# Simulating Natural Conditions in the Laboratory: A Re-Examination of Sexual Isolation between Sympatric and Allopatric Populations of *Drosophila pseudoobscura* and *D. persimilis*

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Simulating natural conditions in the laboratory poses one of the most significant challenges to behavioral studies. Some authors have argued that laboratory "choice" experiments reflect mate choice in nature more accurately than "no-choice" experiments. A recent choice experiment study questioned the conclusions of several earlier studies by failing to detect a published difference in sexual isolation between populations of *Drosophila pseudoobscura*, and suggested their result was more robust because of the more realistic design. Here, we re-examine the methods and analyses of this recent study, and we find there was indeed a difference in sexual isolation between populations of *D. pseudoobscura*. We also conduct a more rigorously controlled choice experiment and, in agreement with previous studies, note that *D. pseudoobscura* females from populations sympatric to their sibling species, *D. persimilis*, exhibit greater sexual isolation than those from allopatric populations. Our results confirm the existence of a geographic pattern in sexual isolation in *D. pseudoobscura*, and we discuss differences in experimental designs in light of the biology of this species.

KEY WORDS: *Drosophila pseudoobscura*; reinforcement; sexual isolation; speciation.

## INTRODUCTION

A major challenge to all laboratory behavioral investigations is how best to simulate conditions encountered by species in nature, especially in studies of species whose ecologies are poorly understood. This problem is particularly acute in studies of many Drosophila species. While the ecologies of some Drosophila species, such as the Hawaiian, cacto-philic, and mycophagous Drosophila, are fairly well described, the ecologies of others, such as the North American species *D. pseudoobscura*, remain almost completely unknown.

Mating behavior of *D. pseudoobscura* has attracted a lot of interest in the past decade because females from populations that co-occur with *D. persimilis* exhibit strong mating discrimination (sexual isolation) against *D. persimilis* males (see e.g., Noor, 1995; Ortíz-Barrientos *et al*., 2004). In contrast, female *D. pseudoobscura* from allopatric populations exhibit weaker discrimination against *D. persimilis* males. This pattern is predicted if the divergence of these species was driven in part by reinforcement, the process whereby natural selection strengthens behavioral discrimination to prevent maladaptive hybridization where species have overlapping ranges. However, most of the studies showing this pattern

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used a "no-choice" mating design: females were paired with heterospecific males individually, and scored for whether they mated. Several studies have shown that choice mating experiments, where females may select between conspecific or heterospecific males, may yield different results from no-choice experiments (e.g., Gupta and Sundaran, 1994; Ryan and Rand, 1993; Wade *et al.*, 1995), and some argue that choice experiments more accurately reflect the natural setting (e.g., Spieth and Ringo, 1983).

Recently, Anderson and Kim (2005) examined this issue in *D. pseudoobscura* and *D. persimilis*. Using choice mating experiments, they failed to confirm published (Noor, 1995) results showing evidence of reinforcement, and concluded there "were no meaningful differences between the isolation indices in sympatric and allopatric strains of the species." They did not even detect a difference in isolation when a distant South American population of *D. pseudoobscura* was used. They suggest that gene flow among *D. pseudoobscura* populations may have eliminated the expected geographic signature of reinforcement.

The results of Anderson and Kim (2005) strikingly contrast multiple studies from multiple laboratories (Dixon Schully and Noor, 2004; Lorch and Servedio, 2005; Noor, 1995; Ortíz-Barrientos *et al.*, 2004; Singh, 1983). This discrepancy could reflect true effects of distinct experimental approaches, or it could suggest that some intrinsic bias was introduced by one experimental design or analysis. We find that the conclusion of Anderson and Kim stems from an incomplete analysis of their data. Following what we believe to be a more appropriate analysis of their data, we find a statistically significant difference in sexual isolation between populations of *D. pseudoobscura* in the direction predicted by reinforcement theory. We also present here results from a more suitable experimental design using two of the same populations used by Anderson and Kim (2005). The results, again, are consistent with reinforcement theory and previous studies (Dixon Schully and Noor, 2004; Lorch and Servedio, 2005; Noor, 1995; Ortíz-Barrientos *et al.*, 2004). We explore these results in light of experimental designs and the biology of this species group.

## MATERIALS AND METHODS

### Reanalysis of Anderson and Kim (2005) Results

Detailed materials and methods executed by Anderson and Kim (2005) are available in their paper.

