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FERTILIZATION IN DROSOPHILA.
I. EVIDENCE FOR THE REGULAR OCCURRENCE OF MONOSPERMY

Berkeley, California

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Submitted for publication in
Developmental Biology

UCRL-10538

UNIVERSITY OF CALIFORNIA

Lawrence Radiation Laboratory
Berkeley, California

Contract No. W-7405-eng-48

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Philip E. Hildreth and John C. Lucchesi

November 2, 1962

FERTILIZATION IN DROSOPHILA.
I. EVIDENCE FOR THE REGULAR OCCURRENCE OF MONOSPERMY[1]

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University of California, Berkeley, California

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INTRODUCTION

Physiological polyspermy is considered a common phenomenon among insects (Wilson, 1924; Wigglesworth, 1947; Rothschild, 1956; Pikó, 1961). Much of this belief rests on the classical works of Henking, 1892, on some species of Hemiptera, Coleoptera, Hymenoptera, Lepidoptera, and Diptera; of Pantel, 1913, on several species of Diptera; of Nachtsheim, 1913, on Apis (Hymenoptera); of Huettner, 1924, 1927, on Drosophila (Diptera); and of Bataillon and Tchou Su, 1928, on Bombyx (Lepidoptera).

During the course of an unrelated investigation, we had occasion to examine freshly laid eggs of Drosophila melanogaster, a large number of which were fixed in stages prior to syngamy. In these eggs polyspermy was conspicuously absent. This observation agreed with that of Hinton and Lucchesi, 1960, made on similar material, but was at odds with the findings of Huettner, 1924, 1927, and of Sonnenblick, 1950. A more thorough investigation of the occurrence of polyspermy in D. melanogaster was therefore undertaken; a second species, D. virilis, belonging to a different subgenus, was also investigated.

In addition, we attempted a reevaluation of some of the early literature dealing with fertilization in insects; this led us, as may be seen in the discussion, to formulate some reservations concerning the universality of physiological polyspermy in this class.

Throughout this report, "polyspermy" means the presence of more than one sperm in an egg. Where convenient, the terms "dispermy" and "trispermy" are used to indicate one or two supernumerary sperms, respectively.

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[1] This work was carried out under the auspices of the U. S. Atomic Energy Commission and was partially supported by National Institutes of Health grants 2G-367(C1) and GF-14,277.

MATERIALS AND METHODS

Stocks

The frequencies of polyspermy were obtained by cytological investigation of meiotic eggs in two species of *Drosophila*. For the investigations on *D. virilis* only wildtype flies were used, but for the studies on *D. melanogaster*, females from two mutant strains (y^2 *B v* w^a and *dor*) inseminated by wildtype Samarkand males provided the eggs for cytological examination. The y^2 *B v* w^a females were homozygous for X chromosomes carrying the genes for yellow body (y^2), bar-shaped eyes (*B*), vermilion-colored (*v*) and apricot-colored eyes (w^a); the latter two genes in this combination produce light-apricot-colored eyes. The X chromosomes also were homozygous for the inversions sc^{S1}, *In49*, sc^8. This stock was kindly supplied to us by Doctor H. J. Muller of Indiana University. The *dor* females have deep-orange-colored eyes (Merrell, 1947) and exhibit a type of female sterility (Merrell, 1947 and Counce, 1956) in which a genotype may produce lethality or permit survival depending on the origin of the cytoplasm in the fertilized egg. This stock was kindly furnished by Doctor Sheila Counce-Nicklas of Yale University.

Collection and Treatment of Fertilized Eggs

Virgin females 2 or more days old were placed with wildtype males of the same age, and eggs were collected over 5-minute periods. The method used is the one described by Hildreth and Brunt, 1962. The great majority of eggs collected by this method were still in meiotic stages at the time of fixation. Immediately after collection the eggs either were placed on coverslips for whole-mount preparation or were immersed in fixative to be embedded and sectioned later.

For autoradiographs wildtype Samarkand females were placed with wildtype Samarkand males, eggs were cultured on tritiated medium (see below), males obtained from that culture were mated with virgin y^2 *B v* w^a females, and eggs were collected for sectioned material as above.

Whole mounts

Fertilized *D. virilis* eggs and y^2 *B v* w^a and *dor* eggs from the *D. melanogaster* stocks were prepared as whole mounts and stained by the Feulgen procedure of von Borstel and Lindsley. Several eggs were placed on a coverslip, cleared with xylol for about 2 minutes, punctured with a fine glass needle, and placed in fixative which was in some cases 95% ethanol and in others modified Kahle's solution. Another method of fixation, which did not require that the eggs be punctured, was also used. The coverslip with its cleared eggs was placed immediately in Bradley-Carnoy solution composed of 1 part concentrated glacial acetic acid : 3 parts 100% ethanol : 4 parts chloroform. This gave very rapid fixation. The eggs in all cases were left in the fixatives for at least 30 minutes before the Feulgen procedure was started. We obtained the best stained figures when the eggs were placed in 1N HCl at room temperature and then were hydrolyzed for 15 minutes in a 60°C water bath. The eggs remained in the stain from 1 to 2 hours.

