MATERNAL EFFECT TRANSFERRED BY INJECTION AND FURTHER ANALYSIS OF TEMPERATURE EFFECTIVE PERIOD FOR TUMOROUS HEAD IN *DROSOPHILA MELANOGASTER*¹

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INITIAL experiments (GARDNER 1948) on the tumorous head phenotype demonstrated a difference in the results of reciprocal crosses. A maternal effect dependent on a substance elaborated in the presence of a homozygous sex-linked gene (*tu-1*) was postulated (GARDNER and WOOLF 1949) to account for this observation. Changes in temperature during the early stages of development were found to alter the effect (GARDNER and WOOLF 1950).

Extensive reciprocal crosses between tumorous head (*tu-h*) and "wild" laboratory stocks (GARDNER and STOTT 1951), laboratory stocks carrying mutants (GARDNER, STOTT and DEARDEN 1952), and flies collected from natural populations (GARDNER and GARDNER 1953) further demonstrated the maternal effect. From most crosses between *tu-h* females and males from other stocks about 30 percent of the first generation progeny showed the tumorous head phenotype compared with about one percent or less from the reciprocal cross between females from most other stocks and *tu-h* males. Variations from this pattern were observed in some crosses. Modifiers and different genetic backgrounds were postulated to account for the differences. Penetrance, expressivity, and relative viability were described in the inbred *tu-h* stock following three years of selection and inbreeding (GARDNER and RATTY 1952). Experiments involving two series of backcrosses (GARDNER 1959) indicated that no persistent cytoplasmic substance is involved and that a single, recessive, sex-linked gene (*tu-1*) controls the maternal effect. An autosomal semidominant gene (*tu-3*) is necessary for any expression of the trait. There is a resemblance between the behavior of tumorous head and that of CO₂ sensitivity for which a genoid was described by L'HERITIER (1951). There are also conspicuous differences. The genoid is an independent virus or plasmagene while two major nuclear genes are involved in the tumorous head complex. No cytoplasmic particle comparable with the genoid of L'HERITIER has been detected in tumorous head flies.

The present experiments were designed to test the hypothesis that a substance elaborated by *tu-h* females carrying the gene *tu-1* in homozygous condition is responsible for the maternal effect. Further analyses of the temperature effective period which is associated with the maternal effect are also included in this report.

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MATERIALS AND METHODS

For the injection experiments, *tu-h* females were etherized and washed in two baths of distilled water to remove adhering food particles, salts, and other substances from the body surface. The flies were then crushed and homogenized with ten times their weight of insect Ringer's solution (1000 ml H₂O, 7.5 gm NaCl, 0.35 gm KCl, 0.21 gm CaCl₂). Crude extracts were lightly centrifuged at 2,800 rpm to remove solid particles and cellular debris. The supernatant was injected into the lateral thorax of wild (Lausanne) virgin females with a Dutky Fest microinjector. Injected females were mated with *tu-h* males, and the progeny were classified and compared with controls.

The standard corn meal, molasses, agar medium with live yeast added was used for all experiments. Flies were maintained at 25°C unless otherwise noted. Percentages are given in the tables with accuracy to the nearest whole number. When reciprocal crosses are symbolized, the female parent is always written first.

The first experiment, carried out to define the temperature effective period more narrowly, was prepared by mass mating 50 *tu-h* females with 50 wild males in dacron laying cages (SIMMONS and GARDNER 1958) and collecting eggs laid during two-hour intervals. Seven successive batches of eggs were deposited on separate petri dishes. Cultures were maintained at 21°C and 45 percent relative humidity except during the four-hour intervals at 30°C. Controls were similar to the experimental cultures but were not subjected to heat treatment. The first petri dish was placed in the 30°C incubator during the first four hours after the eggs were laid, the second was subjected to the higher temperature during the interval from four to eight hours after egg deposition, and so on at four-hour intervals for 24 hours. After eclosion, the adult flies were killed by freezing and classified for abnormal cephalic growths.

