Evaluation of Genotoxicity of Lufenuron and Chlorfluazuron Insecticides in *Drosophila melanogaster* Using a Germ-Line Cell Aneuploidy and Chromosomal Aberrations Test

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Abstract— Acyl ureas, as insect growth regulators, were developed as "safe" and "non-mutagenic" insecticides. Here, using *Drosophila melanogaster* as a model system, three genotoxic modes of action of the acyl ureas were revealed. First, an adapted and modified genotoxicity test revealed genotoxicity in the germ-line cells of treated males. This was characterized as aneuploidy and chromosomal aberrations in brood 1 sperm of parental males that were inherited and appeared phenotypically at statistically significant rates ($P \leq 0.05$) in F1 males. Second, damage to the entire genomic DNA, which was assessed in the adult fly of the isogenic strain w^{1118}, was qualitatively indicated by an apoptosis-associated DNA fragmentation test. Apoptosis-specific internucleosomal cleaved fragments of 180–200 bp (and their multiples) were detected. DNA damage was also quantitatively estimated by the comet assay under a high alkaline condition (pH > 13). From the tail moment criterion of this test, a statistically significant difference ($p < 0.01$) between the treated and untreated flies was observed. Third, we identified point mutations in specific fragments of the *Dmp53* and *Rbf* tumor suppressor genes. Pairwise alignments of the obtained PCR products from pools of treated and untreated flies revealed the occurrence of point mutations as a genotoxic effect of acyl urea treatment. Based on these findings we propose that acyl ureas are multitudinous mutagens having three distinct mutagenic modes of action.

Keywords: Aneuploidy, Drosophila, Genotoxicity, Pesticides.

I. INTRODUCTION

THE intensive use of insecticides leads to chronic exposure to lower doses of these toxicants, which may result in genotoxic stress in humans and other mammals [1]. Therefore, human genotoxicity and cancer risk because of exposure to insecticides represent a prominent hazard to public health. In response to DNA damage, mammals utilize distinct pathways such as apoptosis to obliterate or at least minimize the resulting hazards [2]. Defects in DNA damage response tend to cause genomic instability and may ultimately result in the formation of cancer [3].

A new generation of safe (nonmutagenic) insecticides has been developed that disrupt the normal growth and development of insects and result eventually in the death of the insect pest [4]. Acyl ureas comprise a group of these insect growth regulators that are able to inhibit synthesis of insect chitin [5]. Most of the affected insects die from rupturing of the new malformed cuticle. Mechanisms of mutagenesis and risk assessment of the acyl ureas should be taken into consideration. This task can be accomplished by genotoxicity tests using genetic systems that detect mutations and their mode of formation [6]. Previously it was noted that no potential for mutagenicity was observed with lufenuron tested in vitro in bacteria (Ames test), in mammalian cells systems, and in vivo in a mouse micronucleus test and unscheduled DNA synthesis test with rat liver cells [7].

In this respect, *Drosophila melanogaster* is recommended as a model experimental animal because more than half of all *D. melanogaster* protein sequences are similar to those of mammals [8]. Furthermore, a direct homology between *D. melanogaster* genes and genes that affect human diseases (nearly 75% of genes that cause human diseases are believed to have a functional homologue in the fly) was also indicated [9, 10]. Tester strains are available or can be constructed for determining almost all types of genetic changes, ranging from gene mutations to chromosome rearrangements, in a great variety of cell types of both sexes [11].

The genotype of the somatic aneuploidy test (SAT) strain, developed by [12], is zw-/w+Y for the male and zw-homozygous for the female (w- is the sex-linked mutation w11E4, which is a deletion of the white locus; z is the sex-linked mutation zeste). Both male and female are homozygous for the recessive mutation sepia eye (se) on the third chromosome. The Y chromosome carries a small piece of the X chromosome that includes the w+ (the wild type gene of the color).

The w^{1118} flies have white eyes because of a deletion in the sex-linked white gene and therefore cannot revert to wild type. The white gene is normally located on the X chromosome, but it does not have to produce an eye color. Flies homozygous or hemizygous for w^{1118} have white eyes, whereas those heterozygous for w^{1118} and wild type have the normal red eyes. For aneuploidy and chromosomal aberrations, when germ-line cells are affected by this mutagenic mechanism, fetal abortions

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or offspring with typical syndromes are produced [13]. With D. melanogaster as a genetic model, there are certain mutagenicity test systems to detect aneuploidy and chromosomal aberrations in germ-line cells and in somatic cells, dominant lethal mutations, sex-linked and autosomal recessive lethal mutations, and translocations [14-16].

