Finally, the present experimental data show the effect of colored light due to the difference in *light wavelength* on the development of a phenotypic trait from a genotype. The results are in accordance with those in *Drosophila* by Dobzhansky and Spassky (1944), Gupta and Lewontin (1982), in plants by Clausen *et al.* (1948), in human-beings by De Lorenzo *et al.* (1999), and Van't Veer and Bernards (2008). The present data confirm the results reported by Berry-Wingfield *et al.* (2010) using different colored light and analyzing *only* the *data on parental* iso-female lines of *D. melanogaster*, and *not* the hybrids (F_1's and F_2's). The data suggest that further research should be carried out on an individual genotype basis for the development of a phenotypic trait.

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Evaluation of ethidium bromide effects in the life cycle and reproductive behavior of *Drosophila melanogaster*.

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Abstract

Ethidium bromide (EB) is an intercalating agent of nucleic acids. For this reason it is generally used in molecular biology and in structural studies of DNA and chromatin. Many scientists have demonstrated that this chemical can have mutagenic properties in some living organisms, including *Drosophila melanogaster*. However, most of them used concentrations up to a thousand times higher than those used used in methods of molecular biology for nucleic acid staining after electrophoresis. In the present work we verified the effect of Ethidium Bromide in all phases of development (egg, larva, pupa, and adult) of ten generations of *Drosophila melanogaster* exposed to the chemical treatment (F1, F3, F6, and F10). Moreover, we analyzed the time spent for pre-copulation and copulation. The results show that ethidium bromide interferes in the viability of eggs, larvae, pupae, and adults of *Drosophila melanogaster*. On the other hand, the behavior related to reproduction showed significant differences between the groups exposed to 30 µM EB and 1µ M EMS (ethylmethanesulfonate) and the control group in terms of the time spent in copulation. So, the data suggest on one side that ethidium bromide interfered in developmental genes, causing in some individuals inviability to reach the adult phase, and on the other side that it can interfere in the fruit fly behavior, acting as a neurotoxic agent.

Introduction

Most of the chemical substances do not have a proper hazard classification. In this context, environmental monitoring is essential for identification of toxic products. Because of that, in the last years occurred a significant growth in the interest for studying the effects of substances to which people are daily exposed (Itoyama *et al.*, 1998). The effects of those drugs have been analyzed in several organisms, including bacteria, yeast, plants, and animals, besides humans, whenever it is possible (Timson, 1977; Leonard *et al.*, 1987).

When the organisms cannot avoid the exposure to a poisonous agent, the biological effects in response to these agents always happen after biochemical and cellular events. Accordingly, the cellular and biochemical parameters have a great potential to be used as stress indicators to evaluate the physiological conditions of an organism (Stegman *et al.*, 1992). Organisms try to overcome these stressful conditions by the activation of genes to produce specific proteins (Nazir *et al.*, 2003a), and the expression of such genes can minimize the stress effects (Atkinson and Walden, 1985).

Biochemical changes can often be translated as modifications in the morphology, behavio,r or metabolic pathways analyzed in a species known as a bioindicator, chosen for its sensibility or tolerance to several parameters, such as organic pollution or other kinds of pollutants (Washington, 1984).

In the last decades, the use of animal tissues for toxicological tests involves two fundamental concepts: science and ethics, leading to a search for alternative approaches. Nowadays, species of *Drosophila* are model organisms for toxicological studies, since they are well defined in terms of their genetics, biological development, and genome (Mukhopadhyay *et al.*, 2004). Additionally, fruit flies have high sensibility to toxic substances, and they are insects of easy maintenance in the laboratory, feeding mainly on bacteria and yeast that participate in the fermentation of carbohydrate rich substrates, such as decomposing fruits (Almeida *et al.*, 2001). Besides, the use of *Drosophila* is recommended by the European Center for Validation of Alternative Methods, which promotes the scientific and regular acceptance of alternative experimental methods (Mukhopadhyay *et al.*, 2004).

The use of insects, specifically *Drosophila*, for biomonitoring of genetic damages caused by chemical agents has traditionally been done more than 50 years. However, during the last years, experiments using *Drosophila* are related to the identification of carcinogens and as a model for the study of mutagenicity mechanisms induced by chemicals (Vogel *et al.*, 1999).