In brief, individuals derived from 4 populations of *D. persimilis* were compared for their sexual isolation from individuals from 4 populations of *D. pseudoobscura*: in each comparison, one *D. pseudoobscura* population was sympatric to the *D. persimilis* population being tested, two *D. pseudoobscura* populations were allopatric but still within North America (Flagstaff, Arizona, and Mesa Verde, Colorado), and one was from Bogota, Colombia (far allopatric). They confined groups of 12 virgin males and 12 virgin females of each species, 7 days post-eclosion in a single Elens and Wattiaux (1964) mating chamber and observed the numbers of copulations of each type for 60 minutes. Four replicate chambers were observed for each combination of species.

They then calculated a "joint isolation index" for both sexes and both species (Merrell, 1950) to measure sexual isolation:

$$I = (x\text{AA} + x\text{BB} - x\text{AB} - x\text{BA})/N$$

where $x$AA, $x$BB, $x$BA, and $x$AB represent the number of matings between *D. pseudoobscura* females and males, *D. persimilis* females and males, *D. pseudoobscura* females with *D. persimilis* males, and *D. persimilis* females with *D. pseudoobscura* males, respectively. $N$ is the total number of matings. $I$ ranges from 1.00 (total isolation, all homogamic matings), through 0 (random mating), to −1.00 (all heterogamic matings).

We present these results as well as our own analyses using two other statistics. The first is the simple proportion of mating *D. pseudoobscura* females that mate with *D. persimilis* males. This was measured as

$$\%\text{Ps}♀ \times \text{Per}♂ = x\text{BA}/(x\text{BA} + x\text{AA})$$

Our second analysis used PSI, a measure of sexual isolation introduced by Rolan-Alvarez and Caballero (2000) which ranges from 0–1 where 0 is complete sexual isolation. We used the formula

$$\text{PSI} = \frac{(x\text{BA})\text{N}}{(x\text{AA} + x\text{BA})(x\text{BA} + x\text{BB})}$$

Unlike the joint $I$, this measure specifically examines the sexual isolation of *D. pseudoobscura* females from *D. persimilis* males, and it also controls for confounding effects of mate propensity. Although males of these species approach females more than they approach other males (e.g., Gowaty *et al.*, 2003), sexual isolation between these species is primarily mediated by female discrimination rather than male

discrimination, as illustrated by intense and unrelenting courtship by males towards unreceptive heterospecific females in both choice and no-choice settings (e.g., Merrell 1954; Noor 1996; Streisinger 1948). Given that all *D. persimilis* are derived from populations sympatric to *D. pseudoobscura*, one should test the reinforcement hypothesis by focusing on the discrimination exhibited by *D. pseudoobscura* females from population sympatric to vs. allopatric to *D. persimilis*, as we have done.

Using the Anderson and Kim (2005) dataset, we calculated all of these measures of sexual isolation for all combinations. Because of the non-normality of the distributions, we rank ordered the combinations for each pairing with a *D. persimilis* strain, and performed a nonparametric Mann–Whitney *U*-test to evaluate whether, across *D. persimilis* strains assayed, females from sympatric populations of *D. pseudoobscura* tended to exhibit stronger isolation than females from the allopatric populations of *D. pseudoobscura*.

### Mating Experiments

In addition to reanalyzing the results of Anderson and Kim (2005), we also performed a set of mating experiments employing a choice design. We focused on the Mather and Flagstaff populations of *D. pseudoobscura* because, in our reanalysis, these were the only two for which it appeared the reinforcement prediction of greater discrimination by sympatric population females may not be fulfilled. All experiments were conducted at 21°C.

To remove confounding effects of inbreeding on mating propensity, we crossed pairs of lines, and used their $F_1$ progeny for our behavioral studies. We crossed the Mount St. Helena 1993 and Mount St. Helena 3 strains of *D. persimilis*, the Mather 97 and Mather 16 strains of *D. pseudoobscura*, and the Flagstaff 6 and Flagstaff 14 strains of *D. pseudoobscura*. All flies used in behavioral experiments were eight days posteclosion. On the seventh day after eclosion, a single *D. persimilis* male and a single *D. pseudoobscura* male were confined together in a 8-dram food-containing vial. The next day, a single *D. pseudoobscura* female was added to the vial without anesthesia, and the three flies were observed for 10 minutes. Following copulation of one male to one female, the unmated male was removed and genotyped for a microsatellite marker bearing a fixed difference between *D. pseudoobscura* and *D. persimilis* (DPSX002: see Noor *et al.*, 2000), and the mated male was inferred. As such, no manipulations were performed on the flies themselves that could affect their behavior prior to the experiment, such as wing-clipping (see Anderson and Kim, 2005).

