EXPERIMENTS ON THE INCIPIENT SPECIES OF THE *DROSOPHILA PAULISTORUM* COMPLEX¹

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Drosophila paulistorum Dobzhansky and Pavan is a superspecies which consists of at least five races or incipient species, living in tropical Central and South America (DOBZHANSKY and SPASSKY 1959; DOBZHANSKY, EHRLMAN, PAVLOVSKY and SPASSKY 1964). An ethological (sexual) isolation and a sterility of hybrid males impede the gene exchange between the incipient species. Although the ethological isolation varies in intensity from strain to strain, apparently no interbreeding takes place where the geographic distribution areas of two or even three incipient species overlap (EHRMAN 1965). The process of speciation has evidently been completed in these overlap zones. On the contrary, in northern Colombia there is found a Transitional race, some strains of which give fertile hybrids with more than a single incipient species. MALOGOLOWKIN-COHEN, SIMMONS and LEVENE (1965) have described also transitional strains, which give fertile hybrids with more than one incipient species. The differences between the Transitional race and transitional strains is that the former has its own geographic distribution area, while the latter occur in populations in which other strains do not show a transitional character. And finally, DOBZHANSKY and PAVLOVSKY (1966) have, in a preliminary communication, described the spontaneous origin in a laboratory strain of a hybrid sterility with strains with which it was formerly fertile. Experiments designed to elucidate some of the above complex and varied behavior are reported in the present article, as well as in the accompanying work of WILLIAMSON and EHRLMAN (1967).

MATERIALS AND METHODS

Lists of localities in which the progenitors of the strains of *D. paulistorum* kept in our laboratory were collected can be found in DOBZHANSKY and SPASSKY (1959) and in DOBZHANSKY, EHRLMAN, PAVLOVSKY and SPASSKY (1964). Additional strains have been obtained through the kindness of DR. S. B. PIPKIN, collected by her at Rio Raposo, Colombia (June 1963), Almirante, Panama (April 1964), and Marco, Amazonas, Brazil, vicinity of Leticia, Colombia (June 1964).

Since the incipient species of the *D. paulistorum* complex cannot be distinguished by inspection of their external morphology, their classification is made routinely as follows. After the females collected in nature are received in the laboratory, they are allowed to produce progenies in single cultures. Then the strains from a given locality are intercrossed chainwise, i.e., $A \times B$, $B \times C$, $Y \times Z$, $Z \times A$. If all the crosses produce fertile progenies, the strains are pooled

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together into a single or several strains that can be maintained. If a break in the chain occurs, additional tests are made to separate the strains into two or more groups belonging to different incipient species. Finally, to determine to which incipient species a new strain belongs, it is crossed to five standard "testers," i.e., known strains of the Centro-American, Amazonian, Orinocan, Andean-Brazilian, and Guianan incipient species (the Guianan has recently been raised to the rank of a full species, KASTRITSIS and DOBZHANSKY 1966). About ten virgin females of the tester or of the tested strains are crossed to ten or more males of the other; if after about a week no larvae appear, the flies are transferred without etherization to a fresh culture, and are given another chance to produce offspring. A second negative outcome is taken as evidence that different incipient species are involved. If offspring are produced, the hybrids are allowed to hatch, and a group of ten or more females and males are tested for fertility (of the hybrid males, since the females are always fertile) in the same way as the original cross. As a rule, the results of the tests are unambiguous, a fertile progeny being produced with one and only one testing strain.

RESULTS

A review of the crosses: Table 1 summarizes the results of the intercrosses of the incipient species. The geographic origins of the strains tested is shown in Figure 1. Three strains, namely Llanos, Raposo, and Marco, are not included among the data in Table 1, since the behavior of these strains is considered separately. Table 1 supersedes and corrects the lists published in DOBZHANSKY and SPASSKY (1959) and in DOBZHANSKY, EHRLMAN, PAVLOVSKY and SPASSKY (1964), but it does not include the crosses reported by CARMODY *et al.* (1962) (since these authors did not test the fertility of the hybrids) or those of EHRLMAN (1965), MALOGOLOWKIN (1963) and MALOGOLOWKIN, SIMMONS and LEVENE (1965).

TABLE 1

Numbers of crosses yielding no progeny (O), fertile female but sterile male hybrids (S), or fertile hybrids (F). C. Amer. = Centro-American, Trans. = Transitional race

Female	Male	O	S	F	Female	Male	O	S	F
C. Amer.	C. Amer.	..	..	19	Andean	Andean	..	..	44
C. Amer.	Amazonian	32	7	..	Andean	C. Amer.	1	22	..
C. Amer.	Orinocan	4	19	..	Andean	Amazonian	34	27	..
C. Amer.	Andean	4	22	..	Andean	Orinocan	7	31	..
C. Amer.	Guianan	..	2	..	Andean	Guianan	2	11	..
C. Amer.	Trans.	2	11	7	Andean	Trans.	..	16	34
Amazonian	Amazonian	1	..	152	Guianan	Guianan	..	..	2
Amazonian	C. Amer.	33	4	..	Guianan	C. Amer.	3	1	..
Amazonian	Orinocan	131	11	..	Guianan	Amazonian	6	..	..
Amazonian	Andean	59	14	..	Guianan	Orinocan	6	1	1
Amazonian	Guianan	17	5	..	Guianan	Andean	..	5	..
Amazonian	Trans.	26	7	..	Guianan	Trans.	4	2	..
Orinocan	Orinocan	1	..	111	Trans.	Trans.	..	3	15
Orinocan	C. Amer.	4	19	..	Trans.	C. Amer.	7	8	7
Orinocan	Amazonian	258	18	..	Trans.	Amazonian	20	7	..
Orinocan	Andean	7	27	..	Trans.	Orinocan	7	6	..
Orinocan	Guianan	13	1	..	Trans.	Andean	..	18	39
Orinocan	Trans.	16	15	..	Trans.	Guianan	..	3	..