Genotoxic substances such as diethylestilbestrol, diphenylhydantoin, imipramine, testosterone, and tolbutamide have shown a high teratogenic potential, whose effects were evident in muscles and neurons in *Drosophila melanogaster* (Bournais-Vardiabasis *et al.*, 1983). The presence of varying ethanol concentrations in the culture medium of *Drosophila* caused the malformation of legs (segments lacking, absence of the legs or deformed), wings, dumbbells, and melted buccal parts (Ranganathan *et al.*, 1987). Cypermethrin, a potent insecticide, promoted the significant increase in DNA damage in the cells of the medium and previous cerebral ganglia (Mukhopadhyay *et al.*, 2004).

The genus *Drosophila* is found in six of the seven zoogeographic areas of the Earth (with exception of Antarctica). *Drosophila melanogaster*, as the other ones from genus *Drosophila*, has a complete metamorphosis, passing through all the developmental stages. The female lays eggs that hatch as larvae. These larvae pass for three stages and then, get into the pupal stage. Pupae stay attached to the glass wall in an artificial system. After a few days**,** from the pupae emerges the flying adult. Among thousands of residues generated by research laboratories, we have chosen to analyze the toxic effects of ethidium bromide (EB). That is the common name for 3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide, an intercalating agent used in methods of molecular biology. There are no studies focusing on the effect of EB in the developmental stages of *Drosophila melanogaster*. However, the effects of this chemical have been tested in other organisms.

Nishiwaki *et al.* (1974) pointed out that in mice EB acts as an inhibitor of RNA-dependent DNA polymerase activity, and for this reason it can be considered as an antitumoral agent. Furthermore, Heinen (1978) showed that EB inhibits cell growth in tissue culture, even at very low concentrations. However, in spite of this, EB is not used as an antitumoral agent because it has mutagenic capacity in some organisms. Results in bacteria show that EB is an effective frameshift mutagen if it is metabolically activated by liver microsomes (McCann *et al.*, 1975). Sea urchin eggs exposed to water containing 50 µM of EB developed chromosomal abnormalities and failed to divide normally (Vacquier and Brachet, 1969). Experiments in bacteria showed that EB is an effective frameshift mutagen if it is metabolically activated by liver microsomes (McCann *et al.*, 1975). Experiments reported by Nass (1972) indicated that the growth of mouse fibroblasts and hamster kidney cells are inhibited by 0.3-13 µM of ethidium, and that mitochondrial, not nuclear, DNA synthesis was inhibited by ethidium.

In *Saccaromyces cerevisiae* EB acts as a strong inducer of *petite* mutants (Slonimski *et al.*, 1968). Its action is based on the inhibition of mitochondrial nucleic acid and protein synthesis and is probably due to specific intercalations between the base pairs of mitochondrial DNA (Perlman and Mahler, 1971).

In previous work (Ouchi *et al.*, 2007), we have analyzed the effect of EB in productivity, protein profile, and phenotypical changes. The present work involved the exposure of ten generations of *D. melanogaster* to EB and intended to analyze its effect in the developmental phases of the insect. For specimens of F10, we also analyzed the effect of EB in sexual behavior, measuring duration of pre-copulation and copulation.

Materials and Methods

Stocks

Specimens of *Drosophila melanogaster* were collected at São José do Rio Preto (State of São Paulo, Brazil) and identified at the *Drosophila* Systematic Laboratory from our Institute. Two lines have been used in this work. One of them originated from one female (isofemale line). The other one was called massal line, because it has been originated by six females, having therefore higher genetic variability. Both stocks were maintained in a temperature-controlled chamber at 24ºC ± 1ºC.

Exposure to ethidium bromide

We used three different concentrations of EB (1, 5, and 30 µM) and two control groups. 1 µM of EB corresponds to the concentration used for visualization of nucleic acids, a solution that is frequently disposed in the drain without chemical neutralization. For the positive control we used 1 µM EMS (Ethylmethanesulfonate – *Acros Organics*), a mutagen, whereas the negative control was fed with uncontaminated culture medium. The chemicals were fully mixed with 50 mL of warm (45ºC) banana-agar medium, and then poured into 250 mL glass bottles. For each treatment, four replicates were prepared; three of them were used for the productivity experiments (Ouchi *et al.*, 2007) and the fourth one for an experiment of larval viability (not shown in this article). Ethidium bromide was purchased from *Promega*.

