be selected to reduce mating selectivity, the mating of island males with mainland females with occur more frequently than the reciprocal mating; Kaneshiro 1980; Kaneshiro and Giddings 1987.) These similarities between *D. santomea* and *D. mauritiana* may reflect common, but poorly understood, evolutionary processes associated with island colonization.

The Biological Realism of Mating Tests

Although the opportunity for choice clearly allows the operation of all possible mechanisms contributing to sexual isolation among these species, we cannot say whether multiple-choice designs are more realistic models of nature than designs in which individuals of only one sex have a choice or those in which no individual has a choice. The suggestion that multiple-choice designs are most realistic (Spieth and Ringo 1983; Alipaz et al. 2005) may come from the observation that some species of *Drosophila* mate on food resources in nature, where flies of several species may congregate. However, this alone does not justify a particular experimental design, not least because we know nothing about the circumstances in which mating occurs in the wild. We do not know, for example, if food resources might be occupied by both species simultaneously, whether females are courted by males of both species simultaneously, whether females evaluate males individually or by comparison, and whether the sex ratio in the mating habitat is approximately equal. Observing flies of the *D. pseudoobscura* group mating in nature, Noor and Ortiz-Barrientos (2005) found that females mated far more frequently when encountering single than multiple males, suggesting that a no-choice design may be the most realistic model of mating in the wild. (One might object that in this case flies were observed at bait buckets, which are not natural aggregations. However, even if densities were artificially high in this study, females still did not choose between simultaneously courting males, so the conclusion that a no-choice situation obtained is probably conservative.)

In addition, interspecific differences in microecology may produce sexual isolation via habitat isolation. Alipaz et al. (2005, p. 421) implied that this might not occur in *Drosophila*, ''Only in selected cases can laboratory experiments approach a reasonable reenactment of secondary contact in nature. Sexual isolation in nonsocial insects may be one such example because the context may be less influenced by ecological forces.'' But if different species gravitate to different food resources or prefer to mate in different microhabitats, ecology can have a profound effect on the opportunity for interspecific mating. A famous example is the tephritid fly *Rhagoletis pomonella*, which is nonsocial but in which the two host races mate preferentially on different fruits (Feder et al. 1994). In the cactophilic species *D. mojavensis*, sexual isolation between two geographically disparate group of populations was observed when flies were reared on laboratory food, but this isolation disappeared when flies were reared on their natural substrate, fermenting cactus (Brazner and Etges 1993). This effect may result from the effects of larval substrate on adult epicuticular hydrocarbons, which act as contact mating pheromones (Stennet and Etges 1997; Etges and Ahrens 2001). Although the larval substrates of *D. yakuba* and *D. santomea* in the wild are unknown, the latter species probably breeds at least occasionally on figs of *F. chlamydocarpa fernandesiana* (Llopart et al. 2005), and further work on sexual isolation might involve rearing flies on those fruits.

Incongruence Between Laboratory and Field Hybridization

Only rarely can we measure sexual isolation directly in nature (e.g., Cruz et al. 2004; Malausa et al. 2005). Is there any way, then, to know if laboratory experiments on sexual isolation are reasonable mimics of conditions in the wild? One solution involves studying the genotypes of F_1 hybrids in the wild. Because the mother of a hybrid can be determined by using species-specific X-linked or mitochondrial markers, one might get an idea of the degree and direction of sexual isolation by determining the relative frequency and genotypes of hybrids formed by the two types of matings compared to pure-species individuals in an area of overlap (this, of course, assumes equal viability of all genotypes). In our sampling of the forest habitat on São Tomé, we collected 76 interspecific hybrids. Of these, three were backcross individuals and the remaining 73 were F_1 male hybrids. All of these 73 individuals had a *D. santomea* mother (see Llopart et al. 2005). This is exactly the opposite of what one would predict from our mating tests, which show that the mating between *D. santomea* females and *D. yakuba* males is much rarer than the reciprocal mating. This disparity in the frequencies of reciprocal hybrids probably does not reflect viability differences, at least in the egg-to-adult stage, as these hybrids are highly viable in the laboratory (Coyne et al. 2004). As Llopart et al. (2005) noted, this disparity must reflect either the relative density of species in the area of overlap (females of the rarer species may show more interspecific mating), some ecological difference between the sexes, a drastic difference in viability of reciprocal-cross hybrids, or another unknown factor that drastically affects sexual isolation in the wild. Our one experiment altering species frequency showed no significant effect on sexual isolation (it did affect mating preferences), although increasing the relative frequency of *D. yakuba* must perforce increase the number of hybrids between *D. yakuba* males and *D. santomea* females. Our results suggest, however, that frequency differences of an enormous magnitude would be necessary to produce the relative frequencies of reciprocal F_1 hybrids found in nature (Llopart et al. 2005). Even in this case, however, one must still explain why naturally occurring hybrids would be produced only in areas where species densities differ drastically, as well as why we find no hybrids who had *D. yakuba* mothers.

