RESEARCH ARTICLE

Is premating isolation in *Drosophila* overestimated due to uncontrolled factors?

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Abstract

Sexual isolation in *Drosophila* is typically measured by multiple-choice mating tests. While many environmental variables during such tests are controlled by the researcher, there are some factors that are usually uncontrolled. We demonstrate, using *Drosophila melanogaster* and *D. pseudoobscura* flies, that the temperature of rearing, preadult density, and level of consanguinity, can all produce differences in mating propensity between genetically equivalent flies. These differences in mating propensity, in turn, can give rise to statistically significant results in multiple-choice mating tests, leading to positive isolation values and the artifactual inference of sexual isolation between populations. This fact agrees with a nonrandom excess of significant positive tests found in a review of the literature of *Drosophila* intraspecific mating choice. An overestimate of true cases of sexual isolation in *Drosophila* in the literature can, therefore, not be ruled out.

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Introduction

Sexual isolation is the main premating reproductive isolation mechanism, and it can be viewed as some sort of incompatibility or disharmony between the sexual behaviour of two species that favours homogamic versus heterogamic matings or, alternatively, as the development of genetic systems determining the choice of conspecific individuals during mating. The finding of some type of mating tendencies between lines, strains or populations of any given species can be envisaged as a tendency to incipient speciation. This is the basis of the species recognition concept of Paterson (1985).

Drosophila is perhaps the most studied genus regarding the development of premating isolation through modification of sexual behaviours in natural or genetically manipulated populations (Spieth 1968; Parsons 1973). Incipient sexual isolation is mainly measured by a multiple-choice mating test (Merrell 1949; Reed and Reed 1950), which consists of placing equal numbers of males and females of two types in a mating chamber in which the numbers of homogamic and heterogamic matings occuring during a fixed period of time are recorded. This fixed period of time, which is set by the researcher, typically ranges between 20 and 120 min, depending on the mating propensities of the flies being studied, and the particular experimental design. Also, variable from study to study is the percentage of flies that mate within this time period, which varies between 50 to 100%. Mating preference is usually inferred when homogamic matings significantly exceed heterogamic ones, which is statistically confirmed by a 2×2 contingency chi-square or by some of the published isolation indexes. The most common of these is the Malogolowkin-Cohen index, which calculates the number of homogamic minus heterogamic matings and divides it by the total number of matings, giving positive values in the case of an excess of homogamic matings, and negative values in the opposite case (Malogolowkin-Cohen *et al.* 1965).

Despite the common use of the multiple choice mating test to detect discrimination and choice, there is a factor

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that can give rise to an erroneous interpretation of the obtained data. Casares *et al* (1998) have shown that if the two types of males and females differ in their mating propensities, and the number of matings occuring in the tests are close to 100% of all possible matings, then the choice conditions are not maintained and there is a high risk of obtaining significant results that do not correspond to true choice and falsely suggest assortative mating. In other words, mating propensity differences between the studied genotypes can produce significant departures from random mating between different types of flies that could then be erroneously interpreted as evidence of incipient sexual isolation. Moreover, differences in mating propensity between populations, lines and particular genotypes are common and have been recorded many times in *Drosophila* literature (e.g. Spiess 1970; Spieth and Ringo 1983). Variability for male and female mating propensity also occurs within populations (Carracedo *et al.* 1991; Casares *et al.* 1992, 1993), and populations readily respond to artificial selection for these traits (Manning 1968; Cook 1973; Cade 1984; Piñeiro *et al.* 1993).

At this point, one can ask whether the evidence of positive assortative mating in some of the published *Drosophila* studies – in which positive assortative mating was inferred from results of multiple choice mating tests – could in fact be an artifact due to differences in mating propensity rather than a case of genetically controlled mating choice. Unfortunately, there is no way to verify if significant multiple-choice test experiment results were in fact affected by differences in mating propensity of the participant genotypes, so we are forced to accept them as possible cases of incipient reproductive isolation. There is, however, an alternative way of addressing this issue by examining studies in which multiple-choice tests yielded nonsignificant results. In these cases, the authors detected no evidence of discrimination and choice. It follows from this that if matings occured randomly, it should be equally probable to find, within the nonsignificant tests, a slight excess of homogamic or heterogamic matings, that is, equal numbers of positive and negative isolation indexes. With this aim, we have reviewed the bibliography of *Drosophila* regarding the use of the multiple-choice test for assessing the appearance of intraspecific sexual isolation. Then, we have discarded those papers in which clear, positive assortative mating was found and linked to behavioural observations. The remaining papers (table 1) include tests between lines artificially selected for different traits, geographical populations, wild and laboratory stocks, founder-flush events, and drift lines, most of them using *D. melanogaster* and *D. pseudoobscura*.