FIGURE 1.—The known geographic distribution of the incipient species of the *Drosophila paulistorum* complex.

Among the 1479 crosses listed in Table 1, 345 are between strains belonging to the same incipient species. All but two easily gave fertile progenies. The exceptions are the crosses Bucaramanga-8 female $\times$ Simla-N male and Cucuta-B female $\times$ Belem-A male, which in repeated tests failed to produce progeny. The first two belong to the Orinocan and the other two to the Amazonian incipient species, as shown by their having produced fertile offspring in several crosses with other strains of the respective groups.

Intercrosses of different incipient species either fail to produce progeny because no matings occur (0 in Table 1), or produce apparently vigorous hybrids which are sterile as males (S), and only exceptionally fertile hybrids of both sexes (F). The fertile exceptions mostly involve strains of the Transitional race, but also

the cross of a Guianan strain to an Orinocan from Panama (EHRMAN 1962). The crosses involving Amazonian females and males of the other forms are the most refractory ones, only 41 out of 307 having produced hybrids. The Andean and the Centro-American females are less discriminating, 141 out of 185 crosses with the former, and 68 out of 110 crosses with the latter having given hybrid offspring, in which the hybrid males were sterile except when Transitional race fathers were involved. The Orinocan race females are intermediate in this respect, 80 out of 378 crosses having given F_1 progenies.

If the Transitional race is disregarded, the results summarized in Table 1 may be said to show the typical or standard behavior of the incipient species—fertile hybrids within and no hybrids at all, or hybrids which are sterile as males, in the crosses between the incipient species. As to the Transitional race, it is both cytologically and genetically an offshoot of the Andean-Brazilian (DOBZHANSKY and PAVLOVSKY 1962; KASTRITSIS 1966). The Santa Marta strain of the Transitional race has however peculiar properties (EHRMAN and WILLIAMSON 1965; WILLIAMSON and EHRMAN 1967).

The hybrid females can be backcrossed to males of either parental form. The backcross progenies consist again of fertile females and sterile males. EHRMAN (1960) studied the hybrids between the Centro-American, Amazonian, and Andean-Brazilian forms, in which the chromosomes were marked by suitable mutant genes. She found that the sterility of the backcross males depends on the chromosomal constitution of their mothers, the presence of any one foreign chromosome (the X, second, or third) being sufficient to make the sons of the females which carry such chromosomes sterile. An exception to this rule was however found in the hybrids between the Transitional and the Centro-American races; here, the presence in a mother of a race-foreign third chromosome does not make her sons sterile (EHRMAN 1962b). Further exceptions of the same sort are described below.

Change in the behavior of the Llanos strain: A sample of *D. paulistorum* was taken on March 19, 1958, at Chichimene, south of Villavicencio, in the Llanos of Colombia. Four strains, each derived from a single female, were established; three of them proved to belong to the Andean-Brazilian and one to the Orinocan incipient species. It is with the last named that we are here concerned. The early tests (carried out by MR. SPASSKY in the autumn of 1958) suggested that the Llanos-A strain was transitional (in the sense of MALOGOLOWKIN 1963, 1965, i.e., giving fertile hybrids with more than one incipient species). Thus, while Llanos females crossed to males of certain other Orinocan strains gave fertile hybrids, the reciprocal crosses gave sterile hybrid males. Llanos males gave fertile sons when crossed to a strain of the Centro-American (Honduras) and of Transitional (Santa Marta) races, while Llanos females gave, as expected, sterile sons in crosses with other Centro-American strains. To clarify the situation, the crosses were repeated in 1959, using more flies. This time fertile male hybrids were obtained in all crosses with all Orinocan strains then available, while the cross to Honduras yielded sterile males. The Llanos-A strain was accordingly classified as an ordinary member of the Orinocan group; as seen in retrospect, it seems

possible that only a part of the hybrid males were fertile and others were sterile. In 1959 and 1960 the strain was examined cytologically, and was found to carry some chromosomal inversions not present in any other strain studied (DOBZHANSKY and PAVLOVSKY 1962).

