the ground truth. The major fracture trends, viz. E–W, NE–SW and NW–SE and the associated smaller lineaments and their permeabilities are varying at different places and concentrated in the eastern side. In addition to the dissection of the dykes at different places, the connectivity of the fractures in the subsurface had allowed the water table to be percolated down into the deeper extents, especially around the epicentre. Extensive research on the interaction of hydrological activities and seismicity had shown that pore-pressure changes due to reservoir filling or water-level changes in a suitable geologic setting can generate seismicity[22]. Therefore, it is concluded that significant amount of base flow in this part of the area had resulted in hydro-loading, following heavy rains during the monsoon period of 2010 and was responsible for the micro-seismicity.

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Effect of phenytoin on development and life-history traits of *Drosophila melanogaster*

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Phenytoin is an antiepileptic drug which has been used in the present study to assess its effect on developmental toxicity from egg to adult eclosion and also life-history traits in *Drosophila melanogaster*. Flies were reared on media supplemented with different doses of phenytoin. The dose-dependent developmental delay, reduced pupal and adult size reduced adult eclosions, minimum larval, adult mortality, but significant pupal mortality was observed with incomplete pupal eclosion. The data show reduction in fecundity for life-history traits, but differences are not statistically significant for fertility and lifespan. The present study reveals that on exposure to phenytoin pre-adult stages are prone to developmental toxicity than the treated adult.

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Keywords: *Drosophila melanogaster*, life-history traits, phenytoin, developmental toxicity.

PHENYTOIN is a first-line antiepileptic drug (AED) which is being widely prescribed throughout the world for more than 70 years as an anticonvulsant to treat epilepsy and seizures, both in acute and chronic conditions. Control of epilepsy with phenytoin can be a difficult and lengthy process because of the narrow therapeutic index of the drug and the wide inter-individual range of doses required[1]. Children born to mothers taking antiseizure drugs have an increased risk (twofold) of congenital malformations. Use of phenytoin during pregnancy has been implicated in Foetal hydantoin syndrome (skeletal, CNS, limb and orofacial defects)[2].

Drosophila melanogaster has been a model for examining fundamentally important problems in biology, especially developmental biology, neurobiology and neuropharmacology[3,4]. The use of animal tissues for toxicological tests now involves two fundamental concepts: science and ethics, leading to a search for alternative approaches. Nowadays, species of *Drosophila* are model organisms for toxicological studies, as they are well defined in terms of their genetics, biological development and genome. Besides, their use is recommended by the European Centre for Validation of Alternative Methods, which promotes the scientific and regular acceptance of alternative experimental methods[5]. Once a molecule is identified as a potential drug, detection of adverse drug reactions is one of the key components of its development. *D. melanogaster* is used to screen for reproductive fitness and developmentl toxicity. Compared with other non-mammalian models, *D. melanogaster* has many similarities with the mammalian reproductive system, including putative sex hormones and conserved proteins involved in genitourinary development. Furthermore, *D. melanogaster* would present significant advantages in time efficiency and cost-effectiveness compared to mammalian models.

Genotoxic substances such as diethylstilbestrol, diphenylhydantoin, imipramine, testosterone and tolbutamide have shown a high teratogenic potential, whose effects were evident in muscles and neurons in *D. melanogaster*[6]. To understand the effect of phenytoin on *D. melanogaster*, the drug was administered separately to pre-adult (eggs) and adult flies. *D. melanogaster* is being studied for its potential to aid in identifying priority chemicals for teratologic study[7].

The method encompasses treating the entire metamorphosis period, i.e. from the egg through the larval stages to pupa formation, by incorporating the test chemical into the medium. Adult females lay eggs that hatch as larvae after a day. These larvae grow tremendously over the next four days as they voluntarily consume food and molt twice. During the final larval instar, larvae stop eating, leave the food (wander) and form a puparium, signalling the onset of metamorphosis. The duration of metamorphosis is four days, after which the adult fly emerges. Thus, the life cycle of the fly is such that developmental exposure and consumption of phenytoin-containing food are voluntary, unlike gestational mammals and occurs mostly during the larval stage of development. Developmental toxicity was evaluated based on the number of days taken for development and number of adults eclosed after treating with the drugs in preadult stage. Adult flies were systematically examined under a binocular microscope for external morphological anomalies.