For detection and quantitative estimation of induced aneuploidy and chromosomal aberrations, germ-line cells of male D. melanogaster of a specific strain were used as a target for this task, and a D. melanogaster genotoxicity test was developed, standardized, and used. This developed test aims to be a feasible and reliable test for detecting inheritable mutations. Because DNA damage is a well-recognized inducer of carcinogenesis, the aim of this study was to reveal genotoxic modes of action of two of the acyl ureas insecticides— lufenuron (LUF) and chlorfluazuron (CHLO)—and to assess their mutagenicity risks by using D. melanogaster as a genetic model.

First, DNA damage effects of both LUF and CHLO were assessed qualitatively using the developed strain of D. melanogaster by the DNA laddering assay and quantitatively by the comet assay. Second, the nucleotide sequence of certain fragments of the two highly familiar tumor suppressor genes— Dmp53 (variants b and c) and DmRbf—were examined for induced point mutations..

II. METHODS AND MATERIALS

Chemicals

LUF (1-[2,5-Dichloro-4-(1,1,2,3,3,3-hexafluoropropoxy) phenyl]-3-(2,6-difluorobenzoyl)urea) was purchased from Novartis Animal Health Inc. (USA), while CHLO (1-[3,5-dichloro-4-(3-chloro-5-trifluoromethyl-2-pyridyloxy)phenyl]-3- (2,6-difluorobenzoyl)urea) was purchased from Shijiazhuang Jitai Sanmu Pesticide Chemical Industry Co., Ltd. (China). Colchicine was obtained from El Nasr Pharmaceutical Chemicals (ADWIC) (Egypt).

Strains

The Drosophila strains used, SAT and w[1118], were from well-established colonies at the Genetics Department, Faculty of Agriculture, Ain-Shams University, Egypt, and Zoology Department, Faculty of Science, Cairo University. The SAT strain was originated from Institute of Genetics, Biological Research Centre, H-6701 Szeged, Hungary. The w[1118] strain was originated from Bloomington Drosophila stock center (Stock no. 5905, FlyBase ID: FBst0005905).

The Drosophila SAT strain of genotype zw-/w+Y for male and zw-/zw for female was used for the development of a germ-line cell aneuploidy test to be used for detection of the inheritable mutations. Flies from the isogenic strain w[1118] (FlyBase ID: FBst0005905) were used for assessment of DNA damage and point mutations resulting from exposure to the tested putative mutagens.

All strains and crosses of normal and treated Drosophila were kept at a density of 100 flies per rearing bottle with controlled temperature at $25 \pm 1°C$, a 16:8 h light-dark photoperiod, and ambient relative humidity (40–70%). Detailed descriptions of standard Drosophila colony and rearing techniques are given by [17]. One-day-old adult flies were collected from an established laboratory colony and then regularly transferred to freshly prepared medium every 3–4 days. One hundred flies were used in each experimental group.

Drosophila melanogaster strains were maintained on SDM (Standard Drosophila Medium) containing agar, corn meal, sucrose and yeast at 22 ± 1 °C in laboratory conditions.

Toxicity

Seven concentrations (10, 15, 20, 25, 30, 35, and 40 ppm incorporated into the feeding medium) were used for determining the LC_{50} value for each insecticide. Third instar larvae (n = 100) were placed for 24 h into the rearing bottles containing a feeding medium incorporated with different concentrations of the acyl ureas. The concentration that caused 50% lethality of the flies was determined graphically using the log-probit analysis (Litchfield and Wilcoxon 1949). LC_{50} was obtained by plotting probit survival against acyl ureas concentrations and fitting points by linear regression. The LC50 was determined to be 38 ppm and 48 ppm for LUF and CHLO, respectively.

Treatment with acyl ureas

Late third instar larvae (72–76 h aged) were exposed to the LC50 of the tested acyl ureas incorporated into the feeding medium for 24 h. The treated larvae were floated from the feeding medium with 20% glycerol and transferred into new rearing bottles containing the normal feeding medium (no acyl ureas included) and held through pupation and then until adult emergence. Ten groups of 20–30 treated parental males were crossed with 40–60 normal untreated virgin females to obtain females inseminated with the first brood (Br1) of sperm. The treated males were recrossed to new normal untreated virgin females under the same conditions to obtain females inseminated with the second brood (Br2) of sperm. The daily emerged flies of this F1 progeny produced from the mentioned crosses were screened for the presence of abnormal progeny (i.e. white-eyed males or sepia-eyed females). Each abnormal male (white-eyed) was then separately crossed with four normal untreated virgin females to test whether it was fertile or sterile, as indicated from hatchability of the eggs laid by these crossed females. Treatment with the known aneugen colchicine was completed by immersion of the third instar larvae for 2 h in 1 mM solution of this chemical, as recommended in [16, 18].

