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Utilization of Mating Behavior as a Parameter to Understand Adaptive Response in *Drosophila melanogaster* using Ethyl Methanesulfonate and Methyl Methanesulfonate

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Abstract

Monofunctional alkylating agents, Ethyl Methanesulfonate (EMS) and Methyl Methanesulfonate (MMS) were used to understand adaptive response utilising mating behaviour as a parameter in *D. melanogaster*. Selected conditioning and challenging doses of EMS (0.5mM and 15mM) or MMS (0.1mM and 3mM) by larval feeding were tested employing different combinations of crosses. The results have revealed that both EMS and MMS affected courtship elements significantly in different combinations of crosses. Nonetheless, significant increases in orientation, tapping, wing vibration and licking were observed when both males and females were treated with a challenging dose of MMS compared to EMS ($p<0.05$). *On par* with this, were also the results of female rejection elements in both the tested chemicals. When conditioning and challenging doses were given after 2 hours of time lag between them to 48±4h or 72±4h aged larvae of *D. melanogaster*, the results showed that male and female courtship elements significantly reduced compared to the additive effect of respective agents. Similarly, the courtship latency and copulation latency were significantly decreased in contrast to copulation duration which was significantly increased ($p<0.05$). Thus the results demonstrate the presence of adaptive response in *D. melanogaster* using courtship elements and the authors opine that mating behaviour can be used as a parameter to analyze adaptive response in *D. melanogaster* within a short period of time compared to other test procedures.

Keywords: Adaptive Response, Challenging Dose, Combined Dose, Conditioning Dose, Courtship Elements, *Drosophila melanogaster*, EMS, MMS, Mating Behaviour

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1. Introduction

Over a century ago, the first description of the courtship behaviour of *Drosophila* was given by Sturtevant[1]. Since then, this parameter was intensively studied and detailed courtship behaviour of *D. melanogaster* was described by Spieth[2] and Bastock and Manning[3]. Despite over 75 years of intensive investigation of our understanding of the genotoxicity[4] in *D. melanogaster*, the utility of mating behaviour in genotoxicity is rudimentary. Very recently, Vasudev and his co-workers proved beyond doubt the utility of *D. melanogaster* courtship behaviour as a parameter to understand toxicity by using standard mutagens-EMS[5] and MMS[6]; and also a pesticide- Dithane M-45[7]. These agents are having a lot of literature with reference to genotoxicity in the spectrum of test systems and mutagenicity, in particular, has been demonstrated in *E. coli*, *D. melanogaster* and mouse and in *in vitro* systems of CHO, human lymphocytes, V-79 (*Cf.* Sega[8]; Khalandar[9]) using EMS and MMS.

Historically, behaviour analysis revealed that how *fruitless* and *doublesex* of *D. melanogaster* regulate courtship in males and females expressing mutations at the respective loci[10]. Nonetheless, recent analysis found that 7 (31.8%) of the 22 human genes were essential for male fertility in *D. melanogaster*[11]. As far as an adaptive response is considered since its discovery[12], a lot of work has been carried out starting from viruses to mammals including human systems using varied protocols[13] except for the mating behaviour of *D. melanogaster.* Hence, present studies have been taken up to 1) understand the utility of mating behaviour in adaptive response 2) get a picture of the interaction of genes and environmental agents in mating behaviour 3) extrapolate the results of this with the human systems, if any, to draw better conclusions on the genetics of attraction between mating pairs and 4) foresee the mating behavioural abnormalities of *D. melanogaster* due to the exposure to environmental agents, in humans to screen the connection between male infertility etc., which is very important in these days.

2. Materials and Methods

In all experiments, the OK strain of *D. melanogaster* has been used as the material and the same has been obtained from the *Drosophila* Stock Centre, Department of Zoology, University of Mysore, Mysore. The flies were maintained in the BOD incubator at 24±1 °C with a normal wheat cream agar medium. The well-known monofunctional alkylating agents, Ethyl methanesulfonate (EMS: CAS No. 62-50-0) and Methyl methanesulfonate (MMS: CAS No. 66-27-3), were obtained from Sigma Chemical Company, USA and were used as the genotoxic agents to study the courtship behaviour and occurrence of adaptive response in *D. melanogaster*. Other routine chemicals were from BDH Chemical Ltd., Mumbai.

2.1 Preparation of Media

- **Control Media**: Normal standard wheat cream agar food medium was used as control. This was prepared according to the procedure of Vasudev and Gurushankara[14].
- **Chemical Media**: The required concentrations of EMS or MMS in food medium were prepared according to the method of Vasudev and Krishnamurthy[15]. This was distributed to food vials in equal quantities and was used as chemical media for all experiments.
- **Delcour Media**: Delcour[16] technique was employed to prepare this media.
- **Collection of Eggs**: The modified procedure of Delcour was used to collect the same age of eggs and larvae[15].

2.2 Concentrations Selected

As has been in practice to understand the adaptive response, there is the necessity for the selection of conditioning and challenging doses of selected agents[17]. From the pilot toxicity studies of Divyashree *et al.,*[5], 0.5mM as the conditioning dose and 15mM of EMS as a challenging dose were selected. Similarly, from the studies of Divyashree and Vasudev[6] 0.1 and 3 mM as conditioning and challenging doses of MMS were employed in the present studies.

2.3 Treatment Schedules

Treatment schedules adapted for the present study are as follows:

Control: Eggs were allowed to develop in a normal food medium.

Conditioning Dose (L): The larvae were fed with media containing 0.5mM of EMS or 0.1mM of MMS and allowed to develop in the same media for the rest of life.

Challenging Dose (H): The larvae were fed with media containing 15mM of EMS or 3mM of MMS and allowed to develop in the same media for their rest of life.

Combined Treatment (L-H): In this treatment schedule, the larvae were fed with conditioning dose of 0.5mM or 0.1mM of EMS or MMS for 48±4h. After 2h of time lag, the same larvae were transferred to the vials containing a challenging dose (15mM or 3mM) of EMS or MMS and allowed to develop in the same media for the rest of life. Similar treatment schedules were carried out using 72±4h aged larvae.

2.4 Experimental Protocols

- **Control:** Untreated male and female flies were released into mating chambers.
- **Female Treated:** Females treated with EMS (0.5mM, 15mM or 0.5mM+15mM) or MMS (0.1mM, 3mM or 0.1mM+3mM) were released with control male into the mating chamber.
- **Male Treated:** Males treated with EMS (0.5mM, 15mM or 0.5mM+15mM) or MMS (0.1mM, 3mM or 0.1mM+3mM) were released with control female into the mating chamber.
- **Both Treated:** Both male and female flies treated with EMS (0.5mM, 15mM or 0.5mM+15mM) or MMS (0.1mM, 3mM or 0.1mM+3mM) were released into the mating chamber.

