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LACK OF MUTAGENIC INFLUENCE OF TEMPERATURE
SHOCK ON PRESYNGAMIC SPERM NUCLEI

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Lack of mutagenic influence of temperature shock
on presyngamic sperm nuclei

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Running Head: Temperature and mutation rate.

SUMMARY

In order to test for the influence of temperature shock on lethal mutation frequency in the presyngamic sperm nucleus, freshly laid fertilized eggs of Drosophila melanogaster were subjected to short periods of very high ($\approx 37^{\circ}\text{C}$) or very low ($\approx 0^{\circ}\text{C}$) temperatures. The frequencies of recessive X-chromosome lethals recovered were not significantly different from that of the control frequency. Thus, temperature shock administered to the sperm after it has penetrated the egg but before syngamy has occurred does not change the recessive lethal mutation frequency. Both extremes of temperature shock to the eggs did result in great increases in sterility of F_1 females which arose from them. It is suggested that the sterilizing effect of temperature is the result of an influence on polar plasm, pole cells, or polar nuclei.

INTRODUCTION

The influence of temperature on mutation rates in the male germ cells of Drosophila melanogaster has been the subject of several investigations, with contradictory results. Such germ cells have been exposed to various temperatures and for varying periods either at some stage of spermatogenesis, as mature sperm in the male, or as mature sperm while in the storage organs of the female. Literature pertaining to the temperature effect has been well reviewed by Plough (1941) and Sheldon (1958). It is the purpose of this report to consider the effect of temperature on the mutation rate in still another stage of the sperm, namely, after it has penetrated into the egg but before syngamy has occurred.

When penetrated by a sperm, the egg of Drosophila melanogaster is normally ready for its chromosomes to undergo maturation divisions; it is generally believed that, at this time, they are in metaphase I of meiosis (see Sonnenblick, 1950). During the next few minutes meiosis is completed, with the formation of a female pronucleus; concurrently the sperm migrates, undergoes morphological changes, vesiculates, and forms a male pronucleus in juxtaposition to its female counterpart. The diploid nuclear condition is established at syngamy.

Some earlier findings in our laboratory suggested the possibility that during transformation from a very compact structure into a pronucleus, the sperm might be sensitive and its genes caused to mutate if subjected to abnormal temperatures. The experiments to be described were conducted to investigate the influence of very high and very low temperatures on recessive-lethal—mutation rates of X chromosomes in the sperm while in the presyngamic stage in the egg cytoplasm.

MATERIALS AND METHODS

Flies for the experiments were obtained from two stocks, one a wildtype (\pm) Samarkand strain and the second a mutant strain bearing on the X chromosome the genetic markers \underline{y} (yellow body), \underline{w}^a (apricot eye color), \underline{v} (vermilion eye color), \underline{B} (bar-shaped eyes), and to suppress crossing over, the inversions \underline{sc}^{S1} , $\underline{In49}$, \underline{sc}^8 . Both stocks have been maintained in mass culture in our laboratory for several years. Virgin $\underline{y} \underline{w}^a \underline{v} \underline{B}$ females and virgin \pm males were collected and stored separately in culture vials for two days. The males were then shaken in with the females (30-40 pairs per vial) and left for 3-5 hours, during which time matings would occur. It is known that females may retain fertilized eggs for several hours and that these eggs will develop normally. When such eggs are laid they will be in various stages of embryogenesis. Since we wished to shock fertilized eggs in presyngamic stages, eggs collected over the first hour were discarded or used as controls; many of the eggs might have been retained and thus be in late embryonic stages of development. When eggs were laid in rapid succession collections were made over 5-minute periods, at room temperature, following the method outlined by Hildreth and Brunt (1962). Eggs for the control series were immediately placed in culture bottles that contained molasses medium, while eggs to be treated were either immediately subjected to temperature shock or aged at 25°C for 10 minutes prior to treatment. After being shocked the eggs were transferred to culture bottles and henceforth all conditions were the same for both treated and control groups.

