INDUCTION OF HYBRID STERILITY IN NONHYBRID MALES OF *DROSOPHILA PAULISTORUM*

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Received September 7, 1966

THREE different kinds of hybrid sterility of males occur within the superspecies *Drosophila paulistorum* Dobzhansky and Pavan, and a fourth one will be described in the present paper. The superspecies consists of at least five races or incipient species (DOBZHANSKY and SPASSKY 1959). The F_1 hybrids between these races are fertile if females and sterile if males. This is a sterility of the genic type, since the degenerative changes begin before meiosis, and the meiotic metaphases show at least some paired chromosomes (DOBZHANSKY, unpublished observations).

The second kind of sterility occurs in males in backcross progenies. This sterility depends upon the genetic constitution not of the males themselves but rather of their mothers. As a rule, all the sons of a female carrying any mixture of the chromosomes of the parental races are sterile, even though some of these sons themselves carry only the chromosomes of a single race (EHRMAN 1960). This sterility operates via a maternal effect, the genes responsible being distributed in all three pairs of chromosomes which *D. paulistorum* possesses. The sterilities of the F_1 hybrid and of the backcross males are due obviously to different causes, since F_1 hybrids are sons of pure rather than of hybrid mothers. The different causation is shown also by the fact that the two kinds of sterility are sometimes dissociated; although in most crosses both the F_1 and the backcross males are sterile, in some exceptional cases the F_1 may be sterile and backcrosses fertile, or vice versa (EHRMAN 1962a).

The third kind of sterility is known thus far only in hybrids between strains of the Transitional race, the ancestors of which were collected at Santa Marta and at Mesitas, Colombia, respectively. The male progeny from the cross Santa Marta female × Mesitas male are sterile, while the reciprocal cross gives fertile progeny of both sexes. When the hybrid females from the Santa Marta female × Mesitas male are backcrossed to Mesitas males, sterile male progenies are obtained in six successive generations (EHRMAN 1963, 1964a). In the seventh backcross to Mesitas, fertile sons are finally obtained. The sterility appears to be due to interactions between the Santa Marta cytoplasm and the Mesitas Y chromosome. Whatever factor is transmitted by the Santa Marta cytoplasm, it is eventually overcome by the Mesitas genome.

The following working hypothesis is worthy of testing: Suppose that the Santa Marta strain carries a substance, or an associated microorganism, a symbiont or

a parasite of some kind, which is regularly transmitted in this strain from generation to generation via the egg cytoplasm. Suppose further that the genome of the Santa Marta strain and the cytoplasmic associate are coadapted, so that the viability and fertility of the flies are not adversely affected by the association. There is, however, no such mutual adaptation between the associate of the Santa Marta strain and the genome of the Mesitas strain, particularly the Y chromosome of the latter. What results is a sterility of the male progeny in the Santa Marta female × Mesitas male cross. This sterility continues for as many generations as it takes to have the cytoplasmic associate of the Santa Marta strain suppressed by the Mesitas genome (EHRMAN 1964b).

This hypothesis was first tested by an attempt to induce the sterility by injection. Can the agent which causes the sterility be transmitted by infection, as well as through the egg cytoplasm? Females of the Mesitas strain were injected with homogenates of Santa Marta flies, or of Santa Marta female × Mesitas male hybrid flies. The injected females were allowed to incubate the "infection" and then crossed to Mesitas males. Their offspring had only Mesitas genes, but the genetically nonhybrid male offspring were nonetheless sterile. Injection of Santa Marta females with similar homogenates leads to no sterility (EHRMAN and WLLIAMSON 1965). This induced, or "infectious" sterility seems to be the fourth kind of sterility found in *D. paulistorum;* in contrast to the sterility in the hybrids, this fourth type is confined to the progeny of the injected mothers, and is not transmitted to further generations.

The purpose of the present communication is to report on additional experiments undertaken to elucidate the mechanics of this infectivity.

