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Sex ratio distortion in *D. simulans* after treatment with quinacrine

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Summary

A significant excess of female offspring resulted when pregnant females of the *D. simulans* species were left to lay their eggs on food containing quinacrine. The same result occurred in a subsequent experiment in which male parents were injected with quinacrine. This was the first time that a chemical compound showing an affinity with DNA *in vitro* altered the sex ratio in a consistent manner. This effect had been predicted based on the unique quinacrine fluorescent staining pattern in *D. simulans*, in which only the Y stains intensely in mitotic chromosomes. It seems that treatment acts on spermatids or spermatozoa causing decreased functioning of the Y-bearing ones, resulting in an excess of female offspring. Interestingly, the species *D. mauritiana* and *D. melanogaster*, although very closely related to *D. simulans*, do not have its staining pattern and as predicted did not respond to treatment; therefore, the important parameter appears to be related to the staining pattern of mitotic chromosomes and not to the phylogenetic relationship.

1. Introduction

Observations on sex ratio distortion, meiotic drive and nondisjunction have been reported in different *Drosophila* species (Zimmering, 1976). The causal factors include chromosomal aberrations or chromosomal additions, physical factors such as temperature and X-rays, and chemicals. The chemical factors include experiments with actinomycin D (Felix, 1969), organic mercury compounds (Ramel & Magnusson, 1969), and monosodium glutamate (De la Rosa *et al.* 1972). These, however, had not been applied to the *D. simulans* species.

While classifying species of the melanogaster group using different criteria (Tsakas & Tsacas, 1984), it was noted that *D. simulans* had an unusual quinacrine fluorescent staining pattern in mitotic chromosomes when compared either to members of the melanogaster subgroup or to its closely related sibling species *D. mauritiana* and *D. melanogaster*. This consisted of the Y being the only mitotic chromosome which becomes intensely fluorescent in most of its length while the X and the autosomes do not (Lemeunier, Dutrillaux & Ashburner, 1978). This observation combined with the conclusions reached in the extensive research *in vitro* on quinacrine binding with DNA by Comings *et al.* (1975) provided the motivation for further research to determine if quinacrine might disturb meiosis and/or mitosis. Among the conclusions of Comings *et al.* are the following: (*a*) quinacrine binds to chromatin by intercalation of the three planar rings with the large group at position 9 lying in the small groove of DNA; (*b*) DNA containing guanine quenches fluorescence, highly AT-rich DNA enhances fluorescence, and this effect persists at the concentration of DNA in the metaphase chromosome; (*c*) histones, which probably bind in the large groove of DNA, have little effect on the ability of DNA to quench quinacrine fluorescence; (*d*) salts, as well as compounds like spermine which bind in the small groove of DNA, inhibited quinacrine binding and quenching. We questioned whether treatment of *D. simulans* with quinacrine might cause a sex-ratio distortion in favour of female offspring. Our experiments took place at three different laboratories. The first three experiments dealt with treatment by addition of quinacrine to the food supply. Based on the results of these, a further experiment was performed to determine if the same effect would be observed when male parents received treatment through injection of quinacrine close to their reproductive systems.

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2. Materials, methods and results

(i) *Treatment through addition of quinacrine to the food supply*

Experiment I (*Agricultural College of Athens*). The female : male ratio in 500 newly emerging offspring was counted before treatment in the three species; the number in parentheses corresponds to the C.N.R.S. collection number: *D. simulans* (260·1) 0·492 : 0·508, *D. melanogaster* (231) 0·494 : 0·506 and *D. mauritiana* (163·1) 0·504 : 0·496.

Treatment consisted of 0·4 mg of a 0·5% aqueous solution of quinacrine dehydrochloride (Sigma Q 0250/catalogue 1986) added to 10 cc of Drosophila food in each vial. This dosage was established after many trial experiments to determine the amount sufficient to produce a significant response while avoiding the potential of quinacrine for toxicity. The food composition was without live yeast addition and as follows: 900 ml H_2O, 100 ml tomato juice, 12 g agar-agar, 125 g sugar, 112 g cornmeal, 2·5 g powdered yeast, 4 g nipagine in 25 ml alcohol. Temperature was maintained at 19 °C.

