Papéis Avulsos de Zoologia
Museu de Zoologia da Universidade de São Paulo

ARTICLE

Genotoxicity of Imidacloprid on the forensically important species *Chrysomya albiceps* (Wiedemann, 1819) (Diptera: Calliphoridae), evaluated by Comet assay

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Abstract. The increasing use of chemical pesticides for crop protection has led to irreversible DNA damage in many animal species, particularly soil-dwelling insects. In this study, we tested a protocol of Comet Assay to detect DNA damage in the hemolymph cells of *Chrysomya albiceps* larvae (Diptera: Calliphoridae) exposed to the neonicotinoid Imidacloprid (IMI). Third-instar larvae were fed on freeze-dried liver substrate rehydrated with one of the following treatments: (i) distilled water (negative control), (ii) imidacloprid (IMI) (0.4980 and 0.9360 mM), and (iii) cyclophosphamide 3.83 mM (positive control). We estimated the Damage Index (DI) and the Damage Frequency (DF) in 300 cells per treatment. According to the shape of the comets, the damage in cells exposed to biocides was significantly higher than the negative control. The DF was also consistently higher in the cells of intoxicated insects. The Comet assay in the haemolymph cells of *C. albiceps* larvae proved to be effective for detecting DNA damage, providing new evidence for genotoxicity. The increasing use of chemical pesticides for crop protection has led to irreversible DNA damage in many animal species, particularly in soil-dwelling insects. Third-instar larvae were fed freeze-dried liver substrate rehydrated with one of the following treatments: (i) distilled water (negative control), (ii) Imidacloprid (IMI) at concentrations of 0.4980 mM and 0.9360 mM, and (iii) cyclophosphamide at 3.83 mM (positive control). We estimated the Damage Index (DI) and Damage Frequency (DF) in 300 cells per treatment. According to the shape of the comets, DNA damage in cells exposed to biocides was significantly higher than in the negative control. The DF was also consistently higher in the cells of intoxicated insects. The Comet Assay in the hemolymph cells of *C. albiceps* larvae proved effective for detecting DNA damage, providing new evidence of the genotoxic effects of IMI on a necrophagous insect species. We discuss the advantages and limitations of the protocol in the context of environmental forensic entomotoxicology.

Keywords. Entomotoxicology; Blowflies; Forensic entomology; Neoticotinoids.

INTRODUCTION

Exposure to toxicants can alter the development, feeding and behaviour of necrophagous insects, which could influence carrion decomposition and their use in forensic entomology. Several compounds interfere with larval development, which can produce errors in estimating the minimum post-mortem interval (min PMI) using methods strictly based on the insect life cycle (Estrada *et al.,* 2009). Larvae fed on contaminated carcasses can also exhibit modified mobility and dispersal, which can influence the likelihood of sampling entomological at the scene of the crime (Jales *et al.,* 2021).

Such questions nurtured the development of forensic entomotoxicology, a branch of forensic entomology that validates the use of insects as: (i) an indirect source of toxicological evidence in the absence of direct matrices, such as blood,

Pap. Avulsos Zool., 2026; v.66: e202666010
https://doi.org/10.11606/1807-0205/2026.66.010
https://revistas.usp.br/paz
https://www.scielo.br/paz
Edited by: Carlos José Einicker Lamas
Received: 17/07/2025
Accepted: 24/10/2025
Published: 03/03/2026

ISSN On-Line: 1807-0205
ISSN Printed: 0031-1049
ISNI: 0000-0004-0384-1825

urine, soil or water, and (ii) Indiscriminate pesticide use has sparked new insights into environmental crimes (Goff & Lord, 2001).

The use of broad-spectrum insecticides for controlling insects of medical, veterinary, and agricultural importance has stirred novel perspectives on environmental crimes, so that a branch of forensic entomology has emerged, that is, environmental forensic entomotoxicology (EFF). EFF employs insects as indicators of environmental pollution, bioaccumulation of heavy metals in trophic webs, and deleterious effects on non-target organisms (Hodecek, 2020). For example, drug absorption, distribution, and excretion may occur at different rates in biological systems, resulting in a quantity of toxicant in an insect that may differ from its concentration in the environment (Goff & Lord, 2001).