Data from treated flies can be compared with those from concurrent untreated flies using statistical tests. The external development of flies eliminates the complications of maternal–placenta–foetal interactions seen in mammalian studies[8]. Reproductive fitness was measured in terms of mating propensity, fecundity, fertility and longevity in treated adult flies. The report on methotrexate reproductive adverse events in multiple animal models, including fruit flies, has been a proof for the use of the *D. melanogaster* model[9]. The intensive toxicity was evaluated by observing phenotype of adults resulting after drug treatment and mortality in treated flies.

The fly stocks were routinely cultured in standard wheat cream agar medium in uncrowded condition at 22 ± 1°C (rearing temperature), 12 : 12 h light and dark periods and relative humidity of 70%. The *D. melanogaster* (oregan k) stocks were obtained from National *Drosophila* Stock Centre, Mysore, India. The test flies were cultured in wheat cream agar medium along with different concentrations of the epileptic drug phenytoin – 5, 10 and 15 mg/ml of the medium.

Phenytoin (5,5 diphenylhydantoin sodium salt) 99%, CAS no: 630-93-3 was obtained from Sigma-Aldrich, dissolved in ethanol and water (1 : 1) and added to the medium. A modified protocol[10] was used for drug standardization. Standardization of doses was carried out on adult mortality for seven days using three doses, viz. low dose –5 mg/ml, mid dose –10 mg/ml and high dose –15 mg/ml of medium to treat the flies.

For preadult treatment, the drug was added to a wheat cream agar medium in different doses (5, 10 and 15 mg/ml). The control cultures were raised on the same diet without addition of the drug. Virgin females and unmated males which were grown for five days were collected and maintained separately in order to age and then transferred to phenytoin-containing medium alongside a control. The 5 ml of the medium was placed in 25 × 100 mm sample tubes and a pair of flies was transferred to each vial. Likewise three successive transfers were made into fresh food containing phenytoin once in two days. Flies were allowed to lay eggs on the medium containing phenytoin in three different doses alongside the control and the number of eggs laid was recorded. Dilute yeast was added to the same set of vials for the eggs to hatch, complete larval and pupal development and for the

eclosion of adults[11]. The number of adult eclosed was recorded from the treated (all three doses) and untreated (control) vials. Simultaneously, the same sets of vials were assessed for the developmental time[12] (recorded in number of days). Adult flies are systematically examined under a binocular microscope for external morphological anomalies. Size of larva, pupa and adult was measured using micrometry. Adult flies were maintained till the mortality and longevity were recorded.

For the treatment of adults, unmated male and virgin female flies were collected in an uncrowded condition and were reared separately for two days. The flies were fed with wheat cream agar medium containing phenytoin in three different doses (5, 10 and 15 mg/ml) for three days. These treated flies were analysed for life-history traits such as mating propensity, fecundity, fertility and longevity[13]. The experimental trials for all three doses were analysed for four different sets, namely untreated male × untreated female (C), treated male × untreated female (T_1), untreated male × treated female (T_2) and treated male × treated female (T_3).

To assess mating propensity, virgin females and unmated males were collected on the day of eclosion anaesthetized with ether to facilitate sorting of the sexes and stored in food vials. Flies were aged for five days in food vials for sexual maturity. For each set of experiments, a single male and female (single pair mating) was allowed to mate in empty glass vials (30 samples) to record the duration of courtship and copulation. The time taken by male to mount on female (courtship duration), the time from mounting to detaching (copulation duration) and the mating activity were observed for 60 min. The pairing of flies from the time of mounting to detaching was recorded. The pairs which do not mate within a stipulated time of 60 min were discarded.