Four pregnant females were placed in each treatment vial and discarded after 8 days. Their offspring were sexed and counted as they emerged. Eight vials comprised one set, and the results of each set are presented in Table 1. *D. simulans* showed a significant excess of females in all four of the sets tested, while the sibling species *D. mauritiana* and *D. melanogaster* showed no significant change in their sex ratios. This intriguing response by *D. simulans* was further investigated in the following experiments.

Experiment II (*Agricultural College of Athens*). This experiment followed the same laboratory conditions, food and dosage of quinacrine as Experiment I, with the exception that only *D. simulans* was under study. A control was established by first placing four pregnant females in a vial without quinacrine, where they were left for 8 days before discarding. Their offspring were sexed and counted as they emerged. The same four females were transferred to a treatment vial and left for an additional 8 days before being discarded. Their offspring were sexed and counted as they emerged and constituted the treated offspring. A set consisted of eight control vials and eight treatment vials, and a total of four sets were performed. Table 2 gives the results, and shows that whereas no significant discrepancy in the sex ratio of the control offspring occurred, there is a significant excess of females in the treated offspring.

Experiment III (*C.N.R.S. Gif-sur-Yvette*). The material and dosage of quinacrine remained the same as in Experiment II. Food composition was as follows: 1 l H_2O, 20 g agar-agar, 131 g sugar, 101 g cornmeal, 14 g powdered yeast, 5 g solution of nipagine in alcohol. Temperature was maintained at 23 °C.

This experiment differed in sequence and length of laying period from Experiment II. First, four pregnant females were left to lay eggs in treatment vials for 5 days before removal. Their offspring were sexed and counted as they emerged and constituted the treated offspring. The same four females were immediately transferred to control vials for a 5-day period before being discarded. Their offspring constituted the control and were sexed and counted as they emerged. A set consisted of six treatment vials and six control vials, and a total of ten sets were performed.

In addition, realizing that the ageing factor might increase the incidence of female offspring, a small control was established to take this parameter into account. Each set of the major experiment had a small control attached consisting of four pregnant females who were first placed in one vial and then transferred

Table 1. *Results of Experiment I: treatment through addition of quinacrine to the food supply of* D. simulans, D. mauritiana, *and* D. melanogaster

	Sets	Females	Males	Probabilities
D. simulans	1	113	87	$0·05 > P > 0·02$
	2	149	113	$0·01 > P > 0·001$
	3	152	109	$0·01 > P > 0·001$
	4	134	99	$0·02 > P > 0·01$
Total		548	408	$0·01 > P > 0·001$
D. mauritiana	1	115	119	$0·70 > P > 0·50$
	2	100	109	$0·70 > P > 0·50$
Total		215	228	$0·50 > P > 0·30$
D. melanogaster	1	189	183	$0·70 > P > 0·50$
	2	147	136	$0·50 > P > 0·30$
Total		336	319	$0·50 > P > 0·30$

The numbers of female and male offspring observed in each set and the corresponding totals for each species are given. The last column gives the χ^2 probabilities composing the sex ratios after treatment with those observed in the control series, previously estimated (see text).

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Table 2. *Results of Experiment II: treatment through addition of quinacrine to the food supply of* D. simulans

	Control		Quinacrine		
Set	Females	Males	Females	Males	Probabilities
1	136	134	145	104	$0{\cdot}10 > P > 0{\cdot}05$
2	155	159	152	118	$0{\cdot}10 > P > 0{\cdot}05$
3	122	116	150	101	$0{\cdot}05 > P > 0{\cdot}02$
4	119	117	140	96	$0{\cdot}05 > P > 0{\cdot}02$
Total	532	526	587	419	$0{\cdot}001 > P$

The numbers of female and male offspring observed in each set and the totals are presented accompanied by the probabilities estimated using the chi-square homogeneity test.

according to the time sequence of the set to another vial. They received no treatment, only their offspring were sexed and counted at each stage. In this control no significant excess of female offspring appeared at either stage of the experiment in any of the sets.