Maintenance of generations

For each bottle, twelve males of the *Drosophila melanogaster* stock were joined to the same number of virgin females. The culture medium was used as substrate for feeding, and females were allowed to oviposit for 6 days. After that, the adults were removed from the bottle for quantification. Ten days after the parents were added to the glass bottle, the new generation initiated its emergence

(F1).   In the fifth day, that corresponds to the maximum emergence of the control group, twelve males and virgin females were isolated from each replicate and then transferred to a new glass bottle, maintaining the same conditions, in order to originate F2 (the second generation).   The same procedure was repeated until the tenth generation.

Viability from egg to adult

A couple from the fourth replicate of each treatment of F10 from the isofemale line was separated, keeping the males separated from the females.   Each *Drosophila* stayed individually in glass tubes for five days until they reached their sexual maturity.   After this period, males and females from each treatment were mixed in the same glass tube where they were allowed to copulate for 24 hours.   Afterwards, we removed the male and allowed the female to lay eggs for 24 hours on a spoon containing 3 mL of agar-sucrose medium.   The eggs for the four replicates were counted with a stereoscopic microscope (*Carl Zeiss*).   The spoons containing eggs were transferred to a 250 mL glass bottle containing 50 mL of banana-agar culture medium, containing or not EB or EMS.   These experiments allowed us to count how many pupae and adults were viable.

Viability from larva to adult

The fourth replicate was also used to collect larvae for viability experiments, accomplished for F1, F3, F6, and F10 of the isofemale line.   Ten glass tubes containing 7 mL of treated banana-agar culture were used for each treatment, and to each one we added ten larvae.   After a few days, adults initiated their emergence and were analyzed quantitatively and morphologically in a stereoscopic microscope.

Viability from pupa to adult

Three replicates were used for experiments of productivity (not shown).   After fifteen days (time reserved to collect productivity information), some pupae remained attached to the wall of the glass without emerging as adults.   These pupae were counted for F1, F3, F6, and F10 from the isofemale line and for F1 and F10 of the massal line.

Sexual behavior

For each treatment, 24 couples were divided in six glass tubes, containing banana-agar culture medium.   Adults from both sexes were maintained isolated for five days in order to reach sexual maturity.   For each day, a negative control was analyzed for each treatment.   At the time of measurements every day, parameters of temperature, brightness, and period were the same.   After females and males were mixed the pre-copulation times were logged.   When each couple started to mate, this copulation time was logged, too.

Statistical analysis

The data of pre-copulation and copulation time were analyzed using Student´s T test ($p < 0.05$) by the software BioEstat 4.0 (Ayres *et al.*, 2005; Zar, 1999).

Results

In order to verify the action of EB in the different developmental stages of *Drosophila melanogaster*, we have analyzed the viability from larvae to adults of the isofemale strain (Table 1) and also pupae that did not emerge in the isofemale and massed lines (Table 2).

Table 1. Larval viability (%) from the isofemale strain of *D. melanogaster* after exposition to EB and EMS.

Treatment	Initial number of larvae	Generations				Average
		F1	F3	F6	F10	
Control	100	89	93	92	84	89.5
1µM EB	100	82	94	95	73	86.0
5µM EB	100	86	97	89	79	87.8
30µM EB	100	84	92	81	71	82.0
1µM EMS	100	74	90	86	80	82.5

Table 2. Pupal viability (%) from isofemale and massal strains of *D. melanogaster* after exposition to EB and EMS.

Treatment	Isofemale Strain				Massal Strain	
	F1	F3	F6	F10	F1	F10
Control	98.7	93	99.6	99.5	98.5	99.4
1µM EB	98.1	94	99.4	99.3	94.7	98.1
5µM EB	97.7	97	99.3	99.1	97.9	96.8
30µM EB	98.2	92	99.1	98.7	92.0	93.7
1µM EMS	93.5	90	98.7	97.1	97.4	93.7

Table 3. Egg to adult viability for F10 from the isofemale line from *D. melanogaster* exposed to EB and EMS.