Development of a germ-line cell aneuploidy and chromosomal aberrations test

A specific D. melanogaster strain was originally developed for detection of somatic cell aneuploidy in parental male eyes as microscopic colored mosaics, (i.e., SAT) by [12]. In the present work, this test was modified and adapted to develop a genetic test for inheritable mutations, (i.e., germ-line cell aneuploidy and chromosomal aberrations test, or GAT) in parental males of D. melanogaster. These mutations appear as abnormal colors of the whole eye (a macroscopic phenotype) in the emerged males and females of F1 progeny because of exposure of the parental males as third larval instars to the LC_{50} of the acyl ureas LUF (38 ppm) and CHLO (48 ppm) for 24 h.

This SAT strain, which has been adapted in the present work, is based on the observation of the eyes of the emerged

parental males and detecting a few microscopic colored mosaics of yellow and white cell clones that represent XYY and XO cell descendants of a single XY cell (during development up to the formation of the parental adult male) in which nondisjunction took place in the developing eye primordium.

On the other hand, the color of the whole eye in the SAT strain of *D. melanogaster* is the trait of concern in the GAT test. The genetic structure controlling this trait is w-/w+Y; se/se in males and w-/w-; se/se in females. When the adult male or female carries only w-, it will have white eyes because of the lack of w+, which regulates eye pigment formation. The opposite is true with the adult carrying only w+, which will have pigmented eyes. The recessive mutation se, located on the third chromosome, was originally introduced into the strain [19] to modify the colors. Therefore, when the w+ gene and two doses of se are present, as occurs in the normal males of this strain, a sepia-eyed phenotype is produced; otherwise, the eye color will be white, as in the normal females of this strain. Hence, GAT is based on the detection, in F1 progeny adults, of the abnormal phenotype sepia-eyed females (because of the presence of the nondisjunctioned w+Y chromosome or its w+ locus) and white-eyed males (because of the absence of the w+Y chromosome or w+ locus). Also, the sterility of the F1 male, which indicates the loss of the X or Y chromosome, is included as an abnormal case. Figure 1 shows eye colors of the normal male and female, and the GAT-produced mutated male and female D. melanogaster. Figure 2 shows scheme for the abnormal gametes produced by the treated parental males, the expected F1 progeny phenotype, and the mutation type.
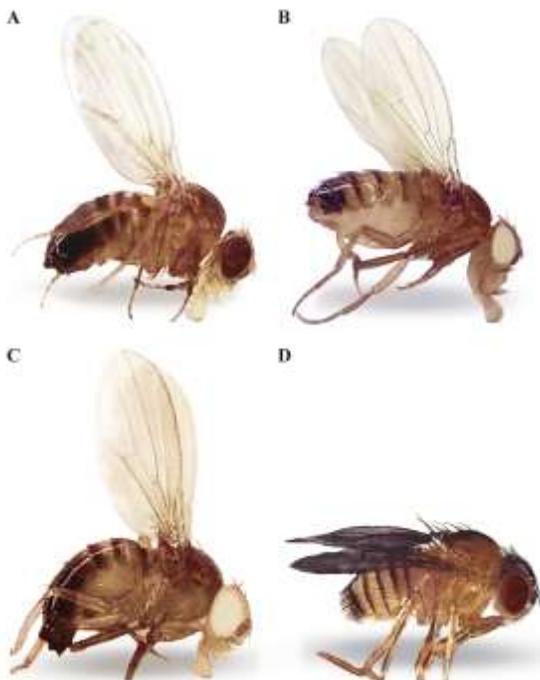