The results of Experiment III are presented in Table 3. This time, when the sequence was treatment vials first followed by the control vials, both the treated and the control offspring showed a significant excess of females. This indicated a 'carry-over' effect of quinacrine which prompted a further experiment.

(ii) *Treatment by injection of quinacrine*

Experiment IV (*University of Athens*). This experiment was performed to determine the effect of quinacrine on the offspring of male parents. The material used was *D. simulans*. Third-instar larvae and adult stages were found to be the most suitable for allowing an adequate number of survivors while permitting the closest access to the reproductive system with minimal risk of damage to it. Treatment consisted of injection of approximately 0·2 μl of a freshly prepared, diluted concentration of 0·5% quinacrine solution. The glass injection needle was handmade and therefore the amount is noted as approximate. In order to determine if injection alone could have an effect on the sex ratio, a small experiment using an injection of isotonic insect solution was made first. No effect was observed. The food composition was the same as in Experiment I, and the temperature was maintained at 23 °C.

Adult males: injected between the 4th and 5th abdominal segments

In order to focus the quinacrine treatment on the newly formed spermatide and spermatozoa (Chandley & Bateson, 1962), the following procedure was applied in adult males. They were left in vials containing twice their number of virgin females for a period of one day with the hope that their mature spermatozoa would be removed. Following their removal from the vials, they received treatment and then remained together for 2

Table 3. *Results of Experiment III: treatment through addition of quinacrine to the food supply of* D. simulans

	Quinacrine			Control		
Sets	Females	Males	Probabilities	Females	Males	Probabilities
1	398	355	$0{\cdot}20 > P > 0{\cdot}10$	490	470	$0{\cdot}70 > P > 0{\cdot}50$
2	429	425	$0{\cdot}95 > P > 0{\cdot}90$	515	402	$0{\cdot}01 > P > 0{\cdot}001$
3	400	378	$0{\cdot}50 > P > 0{\cdot}30$	459	343	$0{\cdot}001 > P$
4	475	397	$0{\cdot}01 > P > 0{\cdot}001$	426	398	$0{\cdot}50 > P > 0{\cdot}30$
5	368	307	$0{\cdot}02 > P > 0{\cdot}01$	462	377	$0{\cdot}01 > P > 0{\cdot}001$
6	462	385	$0{\cdot}01 > P > 0{\cdot}001$	408	383	$0{\cdot}50 > P > 0{\cdot}30$
7	416	328	$0{\cdot}01 > P > 0{\cdot}001$	443	362	$0{\cdot}01 > P > 0{\cdot}001$
8	457	434	$0{\cdot}50 > P > 0{\cdot}30$	430	389	$0{\cdot}20 > P > 0{\cdot}10$
9	356	306	$0{\cdot}10 > P > 0{\cdot}05$	424	403	$0{\cdot}50 > P > 0{\cdot}30$
10	465	363	$0{\cdot}001 > P$	456	407	$0{\cdot}10 > P > 0{\cdot}05$
Total	4226	3678	$0{\cdot}001 > P$	4513	3934	$0{\cdot}001 > P$

The number of female and male offspring observed in each set, the totals and probabilities are presented. In this experiment the sequence was reversed and the control showed a 'carry-over' effect, therefore the probabilities are estimated using the chi-square test based on the previously determined female : male ratio.

Note: No excess of male offspring occurred at any stage of any set, while a significant excess of females occurred in nine stages, two with a probability between 0·02 and 0·01, six between 0·01 and 0·001, and two smaller than 0·001.

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Table 4. *Results of Experiment IV treatment by injection of quinacrine to male parents of* D. simulans *when in adult or larval stage*

Percentage of quinacrine	Age at injection	Crosses	Females	Males	Probability
0·05	Adult, unknown age	5	365	273	$0{\cdot}001 > P$
0·025	Adult, unknown age	9	304	238	$0{\cdot}01 > P > 0{\cdot}001$
0·017	Adult, unknown age	6	259	235	$0{\cdot}25 > P > 0{\cdot}10$
0·05	Adult, 1–2 days old	6	676	394	$0{\cdot}001 > P$
0·05	Adult, 9–10 days old	9	638	656	$0{\cdot}95 > P > 0{\cdot}90$
0·05	Larvae	7	639	391	$0{\cdot}001 > P$
0·025	Larvae	8	478	390	$0{\cdot}01 > P > 0{\cdot}001$