The same set of vials which was used to observe mating propensity was used to assess the fecundity. Soon after mating, males from each pair were separated and females were transferred into separate vials containing fresh food medium. Fecundity was assayed by counting the number of eggs laid by the female. Likewise, each replicate was transferred successively to the next set of fresh food vials containing medium every alternate day for six days. Immediately after each transfer, the vials were checked for the eggs laid and were counted under stereomicroscope.

The same set of vials that was used to assess fecundity was used to assess fertility (total number of adults emerged). The eggs were allowed to hatch and dilute yeast was added till pupation. Further, the number of flies that emerged from all the experimental trials for each dose was recorded[13].

Longevity was assessed simultaneously using the same set of flies along with fecundity and fertility. All the vials were observed from the day of adult emergence to record the lifespan of the flies.

One-way ANOVA was performed for the said life-history parameters, namely courtship duration, copulation duration, fecundity, fertility and longevity. Post-hoc Duncan's Multiple Range Test was conducted to record the significant differences. The analysis was performed using the Statistical Presentation System Software Package, SPSS version 15.0.

Using *D. melanogaster*, the effects of growth on medium supplemented with 5, 10 and 15 mg/ml phenytoin were determined alongside a control. The differences are not statistically significant for fecundity (Figure 1 *a*) in all the doses when compared with control. Significant difference was observed for adult eclosion in high dose ($P < 0.001$) when compared with low dose and control (Table 1).

The mean developmental time from egg to adult emergence was 11.90 days in control (Figure 1 *b*). The figure also shows extreme values in the mean development time of 16.70 days in high dose and 14.80 days in mid dose, but the differences are not statistically significant at low dose when compared with control.

The differences are not statistically significant for size (mm) between treated and control larvae, whereas pupal size was statistically significant (Figure 2 *a*) in mid dose (2.15 mm) and high dose (2.16 mm) of phenytoin when compared to control; but there were no differences at low dose. Interestingly, Table 2 shows that the mean adult size is significantly reduced in 10 mg/ml (1.88 mm) and 15 mg/ml (1.89 mm), but not observed in 5 mg/ml when compared to control. Drastic changes in size variation were observed and recorded in treated flies for pupal and adult size (Figure 3 *a* and *c*).

The eggs treated with higher doses have shown significant mortality rates for pupa than larva and adult. The percentage of pupal mortality rate was significant at 41.42 and 40.66 in 10 and 15 mg/ml of medium respectively (Figure 2 *b*), whereas larval and adult stages have not shown significant mortality. Interestingly, the increased pupal mortality was observed with typical pattern of partial eclosion (Figure 3 *b*).

Table 3 reveals the mean courtship duration assessed on exposure to phenytoin for three different doses in untreated and treated experimental trails. The observed differences are not statistically significant for courtship duration in C, T_1, T_2 and T_3 trials on exposure to different doses of phenytoin. The mean copulation duration of both untreated and treated trials on exposure to 5, 10 and 15 mg/ml phenytoin is not statistically significant (Table 3).

The mean fecundity in different experimental trials (C, T_1, T_2 and T_3) on exposure to different doses of phenytoin is depicted in Table 4. The data reveal reduced mean number of eggs produced in T_3 trial where both males and females are treated on exposure to 10 and 15 mg/ml of medium compared to T_1 and T_2. The differences are significant between treated trials and control at higher