Fig. 1: Eye colors of the normal male (sepia-eyed, A) and female (white-eyed, B), and the GAT-produced mutated male (white-eyed, C) and mutated female (sepia-eyed, D) *D. melanogaster*
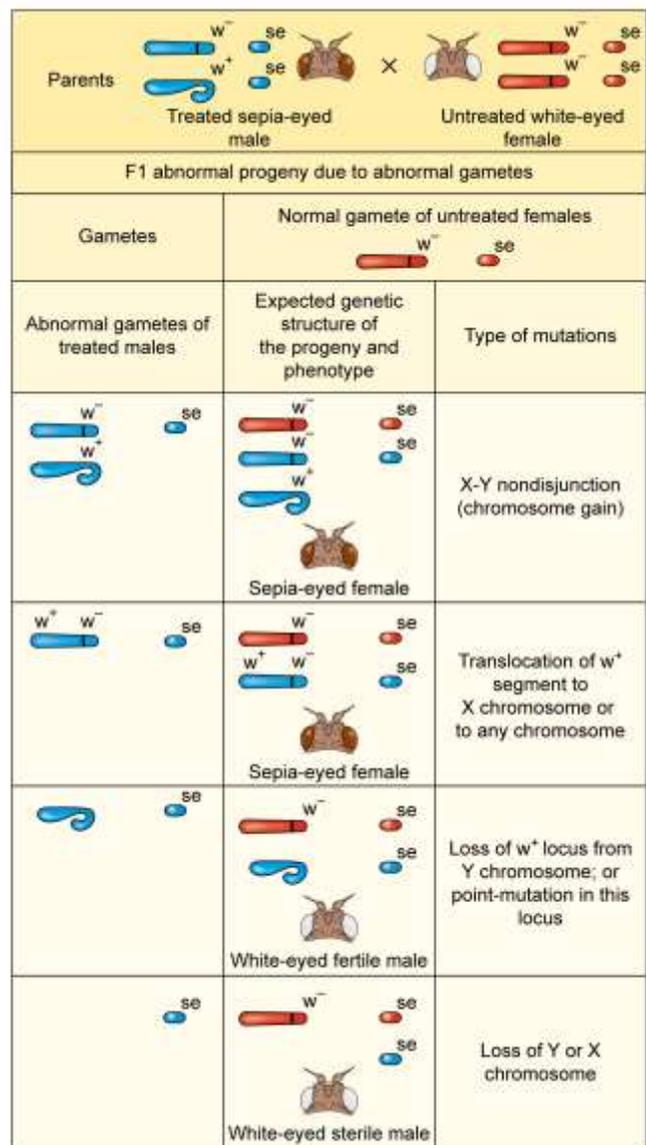

Fig. 2: Scheme for the detection and mode of aneuploidy and chromosomal aberrations in adult *D. melanogaster* using the developed GAT

The modification and adaptation of SAT to be used as GAT enables detection and quantitative estimation of the frequency of induced aneuploidy and chromosomal aberrations in male germ-line cells.

Standardization of the developed GAT

The validity of the developed GAT as a genotoxicity test for detecting aneuploidy and chromosomal aberrations in the germ-line cells of male *D. melanogaster* was tested. In this case, the known aneugenic chemical colchicine was used, as recommended by [16, 18] (see Materials and Methods). The obtained data show that colchicine has considerable aneugenic potential; it causes the production of white-eyed sterile (mutated) males and sepia-eyed (mutated) females with a highly significant difference ($p < 0.001$) when compared with that of the negative control.

Table 2 shows effect of LUF and CHLO on aneuploidy and chromosomal aberrations in germ-line cells of *D.*

melanogaster, tested by GAT using chi-square statistics (χ^2-test)

Therefore, GAT is a feasible and reliable test for the detection and quantitative estimation of the mutagenic potential of chemicals, such as the acyl ureas insecticides in this study, to induce aneuploidy and chromosomal aberrations in germ-line cells of parental male *Drosophila*. When GAT is compared with SAT, it is revealed to be more feasible and reliable for different parameters than SAT.

Alkaline comet assay

The extent of DNA damage in all types of cells of the isogenic strain (w[1118]) of *Drosophila* adult that developed from the untreated and treated third larval instar was assessed. The adult flies were frozen in liquid nitrogen, around 100 flies were gently homogenized into powder, and then an alkaline comet assay as described by [20] was utilized.

Evaluation of DNA was visualized with fluorochrome stain of DNA with the fluorescent microscope and a 40X objective (depending on the size of the cells being scored). A Komet™ analysis system 4.0 developed by Kinetic Imaging, LTD (Liverpool, UK) linked to a CCD camera was used to measure the length of DNA migration (tail length, in µm) (TL) and the percentage of migrated DNA (DNA %). Finally, the program calculated tail moment. Fifty to one hundred randomly selected cells were analyzed per sample (at least 25 cells per slide and 3 slides per treatment were evaluated). Three different parameters were used as indicators of DNA damage: tail moment (TM) (arbitrary units), tail DNA (%), and tail length (mm). These parameters have been described previously in detail [21].

DNA fragmentation assay

DNA from 50 flies was isolated using a salting-out extraction method as described by [22]. It was quantitated at 260 nm, electrophoresed on a 1.5% agarose gel in TBE buffer, and visualized after ethidium bromide staining using GelDoc XR (BioRad Laboratories, Inc).