In all the above experimental protocols the qualitative and quantitative aspects of mating elements of males and females were analysed and recorded. Twenty-five pairs were observed for each experiment, and we repeated the experiments thrice. We recorded 75 observations each for treated and control groups.

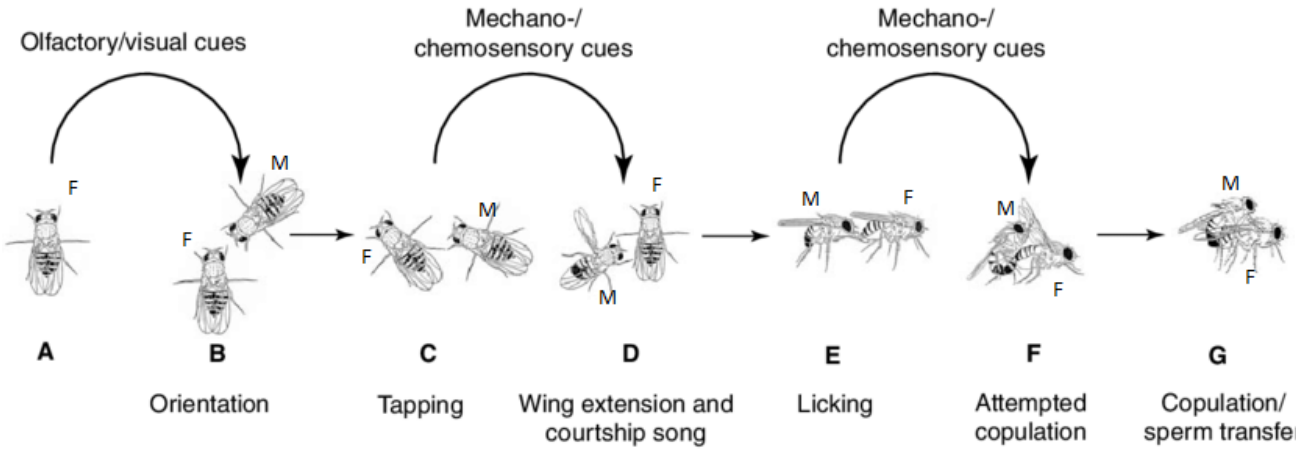


Photo 1. Courtship steps performed by male *D. melanogaster*. Image modified from Billeter *et al.*,[18] (M- male; F- female).

2.5 Rate of Development and Viability

To understand the adaptive response of EMS and MMS with respect to the rate of development and viability, an equal number of eggs (100) and same age (±6h) eggs that were collected from Delcour technique, were placed into each bottle containing media of different treatment schedules of EMS or MMS and allowed to develop. For combined treatment, after treatment of conditioning dose for 48±4h, the larvae were floated out from the food media using 0.7% NaCl solution (Graf *et al*[19]). 100 larvae were transferred into quarter-pint milk bottles containing standard *Drosophila* food mixed with a challenging dose of EMS or MMS after a 2h time lag. Control larvae were placed in quarter-pint milk bottles containing normal food only. Then larvae were maintained in the same bottles throughout their larval period in BOD incubator. The emerged flies were counted and sexed every day from the eclosion of flies to the last day of emergence in each treated group of EMS or MMS. Similar experiments were carried out using 72±4h aged larvae.

2.6 Isolation of Virgin Flies for Mating

The virgin females and bachelor males were isolated within three to four hours of the adult fly emergence

from the pupated bottles and maintained separately in a normal medium. They were aged for five days to observe the courtship behaviour.

2.7 Courtship Behavioural Assay

Courtship assay was conducted during the morning hours (7-10 AM) every day in a room with a temperature of 24±1 °C under normal laboratory light conditions. In controls, the single virgin female was introduced into the Elens-Wattiaux[20] mating chamber without etherizing them, using an aspirator. A bachelor male was added to it and allowed to acclimatize to the chamber for 30 sec. The details of courtship behaviour were directly observed through a hand lens of 10x magnification. The respective flies were introduced into the observation chamber depending upon the experimental protocols mentioned above. Courtship activities such as orientation, tapping, wing vibration and licking which are male-specific activities[10] and ignoring, extruding, and decamping which are female-specific activities[21] and copulation duration were qualitatively and quantitatively recorded separately in the control and treated groups. From these data, courtship latency and copulation latency were analyzed and tabulated. Continuous observations were made for 30 minutes, then if there had been no copulation, that was considered as unmated, and that pair was replaced by fresh pair. The mating chamber was washed every time after each observation to remove sex pheromones of previous couples if any.

2.8 Statistical Analysis

The data obtained from the individual experiments were subjected to statistical analysis to determine the significance level between the control and treatment groups. The data were expressed as Mean±SE and compared using a one-way Analysis of Variance (ANOVA). Comparisons among groups were analyzed according to Tukey's post hoc test using the SPSS statistical package version 23.0.

3. Results

Challenging dose of EMS or MMS has significantly increased rate of development and reduced viability compared to conditioning dose and control (Table 1, $p<0.05$). On the other hand, the same table reveals that when combined treatment of EMS or MMS was given at 48±4h or 72±4h, the rate of development was significantly reduced whereas viability was increased compared to the additive effect (Table 1, $p<0.05$). Furthermore, there was a significant difference between 48±4h and 72±4h treatments. Whether it was male or female or both treated, the male courtship elements were significantly increased both in conditioning and challenging doses of EMS or MMS except the licking element in the male treatment of EMS or MMS compared to controls (Tables 2-7, $p<0.05$). It is interesting to note that in the combined treatments of EMS or MMS at 48±4h or 72±4h aged larvae, all the male courtship elements were significantly decreased compared to additive effect. The courtship latency and copulation latency have been shown to be significantly extended by conditioning and challenging doses of EMS or MMS compared to controls in all the experimental treated groups. Contrary to this, the combined treatments to 48±4h or 72±4h aged larvae revealed significantly reduced frequencies of courtship latency and copulation latency compared to the additive effect (Tables 2-7, $p<0.05$). The conditioning dose of 0.5mM and challenging dose of 15mM EMS produced copulation duration of 17.63±0.11min and 11.47±0.13min respectively in both male and female treated groups compared to 19.35±0.05min of control (Table 4, $p<0.05$). Similar results were obtained when MMS was used as conditioning and challenging doses (Table 7, $p<0.05$). However, in the combined treatments of EMS or MMS of different combinations of crosses, the copulation duration was significantly increased at 48±4h or 72±4h aged larvae compared to respective challenging doses (Table 8, $p<0.05$). For example, in the case of combined treatment of EMS, the copulation duration was 13.77±0.05 min compared to 11.47±0.13 min of challenge dose at 48h larval age. However, in the combined treatment of MMS, the copulation duration was 12.44±0.07 min compared to 10.44±0.25 min of challenge dose at the same duration of treatment. The studied female rejection elements such as ignoring, extruding, and decamping showed a significant increase in both conditioning and challenging doses of respective agents compared to controls (Figures 1 and 2; $p<0.05$). In contrast, in combined treatments at 48±4h or 72±4h, frequencies of rejection elements were significantly reduced compared to challenging doses of tested agents (Figures 1 and 2; $p<0.5$).