Neonicotinoid pesticides, such as imidacloprid ($C_9H_{10}ClN_5O_2$), are used worldwide and have raised concern due to their adverse effects on non-target insects, particularly on soil-dwelling larvae of Diptera and Coleoptera (Wang *et al.,* 2023). Imidacloprid can simulate the action of acetylcholine and interfere with the nervous activity of insects, thus resulting in death due to long-term over-excitation (Li *et al.,* 2012). When applied in the field, only a small part reaches its biological targets; most particles remain in the soil or the water until degradation or bioaccumulation. Over 90% of neonicotinoids could move off-site and persist in non-target environmental media (Cavallaro *et al.,* 2023).

Dung is a protein source and breeding site for forensically important species of Muscidae, Sarcophagidae, and Calliphoridae (Diptera). *Chrysomya albiceps* (Wiedemann, 1819) (Calliphoridae) was a dominant species in cattle dung in Spain, and occurred in urban areas, rainforests, dry forests, and agro-ecosystems (Vasconcelos *et al.,* 2015, 2016; Carmo *et al.,* 2021), the Amazon Forest (Souza & Guimarães, 2022), and Europe (Hodecek *et al.,* 2024). It is also one of the most frequent species found in human cadavers in many countries (Oliveira & Vasconcelos, 2010). *C. albiceps* is a model species in forensic entomotoxicology because a diversity of toxic substances, such as malathion and parathion, can be detected and quantified in its tissues (Gosselin *et al.,* 2011).

However, empirical data on the damage caused by xenobiotics, such as insecticides, at the cellular level are scarce. Sub-lethal and sub-organismal level effects (*e.g.,* small turnover rate for proteins, DNA fragmentation, etc.) can influence energy metabolism, fitness, and reproductive success, which leads to population-level effects (Jha, 2008). Genotoxic compounds alter the structure and the information content of DNA, and interfere, temporarily or not, directly or indirectly, in normal processes in the cells (Jha, 2008; Phillips & Arlt, 2009; Gajski *et al.,* 2019; Menz *et al.,* 2023). In the case of soil-dwelling insects, exposure to biocides released into the environment may result in unrepaired DNA strand breaks, leading to mutations and changes in normal cell processes (Phillips & Arlt, 2009; Chatterjee & Walker, 2017).

The Comet assay (CE), or single-cell gel electrophoresis (SCGE), is a simple method for measuring DNA strand breaks in eukaryotic cells because it is a sensitive, rapid and relatively inexpensive technique, amenable to any species (Jha, 2008; Chatterjee & Walker, 2017). It combines agarose electrophoresis with fluorescence microscopy to observe and quantify DNA strand breaks at the level of single cells. Cells with damaged DNA exhibit increased migration of the chromosomal DNA from the nucleus in an electric field, and the migration pattern has a typical 'comet' shape, consisting of a head and a tail. The bulk DNA, also called nucleoid ("head"), moves from the cathode to the anode during electrophoresis more slowly than the short, broken DNA fragments ("tail"), resembling a comet (Hartmann *et al.,* 2003; Møller *et al.,* 2020; Collins *et al.,* 2023).

The Comet assay has advantages in toxicological research because various cell types can be used without prior knowledge of their karyotype or genome structure (Jha, 2008; Lapuente *et al.,* 2015). Since its pioneering use as a model to detect DNA damage using the comet assay (Gaivão & Sierra, 2014), *Drosophila melanogaster* Meigen, 1830 (Diptera: Drosophilidae) has been exposed to xenobiotics, natural radiation, and urban pollution (Augustyniak *et al.,* 2016; Verçosa *et al.,* 2017; Santana *et al.,* 2018). The Comet assay has also been used to evaluate the fate of insecticides in the environment using dung beetles (Coleoptera: Scarabaeidae) as models (Cavallaro *et al.,* 2023). This information can help to understand cellular responses to detrimental factors and to predict negative effects at both the organism and population levels.