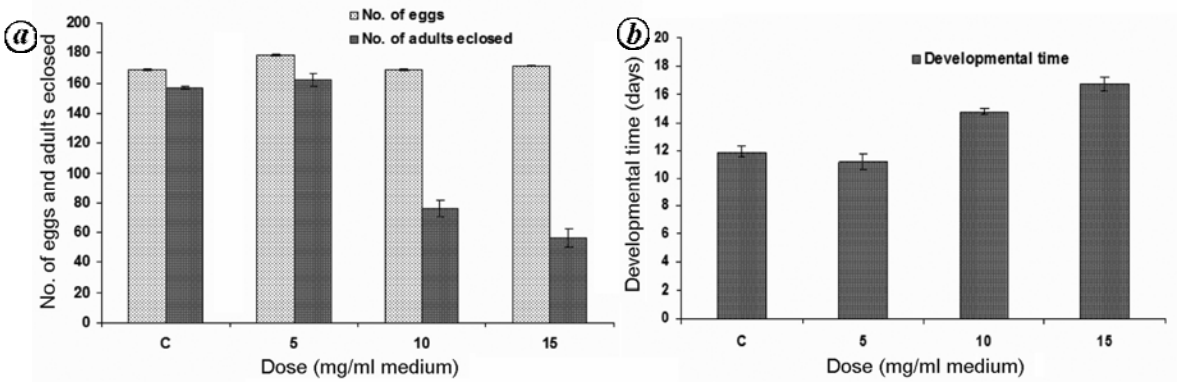

Figure 1. Mean (± SE) number of eggs and adults eclosed (***a***) and development time (***b***) on exposure to phenytoin.

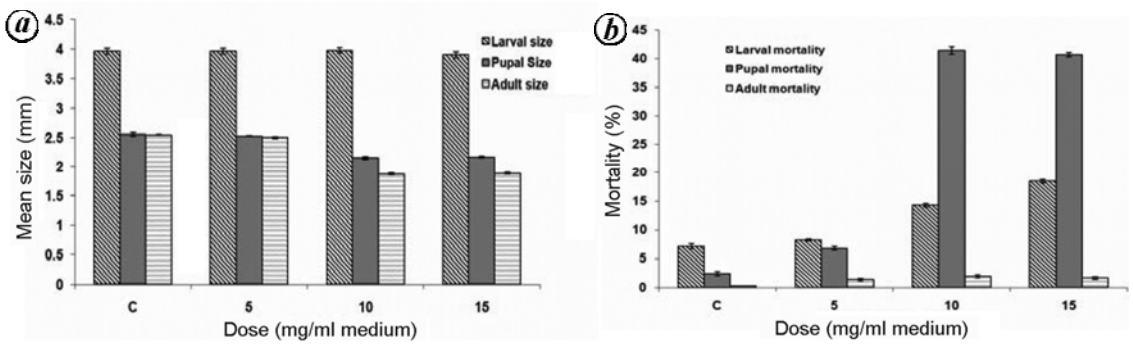

Figure 2. Mean (± SE) size (mm) (***a***) and mortality (%) (***b***) of larva, pupa and adult on exposure to phenytoin.

Table 1. Mean (± SE) number of eggs, adults and developmental time (days) on exposure to phenytoin

	Traits		
Dose↓	No. of eggs	No. of adults eclosed	Developmental time
Control (c)	168.80 ± 0.72	156.50 ± 0.64	11.90 ± 0.40
5 mg/ml (l)	178.60 ± 0.51	162.20 ± 4.58	11.20 ± 0.55
10 mg/ml (m)	168.80 ± 0.74	76.10 ± 5.43	14.80 ± 0.20
15 mg/ml (h)	171.30 ± 0.36	56.40 ± 5.83	16.70 ± 0.47
ANOVA	$F = 1.615$	$F = 47.341$	$F = 26.266$
	$P > 0.203$	$P < 0.001$	$P < 0.001$
DMRT	c/l, c/h, l/m, l/h	c/l, c/m, c/h, l/m, l/h, m/h	c/m, c/h, l/m, l/h, m/h

l, Low dose; m, Mid dose; h, High dose. DMRT, Duncan's Multiple Range Test.

dose. The fecundity has reduced in T_3 followed by T_1 and T_2 in 15 mg/ml. Differences are not statistically significant for 5 mg/ml of phenytoin exposure in all the four experimental trials.