Table 1. The presence of adaptive response in developmental time and viability of *D. melanogaster* observed after combined treatment of EMS or MMS

Treatment	EMS				MMS			
	MDT for group		% Viability		MDT for group		% Viability	
Control	10.03±0.02[a]		95.44±0.29[a]		10.03±0.02[a]		95.44±0.29[a]	
Conditioning (L)	10.32±0.10[a]		93.44±0.29[b]		10.43±0.04[a]		92.44±0.29[b]	
Challenging (H)	16.76±0.26[d]		53.22±0.11[e]		16.07±0.24[d]		51.55±0.29[e]	
Combined (L-48h-H)	12.60±0.34[c]		77.99±0.19[d]		13.11±0.24[c]		72.11±0.48[d]	
Combined (L-72h-H)	11.61±0.09[b]		85.77±0.39[c]		12.31±0.15[b]		79.77±0.11[c]	
# Reduction (or) Increase	% Reduction of MDT		% Increase of viability		% Reduction of MDT		% Increase of viability	
	48h	53.47	48h	46.82	48h	50.52	48h	49.92
	72h	57.12*	72h	41.51*	72h	53.54*	72h	44.60*

Note: EMS: L-0.5mM, H-15mM; MMS: L-0.1mM, H-3mM. Data of 3 independent experiments; 300 eggs were used per experiment and a total of 900 eggs were analyzed per dose. MDT: Mean developmental time (in days). Mean with SE are given. Values with the same superscripts are not significantly different ($p>0.05$), whereas values with different superscripts are significantly different ($p<0.05$) from one another. # significant compared to an additive effect. Formula to analyze the Reduction (or) Increase: (Combined dose (L, H)/Additive (L+H) × 100) – 100. *: $p<0.05$ significant difference between 48h and 72h age groups.

Table 2. Frequency of courtship elements of *D. melanogaster* male treated with conditioning, challenging and combined doses of EMS and mated to normal female

Treatment	Orientation (in no.)	Tapping (in no.)	Wing vibration (in no.)	Licking (in no.)	Courtship latency (in sec.)	Copulation latency (in min.)	Copulation duration (in min.)
Control	4.58±0.07[a]	3.62±0.07[a]	4.64±0.05[a]	2.43±0.04[a]	10.80±0.16[a]	1.51±0.02[a]	19.35±0.05[a]
Conditioning (L) (0.5mM)	5.36±0.26[b]	4.43±0.32[b]	5.91±0.26[b]	2.98±0.37[a]	16.28±0.42[b]	2.87±0.23[b]	18.37±0.11[b]
Challenging (H) (15mM)	25.93±0.27[e]	19.69±0.25[e]	21.23±0.20[e]	14.05±0.25[d]	35.77±0.25[e]	10.66±0.26[d]	12.79±0.03[e]
Combined (L-48h-H)	19.97±0.27[d]	14.88±0.23[d]	17.01±0.20[d]	8.90±0.18[c]	26.05±0.41[d]	6.76±0.25[c]	14.45±0.07[d]
Combined (L-72h-H)	17.09±0.27[c]	12.83±0.22[c]	13.97±0.27[c]	7.81±0.40[b]	20.97±0.27[c]	6.01±0.28[c]	15.59±0.23[c]
#Reduction (%)	48h 36.17	38.30	37.32	47.73	49.95	50.03	_
	72h 45.38*	46.80*	48.52*	54.13*	59.71*	55.58*	_

Note: Data of 3 independent experiments; 25 pairs per experiment were used; Mean with SE are given. Values with the same superscripts are not significantly different ($p>0.05$), whereas values with different superscripts are significantly different ($p<0.05$) from one another. # significant compared to an additive effect. Reduction % = (Combined dose (L, H) / Additive (L+H) × 100) – 100. *: $p<0.05$ significant difference between 48h and 72h age groups.

Table 3. Frequency of courtship elements of *D. melanogaster* female treated with conditioning, challenging and combined doses of EMS and mated to normal male

Treatment		Orientation (in no.)	Tapping (in no.)	Wing vibration (in no.)	Licking (in no.)	Courtship latency (in sec.)	Copulation latency (in min.)	Copulation duration (in min.)
Control		4.58±0.07^{a}	3.62±0.07^{a}	4.64±0.05^{a}	2.43±0.04^{a}	10.80±0.16^{a}	1.51±0.02^{a}	19.35±0.05^{a}
Conditioning (L) (0.5mM)		6.59±0.26^{b}	5.49±0.27^{b}	6.80±0.34^{b}	3.69±0.32^{b}	14.37±0.25^{b}	2.86±0.04^{b}	18.67±0.05^{a}
Challenging (H) (15mM)		27.65±0.25^{e}	22.01±0.25^{e}	23.82±0.27^{e}	15.82±0.15^{e}	32.72±0.20^{e}	12.78±0.28^{e}	13.55±0.04^{d}
Combined (L-48h-H)		21.98±0.23^{d}	15.99±0.17^{d}	19.09±0.26^{d}	9.76±0.22^{d}	23.92±0.28^{d}	8.05±0.23^{d}	15.72±0.26^{c}
Combined (L-72h-H)		18.89±0.20^{c}	14.06±0.26^{c}	15.83±0.26^{c}	8.92±0.20^{c}	18.63±0.22^{c}	6.74±0.26^{c}	16.73±0.26^{b}
# Reduction (%)	48h	35.80	41.85	37.65	49.97	49.20	48.52	_
	72h	44.83*	48.87*	48.30*	54.27*	60.43*	56.90*	_

Note: Data of 3 independent experiments; 25 pairs per experiment were used; Mean with SE are given. Values with the same superscripts are not significantly different ($p>0.05$), whereas values with different superscripts are significantly different ($p<0.05$) from one another. # significant compared to additive effect. Reduction% = (Combined dose (L, H) / Additive (L+H) × 100) – 100. *: $p<0.05$ significant difference between 48h and 72h age groups.