Under the null hypothesis of no mating choice, the values of the isolation index calculated in the 240 nonsignificant tests must be seen as normal deviations around random mating (isolation index of zero). However, in these 240 tests there was an excess of positive (165) versus negative (75) results (a 2 : 1 ratio). What explanations we can offer for this excess (90 cases) of positive but nonsignificant tests? The first and more obvious answer is that in a large number of the 240 tests there were assortative tendencies that were not large enough to attain significance. Another possible answer emerges from the design and data handling of the multiple-choice mating tests. As discussed above, Casares *et al.* (1998) have demonstrated that mating propensity differences can give rise to deviations from random mating and false positive isolation measures. If one of the two competing types has greater male and female mating propensity than the other type, then homogamic matings will exceed heterogamic ones and the isolation index will be positive. A negative value would appear if one of the two types has higher propensity than the other type in one sex, but poorer in the other sex. With this matter in mind,

Table 1. Bibliography used to collect multiple-choice-test results.

Author/s	Date	*Drosophila* species	Origin of lines used
Anderson W. W. and Ehrman L.	1967	*pseudoobscura*	geographic populations
Burnet B. and Connolly K.	1974	*melanogaster*	spontaneous activity (artificial selection)
Dodd D. M. B.	1989	*pseudoobscura*	food adaption (artificial selection)
Dodd D. M. B. and Powell J. R.	1985	*pseudoobscura*	founder-flush
Ehrman L. and Parsons P. A.	1980	*immigrans*	geographic populations
Ehrman L. and Parsons P. A.	1981	*immigrans*	homopopulation isofemales
Henderson N. R. and Lambert D. M.	1982	*melanogaster*	geographic populations
Kilias G. *et al.*	1980	*melanogaster*	environmental adaption
Markow T. A.	1981	*melanogaster*	geo- and photo-tactic selection
Millar C. D. and Lambert D. M.	1986	*pseudoobscura*	geographic populations
Petit C. *et al.*	1976	*melanogaster*	geographic populations
Pot W. *et al.*	1980	*melanogaster*	Adh variants
Powell J. R.	1978	*pseudoobscura*	founder-flush and inbreeding
Ringo J. M. *et al.*	1985	*simulans*	founder-flush
Zouros E. and D'Entremont C. J.	1980	*mojavensis*	geographic populations

one can speculate if higher male and female mating propensities in one type over the other could have produced the observed bias in the distribution of positive and negative isolation measures observed in the literature. This is the starting point of the present work.

Although mating propensities have a genetic basis, it is also known that behavioural measurements are subjected to environmental factors, some of which are well known by *Drosophila* workers: handling, anaesthesia, food quality, temperature, age, etc. Some variables have even been used as controlled treatments, such as prior experience, housing, diet etc. (Pruzan 1976; Ehrman 1990; Etges 1992; Kim *et al.* 1996; Barth *et al.* 1997). The aim of the present work is to investigate if variation in some rearing conditions that are usually uncontrolled by researchers could have produced significant changes in male and female mating propensities to explain the excessive number of positive isolation measures found in the literature. With this purpose, we have chosen three variables which are fairly controlled, or uncontrolled, by researchers: temperature of rearing, preadult density, and level of consanguinity. Rearing temperature can be different for lines, strains, or populations maintained in different chambers or, if in the same chamber, if a temperature gradient exits inside it. Preadult density is a very important factor affecting adult development that is often not controlled by researchers because the great job it represents. Finally, inbreeding can be very different for controls and selection lines, wild populations, manipulated strains and all type of lines maintained without a strict control of their population size in the laboratory.