Although we have made some progress in understanding sexual isolation in the laboratory, we are a long way from knowing how sexual isolation operates in natural populations of these species. This is due, in part, to the limitation of both finding mating pairs and hybrids in nature, as well as to the absence of fitness estimates for hybrid genotypes in the wild. We clearly need similar studies of different pairs of *Drosophila* species to determine whether our conclusions about environment and sexual isolation apply to the genus *Drosophila* as a whole.

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Literature Cited

Alipaz, J., S. Fang, N. Osada, and C.-I. Wu. 2005. Evolution of sexual isolation during secondary contact: genotypic versus phenotypic changes in laboratory populations. Am. Natur. 165: 420–428.

Boughman, J. W. 2002. How sensory drive can promote speciation. Trends. Ecol. Evol. 17:571–577.

Brazner, J. C., and W. J. Etges. 1993. Premating isolation is determined by larval substrates in cactophilic *Drosophila mojavensis*. II. Effects of larval substrates on time to copulation, mate choice, and mating propensity. Evol. Ecol. 7:605–624.

Casares, P., M. C. Carracedo, B. D. Rio, R. Piñeiro, L. García-Flórez, and A. R. Barros. 1998. Disentangling the effects of mating propensity and mating choice in *Drosophila*. Evolution 52:126–133.

Chan, K. M., and S. A. Levin. 2005. Leaky prezygotic isolation and porous genomes: rapid introgression of maternally inherited DNA. Evolution 59:720–729.

Coyne, J. A. 1996. Genetics of sexual isolation in male hybrids of *Drosophila simulans* and *D. mauritiana*. Genet. Res. 68:211–220.

Coyne, J. A., and H. A. Orr. 1989. Patterns of speciation in *Drosophila*. Evolution 43:362–381.

———. 1997. ''Patterns of speciation in *Drosophila*'' revisited. Evolution 51:295–303.

———. 2004. Speciation. Sinauer, Sunderland, MA.

Coyne, J., K. Mah, and A. Crittenden. 1994. Genetics of a pheromonal difference contributing to reproductive isolation in *Drosophila*. Science 265:1461–1464.

Coyne, J. A., S. Y. Kim, A. S. Chang, D. Lachaise, and S. Elwyn. 2002. Sexual isolation between two species with overlapping ranges: *Drosophila santomea* and *Drosophila yakuba*. Evolution 56:2424–2434.

Coyne, J. A., S. Elwyn, S. Y. Kim, and A. Llopart. 2004. Genetic studies of two sister species in the *Drosophila melanogaster* subgroup, *D. yakuba* and *D. santomea*. Genet. Res. 84:11–26.

Cruz, R., M. Carballo, P. Conde-Padín, and E. Rolán-Alvarez. 2004. Testing alternative models for sexual isolation in natural populations of *Littorina saxatilis*: Indirect support for by-product ecological speciation? J. Evol. Biol. 17:288–293.

Ehrman, L. 1961. The genetics of sexual isolation in *Drosophila paulistorum*. Genetics 46:1025–1038.

———. 1965. Direct observation of sexual isolation between allopatric and between sympatric strains of the different *Drosophila paulistorum* races. Evolution 19:459–464.

Endler, J. A. 1992. Signals, signal condition, and the direction of evolution. Am. Nat. 139:125–153.

Etges, W. J., and M. A. Ahrens. 2001. Premating isolation is determined by larval rearing substrates in cactophilic *Drosophila mojavensis*. V. Deep geographic variation in epicuticular hydrocarbons among isolated populations. Am. Nat. 158:585–598.

Feder, J. L., S. Opp, B. Wlazlo, K. Reynolds, W. Go, and S. Spisak. 1994. Host fidelity is an effective premating barrier between sympatric races of the apple maggot fly. Proc. Natl. Acad. Sci. USA 91:7990–7994.

Gilbert, D. G., and W. T. Starmer. 1985. Statistics of sexual isolation. Evolution 39:1380–1383.

Harshman, L. G., and A. G. Clark. 1998. Inference of sperm competition from broods of field-caught *Drosophila*. Evolution 52: 1334–1341.

Jacobs, M. E. 1978. The influence of beta-alanine on mating and territorialism in *Drosophila melanogaster*. Behav. Genet. 8: 487–502.

Kaneshiro, K. Y. 1980. Sexual isolation, speciation, and the direction of evolution. Evolution 34:437–444.

Kaneshiro, K. Y., and L. V. Giddings. 1987. The significance of asymmetrical sexual isolation and the formation of new species. Evol. Biol. 21:29–43.

Kliman, R. M., P. Andolfatto, J. A. Coyne, F. Depaulis, M. Kreitman, A. J. Berry, M. McCarter, J. Wakeley, and J. Hey. 2000. The population genetics of the origin and divergence of the *Drosophila simulans* complex species. Genetics 156:1913–1931.

Krebs, R. A., and K. L. Bean. 1991. The mating behavior of *Dro-*

sophila mojavensis on organ pipe and agria cactus. Psyche 98: 101–109.