Table 4. Frequency of courtship elements of *D. melanogaster* both male and female treated with conditioning, challenging and combined doses of EMS

Treatment		Orientation (in no.)	Tapping (in no.)	Wing vibration (in no.)	Licking (in no.)	Courtship latency (in sec.)	Copulation latency (in min.)	Copulation duration (in min.)
Control		4.58±0.07^{a}	3.62±0.07^{a}	4.64±0.05^{a}	2.43±0.04^{a}	10.80±0.16^{a}	1.51±0.02^{a}	19.35±0.05^{a}
Conditioning (L) (0.5mM)		8.70±0.26^{b}	6.79±0.37^{b}	7.63±0.40^{b}	4.71±0.26^{b}	18.86±0.29^{b}	3.70±0.28^{b}	17.63±0.11^{b}
Challenging (H) (15mM)		30.77±0.25^{e}	24.89±0.36^{e}	26.41±0.28^{e}	18.84±0.25^{e}	38.09±0.26^{e}	13.83±0.24^{d}	11.47±0.13^{e}
Combined (L-48h-H)		25.09±0.24^{d}	17.82±0.36^{d}	20.99±0.36^{d}	11.99±0.41^{d}	28.76±0.39^{d}	9.90±0.42^{c}	13.77±0.05^{d}
Combined (L-72h-H)		21.86±0.37^{c}	14.87±0.35^{c}	17.85±0.35^{c}	9.89±0.34^{c}	24.85±0.39^{c}	8.99±0.35^{c}	14.57±0.12^{c}
# Reduction (%)	48h	36.43	43.75	38.33	49.08	49.49	43.52	_
	72h	44.61*	53.06*	47.56*	58.00*	56.36*	48.71*	_

Note: Data of 3 independent experiments; 25 pairs per experiment were used; Mean with SE are given. Values with the same superscripts are not significantly different ($p>0.05$) whereas values with different superscripts are significantly different ($p<0.05$) from one another. # significant compared to an additive effect. Reduction % = (Combined dose (L, H) / Additive (L+H) × 100) – 100. *: $p<0.05$ significant difference between 48 and 72h age groups.

Table 5. Frequency of courtship elements of *D. melanogaster* male treated with conditioning, challenging and combined doses of MMS and mated to normal female

Treatment		Orientation (in no.)	Tapping (in no.)	Wing vibration (in no.)	Licking (in no.)	Courtship latency (in sec.)	Copulation latency (in min.)	Copulation duration (in min.)
Control		4.58±0.07[a]	3.62±0.07[a]	4.64±0.05[a]	2.43±0.04[a]	10.80±0.16[a]	1.51±0.02[a]	19.35±0.05[a]
Conditioning (L) (0.1mM)		7.07±0.53[b]	5.12±0.12[b]	6.60±0.54[b]	3.47±0.29[a]	17.34±0.56[b]	2.71±0.17[b]	17.88±0.10[b]
Challenging (H) (3mM)		29.15±0.27[e]	23.54±0.25[e]	26.61±0.28[e]	18.71±0.56[d]	37.12±0.69[e]	12.40±0.42[d]	11.44±0.25[e]
Combined (L-48h-H)		22.13±0.22[d]	16.57±0.34[d]	19.47±0.42[d]	11.73±0.14[c]	27.54±0.46[d]	5.27±0.41[c]	13.47±0.39[d]
Combined (L-72h-H)		18.73±0.13[c]	14.82±0.20[c]	16.66±0.19[c]	9.78±0.11[b]	21.20±0.18[c]	4.43±0.05[c]	14.67±0.04[c]
# Reduction (%)	48h	38.90	42.18	41.37	47.11	49.43	65.12	_
	72h	48.28*	48.29*	49.83*	55.90*	61.07*	70.68*	_

Note: Data of 3 independent experiments; 25 pairs per experiment were used; Mean with SE are given. Values with the same superscripts are not significantly different (p>0.05) whereas values with different superscripts are significantly different (p<0.05) from one another. # significant compared to an additive effect. Reduction % = (Combined dose (L, H) / Additive (L+H) × 100) – 100. ***:** p<0.05 significant difference between 48h and 72h age groups.

Table 6. Frequency of courtship elements of *D. melanogaster* female treated with conditioning, challenging and combined doses of MMS and mated to normal male

Treatment		Orientation (in no.)	Tapping (in no.)	Wing vibration (in no.)	Licking (in no.)	Courtship latency (in sec.)	Copulation latency (in min.)	Copulation duration (in min.)
Control		4.58±0.07[a]	3.62±0.07[a]	4.64±0.05[a]	2.43±0.04[a]	10.80±0.16[a]	1.51±0.02[a]	19.35±0.05[a]
Conditioning (L) (0.1mM)		8.25±0.20[b]	6.19±0.17[b]	7.40±0.33[b]	4.42±0.25[b]	16.47±0.36[b]	3.12±0.14[b]	18.57±0.15[b]
Challenging (H) (3mM)		31.57±0.24[e]	26.89±0.21[e]	28.79±0.11[e]	20.76±0.22[e]	36.52±0.47[e]	13.25±0.19[e]	12.32±0.25[e]
Combined (L-48h-H)		24.32±0.23[d]	20.39±0.24[d]	22.66±0.07[d]	13.18±0.17[d]	25.20±0.14[d]	6.43±0.06[d]	14.43±0.04[d]
Combined (L-72h-H)		20.28±0.27[c]	17.25±0.21[c]	19.21±0.22[c]	10.41±0.28[c]	20.77±0.49[c]	5.74±0.07[c]	15.34±0.06[c]
# Reduction (%)	48h	38.92	38.36	37.38	47.65	52.44	60.72	_
	72h	49.07*	47.85*	46.91*	58.65*	60.80*	64.93*	_

Note: Data of 3 independent experiments; 25 pairs per experiment were used; Mean with SE are given. Values with the same superscripts are not significantly different (p>0.05) whereas values with different superscripts are significantly different (p<0.05) from one another. # significant compared to an additive effect. Reduction % = (Combined dose (L, H) / Additive (L+H) × 100) – 100. ***:** p<0.05 significant difference between 48h and 72h age groups.

