A NEW TYPE OF ISOLATING MECHANISM IN DROSOPHILA

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While working on the general problem of sexual isolation in the genus Drosophila, the writer discovered a mechanism which forms a very effective barrier against the exchange of genes between certain members of this genus. It was first detected in the cross *D. buzzatii* ♀ × *D. arizonensis* ♂, two members of the mulleri subgroup of species. It was later found to be operative between other members of this subgroup, as well as between species belonging to other groups. The phenomenon stems from a reaction of the vagina that almost immediately follows copulation, and may be designated the *insemination reaction.* Within a very short time after mating, the vagina begins to enlarge and soon takes on an edematous-like condition, increaseing to three or four times the normal size of this organ in virgin females.

This change occurs in both intraspecific and interspecific crosses, but in intraspecific crosses (homogamic matings) the vagina returns to its normal size in the course of a few hours, while in interspecific crosses (heterogamic matings) it may remain swollen for several days and undergo deleterious changes which prevent it from carrying on its normal functions. In extreme cases, the reaction is so severe that eggs descending from the median oviduct may disintegrate in the vaginal cavity, or, if laid, they are not inseminated and are sometimes abnormal.

The *buzzatii-arizonensis* combination may be used to illustrate this reaction and its consequences. In previous publications from this laboratory, the cross *D. buzzatii* ♀ × *D. arizonensis* ♂ was designated as "incompatible." This term was employed in its usual sense to indicate that such test crosses do not result in the production of offspring. Recent studies on sexual isolation now reveal that the failure to produce hybrids in this cross is not due to the lack of insemination, but is the result of the insemination reaction that follows a successful copulation. When *buzzatii* females were crossed to *arizonenis* males in small mass cultures of ten females and ten males, 88% of the females were found to have been inseminated after an exposure of ninety-six hours, as shown by the presence of sperm in their ventral receptacles. We have tested this cross repeatedly but have never been able to obtain hybrids of any kind, either adults, pupae or larvae.

In studying this phenomenon, we have followed the plan of placing one virgin female with five males in a culture vial, and as soon as copulation had occurred the males and female were separated. Such females were dissected at given intervals of time and their reproductive tracts examined for the presence of sperm and any changes that may have occurred in the vagina.

In homogamic matings the vagina begins to enlarge in about four minutes after copulation, and continues to enlarge until it reaches the maximum size at the end of about one hour. Within twenty-five to thirty minutes after copulation the vagina turns slightly opaque and continues to become more and more so up to the end of the second hour. It then begins to show signs of clearing, and within five or six hours its opacity has completely disappeared. It returns to its normal size and semi-transparent condition by the end of eight hours.

The behavior of the sperm following insemination is a matter of interest. Within five minutes after copulation the sperm begin moving along the ventral side of the vaginal cavity to the point of origin of the ventral receptacle and soon start entering that organ. They continue to pass into the receptacle until it may become packed with these elements. Since in species belonging to this subgroup, the sperm rarely ever enter the spermathecae, there is frequently an excess of sperm left in the swollen vagina. Eventually, these are expelled, together with the rest of the contents of the vagina, in the form of whitish droplets which are easily seen on the surface of the food and which may be removed to a slide and examined under the microscope.[1] Undoubtedly the voiding of the contents of the vagina accounts for the return of that organ to a normal condition by the end of the eighth hour. A majority of these females will, in due time, lay eggs and produce offspring without further inseminations.

In heterogamic matings the behavior of the sperm and the reaction of the vagina are at first similar to those occurring in intraspecific crosses, but certain significant differences soon appear. In the first place, the insemination reaction occurs somewhat more rapidly and the opacity of the vagina is more pronounced. Moreover, the sperm are slower in reaching the mouth of the ventral receptacle and do not begin entering that organ until about forty-five minutes after copulation. A large mass of sperm frequently collects about the opening to the receptacle, and the sperm have difficulty in their attempts to enter its lumen. A few sperm are usually present in the proximal end of the receptacle by the end of two hours, and by the end of three hours a few are at the distal end. While the quantity of sperm which finally reaches this position varies considerably, yet this amount is always very much less than is the case in homogamic matings. In one case, only two spermatozoa were present at the distal end, and in another only six. In practically all cases the sperm are restricted to the distal one-third or one-fourth of the ventral receptacle. In contrast to this condition, the receptacle always contained many sperm in homogamic matings and was frequently recorded as being "full" or "solid" with them.

