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Speciation driven by natural selection in *Drosophila*

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REINFORCEMENT is the process by which natural selection strengthens sexual isolation between incipient species, reducing the frequency of maladaptive hybridization and hence completing reproductive isolation. Although this model of speciation was once widely accepted^{1,2}, its plausibility^{3,4} and experimental support^{5–7} have been recently attacked. Here we provide an example of speciation by reinforcement, in the North American fruitfly *Drosophila pseudoobscura*. The results suggest that females of *D. pseudoobscura* evolved increased sexual isolation from their sibling species, *D. persimilis*, by natural selection against maladaptive hybridization.

Hybrid female offspring of *Drosophila pseudoobscura* and *D. persimilis* are fertile, but hybrid males are sterile, providing a selective disadvantage to mismatching. Small numbers of hybrids have been captured in the field^{8,9}, showing that interspecific mating occurs in nature. *D. pseudoobscura* ranges across the United States from Texas to the Pacific coast. The range of *D. persimilis* is completely contained within that of *D. pseudoobscura*, being found only in the Pacific coastal states¹⁰. *D. pseudoobscura* and *D. persimilis* flies were collected from two areas of California where they co-occur (sympatric populations), Mather and Mount St Helena; and *D. pseudoobscura* was also collected from Provo (Utah), Flagstaff (Arizona) and Tempe (Arizona), three areas where *D. persimilis* is not found (allopatric populations).

Females of *D. pseudoobscura* and *D. persimilis* discriminate against heterospecific males. Males, however, court the two species indiscriminately¹¹. Because reinforcement would only operate in areas where species hybridize, the theory predicts that, when tested with *D. persimilis* males, *D. pseudoobscura* females from sympatric populations should show stronger sexual isolation than *D. pseudoobscura* females from allopatric populations. There are no allopatric populations of *D. persimilis* with which similar comparisons can be made using *D. pseudoobscura* males.

The reinforcement hypothesis was tested by pairing individual *D. persimilis* males with individual *D. pseudoobscura* females derived from either sympatric populations or allopatric populations. In five of six comparisons, significantly fewer interspecific matings occurred with the *D. pseudoobscura* females derived

TABLE 1 Matings of *D. persimilis* males to *D. pseudoobscura* females from sympatric (S) and allopatric (A) populations

| Male: Mather <i>D. persimilis</i> | | | | |
|--|---|----------------|-------|-------------|
| | | Total observed | Mated | Probability |
| (S) | Mather <i>D. pseudoobscura</i> | 200 | 73 | |
| (A) | Tempe <i>D. pseudoobscura</i> | 200 | 72 | 0.5 |
| (S) | Mather <i>D. pseudoobscura</i> | 102 | 16 | |
| (A) | Flagstaff <i>D. pseudoobscura</i> | 102 | 63 | <0.001 |
| (S) | Mather <i>D. pseudoobscura</i> | 100 | 18 | |
| (A) | Provo <i>D. pseudoobscura</i> | 100 | 49 | <0.001 |
| Male: Mount St Helena <i>D. persimilis</i> | | | | |
| | | Total observed | Mated | Probability |
| (S) | Mount St Helena <i>D. pseudoobscura</i> | 147 | 23 | |
| (A) | Tempe <i>D. pseudoobscura</i> | 147 | 51 | <0.001 |
| (S) | Mount St Helena <i>D. pseudoobscura</i> | 107 | 28 | |
| (A) | Flagstaff <i>D. pseudoobscura</i> | 107 | 56 | <0.001 |
| (S) | Mount St Helena <i>D. pseudoobscura</i> | 100 | 33 | |
| (A) | Provo <i>D. pseudoobscura</i> | 100 | 56 | <0.001 |
| Probability across comparisons = 0.031 | | | | |

All stocks and experimental flies were kept at 21 °C with 12-h light/dark cycles. All crosses were done within 1½ years of the flies being collected. Population stocks were constructed by crossing 2–4 isofemale lines from each locality (as many as were available): Flagstaff *D. pseudoobscura* (3 lines), Mather *D. persimilis* (4), Mather *D. pseudoobscura* (3), Mount St Helena *D. persimilis* (4), Mount St Helena *D. pseudoobscura* (2), Provo *D. pseudoobscura* (4), Tempe *D. pseudoobscura* (4). Virgin flies used for the experiments were collected from the population stocks and aged for 8 days after eclosion to reach sexual maturity and receptivity. Single male and female pairs were then placed without anaesthesia in an 8-dram food-containing vial and confined for 24 h. To identify matings, the females' spermathecae and seminal receptacles were dissected and scored for sperm presence. All data were collected blind; the experimenter did not know if the flies derived from sympatric or allopatric crosses. The proportions of matings in sympatric and allopatric populations were compared with independent one-tailed Fisher's exact tests of independence, and the probabilities are given. To develop a statistical test of the entire table, one of the numbers from each comparison (either the number of matings with the sympatric females or allopatric females) was chosen with equal probability and summed over all six comparisons. This bootstrapping procedure was repeated 10,000 times to obtain a null distribution for the number of matings. The proportion of summations that was less than or equal to the total number of sympatric matings (191) is reported as a probability statistic.