Given the growing importance of environmental forensic entomotoxicology, we aimed to: (i) test a protocol for using the comet assay using *Chrysomya albiceps* larvae as a target model; (ii) detect genotoxic effects, measured by DNA damage, caused by imidacloprid in haemolymph cells, and (iii) assess the validity of the comet assay as a technique to be used in toxicological and forensic protocols.

MATERIAL AND METHODS

We used *C. albiceps* larvae from a standardized laboratory colony under controlled conditions of temperature (24 ± 2°C), relative humidity (70% ± 5%), and photoperiod (12:12 light: dark). The colony has been established for at least five generations from samples collected in rainforest fragments in Recife (08°22′54″S, 34°56′53″W), Northeastern Brazil.

Because there are no published protocols for the Comet assay using larvae of *C. albiceps,* we based our protocol on techniques developed for *D. melanogaster* (Verçosa *et al.,* 2017; Amorim *et al.,* 2020). We carried out pilot tests using different sample sizes (5, 15, and 35 larvae) for collecting haemolymph cells of *C. albiceps* larvae to provide sufficient biological material. The most consistent quality and quantity of haemocytes was obtained from a sample of 35 larvae at 3rd instar of homogeneous size and weight.

For the experimental set-up, the larvae were allowed to feed for 24 h on a treatment medium composed of freeze-dried bovine liver (10 g), on which we adminis-

tered 20 mL of one of the following treatments: (i) distilled water (negative control); (ii) imidacloprid (IMI) (0.4980 and 0.9360 mM) (Bayer Evidence® 700 WG, 700 g/kg, 70% m/m); and (iii) cyclophosphamide 3.83 mM (positive control). All chemicals were diluted in distilled water. The choice of IMI concentrations followed the recommendations of Sousa *et al.* (2019) and Frantzios *et al.* (2008), who verified genotoxic effect of the compounds on *D. melanogaster.* Cyclophosphamid has been used in comet assays as a positive control due to its potent genotoxicity to insect cells, so that the maximum damage can be visually defined.

We then proceeded with the comet assay, as summarized in Fig. 1. Haemocytes from larvae fed on each treatment were extracted from a pool of 35 individuals per replicate (three replicates in total). The larvae were transferred to a well of an excavated plate containing 150 µL of EDTA to prevent haemolymph clotting. The cell pool (0.5 mL) was transferred to 1.5 mL microtubes and centrifuged at 3,000 rpm for 3 min. Then, 100 µL of the supernatant was discarded, and 100 µL of new EDTA was added, and the procedure was repeated three times. Cuticle breakdown of 35 larvae resulted in 60 µL of haemolymph per replicate, which were homogenized with a 0.5% low-melting agarose solution (LM agarose) at 37°C, in the dark.

The homogenized material was applied to four histological slides, previously washed and bathed in standard 1.5% agarose (diluted in Phosphate-Buffered Saline pH 7.4) and dried at 24°C for 48 h. The slides were covered with coverslips and kept at 4°C for 10 min. They were then immersed in a lysis solution (2.5 M NaCl; 100 mM EDTA; 1 M NaOH; 1% Tris pH 10; 1% Triton X-100 and 10% DMSO) at 4°C for 48 h. Subsequently, the slides were aligned in a 40 cm electrophoresis vat filled with buffer (1 M NaOH; 200 mM EDTA pH > 13) and immersed at 4°C for 20 min. The slides were subjected to an electrophoretic run at 40 V and 300 mA for 20 min. The slides were transferred to a neutralization solution (0.4 M Tris-HCl, pH 7.5) for 15 min, fixed in ethanol for 5 min, air-dried and stored at 4°C until observation under microscope.

To visualize the nucleoids the material was stained with 50 µL of GelRed (Biotium) diluted in purified water (1:500) and observed under a fluorescence microscope Zeiss-Imager M2, equipped with the Alexa-Fluor 546 filter, under 400× magnification. Comets were classified according to Ostling & Johanson (1984) e Møller *et al.* (2023) (Fig. 2). It consists of a grade of the integrity of the "head" and the length of the "tail" of nucleoids, assigning comparative values from 0 (intact), 1 (minimal), 2 (intermediate), 3 (intense) and class 4 (maximum damage). One hundred cells were observed for each replicate, totaling 300 nucleoids per treatment.

