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Rotenone induced alterations in lifecycle parameters and compound eye morphology of Drosophila melanogaster

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Abstract

Rotenone is a widely used natural pesticide and piscicide and a potent inhibitor of mitochondrial complex I (NADH-quinone reductase) that elicits the degeneration of dopaminergic neurons. Present study explored the adverse effect of rotenone at concentrations far below the reported LC_{50} on several life cycle parameters and on the compound eye structure of a non-target insect, *Drosophila melanogaster*. Newly hatched first instar larvae were introduced in *Drosophila* food medium containing different concentrations (50, 100, 150, 200, 250 µg/L) of rotenone. Different life cycle parameters were recorded. Emerged adult flies were collected and studied under Scanning electron microscope. Student's t-test was performed to explore significant difference and changes in both life cycle parameter and eye architecture. Treated groups showed significant delay ($p<0.05$ level) in larval duration, pupal duration, and in emergence of flies when compared with the control groups. Delay in life cycle parameters significantly increased with increase of rotenone exposure. Interestingly, the compound eye structure of the treated flies was seen to be affected by rotenone. Thus these results clearly demonstrate that chronic exposure to rotenone can alter the structure and lifecycle of a non-target organism like *Drosophila at* sub-lethal concentrations. Thus rotenone in *Drosophila* at sub-lethal concentrations might be the reason for developmental toxicity.

Keywords: Rotenone, *Drosophila*, ommatidia, SEM

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Introduction

Rotenone is an insecticide and piscicide having natural origin, derived from certain plant members of pea family found in South America and Southeast Asia. It is widely used as a garden insecticide and in organic farming as a natural, botanical insecticide though it is better known for its use as piscicide. The basic mechanism of action of rotenone involves inhibition of mitochondrial enzyme NADH-dehydrogenase that leads to cell death. Rotenone causes effective toxicity on target organisms like fishes (1) and is also known to increases mitochondrial superoxide release (2). Chronic exposure of rats to rotenone induces PD-like symptoms, including dopaminergic neurodegeneration and the occurrence of cytoplasmic inclusions similar to Lewy bodies (3). Recent studies show that chronic exposure of *Drosophila* to rotenone recapitulates key features of Parkinsonism, including selective loss of dopaminergic neurons and manifesting locomotor deficits (4). According to Environmental Protection Agency, USA (5), the ecological risk assessment considers 200 ppb (200 µg/L) and 50 ppb (50 µg/L) to be the maximum potential exposure for exposed aquatic organisms in lake/reservoirs/ponds and river respectively. While 40 ppb (40 µg/L) is reported to be more than adequate to kill most species of fish, it is

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also known to causes severe effects on aquatic non target organisms. Interestingly, EPA has not yet estimated any Risk Quotient for terrestrial non-target insects. However, based on a single contact study in honey bees, technical grade rotenone is classified as practically non-toxic on acute exposure basis to non-target terrestrial insects (LD50>>60 µg/ai per bee).

But till date no report on chronic exposure to rotenone has been available for terrestrial insects. Hence the present study explores the undesired effects of chronic rotenone exposure to rotenone in non-target insect model, *Drosophila melanogaster*. Several research works have used *Drosophila* model to describe the adverse effects several chemicals. Arsenic containing hydrocarbon causes developmental toxicity (6). It is also reported that 4- phenylbutyrate (PBA) in the food medium causes an increase in life span in *Drosophila melanogaster* (7). Phenol intoxication is also known for altered life span and fertility rate of *Drosophila melanogaster* (8). Fluoridated chemicals like Cryolite, sodium fluoride, Flubendiamide are seen to cause severe anomalies on selected life cycle parameters in this insect (9)(10), whereas organophosphate, like Acephate, have successfully demonstrated architectural alterations in the compound eye structure of the same model insect (11). Interestingly fluorides not only induce structural alterations in the eye of exposed *Drosophila* but the alterations or changes were found to manifest trans-generational transmission to the consecutive generations (12).

Drosophila is very useful in environmental monitoring studies for assessment of the potential of chemicals related to environmental pollution and now *Drosophila* is also considered as a model organism in several toxicological experiments (13). It is a holometabolous insect that completes its total life cycle within 2 weeks at (25 ± 1) °C (14). Female fly lays nearly 400 eggs that hatch after 12-15 hours and the resulting 1st instar larvae, which molt twice at about 24 and 48 hours. During the period of development the flies feed on the sugar content of fruits and also on the by-products that are produced through decomposition by micro-organisms.