In homogamic matings the vagina may soon return to a normal condition, as stated above, but in heterogamic matings its swollen condition may last for several days, accompanied by certain significant changes. One of the first differences noted is a change in color. At the end of the third hour after copulation the vagina turns slightly brownish and remains so up to about the twenty-fifth hour, when it often appears blackish under transmitted light. By forty hours the contents of the vagina become localized and sharply delimited, occupying the antero-dorsal part of the cavity. As time goes on this *reaction mass* becomes reduced in size and pear-shaped with the smaller end directed posteriorly. Further reduction may occur until its size does not constitute more than one-fourth the volume of the vagina. In some cases the reaction mass may undergo disintegration and entirely disappear, although usually traces of it can be detected. Even in such cases the vagina is left abnormal in appearance. In the *buzzatii-arizonensis* cross the reaction mass is usually present in females dissected on the seventh day after copulation.

Since only a part, and usually a very small part, of the sperm received by the female ever succeeds in entering the ventral receptacle, the question arises as to what becomes of those remaining in the vagina. Motile sperm are easily seen in this organ and have been detected in practically every case up to about the seventh hour after copulation. They have not been observed after this period, although the vagina of one female of the eighth hour contained a group of non-motile sperm located on the ventral side of its cavity. No evidence was obtained that the excess sperm had been expelled as in homogamic matings, but this remains as a possibility. It may be that these sperm are absorbed by the reproductive tract, as has been suggested for certain other insects.[2]

The sperm in the distal end of the ventral receptacle remain alive for at least 160 hours, and perhaps longer. There is, however, no evidence in the *buzzatii-arizonensis* cross that these sperm ever inseminate the eggs. The first egg discharged from the ovary in this series of dissections was forty-eight hours after copulation. It was just entering the median oviduct when first seen. One female dissected at fifty-six hours had laid five eggs, and two dissected at seventy-two hours had each laid one egg. These eggs were checked by the smear technique,[3] but none was found to contain sperm. The females had laid a considerable number of eggs by ninety-six hours, but most of these were transparent, like eggs laid by virgin females. A total of 105 of these eggs were checked for the presence of sperm and all were found not to have been inseminated. Some of the eggs break down and go to pieces in the vagina, and its cavity may become full of their debris. In one case, parts of three eggs were found in the vaginal cavity, and eggs in various degrees of disintegration were sometimes observed.

The intraspecific matings of nine different species of the mulleri subgroup have been examined for the presence of the insemination reaction and without exception all showed that it occurred in them. In interspecific crosses between these species, the reaction was found to occur in all combinations in which copulation had been successful, irrespective of whether or not such matings had resulted in the production of hybrids. Successful copulations did not take place in a majority of the combinations tested, but in all crosses in which it did occur, the history of the insemination reaction was essentially the same as outlined above for the *buzzatii-arizonensis* cross. In the few cases in which hybrids were produced, the reproductive tract of the female had recovered sufficiently to allow some of the eggs to become inseminated. In most crosses, however, the number of hybrids produced was relatively small, and these were sometimes abnormal or else the zygotes never developed beyond the mid-larval stage. The most successful cross, in so far as the production of hybrids is concerned, was between the two most closely related forms, *D. mojavensis* and *D. arizonensis.*

The hybrids from the cross *D. mojavensis* ♀ × *D. arizonensis* ♂ are fertile, and pair mating tests show that approximately 75% of the females produce offspring, although the average number of individuals per culture is relatively low. In one experiment, six-day-old *mojavensis* females were crossed to *arizonensis* males of the same age in small mass matings, and four days later the females were dissected and their seminal receptacles examined. It was found that 93% of these females had been inseminated, and of this number, the vagina was clear and normal in twenty-seven cases, clear and nearly normal in thirty-three others, and opaque and abnormal in the thirty-three remaining specimens. The ventral receptacles of all the females of groups one and two contained motile sperm, and these are the females that are responsible for the production of the hybrids. It is doubtful if the vagina in any of the females belonging to the third group would ever recover sufficiently to enable them to lay fertile eggs.