MATERIALS AND METHODS

The following strains of *Drosophila paulistorum* were used in the experiments to be described in this paper: (1) Santa Marta, Colombia, Transitional race (DOBZHANSKY and SPASSKY 1959), collected by DRS. H. L. CARSON and M. WASSERMAN in 1956; (2) Mesitas, west of Bogota, Colombia, Transitional race (EHRMAN 1963), collected by DR. A. HUNTER in 1962; (3) Goofy Lake, south of Panama City, Panama, Amazonian race, collected by DR. S. B. PIPKIN in 1962. A strain from Goofy Lake carrying an autosomal recessive eye color mutant "orange" was used; this mutant was isolated by MRS. O. PAVLOVSKY; (4) Llanos, Chichimene, south of Villavicencio, Colombia (see DOBZHANSKY and PAVLOVSKY 1966), collected by TH. DOBZHANSKY in 1958; (5) Raposo, on the Pacific coast of Colombia (DOBZHANSKY and PAVLOVSKY 1966) collected by DR. S. B. PIPKIN, 1963, and (6) an autosomal recessive ebony mutant in the Lancetilla, Honduras, strain of the Centro-American race, (MALOGOLOWKIN and ERHMAN 1960). A nonmutant wild-type strain from the same locality was also used.

As representatives of the other *D. paulistorum* races we selected: (7) Caripe, Venezuela of the Orinocan incipient species; (8) Angra, Brazil, Andean-South Brazilian; (9) Belem, Brazil, Amazonian, and finally (10) Apoteri, Guianan. (For more details about these strains see DOBZHANSKY and SPASSKY 1959 and DOBZHANSKY *et al.* 1964).

For injection experiments, flies were routinely homogenized in cold 0.24 M sucrose, buffered with 0.05 M Tris-HCl, pH 7.4, at the rate of 0.01 ml per fly. The homogenate was centrifuged at 3,000 rpm for 15 minutes at 0°C and the supernatant pipetted into a sterile tube and kept in an ice bath. Virgin females were injected between the third and fourth abdominal sternites. The amount of supernatant injected into a fly was controlled by using the extension of the fly's proboscis as a criterion; this occurs when approximately 0.2–0.3 μl of the liquid is injected.

Slight modifications of this procedure were used to test different tissues; these will be discussed in the text.

The injected flies were placed in paper cones in yeasted food vials, held until it was certain they had survived the injection, then sent to one of us (L. E.) for further handling. Following their arrival the injected females were held for five days, placed in a culture bottle with their own strain of males, and transferred every two days until the end of oviposition. As the F_1 progeny emerged, the males were tested for fertility in mass cultures. The testes of some males were removed in Waddington's physiological insect saline and examined for the presence of motile spermatozoa and testicular development.

RESULTS

Experiments with Santa Marta (S) and Mesitas (M) strains: Sex of the donor of the sterility factor: The first experiments (preliminary report in EHRMAN and WILLIAMSON 1965) were made with homogenates prepared from F_1 hybrid flies from the cross S female × M male (both sexes of the hybrids ground together), and from nonhybrid males and females of the S strain. The former homogenates included sterile males and the latter, fertile ones. The results shown in lines 1 and 2 of Table 1 were the same as before. The homogenates, when injected into M females caused the latter eventually to produce sterile sons.

An extension of these experiments using precisely the same techniques (see (EHRMAN and WILLIAMSON 1965), indicates that the injections of homogenates

TABLE 1

Injection experiments and fertility tests of male progenies

No.	Donor	Recipient	Crossed to	Male progeny
Whole flies				
1	S♀,♂	M♀	M♂	Sterile
2	F_1(S♀ × M♂)♀,♂	M♀	M♂	Sterile
3	S♀	(41)M♀	M♂	(400)Fertile
4	S♂	(26)M♀	M♀	(150)Sterile
5	F_1(S♀ × M♂)♀	(19)M♀	M♂	(200)Fertile
6	F_1(S♀ × M♂)♂	(20)M♀	M♂	(150)Sterile
Hemolymph				
7	S♀	(32)M♀	M♂	(400)Fertile
8	S♂	(15)M♀	M♂	(100)Fertile
9	F_1(S♀ × M♂)♀	(15)M♀	M♂	(100)Fertile
10	F_1(S♀ × M♂)	(23)M♀	M♂	(650)Fertile
Testes and paragonia				
11	F_1(S♀ × M♂)	(67)M♀	M♂	(1000)Sterile
Paragonia alone				
12	F_1(S♀ × M♂)	(37)M♀	M♂	(100)Sterile
Whole flies				
13	[M♀← F_1(S♀ × M♂)]F_1♀,♂	(27)M♀	M♂	(300)Fertile
14	[M♀← F_1(S♀ × M♂)]F_1♀♂	(27)M♂	M♀	(300)Fertile
15	[M♀← F_1(S♀ × M♂)]F_1♂	(33)M♀	M♂	(300)Fertile
Testes and paragonia				
16	M♀← F_1(S♀ × M♂) F_1	(17)M♀	M♂	(550)Fertile

M = Mesitas; S = Santa Marta; ← injected into; approximate numbers of the flies injected or tested for fertility are shown in parentheses.

prepared from females alone do not result in the production of sterile male progenies one generation later (lines 3, 5). Extracts prepared from males and females together or from males alone do give sterile sons when injected into Mesitas females (lines 4, 6). The use of males as recipients always gives negative results (WILLIAMSON and EHRMAN 1966).