In 1963 new strains of *D. paulistorum* were collected in Venezuela, Trinidad, and British Guiana. The Llanos-A strain was employed as one of two testers for the Orinocan incipient species (the other tester was a strain from Caripe, Venezuela). Several new strains gave sterile male hybrids with Llanos as well as with other incipient species (these strains were not then tested with Caripe). The conclusion was drawn that these strains belonged to a new race of their own, which was named Calypso (DOBZHANSKY, EHRLMAN, PAVLOVSKY and SPASSKY 1964). This proved to be wrong, since subsequent tests of the supposed Calypso with Orinocan strains other than Llanos produced fertile hybrids. It was the Llanos strain which now evinced an unexpected behavior, since it produced sterile F_1 hybrid males when crossed, either as a female or as male, to all other Orinocan strains available in the laboratory. Table 1 in DOBZHANSKY and PAVLOVSKY (1966) lists the outcomes of 127 crosses in which one of the parents was Llanos. Of these, 45 were crosses with Orinocan strains; all of them produced hybrids, and in all cases the hybrid males were sterile. Of the remaining 82 crosses of Llanos with incipient species other than the Orinocan, 52 failed to produce any progenies, and 30 gave progenies with sterile males.

From 1963 on, the "new" Llanos strain has behaved differently from the Llanos-A strain of 1958 and 1959. Yet the "new" strain carries the same unique inversions as the old, and until mid-1964, when the Marco strain was received, there was no other strain in our laboratory with which the new Llanos produced fertile hybrids. Some change has evidently occurred in the Llanos strain between 1959 and 1963. (The cytology of *D. paulistorum* will be described in a forthcoming paper of DR. C. KASTRITSIS).

Fertility and sterility in backcross progenies of the new Llanos and an Orinocan strain: An autosomal recessive mutant orange (possibly a homologue of cinnabar in *D. melanogaster*) has been found in an Orinocan strain, the ancestors of which were collected at Georgetown, British Guiana. The crosses Llanos female $\times$ orange male, and orange female $\times$ Llanos male both produce progenies; 29 F_1 males from the first and 25 from the second cross were tested for fertility with groups of orange or wild-type Georgetown females, and all proved sterile; dissection of their testes and examination under phase-contrast microscope revealed gross abnormalities in the spermatogenesis.

The F_1 hybrid females from both reciprocal crosses were mated to orange males; 97 wild and 86 orange-eyed females, and 69 wild and 66 orange males were counted in the backcross progenies. The fertility of the backcross males was first examined in mass tests, i.e., in 6 to 12 backcross males per culture mated with similar numbers of virgin females. All the cultures of such group tests yielded progenies, though sometimes small ones. This behavior differs from that of the crosses between other incipient species, which produce all sterile backcross males. To find out whether all, or only a part of the backcross males were fertile,

individual tests were arranged. Single males were mated to groups of five virgin females; if no larvae appeared in about a week, the flies were given another chance in a new culture for about one week more. Of the 20 orange-eyed backcross males so tested seven were fertile and 13 sterile, while among 25 normal-eyed ones three were fertile and 22 sterile.

Second and third backcrosses were raised, using mostly wild-type hybrid females and orange-eyed males from the stock. The fertility of the backcross males was examined in group tests only; the cultures with orange males were all fertile, while those with wild-type males produced usually only scant progenies.

Fertility and sterility in crosses of the new Llanos to other incipient species: Attempts were made to cross the new Llanos strain to the orange-eyed mutant of the Amazonian (of Panamanian origin), the veinlet mutant of the Transitional (from Bucaramanga, Colombia), and the Plum mutant of the Guianan (Georgetown) incipient species. No offspring was obtained in either of the reciprocal crosses, nor in the cross of orange mutant females (from Cucuta, Colombia) to Llanos males. The crosses Llanos females $\times$ orange Amazonian (Cucuta) males, and Llanos females $\times$ Spot (Centro-American) males and the reciprocal, gave fertile female and sterile male F_1 hybrids. Backcrosses were made of the hybrid females to males of both parental strains; the backcross progenies consisted of fertile females and completely sterile males. This is what can be characterized as a "normal" behavior in crosses between incipient species of *D. paulistorum*.

The crosses of Andean-Brazilian dark-eye mutant (from Apoteri, British Guiana) females $\times$ Llanos males, and the reciprocal, gave, as expected, fertile female and sterile male F_1 hybrids. The females were backcrossed to dark-eye (recessive) males, and produced a total of 93 normal and 101 dark-eye females and 78 normal and 85 dark-eye males. The males were tested for fertility by group tests. The normal-eyed males were all sterile, and on dissection showed no motile sperm in their testes. The dark-eye males were also sterile, except that one of the cultures produced a few larvae and pupae. On dissection, the males showed motile sperm. A second backcross was therefore arranged, of normal-eyed females from the first backcross to dark-eye males from the stock. In the progeny, the normal-eye males were all sterile and had no motile sperm, while at least some of the dark-eye males were fertile and produced abundant progenies. Such fertility of backcross males is "abnormal," but whether or not the Llanos strain would have shown this behavior also before it became sterile with the Orinocan strains is impossible to determine.