The mean fertility is represented in Table 4 for three doses of phenytoin exposure (5, 10 and 15 mg/ml) of untreated and treated experimental trials. The results show negligible difference in the number of adults emerging in the trials C, T_1, T_2 and T_3, where both sexes treated or untreated, or either the males or females are used in all these experimental trials for different doses of phenytoin exposure. According to ANOVA, the differences were not statistically significant for both treated and untreated experimental trials on exposure to all the doses of phenytoin.

Table 4 also shows the mean longevity of flies which are treated and untreated for different doses of phenytoin, and difference has not been observed for lifespan. Longevity was not affected much – it relies on an average of 90 days in 10 mg/ml and 92 days in 15 mg/ml of phenytoin exposure.

Exposure to anticonvulsant drugs during pregnancy is one of the most common potentially teratogenic exposures, occurring in 1 in 250 of pregnancies[14]. The embryotoxic and teratogenic effects of phenytoin have also been experimentally demonstrated in rodents[15,16]. Phenytoin and related proteratogens, including the sedative drug thalidomide, are bioactivated by embryonic prostaglandin H synthases, lipoxygenases and related enzymes to a free radical intermediate, which causes

Table 2. Mean (± SE) morphomeristic traits and percentage of morphometric traits of *D. melanogaster* on exposure to phenytoin

	Morphomeristic traits (mm)			Morphometric traits (%)		
Dose	Larval size	Pupal size	Adult size	Larval mortality	Pupal mortality	Adult mortality
Control	3.96 ± 0.053	2.56 ± 0.032	2.55 ± 0.009	7.14 ± 0.422	2.42 ± 0.334	0.29 ± 0.036
5 mg/ml	3.96 ± 0.051	2.53 ± 0.008	2.50 ± 0.019	8.26 ± 0.204	6.78 ± 0.32	1.34 ± 0.253
10 mg/ml	3.98 ± 0.047	2.15 ± 0.022	1.88 ± 0.012	14.28 ± 0.339	41.42 ± 0.667	1.98 ± 0.279
15 mg/ml	3.90 ± 0.055	2.16 ± 0.013	1.89 ± 0.018	18.54 ± 0.326	40.66 ± 0.354	1.62 ± 0.252

Table 3. Mean (± SE) mating propensity of *D. melanogaster* on exposure to phenytoin

Traits →	Courtship duration			Copulation duration		
Dose → (mg/ml) Trial ↓	5	10	15	5	10	15
C	6.00 ± 0.93	5.40 ± 0.87	4.70 ± 0.47	21.80 ± 0.96	22.50 ± 0.85	23.30 ± 0.76
T_1	5.20 ± 0.81	5.56 ± 0.84	5.40 ± 1.09	22.60 ± 0.76	23.02 ± 0.67	21.80 ± 0.85
T_2	6.30 ± 1.12	6.02 ± 0.72	5.90 ± 0.78	21.80 ± 0.72	21.90 ± 0.89	23.10 ± 0.83
T_3	4.40 ± 0.65	5.62 ± 0.75	5.50 ± 0.50	22.70 ± 0.75	22.20 ± 0.76	23.12 ± 0.97
ANOVA	$F = 0.905$	$F = 0.114$	$F = 0.436$	$F = 0.326$	$F = 0.372$	$F = 0.645$
	$P > 0.448$	$P > 0.951$	$P > 0.729$	$P > 0.806$	$P > 0.774$	$P < 0.591$
DMRT	C/T_1, C/T_3, T_1/T_2, T_1/T_3, T_2/T_3	C/T_2, T_1/T_2, T_2/T_3	C/T_1, C/T_3, C/T_2	C/T_1, C/T_3, T_1/T_2, T_2/T_3,	C/T_1, C/T_2, C/T_3, T_1/T_2, T_1/T_3, T_2/T_3	C/T_1, T_1/T_2, T_1/T_3

C, Untreated♂ × Untreated♀; T_1, Treated♂ × Untreated♀; T_2, Untreated♂ × Treated♀; T_3, Treated♂ × Treated♀.