According to Rabinowitz (1941), at 24°C freshly laid fertilized eggs will have reached a stage from telophase II of meiosis to conjugation of the pronuclei at about 13 minutes, and the first mitotic division will occur at about 23 minutes. In the preliminary experiments, in order to shock the sperm nucleus in the late stages just prior to syngamy, the eggs were aged for 10 minutes, then placed in the freezing compartment of a refrigerator (temperature range of -2 to -10°C) or in a paraffin oven (range of +37 to +45°C) and left for periods of 5, 10, or 15 minutes. Egg mortality was high, as was also F₁ female sterility (18-24% at low and 37-45% at high temperatures), and the lethal frequencies, as indicated by only a few hundred tested chromosomes, were not different from those of the controls.

In order to reduce egg mortality and F₁ female sterility, less extreme temperatures were used in subsequent experiments; and to lengthen the period of temperature shock to the sperm nucleus, aging of eggs prior to treatment was discontinued. The freshly laid eggs (0-5 minutes old) were immediately shock treated for 15 minutes in a tray just below the freezing compartment where the temperature was 0 ± 5°C or in the paraffin oven at 37 ± 3°C. In order for us to estimate what percentage of the eggs would be in meiosis when treated, eggs were collected during 5-minute periods, fixed during the next 15 minutes, then prepared and stained by the Feulgen whole-mount method (see Hildreth and Lucchesi, 1963). Of 190 fertilized eggs, 75% (143) were in presyngamy stages and most of the rest in early cleavage stages, although a few were blastulae. Thus the majority of the eggs would be treated during presyngamy stages but some would be in cleavage when treatment was initiated.

On any one day of experimentation as many as 24 collections were made from one group of females. Even if fertilization occurred immediately after the parents were placed together and the egg was retained by the female until just prior to shock treatment, no egg, or zygote, could have been more than 8-1/2 hours old when treated. Since the females were stimulated to oviposit rapidly, it is unlikely that any eggs were retained for so long a period; the cytological evidence also supports this contention.

The mating scheme is shown in Fig. 1. The F_1 females were collected and placed individually with two $\underline{y} \underline{w}^a \underline{v} \underline{B}$ males (supplemented from stock cultures) in small culture vessels (creamers) 15 days after the eggs from the P females had been placed in culture bottles. Fourteen days later the F_2 cultures were examined and classified. A culture was scored as OK (non-lethal) if it contained one or more \pm males, lethal (l) if it contained 35 or more flies but no \pm male, or doubtful (df) if it contained fewer than 35 flies but no \pm male. Doubtful cultures were recultured by again mating the F_1 female, or any of her heterozygous daughters, with $\underline{y} \underline{w}^a \underline{v} \underline{B}$ males in an attempt to recover the \pm chromosome. In some cases cultures remained doubtful because the F_1 female was dead and there were no heterozygous daughters available for mating. If the F_1 female produced no offspring (larvae-adult), the culture was scored as sterile (st) but the female was remated for further classification. In some of the sterile cultures the female had died and thus could not be retested; these cultures remained as steriles but are treated as a separate group in the statistical analysis (Results and Discussion).

RESULTS AND DISCUSSION

The results are presented in Table I; totals for the control series also include the data from the preliminary experiments. The lethal frequencies are lower in both treated series than in the control series but not significantly so, as is shown by the homogeneity test. The greatest difference is between the control and cold shock groups, but even here the probability is 0.1-0.2 ($\chi^2 = 1.90$, d. f. = 1) that chance fluctuations could account for the deviations. The results for the treated series were compiled from 64 experiments (the controls from an additional 30), each carried out on a different day. No attempt was made to collect eggs from individual females; therefore, eggs fertilized by the sperm from a single male were undoubtedly distributed among the samples that were treated at either high or low temperature or used as controls. Thus, if a male had among his sperm a cluster of lethal mutants that arose by gonial replication of a single mutant, these lethal mutants could be distributed randomly among the control and treated series in any one experiment. If there were such clusters the mutation rate would be elevated, and if there were an unequal distribution the results would be biased. The distribution of lethals among the 64 experiments is shown in Table II. In 23 of the experiments there were clusters of lethals and in 21 of these two or three of the series were involved. How would the lethal frequencies from the total data compare with the frequencies in only those experiments in which no lethal or a single lethal was recovered? The totals for 41 such experiments yield lethal frequencies of 6/2480 chromosomes in the control, 6/2263 in the heat, and 4/2230 in the cold-treated series. This