PCR amplification and sequence analysis

A standard PCR protocol was applied on the extracted DNA pools from 50 flies from both the treated and untreated isogenic strain of *Drosophila* (w[1118]) samples. Table 1 shows the sequences of the primers designed to amplify the target genes *Dmp53* (two variants, *Dmp53b* and *Dmp53c*) and *Rbf*. These primers were designed using Primer3 (http://www.ncbi.nlm.nih.gov/tools/primerblast/), obtained and synthesized from Bioneer (Seoul, Korea).

TABLE 1: THE NUCLEAR SEQUENCES AND DROSOPHILA GENE ACCESSIONS NUMBERS ON WHICH THE DESIGN WAS BASED.

Gene	Primers	Sequences	Expected product size (bp)	Designing based on accession no.
Rbf	F	TCTGGCACATCTTTGAGCAC	233	NM_080297.2
	R	CGGGTTGTAAGGAGTTGCAT		
Dmp53b	F	TGTATCGGGCGAAAAGAAAC	247	NM_206544.2
	R	CTCGGCTATCATTGCTCTCC		
Dmp53c	F	GGTGGCCACTACGATTCTGT	215	NM_001170223.1
	R	AATTCCGATCCCGATACCTC		

PCR amplifications were carried out using 2 µl sample DNA extracts in a total volume of 50 µl. Reactions were carried out using the ready-made master mix DreamTaq DNA polymerase (Fermentas, Thermo Scientific) supplied in 2X DreamTaq Green Buffer. Samples were initially denatured at 95°C for 5 min. PCR was monitored for 39 cycles with denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 20 s were performed. A final extension at 72°C for 10 min was necessary for complete amplification.

The PCR products of the *Dmp53* and *DmRbf* were cleaned up using a QIAquick PCR purification kit (QIAGEN, Germany). The purified PCR products were then subjected to DNA sequencing to determine mutations and the percentage of identities in nucleotide sequences of the amplified fragments of both *Dmp53* gene variants and of *Rbf*. The nucleotide sequences were determined by automated DNA sequencer - 3730xl DNA analyzer from Applied Biosystems using BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems). Nucleotide sequence analysis was performed with Genetyx software version 7.3.0 (GENETYX, Tokyo, Japan). The nucleotide homology search for the DNA sequence was performed by BLAST on the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence alignment analysis was performed with the ClustalW program [23] and the BioEdit v7.1.3 program (http://www.mbio.ncsu.edu/bioedit/bioedit.html).

Statistical analysis

Statistical analysis for comet assay data parameters (tailed cells, tail length, % DNA, and tail moment) was done using one-way ANOVA. Data from five replicas of each group were analyzed, and the significance of difference was determined by GraphPad Prism® software (GraphPad Software, Inc., USA). Results of the germ-line cell aberrations test and the accompanying *p* values were analyzed using chi-square statistics (χ^2-test) performed with SPSS software (version 15; SPSS, Chicago, IL).

III. RESULTS AND DISCUSSION

Referring to the genotoxic stress of the insecticide stressor, some effects, including several common mechanisms of mutagenesis, may be developed. These include formation of DNA adducts, damage to DNA structure, inhibition of DNA repair systems, and disturbance of genetic and cell cycle processes. Under these stressful conditions, apoptosis is triggered [24, 25]. To minimize LUF and CHLO risk to

humans, these insecticides' identification, genotoxicity mechanisms, and risk assessment are necessary for the protection of public health.

The obtained data in the present work is outlined below in an attempt to shed some light on the genotoxic modes of action of the tested acyl ureas LUF and CHLO in *D. melanogaster* and also for risk assessment of these putative mutagens.

Assessment of germ-line cell aneuploidy and chromosomal aberrations

The quantitative evaluation of the mechanism of mutagenicity of the insecticides under investigation in the present work examined the deleterious effects of aneuploidy by using a test for germ-line cell aneuploidy and chromosomal aberrations (inheritable mutations) with *D. melanogaster* as a test animal. The eye color trait in *D. melanogaster* is the phenotype used as the indicator in this test; this is a sex-linked trait, and its gene is carried on the X chromosome [26].

Use of GAT for detection and quantitative estimation of mutagenicity of LUF and CHLO

It has been indicated that LUF and CHLO are not toxic to adult *Drosophila* but are toxic to larvae and pupae [27] because they act as anti-chitin formation compounds in these immature stages. The larvae and pupae have a clearly observed malformed cuticle that leads to death [28].