Sectioned material

The eggs were first dechorionated in commercial Clorox (sodium hypochlorite 5.25% by weight, inert ingredients 94.75%) then transferred with a needle or brush to vials of Bradley-Carnoy solution. After fixation the eggs were embedded in paraffin, sectioned at 4 μ, affixed serially to slides, and stained with azur-eosin-Giemsa or by the Feulgen method. The azur-eosin-Giemsa is not specific for DNA and stains the sperm tails as well as heads, although differentially. Only $y^2 B\ v\ w^a$ eggs from the *D. melanogaster* stocks were sectioned.

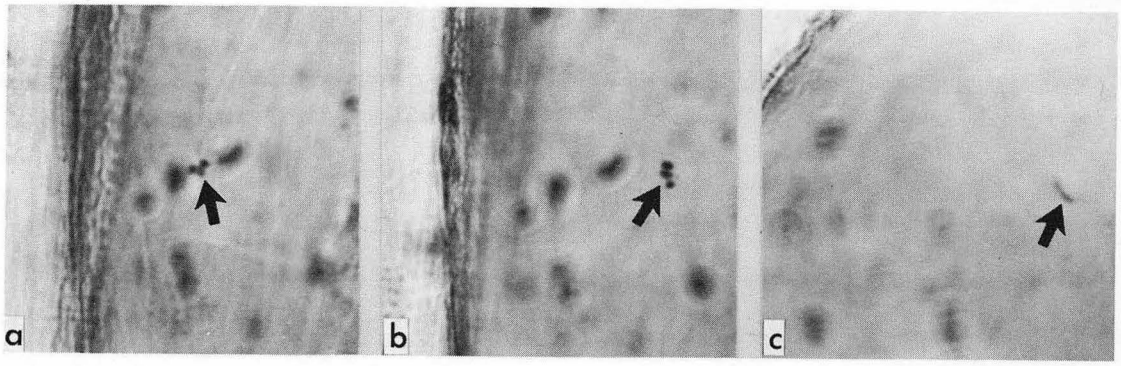
Autoradiographs

Fifty-five freshly laid eggs obtained from wildtype Samarkand females placed with wildtype Samarkand males (see above) were put immediately on culture medium that contained tritiated thymidine. The medium contained 1.2 ml of cornmeal-agar-molasses mixture to which had been added 0.5 ml of modified Eagle's solution, 0.5 ml of 1% agar, and 0.2 ml of tritiated thymidine solution (Schwarz Bioresearch Inc., sp. act. 3.0 Ci/mM) with a total activity of 100 μCi. The entire development of the individuals through the time of pupation occurred on this medium. The male pupae were transferred to and allowed to eclose in vials that contained nonradioactive medium. Thus the males could not have mated until placed with the appropriate females. Nine males were placed with 20 virgin $y^2 B\ v\ w^a$ females in a single vial where they remained for about 2 hours. Eggs were then collected over 5-minute intervals.

The eggs were fixed and sectioned as above and stained by the Feulgen method, and then autoradiographs were prepared by the film-stripping technique. Kodak fine-grain autoradiographic film AR.10 was placed on the slides and exposed for 3 weeks; this exposure period was found to be satisfactory, as evidenced by the fact that each of the stained sperm heads in the egg sections had a cluster of grains over it. Immediately after the eggs had been collected the females were killed and the seminal receptacles with their isotope-bearing sperm were smeared on slides. Autoradiographs were then prepared and exposed for 3 weeks. These sperm smears were examined with a phase-contrast microscope to determine the frequency of radioactive sperm and the average number of grains over each sperm.

RESULTS

The original observations were made on the Feulgen-stained whole mounts of the $y^2 B\ v\ w^a$ eggs. Since in the Feulgen procedure only the DNA is stained, the sperm heads and meiotic or mitotic chromosomes could be observed as reddish-purple objects in colorless cytoplasm (Fig. 1). Three dispermic (2.9%) and 100 monospermic eggs were found among 103 fertilized eggs in meiotic stages. In no instance was a higher degree of polyspermy observed. In each case of dispermy the two sperm were in approximately the same stage of condensation, but one sperm was approaching the female meiotic chromosomes while the second was located near the anterior tip of the egg. One egg was in anaphase I, the second in telophase I, and the third in metaphase II of meiosis. The fact that polyspermy was rare led us to perform