Lack of sexual isolation between the new Llanos and Orinocan: Although strains of the same incipient species from different localities often show slight preferences for homogamic matings, and thus rudiments of sexual (ethological) isolation, this rarely even approaches the degree of such isolation between strains of different incipient species (CARMODY *et al.* 1962). We have seen above that the new Llanos strain gives sterile F_1 hybrid with the orange-eyed mutant of the Orinocan race. Does it show a sexual isolation from orange? Tests were arranged by "male-choice" method; 10 females of Llanos and 10 females of orange were

confined with 10 males of Llanos, or of orange, in vials for several hours. The dissection of the females showed the following (+ indicates insemination and — virginity):

Males	Llanos		Orange		Isolation index
	+	—	+	—	
Llanos	56	34	36	54	+0.22
Orange	44	47	44	48	—0.06

There may be a weak preference for homogamic matings with Llanos males, but none with orange males. In acquiring sterility with Orinocan strains, Llanos did not simultaneously acquire any pronounced sexual isolation. At the same time, it remained strongly isolated from the Andean-Brazilian and the Centro-American incipient species (see Table 3 in DOBZHANSKY and PAVLOVSKY 1966).

The Raposo population: In June 1963, 44 females of *D. paulistorum*, collected by DR. S. B. PIPKIN at Rio Raposo, in western Colombia, gave rise to as many strains, which were intercrossed chainwise, all gave fertile hybrids, and were consolidated into 4 strains, Raposo-A, B, C, and D.

The standard crosses with a variety of strains of different incipient species gave the following results (O = no progeny, S = sterile males in F_1 , F = fertile male hybrids):

Crossed to	O	S	F	Crossed to	O	S	F
C. Amer	..	8	..	Andean	..	17	..
Amazonian	23	3	..	Guianan	2	..	..
Orinocan	..	20	..	Transitional	6	10	..

Although Raposo crosses easily to Centro-American, Orinocan, and Andean strains, no cross has produced fertile F_1 hybrid males. It seems, then, that Raposo is a race or an incipient species of its own. Nothing more was done with Raposo strains until 1965, when an analogy between the behavior of Raposo and of the new Llanos strain began to be suspected. As shown above, Llanos gives sterile F_1 but at least some fertile backcross hybrids with Orinocan strains, and shows no sexual isolation from the latter. Experiments were accordingly arranged, crossing Raposo to mutant strains of other incipient species. The following results were then obtained:

- A. Plum (Guianan) ♀ × Raposo ♂ —no progeny.
- B. Raposo ♀ × Plum (Guianan) ♂ — F_1 males sterile; Plum and wild-type females backcrossed to Raposo males gave progenies with sterile males.
- C. Raposo ♀ orange (Amazonian) ♂ —no progeny.
- D. Orange (Amazonian from Cucuta, Colombia) ♀ × Raposo ♂ — F_1 males sterile. Two backcrosses to orange gave progenies in which most of the males were sterile (by group tests), except for one second backcross batch of orange-eyed males which produced a small progeny after two transfers to fresh cultures.
- E. Raposo ♀ × orange (Orinocan) ♂ — F_1 males sterile; backcross to orange gave a progeny in which at least some of the males were fertile.
- F. Orange (Orinocan) ♀ × Raposo ♂ — F_1 males sterile; backcross to orange gave a progeny in which orange-eyed males were fertile but normal-eyed ones sterile. In the second backcross to orange, at least some orange as well as normal-eyed males were fertile (in group tests).

G. Raposo ♀ × dark eye (Andean-Brazilian) ♂— F_1 males sterile; backcross to dark eye gave a progeny in which dark-eyed males were fertile and normal-eyed ones sterile. Second and third backcrosses were made using both normal and dark-eyed first backcross females and dark-eyed or normal (Raposo) males respectively. The dark-eyed males in the progenies were fertile and the normal-eyed were sterile.

H. Dark eye (Andean-Brazilian) ♀ × Raposo ♂— F_1 males sterile; two backcrosses to dark-eyed males gave fertile dark-eyed and sterile normal-eyed males.

I. Veinlet (Transitional) ♀ × Raposo ♂—no progeny.

J. Raposo ♀ × veinlet (Transitional) ♂— F_1 males sterile; first backcross to veinlet gave all sterile males in the progeny; second backcross to veinlet gave also all sterile males.

K. Raposo ♀ × ebony (Centro-American, Lancetilla, Honduras) ♂ and the reciprocal— F_1 males sterile. First and second backcrosses to ebony males gave progenies in which both ebony and wild-type males were fertile by group tests. Tests of single males from the progeny of the first backcross were therefore made; of the 20 ebony males, 10 were fertile and 10 sterile; of the 10 normal males, 5 were fertile and 5 sterile.

Sexual behavior of Raposo flies: The above described results of crosses and fertility tests of hybrids between Raposo of other strains are unusual. Crosses with three incipient species, Orinocan, Andean-Brazilian, and Centro-American all give sterile F_1 males, but at least some fertile males in the backcrosses. Raposo is not however a regular member of the Transitional race, since it is hard to cross and gives sterile hybrids with other strains of that race. Tests of sexual preferences were arranged to throw more light on the situation. The orange-eye mutant of the Orinocan, dark-eye of the Andean-Brazilian, and ebony of the Centro-American (Lancetilla, Honduras) incipient species were used. "Male choice" technique was chosen, with 10 virgin females of each of two strains and 10 males of one of them per vial. The time of exposure varied from 3 to 9 hours, to obtain roughly 50% of the females inseminated. The results are summarized in Table 2.

It is evident that Raposo is decidedly, though far from completely, isolated sexually from the Orinocan and Andean-Brazilian strains tested, but shows no isolation at all from the Centro-American strain. In short, as far as its sexual behavior is concerned, Raposo is a member of the Centro-American incipient species, just as the new Llanos continues to be a member of the Orinocan incipient species.

