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Evaluation of the Effects of a Single Exposure to Ethidium Bromide in *Drosophila melanogaster* (Diptera-Drosophilidae)

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Each year new chemicals enter in the market and generate an increasing volume of residues, raising health and environmental concerns. Although many of those compounds are known as toxic, a significant proportion is potentially dangerous and does not have a proper hazard classification, and can generate harmful biological effects. Biochemical changes can be often translated as modifications in the morphology, behavior, or metabolic pathways, analyzed in a species known as bioindicator (Washington 1984). In this ecological context, biomonitoring can be defined as the use of systematic responses of live organisms in order to evaluate the environmental changes, generally caused by human actions (Mathews et al. 1982).

Concerning biomonitoring, over the past decade, issues such as animal handling and care in toxicology research and testing became one of the fundamental concerns for both science and ethics. Emphasis has been given to the use of alternatives to mammals in testing, research and education. *Drosophila melanogaster* is the most widely used insect model because of its well-elucidated genetics and developmental biology. Moreover, the use of *Drosophila* has been recommended by the European Center for the Validation of Alternative Methods (ECVAM) with the purpose of reducing, refining or replacing the use of laboratorial animals (Benford et al. 2000).

Among thousands of residues generated by research laboratories, we choose to analyze the potential toxic effects from Ethidium Bromide (EB). That is the common name for 3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide, an intercalating agent usually used in molecular genetics and in structural studies of DNA and chromatin. Heinen (1978) showed that EB inhibits cell growth in tissue culture, even at very low concentrations; but in spite of this, EB is not used as an antitumoral agent because it has mutagenic capacity in some organisms. The 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 or more of ethidium bromide developed chromosomal abnormalities and failed to divide normally (Vacquier and Brachet 1969). In mice, EB apparently has little or no access to nuclear DNA, at least in vivo, while it intercalates perfectly well with isolated nuclear DNA in vitro (Pack and Loew 1978).

Drosophila has fulfilled a dual function in the field of genetic toxicology: it has been used for short-term tests for identifying carcinogens and also as a model for studies of the mechanisms of mutagenesis induced by chemicals (Vogel et al. 1999). EB is commonly used in techniques of molecular biology, but the intercalating property is potentially harmful for living beings. So, we decided to verify possible toxic effects that EB could cause in humans who manipulate this compound every day, using *D. melanogaster* as a bioindicator. We choose to analyze a range of concentrations compatible with those used in laboratories to stain acid nucleic agarose gels. Even though EB is considered as a mutagenic compound, it is not classified as

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a possible carcinogenic agent for humans by IARC (International Agency for Research on Cancer).

In the present study we investigated the influence of different concentrations of EB on productivity, morphological alterations and biochemical analyses based on beta-naphthyl esterase activity, using *D. melanogaster* as a bioindicator. Furthermore, we verified the action of EB in two developmental stages of this insect's life cycle.

Materials and Methods

Specimens of *Drosophila melanogaster* were collected in May 2005, at São José do Rio Preto (State of São Paulo, Brazil), using suitable traps (Medeiros and Klaczko 1999). The species' identification was carried out at the Laboratory of *Drosophila*'s Systematic of our Institute. One female originated the isofemale stock which was maintained in a temperature controlled chamber at 24 ± 1°C.

We used three different concentrations of EB (1, 5 and 30 μM) and two control groups. The lowest concentration corresponds to that one used for visualization of nucleic acids. For the positive control we used 1 μM EMS (Ethyl methanesulfonate), a mutagenic chemical, whereas the negative control was not exposed to EB or to EMS. The chemicals were fully mixed with warm (45°C) 50 mL of the banana-agar culture medium, and then poured into a 250-mL glass bottle. For each treatment four replicates were prepared; three of them were used for the productivity experiments and the fourth one for the experiment of larval viability.

For each bottle, 12 males of the *D. melanogaster* stock were joined to the same number of virgin females. The treated culture medium was used as substrate for feeding, and females were allowed to oviposit for 6 days. After that, the adults were removed. Ten days after the parents were added to the glass bottle, the new adult generation initiated its emergence. During 15 days the adults were counted twice a day and morphologically analyzed with a stereoscopic microscopy (*Carl Zeiss*). Some adult females and males, and all the flies that displayed morphological alterations, were kept frozen at –20°C for later electrophoretical analysis. At the end of that period, pupae that did not emerge as flies and remained attached to the bottle wall were counted, and this allowed measuring the effect of EB on the insect viability.

All data of daily productivity were expressed as the proportion of flies emerged each day divided by the total produced per treatment. The proportion of flies carrying malformations was calculated as the ratio of the total number of *Drosophila* emerged on each treatment. The statistical analyses were performed using the program BioEstat 4.0 (Ayres et al. 2005). In order to the compare control and the treated groups, in first instance we applied the χ^2 homogeneity test for two multinomial populations (Mood et al. 1974). Subsequently, for multiple comparisons of pair proportions we used the Normal Approach Z for independent samples (Moore 2005), using a $p \leq 0.05$ significance level.