In this study the authors wish to demonstrate the effects of rotenone on selected life cycle parameters and on the structure of compound eye of Dipteran model organism *Drosophila melanogaster*. As a non-target organism *Drosophila* is used, similar to other toxicological studies (15) (19) (20).The organisms can be exposed to the test chemical through various routes, like cutaneous, oral, respiratory, etc.

In the present study the flies were exposed to different concentrations of rotenone much below the reported acute LD_{50} (LD_{50} in another Dipteran insect, honey bee is LD50>>60 µg/ai, EPA, 2007). In the present study treatment concentrations used were between 50µg/L (50ppb) to 250µg/L (250 ppb) and the chemical was applied with standard *Drosophila* food. Hence the study is targeted to explore the effects of chronic sub lethal exposure of rotenone in the non-target insect model. Interestingly the study successfully demonstrated that selected life history traits differentially responded to different treatment concentrations of rotenone and in addition also depicted adverse impact of the chemical on compound eye architecture of the model non-target insect, *Drosophila melanogaster*.

Methodology

Maintenance of Fly

Drosophila melanogaster Oregon R strain, was maintained in laboratory within environmental chamber at (22 ± 1) °C in Standard Drosophila medium (SDM) which was prepared using agar-agar, maize powder, yeast, and sucrose etc. at a particular concentration and boiled until required consistency was achieved. Later, minute amount of Propionic acid and Nepagin powder dissolved in rectified spirit were added so as to protect the culture plates from fungal and/or mite infection.

Chemicals Used

In this study, rotenone (Calbiochem, Merck) [2R,6aS,12aS)-1,2,6,6a,12,12a-hexahydro-2-isopropenyl-8,9 dimethoxychromeno[3,4-b]furo[2,3-h] chromen-6-one] with molecular formula $C_{23}H_{22}O_6$ was used as the test chemical. The culture media containing graded concentrations of the chemical was prepared and *Drosophila melanogaster* were maintained in such culture vials to evaluate the effects of rotenone on selected life cycle parameters as well as on the compound eye architecture in *Drosophila melanogaster*.

Treatment Schedule

Different concentrations of rotenone (50, 100, 150, 200, 250 µg/L) were prepared in SDM in respective culture

plates into which first instar larvae were introduced. Triplicate sets of each treatment group were employed in the study. The larvae were subjected to chronic exposure to rotenone through food and were allowed to emerge as adults. Triplicate sets of Control along with each treatment group were maintained for comparison.

Life cycle study

Selected life cycle parameters of *Drosophila melanogaster* were studied in different treatment categories and compared with the control counter parts.1stinstar larvae (20 in number) were introduced in each culture plate and 3 such culture plates were taken for each treatment group including the control. Thereafter the day of pupation was noted for all treatment groups which were followed by recording the day of emergence of adults and the number of emerging flies in each treatment category. The data obtained were compared with the control. The emergence of flies was recorded till all the flies emerged. From the obtained data, variation in larval duration, pupal duration and the emergence of flies if any, between control and different treatment groups were analyzed.

Preparation for SEM

Adult flies were collected randomly from each treatment group and were fixed in 2.5% glutaraldehyde for 2 hours and then dehydrated with graded alcohol. Finally gold coating was done using IB-2 Ion Coater (EIKO ENGINEERING) to examine the outer structure of compound in *Drosophila melanogaster* eye to reveal morphological alteration under Scanning Electron Microscope (S-530 HITACHI) following the method of Podder et al. 2012 (15), and Dutta et al. 2014 (11).

Observation of compound eye under compound microscope to analyze the percentage of occurrence of eye alterations

The adult flies were randomly collected from each treatment group which is in triplicate and were preserved in 70% alcohol. Thereafter flies were observed under compound microscope to measure the percentage of eye alterations compared with the control flies.

Statistical analysis

Student's t-test was performed to explore significant difference, if any, in life cycle parameters and percentage of eye deformities among all experimental groups. $p<0.05$ was considered as statistically significant.