10 microns

ZN-3447

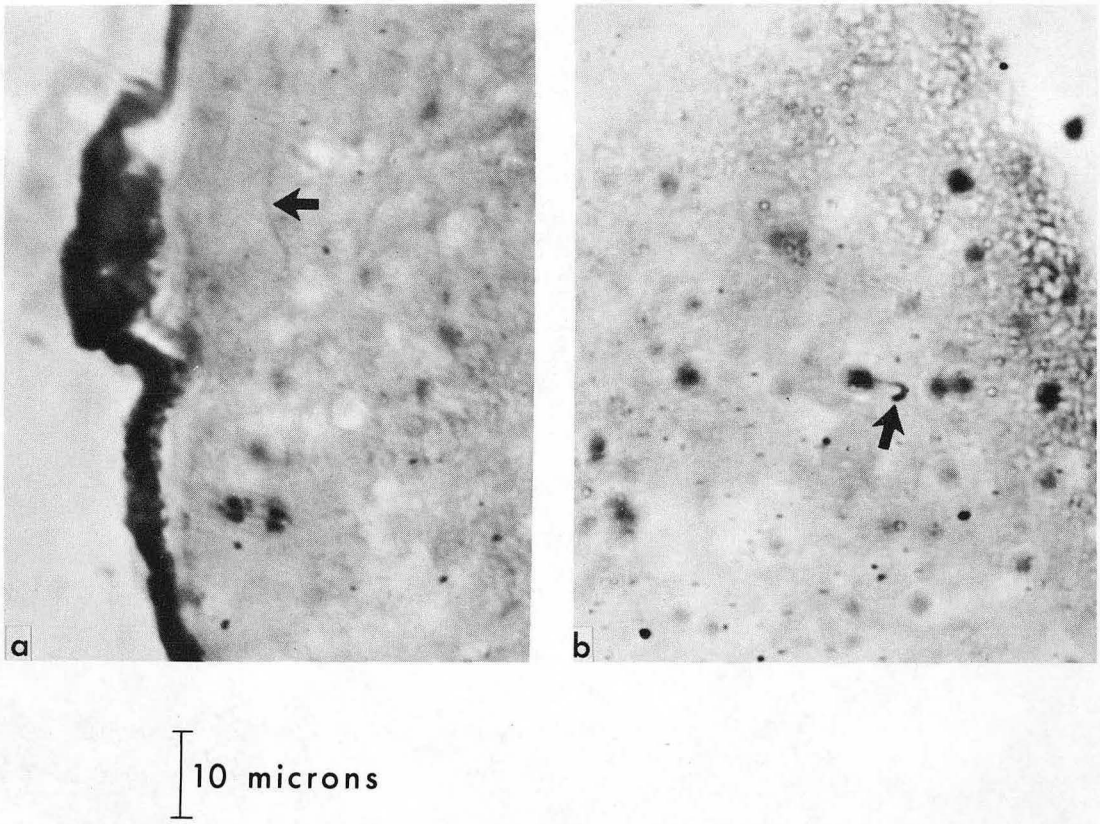
Fig. 1. Feulgen-stained whole mount of D. melanogaster egg in Telophase I: (a) outer figure; (b) inner figure; (c) sperm head.

a similar experiment with another species, D. virilis. In this species 87 fertilized eggs in meiotic stages were examined; every one was found to be monospermic.

Since the earlier reports by other investigators on polyspermy in Drosophila were based on observations of sectioned eggs, we undertook an examination of sectioned $y^2B\ v\ w^a$ eggs some of which were stained with azur-eosin-Giemsa and others with the Feulgen method. A total of 62 fertilized eggs in meiotic stages were examined. The 28 Feulgen-stained eggs were all monospermic and, among the 34 eggs stained with azur-eosin-Giemsa (Fig. 2), 31 were monospermic and 3 were found to be dispermic; thus the frequency of dispermy in the sectioned material (4.8%) was similar to that in the whole-mount material. In each of the three dispermic eggs the two sperm were in about the same stage of condensation. In two of these eggs one sperm was near the meiotic figure and the accessory sperm at the anterior tip of the egg. In the third egg the accessory sperm had migrated past the meiotic figure to the posterior part of the egg. In the azur-eosin-Giemsa sections fragments of sperm tails were readily discernible and could be distinguished from the sperm heads. A composite camera lucida drawing (Fig. 3) was made of the tail fragments from all sections of one egg. Most of the fragments could be connected directly with fragments in adjacent sections to yield one long tail. Because of contraction or breakage of the tail there were some fragments that were too short to connect together, but they obviously were parts of the one tail.