The Marco strain: Three of the four strains collected by DR. S. B. PIPKIN at

TABLE 2

Numbers of inseminated (+) and uninseminated (—) females in tests of Raposo with Orinocan (OR), Andean-Brazilian (AB) and Centro-American (CA) strains

Females	Males	Homogamic		Heterogamic		Isolation index
		+	—	+	—	
Raposo + OR	Raposo	36	8	14	31	+0.45
Raposo + OR	OR	23	6	4	24	+0.69
Raposo + AB	Raposo	18	9	6	21	+0.50
Raposo + AB	AB	25	2	11	18	+0.42
Raposo + CA	Raposo	61	37	60	36	—0.002
Raposo + CA	CA	53	37	52	35	—0.01

Marco, Brazil, belonged to the Andean-Brazilian incipient species. The fourth has a unique behavior, to be described. It is relevant to note that these strains were received in our laboratory in June 1964, i.e., after the "new" behavior of the Llanos strain was discovered. The tests with various strains of the different incipient species gave the following results:

Crossed to	O	S	F	Crossed to	O	S	F
C. Amer.	..	1	..	Guianan	3	2	..
Amazonian	9	..	..	Transitional	18	9	1†
Orinocan	..	13	1*	Raposo	..	1	..
Andean	9	15	..	Llanos new	..	..	2

* Not confirmed on retest. † One male hatched.

The only strain of *D. paulistorum* with which Marco gives fertile F_1 hybrids is the new Llanos. This has been checked both with the wild-type Llanos and with a rough-eye mutant found in the latter. The backcrosses of the F_1 females to rough gave the expected segregation for rough; 13 rough-eyed backcross males were tested for fertility in individual tests, and 12 were fertile; all 15 of the 15 wild-type males tested were also fertile.

Thus far Marco seems to be a strain found in nature which is identical with the new Llanos arisen in the laboratory. Further tests have however disclosed slight differences. These results may be reviewed in summary form as follows:

A. Marco ♀ × orange (Orinocan) ♂— F_1 males sterile. In the first backcross to orange, 24 wild-type and 25 orange males were group-tested for fertility, and proved sterile, although on dissection some males showed motile sperm. In the second and third backcrosses of wild-type females to orange males, several dozen wild-type and orange males were group-tested; some cultures were slightly fertile and others completely sterile.

B. Orange (Orinocan) ♀ × Marco ♂—results parallel to A, except that in the third backcross all of the test cultures of the hybrid males were at least somewhat fertile.

C. Marco ♀ × dark eye (Andean-Brazilian) ♂— F_1 males sterile. The first backcross to dark eye gave sterile sons (12 wild-type and 10 dark-eyes tested, one culture produced a single pupa). The second backcross gave also sterile sons (18 wild-type and 24 dark-eyes group tested). The third backcross still gave sterile wild-type (33 tested) but some fertile dark-eye males (14 tested).

D. Dark eye (Andean-Brazilian) ♀ × Marco ♂— F_1 males sterile. The first backcross gave sterile sons (15 tested). In the second backcross the wild-type males were sterile (47 tested), but some of the dark-eyed males were slightly fertile (31 tested). In the third and fourth backcrosses the wild-type males were sterile (more than 100 group-tested).

E. Marco ♀ × ebony (Centro-American) ♂— F_1 males sterile.

F. Ebony (Centro-American) ♀ × Marco ♂— F_1 males sterile. The first backcross gave completely sterile wild-type and ebony males with highly abnormal spermatogenesis, showing nothing like normal spermatozoa (19 group-tested).

Sexual behavior of Marco flies: The sexual preferences of the Marco strain were tested by the same "male-choice" technique as used for the new Llanos and for Raposo. The results are summarized in Table 3. Marco and the new Llanos intercross as freely as do females and males of the same strain. Since the new Llanos intercrosses freely also with the orange mutant strain of the Orinocan incipient species (Table 2 in DOBZHANSKY and PAVLOVSKY 1966), it was to be

TABLE 3

Numbers of inseminated (+) and uninseminated (—) females in tests of Marco with the new Llanos (L), Orinocan (OR), Andean-Brazilian (AB), and Centro-American (CA) strains

Females	Males	Homogamic		Heterogamic		Isolation index
		+	—	+	—	
Marco + L	Marco	27	32	35	29	—0.09
Marco + L	L	51	28	39	39	+0.13
Marco + OR	Marco	25	5	1	26	+0.91
Marco + OR	OR	26	2	5	23	+0.68
Marco + AB	Marco	23	6	6	21	+0.56
Marco + AB	AB	27	1	0	27	+1.00
Marco + CA	Marco	28	14	12	31	+0.41
Marco + CA	CA	33	16	5	40	+0.72

expected that Marco will do the same with orange. Table 3 shows that the expectation is not fulfilled at all—there is actually a very pronounced isolation between Marco and orange. Marco gives a pronounced isolation also with the Andean-Brazilian incipient species, and a rather less pronounced one with the Centro-American one.