Results of Life Cycle

Effects of rotenone on larval duration of Drosophila melanogaster

Effect of different concentrations (50, 100, 150, 200 and

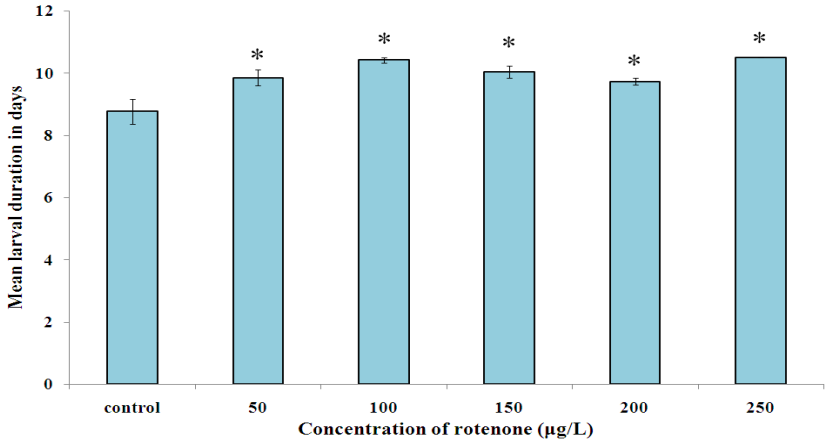


Figure A. Variation in the larval duration of *Drosophila melanogaster* due to exposure to different concentrations of rotenone. *D. melanogaster* larvae were fed with food containing different concentrations of rotenone (50, 100, 150, 200, 250 µg/L). Control sets of insects maintained in normal food were used for comparison. The data expressed as the mean larval duration in days. The data represent mean ± standard error (SE) of three pooled determinations. Each pool consisted of 20 larvae. The vertical line represents standard error (SE). The * denotes significant change when compared with the control.

250µg/L) of test chemical rotenone was observed on the mean larval duration when compared with the control set. Time taken by the larvae from 1st instar stage up to pupation was considered as larval duration. When different treatment groups were compared with control group, each treatment category manifested significant increase ($p<0.05$) in larval duration in comparison with control group having the duration of 8.76±0.4 days. The increased larval duration in flies treated with 50, 100, 150, 200 and 250µg/L of rotenone were 9.85±0.25, 10.415±0.085, 10.025±0.195, 9.7275±0.1025 and 10.5±0.001 days respectively (Figure A). All the treatment concentrations were found to lengthen the larval duration but no dose dependent increase in response was seen.

Effect of rotenone on pupal duration of D. melanogaster

Pupal duration is considered as the time taken by the insect from the formation of the pupal case till the emergence of the adult fly. When pupal duration of insects from different treatment groups was compared with the control set, only slight variations were observed (Figure B). Although a small increase was seen in each treatment set compared to control. The mean pupal duration of control fly was 6.58±0.37 days, while the treated flies show increased pupal duration. The pupal duration of treated flies were 7.55±0.15, 7.44±0.11, 7.63±0.03, 7.53±0.035 and 7.87±0 days in response to 50, 100, 150, 200 and 250µg/L treatments respectively. The increased pupal duration of treatment categories were statistically significant ($p<0.05$) when compared with the control group. Similar to the larval duration, there was no dose dependent increase in the pupal durations though all the treatment categories manifested increased pupal duration.

Effect of rotenone on the duration of the emergence of D. melanogaster

Adult emergence duration is the time taken by first instar larvae to emerge as adult flies. The mean adult emergence duration was recorded as 14.14 ± 0.56days in control, whereas the parameter for treated groups was found to be delayed (Figure C). Durations of adult emergence in treatment categories were 16.4 ± 0.1, 16.77±0.11, 17.65 ±1.15, 16.235 ±0.095 and 17.375 ±0 days in response to 50,100,150,200 and 250 µg/L treatments respectively. With the increase in treatment concentrations in case of the first three treatment categories, an increase in emergence time was seen. But further increases in exposure fail to manifest any dose dependent increase. But the increased time taken for adult emergence of *Drosophila melanogaster* after treatments (in different treatment categories) is statistically significant ($p<0.05$) when compared with control flies.