Crossing of the treated parental w-/w+Y males with untreated w- homozygous females, where both are homozygous for se, is expected to produce abnormal F1 progeny of the various phenotypes sepia-eyed female and fertile or sterile white-eyed males. Production of the referred phenotypes depends on the types of mutation occurring in sperm of the crossed parental males. These may include aneuploidy as a result of non-disjunction or loss of X- and Y-chromosomes. Also, chromosomal aberrations may be included, such as loss of the w+ gene from the Y chromosome (or point mutation in this gene leading to loss of its function in eye pigmentation) or translocation of the w+ segment to X- or any other chromosome.

When the developed GAT was used for assessment of the mutagenicity of the acyl ureas LUF and CHLO, cases of aneuploidy and chromosomal aberrations were detected and quantitatively estimated by comparison to a negative control. Also, colchicine-treated flies were used as a positive control, serving as an indicator for the validity of the test in each replicate. The data show that a significant number ($p < 0.05$) of F1 males having the abnormal white eye color resulted from Br1 germ-line cells of treated parental males. In contrast, this abnormality was not observed in F1 males produced from Br2 ($P > 0.05$). Also, the number of abnormal sepia-eyed F1 females produced from both Br1 and Br2 germ-line cells of treated parental males is insignificantly different from that of the negative control ($P > 0.05$,).

The mutagenic changes during spermatogenesis and production of certain abnormal gametes in parental males that lead to formation of F1 white-eyed fertile males seems to be caused by loss of the w+ segment from the Y chromosome, or to point mutation in the w+ gene itself, causing loss of its function in eye pigmentation. In contrast, the insignificant results of mutagenicity in F1 females (Table 2) indicate the insignificant production of abnormal gametes with X-Y nondisjunction or with translocation of the w+ segment to any chromosome in parental males.

The results of GAT on treated parental male *Drosophila* in this study indicate that acyl ureas are mutagenic to the germ-line cells of Br1, but not to those of Br2. The occurring mutations are therefore inherited from parental males to males and females of F1 progeny.

In *Drosophila* (at 25ºC), spermatogenesis starts from the third larval instar and continues thereafter in pupae and adults; therefore, the emerged adult males have mature sperm [29, 30]. At the time of exposure of the parental third larval instar to LUF and CHLO, different developmental stages of spermatogenesis can be observed inside the sperm tubes (follicle). Therefore, during the first 5 days after emergence of these males, they inseminated the crossed untreated virgin females with the Br1 sperm that were in the maturation and reduction stages at the time of their exposure to LUF and CHLO. During this time, spermatocytes undergo the two meiotic divisions to produce spermatids and also the transformation of spermatids into spermatozoa. On the other hand, during the next 5 days after emergence, these males will inseminate the untreated virgin females with the Br2 sperm. These sperm were in the growth stage at the time of their exposure to LUF and CHLO, during which time the primary spermatogonia (enclosed in cysts) divide and increase in size to form spermatocytes.

Therefore, the germ-line cells undergoing the two meiotic divisions of Br1 are more sensitive to LUF and CHLO at the time of exposure, and exhibited mutagenic responses. This is in comparison to those of Br2, where they exhibited only mitotic divisions at the time of exposure. Hence, and according to the GAT used, it can be concluded that the acyl ureas insecticides LUF and CHLO tested in this study have variable mutagenic potential on the different stages of development of the germ-line cells of male *Drosophila,* causing aneuploidy and chromosomal aberrations.

TABLE 2: EFFECT OF LUF AND CHLO ON ANEUPLOIDY AND CHROMOSOMAL ABERRATIONS IN GERM-LINE CELLS OF D. MELANOGASTER, TESTED BY GAT.

Treatment	Broods	No. of tested males	White-eyed males (mutants)	Frequency	X^2	p	No. of tested females	Sepia-eyed females (mutants)	Frequency	X^2	P
Control	Br1	3690	3	0.108	-	-	4158	4	0.24	-	-
	Br2	3077	4	0.194	-	-	3524	6	0.20	-	-
Colchicine	Br1	2115	18	0.85	22.101	0.001***	2246	21	0.93	26.385	0.001*
	Br2	2725	27	0.99	20.151	0.001***	2864	31	1.08	22.827	0.001*
LUF	Br1	4069	12	0.294	4.576	0.032*	4540	6	0.132	0.244	0.621
	Br2	5028	11	0.219	0.814	0.367	5183	7	0.135	0.174	0.676
CHLO	Br1	3240	9	0.278	3.853	0.050	3428	3	0.088	0.015	0.901
	Br2	2885	9	0.312	2.266	0.132	2606	7	0.269	0.685	0.408

* significant, and *** highly significant, difference from the negative control at $p < 0.05$ and $p < 0.001$, respectively, using X^2-test.