As mentioned before, the fourth replica was used to collect larvae for viability experiments. 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 quantified and analyzed morphologically in a stereoscopic microscope.

The pattern of total proteins, as well as alpha and beta naphthyl esterase activities were verified by electrophoresis, with a specific staining for each one in the same polyacrylamide gel. For these experiments we used some males and females of the negative control and also flies displaying morphological alterations. Each fly was homogenized individually in 0.2M Tris–HCl pH 8.8 buffer. The extracts were centrifuged at 10.000 rpm using a MiniSpin (Eppendorf) centrifuge for 3 min. For electrophoresis we used 10 μL of the extract and 0.1M Tris-Glicine pH 8.3, setting the voltage to 180 V during 4 h. The gels were stained using twice: first for alpha and beta-naphthyl esterase activity, using α and β-naphthyl acetate as substrates, according to Galego et al. (2006) for 90 min. After that, the gel was submerged in a solution of 20% ethyl alcohol and 20% acetic acid for 1 h, and subsequently stained with Coomassie Brilliant Blue R-250. Alpha naphthyl esterase activity is visualized as a black spot, beta naphthyl esterase activity as a red band, and other proteins as blue bands.

Results and Discussion

In order to analyze the effect of the exposition of *D. melanogaster* to EB, the productivity was measured along 15 days. In the data of the three replicates we applied the "Chi-Square" test and the results revealed that all differences were significant compared to the control. Following that, we verified the comparison of pair proportions, as shown in Table 1, where the asterisks point to significant differences between the exposed groups and the negative control. Accordingly, we can notice that most of the days for the treatments with EB and EMS demonstrated significant differences on productivity. Remarkably, the groups exposed to 1 and 5 μM EB have a different profile with delayed emergence, and suggests that it would continue beyond the 15th day.

The alterations in the normal morphological patterns affected mainly tergites and wings. However, other malformations were found, such as the absence of one paw and different body pigmentation. These alterations are not shown in this work.

Table 1 Mean proportion of daily productivity (from three replicates) for each treatment

Treatment										
Daily Productivity	Control		1 μM EB		5 μM EB		30 μM EB		1 μM EMS	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1st	0.01	0.01	0.01	0.01	–	–	–	–	–	–
2nd	0.07	0.03	0.06	0.05	0.03*	0.01	0.01*	0.01	0.01*	0.01
3rd	0.14	0.03	0.07	0.01	0.11*	0.03	0.07	0.05	0.15	0.05
4th	0.30	0.03	0.22*	0.05	0.24	0.02	0.11*	0.05	0.14*	0.02
5th	0.38	0.05	0.24*	0.05	0.21*	0.05	0.54*	0.05	0.51*	0.04
6th	0.05	0.02	0.18*	0.05	0.22*	0.05	0.24*	0.03	0.18*	0.05
7th	0.02	0.01	0.06*	0.02	0.07*	0.04	0.02	0.03	0.01	0.01
8th	0.01	0.01	0.07*	0.02	0.03*	0.01	0.01	0.01	0.01	0.01
9th	0.01	0.01	0.03*	0.02	0.02	0.02	0.01	0.01	0.01	0.01
11th	0.01	0.01	0.03*	0.03	0.03*	0.03	–	–	0.01	0.01
13th	–	–	0.03*	0.03	0.03	0.03	–	–	–	–
15th	–	–	0.01	0.01	0.01	0.02	–	–	–	–

* Significant differences compared to the negative control ($p \leq 0.05$)

However, we cannot analyze just one parameter to infer if the chemicals are exerting their toxicity properties on this species. So, other points were verified (shown on Table 2). It is noteworthy that the group exposed to 30 μM of EB has the same amount of days with significant differences as EMS, when compared to the control. In spite of this, the group exposed to 30 μM EB and the control has a similar frequency of malformations. The larval viability was lower in 30 μM EB compared to the negative control, and the total productivity was 17% lower. For the other treatments (1 and 5 μM EB and 1 μM EMS) the larval viability was always lower than for the control. However, the frequency of alterations was reached at four times larger than the control. Moreover, some pupae did not emerge as adult flies, and the groups exposed to 1, 5 and 30 μM EB and EMS revealed a larger emergence than the control. These experiments revealed that the exposition of EB was not dose-dependent, as the proportion of alterations, and larvae and pupae viability do not display a clear trend with EB concentration. The viability from egg to larvae was more affected by the chemical.

The curve of daily total productivity of the group exposed to the highest EB concentration (30 μM) is similar to that obtained in the presence of EMS (Fig. 1). Moreover, the proportion of alterations was similar for the groups exposed to 1 and 5 μM of EB and EMS. However, in *D. melanogaster* these effects were not dose-dependent. Ohnishi (1977) reported that adult specimens of male *Drosophila melanogaster* fed with EMS presented a high frequency of recessive lethal mutations and also polygenic mutations affecting viability. This could be an explanation for the results observed in some concentrations of ethidium bromide and EMS. EMS is known to produce base-pair substitutions and chromosome changes (Mukai 1970). Mutation and chromosome breaking effects have been reported by Alderson (1965), Epler (1966) and Jenkins (1967).