TABLE 2 Comparison of *D. persimilis* male courtship of sympatric and allopatric *D. pseudoobscura* females

| Female | Courtship element | Mean (s.e.) |
|--|---------------------------------|-------------|
| Mount St Helena <i>D. pseudoobscura</i> | Courtship latency | 81 (17.2) |
| | Number of courtships | 7.0 (1) |
| | Number of attempted copulations | 5.4 (0.7) |
| Flagstaff <i>D. pseudoobscura</i> | Courtship latency | 61 (9.6) |
| | Number of courtships | 5.9 (0.8) |
| | Number of attempted copulations | 4.9 (0.7) |

Flies for these experiments were collected and aged as described above. Single male and female pairs were placed without anaesthesia in 8-dram food-containing vials and observed for 10 min. Courtship latency was defined as the time in seconds from introduction of the male until the male performed the wing vibration characteristic of courtship²¹. Courtships were scored when the male performed a wing vibration towards the female or attempted to copulate. Each episode terminated when the male no longer oriented to the female for 5 s. Observations were concluded after 10 min, and each pairing was replicated 25 times.

TABLE 3 Matings of *D. pseudoobscura* males to *D. persimilis* females from populations where it is rare (R) or abundant (A)

| Male: Flagstaff <i>D. pseudoobscura</i> | | | |
|---|----------------|-------|-------------|
| Female | Total observed | Mated | Probability |
| (R) Mount San Jacinto <i>D. persimilis</i> | 100 | 10 | |
| (A) Mather <i>D. persimilis</i> | 100 | 40 | <0.001 |
| (R) Mount San Jacinto <i>D. persimilis</i> | 100 | 20 | |
| (A) Mount St Helena <i>D. persimilis</i> | 100 | 17 | 0.36 |

from the sympatric populations (see Table 1). The sixth comparison showed no difference. The overall results are consistent with the expectation of the reinforcement hypothesis.

To assess whether this pattern could have resulted from *D. persimilis* male preference for allopatric *D. pseudoobscura* females, rather than differences in female *D. pseudoobscura* discrimination, measures of courtship of *D. persimilis* males with *D. pseudoobscura* females from a sympatric population and an allopatric population were quantified (see Table 2). In both tests, males courted and attempted to copulate immediately, showing no significant difference in courtship of sympatric versus allopatric *D. pseudoobscura* females.

Another prediction of reinforcement is that it should be stronger in the rarer species, because rarity increases the probability of mating with the wrong species and hence increases the strength of selection to reduce hybridization¹². An isofemale line

of *D. persimilis* was collected in 1993 from Mount San Jacinto, where the species is very rare (C. Babcock and W. W. Anderson, personal communication). *D. persimilis* is about as common as *D. pseudoobscura* at Mount St Helena¹³, and is more common at Mather¹⁴. Thus the reinforcement hypothesis predicts that *D. persimilis* females from Mount San Jacinto should show more discrimination than *D. persimilis* females from Mather and Mount St Helena against mating with *D. pseudoobscura* males.

To test this prediction, individual *D. pseudoobscura* males were paired with individual *D. persimilis* females derived from Mount San Jacinto and with *D. persimilis* females from the populations where *D. persimilis* is common. Mount San Jacinto females mated with *D. pseudoobscura* males significantly less often than did Mather females (see Table 3), supporting the reinforcement prediction. The results were not significantly different for the Mount St Helena population, but this outcome, too, is expected because *D. persimilis* is not as common at Mount St Helena as it is at Mather.

This study addresses two common criticisms of all previous studies of reinforcement. First, previous studies have dealt almost exclusively with species that are not known to hybridize in nature or produce some fertile hybrid offspring^{5,6}. Unless these conditions are met, there is no chance for gene flow and the operation of reinforcement; indeed the absence of gene flow in sympatry means that the two taxa were already good species^{15,16}. Second, the pattern of greater mating discrimination in areas of species overlap, which is often used to support reinforcement, could also result from the fusion or extinction of less isolated populations in sympatry⁷. This explanation seems unlikely in these species because high levels of gene flow apparently exist between *D. pseudoobscura* populations^{17,19}, reducing the possibility of populational fusion/extinction.

These results suggest that female *D. pseudoobscura*, and perhaps *D. persimilis*, evolved increased sexual isolation in response to the maladaptive results of mismating with heterospecific males in sympatry. It is unlikely that genetic drift or some other selection pressure caused both sympatric populations to evolve higher mating discrimination without any similar changes arising in the three allopatric populations. It is possible that an unknown third variable could explain these results; for example, an ecological variable could simultaneously allow *D. persimilis* to coexist within the range of *D. pseudoobscura* and also select for aberrant mating behaviour within *D. pseudoobscura* that fortuitously reduces heterospecific mating. Such a possibility, however, seems rather *ad hoc*, as it could produce changes in either direction. Reinforcement is a more parsimonious hypothesis because the conditions for its operation are known to occur and the specific prediction of direction is satisfied in these results.

Studies of the genetics of the reinforcement effect can now be performed. Previous genetic studies of isolating mechanisms might describe genetic changes that occurred only after speciation was completed²⁰. Investigating the genetic basis of the increased sexual isolation in *D. pseudoobscura* provides the rare opportunity to study genetic changes directly involved in the completion of speciation. □

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