Table 7. Frequency of courtship elements of *D. melanogaster* both male and female treated with conditioning, challenging and combined doses of MMS

Treatment		Orientation (in no.)	Tapping (in no.)	Wing vibration (in no.)	Licking (in no.)	Courtship latency (in sec.)	Copulation latency (in min.)	Copulation duration (in min)
Control		4.58±0.07[a]	3.62±0.07[a]	4.64±0.05[a]	2.43±0.04[a]	10.80±0.16[a]	1.51±0.02[a]	19.35±0.05[a]
Conditioning (L) (0.1mM)		10.66±0.24[b]	7.38±0.43[b]	8.87±0.15[b]	5.11±0.19[b]	18.45±0.47[b]	3.96±0.07[b]	16.69±0.24[b]
Challenging (H) (3mM)		34.62±0.42[e]	28.86±0.35[e]	31.76±0.30[e]	24.17±0.19[e]	39.70±0.70[e]	14.40±0.42[e]	10.44±0.25[e]
Combined (L-48h-H)		27.28±0.24[d]	23.19±0.22[d]	25.33±0.23[d]	14.56±0.23[d]	31.17±0.17[d]	8.46±0.09[d]	12.44±0.07[d]
Combined (L-72h-H)		23.31±0.19[c]	20.77±0.47[c]	21.54±0.46[c]	12.34±0.07[c]	28.72±0.15[c]	7.27±0.23[c]	13.51±0.07[c]
# Reduction (%)	48h	39.75	36.00	37.65	50.27	50.61	53.92	_
	72h	48.52*	42.68*	46.98*	57.85*	46.39*	60.40*	_

Note: Data of 3 independent experiments; 25 pairs per experiment were used; Mean with SE are given. Values with the same superscripts are not significantly different (p>0.05) whereas values with different superscripts are significantly different (p<0.05) from one another. # significant compared to an additive effect. Reduction % = (Combined dose (L, H) / Additive (L+H) × 100). *: p<0.05 significant difference between 48h and 72h age groups.

Table 8. Percentage increase in copulation duration in *D. melanogaster* after combined doses of EMS or MMS at 48 and 72h larval age compared to the challenging dose

Groups	EMS					MMS				
	Challenging dose (15mM) (A)	Combined treatment of conditioning and challenging dose (0.5+15mM) (B)		% Increase (C)		Challenging dose (3mM) (A)	Combined treatment of conditioning and challenging dose (0.1+3mM) (B)		% Increase (C)	
		48h	72h	48h	72h		48h	72h	48h	72h
Male treated	12.79±0.03	14.45±0.07[a]	15.59±0.23[b]	12.97	21.89[c]	11.44±0.25	13.47±0.39[a]	14.67±0.04[b]	17.74	28.23[c]
Female treated	13.55±0.04	15.72±0.26[a]	16.73±0.26[b]	16.01	23.46[c]	12.32±0.25	14.43±0.04[a]	15.34±0.06[b]	17.12	24.51[c]
Both male and female treated	11.47±0.13	13.77±0.05[a]	14.57±0.12[b]	20.05	27.02[c]	10.44±0.25	12.44±0.07[a]	13.51±0.07[b]	19.15	29.40[c]

Note: Data from Tables-2-7; Percentage of increase was calculated by using formula C = (B/A × 100) – 100. **a:** p<0.05 significant difference between challenging dose and 48h combined treatment **b:** p<0.05 significant difference between challenging dose and 72h combined treatment **c:** p<0.05 significant difference between 48h and 72h age groups.

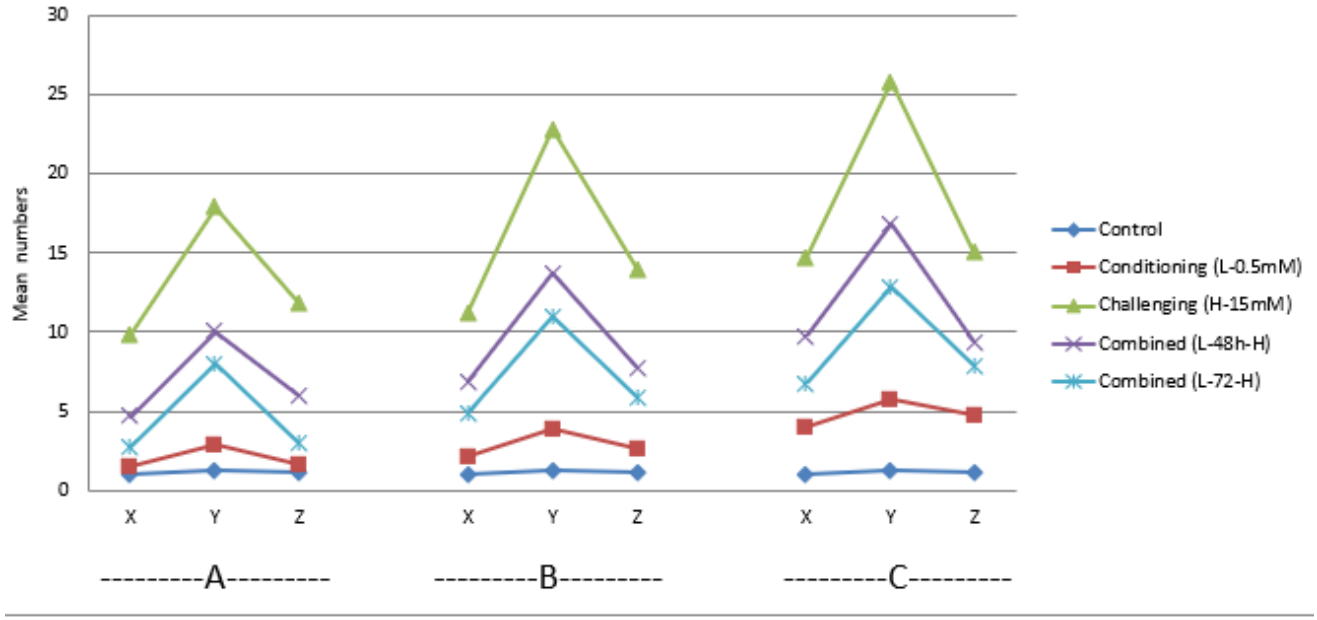


Figure 1. Female rejection courtship elements of *D. melanogaster* were observed after conditioning, challenging and combined treatment of EMS. **(A)** Treated male × Normal female **(B)** Treated female × Normal male **(C)** Treated male × Treated female; X- Ignoring; Y- Extruding; Z- Decamping.