Treatment	F10			
	Eggs laid (24h)	Pupae	Adults	% emerged adults
Control	127	111	111	87.4
1 µM EB	161	139	139	86.3
30µM EB	109	74	74	67.8
1µM EMS	130	43	43	36.2

Table 1 shows that in the first generation, the exposed animals had a lower viability than the control group. Even having small differences in viability, some morphological alterations were found in adults for the groups exposed to 1 and 5 µM of EB: one male with morphological alteration in wings and two males with morphological alterations in wings, respectively. This fact shows that even those insects that emerged might have suffered some type of gene alteration.

In F3, we could notice that the viability from larva to adult was not always higher in the control group, as verified for F1. For F6, a similar result to that described previously for F3: for the group exposed to 1 µM of EB the larval viability was higher than for the negative control. However, this fact was not observed in the same experiment for the 10th generation, where the larval viability was always smaller in the exposed groups. Considering the average of all generations, the larval viability was, in all treated groups, lower than for the control. We also observed that some flies started their emergence, but stopped in the middle of the process. So, we noticed that not only larvae were affected by the chemical treatments, but pupae were, too.

This fact was confirmed by the experiment involving the viability from pupae to adults, showed in Table 2. There we can see that the viability of pupae for the groups treated with EB and EMS were, in all conditions, slightly lower than that from the negative control group, in F1, F6, F10 of isofemale strain and for F1 and F10 of massed strain. If we compare the isofemale and massed lines for F10, we notice that the massed line, for the same concentration of EB and EMS, had a lower viability.
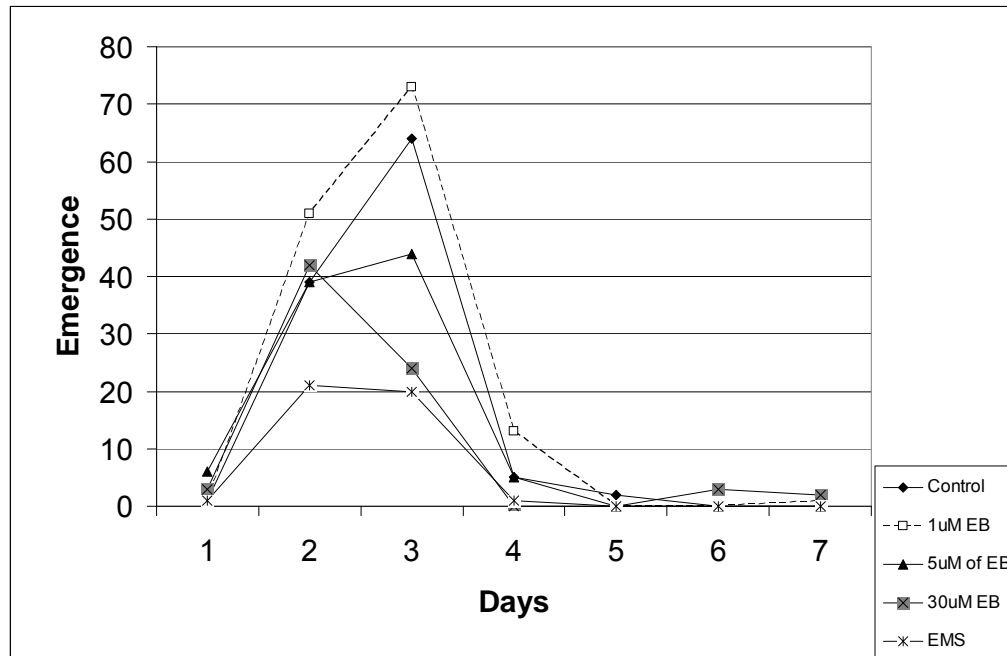

Figure 1. Pattern of daily emergence (egg viability).

Table 4. Average time expressed as minutes (') and seconds (") of pre-copulation and copulation for F10. The asterisks (*) represents the process (pre-copulation or copulation) and the treatment that showed significant difference compared to the control.