Materials and methods

The influence of rearing temperature and preadult density on mating propensity was examined independently in *D. melanogaster* and in *D. pseudoobscura*. For *D. melanogaster*, we used the Akayu population supplied by the Umea Stock Center of *Drosophila*. To measure male and female mating propensity in the different treatments, it was necessary to use a tester line: we used a wild *D. melanogaster* line (melB), which has been in our laboratory for more than ten years. The Tucson Stock Center provided us with the 14011–0121.35 (ps35) and 14011–0121.89 (ps89) *D. pseudoobscura* stocks. The first was used as the experimental population, and the second as the tester line.

To determine optimal and suboptimal larval densities, we carried out a prior experiment by seeding known numbers of fertilised eggs in 60 cm^3 vials filled with 20 ml of food. We recorded the mean developmental time and size of the emerged flies. A preadult density of 200 larvae per vial was found to be optimal, while 600 larvae per vial resulted in high larval crowding and poor developmental conditions. Therefore, the effects of preadult density on mating propensity were examined using adults developed from cultures at 200 and 600 eggs per vial, reared at 21°C.

The influence of rearing temperature was examined in vials with a fixed density of 200 larvae that were placed in three different chambers at 18, 21 and 25°C for *D. melanogaster*, and in chambers at 18 and 25°C for *D. pseudoobscura*. When preadult development was over, and adults started to emerge, all vials were moved to a chamber at 21°C.

Finally, the effect of the level of endogamy was investigated by evaluating the mating behaviour of a line derived from the *D. melanogaster* Akayu population after eight generations of full-sib mating (inbreeding coefficient, $F = 0.83$). In this experiment, the density was 200 larvae per vial and temperature 21°C.

Female mating propensity was measured as the time from the first courtship behaviour to the start of copulation, following Carracedo *et al.* (1991). Adults of the Akayu population coming from different treatments and from the melB line were collected in the first two hours after emergence, and each sex was aged separately in groups of five in vials with food at 21°C. When adults were four days old, a female to be tested and two tester males were transferred without anaesthesia to an empty vial, and observed continuously for a maximum of 30 min. The time to copulation or the failure to mate was recorded. Male mating propensity was measured in a similar manner, but in this case a male to be tested was paired with one tester female. Around 200 females were examined for each temperature, and 100 for the remaining treatments. The same methodology was followed for the ps36 and ps89 *D. pseudoobscura* lines.

The time to copulation values in seconds were $\log_{10}$ transformed to generate an approximately normal distribution (Dow 1976), and then regressed against probit values. The resulting regression line was used to calculate the mean time and error variance of mated and unmated individuals (details in Casares *et al.* 1992).

To carry out the multiple-choice mating test, flies of alternative types were either marked in the first two hours after emergence by placing a small dot of white non-toxic paint in the middle of their thorax, or left unmarked. Although this manipulation has previously been shown to have no effect on mating propensity (Casares *et al.* 1998), marking was rotated between types to avoid any undesirable effect. Flies were stored in vials with food in groups of five at 21°C. Four days later, twelve pairs of each type were placed in a 12 cm diameter petri dish and observed for 60 min. No anaesthesia was used. Copulating pairs were aspirated and the male and female types identified. Non-copulating pairs were discarded. Ten replicates were done.

Results and discussion

Table 2 shows the mating times in $\log_{10}$ seconds for the experimental lines of *D. melanogaster*. In both sexes, the increase of rearing temperature resulted in greater mating propensity of the adults, as indicated by the shorter copulation times. Increasing preadult density had a negative effect on mating propensity, mainly in females. The inbred line also showed a reduction in mating propensity relative to its base population.

Table 3 shows the results for *D. pseudoobscura*. Again, we found that increasing rearing temperature and decreasing preadult density generally resulted in shorter time to copulation, with females being more affected by the change of rearing temperature, and males by change in preadult density.

These observed differences in female and male mating propensities suggest that a multiple-choice mating test carried out with flies drawn from these experimental lines could result in a significant isolation index and a subsequent artifactual inference of incipient reproductive isolation between the lines. We note that the increase or decrease in mating propensity affects both sexes in the same direction, so multiple-choice tests would produce positive results.