The Damage Index (DI) was calculated as follows: $DI = 0x(N_0) + 1x(N_1) + 2x(N_2) + 3x(N_3) + 4x(N_4)$, so that it ranges from a minimum of zero – in case no DNA damage is observed in any cell (level 0) – to 400, which corresponds to all cells (N = 100) under the maximum DNA damage (level 4). The Damage Frequency (DF%) = $[(N_T - N_0).100] / N_T$, where N_1, N_2, N_3, N_4 represent the total number of damages 0, 1, 2, 3, and 4, respectively; N_T is the total number of damages; and N_0, the total number of zero damages. The Damage Frequency varied from 0 to 100%.

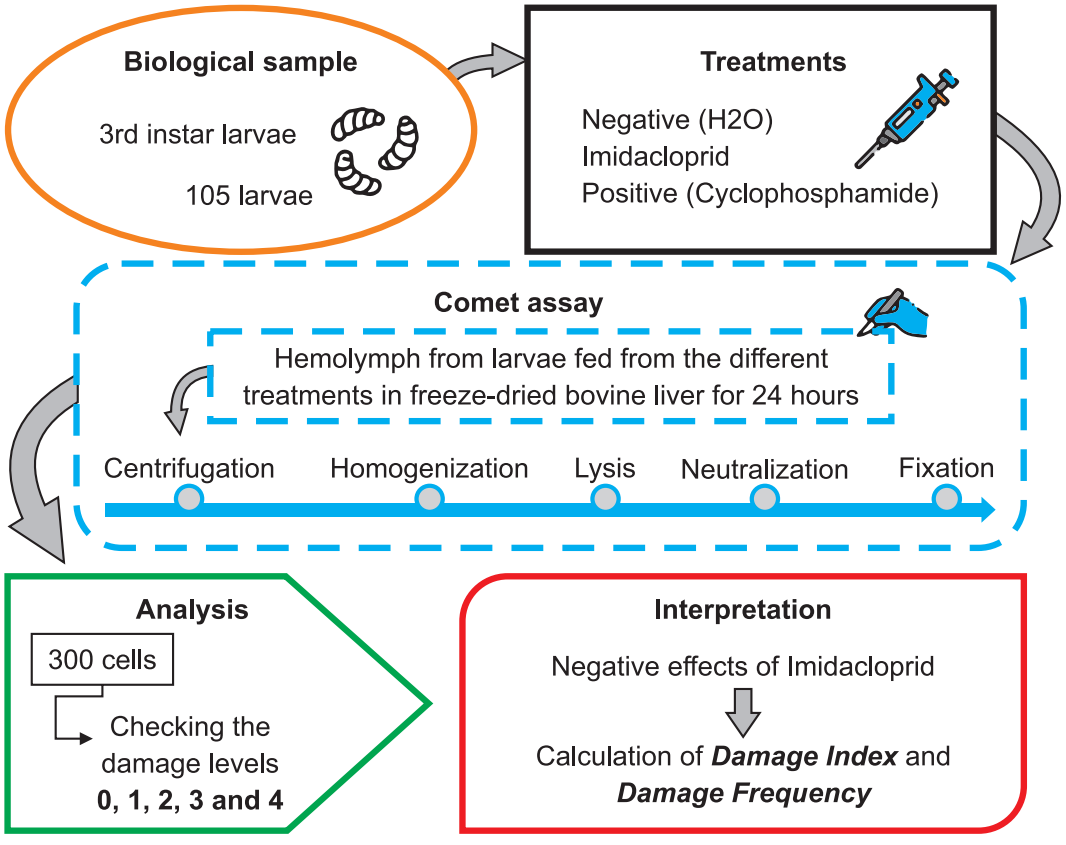

Figure 1. Steps of the comet assay in haemolymph cells of *Chrysomya albiceps* larvae exposed to concentrations of Imidacloprid (IMI).