The pattern of emergence in the presence of 1 and 5 μM of EB shows a significant increase compared to the negative control. Mukai (1964) reported a similar effect for EMS in the same species. According to him, when all the mutations are located on the same chromosome, the viability is high, even showing overdominance of the mutants; but it would be low, showing a partial dominance, when the mutants are distributed between both homologs.

In order to understand biochemical changes induced by EB, the pattern of beta naphthyl esterase activity was

Table 2 Summary of results after a first exposition to EB and EMS

Treatment	Total number of emerged adults	Proportion of larval viability		Proportion of pupal viability		Proportion of alterations	
		Mean	SD	Mean	SD	Mean	SD
Control	463	0.89	0.18	0.987	0.004	0.004	0.007
1 μM EB	752	0.82	0.16	0.981	0.008	0.014	0.005
5 μM EB	917	0.86	0.13	0.976	0.009	0.016	0.004
30 μM EB	386	0.84	0.12	0.981	0.004	0.004	0.006
EMS	288	0.74	0.22	0.937	0.009	0.015	0.008

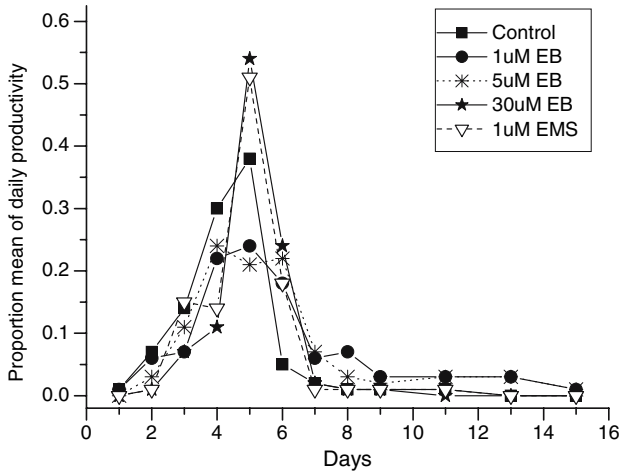

Fig. 1 Proportion of daily productivity emergence (females and males) for a single exposure of *Drosophila melanogaster* larvae and pupae to EB and EMS

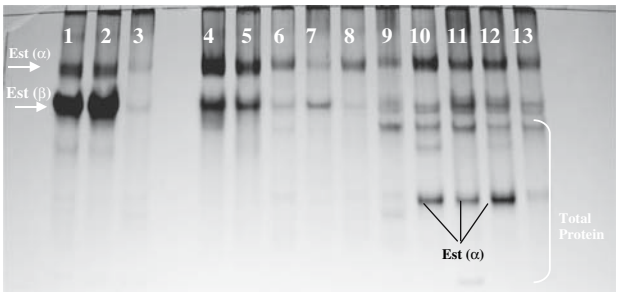

Fig. 2 Electrophoresis gel showing esterases activities and the pattern of total proteins for adult specimens. Est(α): esterase activity in the presence of alpha naphthyl acetate; Est(*β*): esterase activity preferentially in presence of beta naphthyl acetate. Both esterase activities were distinguished by the color expressed in the presence of substrates (not shown). *1,2* Control males; *3* males with wings' alterations; *4,5* control females; *6–13* females with wings' alterations

analyzed by electrophoresis, of some normal and abnormal females and males (Fig. 2). For both sexes the activity of beta naphthyl esterase was lower than for the normal flies. According to Marcos et al. (1981), 3 mM of EB can induce dominant lethals, sex-linked recessive lethals, non-disjunction, loss of X or Y chromosomes and translocations between the second and third chromosomes. Moreover, since we performed two different stains in the same gel, the presence of spots not found among normal flies suggests that some other proteins are produced or modified by the exposure to EB.

However, for the same alteration, in wings for example, different patterns are observed (specimens 6–13, Fig. 2). Goncharova et al. (1988) revealed that *D. melanogaster* cells can have an individual sensitivity to the presence of a mutagen. The different sensibility of separate individuals to mutagens reflects the existence of cryptic genetic variability in *Drosophila* strains.

In conclusion, *D. melanogaster* is a reliable model organism for many areas of knowledge, including genetic, behavior, biomonitoring and mutagenesis. So, according to our data, we can conclude that EB, even in low concentrations, can induce toxic effects in *D. melanogaster* in terms of productivity, morphological and biochemical parameters, presumably due to its genotoxic properties. We suggest that humans that work with EB and are chronically exposed should protect themselves when manipulating this chemical, since toxic effects could appear after a significant delay, and EB should be chemically neutralized before its disposal.

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