One hundred replicates of each of the following combinations were examined: *D. persimilis* male + Mather *D. pseudoobscura* male + Mather *D. pseudoobscura* female, and *D. persimilis* male + Flagstaff *D. pseudoobscura* male + Flagstaff *D. pseudoobscura* female. Equal numbers of each combination type were assayed each day. As the Mather population bears both species while the Flagstaff population is allopatric and contains only *D. pseudoobscura*, reinforcement theory predicts that *D. persimilis* males should have lower mating success to Mather females than to Flagstaff females. This hypothesis was tested using a Fisher's exact test.

## RESULTS AND DISCUSSION

### Reanalysis of Anderson and Kim (2005) Results

Before presenting our own experimental results, we explore aspects of the design and analysis of the Anderson and Kim (2005) study. These authors used a "multiple choice" design, where multiple males and females of both species were confined together. They then assessed the numbers of each of four types of mating (e.g., *D. pseudoobscura* male × *D. persimilis* female: see Table I). From these numbers, they calculated a "joint isolation index" (Joint *I* in this case) that computes the total behavioral isolation between the two strains tested.

We suggest the analysis of Anderson and Kim (2005) is inappropriate because their measure pools behavioral discrimination by *D. pseudoobscura* females with that by *D. persimilis* females. Anderson and Kim (2005) noted that "*Drosophila pseudoobscura* females strongly discriminated against *D. persimilis* males, while *D. persimilis* females were less discriminating." As a result, the variation in the joint isolation index among strains was influenced more by the variation among *D. persimilis* females than the behavior of *D. pseudoobscura* females. However, the behavior of *D. persimilis* females cannot be studied in terms of sympatry vs. allopatry, since all *D. persimilis* populations are sympatric to *D. pseudoobscura*, so the variation studied was not relevant to reinforcement in this system. In contrast, the claim of reinforcement in this species group is specific to the discriminatory behavior of *D. pseudoobscura* females (e.g., Noor, 1995), and this behavior was not partitioned out in their analysis.

**Table I.** Sexual Isolation between *D. persimilis* (Per) and *D. pseudoobscura* (Ps) in Sympatry (sym) and Allopatry

| | Per♀×Per♂ | Per♀×Ps♂ | Ps♀×Per♂ | Ps♀×Ps♂ | JointI | %Ps♀×Per♂ | PSI(Ps♀) | Rank |
|---|---|---|---|---|---|---|---|---|
| *D. persimilis* Death Valley, CA × | | | | | | | | |
| *Ps* Death Valley, CA (sym) | 39 | 2 | 2 | 45 | 0.909 | 4.26 | 0.091 | 1 |
| *Ps* allopatric pop1 | 43 | 2 | 4 | 42 | 0.868 | 8.70 | 0.168 | 2 |
| *Ps* allopatric pop2 | 40 | 1 | 4 | 40 | 0.882 | 9.09 | 0.176 | 3 |
| *Ps* allopatric (Bogota) | 39 | 2 | 9 | 37 | 0.747 | 19.57 | 0.355 | 4 |
| *D. persimilis* James Reserve, CA × | | | | | | | | |
| *Ps* James Reserve, CA (sym) | 18 | 17 | 0 | 45 | 0.575 | 0.00 | 0.000 | NA |
| *Ps* allopatric pop1 | 19 | 7 | 0 | 43 | 0.797 | 0.00 | 0.000 | NA |
| *Ps* allopatric pop2 | 26 | 13 | 0 | 43 | 0.683 | 0.00 | 0.000 | NA |
| *Ps* allopatric (Bogota) | 16 | 2 | 0 | 46 | 0.938 | 0.00 | 0.000 | NA |
| *D. persimilis* Mather, CA × | | | | | | | | |
| *Ps* Mather, CA (sym) | 41 | 4 | 2 | 45 | 0.870 | 4.26 | 0.091 | 2 |
| *Ps* allopatric pop1 | 35 | 10 | 3 | 44 | 0.717 | 6.38 | 0.155 | 3 |
| *Ps* allopatric pop2 | 31 | 11 | 1 | 43 | 0.721 | 2.27 | 0.061 | 1 |
| *Ps* allopatric (Bogota) | 36 | 6 | 7 | 40 | 0.708 | 14.89 | 0.308 | 4 |
| *D. persimilis* Spray, OR × | | | | | | | | |
| *Ps* Spray, OR (sym) | 25 | 15 | 0 | 48 | 0.659 | 0.00 | 0.000 | 1 |
| *Ps* allopatric pop1 | 17 | 4 | 1 | 44 | 0.848 | 2.22 | 0.081 | 2 |
| *Ps* allopatric pop2 | 38 | 9 | 3 | 42 | 0.739 | 6.67 | 0.150 | 4 |
| *Ps* allopatric (Bogota) | 26 | 10 | 2 | 44 | 0.707 | 4.35 | 0.127 | 3 |

Raw mating scores are presented as well as the Joint Isolation Index (JointI), percentage of mating *D. pseudoobscura* females that mated with *D. persimilis* males (%Ps♀×Per♂), index of sexual isolation of *D. pseudoobscura* females (PSI), and rank of PSI within each set of experiments with *D. persimilis* males (Rank).