Because visual interpretation of the comet shape could be subjective, all categorizations from 0 to 4 were performed in a double-blind manner. Two people analyzed each slide, and only fully matched diagnoses were considered. For the sake of simplicity, we refer to concentrations of imidacloprid as "low" and "high" to facilitate a preliminary interpretation of dose-response. The mean values of DI and DF% in the treatments were compared using one-way ANOVA followed by the Bonferroni post-test, which provides a conservative adjustment for multiple comparisons and reduces the likelihood of errors when evaluating differences between treatment means, using the Stata 14.2 program, with 5% significance level.

RESULTS

When data from all treatments were combined, we designed a visual scale from 0 to 4, based on the increasing level of DNA damage in the haemolymph cells of *C. albiceps* larvae (Fig. 3). Based on this calibration, the levels of cellular damage in our study were classified into one of five categories: 0, 1, 2, 3, or 4.

IMI induced severe DNA damage in the haemolymph cells at the concentration of 0.0468 mM, ranking a large proportion of cells into classes 3 and 4 (25.67% and 42.0%, respectively). However, no clear dose-response relationship was observed between the two concentrations tested, suggesting a possible non-linear effect, as the higher concentration tested (0.0936 mM) showed a reduced effect in class 3 (20.0%) and class 4 (31.3%). In the negative control, 89.7% of cells remained undamaged (class 0), with maximum damage level (class 4) recorded in only 2.3%, confirming a low baseline genotoxicity for comparison with chemicals (Table 1).

Both IMI concentrations induced high levels of DNA damage in the haemolymph cells of *C. albiceps* larvae. The Damage Frequency in the cells exceeded 78.0% in both treatments, in sharp contrast with the negative control (± 10%), indicating a significant genotoxic effect at both concentrations ($P < 0.001$). The Damage Index was