This brief account of the insemination reaction in the mulleri subgroup raises the question as to its possible occurrence in other species of the genus. In past studies on members of the virilis group we had, from time to time, observed this reaction in interspecific crosses, but had not realized its significance. With the knowledge gained in studying the mulleri series, we reexamined the virilis group and found that the reaction also occurs among members of that group. However, the insemination reaction is rather inconspicuous in homogamic matings and could be overlooked. The reaction is quite obvious in the heterogamic matings, although not so striking as in the mulleri series.

Mr. Marshall R. Wheeler has undertaken a study of all available species of Drosophila in the laboratory, with the view of determining the extent of the insemination reaction among other members of the genus. He studied the intraspecific crosses only and used the method given above for obtaining a timed series of stages. In the list given below, members of the virilis and mulleri series were examined by the writer, all others were studied by Mr. Wheeler, to whom I am indebted for the privilege of including his results in advance of publication. The several species examined are given in their systematic order as outlined by Sturtevant.[4] The terms present and absent are used to indicate whether or not the insemination reaction was found to occur in the different species. The list is not complete, but gives all forms thus far examined.

Subgenus *Pholadoris:* present in *D. victoria.* Subgenus *Sophophora:* 1. saltans group, absent in *D. prosaltans.* 2. willistoni group, absent in *D. nebulosa.* 3. melanogaster group, absent in *D. melanogaster.* 4. obscura group, absent in *D. pseudoobscura.* Subgenus *Drosophila:* 1. quinaria group, present in *D. transversa, D. innubila, D. subpalustris, D. suboccidentalis* and *D. subquinaria.* 2. guttifera group, present in *D. guttifera.* 4. virilis group, present in *D. virilis, D. americana* and *D. texana.* 6. tripunctata group, absent in *D. tripunctata.* 7. funebris group, present in *D. funebris.* 8. repleta group, absent in *D. hydei;* present in *D. mercatorum, D. pararepleta, D. hexastigma* and *D. gibberosa.* 8a. mulleri subgroup, *D. anceps, D. aldrichi, D. arizonensis, D. buzzatii, D. hamatofila, D. mojavensis, D. peninsularis, D. mulleri* and *D. ritae,* present in all nine. 10. melanica group, present in *D. melanica.* 13. cardini group, absent in *D. cardini.* 14. immigrans group, present in *D. immigrans.* Miscellaneous forms, present in the two subspecies, *D. pallidipennis pallidipennis* and *D. p. centralis.*

This list shows that the insemination reaction was found to occur in twenty-eight of the thirty-five species examined. On the basis of this study, it seems safe to predict that it is present in all members of the quinaria and virilis groups and the mulleri subgroup, but probably absent in all members of the subgenus *Sophophora.* The repleta group presents an interesting situation. The reaction was found to be present in thirteen of the fourteen species examined, with *D. hydei* representing the exception. There is incomplete evidence that some of the forms closely related to *hydei* also do not have the reaction. If this turns out to be true, it will be possible to divide the large repleta group into two main divisions, one with and one without the reaction.

It is legitimate to speculate on the nature of the insemination reaction and its possible rôle in Drosophila speciation. It is well at first to restate briefly just what happens after copulation has taken place. The introduction of the semen with its contained spermatozoa into the vagina is followed almost immediately by a reaction of the mucous membrane which secretes a relatively large amount of fluid into the cavity. This is what brings about the characteristic swelling of the vagina. This membrane must be hypersensitive to the foreign protein, and, since the reaction occurs after the first copulation, its hypersensitiveness must be an inherited character. Whether the spermatozoa *per se,* or the semen, or both are responsible for calling forth the reaction is a matter of considerable interest. We have observed, from time to time, that in some crosses the male had failed to deliver spermatozoa with the semen at the time of copulation, and yet the typical insemination reaction had occurred. The most careful examination of the freshly formed reaction mass in such cases failed to disclose the presence of sperm. Moreover, no evidence was found of any form of lysis that might have been responsible for the absence of sperm.[5]