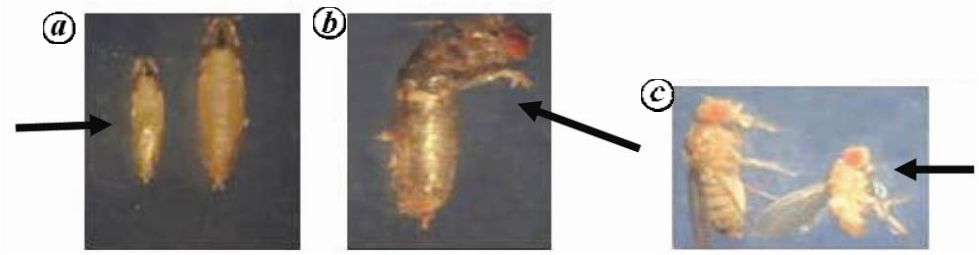

Figure 3. Phenotypic variations. ***a***, Reduced pupal size; ***b***, Pupal mortality; ***c***, Reduced adult size of *D. melanogaster* on exposure to phenytoin.

embryonic oxidative stress, hydroxyl radical formation and oxidative damage to DNA and other cellular macromolecules in embryonic tissues[17]. A study reported that dose-dependent increase in embryopathies was caused by phenytoin by both maternal and embryonic analyses[18].

Prenatal exposure to AEDs in humans results in a wide range of developmental abnormalities, including growth deficiency, developmental delay, reduced size, permanent neurobehavioural abnormalities and foetal death[19]. The frequency of major malformations, growth retardation and hypoplasia of the midface and fingers, known as anticonvulsant embryopathy, is increased in infants exposed to anticonvulsant drugs *in utero*, but it is not known whether the abnormalities were caused due to maternal epilepsy or by exposure to anticonvulsant drugs[20,21]. It is also still unknown if phenytoin has a higher risk than newer AEDs[22].

Phenytoin exposure decreased maternal weight during gestation as detected in rats[23,24]. Consistent with these observations are studies that found increased resorptions and decreased litter size in rabbits[25] and decreased litter weight in rats[26]. Decreased foetal weight was observed in mice[27] as well as decreased whole brain[28]. Consistent with the symptoms of foetal hydantoin syndrome in humans, the teratogenicity (cleft palate) of phenytoin was documented in mice[29]. A possible correlation with this observation is a detection of altered craniofacial gene expression in embryonic mice[30]. Similar results have been expressed in *D. melanogaster* on exposure to phenytoin with drastic dose-dependent developmental delay (Figure 1 *b*), reduced adult eclosion (Figure 1 *a*) and reduction in pupal and adult size (Figure 2 *a*), with significant increase in larval and pupal mortality which in turn has led to reduction in adult eclosion.

Thus the result emphasizes *D. melanogaster* as a useful model system to uncover the complex etiology of development. The metabolic activation of phenytoin to a reactive intermediate has been proposed to account for the teratogenicity and possible genotoxicity[2]. Antiepileptic medication use affects reproductive endocrine function, but its impact on fertility is not well known[31]. Men on AEDs have been reported to have more sperm abnormalities

Table 4. Mean (± SE) life-history traits of *D. melanogaster* on exposure to phenytoin