We present their Table I here, along with additional calculations (see our Table I). Of specific interest to reinforcement theory is whether *D. pseudoobscura* females from populations sympatric to *D. persimilis* exhibit greater discrimination than *D. pseudoobscura* females from allopatric populations. As such, one should confine analyses to the behavior of *D. pseudoobscura* females. We do this, with identical results, using two metrics (see Materials and Methods), and we exclude the pairings with *D. persimilis* males from James Reserve, CA, because none of these males ever mated with *D. pseudoobscura* females (see also below evidence for inbreeding depression in this strain).

We observe that females from sympatric strains of *D. pseudoobscura* exhibit the highest sexual isolation when tested against *D. persimilis* males from Death Valley, CA, and Spray, OR, and the second highest sexual isolation of strains when tested against *D. persimilis* males from Mather, CA. The differences among strains are not individually statistically significant, but this is largely a function of the very small number of *D. pseudoobscura* females that mated interspecifically from any population. To bypass this difficulty, we performed an analysis of the full dataset, correcting for potential complications caused by

the use of different *D. persimilis* strains. If we rank each *D. pseudoobscura* strain based on its relative isolation from each *D. persimilis* strain and use a Mann–Whitney $U$-test to compare these ranks (sympatric vs. allopatric), we find that the lines from sympatric *D. pseudoobscura* populations exhibit a pattern of significantly higher sexual isolation than the lines from allopatric populations ($p = 0.046$). This is the pattern predicted by reinforcement theory and is consistent with earlier studies (Dixon Schully and Noor, 2004; Noor, 1995; Ortíz-Barrientos *et al.*, 2004), but appears to contradict the conclusion of Anderson and Kim (2005).

Interestingly, using this analysis, we also observe that the isolated Bogota population of *D. pseudoobscura* generally exhibits the *weakest* sexual isolation from *D. persimilis* males. This result is also consistent with two other studies (Lorch and Servedio, 2005; Singh, 1983), but contrary to the conclusion of Anderson and Kim (2005).

The reason for the apparent contradiction is simple: reinforcement should have been most effective in increasing the sexual isolation of *D. pseudoobscura* females in populations sympatric to *D. persimilis* relative to *D. pseudoobscura* females from allopatric populations. However, because all

populations of *D. persimilis* are sympatric to *D. pseudoobscura*, reinforcement could not have created the predicted differences among sympatric vs. allopatric populations in this species. As such, the use of any isolation index which conflates the sexual isolation between individuals of the two species was inappropriate for examining the question of reinforcement in this system. Effectively, the "signal" from reinforcement in *D. pseudoobscura* was masked by the "noise" from *D. persimilis* in their study.

The results of the Anderson and Kim (2005) study do fail to confirm the preliminary evidence for reinforcement of female discrimination in *D. persimilis* females (Noor, 1995). Indeed, females from this species appeared to be generally *less* discriminating than *D. pseudoobscura* females. This result is curious because all *D. persimilis* populations are sympatric with *D. pseudoobscura*, so one could speculate that selection for discrimination might be stronger. This lack of evidence for reinforcement in *D. persimilis* may reflect errors in the estimates of relative species abundances using capture data to artificial baits or unknown genetic or environmental variables affecting behavioral discrimination in one species but not the other.

### Experimental Design Issues, and New Choice Test of Sexual Isolation between Sympatric and Allopatric Populations of *D. pseudoobscura* and *D. persimilis*

Although providing females with a simultaneous choice of mates could reflect the natural mating setting more accurately (though we argue against this later), Anderson and Kim (2005) failed to repeat a different natural aspect of the earlier studies: the previous studies used outbred flies produced by crosses between inbred lines (Lorch and Servedio, 2005; Noor, 1995; Ortíz-Barrientos *et al.*, 2004). Instead, Anderson and Kim (2005) used single laboratory strains for their assays, many of which had been in culture for nearly a decade and likely suffer inbreeding depression. This inbreeding may have caused the low mating success exhibited by *D. persimilis* males from James Reserve, CA, to both their own females and to *D. pseudoobscura* females (see Table I). Consistent with inbreeding depression, the lower conspecific mating rate of their James Reserve strain *D. persimilis* males compared to the conspecific mating rates of other *D. persimilis* strains is statistically significant (Mann–Whitney $U$, $p = 0.018$).