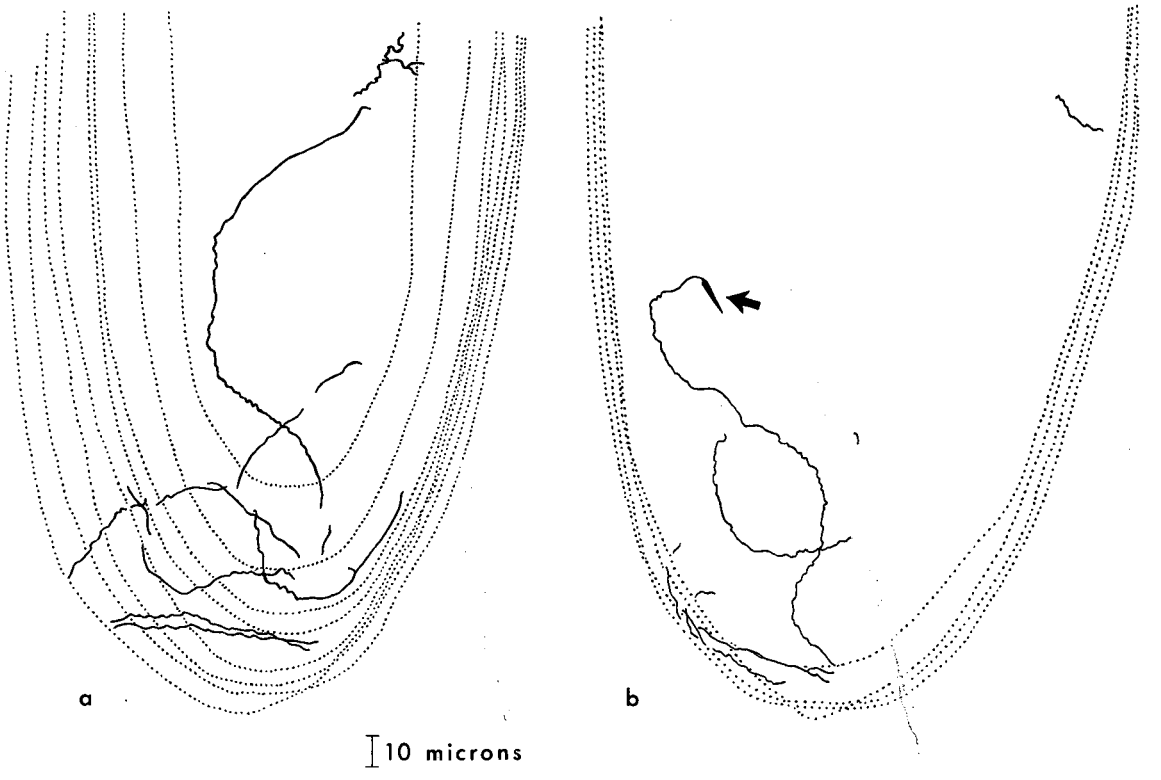
Because of the discrepancies between our results and those of other investigators it was deemed advisable to examine another strain of D. melanogaster. We chose the mutant deep-orange (dor). When homozygous dor females mate with dor/Y males the dor sons exhibit early lethality. When females of the same type mate with wildtype males some of the dor sons undergo a longer embryonic development before death occurs. The longer period of development in the latter case has been attributed to the influence of dor^+ accessory sperm present in the cytoplasm of the egg (Counce, 1956). For this series we again used the Feulgen whole-mount method. Among 42 fertilized eggs in meiotic stages 38 were monospermic and four were dispermic (9.5%). This frequency of dispermy is higher than in the $y^2B\ v\ w^a$ eggs, but the sample is small and a statistical analysis at this time would be worthless. However, once again, never more than two sperm were present.

There still remained, as an explanation for the discrepancy between these results and those of other authors, the possibility that accessory sperm were being chemically degraded so rapidly that we were not able to stain them with our technique although they might have been stainable with Heidenhain's iron hematoxylin. If this degradation occurs, then the Feulgen technique is not good for a study of polyspermy frequencies. We decided to combine the Feulgen technique with tritiated thymidine labeling of the sperm to resolve this problem. The only tritiated thymidine in the eggs examined then would be brought in by the sperm heads, not by the tails, as the isotope would be incorporated into DNA only. Therefore, if every stained sperm were labeled and every labeled sperm were stained there would be little doubt about the usefulness of the Feulgen stain in these studies. The sectioned material was examined and only those slides which had flat and complete sections were stained and film-stripped. After 3 weeks' exposure the slides were developed and examined microscopically. All slides that had more than four sections missing, heavy background or folds, or other film impairments were discarded.