DNA damage

DNA damage is considered a reflection of inadequate function of repair pathways in an organism that is a consequence of DNA adduct formation resulting in DNA strand breaks, formation of alkali-labile lesions, and crosslinks [31, 32]. DNA damage (as a second genotoxic mode of action) in the whole body cells of the acyl ureas-treated adult w^{1118} strain *Drosophila* flies treated as third larval instars that were used in these assays was qualitatively detected by an apoptosis-associated DNA fragmentation test and also quantitatively estimated by the comet assay.

Apoptosis-associated DNA fragmentation

The genotoxic effect of the acyl ureas LUF and CHLO was further assayed qualitatively for apoptosis-associated DNA fragmentation in the whole body cells of treated parental adult *Drosophila* flies. Figure 3 shows the tested acyl ureas induced a ladder-like pattern of newly appeared electrophoretic bands on the agarose electrophoretogram of the whole genomic DNA extracted from acyl ureas-treated flies.

The results show that during apoptosis, cleavage of chromatin DNA into inter-nucleosomal fragments of 180–200 bp and multiples occurs [33]. In the case of treatment with LUF and CHLO acyl ureas, most of the new bands detected are around these molecular sizes and their multiples). These results suggest that treatment with the tested acyl ureas results in the chromatin DNA fragmentation characteristic of cells during apoptosis. As mentioned above, this is because of inadequate function of repair pathways; a condition that leads to induction of apoptosis [33].
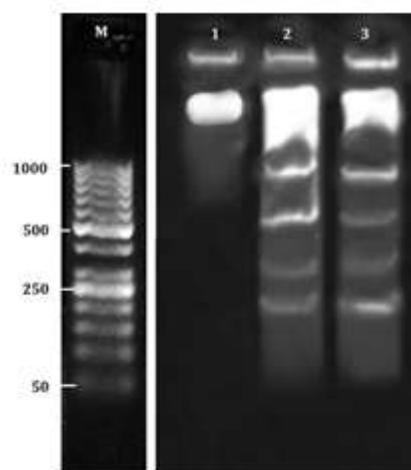

Fig. 3: Agarose gel electrophoresis of induced apoptosis-associated DNA fragmentation in the whole body cells of parental adult isogenic strain w[1118] Drosophila exposed as third larval instars to the LC50 of the acyl ureas LUF and CHLO incorporated into the feeding medium. Lane M: 50 to 1000 bp DNA ladder (Fermentas); Lane 1: control genomic DNA; Lanes 2 & 3: flies treated with LUF and CHLO, respectively.

Single-cell gel electrophoresis; comet assay

DNA damage was estimated quantitatively in whole body cells of acyl ureas-treated parental adult flies using an alkaline comet assay. Under these strong alkaline conditions (pH > 13), the comet assay can quantitatively measure DNA damage in the form of single-strand breaks, double-strand breaks, alkali-labile sites (primarily apurinic and a pyrimidinic sites), incomplete excision repair sites, and DNA cross links [20]. The results are presented in Table 3, and the data include the tail length (µm), percentage of DNA concentration in the tail, and the calculated tail moment, according to [20]. The obtained tail moment values of the treated parental flies are multiples those of the control flies with a highly significant difference ($p < 0.01$ and $p < 0.001$) (Table 3). This indicates a high genotoxic potential capable of causing DNA damage in these insecticides. Therefore, we suggest also taking into consideration the number of tailed cells in comet scoring, as

recommended by some authors [34, 35]; in this case, the indicated genotoxic potential could be verified.

Table 3 shows the quantitative evaluation of the DNA damage caused by LUF and CHLO in whole body cells of parental adults of the isogenic strain w[1118] of *Drosophila* using comet assay.

TABLE 3: Quantitative estimation by comet assay of the DNA damage, expressed as tail moment in whole body cells of parental adults of the isogenic strain w[1118] of Drosophila exposed as third larval instars to the LC50 of the acyl ureas LUF and CHLO incorporated in the feeding medium.

Treatment	No. of samples	No. of cells observed	Tailed cells %	Tail length (μm)	Tail DNA %	Tail moment
Control	5	500	4.4±0.24	1.50±0.04	1.61±0.06	2.50±0.14
LUF	5	500	9.4±0.93*	5.89±0.91*	5.31±0.71*	43.24±5.98*
CHLO	5	500	11.4±1.2**	5.88±0.71*	5.77±0.98*	43.67±9.31*

*Significant and **highly significant difference from the negative control $p < 0.01$ and $p < 0.001$, respectively.