The number of crosses and the number of female and male offspring observed are presented, accompanied by the probabilities estimated using the chi-square test based on the previously determined female : male ratio.

days in order to allow them time to recover and for quinacrine to act. Single crosses were then performed with virgin females for a duration of 2 days. After this the male was discarded while the female remained for 10 more days in the same vial to lay eggs. The offspring were sexed and counted as they emerged. The results are presented in Table 4, which shows that in the experiment on adult males of unknown age a significant sex-ratio distortion in favour of females occurred when the two higher concentrations of quinacrine were used. The weakest concentration did not significantly affect the sex ratio and was eliminated from the following treatments. In the final experiment on adults of known age, which was carried out to determine if the ageing factor has an effect, an even more significant excess of female offspring occurred after treatment of the 1- to 2-day-olds, while none occurred after treatment of the 9- to 10-day-old adults.

Larvae: injection site centred between the two respiratory trachea, at the depth of and in line with the gonads

The male larvae received treatment. When they emerged as adults single crosses were performed with virgin females and the pairs were left together for 2 days. After this the male was discarded while the female remained for 10 more days in the same vial to lay eggs. These offspring were sexed and counted as they emerged. The results are presented in Table 4, which shows that a significant sex-ratio distortion in favour of female offspring occurred with both of the concentrations used, but the effect was greater in the higher one.

3. Discussion

The experimental addition of quinacrine to the food supply resulted in a significant sex-ratio distortion in favour of females. It might have been expected that *D. mauritiana* and *D. melanogaster*, as closely related sibling species to *D. simulans*, would respond similarly to this treatment. However, this did not occur as had been anticipated in this case, due to the difference in their quinacrine staining pattern. The most important parameter for this response to quinacrine appears to be the staining pattern of mitotic chromosomes, especially sex chromosomes, and not the phylogenetic relationship between species.

Our curiosity was aroused when we noticed that in Experiment III, in which the sequence of control and treatment was reversed, a sex-ratio distortion in favour of females also occurred in the offspring of the control vials. In this case the only contact with quinacrine was through the mothers' previous 5-day stay in the treatment vials. A probable explanation for this 'carry-over' effect is the exposure of the pregnant females to quinacrine through contact with the food via the gonopod or pretarsus, by consumption of the food, or via the respiratory system. Through these entrances it seems that quinacrine affects the sperm stored in the spermatheca and therefore affects the final meiotic product. This observation prompted further research to determine what effect injection of quinacrine near the male reproductive system would have. The results of Experiment IV show that males, whether treated as adults or larvae, gave excess female progeny, and the higher concentration of quinacrine resulted in the greater effect. Also, the ageing factor appeared to be crucial. The youngest adult males tested showed the greatest effect, while the oldest tested did not show any sex-ratio distortion. An explanation for this may be that penetration of quinacrine into the testes of the older males is reduced due to the sclerotization of the tissues. It is interesting to note that the sex-ratio distortion seen in adults of unknown age falls between that of the youngest and oldest adults tested.

In summary, treatment with quinacrine appears to act on spermatide or spermatozoa, causing decreased functioning of the Y-bearing ones. Evidence for this effect is most clear in the case of mature sperm, as seen in the 'carry-over' effect in Experiment III and in the sperm-bundles stage of third-instar larvae.

This was the first time that a chemical compound showing an affinity with DNA *in vitro* has been found

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to alter the sex ratio in a consistent way. The effect had been predicted based on the unique quinacrine fluorescent staining pattern of mitotic chromosomes of *D. simulans*. Perhaps a sex-ratio distortion may be prompted through quinacrine treatment in other organisms according to their staining pattern of mitotic chromosomes. Such a case might be that of humans, in which the Y chromosome stains intensely with quinacrine throughout all stages of meiosis from spermatogonia to mature spermatozoa (Pearson & Bobrow, 1970).

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