Lachaise, D., M. Harry, M. Solignac, F. Lemeunier, V. Bénassi, and M.-L. Cariou. 2000. Evolutionary novelties in islands: *Drosophila santomea* a new *melanogaster* sister species from São Tomé. Proc. R. Soc. Lond. B 267:1487–1495.

Levene, H., and T. Dobzhansky. 1945. Experiments on sexual isolation in *Drosophila*. V. The effect of varying proportions of *Drosophila pseudoobscura* and *Drosophila persimilis* on the frequency of insemination in mixed populations. Proc. Natl. Acad. Sci. USA 31:274–281.

Lewontin, R. C., D. Kirk, and J. F. Crow. 1968. Selective mating, assortative mating, and inbreeding: definitions and implications. Eugenics Q. 15:141–143.

Llopart, A., S. Elwyn, D. Lachaise, and J. A. Coyne. 2002. Genetics of a difference in pigmentation between *Drosophila yakuba* and *D. santomea*. Evolution 56:2262–2277.

Llopart, A., D. Lachaise, and J. A. Coyne. 2005. An anomalous hybrid zone in *Drosophila*. Evolution 59:2602–2607.

Malagolowkin-Cohen, C., A. Solima-Simmons, and H. Levene. 1965. A study of sexual isolation between certain strains of *Drosophila paulistorum*. Evolution 19:95–103.

Malausa, T., M.-T. Bethenod, A. Bontemps, D. Bourguet, J.-M. Cornuet, and S. Ponsard. 2005. Assortative mating in sympatric host races of the European corn borer. Science 308:258–260.

Merrell, D. J. 1954. Sexual isolation between *Drosophila persimilis* and *Drosophila pseudoobscura*. Am. Nat. 88:93–99.

Noor, M. A. F. 1995. Speciation driven by natural selection in *Drosophila*. Nature 375:674–675.

———. 1996. Absence of species discrimination in *Drosophila pseudoobscura* and *D. persimilis* males. Anim. Behav. 52: 1205–1210.

Noor, M. A. F., and D. Ortiz-Barrientos. 2005. Simulating natural conditions in the laboratory: a re-examination of sexual isolation between sympatric and allopatric populations of *Drosophila pseudoobscura* and *D. persimilis*. Behav. Genetics. 35: *In press*.

Nosil, P., B. J. Crespi, and C. P. Sandoval. 2002. Host-plant adaptation drives the parallel evolution of reproductive isolation. Nature 417:440–443.

Patterson, J. T., and W. S. Stone. 1952. Evolution in the genus *Drosophila*. Macmillan, New York.

Pérez-Figueroa, A., A. Caballero, and E. Rolán-Alvarez. 2005. Comparing the estimation properties of different statistics for measuring sexual isolation from mating frequencies. Biol. J. Linn. Soc. 85:307–318.

Pitnick, S. 1993. Operational sex ratios and sperm limitation in populations of *Drosophila pachea*. Behav. Ecol. Sociobiol. 33: 383–391.

Rice, W. R. 1989. Analyzing tables of statistical tests. Evolution 43:223–225.

Rolán-Alvarez, E. 2004. Evolution of asymmetry in sexual isolation: a criticism of a test case. Evol. Ecol. Res. 6:1099–1106.

Rolán-Alvarez, E., and A. Caballero. 2000. Estimating sexual selection and sexual isolation effects from mating frequencies. Evolution 54:30–36.

Shelly, T. E. 1989. Waiting for mates: variation in female encounter rates within and between leks of *Drosophila conformis*. Behaviour 111:34–48.

Sokal, R. R., and F. J. Rohlf. 1995. Biometry. 3rd ed. W. H. Freeman, San Francisco, CA.

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

Stennett, M. D., and W. J. Etges. 1997. Premating isolation is determined by larval rearing substrates in cactophilic *Drosophila mojavensis*. III. Epicuticular hydrocarbon variation is determined by use of different host plants in *Drosophila mojavensis* and *Drosophila arizonae*. J. Chem. Ecol. 23:2803–2824.

Tan, C. C. 1946. Genetics of sexual isolation between *Drosophila pseudoobscura* and *Drosophila persimilis*. Genetics 31:558–573.

Watanabe, T. K., and M. Kawanishi. 1979. Mating preference and the direction of evolution in *Drosophila*. Science 205:906–907.

Wirtz, P. 1999. Mother species-father species: unidirectional hybridization in animals with female-choice. Anim Behav 58:1–12.

Wood, D., and J. M. Ringo. 1980. Male mating discrimination in *Drosophila melanogaster*, *D. simulans*, and their hybrids. Evolution 34:320–329.

Wu, C.-I., H. Hollocher, D. J. Begun, C. F. Aquadro, Y. Xu, and M.-L. Wu. 1995. Sexual isolation in *Drosophila melanogaste*r: a possible case of incipient speciation. Proc. Natl. Acad. Sci. USA 92:2519–2523.

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