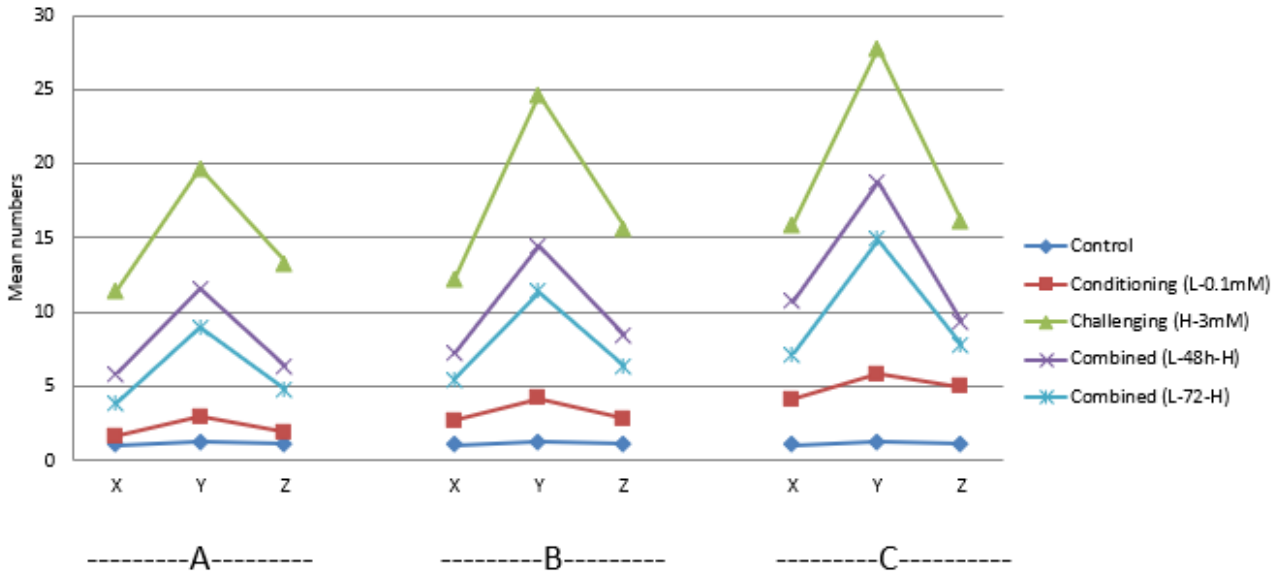


Figure 2. Female rejection courtship elements of *D. melanogaster* were observed after conditioning, challenging and combined treatment of MMS. **(A)** Treated male × Normal female **(B)** Treated female × Normal male **(C)** Treated male × Treated female; X- Ignoring; Y- Extruding; Z-Decamping.

4. Discussion

Because of experiments of Muller[22] and Luning[23], it was possible to use viability and rate of development as parameters of toxicity and the same has been demonstrated in the present studies by using conditioning and challenging doses of EMS or MMS (Table 1, $p<0.05$). What is surprising in these investigations is that when a conditioning dose is given prior to challenging, the rate of development has been reduced and the viability has been increased irrespective of treatment schedules i.e., to 48h or 72h old larvae showing the reduced effect of challenging dose. After 45 years of adaptive response research[12], this is the first report to show the presence of adaptive response using toxicity parameters such as rate of development and viability in *D. melanogaster,* even though many reports are available on the existence of this phenomenon employing mutagenicity parameters (*Cf.* Khalandar and Vasudev[17]). However, Samson and Cairns[12] and Jeggo *et al.,*[24] showed in *E. coli*, the presence of adaptive response in lethality using MNNG. Similarly, Samson and Schwartz[25] in CHO revealed that repeated pre-treatment with low doses of MNNG significantly elevated survival of cells toxicity induced by challenge dose. Karran *et al.,*[26] employing MNNG and MNU analysed the occurrence of adaptive response in human fibroblast cell survival. Morohoshi and Munakata[27] have also reported that the cells of *Bacillus subtilis* grown in MNNG at a non-toxic concentration acquired resistance to lethality when given the toxic dose. Laval[28] demonstrated that pre-treatment of H_4 (rat hepatoma) cells for 48h with non-toxic doses of alkylating agents: - MMS and MNNG, rendered the cells more resistant to the toxic effects of these compounds. Even in *Vibrio cholerae*, resistance to the lethal effects of challenging doses was shown by Bhattacharya[29] and Basak[30].

Mating behaviour is a complex trait with multiple interacting units having small individual effects whose expression depends on the environment. Male courtship behaviour is composed of fixed stereotypical sequences of action patterns and thus is an innate program that can be generated practically without the involvement of learning[3,31,32]. When giving the full description of courtship elements of males by Spieth[2] and Bastock and Manning[3] classified into three basic elements namely (1) orientation (2) vibration and (3) licking culminating in copulation. Recent workers divided the ritual of male courtship behaviour into six elements that occur in a fixed order: orientation, tapping, wing vibration, licking, attempt to copulate and copulation (*Cf.* Villella and Hall[10]). Further, Hall[33] and Greenspan[34] showed that individual components of the behaviour can be quantified and separated and hence these elements were used in the present investigations to understand adaptive response and the effects of environmental agents such as EMS and MMS. Further, the present results, of the control and treated groups were like that described by earlier workers. This means to say that these agents did not affect the courtship behaviour qualitatively. However, when the data were analysed, the authors were able to show quantitative differences. Probable reasons for much difference are discussed in the foregoing section.

Numerous genes are involved in the differences in morphological, physiological, and behavioural aspects of males and females and undoubtedly reflect sex-specific activities, especially in courtship behaviour. Studies on courtship behaviour using two genes namely *fruitless (fru)* which is expressed in males and *double sex (dsx)* which is expressed in females in *D. melangoster* showed how *fru* and *dsx* regulate courtship in males and females expressing mutations[10,35,36]. Nonetheless, given the complexity of *Drosophila* courtship behaviour, it is not surprising that mutation in genes affects multiple biological processes including mating behavior[37,38]. These also include mutations in genes required for normal development. Therefore, recently the study of courtship behaviour has been widely used to explore the genetic basis and the role of environmental factors on fly reproductive behaviour, as *D. melanogaster* is one of the best model systems and the results of which can be extrapolated to the human system[10,39,40].