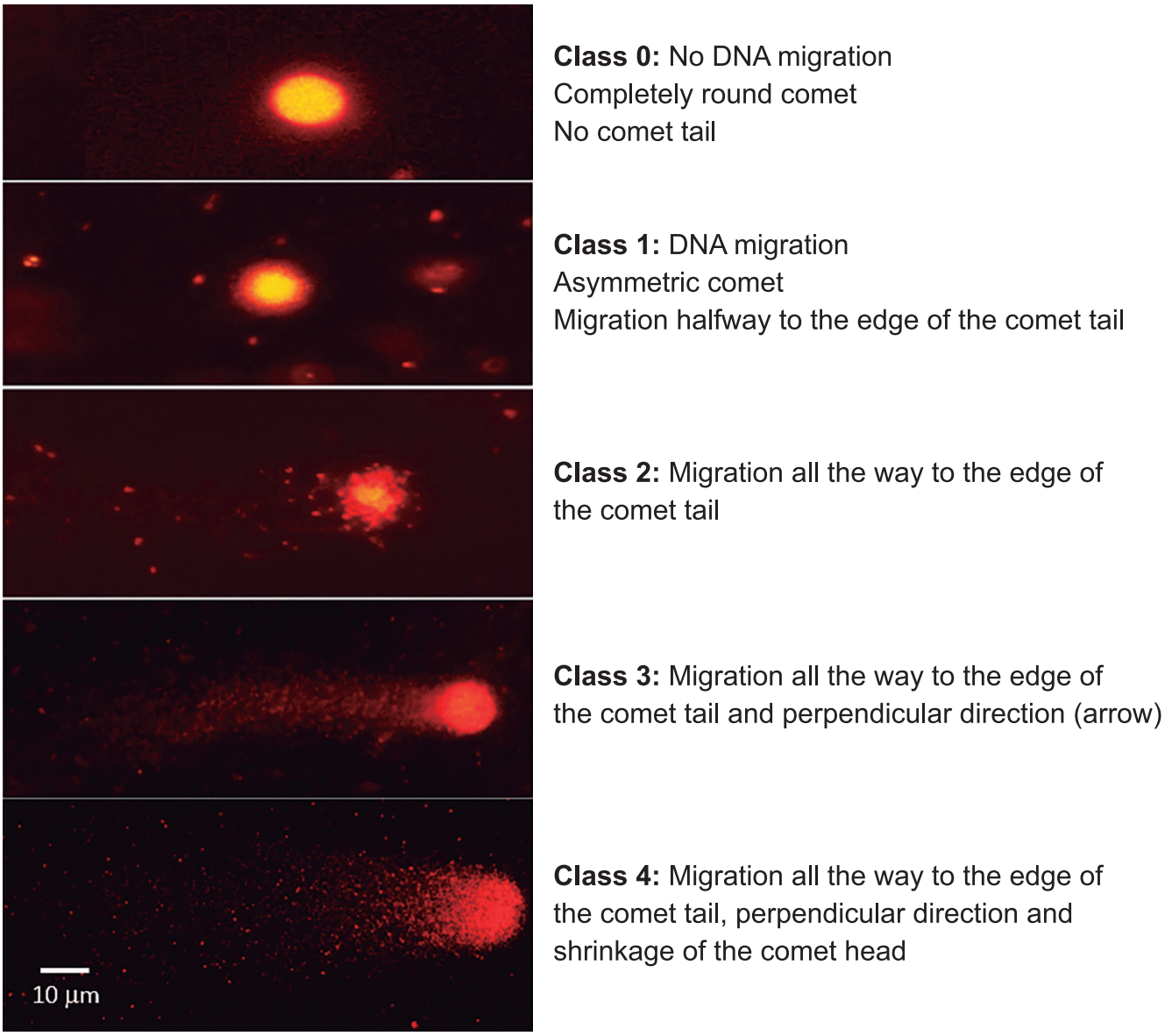

Figure 2. Representative adapted images of DNA damage observed using the Comet assay grading system described by Ostling & Johanson (1984) and Møller *et al.* (2023).

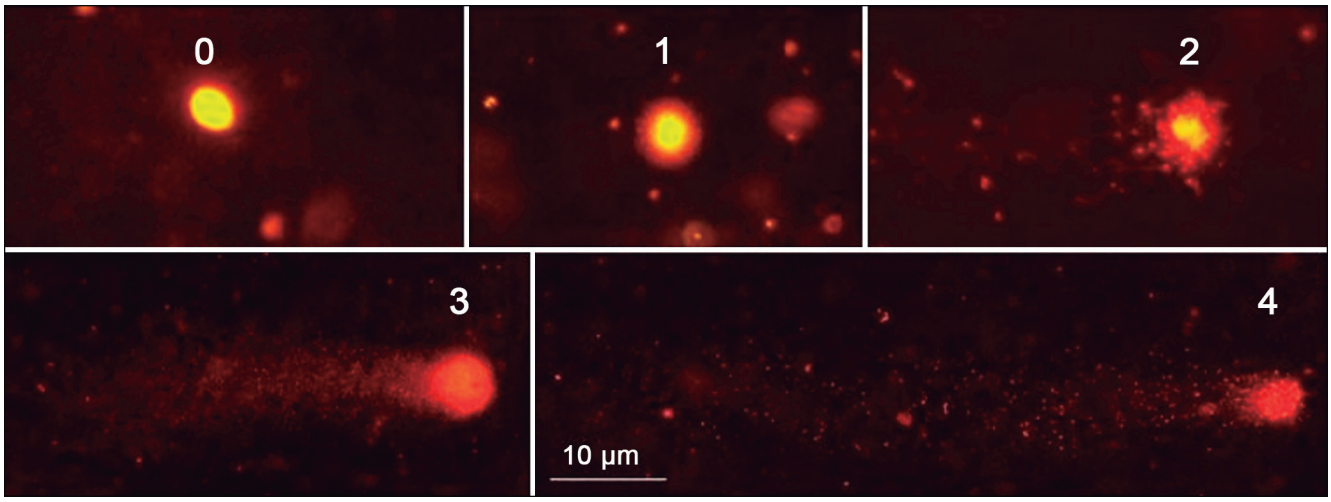

Figure 3. Images of GelRed-stained nucleoids obtained from *Chrysomya albiceps* larvae. Class zero (0) represents the absence of genetic damage and classes 1 to 4 indicate increasing DNA damage (increase in the comet's tail and decrease in its head).