The Santa Marta strain: This strain is descended from a single female collected in 1956 by Drs. H. L. CARSON and M. WASSERMAN (DOBZHANSKY and SPASSKY 1959). It is a representative of the Transitional race, which produces hybrids both with some Centro-American and Transitional race strains. It gives, however, a sterility of a special kind with Mesitas and Palmira strains of the Transitional race (EHRMAN 1963; WILLIAMSON and EHRMAN 1967). The results of crosses of Santa Marta to a variety of strains can be summarized as follows:

Crossed to	O	S	F	Crossed to	O	S	F
C. Amer.	..	..	14	Amazonian	12	4	..
Transitional	..	4	4	Guianan	1	1	..
Orinocan	3	12	..	Andean	..	35	..
Raposo	..	2	1	Marco	..	2	..

More detailed studies have been made of the crosses with Centro-American, Raposo, Orinocan, and Andean strains. The results are as follows:

A. Santa Marta ♀ × ebony (Centro-American) ♂— F_1 males fertile. F_1 females testcrossed to ebony gave an 80 wild-type: 63 ebony segregation. Of the 19 wild-type males tested individually, one was sterile, two died prematurely and 16 were fertile; of the 10 ebony males all were fertile.

B. Ebony (Centro-American) ♀ × Santa Marta ♂—results as in A. Of the 20 wild-type backcross hybrid males, 18 were fertile, and of the 10 ebony males 7 were fertile and 3 died before the tests were completed.

C. Santa Marta ♀ × veinlet (Transitional from Bucaramanga, Colombia) ♂— F_1 males sterile, testcross males sterile on mass tests.

D. Veinlet ♀ × Santa Marta ♂—No progeny.

E. Santa Marta ♀ × plexus-like (sex-linked recessive in a Raposo strain) ♂— F_1 males sterile. Backcross of F_1 females to plexus-like gave 59 wild-type and 40 plexus-like flies. Of the 10 wild-type backcross males tested individually only one was fertile; of the 10 plexus-like males

7 were fertile and 3 sterile. The backcross to Santa Marta gave 3 sterile and 1 fertile plexus-like and 4 fertile and 3 sterile wild-type males.

F. Plexus-like (Raposo) ♀ × Santa Marta ♂— F_1 males fertile in mass test. F_1 females were testcrossed to plexus-like and to wild-type Santa Marta males. From the first of these testcrosses 10 plexus-like males were tried out for fertility, 5 were fertile, 2 sterile, and 3 died; of the 10 wild-type males 5 were sterile, 2 fertile, and 3 died. From the second testcross 9 wild-type males were tested, 4 were fertile, 2 sterile, and 3 dead.

G. Santa Marta ♀ × dark-eye (Andean-Brazilian) ♂— F_1 males sterile. From the first backcross to dark-eye 23 wild-type and 10 dark-eye males were tested in mass tests, and proved sterile. From the second backcross 35 wild-type and 20 dark-eye males were mass-tested, and proved likewise sterile. In the third backcross wild-type males were sterile and dark-eye males were fertile in mass tests.

H. Dark eye (Andean-Brazilian) ♀ × Santa Marta ♂—Results entirely analogous to those in G.

I. Santa Marta ♀ × orange eye (Orinocan) ♂— F_1 males sterile. The first backcross to orange gave sterile wild-type (11) and fertile orange (4) males in mass tests. The second backcross to orange gave in mass tests both wild-type (19) and orange (18) males fertile.

J. Orange (Orinocan) ♀ × Santa Marta ♂—Results entirely analogous to those in I.

Sexual behavior of Santa Marta flies: CARMODY *et al.* (1962) tested the Santa Marta strain with a variety of others, and found a remarkable variety of results. Santa Marta shows no isolation from Bucaramanga, but a pronounced one from Palmira (Transitional race strains). With the Centro-American strains (Honduras, Costa Rica) the isolation is weak but statistically significant. The behavior with Andean-Brazilian strains is most erratic: no isolation with Tingo Maria when the Santa Marta strain is used as male, but a strong isolation with Tingo Maria males; complete isolation from Angra (males of the Angra, Brazil, strain), moderate isolation from Santo Domingo (Equador) and Santa Cruz (Bolivia) strains, when these latter are used as males.

The results of new tests are summarized in Table 4. There is a slight, but significant, preference for homogamic matings both with Raposo (the plexus-like mutant), and with Centro-American flies (ebony mutant, from Honduras). This shows once more the independence of the hybrid sterility and the sexual behavior as isolating mechanisms; indeed, as shown above, Santa Marta gives fertile hybrids in both reciprocal matings with Centro-American, but sterile males with Raposo when the latter are used as fathers. With the Orinocan incipient species (orange mutant from Georgetown, Guiana), a strong sexual isolation is obtained.