ZN-3448

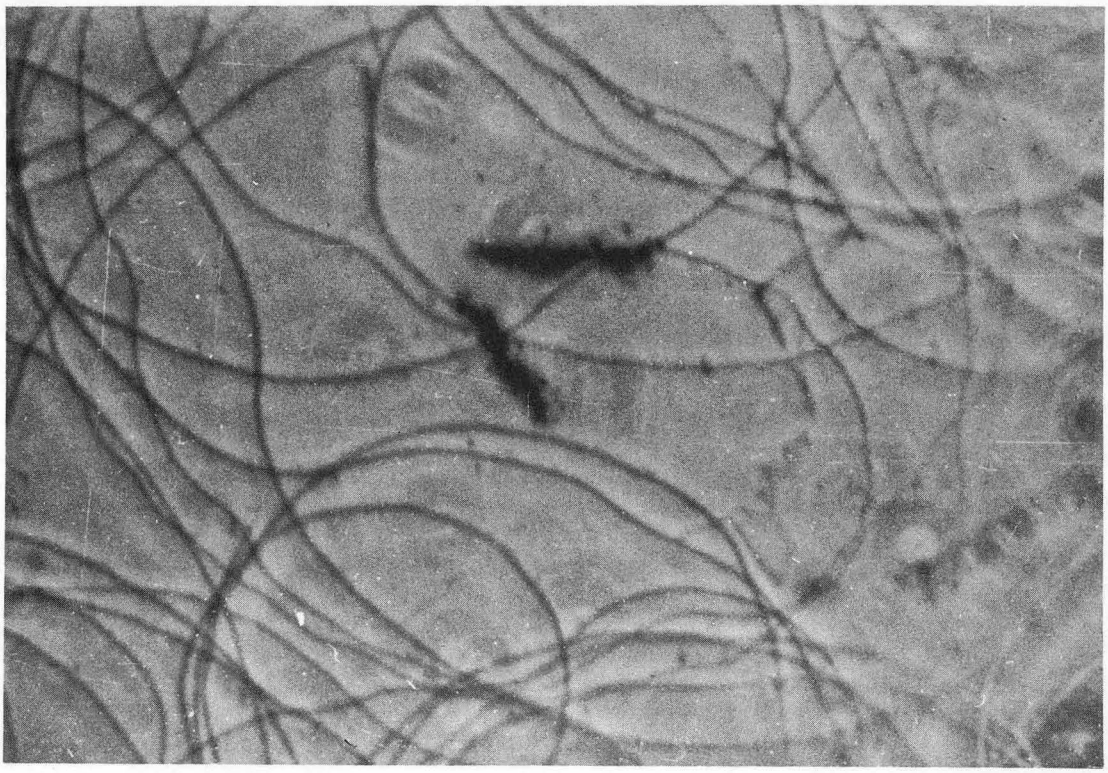
Fig. 2. Sections of D. melanogaster egg stained with azur-eosin-Giemsa: (a) Anaphase I; note the section of sperm tail above the meiotic figure; (b) sperm head.



MUB-1416

Fig. 3. Camera lucida composite of a sperm. For clarity the two halves of the egg are drawn separately: (a) tail fragments were found in every section; (b) only those sections which contained tail fragments are represented. Note the sperm head.

The average number of sections per egg was 26. There were 20 eggs (one per slide) that met our standards. First, we examined the sperm smears from the seminal receptacles of the females; every one of the sperm had a grain cluster over it (Fig. 4), which meant that each had been labeled with the tritiated thymidine. The number of grains per sperm ranged from 26 to 49 with an average of 36.1. We then examined 196 grid units (30 square microns per unit; magnification 1350X) in each of four preselected areas between sections, on every one of the 20 slides, to determine background count and the distribution of clusters which could not be attributed to labeled sperm. These distributions are shown in the second row of Table I. Next, every egg section was examined for the presence of Feulgen-positive sperm. Nineteen eggs had one stained sperm each; and there was only one which contained a second Feulgen-positive sperm. Each of the 21 sperm had a grain cluster associated with it (Fig. 5). The distribution of grains per sperm is shown in the first row of Table I. The number of grains formed for a given exposure time is decreased as the distance between the isotope source and the film is increased. Since in the egg sections there is cytoplasm between the sperm and the film, the distance between these partially reduces the grain formation. Thus the number of grains per sperm in the sectioned material is less than in the sperm smears. Approximately 97% of the background clusters contain seven or fewer grains and about 91% of the stained sperm have clusters of more than seven grains. We then scanned every egg section thoroughly for clusters that contained eight or more grains. We assumed that any unstained sperm would have a distribution of grains similar to that in the stained sperm. If this were correct we might recognize about 91% of the accessory sperm. However, as can be seen in the last row of Table I, there was only one cluster in a Feulgen-negative area that had more than eight grains. The frequency of such clusters is no higher than in the areas between sections, therefore we may conclude that there were no accessory sperm in these eggs. Table II summarizes the frequencies of dispermy in our various series.



10 microns

ZN-3449

Fig. 4. Autoradiograph of sperm from the seminal receptacle of a female.