For the next experiment, designed to determine whether longer periods at higher temperature are more effective than four-hour intervals, another series of cultures was obtained as described above for the first experiment. Six experimental cultures were treated in the same way but all six were placed in the 30°C incubator immediately after egg deposition and left for different periods of time. The first was left four hours, the second, eight, and so on at increases of four hours until the last group which remained for 24 hours. Controls were maintained at 21°C and 45 percent relative humidity.

When it was shown that the abnormal growths being investigated could be altered by temperature changes during four-hour intervals in the early developmental period, a comparative study was made of the metabolic rates of developing larvae from different cultures during the first 24 hours after egg deposition. One hundred eggs were collected from each of the following: *tu-h* stock, wild stock, *tu-h* × wild, and wild × *tu-h* (female parent is always written first). Oxygen uptake during the first 24 hours after egg deposition was determined with a Warburg apparatus. Readings were taken at 30-minute intervals.

THOMPSON and TENNANT (1953) found by alternating wet and dry exposures that *Drosophila* larvae have a higher respiration rate in saturated air

than in dry air. Since humidity effects are closely allied with temperature effects, these two environmental factors were controlled as well as possible during the first 24 hours of development. Tests were made at 20°C with 20, 45, 52, and 66 percent relative humidity and at 30°C with 21, 45, 53, and 68 percent relative humidity. Eggs were laid at the temperature and relative humidity indicated, parent flies were removed, and cultures were allowed to remain under the same environmental conditions for 24 hours. Cultures were then removed to another incubator maintained at 24°C and 40 percent relative humidity. After eclosion the adults were classified for abnormal growths.

The atmospheric relative humidity which was recorded did not represent the effective relative humidity during the larval stage. Eggs were laid on the surface of the medium where the relative humidity of the air could have an influence, but when the larvae emerged from the eggs they went immediately into the medium which was saturated with moisture. The larvae were probably not influenced by the air above the medium under the conditions of this experiment. All cultures were as nearly alike as possible, with respect to the water content of the medium.

EXPERIMENTAL RESULTS

The results of the first injection experiment are summarized in Table 1. Four wild (Lausanne) females, injected with extract from adult *tu-h* females and mated with *tu-h* males, produced a total of 425 progeny with 76 or 18 percent abnormal. From two control matings, which were similar except that the wild females were not injected, 276 progeny were produced with two (one female and one male) abnormal. Another control experiment was carried out in which Lausanne females were punctured with the injection needle, but no extract was released. Four (three females and one male) of the 207 progeny were abnormal. When insect Ringer's solution was injected into Lausanne females, ten (eight females and two males) of the 264 progeny were abnormal. Results from controls were within the range obtained from previous matings between Lausanne females and *tu-h* males. Lausanne apparently has a genetic background which may increase the percentage of abnormal flies from this type of mating to as much as

TABLE 1

F₁ progeny from crosses between wild (Lausanne) females, injected with extract from tu-h females and crossed with tu-h males. Results from controls and reciprocal crosses are included

Treatment†	Abn. females	+ females	Abn. males	+ males	Percent* abnormal
Wild injected × <i>tu-h</i>	34	179	42	170	18
Control (not injected)	1	125	1	149	1
Punctured	3	96	1	107	2
Ringer's injected	8	132	2	122	4
<i>tu-h</i> × Lausanne (not injected)	67	150	77	171	31

* Percentages given with accuracy to nearest whole number.

† Female parent is always written first.

three or four percent of abnormal flies compared with less than one percent for most wild stocks. The small percentages (less than one percent) of abnormal flies from wild females and *tu-h* males were explained (GARDNER and WOOLF 1949) on the basis of independent action of gene *tu-3*. From the reciprocal cross between *tu-h* females and wild (Lausanne) males, 144 of the 465 flies were abnormal. This result is comparable with previous large-scale matings between *tu-h* females and Lausanne males.