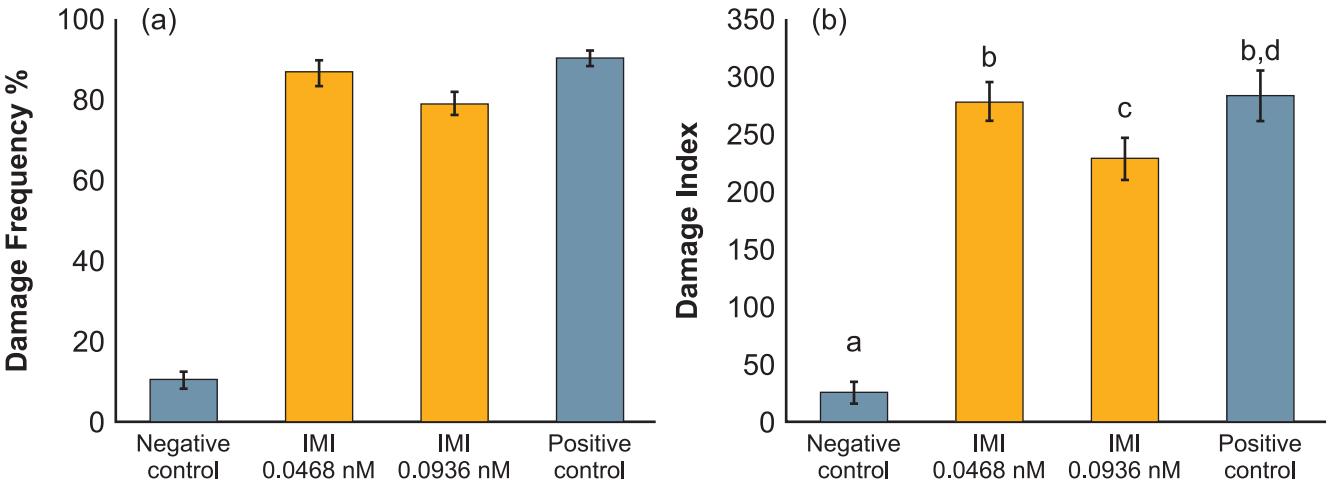

Figure 4. Mean Values (± SD) of Damage Index (a) and the Damage Frequency (b) after exposure of *Chrysomya albiceps* larvae to the treatments with distilled water (negative control), Imidacloprid (IMI), and cyclophosphamide (positive control). In (b) different letters indicate statistically significant differences.

also considerable for both treatments (approximately 270 for 0.0468 mM and 220 for 0.0936 mM) (Fig. 4a).

The Damage Index showed that the negative control group differed significantly from all others ($P < 0.001$). The 0.0468 mM IMI treatment differed significantly from the 0.0936 mM IMI treatment ($P < 0.001$), but its effect did not differ markedly from the positive control ($P = 0.142$). Despite the intense genotoxic response observed at both IMI concentrations, the higher dose (0.0936 mM) exhibited a slight reduction in the Damage Index, suggesting a possible non-monotoxic effect. Notably, the 0.0468 mM concentration induced damage comparable to the positive control, while the higher concentration differed statistically from it ($P < 0.001$), indicating that intermediate concentrations may cause greater genetic damage (Fig. 4b).

DISCUSSION

Toxicants can cause deleterious effects at multiple levels of biological organization, ranging from populations across generations to individual molecules within a living cell. Here, we assessed the validity of the comet assay (CE) to detect DNA damage caused by a commonly used biocide in the haemolymph cells of *C. albiceps* larvae, the most widely used insect species in forensic entomology. The findings can be incorporated into basic (*e.g.,* carrion ecology) and applied entomology, as exemplified by environmental forensic entomotoxicology.

Blow fly larvae develop in the necrobiome, a patchy and ephemeral ecosystem represented by the interactions between the biota and the decomposing organic animal matter in terrestrial environments (Benbow & Pechal, 2019). High residues of Imidacloprid have been detected at different concentrations in many matrices, including soil and freshwater resources, and have increased dramatically over the last 20 years (He *et al.,* 2024). Nevertheless, CE protocols have prioritised non-insects to detect genotoxic effects of imidacloprid, such as the freshwater crustacean *Ceriodaphnia dubia* Richard, 1894 (Raby *et al.,* 2018).