F10 isofemale line		
Treatment	Pre-Copulation	Copulation
Control	9'02"	19'36"
1 µM of EB	7'36"	22'22"
Control	13'15"	19'15"
5 µM of EB	10'56"	20'35"
Control	16'14"	18'48"
30 µM of EB	14'08"	21'01" *
Control	17'15"	21'50"
1 µM of EMS	12'15"	20'30" *

In Table 3, we show the effect of the chemical treatment in all the developmental stages. This experiment shows that EB and EMS affected mainly eggs and larvae, since from all the pupae emerged adults. Moreover, we quantified the pupae by the day that emerged in adults' emergence of the groups exposed to 30 µM EB and 1 µM EMS occurred one day before the others treatments, and their productivity was lower than the control, as shown by Figure 1.

The pre-copulation and copulation times are shown in Table 4. We can observe that the group exposed to 30 µM EB had a significantly larger time for copulation when compared to the control. However, for the group exposed to 1 µM EMS, the spent time was smaller than in the control. Both differences were significant ($p \leq 0.05$).

Discussion

Until the decade of 1980, there are no available works related to the action of EB in *Drosophila*. Marcos *et al.* (1981a) tested the genotoxic effects of EB in *Drosophila melanogaster*, using wild-type males. The concentrations that they used were in the range from 0.03 to 3 mM, many times larger than those used in our experiments. Toxicity tests were performed and detected that $LC_{50} = 2.16$ mM, for a 48h exposure. EB induced a significant increase in sex-linked recessive lethals (1.01% at 3 mM), and induced dominant lethals to a significant extent (Marcos *et al.*, 1981b).

Our results showed that in the experiments of viability from larvae to adults and pupae to adults, in ten generations, some alterations had happened during the development of the insects. These effects were observed by the higher amount of inviable larvae, which did not complete their development or failed to emerge as adults. Moreover, some flies emerged with wing alterations. Ranganathan *et al.* (1987) tested ethanol for teratogenicity in *Drosophila melanogaster* and reported malformations involving the legs and wings. Also, by exposing larvae to ethanol, the developmental stage sensitivity was investigated, showing also harmful effects. Genotoxic effects of griseofulvin, an antimycotic agent widely used in dermatophytoses, were studied by Tripathy *et al.* (1996) in the somatic and germ line cells, on third and second instar larvae of *Drosophila melanogaster*. Second and third instar larvae, exposed to acrylamide, considered to be a carcinogen, displayed genotoxic effects in *Drosophila melanogaster*, by the wing mosaic assay and the sex-linked recessive lethals test. It was observed that acrylamide is both mutagenic and recombinogenic in the wing disc cells and induces sex-linked recessive lethals (Tripathy *et al.*, 1991).

As mentioned above, some pupae started their emergence, but died in the middle of the process, leaving the body partially out of the pupal case. A similar result was observed by Sousa-Polezzi and Bicudo (2004), analyzing the effect of Phenobarbital (PB) in the development of *Aedes aegypti* (Diptera, Culicidae), suggesting that PB may affect the nervous system.

Analyzing the viability of laid eggs for the tenth generation of the isofemale line, we observed that it was lower in the exposed groups (Table 3). Moreover, we can see that there was a decrease of viability as EB concentration increased. Concerning the groups exposed to EMS, viability was lower than for those treated with EB. In the present study, EMS was used as a positive control, since it is a known mutagenic product (Griffiths *et al.*, 1998), being used as a parameter for the data obtained with ethidium bromide. Marcos *et al.* (1981a) carried out similar experiments analyzing the influence of EB and egg viability counting laid eggs and emerged flies. The number of viable eggs was inferior to the number of laid eggs (when compared to the control group), in agreement with our results. Our experiment allowed us to analyze all the stages, and it is possible to verify that the highest effect affected both eggs and larvae, because all the pupae emerged as adults. Several cellular divisions, mitoses and meiosis characterize the egg phase. Marcos *et al.* (1981a) have pointed out that EB act as a mitotic and meiotic poison and it even blocks the process of spermatogenesis.