In courtship behaviour, the first basic element is orientation where a male finds a female and he directs his body axis directly towards her[2,41-43] using chemical, visual, and olfactory signals[32,41]. In eye colour mutants, because of defects in vision which tend to affect orientation in relation to females in turn in the termination of tracking and consequently the whole of courtship is affected[43]. Gene *fru* is expressed in 15% of Olfactory Receptor Neurons (ORNs) on the third antennal segment, which innervates olfactory trichoid sensilla[44,45], if blocked, it reduces male courtship. Thus, shows that these are important in detecting sex pheromones. ORNs that express *ir84a* (ionotropic glutamate receptors) have been shown to co-express *fruM*. *ir84a* mutant males court females at diminished levels, while restoring

ir84a function re-establishes normal courtship levels implicating *ir84a* in the regulation of male courtship[46]. Further, cuticular hydrocarbons are also known to play an important role as contact pheromones in *D. melanogaster* mate recognition[47,48]. In *D. melanogaster* many studies have shown that some female-specific long-chain hydrocarbons especially C27 dienes are attractive substances for males and some of the male components, cis-Vaccenyl acetate (cVA) and 7-tricones (7:C23) are shown to reduce the female attractiveness[47,48]. It is also shown that dienes produced only in females, stimulate male courtship[47,49,50]. The monoenes (7 tricosene; 7T) and 5 tricosene (5T) produced in males tend to inhibit male courtship[48-50]. Further, cVA sensed by Or67d ORNs in both males and females inhibits male courtship[51,52]. 7, 11-heptocosodiene[49], a 27-carbon (27C) hydrocarbon and 9 pentacosadiene (25C) produced in the females, have been shown to stimulate males to court target flies[53]. This is because olfactory receptor genes (*ors*) of odour receptors in the form of olfactory epithelia on antennae containing 1200 Olfactory Receptor Neurons (ORN) and maxillary palps[54,55] are expressed. From these discussions, it can be inferred in the present studies that EMS or MMS has significantly affected the genes and pheromones, in terms of olfactory and visual signals which might have heightened levels of orientation towards females (Tables 2-7; $p<0.05$). Similar results were demonstrated by using different chemicals in *D. melanogaster* [5-7,56-58]. But it is interesting to note that when the flies were pretreated with a conditioning dose of EMS or MMS followed by a challenging dose of the same agent with a time gap of 2h in different combinations of crosses at 48h or 72h treatments showed a 35.80 to 49.07 % reduction in bouts of orientation. These values are significant compared to additive effects (Tables 2-7; $p<0.05$). As has been demonstrated earlier, the adaptive dose has triggered the repair mechanism to attenuate the damage caused by the challenge dose.

After approaching the female, the male taps the female's abdomen with his foreleg using the pheromonal cues for gender recognition[2,59,60]. After courtship initiation, the male again uses pheromonal cues by licking the female's genitalia[2,43]. Thus, gender recognition is basically believed to be via non-volatile pheromones largely by the Gustatory Receptor Neurons (GRNs) of the foreleg[41]. Studies suggest that the detection of female-enriched long-chain Cuticular Hydrocarbons (CHCs) stimulates male-female courtship, while the detection of male-enriched CHCs promotes male-male repulsion[61]. To date, only three gustatory receptors, Gr39a are taught to detect female-enriched CHCs[62], while Gr32a and Gr33a serve to detect male-enriched CHCs[63]. GRNs expressing each of these Grs do not express *fru*. But *pickpocket-23 (ppk23)* and *pickpocket-29 (ppk29)* genes are expressed in *FruM*-positive GRNs of the foreleg tarsi[64]. There appears to be twice the number of *ppk 23*$^{+}$ neurons in the male foreleg when compared to the female[64]. Males with silenced *ppk 23*$^{+}$ neurons display defects in courtship towards females[64]. In another study, it has been shown that tarsi of males possess one-third more taste bristles than do females[65,66]. All these points prove that there is a change in gustatory behaviour if GRNs are altered. While contacting between the male tarsus and a female abdomen provokes real-time activation of P1 neurons, the presence of cVA attenuates this response[67] demonstrating the role of P1 neurons in integrating gustatory and olfactory information ultimately acting as a switch for courtship initiation. Thus in the present study, treatment with EMS or MMS has resulted in defects in tapping and licking elements of courtship behaviour in *D. melanogaster* (Tables 2-7), which can be linked to the influence of EMS or MMS on gustatory receptor neurons. Partial overcoming of this abnormality was possible by pre-treating the flies with a conditioning dose of challenged individuals which resulted in a 36 to 58.65 % reduction in 48 or 72 h treated groups (Tables 2-7). This is because of the presence of adaptive response.

According to Ewing[68], wing vibration, in which the male extends the wing nearest to the female and vibrates it up and down for brief periods, is the most important element of courtship behaviour in stimulating the male[59,60] and responses are induced by female hydrocarbons[69,70]. This vibration is an acoustic signal (love song) to attract females[71,72]. To generate a bonafide courtship song requires the expression of male-specific isoforms of both *dsx (DsxM)* and *fru (FruM)* in the CNS[73,74]. Investigations of Villella *et al.*,[75] demonstrated that song generated by *fru* mutant was aberrant. These findings are supported by the present results, where it has been revealed that the strong mutagen-EMS or MMS could cause mutations in fru gene to induce defects in wing vibration in different combinations of crosses to result in abnormal song (Tables 2-7). This has been to some extent overcome by using pre-treatments of conditioning doses of EMS or MMS. This has resulted in the production of almost normal wing vibrations compared to the additive effect, thus demonstrating the presence of an adaptive response.

Courtship latency is the time elapsing between the introduction of the flies into the chamber and the beginning of courtship. It is also known that *Drosophila* males with high vigour must perform the same courtship act many times to non-receptive females and vice versa. It is clear from the present investigations that treatment with EMS or MMS resulted in not maintaining the high vigour of males and loss of receptivity in females. These results are *on par* with the earlier works (*Cf.* Divyashree and Vasudev[6]). In support of these findings[76-78] have demonstrated that female fly mutants for *desat1* have fewer pheromones and are less attractive to wild-type males leading to increased courtship latency. Furthermore, the knockdown of *desat F* in oenocytes and fat body led to a large decrease in female pheromone product and to less courtship from wild-type males[79]. Nonetheless, knockdown of *desat1* female, the wild type males have courtship latency of 4 times higher. Males attempting copulation and males successful in copulation were fewer (~45% and ~75% respectively) and the times required for these behaviours were increased (+11.2% and +28% respectively). *On par* with this, in the previous studies with *desat1* mutant, the 4-fold decrease in both 7 monoenes and 7, 11 dienes in females were accompanied by a 50% increase in courtship latency[77]. In the study of oenocytes[80], the effect of *desat1*, knockdown is followed by a 78% decrease in 7-monoenes and also a 90% decrease in 7,11-dienes and a 4-fold increase in courtship latency. As has been said, EMS and MMS proved beyond doubt that these induce a high frequency of mutations in different test systems. Thus, in the present studies, EMS or MMS caused an effect on respective behavioural genes to induce increased courtship latency (Tables 2-7). However, when the flies were pre-treated with a conditioning dose prior to the challenge dose in different crosses, resulted in reduced courtship latency. This is evidence for the presence of adaptive response in courtship latency.