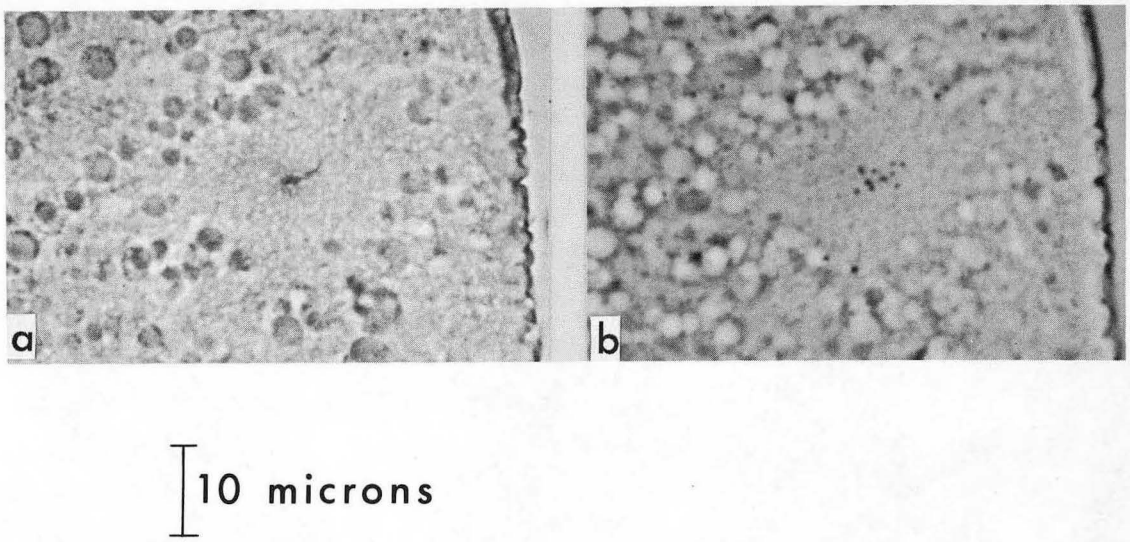
Table I. Frequency of dispermy: stained and labeled material.

Number of grains per grid unit (30 μ^2)	Clusters over Feulgen-positive sperm[a]	Background clusters between sections[b]	Clusters over sections in Feulgen-negative areas[c]
4	-	81	
5	1	26	
6	-	10	
7	1	4	
8	-	-	-
9	2	1	-
10	4	2	-
11	1	-	1
12	2	-	-
13	-	-	-
14	2	-	-
15	1	-	-
16	-	-	-
17	1	-	-
18	-	-	-
19	2	-	-
20	-	-	-
21	1	-	-
22	-	-	-
23	-	-	-
24	-	-	-
25	3	-	-

[a]Twenty eggs examined: one was dispermic.

[b]15,680 grid units examined (780 per slide).

[c]Many more than 100,000 grid units examined.



ZN-3450

Fig. 5. Autoradiograph of egg section showing: (a) the sperm head in its characteristic cytoplasmic island; (b) a slightly higher focus of the same section, at the level of the grain cluster.

Table II. Summary of dispermy frequencies.

Genotype of P females	Number of fertilized meiotic eggs	Number of sperm per egg: 1	2	3 or more	Dispermy (%)	Material*
D. melanogaster						
(1) $\frac{y^2 B v w^a}{y^2 B v w^a}$	103	100	3	0	2.9	WM, F
	28	28	0	0	0.0	S, F
	34	31	3	0	8.8	S, G
[Total]	[165]	[159]	[6]	[0]	[3.6]	
(2) $\frac{y^2 B v w^a}{y^2 B v w^a}$	20	19	1	0	5.0	S, F, H^3-T
(3) $\frac{dor}{dor}$	42	38	4	0	9.5	WM, F
D. virilis						
wildtype	87	87	0	0	0.0	WM, F

*WM = whole mounts; S = sections; F = Feulgen stain; G = azur-eosin-Giemsa stain; H^3-T = tritiated thymidine.

DISCUSSION

In contradiction to the earlier literature, in which polyspermy is stated to occur normally, our findings lead us to believe that polyspermy in Drosophila is not the frequent or normal result of fertilization. Huettner, 1924, examined 300 D. melanogaster eggs and found nine to be momospermic, 291 to have more than one and a few to have more than 30 sperm; in 1927 he stated "One can usually count a dozen or more sperms in the anterior region of most eggs." Sonnenblick, 1950, states "Polyspermy is the general rule in Drosophila, each egg usually being penetrated by more than one spermatozoön, some receiving thirty or more sperms. The latter condition is probably an aberrant one, since about five to eight sperms are typically found in eggs." Since the time of publication of these papers, polyspermy in Drosophila has been considered the general rule. How can the results of these investigations be reconciled with ours? Counce in 1959 reported cases of polyspermy in nine species of Drosophila, with five or six sperm per egg usually being the least in any of the species. Her estimates, and also those of the previously mentioned authors, were based on sectioned eggs stained with iron haematoxylin. When our results were brought to her attention she reexamined her material, checking specifically for sperm heads and concluded that never more than two were present in any egg (personal communication). It is probable that the staining technique is responsible for the differences in the degrees of polyspermy observed by the various investigators. Iron haematoxylin is not a specific stain for DNA, and stains sperm tails as well as other material. Huettner, 1924, states "The tail of sperms stain slightly with iron haematoxylin in the testis of Drosophila. It is interesting, therefore, to note that in the egg they stain as conspicuously as the chromatin or the yolk granules." The sperm length varies considerably among the various species of Drosophila (Yanders and Perras, 1960). The length of D. melanogaster sperm is approximately 1.75 mm (Cooper, 1950, and Yanders and Perras, 1960). Thus the sperm is about three times the length of the egg and much, if not all, of the tail penetrates the egg at the time of fertilization. The sperm of D. virilis is probably at least 5 mm long (A. F. Yanders, personal communication) and may be seen as a large coil at the anterior end of the fertilized egg in the early stages of meiosis. Fragments of these tails and other non-DNA-bearing material in the iron-haematoxylin-stained egg sections might erroneously be thought to be sperm heads, especially if one believes that polyspermy exists.