Drosophila paulistorum females are, thus, not sterilized themselves by this infection; rather, they serve as "carriers," transmitting it to their progeny. The sterility-inducing factor which must be present in tissues of the females's body is not infectious when extracted from it. The males, on the other hand, present the opposite situation. Males are sterilized naturally, by crosses, or artificially, if their mothers are injected. Homogenates prepared from the bodies of males give positive results.

Tissue of the donor of the sterility factor: Additional information regarding the tissue in which this artificially transferable factor resides was obtained from injection experiments in which either the hemolymph alone, or the reproductive organs alone (testes and the paragonia) were employed as possible sources of the factor.

The procedure followed for the hemolymph extractions and injections was that described by WILLIAMSON (1965, 1966). In the present report, a pool of hemolymph was collected from pure Santa Marta females and injected into pure Mesitas females. The injected females were kept alone in food vials for ten days, and then mated *en masse* to Mesitas males. The results of these injections are given in Table 1, line 7. There were fertile males in all the broods of the progeny.

In addition to the pooled hemolymph injections, individual Mesitas females were also injected with the hemolymph from a single fly, with that of pure Santa Marta males and F_1 hybrid males and females from the cross Santa Marta females × Mesitas male. These females were handled in like manner as those injected with the pooled hemolymph. The data for the latter three sets are given in lines 8, 9 and 10 of Table 1. Here, too, there were fertile sons in all the broods. It is clear, then, that the factor responsible for the production of sterile sons by Mesitas females injected with an extract prepared from whole F_1 males (S × M) does not exist, at least not in an infective form, or not in sufficient quantity, in the hemolymph.

For the experiments employing testes and paragonia, these structures were dissected in cold 0.24 M sucrose, Tris buffered at pH 7.4, cleaned as far as possible of fat bodies and other extraneous tissue, and transferred to a small glass homogenizer containing 0.1 ml of the sucrose solution. From this point the technique followed was that described previously (EHRMAN and WILLIAMSON 1965). Mesitas females were injected and mated two days later with Mesitas males, kept at room temperature, and transferred to fresh medium every two days for as long as they yielded progeny. Twelve days after the injections were performed, these Mesitas females began producing only sterile sons, and continued to do so in their ensuing broods. This experiment has been repeated using a newly prepared extract with identical results (line 11, Table 1). Line 12 of the table indicates that paragonia (the paired accessory glands) extracts alone produce the same result

(these same extracts cause the death of *D. melanogaster* females). Some of the spermatocytes of the sterile sons produced by mothers that previously received paragonial injections, are grossly abnormal. Many are polynuclear, all oversized as if swollen, and yet a small quantity of motile "sperm" is produced (see Figure 1). This is evidently nonfunctional. The sterility is due to a disturbance of spermatogenesis apparently resembling that in the male hybrid between incipient species of *D. paulistorum* (DOBZHANSKY, unpublished data).

Electron microscopic studies of the paragonia reveal the presence of a unique, large filamentous RNA virus which is extruded in great quantities into the seminal fluid (DR. B. TANDLER, Sloan-Kettering Institute for Cancer Research, personal communication; these studies will form the basis of a separate report).

DR. JOHN B. NELSON, Rockefeller University, kindly examined this *Drosophila paulistorum* material for possible symbiotic bacteria with negative results; he utilized Feulgen, Giemsa and Wayson stains. One of us (D. L. W.) looked for spirochetal infection, again with negative results. DR. JOHN P. KRAMER, Cornell University, looked for microsporidial infections and discovered that some strains are indeed infected. This endoparasite, however, did not prove to be involved in the hybrid sterility described here (but see STALKER and CARSON 1963 and EHRMAN 1964b).