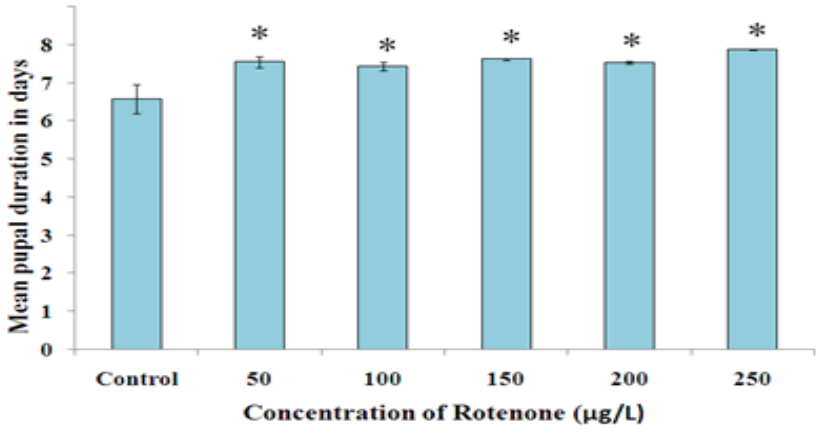


Figure B. Variation in pupal duration of *Drosophila melanogaster* after exposure to different concentrations of rotenone. *D. melanogaster* larvae were maintained in food containing different concentrations of rotenone (50, 100, 150, 200, 250 µg/L). Control sets of insects maintained in normal food were used for comparison. The data expressed as the mean pupal duration in days. The data represent mean ± standard error (SE) of three pooled determinations. Each pool consists of 20 larvae. The vertical line represents standard error (SE). The * denotes significant change when compared with the control.

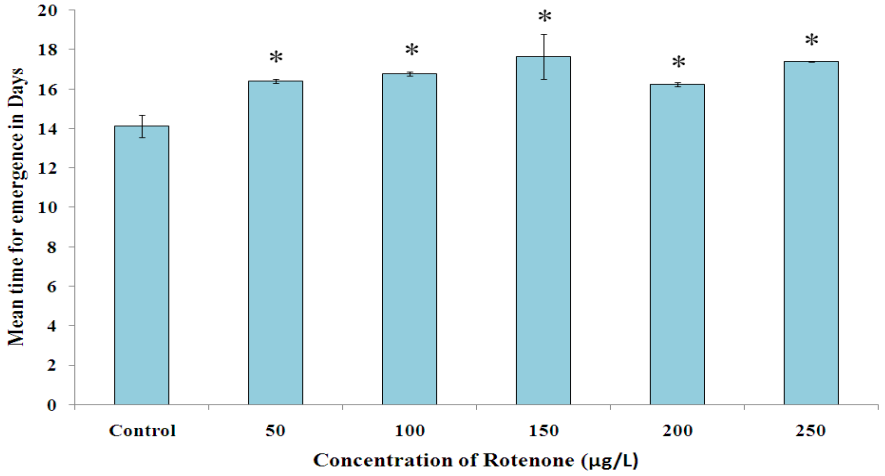


Figure C. Variation in duration till emergence of adult fly, *Drosophila melanogaster* after treatment with different concentrations of rotenone. *D. melanogaster* larvae were fed with different concentration of rotenone (50, 100, 150, 200, 250 µg/L). These larvae were allowed to pupate and the pupae were subsequently allowed to emerge as adult flies. Control sets of insects maintained in normal food were used for comparison. The data are expressed as the mean time taken for emergence in days. The data represent mean ± standard error (SE) of three pooled determinations. Each pool consisted of 20 larvae. The vertical line represents standard error (SE). The * denotes significant change when compared to control.