The Feulgen stain is specific for DNA (therefore does not stain sperm tails), and eliminates the errors that might be introduced by the non-specific staining of iron haematoxylin. Our conclusion is that in Drosophila monospermy, not polyspermy, is the general rule; dispermy occurs in low frequencies, as has been shown cytologically. There is also genetic evidence for occasional double fertilization of binucleate eggs in Drosophila (for example, Stern and Sekiguti, 1931; Goldschmidt, 1937), but these events seem to be quite rare. We do not wish to imply that trispermy or a greater degree of polyspermy does not exist in Drosophila. It is possible that such a degree of polyspermy may be found to occur in some strains, or in some mutant genotypes because of a breakdown in the barrier to polyspermy, or that it may occur rarely as a chance phenomenon in an essentially monospermic strain.

Concerning other insects, accounts of regular monospermy have appeared in sporadic fashion in the literature. They encompass several orders: Homoptera: Pseudococcus citri (Schrader, 1922), Pseudococcus obscurus (Nur, 1962), Pyrilla perpusilla (Sander, 1956); Lepidoptera: Dacrisia virginica (Johannsen, 1929); Diptera: Sciara impatiens (Carson, 1946); Hymenoptera: Paracopidosomopsis floridanus (Patterson, 1917), Copidosoma gelechiae (Leiby, 1922), Habrobracon juglandis (Speicher, 1936). When grouped in this fashion, these exceptions from the "rule of physiological polyspermy" in insects are impressive and seem to warrant a reevaluation of some of the early literature responsible for the formulation of this rule.

A critical review of Henking's 1892 paper reveals that this author furnished data for only two of the 14 species considered. In Pyrrhocoris apterus (Hemiptera) he found 47 monospermic, 38 dispermic, and 5 trispermic eggs (about 50% polyspermy); in Angelastica alni (Coleoptera) he found 85 monospermic, 13 dispermic, and 3 trispermic eggs (about 20% polyspermy). It should be noted that more than 85% of the polyspermic eggs just mentioned contained only one supernumerary sperm. In the case of an additional four species, Henking limited himself to purely qualitative considerations; of Donacia sp. (Coleoptera) he said that one or two sperm penetrate in the yolk mass; of Adimonia tanaceti (Coleoptera), frequently two sperm had penetrated; of Bombyx mori (Lepidoptera), one as well as two sperm per egg were observed; of Lasius niger (Hymenoptera), he said that frequently although not always several sperm penetrate into the egg.

Pantel's observations (1913) were made on fresh dipteran eggs removed from the uterus and examined in saline solution. He diagrammed many instances in which eggs exhibited packets of sperm tails variously caught in the micropylar apparatus; but by his own testimony, he did not attempt to determine the number of sperm heads present in these eggs.

Nachtsheim, 1913, states that in Apis mellifera (Hymenoptera) the number of sperm penetrating the egg normally fluctuates between three and seven. A justification for repeating his work may be provided by the following theoretical considerations: in Habrobracon juglandis (Hymenoptera) the frequency of dispermic eggs is of the order of 1 to 2% (Speicher, 1936). In this species, the frequency of gynanders with androgenetic male parts is 0.6% (Whiting, 1943); therefore, in approximately one-half the dispermic eggs the supernumerary sperm is allowed to undergo mitotic divisions. This is not surprising, since the egg cytoplasm is compatible with the early cleavage of haploid nuclei (giving rise to males) as well as the cleavage of diploid nuclei (giving rise to females). The same cytoplasmic compatibility is found in Apis; here the frequency of gynanders with androgenetic male parts is 1.34% (Rothenbuhler, Gowen and Park, 1952), almost twice that found in Habrobracon. Therefore, one might also expect the frequency of polyspermy in Apis to be about twice that found in Habrobracon, i.e., of the order of 4 to 5% dispermy, and not several hundred times as great, as claimed by Nachtsheim.