Traits →	Fecundity			Fertility			Longevity		
Dose → Trials ↓	5	10	15	5	10	15	5	10	15
C	186.50 ± 2.43	180.50 ± 2.43	182.50 ± 1.23	180.30 ± 3.10	178.30 ± 3.10	179.01 ± 0.97	89.12 ± 1.63	93.14 ± 1.28	86.30 ± 0.36
T_1	180.70 ± 3.35	177.50 ± 3.61	140.61 ± 5.73	172.90 ± 4.34	166.30 ± 5.67	123.34 ± 4.08	93.56 ± 0.86	88.94 ± 1.97	89.20 ± 1.87
T_2	171.70 ± 5.01	174.20 ± 4.49	154.35 ± 5.57	161.80 ± 4.79	158.40 ± 4.19	131.03 ± 4.34	87.04 ± 1.76	85.28 ± 1.49	86.40 ± 0.43
T_3	180.03 ± 4.30	156.70 ± 6.42	112.41 ± 5.03	164.38 ± 4.78	144.10 ± 7.02	92.56 ± 5.52	90.14 ± 1.43	90.76 ± 0.65	92.56 ± 1.74
ANOVA	$F = 1.789$	$F = 0.575$	$F = 7.298$	$F = 1.299$	$F = 0143$	$F = 4.910$	$F = 0.802$	$F = 0.675$	$F = 1.232$
	$P > 0.167$	$P > 0.635$	$P < 0.012$	$P > 0.290$	$P > 0.934$	$P < 0.002$	$P > 0.103$	$P > 0.342$	$P > 0.421$
DMRT	C/T_1, C/T_2, C/T_3, T_1/T_2, T_2/T_3	C/T_1, C/T_2, C/T_3, T_1/T_2, T_1/T_3, T_2/T_3	C/T_1, C/T_2, C/T_3, T_1/T_3, T_2/T_3	C/T_1, C/T_2, C/T_3, T_1/T_2, T_1/T_3, T_2/T_3	C/T_1, C/T_2, C/T_3, T_1/T_2, T_1/T_3, T_2/T_3	C/T_1, C/T_2, C/T_3, T_1/T_2, T_1/T_3, T_2/T_3	C/T_1, C/T_2, C/T_3, T_2/T_1, T_2/T_3, T_3/T_1	C/T_1, C/T_2, C/T_3, T_2/T_1, T_2/T_3, T_3/T_1	C/T_1, C/T_2, C/T_3, T_2/T_1, T_2/T_3, T_3/T_1

C, Untreated♂ × Untreated♀; T_1, Treated♂ × Untreated♀; T_2, Untreated♂ × Treated♀; T_3, Treated♂ × Treated♀.

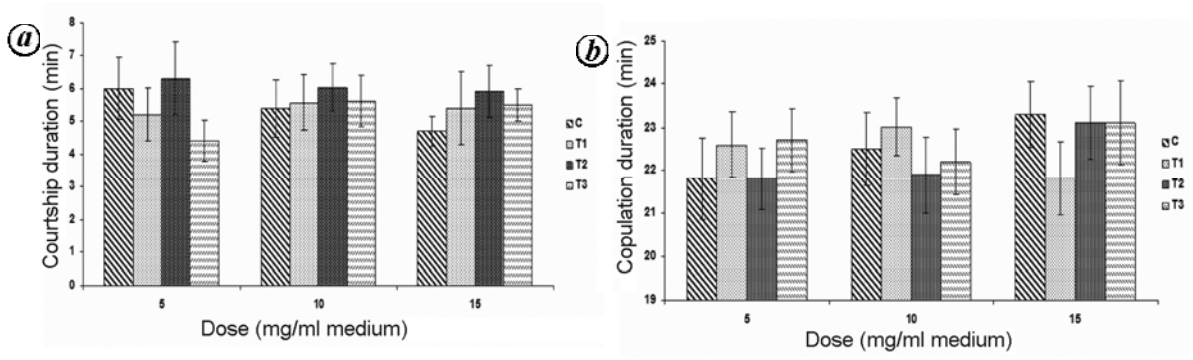

Figure 4. Mean (± SE) courtship duration (***a***) and copulation duration (***b***) for three doses of phenytoin in different experimental crosses.