distribution is also random ($p = 0.8-0.9$, $\chi^2 = 0.39$, d. f. = 2). From these data there is no evidence that either heat or cold shock influences the mutation rate within the temperature ranges and conditions described. It is debatable whether more extreme temperatures would demonstrate a change in mutation frequencies, as during the presyngamic stage, temperatures that might induce lethal mutations would, perhaps, also kill the egg. The interval between the time that the sperm enters the egg and the time that syngamy is accomplished is quite brief; therefore, long periods of shock treatment (at less traumatic temperatures) to the sperm nucleus are not possible. This is a disadvantage not shared, for example, by the mature sperm which may be maintained in a viable condition and treated over a few weeks (see Byers and Muller, 1952).

The greatest effect of temperature shock was on the frequency of sterility among the F_1 females (Table I). The interesting aspect is that it was not the females themselves that received the shock treatment but it was the eggs from which they arose. The frequency of sterility was increased greatly by both extremes in temperature; because a homogeneity test yields a χ^2 value of more than 256 (d. f. = 2, $p =$ much less than 0.0001) it is highly unlikely that such deviations between the series would occur by chance alone. Even if one compares the two series with the least deviation between them, the χ^2 value is greater than 82. It is possible that accidental deaths of F_1 females, especially if in higher frequency in the treated than in the control series, could result in a bias and cause the highly significant deviations obtained. Only in the later experiments, in our records,

did we distinguish between sterile-alive females that could be remated for further testing and sterile-dead females that could not be. Among more than 5000 cultures tested in each series the heat treated had 1.3%, the control 1.5%, and the cold-treated series 1.2% classified as sterile-dead. A homogeneity test of these data yields a probability of 0.2-0.3 ($\chi^2 = 2.56$, d. f. = 2) that the deviations could have been caused by chance fluctuations. Thus temperature shock does not appear to have been a causative factor for these deaths, whether they were accidental (e. g., being stuck in the medium, or overetherized) or were a result of poor viability. If it is assumed that the above frequencies are characteristic of the total data, then about 39% of the sterile cultures in the control, 14% in the heat-treated, and 19% in the cold-treated series resulted from these early deaths. The proper classification of these sterile-dead cultures is in doubt; in fact, they might have had a distribution among the OK, 1, df, and st classes similar to that among the total data. If, because of uncertainties in classification, the sterile-dead cultures are eliminated from the sterile group, the discrepancies in sterile frequencies are increased. As was seen above, when the sterile-deads were included in the homogeneity test, a χ^2 value of approximately 256 was obtained; when they were excluded from the sterile class, the χ^2 value was increased to approximately 378. This increase in significance results from the fact that even though the sterile-dead cultures were distributed at random among the treated and control series, they accounted for a much higher proportion of the total number of sterile cultures among the control than among the other series. There can be little doubt that the increase in sterility is caused