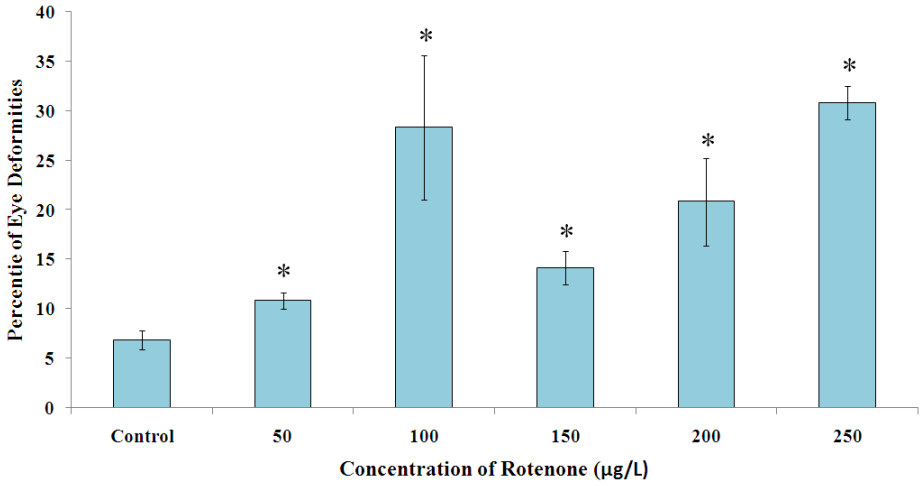


Figure D. Bar diagram showing altered percentage of eye deformities in *D. melanogaster* compared with control at different treatment concentration of rotenone. Maximum percentage of alteration (30.833±1.666) is exhibited in case of adult flies which collected from 250 µg/L treatment category whereas minimum alteration (6.86± 0.933) is exhibited by control group. The * denotes significant change in comparison to control.

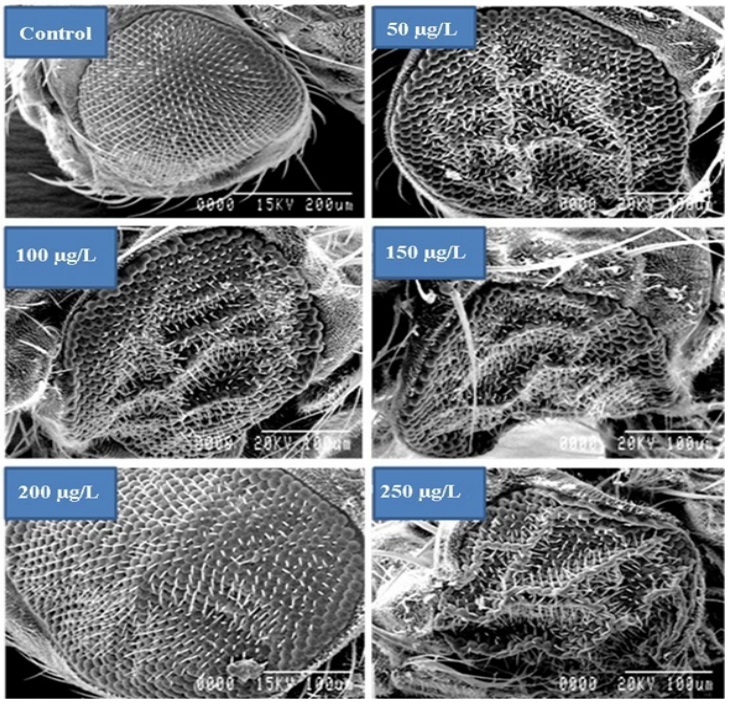


Figure E. SEM of compound eye (at 300X) exhibiting altered bristle orientation and disrupted ommatidial structure in *Drosophila melanogaster* exposed to 50, 100, 150, 200, 250 µg/L rotenone. SEM of control eye is used for comparison.

Result For SEM

Examination under Scanning Electron Microscope (SEM) after gold coating revealed that around 800 ommatidia (simple eyes) arranged in a regular crystalline pattern constitute the compound eye of *Drosophila melanogaster*. There is also an even distribution of mechano-sensory bristles between the ommatidia in the control flies as can be seen in (Figure E) at 300 X magnification. In the present study, treatment with rotenone is seen to disrupt the regular crystalline pattern of the ommatidial arrangements. As the treatments with chemical concentrations were increased (50,100,150,200,250µg/L), scanning electron microscopy revealed higher magnitude of morphological abnormalities in the compound eye of fruit fly. Flies treated with50 ppb (µg/L) exhibited a small groove in the middle portion of the eye with minimum bristle disorientation. Higher doses of treatment (100, 150, 200, 250 µg/L) showed greater number of groves and deeper ridges and altered bristle arrangements (Figure E).

Result for percentage of eye deformities

The percentage of eye alterations when studied in male female together shows that, flies receiving 50 µg/L and 100 µg/L treatment of rotenone have lesser degree of eye alteration (10.83± 0.833 and 28.33± 7.264 respectively) than the flies exposed to 150, 200 and 250 µg/L rotenone treatment where much greater percentage of major alterations were found (14.1666±1.666, 20.833±4.409 and 30.833±1.666 respectively).As expected only 6.86± 0.933 percent of control flies manifested some alterations (figure D). All the treatment categories successfully demonstrated a significant increase ($p<0.05$) in deformity incidence though no dose dependent response was apparent.