Copulation latency indicates the vigour of males and receptivity of female[7,56,81]. It also shows the time required for males and females to initiate copulation. The higher the vigour of males and receptivity of females, the shorter the copulation latency and vice versa. Further, the role of 7,11-dienes on the frequency and rapidity of wild-type males to mate has also been reported[76,77,82]. It was also shown that increased copulation latency and fewer copulation attempts when female diene levels were very low due to *desat1* mutation in which both 7-monoenes and 7,11-dienes were affected[77]. This element has been significantly extended by the mutagen EMS or MMS in the experimentally treated groups (Tables 2-7; $p<0.5$). Contrary to this, is the result of combined treatment at 48h or 72h, where there is a significant reduction of copulation latency ($p<0.05$) demonstrating adaptive response.

Copulation duration is the time between initiation of copulation to termination of copulation of each pair. During the copulation, sperms from the male are transferred to the female reproductive tract and therefore, the duration of copulation has a lot of significance in an animal's life. Long duration of copulation results in storing a large number of sperms, which is advantageous for the flies to lay a large number of fertilized eggs and to continue the generation. In the present investigation, the treatment of EMS or MMS resulted in a significant reduction of time to transfer the sperms in the challenge as well as conditioning doses compared to control (Tables 2-7, $p<0.05$), thus may result in reduced fertility. In contrast to this, when combined doses of EMS were given, the copulation duration has been increased to 13.77±0.05 from 11.47±0.13 min of challenging dose in both male and female treated groups which is significant (Table 8; $p<0.05$). Similarly, in the combined treatment of MMS, the copulation duration has been increased from 10.44±0.25 of challenge dose to 12.44±0.07 min (Table 8; $p<0.05$). This is evidence for the presence of adaptive response in courtship duration.

Receptive females appear to adapt a passive role in courtship by slowing down with occasional stops ceasing to reject behaviours and opening their vaginal plate for copulation[10,33,83-85]. However unreceptive females may show a variety of rejection responses including wing flicking, kicking, or fending with legs, ignoring, extruding and decamping[60,84,86-87]. The frequency of decamping depends on the degree of receptivity of female[88]. Even though, the higher number of bouts of rejection elements of females such as ignoring, extruding, and decamping were noticed in the challenging dose of EMS or MMS, in combined treatments of 48h or 72h the bouts of rejection elements were significantly reduced, accounting for early receptivity of females compared to the challenge dosed females (Figures 1,2; $p<0.05$). This effect is due to the adaptive dose which induced a repair mechanism as has been demonstrated by earlier workers and discussed at length[13,89].

Thus, the picture that emerges from the above discussion is that EMS or MMS induces drastic effects on the different elements of mating behaviour in different combinations of crosses. Such drastic effects of EMS or MMS are like the ones that have been demonstrated by earlier workers (*Cf.* Sega[8]; Khalandar[9]). At this point, we must mention that *Nerd*, an EMS-induced mutation reduces male mating success and attempted copulation is significantly decreased compared with wild-type males and copulation is rarely observed. Further, in *Nerd* males, the amount of the male-predominant cuticular pheromone 7-T is lower than in wild-type males[90]. The present study is in support of the said observations. This is the direct evidence to understand the effect of environmental mutagens like EMS and MMS on gene/s resulting in behavioural abnormalities. However, when EMS or MMS conditioning dose was given 2h prior to the challenge dose showed the reduced frequencies of both male and female elements of mating behaviour in different combinations. Thus, unequivocally presenting the evidence of adaptive response. Employing three alkylating agents EMS, MMS and ENU, Kaya *et al.*,[91] have shown the occurrence of adaptive response in wing disc cells of *D. melanogaster* larvae. They used the conditioning dose of 0.005 or 0.02mM EMS or 0.001 or 0.002mM of MMS or 0.0002 or 0.0001mM of ENU and treated for 24h to 2 days old larvae (48±4h). These treated larvae were challenged with a challenging dose of 0.5mM EMS; 0.1mM of MMS or 0.01mM of ENU, until the end of development. The results of the wing spot test analysis revealed that the adaptive doses clearly reduced the frequency of wing spots induced by the challenging dose. Similarly, the larval pre-treatment with the adaptive dose of EMS (0.05mM) resulted in the resistance of germ cells to a higher dose of EMS (1mM). Specifically increasing the offspring production, decreasing dominant lethality in F_1 as well SLRLs in F_2 significantly compared with the effect of challenge dose alone[92]. The results of wing spot and dominant lethal assays in *D. melanogaster* indicated a reduction in the percentage of wing spots or damages in the combined treatment of EMS or MMS[93] or ENU or MNU[94] compared to respective challenging doses.

Concordant with this is that when a conditioning dose of different mutagens was given prior to a challenging dose of the same agent with a time lag, resulted in reduced mutations in *E. coli, V. cholora*, *S. typhi*, human lymphocytes, *D. melanogaster* (*Cf.* Khalandar[9]). Nonetheless, there is the reduction of chromosomal aberrations when a similar conditioning dose was given in the case of *V. faba*, *H. valgare*, *P. pictus*, mice, human lymphocytes, V79, CHO (*Cf.* Khalandar[9]). In all the experiments, including the present it is interesting to note that both ethylating and methylating agents are efficient inducers of adaptive response. These results are discussed to mean that the adaptive dose has induced the production of repair enzymes which in turn acted on the drastic effects of challenging dose, resulting in less drasticity[13,89,95].

When the data of previous studies[5,6] and the present one are pooled together, it is clearly demonstrated that elements of mating behaviour which is having many advantages including the short duration can be used to analyse toxicity on one hand and adaptive response on the other. Further studies are warranted to prove beyond doubt the utility of mating behaviour using different environment agents because mating behaviour is important in reproductive success and the production of successful generations.

5. Conclusion

This study systematically demonstrated the influence of environmental mutagens EMS and MMS on the courtship behaviour of the Fly model. These agents significantly influenced the male courtship elements such as orientation, tapping, vibration, licking, copulation latency and courtship latency and female rejection elements. Even courtship duration has been drastically reduced. Contrary to this, when the conditioning dose was given prior to the challenging dose the effects of challenging doses were reversed significantly, thus demonstrating the presence of adaptive response. These experiments also revealed that both ethylating and methylating agents are efficient inducers of adaptive response. Nonetheless, these studies also give us an idea of how the genes controlling courtship behaviour with the influence of environmental agents alter the courtship behaviour. This is the first report as far as the knowledge of the authors goes about the use of courtship behaviour, which is short duration, easy to handle and economical, compared to other parameters of *Drosophila melanogaster*, in adaptive response. Hence, it can be concluded that courtship behaviour can be used as a parameter to understand adaptive response which is important in these days of

extensive use of drugs for different therapies and the increase of environmental pollutants.

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