The extract injection experiments on Lausanne females were repeated under carefully controlled conditions. Results of the second experiment are summarized in Table 2. Nineteen percent of the progeny of the injected females were abnormal compared with three and four percent from the two controls.

Injection experiments similar to those with Lausanne were conducted with the Samarkand wild stock. Among the numerous stocks with which *tu-h* has been outcrossed, Samarkand crosses have shown the lowest expression of tumorous head. Apparently the genetic background in this stock reacts in a different way than that of Lausanne. Results of the experiments involving Samarkand are summarized in Table 3. Extract from *tu-h* females was injected into Samarkand females which were later mated with *tu-h* males. Four percent of the progeny were abnormal. No abnormal flies were obtained from the control cross between uninjected Samarkand females and *tu-h* males. Samarkand females injected with Ringer's solution produced no abnormal flies. Extract from larval *tu-h* flies injected into Samarkand females did not alter the results, but females injected with extract from *tu-h* eggs and mated with *tu-h* males produced three percent of

TABLE 2

Results from second experiment similar to that reported in Table 1 in which extract from tu-h females was injected into wild (Lausanne) females which were crossed with tu-h males

Treatment	Abn. females	+ females	Abn. males	+ males	Percent abnormal
Wild injected $\times$ <i>tu-h</i>	41	122	17	127	19
Control (not injected)	1	55	3	72	3
Ringer's injected	8	132	2	122	4

TABLE 3

F₁ progeny from crosses between wild (Samarkand) females, injected with extract from tu-h females and crossed with tu-h males. Results from controls and reciprocal crosses are included

Treatment	Abn. females	+ females	Abn. males	+ males	Percent abnormal
Wild injected $\times$ <i>tu-h</i>	9	247	8	212	4
Control (not injected)	0	1,041	0	988	0
Ringer's injected	0	206	0	178	0
<i>tu-h</i> larval extract injected	0	66	0	76	0
<i>tu-h</i> egg extract injected	4	90	2	85	3
Wild (Lausanne) extract injected	0	101	0	110	0
<i>tu-h</i> $\times$ Samarkand	16	146	2	176	5

abnormal flies. Extract from Lausanne wild females was injected into Samarkand females which were mated with *tu-h* males. No abnormal flies were produced. Five percent of abnormal flies came from the reciprocal cross between *tu-h* females and Samarkand males.

Results from the first experiment designed to test the temperature effective period are summarized in Table 4. The maternal effect could be greatly increased by changing the temperature from 21°C to 30°C at any of the four-hour intervals between the 8–12 and the 20–24-hour periods after the eggs were deposited. Cultures subjected to increased temperature during the first two intervals (0–4 and 4–8 hours after egg deposition) were not significantly different from the control maintained constantly at 21°C. Results of the next experiment in which the cultures were left at 30°C for different time periods are summarized in Table 5. These results were not significantly different from those of the first experiment (Table 4), indicating that a four-hour interval was sufficient for the full effect.

Results of the oxygen consumption experiment are summarized in Table 6 and shown graphically in Figure 1. Progeny of the two reciprocal crosses (*tu-h* × wild and wild × *tu-h*) show no significant difference in oxygen consumption. Neither

TABLE 4

F₁ progeny from crosses between tu-h females and Lausanne males subjected to 30°C at four-hour intervals during first 24 hours after egg deposition

Time period	No. eggs	Adults classified				Percent abnormal
		Abn. females	+ females	Abn. males	+ males	
0–4 hours	136	17	27	6	24	31
4–8 hours	123	9	14	13	24	37
8–12 hours	174	24	21	47	7	72
12–16 hours	128	30	4	22	13	75
16–20 hours	154	40	12	21	15	69
20–24 hours	123	18	12	14	8	62
Control	131	10	24	21	34	35

TABLE 5

F₁ progeny from crosses between tu-h females and Lausanne males subjected to different time periods at 30°C, each interval beginning with time of mating