Some other studies have focused on the effects of some chemical products in adults and in the different stages of development, using *Drosophila* as a bioindicator. Akins *et al.* (1992) revealed that some heavy metals, such as lead and cadmium, caused in *Drosophila melanogaster* a developmental delay at the phase from larva to pupa. In larvae of *D. melanogaster* exposed to sodium azide (a potent mutagenic product), it was observed the induction of somatic mutations and mitotic recombination in the wing cells (González-César and Branch-Morales, 1997). The same effect was observed when the tests were performed with methyl parathion, azametyphos, dichlorvos, and diazinon (Ekebas *et al.*, 2000). Until now, there are no studies focusing on the effect of EB in all developmental stages of *Drosophila melanogaster*.

It is noteworthy that the groups exposed to 30 µM EB and 1 µM EMS had emergence in the 6th and 7th days, whereas the others already stopped at the 5th day.

Itoyama *et al.* (1995) reported similar delays, analyzing the influence of caffeine in *Drosophila prosaltans*. In larvae of *Telmatoscopus albipunctatus* (Diptera – Pshychodidae), Sehgal and Simões (1977) verified that caffeine caused a significant delay of development and high mortality.

In the literature there are some works that verified the reproductive performance through the behavior, since some drugs can act on the nervous system. Itoyama *et al.* (1995) studied the effects of caffeine in mating of *Drosophila prosaltans*, based on the observation of the duration of pre-copulation and copulation. Statistically, they have found a difference only in the pre-copulation time. Nazir *et al.* (2003b) observed that dimethyl sulfoxide in *Drosophila melanogaster* has expressed a toxic effect in hatchability, emergence, fecundity, and in reproductive performance. In order to verify if the behavior could be influenced by EB, we observed the duration of pre-copulation and copulation. Our results showed that all the exposed groups had a smaller duration for pre-copulation than the negative control. The groups treated by EB showed a larger time for copulation, but only for 30 µM EB the difference was significant. In *Drosophila,* the mating movements are complex and follow a characteristic pattern of each species. Previous work (Ouchi *et al.*, 2007) showed that the presence of ethidium bromide caused malformations in *Drosophila*'s body, which could have influenced sexual behavior. Besides, EB could have some influence on the neural system.

In conclusion, our results suggest that EB in low concentrations influenced in phases of development, mainly in eggs, causing in some *D. melanogaster* inviability to reach the adult stage and could have some effects on the neural system.

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Genetic control of color dimorphism in *Drosophila punjabiensis* of montium subgroup.

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Abstract

Drosophila punjabiensis, belonging to the montium species subgroup of melanogaster group, was examined for abdominal melanisation. Females show dimorphism dark or light in coloration, whereas males exhibit monomorphic abdominal melanisation, *i.e.*, all are dark. The color dimorphism is regulated by two alleles of a single autosomal locus, and the light allele is dominant. Thus, *D. punjabiensis* exhibits color dimorphism controlled by a single locus, but its ecological significance is not clear.

Introduction

Abdominal melanisation is a conspicuously variable adaptive trait in many insects including *Drosophila* (Wittkopp *et al.,* 2003; True, 2003; Rajpurohit *et al.,* 2008). In different insect taxa, there are diverse patterns of body melanisation, *i.e.,* (a) several black species of Collembola occur in temperate regions, *i.e.,* Pyrenees, Swiss Alps, and Himalayas (Mani, 1968; Rapoport, 1969); (b) in *D. melanogaster*, a cosmopolitan species, the extent of melanism varies with geographical location (Pool and Aquadro, 2007; Parkash *et al.,* 2008a,b); (c) discrete melanic and non-melanic morphs occur as genetic polymorphism in species of the montium species subgroup (Ohnishi and Watanabe, 1985). The color polymorphism in abdominal tergites was first reported by da Cunha (1949) for *D. polymorpha.* Later on color variations in montium species subgroup *D. rufa* (Oshima, 1952), *D. kikkawai* (Freire-Maia *et al.,* 1954), *D. auraria* (Lee, 1963), and *D. jambulina* (Parkash and Sharma, 1978; Parkash *et al.,* 2009) were described.

The present work is a first report showing the genetic basis of color dimorphism in *D. punjabiensis* of montium species subgroup, through Mendelian crosses between dark selected and