Discusssion

The present study successfully demonstrates the chronic sub-lethal effects of the organic pesticide, rotenone, on life cycle and compound eye of *D. melanogaster*. The variations in several life parameters and alterations in eye morphology are expressed as a function of the treatment. Interestingly, the increase in the duration of different life stages with the increasing treatment concentrations indicate interference of the chemical in the developmental process causing lengthening of the life stages. Though the increase in larval, pupal and adult

durations do not express a clear dose dependent response, yet their involvement in slowing down of development processes is clear Sir2, a member of the sirtuin family of protein acylases is known to deacetylate lysine residues within many proteins and is reported to be associated with life span extension in a variety of model organisms. Several studies have shown that increased expression of the *Drosophila* Sir2 homolog (dSir2) is responsible for lengthening of lifespan in *Drosophila* (16). Arsenic-containing hydrocarbons (AsHC) are also known to cause developmental toxicity in late developmental stages of *Drosophila melanogaster* (6). 4-phenylbutyrate (PBA) is also known to inhibit the activity of histone deacetylase, thus inducing hyperacetylation of histones, hence tending to release histones from their binding to chromatin, with consequent effects on gene transcription. Flies fed on PBA show a global increase in histone acetylation as well as express dramatic alterations in gene expression pattern, including either induction or repression of numerous genes related to ageing (6). Similarly rotenone might also alter the gene expression related to ageing. Thus this might be one of the mechanisms by which the life cycle duration of *Drosophila melanogaster* is increased. Another possibility might be the alterations in TOR pathway. The target of rapamycin (TOR) pathway which is a major nutrient-sensing pathway, when genetically down regulated, is known to be associated with delay in developments that in turn increase the life span duration in evolutionarily diverse organisms including mammals. It is observed that feeding rapamycin to adult *Drosophila* causes life span extension as seen in some TOR mutants (17).This chemical can arrest the organismal growth by inhibition of TOR kinase. Thus above mentioned test chemical rotenone might be acting in the same line to create the developmental delay in life cycle parameters by interfering with signaling pathway (TOR) or by regulating gene expression and protein function. Similar delay in the developmental process has also been observed in *Drosophila* when exposed to other chemicals like fluoridated chemical Cryolite (10), Sodium fluoride (12), and sulfur containing fungicide Thiovit jet (18).

The eye of *D. melanogaster* has been used as a model in several studies across the globe to study developmental patterning. In the present study, *Drosophila* compound eye has been considered as a model to observe rotenone induced changes in neuro-developmental pattern if any. Since both ommatidia and bristles, the two main components composing the eye, are of neuronal origin (19) and they are known to maintain a regular pattern, hence eye serves as an ideal model for validation of chemical induced alterations, if any. The ommatidia and bristles of *D. melanogaster* eye are formed from neuronal precursors and allow for highly sensitive characterization of mutations that alter neural developmental function (20). The CG5961gene functions as component of a multi-component SCF ubiquitin ligase complex. Specific alterations in expression of CG5961in the neuron-rich area of *D. melanogaster* eye is known to lead in disruption of tissue formation and decreased ommatidia and bristle numbers (21). Mutations in the furrowed (fw) gene of *Drosophila* result in defects in the development of the eye and mechanosensory bristles. The eyes are found to get reduced in size, have furrows or crevices in the retina, and show a disturbed patterning of ommatidia (22). Subsequently, targeted over expression of dMyc in *Drosophila* eye is known to result in ommatidial enlargement and roughening of the eye surface (23). Several genes known to be involved in the developmental maintenances of the eye, and any functional alteration in these genes might result in disruption of normal eye structure. Among the various relevant genes, Rab11 and Rab6 are most important ones (24). Processing and transporting rhodopsin are the main functions of these two genes (25). These ommatidia of Drosophila eye are facets that make up the eye which are connected to nervous system. Distinct disorganization and ridge formation in the compound eye from exposure of the larvae to rotenone has occurred in the present study which might be due to abnormal functioning of the nemo gene at the time of differentiation of the eye disc.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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