In the case of Bombyx mori (Lepidoptera), Henking, 1892, simply stated that he had observed the presence of one as well as two sperm per egg. Kawaguchi, 1926*, on the other hand, reported a high degree of polyspermy

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*We are grateful to Professor Y. Tazima for the translation of pertinent parts of this paper.

in this species, about 85% of 217 fertilized eggs containing two or more sperm (one case with eight and one with eleven sperm representing the upper limit of polyspermy in this material). Then, in 1928, Bataillon and Tchou Su stated that every Bombyx egg, when laid, contains 1, 2, or 3 sperm; they added that in this species "normal polyspermy is weak." Although there appears to be some disagreement among these authors regarding the degree of polyspermy in Bombyx, they all conclude that it does occur. It should be noted that Goldschmidt and Katsuki (see Tanaka, 1953) described the occurrence in certain strains of frequent double fertilizations that produced mosaics. These mosaics resulted from an hereditary tendency for the second polar nucleus as well as the pronucleus to be fertilized.

From the foregoing discussion it can be concluded that in a considerable fraction of those insects in which fertilization has been studied cytologically, physiological polyspermy is limited to less than 10% dispermy. In mammals, the incidence of polyspermic eggs can reach values of 3.2% in the rat, 1.2% in the mouse and 1.4% in the hamster (Pikó, 1958; Braden, Austin, and David, 1954; Austin and Braden, 1956, respectively; as quoted in Austin, 1960); polyspermy here is termed pathological. The main distinction between the two types of polyspermy is based on the kind of protection that the egg has devised against the presence of supernumerary sperm in its cytoplasm: in the case of physiological polyspermy, no barrier exists to their entry, they are suppressed or caused to degenerate inside the egg; organisms in which polyspermy is said to be pathological have external barriers against the penetration into the egg of more than one sperm; failure of these barriers almost always brings about the degeneration of the embryo.

Rothschild, 1954, introduced a nomenclature which classifies eggs according to the two types of protection they have against polyspermy: Type I Inhibition represents the defense mechanism of those eggs in which polyspermy is pathological, and Type II Inhibition the defense mechanism of those eggs in which polyspermy is physiological. This nomenclature lends itself to the addition of a third category, Type III Inhibition, which combines features of the other two types and which would apply to the eggs of many if not of all insects, for the following reasons. Those insect species in which the large majority of the eggs are monospermic must have evolved some barriers to the entry of supernumerary sperm. In the absence of a well demonstrated cortical reaction, resulting in the formation of some structure analogous to the fertilization membrane of sea urchins, the necessity that the sperm pass through a small micropyle may be considered such a barrier. Yet, when supernumerary sperm do penetrate the egg, they do not seem to impair its development.

It must be emphasized that Type III Inhibition does not represent a new kind of inhibitory process, yet its formulation seems desirable in that it can be used to set apart insect eggs from the eggs of other animals in reference to their reaction to polyspermy.

SUMMARY

Contrary to the widespread belief that polyspermy is a normal characteristic of fertilization in Drosophila, we obtained evidence indicating that, as a rule, only one sperm is present per fertilized egg in two species investigated.

Feulgen-stained whole mounts of D. melanogaster and D. virilis meiotic eggs, and serially sectioned eggs of D. melanogaster stained with Feulgen or with azur-eosin-Giemsa were examined. No polyspermy was observed among 87 fertilized eggs of D. virilis; among 165 fertilized eggs of D. melanogaster, only six were found to be dispermic.

An autoradiographic series in which we combined tritiated thymidine labeling and Feulgen staining of sperm fully supported the validity of the Feulgen technique as a means of determining the degree of polyspermy in Drosophila. In this series each sperm was found to be Feulgen-positive and also labeled. Nineteen of 20 eggs examined were monospermic, one was dispermic.

A survey of the literature dealing with insect fertilization has led us to conclude that in a large number of insect species (1) physiological polyspermy does not occur and (2) when accessory sperm do occasionally enter the egg, pathological effects of polyspermy need not be manifested. The reaction to polyspermy of these species should be considered as separate from Type I Inhibition (found in those eggs in which polyspermy is pathological) and from Type II Inhibition (found in those eggs in which polyspermy is physiological). We propose to name this reaction Type III Inhibition, with the stipulation that it does not involve a new kind of inhibitory process against polyspermy but simply combines features of the other two types of inhibition.

ACKNOWLEDGMENTS

The authors wish to thank Doctor Curt Stern for his valuable advice and encouragement during the course of the experiments and preparation of the manuscript. We also wish to thank Mrs. Carol Konrad for her instruction in preparing the autoradiographs, and Miss Cole Brunt, who sectioned the eggs and provided other technical assistance.

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†The references thus marked were kindly brought to our attention by Dr. S. Counce-Nicklas.

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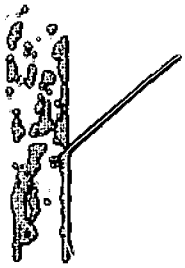
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