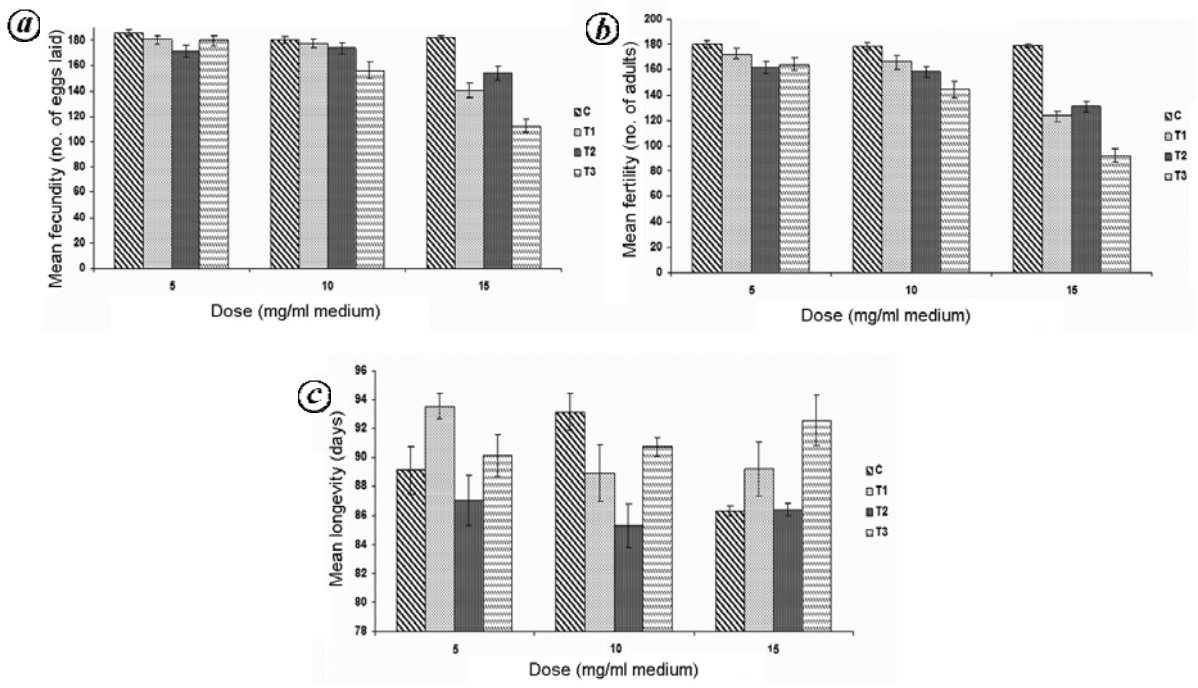

Figure 5. Mean (± SE) fecundity (***a***), fertility (***b***) and longevity (***c***) for three doses of phenytoin in different experimental crosses.

and lower sperm motility and also fertility rates have reduced after long-term treatment with different AEDs[32]. The effect of AED use on the number of children has not been widely studied; the number of children was lower in patients on AEDs than in untreated patients, or subjects without epilepsy. Women with epilepsy, particularly

those taking AEDs, are at increased risk for endocrine dysfunction and infertility[33]. It has been claimed that fertility rates have been reduced after long-term treatment with different AEDs[34,35].

The present study shows that there was no significant difference in mating propensity (Figure 4), but reduction in fecundity (Figure 5 *a*) in T_3 experimental trial, where both males and females were treated with higher doses (10 and 15 mg/ml). Also, there was minimum reduction in T_1 and T_2 where either the males or females were treated in each trial, but no effect on fecundity with low dose of phenytoin exposure compared to trial C, where both males and females were untreated. The difference has not been observed for fertility (Figure 5 *b*) in all the experimental trials on exposure to different doses of phenytoin. Longevity (Figure 5 *c*) has not been affected when treated with different doses of phenytoin in all the four trials of treated adult.

Therefore, the fruit fly offers remarkable biological similarity to humans, as a sophisticated genetic tool to screen drugs and drug targets[4]. Phenytoin exhibits adverse health effects at high doses or after chronic use by humans and is lethal when added to the diet of *Drosophila*.

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