TABLE 4

Numbers of inseminated (+) and uninseminated (—) females in tests of the Santa Marta (SM) strain with Raposo (R), Centro-American (CA) and Orinocan (OR) strains

Females	Males	Homogamic		Heterogamic		Isolation index
		+	—	+	—	
SM + R	SM	27	32	14	42	+0.29
SM + R	R	31	20	22	29	+0.17
SM + CA	SM	20	11	15	14	+0.11
SM + CA	CA	20	18	20	21	+0.04
SM + OR	SM	33	11	11	30	+0.47
SM + OR	OR	23	6	4	26	+0.71

DISCUSSION

The genetic status of the superspecies *Drosophila paulistorum* is as complex as it is interesting. The Centro-American, Orinocan, Amazonian, Andean-Brazilian, and Guianan groups of populations have diverged genetically, and have evolved reproductive isolations, safe enough for them to coexist sympatrically without gene exchange. The Guianan is the most distinctive, and seems to deserve being regarded a species of its own (KASTRITSIS and DOBZHANSKY 1966). The status of the others is interesting precisely because they may be considered conspecific or not conspecific with equal propriety. The female hybrids are fertile, thus keeping open at least a potentiality of gene exchange. Another such potentiality seems to be available because of the existence of the Transitional race and transitional strains, although these races and strains occur geographically mostly or only where a single incipient species is living (MALOGOLOWKIN 1963; MALOGOLOWKIN, SIMMONS and LEVENE 1965; EHRMAN 1965).

The nature and the origin of the genetic differences between the incipient species need elucidation. The emergence in the laboratory of the "new Llanos," which gives sterile F_1 male hybrids with strains of the Orinocan incipient species, with which it was fertile a few years earlier, has opened up an hitherto unexpected approach to the problem. The situation shows suggestive similarities to the hybrid sterility induced by "infection" in other strains of *D. paulistorum* (WILLIAMSON and EHRMAN 1967). A plausible hypothesis is that the new Llanos differs from its ancestor by carrying some kind of symbiont or cytoplasmic particle, which permits fertility with the Llanos chromosomal genotype, but causes sterility with other genotypes. This conjecture is favored by the demonstration by WILLIAMSON and EHRMAN that the new Llanos can serve as an inducer of an "infectious" sterility in other strains, while the injection of the new Llanos material is lethal to still other strains. Whether the transformation of the original Llanos strain into the new Llanos involved an infection from the outside, or the original carried a symbiont which became prevalent under laboratory conditions, is an open question.

The emergence of the new Llanos is enhanced in interest because its analogues appear to be found in nature. The Raposo population behaves genetically with respect to the Centro-American incipient species as the new Llanos does to its Orinocan progenitor. The similarity is, to be sure, incomplete. Almost nothing is known about the ability of Raposo to serve as a source of infectious sterility; its behavior in crosses to Orinocan and Andean-Brazilian forms is quite different from that of the new Llanos. But the similarity is more suggestive: Raposo shows no sexual isolation from the Centro-American, just as the new Llanos continues to be Orinocan in its sexual behavior. The emergence of the hybrid sterility did not automatically lead to change in sexual identity. This favors the hypothesis suggested by EHRMAN (1962b and earlier), that the hybrid sterility between the incipient species of *D. paulistorum* is the primary isolating mechanism, while the ethological isolation has developed later, by natural selection in response to the challenge of hybridization.

Another analogue of the new Llanos is the Marco strain. It is the only "natural"

strain that gives fertile hybrids with the new Llanos, and shows no trace of sexual isolation from the latter. Otherwise the two strains are by no means identical. Marco is strongly isolated ethologically from the Orinocan incipient species, while the new Llanos is not so isolated. If Marco arose in nature in a way analogous to the origin of the new Llanos in the laboratory, it had its ethological isolation either present before, or it was superimposed since its origin. Another difference between Marco and the new Llanos concerns the males in the backcross progenies with the Orinocan; these males are mostly sterile when Marco is one of the parents, and fertile when the new Llanos is the parent. The sterilities of the F_1 and of the backcross hybrid males in *D. paulistorum* have been shown by EHRMAN (1962b) to be different in causation, and the data in the present report contain more examples of this independence. Nevertheless, preliminary cytological observations (unpublished) show rather similar disturbances in the spermatogenesis of the F_1 and the backcross hybrids.

The Santa Marta strain is a special case, different from all others. It gives fertile F_1 and backcross hybrid males with Centro-American strains; the F_1 male hybrids which had Santa Marta as mothers, and Raposo or certain Transitional race strains as fathers, are sterile; the reciprocal crosses give fertile F_1 males. Except for its sterility with Raposo, Santa Marta behaves more like a Centro-American than like a Transitional race. With the latter it gives a peculiar hybrid sterility described by EHRMAN (1962a), and the "infectious" sterility discussed by Williamson and Ehrman in their paper in the present issue. The origins of Santa Marta cannot be deciphered from the evidence now available; perhaps more material from northwestern South America could clarify it.

The findings reported in the present article, and in the accompanying one by WILLIAMSON and EHRMAN (1967), suggest a hitherto scarcely suspected method of initiation of species formation. This is the establishment of a symbiotic relationship between the fly and some associated microorganism. This latter becomes integrated with and transmitted regularly through the female sex cells of the host species. The symbiont need not, however, be integrated with the genes of other populations of the host; the hybrids thus become sterile. Natural selection then promotes the formation of an ethological isolation between the populations carrying different symbionts. Whether this is a very special situation confined only to the superspecies *Drosophila paulistorum*, or it is distributed more widely, is for future investigations to discover.

DRS. K. G. McWHIRTER and VALERIO SCALI, working in PROFESSOR E. B. FORD's laboratory at Oxford University, England, have observed perhaps similar phenomena in geographic populations of the butterfly, *Maniola jurtina*. They very kindly permit us to quote their as yet unpublished data.