Lack of "serial" transfer of the sterility factor: An interesting aspect of these experiments concerns the failure of the injected Mesitas females to transmit to their daughters the ability to produce sterile grandsons. When the female siblings of sterile males, in the progenies of injected Mesitas females, are mated with

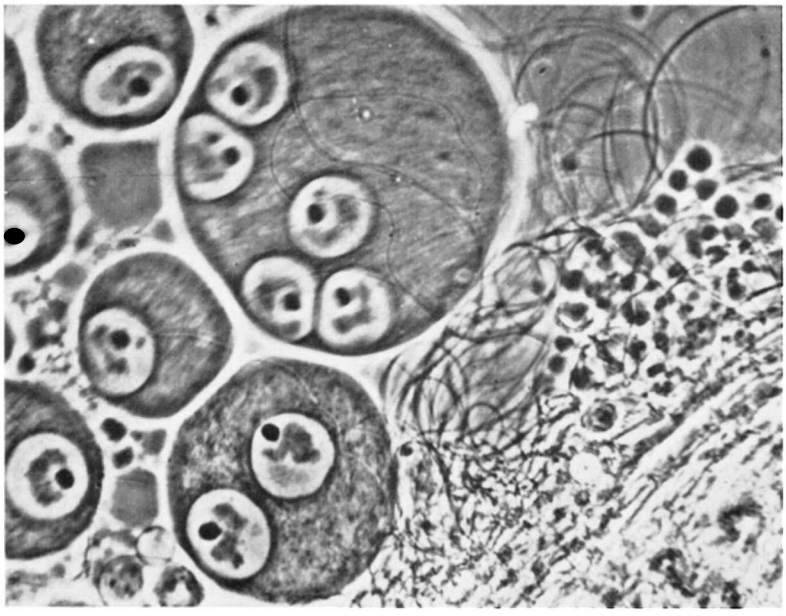

FIGURE 1.—Abnormal spermatocytes and some non-functional but motile "sperm" from a *Drosophila paulistorum* male sterilized by the injection of its mother with homogenates of paragonia (phase microscopy, live material).

Mesitas males, their sons are always fertile (lines 13, 14, 15 of Table 1). The same result is obtained when sterile sons of the injected Mesitas females are used as a possible source of the sterility factor, the prepared homogenate being injected into Mesitas females. Such injected females produce only fertile sons. So, although capable of inducing a Mesitas female to produce sterile sons, the factor does not establish itself in its host to be transmitted genetically (maternally) for more than a single generation. This situation is analogous to that found in the g^- mutant of the CO_2 sensitivity virus, sigma (OHANESSIAN-GUILLEMAN 1959). This mutant of the sigma virus is unable to invade the germinal line of the female into which it is injected, although it can bring about the symptoms of that trait, namely, paralysis and death upon exposure to CO_2; but it is transmitted in typical maternal inheritance when it is present in its stabilized or integrated form (OHANESSIAN-GUILLEMAN 1959, 1963). (We tested four strains of *D. paulistorum* for CO_2 sensitivity (sensitive/resistant): Santa Marta = 0/262; Mesitas = 0/182; Goofy Lake = 0/495; Llanos = 0/169. At the same time a strain (collected at Plattsmouth, Nebraska) of *D. melanogaster* = 150/0.)

To determine whether the infective agent could be recovered from the females into which it had been injected and which had acquired the capacity to produce sterile sons, Mesitas females injected with the extract prepared from the testes plus paragonia described above were homogenized 45 days later, and the extract injected into Mesitas females (line 16, Table 1). The results were again negative. There seems to be no "serial transfer" of the sterility factor.

Thus, hybrid sterility in *D. paulistorum* can only be induced in the sons of injected females, not in subsequent generations.

Incubation time required by the sterility factor: An approximate five-day incubation period must intervene between the injection of the recipient females and the subsequent production of sterile progeny (see EHRMAN and WILLIAMSON 1965, for a discussion of five- and ten-day incubation periods). *D. paulistorum* has approximately a 12-day life cycle from egg to adult at 25°C. However, if the recipient females are crossed to males of their own strain immediately after injection, the production of sterile male progeny begins gradually. Table 2 illustrates this point. Mesitas females were injected with homogenates of testes and paragonia of sterile F_1 males from the cross S female × M male. They were mated immediately after injection (March 4, 1966) to males of their own strain. The percentage of sterile abnormal males in each brood, given in the right-hand column are minimal estimates. Many of the "apparently normal" males are only ostensibly so to the eye of the dissector (L. E., as kindly checked by Professor TH. DOBZHANSKY).