Specific gene mutations

DNA damage resulting from genotoxic stress triggers transcription of genes that limit mutations and promote survival in organisms ranging from bacteria to humans [36]. These are the tumor suppressor genes (TSGs) in which a loss-of-function mutation leads to cellular overproliferation [37]. TSGs are reported to be mutated in human cancers and can cause tumor susceptibility in mice [38, 39].

According to some reports, *Drosophila* contains a group of TSGs [40]. These latter are homologues of known human TSGs, including *P53* and *Rb,* and are denoted as *Dmp53* and *Rbf*, respectively [41, 42]. These vital TSGs (*Dmp53* and *Rbf*) were selected for assessment of mutations as a third mode of genotoxic action in parental adults of isogenic strain w[1118] *Drosophila* because mutations in TSGs leads to an increased rate of resulting mutations under the effects of genotoxic stressors, which is required for DNA damage-induced apoptosis in *Dmp53*, but unlike the mammalian *p53*, *Dmp53* appears to be unable to block the cell cycle in G1 phase [43, 44]. Two variants (b and c) of *Dmp53* were selected in this study to reveal the expected mutagenesis in whole body cells of acyl ureas-treated parental adults. The sequences of the PCR-amplified fragments obtained from the treated samples were aligned, as opposed to the same fragment from the negative control samples (see supporting information). Examination of these sequence alignments shows that different mutations are observed, including substitutions, insertions, and deletions, and in some cases, long sequences of deletions were observed (see supporting information).

On the other hand, for *Rbf,* the increased rate plays a critical role in the regulation of cell proliferation [45]. Rb protein plays an important role in the regulation of cell division, namely, cell death, and its activity is altered in most human tumors [46, 47]. It is able to modulate the action of E2F transcription factors and regulate cell cycle progression [48]. The sequence of this PCR-amplified fragment of *Rbf* in treated flies obtained in this study was aligned, as opposed to that of the negative controls, as shown in supporting information file. This alignment reveals different mutations, including insertions, substitutions, and deletions.

For the two genes *Dmp53* and *Rbf*, since pooled samples were used in the sequence analysis in this study, the obtained data are actually the summation of various mutations in different individuals. Therefore, the assessment of mutagenicity of LUF and CHLO in *Drosophila,* at the gene level, is considered qualitative, and does not show the actual frequency of mutation in individual flies. However, these qualitative results indicate the ability of these insecticides to cause mutations in the tested TSGs in the form of insertions, substitutions, and deletions of one or more nucleotides in the DNA strand. This can be considered, qualitatively, as an indication of the mutagenic potential of the used insecticides to the two TSGs. The occurring mutations in these TSGs may include loss-of-function mutations, leading to the observed levels of mutagenicity assayed in the form of DNA damage and as aneuploidy and chromosomal aberrations.

With regard to the physiology of the cell, more extensive binding has been found with transcriptionally active DNA, due to its open conformation [49]. Many, if not most, adducts have the potential to cause mutation [50]. Mutations arise either during DNA replication at the damaged sites or during DNA repair [51]. Hence, it can be assumed that the acyl ureas LUF and CHLO, as electrophiles, are able to form with DNA, as a nucleophile, DNA adducts. Therefore, they have multitudinous mutagenic potential to cause the observed mutations in *Drosophila* shown through the three indicated genotoxic modes of action: aneuploidy and chromosomal aberrations, DNA damage, and mutations in the selected genes.

The results of this study indicate the acyl ureas LUF and CHLO are genotoxic insecticides, and their genotoxic modes of action include aneuploidy and chromosomal aberrations, DNA damage, and point mutations. However, there have been claims that these acyl ureas are safe, nonmutagenic insecticides, specifically through the Ames test [52] on *Salmonella* [53, 54]. From the few available biochemical data [55-58], it is implied that the biochemical mode of action of acyl ureas is correlated directly or indirectly to regulatory proteins and their regulatory genes. Therefore, it could be hypothesized that acyl ureas are able to directly or indirectly cause multitudinous effects on the body macromolecules of *Drosophila* (and other insects), leading to interruption in performance of body physiology that eventually leads to death.

It is worth mentioning that a direct homology exists between *Drosophila* genes and genes that affect human diseases, and a considerable conservation of genes and pathways affecting key biological processes between flies and humans exists [8, 59, 60]. Therefore, a homology to the deleterious genotoxic effects observed in *Drosophila* with this study may exist for humans exposed to acyl ureas from use of as insecticides, creating the hazard of genotoxicity and potentially also proteotoxic stresses.

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