by the temperature shock. No sterile females were dissected so the condition of their ovaries is not known, although some females did lay sterile eggs. The germ cells originate from nuclei situated in the polar plasm at the posterior end of the egg. In mature oocytes and young zygotes, the polar plasm is visibly distinct from the other cortical regions of the egg (see Sonnenblick, 1950, for embryological development of pole cells and gonads), and even before blastoderm formation an average of 31-40 nuclei are segregated into the polar plasm. During embryogeny the pole cells migrate but do not undergo mitosis until about the 16th hour of development; even if retained for several hours by a female, the oldest egg treated in our experiments would be in a stage of pole cell migration. The mesodermal sheath which envelops the germ cells is formed at approximately 14 hours. Only a few of the original pole cells are destined to become germ cells. Investigations by Geigy (1931) later confirmed by Aboim (1945), showed that ultraviolet irradiation of the blasteme stage embryos could cause partial and even complete castration. Experiments by Poulson and Waterhouse (1960) demonstrated that during the first two hours after fertilization the embryo varies considerably in its sensitivity to ultraviolet irradiation. The effect (absence of gonads, a single gonad, or reduced cell number in gonads) was greatest at the time of pole cell formation, at 89-102 minutes. The least affected period was that before migration of nuclei into the polar plasm (60-75 minutes). After pole cells formed and while they were undergoing mitosis, sensitivity decreased but not to the level observed when only the polar plasm was irradiated.

In view of the above evidence, it is probable that the sterilizing effect of temperature shock was on either the polar plasm, nuclei in this plasm, or pole cells (as opposed to nuclei in the syncytial polar plasm), either in the polar plasm or in their course of migration. Of course, a more generalized effect of temperature on the cytoplasm cannot be ruled out. Whether the treatment in some cases caused castration is not known, and it may be that gonads were formed but contained functionless gametes. The exact nature of the sterilizing effect of temperature awaits further experimentation.

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Table I.

Frequencies of lethal (l), doubtful (df), and sterile (st) cultures among F₂. Figures in parentheses are percentages. Lethal frequencies were derived from OK + l (lethal) and others from total chromosomes tested.

	Age when eggs shocked (min)		<u>P Chromosomes tested</u>				
	<u>Start</u>	<u>End</u>	<u>Total</u>	<u>OK</u>	<u>l</u>	<u>df</u>	<u>st</u>
Control 25°C			13821	13209	44	44	524
					(0.33)	(0.32)	(3.79)
0°C±5	0-5	15-20	10948	10209	24	25	690
					(0.23)	(0.23)	(6.30)
37°C±3	0-5	15-20	9404	8507	26	18	853
					(0.30)	(0.19)	(9.07)
Homogeneity tests:		<u>χ²</u>		<u>d. f.</u>		<u>p</u>	
lethals		1.92		2		0.3-0.5	
doubtfuls		3.99		2		0.1-0.2	
steriles		256+		2		much less than 0.0001	

Table II.

Distribution of lethal mutants recovered from individual experiments.

$\chi^2 = 2.55$, d. f. = 2, $p = 0.2-0.3$.

<u>Individual experiments</u>	<u>Series with lethal mutants</u>	<u>Mutants per series</u>	<u>Mutants in each series</u>		
			<u>Control</u>	<u>High</u>	<u>Low</u>
25	0	0	0	0	0
6	1	1	6	0	0
6	1	1	0	6	0
4	1	1	0	0	4
2	1	2	4	0	0
5	2	1 or more	7	10	0
3	2	1 or more	0	3	4
6	2	1 or more	8	0	6
7	3	1 or more	10	7	10
Total 64			35	26	24
Frequency			0.36	0.30	0.23

P $\underline{y} \underline{w}^a \underline{v} \underline{B} \overset{v}{\text{♀♀}} \times \pm \text{♂♂}$
 fertilized eggs, temperature shocked

F₁ many (1 $\underline{y} \underline{w}^a \underline{v} \underline{B} / \pm \text{♀}$ x 2 $\underline{y} \underline{w}^a \underline{v} \underline{B} \text{♂}$)

F₂ $\underline{y} \underline{w}^a \underline{v} \underline{B} \overset{oo}{\text{++}}$, $\underline{y} \underline{w}^a \underline{v} \underline{B} / \pm \text{♀♀}$, $\underline{y} \underline{w}^a \underline{v} \underline{B} \text{♂♂}$, $\pm \text{♂♂}$

Fig. 1. Mating scheme for detection of recessive lethal mutations in X chromosome of P ± male.

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