The results of multiple-choice tests using flies developed at preadult densities of 200 and 600 larvae per vial are shown in table 4. For each species, the first row of data corresponds to the mating types of the first 12 copulating pairs (first 50% of all possible matings) observed in each mating chamber, and the second row to the matings observed at the end of the test after 1 h of observation. The 2×2 contingency chi-squares for the first 50% of matings are not significant, indicating that matings were at random, as expected from flies taken from the same population. However, the chi-squares for matings after one hour of observation (75% and 85%, respectively, of all possible matings) are significant for *D. pseudoobscura*, and close to significance in the case of *D. melanogaster*. Increasing the observation period would increase the chance of attaining significance. For both species, the corresponding isolation indices are positive, indicating an excess of homogamic matings, a result that would typically be interpreted as evidence for incipient reproductive isolation. In short, results are as expected, that is, mating till 50% were at random, while further elongation of the duration of the test gives rise to results that erroneously suggest incipient sexual isolation, as established in Casares *et al.* (1998).

We conclude that ascertaining sexual isolation in *Drosophila* through the commonly used design of the multiple-choice mating test depends not only on possible

Table 2. Time to copulation in minutes of flies from the same *D. melanogaster* Akayu population reared under different treatments. Raw data were analysed in decimal log of time in seconds. Preadult densities were 200 larvae per vial and rearing temperature 21°C unless otherwise indicated. Sample sizes were around 200 for the temperature tests and 100 for the remaining ones. IC stands for inbreeding coefficient. Within-treatment comparisons were carried out by analysis of variance (F) and Student's t-tests (t).

	Treatments									
	Rearing temperature °C				Inbreeding			Preadult density		
Sex	18	21	25	F (2, > 500)	IC = 0.83	Control	t	200	600	t
Females	5.65	5.27	2.96	21.8***	14.52	5.52	7.1***	5.52	11.53	7.3***
Males	7.80	5.92	3.18	43.9***	7.62	5.27	3.1**	5.78	8.16	2.2*

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Table 3. Time to copulation in minutes of flies from the same *D. pseudoobscura* ps35 line reared under different treatments. Raw data were analysed in decimal log of time in seconds. The rearing temperature test was done from flies developed at density of 200 larvae per vial. The preadult density tests used larvae reared at 21°C. Sizes were around 130 in each test. Within-treatment comparisons were carried out by Student's t-tests (t).

	Treatments					
	Rearing temperature °C			Preadult density		
Sex	18	25	t	200	600	t
Females	12.07	5.03	2.68**	5.78	7.80	1.15
Males	9.81	5.27	1.94	4.09	7.98	2.26*

*$P < 0.05$; **$P < 0.01$.

Table 4. Results of a multiple choice test carried out with virgin flies from the *D. melanogaster* Akayu population (4-day old), and from the ps35 *D. pseudoobscura* line (6-day-old). Flies were reared at preadult densities of 200 and 600 larvae per vial. Ten replicates each with twelve pairs of each type were examined. In the mating types, the female is cited first. Data were taken from the first 50% matings and from completion of the test after one h. Significance was measured by a 2×2 contingency chi-square. The Malogolowkin-Cohen index (I) assess the degree and direction (sign) of sexual isolation.

	Mating types						
	200×200	200×600	600×200	600×600	c^2	P	$I \pm$ s.e.
D. melanogaster							
Matings at 50%	48	34	20	18	0.37	0.54	+ 0.10 ± 0.09
Matings at 60 min	60	41	36	42	3.11	0.08	+ 0.14 ± 0.07
D. pseudoobscura							
Matings at 50%	41	33	26	20	0.01	0.90	+ 0.01 ± 0.09
Matings at 60 min	68	40	48	50	4.08	0.04*	+ 0.15 ± 0.07

genetic differences directly involved in recognition between the examined lines, but also on minor and often uncontrolled variables, such as temperature of rearing, preadult density and inbreeding level. The corollary is that the number of true cases of sexual isolation in *Drosophila* could be less than found in the literature, which agrees with a great stability of the *Drosophila melanogaster* mating system (Lambert and Henderson 1986). This assertion is also supported by our review of published data showing a clear bias towards the occurrence of positive sexual isolation at the intraspecific level in both *D. melanogaster* and *D. pseudoobscura*.

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