Time period	No. eggs	Adults classified				Percent abnormal
		Abn. females	+ females	Abn. males	+ males	
0–4 hours	366	79	215	37	35	32
8 hours	286	64	159	39	24	36
12 hours	421	119	68	161	73	67
16 hours	309	145	55	85	24	74
20 hours	241	100	31	87	23	78
24 hours	263	123	24	64	52	71
Control	578	118	156	128	149	45

TABLE 6

Measurements of oxygen consumed by eggs and larvae of different stocks and crosses given in microliters per egg during four-hour intervals

Time period	Source of samples			
	(1) <i>tu-h</i> × <i>tu-h</i>	(2) + × +	(3) <i>tu-h</i> × +	(4) + × <i>tu-h</i>
(a) 0-4 hours	22.824*	12.896	19.432	25.976
(b) 4-8 hours	18.512	39.496	43.432	47.952
(c) 8-12 hours	12.960	45.312	45.812	53.944
(d) 12-16 hours	46.656	96.712	102.872	115.872
(e) 16-20 hours	30.880	45.168	56.288	53.952
(f) 20-24 hours	31.720	49.208	55.144	58.984

* Oxygen consumption in microliters/per egg/four-hour period.

is there a significant difference between the results of the two reciprocal crosses and those of the wild stock. Analysis of variance showed the time period 12-16 hours (d at the left of Table 6) to be significantly different (one percent level) from c, e, and f. The differences c *vs.* e, f, and e *vs.* f were not significant. The cross 1 (*tu-h* × *tu-h*) sample was significantly different (one percent level) from 2, 3, and 4.

FIGURE 1.—Oxygen consumption of samples of 100 eggs during first 24 hours after eggs were laid (data from Table 6). Upper curve represents the effect of increased temperature during first 24 hours after egg deposition (data from Table 4).

The graph at the top of Figure 1 represents the percentage of abnormal flies produced when cultures from the *tu-h* × wild cross were subjected to 30°C at four-hour intervals (from Table 4). There is a rough parallel between the rate of respiration and the temperature effective period for the tumorous head trait.

Under the conditions of these experiments, no significant differences in head abnormalities were related to humidity, but the relationships previously reported for temperature (GARDNER and WOOLF 1950) were confirmed. The results showed the present level of expression in the tumorous head stock after some 12 years of selection and inbreeding in our laboratory to be about 80 percent at 20°C and 90 percent at 30°C. They also confirmed the difference in reciprocal crosses, upon which the maternal effect hypothesis was based, with two wild stocks (Lausanne and Canton).

DISCUSSION

Transfer of the maternal effect from tumorous head females to wild females by injection of a crude extract supports the hypothesis (GARDNER and WOOLF 1949) that a tangible substance is involved. Previous experiments (GARDNER 1959) have shown that no persistent cytoplasmic factor is carried from generation to generation, but the possibility of a virus or a genoid (L'HERITIER 1951) acting under the control of a gene is not excluded.

The substance injected into the body cavity must be capable of penetrating the developing eggs in the ovary or tubes. KING and BURNETT (1959) have shown from autoradiographic studies that certain chemicals may be taken into the ovary of fruit flies. The *tu-h* substance depends on the sex-linked gene (*tu-1*), and its action is related to the action of the autosomal gene (*tu-3*) which must be present if any expression of the trait occurs in the adult flies. The substance may act by altering the dominance of the autosomal gene (*tu-3*). CAVALCANTI, LAGDEN, FALCAO, and CASTRO (1958) have described the interaction of a nuclear and cytoplasmic component in sex-ratio factor in *Drosophila prosaltans*, and MALOGOLOW-KIN, POULSON, and WRIGHT (1959) have succeeded in transferring a maternally inherited sex-ratio in *D. willistoni*.

From the two injection experiments reported here, 18 and 19 percent of the progeny of injected females and *tu-h* males (simplex for *tu-1* and homozygous for *tu-3*) were abnormal. These results may be compared with 31 percent for the progeny of *tu-h* females (homozygous for *tu-1* and *tu-3*) and wild males. F₁ flies from both of these crosses would carry one *tu-3* gene; those from *tu-h* mothers showed the characteristic maternal effect, and those which were injected with *tu-h* maternal extract showed the same effect in a lesser but significant degree.