A more convincing line of evidence that the sperm are not responsible for the reaction is seen in the results obtained in backcross matings of sterile F_1 males. These males have slightly smaller testes than normal, and spermatozoa are never formed in them. The F_1 males from the cross *arizonensis* ♀ × *mojavensis* ♂ are of this type, and they will sometimes mate with either *arizonensis* or *mojavensis* females. Their sperm-free semen always brings about the typical insemination reaction. It is, therefore, certain that the presence of sperm in the semen is not necessary for the induction of the reaction.

Another point of interest is whether the insemination reaction will be repeated in the vagina of a female which has mated a second time. The answer to this question will depend on the species. In the first place, in forms which do not show the reaction, copulation may occur two or more times, and in some species at relatively short intervals. In species which show a weak reaction (e.g., virilis group), the female may remate two or more times at intervals of several hours, and a reaction of about the same intensity as the first occurs after each mating. Finally, in species in which the reaction is severe the female may never remate, but if she does, it is only after a long period of time, but here too the reaction occurs, provided the copulation has been successful.

Three possible functions may be suggested for the insemination reaction. The first of these refers to its occurrence in homogamic matings. It may have the effect here of preparing the reproductive tract for the fertilization mechanism which is to follow. It should be pointed out, in this connection, that even in forms which show no visible reaction there still may be a change in the mucous membrane which has the same effect. Unfortunately, this suggestion cannot be tested experimentally.

A second possible rôle of the insemination reaction has a more direct bearing on the problem of speciation. In species which do not have this reaction, the female may copulate two or more times in intraspecific matings. In contrast to this, the female usually mates but once in species showing a strong reaction. In the first instance the male in the population could, and probably would, mate with the same female more than once, instead of fertilizing additional females. In the second instance the male would be forced to copulate with more than one female, if he were to mate more than a single time. This might be an advantage in a population with a restricted number of males. If the insemination reaction arose as a mutation, irrespective of any selective value it might have to the species as a whole, it would spread throughout the population. This is because virgin females might be inseminated by males either with or without the factor. Females fertilized by males not carrying the factor may be remated by both kinds of males. However, a female inseminated by a male with the factor is much less likely to be remated. Hence, a female mated by a male not carrying the factor may have progeny by several males, but a female carrying the factor will ordinarily have progeny by only one male. This is a very interesting example of how a mutation, even though it be non-adaptive, would replace its alleles in the population.

A third rôle is revealed in interspecific crosses among forms which have the insemination reaction, for here it has the effect of either reducing the exchange of genes, or preventing such exchanges altogether. The *buzzatii-arizonensis* cross is an excellent example of the complete elimination of gene transfer. Sexual isolation in this cross is weak and copulation occurs almost as frequently (88%) as in the intraspecific cross (97%), and yet, as a result of the insemination reaction, the females are unable to produce offspring.

In conclusion, it should be pointed out that the widespread occurrence of this reaction in the genus would suggest that it may be present in forms other than Drosophila. It is possible that the results reported above may have some bearing on the controversial point regarding the effect of sperm injections on the development of the reproductive tract in immature mammals.[6, 7]

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[1] In 1942 Dr. Wilson S. Stone and the writer observed this same habit in females of the virilis group.

[2] Cragg, F. W., *Ind. J. Med. Res.*, **8,** 32–39 (1920); **11,** 449–473 (1923).

[3] Patterson, J. T., *Science,* **101,** 156 (1945).

[4] Sturtevant, A. H., *Univ. of Tex. Publ.*, **4213,** 7–51 (1942).

[5] I am indebted to Dr. V. T. Schuhardt, of the department of Botany and Bacteriology, for valuable suggestions in preparing this account on the nature of the insemination reaction.

[6] Green-Armytage, V. B., *Proc. Roy. Soc. Med.*, **36,** 105 (1943).

[7] Bacsick, Sharman, and Wyborn, *J. Obstet. Gynaec. Brit. Emp.*, **52,** 334 (1945).