Anderson and Kim (2005) also combined groups of 48 (24 males and 24 females) simultaneously in the mating chamber. They note that, to prevent biases, Casares *et al.* (1998) recommended that observations be discontinued when 50% of potential matings have occurred. Consistent with the recommendation of Casares *et al.* (1998) based on studies of *D. melanogaster*, Anderson and Kim (2005) discontinued observations after 30 minutes. However, *D. pseudoobscura* mates far more quickly than *D. melanogaster*, and 67–96% of potential matings had occurred in this 30-minute period, so biases may have obscured their results (see also Gilbert and Starmer, 1985). We execute our experiment using repeated rounds of single *D. pseudoobscura* females paired simultaneously with one *D. persimilis* male and one *D. pseudoobscura* male, hence preventing any bias resulting from unequal pool size.

For our experiment, we used outbred linecrosses of *D. pseudoobscura* from Mather and Flagstaff and *D. persimilis* from Mount St. Helena. This choice of strains is very conservative, because in our reanalysis of the results of Anderson and Kim (2005), these were the only two populations in which females from the allopatric *D. pseudoobscura* strain exhibited stronger discrimination than the sympatric strain (allopatric population 2 was derived from Flagstaff). In our experiment, all females mated with a male within the first two minutes of the 10-minute confinement. We find that, even when given a choice of potential mates, females derived from the Mather population exhibit stronger behavioral discrimination than females derived from the Flagstaff population: 2/100 Mather *D. pseudoobscura* females mated with *D. persimilis* males while 12/100 Flagstaff *D. pseudoobscura* females mated with *D. persimilis* males (Fisher's exact test, $p = 0.010$). Again, this result is predicted by reinforcement theory and is consistent with earlier studies (Dixon Schully and Noor, 2004; Noor, 1995; Ortíz-Barrientos *et al.*, 2004), but contradicts the conclusion of Anderson and Kim (2005).

### Synthesis: How Does One Conduct Mate-Choice Experiments to Simulate Natural Conditions?

We now turn to the broader question of whether choice or no-choice experiments more accurately reflect the natural setting of these species. Anderson and Kim (2005) justify their choice design by noting, "Ortíz-Barrientos *et al.* (2004) reported that a single *D. pseudoobscura* female was surrounded by multiple males in many areas at Mather, a situation that clearly provides a choice of mates for the female." However, this is a misrepresentation of that paper and of the

data. In fact, Ortíz-Barrientos *et al.* (2004) said, "The rationale of this no-choice design is based on behavioral observations suggesting that females tend to copulate more often in the presence of single males than when multiple males approach them (Noor, unpublished data). Therefore, no-choice experiments should provide a more conducive setting for mating." In the field observations at Mather, 107 females were approached by males singly, and 11 of these females copulated. In contrast, 33 females were approached simultaneously by two courting males, and in every case, the female immediately flew away without mating. The difference between these observations is marginally significant (Fisher's exact test, $p = 0.067$), suggesting females tend to copulate with males that approach singly more often than when courted simultaneously by two males. This difference may result from confusion of courtship signals, and it justifies a sequential encounter model for simulating courtship in nature, rather than confining females with multiple males simultaneously without possibility of escape.

In choosing an experimental design, one must consider the behavior of the species being studied. Courtship in *D. pseudoobscura* is exceedingly short—frequently under five seconds both in the laboratory and in the wild. As such, females must decide "yes or no" for a given male without the option of assessing other males. In this sense, no-choice experiments may reflect nature better than choice experiments (see also Fraser and Boake, 1997); in the latter, two males are unnaturally confined in close proximity to a female who is incapable of fleeing them both, even though fleeing would have been the most likely outcome in nature.

We strongly agree with Anderson and Kim (2005) that "any experimental study is somewhat artificial, and rearing and handling conditions may also affect behavior." Although there is intuitive appeal to thinking that choice experiments more accurately reflect the natural setting, preliminary data contradict this assertion. Further, all experiments, choice or otherwise, must be carefully controlled and carefully analyzed to assure that the questions being studied have been properly addressed. It is too easy to come to an improper conclusion if this is not done.

## ACKNOWLEDGMENTS

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