"This species feeds on grass which, in the wild, is of course infected, as has been fully demonstrated, with many forms of bacteria both Gram positive and Gram negative. In the Isles of Scilly, the mid- and hind-gut of the larvae at all stages contain Gram positive bacteria only, and a very limited range of them. The fore-gut contains Gram negative also immediately after feeding, but this type is eliminated within, say, 15 minutes or so.

Fifth instar larvae from most sites on the English mainland contain in the mid-

and hind-gut Gram negative bacteria only (and a limited range of them). In a few sites from the English mainland, the gut contains both Gram positive and Gram negative types. The distinction between the Scilly and mainland types persists when the larvae are reared on the same grass in the laboratory. It is clear therefore that some antibacterial agent, genetically determined, is secreted by the Scillonian larvae, and one of a different type is secreted by the larvae in most populations from the English mainland. McWHIRTER and SCALI find that the gut of the larvae becomes sterile as a result of each ecdysis, to be reinfected as soon as feeding starts."

Thanks are due to MR. SPASSKY, who has established the strains of *Drosophila paulistorum* kept in our laboratory, and made some of the early crosses. DR. S. B. PIPKIN very kindly sent us the Willistoni-group flies which she collected at Raposo, Colombia, at Marco, Brazil, as well as many collections from Panama. The Santa Marta strain came from the collection of Drs. H. L. CARSON and M. WASSERMAN. At all stages of the work we had the privilege of consultation with DR. L. EHRLMAN, whose work, in cooperation with DR. D. WILLIAMSON, has thrown new light on our findings.

SUMMARY

The origin in the laboratory of a sterility of male hybrids between strains which were previously fertile is described. The strain involved is the "new Llanos," descended from a progenitor collected in the Llanos of Colombia. This strain was giving fertile hybrids with the Orinocan incipient species of *Drosophila paulistorum*, but is now producing sterile male hybrids. In evolving the sterility, the new Llanos has not become sexually isolated from its progenitor. New Llanos males continue to be accepted by Orinocan females, and vice versa.—The population from Raposo, Colombia, shows the same relationship to the Centro-American incipient species as the new Llanos does to the Orinocan one. Alterations of the kind which took place in the Llanos strain in the laboratory occur also in nature. The genetic properties of two other transitional strains, Marco and Santa Marta, are described. The hypothesis is put forward that the process of speciation may be initiated by establishment of new symbiotic relationships between *Drosophila* and a virus or other microorganism.

APPENDIX

by COSTAS D. KASTRITSIS¹

Cytological examination of the Santa Marta, Marco, and Llanos strains of *Drosophila paulistorum* yields results which can be very helpful for the determination of the relationships of these strains with the other strains belonging to the various incipient species. An account of these relationships is given below:

(1) Santa Marta. The strain is considered as belonging to the Transitional "race" on the basis of hybridization tests. If the strain was to be classified on the basis of chromosomal morphology, however, it would have been placed in the Centroamerican rather than the Transitional "race". The Santa Marta strain carries one homozygous inversion in XL, one homozygous inversion in XR, one homozygous inversion in 2L, one homozygous and one heterozygous inversion in 2R, and five heterozygous inversions in the 3rd, all of which are found also in the Centroamerican

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"race", either in monomorphic or in polymorphic condition. Only three of the inversions found in the Santa Marta strain occur in other strains of the Transitional "race" as well. It should be mentioned, however, that Santa Marta shares some of its inversions with the Orinocan and the Andean, but the number of these inversions is not nearly as large as that shared with Centroamerican.

(2) Llanos. The strain was originally placed in the Orinocan "race" on the basis of hybridization tests, but it has changed while in the laboratory, due to the development of some sort of reproductive isolation. Chromosomally, the strain shares one homozygous and two heterozygous inversions with Centroamerican and Transitional, but it is unique in having at least seven heterozygous inversions in the 3rd chromosome, most of which are not found elsewhere, insofar as can be determined. It does carry one homozygous inversion in the 2L chromosome which is found to be monomorphic throughout the Orinocan "race".

(3) Marco. This strain is very closely related to that of Santa Marta, in that they both carry identical XL chromosomes, they share one homozygous inversion in the 2L, one homozygous inversion in the 2R, and two inversions in the 3rd which are heterozygous in Santa Marta but homozygous in Marco. The two strains differ by one inversion in the XR, Marco having the same arrangement as "Standard".

The Marco strain is also related to that from Llanos in that it carries one homozygous inversion in the 2R and one homozygous inversion in the 3rd, both of which are found in the Llanos strain as heterozygous; Marco and Llanos have identical 2L chromosomes. It might be of significance that the homozygous inversion in the 3rd chromosome which the strain from Marco shares with that from Llanos is one of the inversions which was considered to be unique for the latter strain.

The data given above suggest the following conclusions: (1) the Santa Marta strain is very closely related to the Centroamerican "race". (2) the Llanos strain is chromosomally unique but related to the Orinocan "race", and (3) the Marco strain, on the basis of chromosomal morphology, is closely related to the Transitional and Centroamerican "races", and somewhat related to the Llanos strain.

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