Tumorous head flies have now been crossed reciprocally with those of numerous laboratory stocks and wild populations. Most results have followed a fairly uniform pattern. About 30 percent of the progeny from the cross, *tu-h* females × wild males, were abnormal compared with one percent or less from the reciprocal cross, wild females × *tu-h* males. Some variations, presumably due to the genetic background in the stock to which outcrosses are made, have been obtained.

Stephenville females mated with *tu-h* males produced the highest proportion (89 percent) of abnormal flies (GARDNER and STOTT 1951). From the reciprocal cross between Stephenville males and *tu-h* females, 20 percent of abnormal flies were obtained. No abnormal flies have been obtained from the cross: Samarkand females $\times$ *tu-h* males, and only five percent of abnormal flies was obtained from the reciprocal cross. The genetic background in Samarkand is evidently better buffered against these abnormal expressions or more favorable for normal expression as compared with the genotypes of other stocks. Samarkand females injected with extract from *tu-h* females and mated with *tu-h* males produced four percent of abnormal flies. When an extract was prepared from eggs of *tu-h* females and injected into Samarkand females which were subsequently mated with *tu-h* males, three percent of the progeny was abnormal. Injection with *tu-h* larval extract had no effect; the results were similar to the uninjected controls in which no abnormal flies appeared.

Two approaches are being made to obtain data concerning the nature of the substance and the mechanics of the maternal effect. (1) Extracts are being refined and separated into their chemical components. Each component is being tested for transmission of the maternal effect. (2) The temperature effective period is being investigated more critically to see if some aspect of metabolic activity might suggest how the maternal effect operates.

To define the temperature effective period more narrowly than the 24-hour period detected in the previous study (GARDNER and WOOLF 1950), it was necessary to collect eggs rather than to transfer whole cultures to different temperature incubators. Laying cages were used to obtain large numbers of eggs from the same parents within a short time period. The results showed that any four-hour interval between 8–12 and 20–24 hours after the eggs were deposited was as effective as the full 24 hours at the higher temperature. The temperature effective period indicates that some chemical activity associated with the abnormality is in progress during this early period of development.

The over-all pattern of oxygen consumption conformed to that obtained by VAVALA (1954). In our experiments there was no significant difference between the results of the reciprocal crosses (*tu-h* $\times$ wild and wild $\times$ *tu-h*), and these results did not differ from those of the wild stock. The sample from *tu-h* consumed considerably less oxygen than the other three samples. This is probably because of the decreased viability of *tu-h* flies, particularly in the egg and larval stages (GARDNER and RATTY 1952). If there were fewer living larvae in the *tu-h* sample after the initial stage of the experiment compared with other samples, the amount of oxygen consumed would be considerably less than that of the other samples. Comparison between the graphs for the heat sensitive period of the *tu-h* $\times$ wild cross and the high oxygen consumption indicates that the heat sensitive period begins earlier and extends longer than the period of highest oxygen consumption.

SUMMARY

A crude extract, prepared from the bodies of tumorous head females, was injected into wild (Lausanne) females which were subsequently mated with tumorous head males. Eighteen and 19 percent of the progeny from two different

experiments expressed the tumorous head phenotype compared with one to four percent for the controls. This result supports the hypothesis that a tangible, transmissible substance is associated with the maternal effect. Similar injection experiments were conducted with the wild Samarkand stock. Four percent of the progeny from *tu-h* injected Samarkand females crossed with tumorous head males were abnormal compared with none for the uninjected controls. The temperature effective period during the first 24 hours of development was defined more narrowly. Four-hour intervals at 30°C between the 8–12 and 20–24-hour intervals after egg deposition were found to be as effective as longer periods at the higher temperature. Measurement of oxygen consumption during the first 24 hours of development showed a peak for the period 12–20 hours after egg deposition.

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