These results should be compared with those of WILLIAMSON (1965) on the infectious "sex ratio" trait in *D. melanogaster*. This maternally inherited, infectious condition has been correlated with the presence of spirochetes in the hemolymph of such "sex ratio" females (POULSON and SAKAGUCHI 1961). In studying daily brood isolations of newly injected and established "sex ratio" lines, WILLIAMSON noted a failure to transmit the infection to the early broods. This was followed by an increase, and finally by a decrease, in the transmission to

TABLE 2

Examination of the testes of sons of Mesitas females injected with homogenates of testes and paragonia of sterile F_1 males from the cross Santa Marta female × Mesitas male

Brood No. and date	Number examined	Grossly abnormal	Percent abnormal
1 (5-III)	47	3	6.8
2 (6-III)	150	10	6.2
3 (9-III)	159	34	20.0
4 (12-III)	313	72	22.8
5 (15-III)	277	132	47.6
6 (18-III)	258	111	43.0
7 (21-III)	312	170	54.5
8 (24-III)	202	71	35.1
9 (27-III)	253	109	43.1
10 (8-IV)	12	9	75.0

The mothers were crossed immediately after injection on March 4, 1966, to Mesitas males.

TABLE 3

Injection experiments and fertility tests of male progenies

No.	Donor	Recipient females	Crossed to males	Male progeny
Testes and paragonia				
1	F_1(S♀ × M♂)	(23) G	G	(360) Fertile
Whole flies				
2	F_1(G♀ × L♂)♀,♂	(58) L	L	(200) Fertile
3	F_1(G♀ × L♂)♀,♂	(63) G	G	None
4	F_1(G♀ × L♂)♀,♂	(38) M	M	None
5	L♀,♂	(37) G	G	(50) Sterile
6	L♀,♂	(89) M	M	(400) Sterile
7	L♀,♂	(17) S	S	(150) Sterile
8	L♀,♂	(23) C	C	(190) Fertile
9	L♀,♂	(15) H	H	(40) Fertile
10	L♀,♂	(16) A	A	(100) Fertile
11	L♀,♂	(60) B	B	(100) Sterile
12	L♀,♂	(24) X	X	(180) Sterile
13	F_1(L♀ × G♂)♀	(19) L	L	(60) Fertile
14	F_1(L♀ × G♂)♀	(35) G	G	(210) Fertile
15	F_1(L♀ × G♂)♂	(22) L	L	(120) Fertile
16	F_1(L♀ × G♂)♂	(13) G	G	(120) Fertile (50) Sterile
17	F_1(e♀ × R♂)♀,♂	(17) e	e	(200) Fertile
18	F_1(e♀ × R♂)♀,♂	(39) R	R	(350) Fertile
19	F_1(R♀ × e♂)♀,♂	(52) e	e	(350) Fertile
20	F_1(R♀ × e♂)♀,♂	(31) R	R	(325) Fertile

M=Mesitas; S=Santa Marta; L=Llanos; G=Goofy Lake; R=Raposo; *e*=ebony mutant of Centro-American race; C=Caripe; H=Lancetilla, Honduras; A=Angra; B=Belem; X=Apoteri. Approximate numbers of the flies injected or tested for fertility are shown in parentheses.

subsequent broods. Note that in Table 2 the percentage of abnormal and sterile males goes steadily up from the first through the fifth broods.

Experiments with the new Llanos strain: Llanos (Colombia), a new incipient species of the *D. paulistorum* complex, has recently been described by DOBZHANSKY and PAVLOVSKY (1966, 1967); it arose in the laboratory at The Rockefeller University sometime between 1958 and 1963 from the Orinocan race (DOBZHANSKY, EHRMAN, PAVLOVSKY, and SPASSKY 1964). This happened with the retention of several unique inversions not found in any other strain, and rules out the possibility of contamination. It was suspected then that its cytoplasm had been somehow altered under laboratory conditions and we proceeded to use it as a source of material for injection into other strains. Table 3 indicates its effect. First, the deleterious effects on recipient females of injections containing Llanos material should be pointed out: It was only by repeated attempts (i.e., repeated injections into groups of virgin females by D. L. W.) and diligent care of these recipients until they produced offspring that the experiments reported in Table 3 could be completed. The nearly lethal effects of the homogenates of Llanos were especially pronounced when hybrid material involving Llanos was injected (lines 3, 4) and when the Belem (Amazonian incipient species) strain females were the recipients. The Santa Marta-Mesitas injection series recorded in Table 1 was free of this lethal effect on recipient females, while the Llanos series showed it clearly. We may hypothesize that either the infectious material may be present in a greater concentration or it may be qualitatively different in this new incipient species, the Llanos strain. Going one step further, it could even be a causative factor bringing about the incipient speciation itself.

Further evidence, summarized in Table 3 is as follows: Homogenates of pure nonhybrid Llanos material are infectious when injected into the Amazonian incipient species (lines 5 and 11); into the Transitional race (lines 6 and 7); and into the Guianan species, line 12, (see KASTRITSIS and DOBZHANSKY 1966). However, neither the Centro-American (line 9) nor the Andean-South Brazilian incipient species (line 10) are adversely affected by injection of this material. Neither is a strain from the Orinocan incipient species (line 8), the one most closely related to Llanos. The Santa Marta-Mesitas strains do not seem to be infectious when injected into other strains of *D. paulistorum* (line 1) as Llanos is.

Injections of hybrid Llanos homogenates, i.e., of the hybrid with females of the Goofy Lake (Amazonian incipient species) strain is lethal to the recipient Goofy Lake (line 3) or Mesitas (line 4) females. This homogenate does not affect Llanos itself (line 2), nor the reciprocal hybrid (lines 13 and 15). Goofy Lake females have, however, survived injection of homogenates of F_1 hybrid females and of F_1 hybrid males of the reciprocal cross, Llanos female $\times$ Goofy Lake male (lines 14 and 16). The injections of homogenates of hybrid females into Goofy Lake females did not result in production of sterile sons; the injections of homogenates of F_1 sterile hybrid males did result in the sterility of some of the male progeny. The authors are aware that the injections of hybrid Llanos material requires further analysis, but we have so far been unable, despite repeated attempts, to obtain more of these hybrids (see DOBZHANSKY and PAVLOVSKY [1966, 1967] on

the sexual isolation between Llanos and the other *D. paulistorum* incipient species).

Finally, Llanos homogenates as well as Santa Marta-Mesitas ones, produce induced sterility for only a single generation—in the sons of injected mothers. No backcross sons in any backcross generation (using as female parents, the fertile sisters of the sterile sons), displayed this induced male sterility.

Experiments with the Rio Raposo strain: A strain of this species collected at Rio Raposo on the Pacific coast of Colombia failed to give fertile F_1 male hybrids with any other strain in our laboratory. Experiments (DOBZHANSKY and PAVLOVSKY 1966, 1967) showed that Rio Raposo is related as closely to the Centro-American race, as the new Llanos is to the Orinocan race. We therefore attempted an artificial transfer by injection, injecting Rio Raposo material into a Centro-American strain from Honduras as the recipient. This latter strain carries an ebony (*e*) autosomal recessive mutant marker (MALOGOLOWKIN and EHRMAN 1960). Our results were entirely negative (lines 17 through 20, Table 3), unlike those in the Llanos series.

These findings and their significance are discussed in the accompanying article by DOBZHANSKY and PAVLOVSKY (1967), together with their findings on sterility of male hybrids between incipient species.

The authors would like to express their appreciation to PROFESSOR TH. DOBZHANSKY, Rockefeller University, and DR. B. TANDLER, Sloan-Kettering Institute for Cancer Research, for their valuable assistance, criticisms and discussions during the course of this study, to DR. JOHN B. NELSON, Rockefeller University, and DR. JOHN P. KRAMER, Cornell University, for relating the results of their studies of these flies for bacteria and microsporidia, respectively; and we thank DR. C. KASTRITSIS, Texas Technological College, for the photograph appearing as Figure 1. The technical assistance of MRS. ARLENE HALYARD is also gratefully acknowledged. This work was supported under National Science Foundation Grant GB-3000 and a Public Health Service Career Development Award, 5K3 HD-9033-02, to L. EHRMAN.

SUMMARY

An induced, or "infectious," hybrid male sterility is described in *Drosophila paulistorum*. Induction is achieved by the injection of homogenates of certain strains into females of certain other strains. The male progenies of the injected females, coming from eggs deposited after an incubation period of several days, are sterile. Homogenates of whole male flies, of the paragonia, and of the male internal reproductive organs induce sterility, while homogenates of whole female flies or injections of the hemolymph do not. The induced sterility is not carried over into generations beyond the immediate male progeny of the injected females. The injection of the same materials into males neither sterilizes the recipients nor their sons.

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