Among terrestrial invertebrates, the CE was more effective than micronucleus tests in detecting DNA dam-

Table 1. Percentage of cells under each level of damage and standard deviation (SD) in haemolymph cells of *Chrysomya albiceps* larvae subjected to treatments with distilled water (Negative control); concentrations of Imidacloprid (IMI) and Cyclophosphamide (Positive control). Damage levels: zero (0) represents no apparent genetic damage and 1 to 4 are increasing levels of genetic damage.

Treatment	Percentage of cells under each level of damage (mean % ± SD)					TOTAL
	0	1	2	3	4	
Negative control	89.67 ± 2.08	3.67 ± 2.31	0	4.33 ± 4.51	2.33 ± 0.58	100%
IMI 0.0468 mM	13.67 ± 3.06	10.33 ± 2.52	8.33 ± 3.06	25.67 ± 7.51	42.00 ± 2.00	100%
IMI 0.0936 mM	21.33 ± 2.89	16.33 ± 5.51	11.00 ± 2.65	20.00 ± 2.65	31.33 ± 4.93	100%
Positive control	10.00 ± 1.73	14.33 ± 2.89	11.00 ± 5.29	17.67 ± 4.04	47.00 ± 6.08	100%

age caused by IMI in the earthworm *Eisenia fetida* (Zang *et al.,* 2000). Comet assay evidenced that *Apis* spp. populations from agricultural areas exposed to pesticides presented more DNA fragmentation than pesticide-free areas (Hayat *et al.,* 2019). Necrophagous insects, as a trophic group of tremendous ecosystem services, face magnified risks in areas under intensive agriculture, especially in countries with lenient regulation of pesticide registration and application, which is the case of Brazil. This is particularly threatening because *C. albiceps* is an important pollinator of crops in the semi-arid region in Brazil (Carmo *et al.,* 2021).

A landmark achievement occurred when the Organization for Economic Cooperation and Development (OECD) adopted a comet assay guideline for *in vivo* testing of DNA strand breaks in animals (Møller *et al.,* 2020). To our knowledge, this is the first attempt to adjust the CE for forensic entomotoxicology and proved to be effective for detecting genotoxic damage in *C. albiceps* larvae exposed to toxicants. Thus, we propose criteria that display explicitly advantages and weak points for the conditions tested, based on our results and on the available literature (Hartmann *et al.,* 2003; Møller *et al.,* 2020; Collins *et al.,* 2023; Nugnes *et al.,* 2023) (Table 2).

The advantages include its reliability, ease of use, relatively low cost, fast results, reduced ethical restraints (as opposed to *in vivo* experiments), and the adaptability to techniques and equipment available in molecular biology laboratories. Additionally, the comet assay can be used for almost all living organisms and for a variety of cell types (Møller *et al.,* 2020). In this study, the negative and positive controls successfully calibrated the scope of DNA damage, and the scale of damage, from 0 to 4, was consistent. CE detects DNA strand breaks and alkali-labile sites (*e.g.,* apurinic/apyrimidinic sites), alkylated and oxidized nucleobases, DNA-DNA crosslinks, UV-induced cyclobutane pyrimidine dimers, among others (Collins *et al.,* 2023). Despite its sensitivity, further tests are needed to discover the exact nature of the DNA damage.

Variability in DNA damage may reflect individual or physiological differences among larvae, affecting the consistency of comet tail measurements. Standardizing larval age, exposure conditions, and sample handling in future assays can help minimize this variability and improve reliability. Several sources of variability exist, including animal-to-animal, cell-to-cell, or even slide-to-slide, which must be accounted for in the design of experiments and in the interpretation of results (Hartmann *et al.,* 2003). We need to improve some procedures to avoid the formation of additional DNA damage during the processing of samples, especially in the standardization of oral administration, the incorporation of quantitative data (*e.g.,* amount of DNA in the tail), and in the use of statistical analyses.

Møller *et al.* (2014) alert that it is important to guarantee that the replicates are not nested or a set of subsamples. To achieve that, our experimental unit consisted of 35 larvae, from different rearing cages and from different generations, to warrant independent observations. Since no data is available for *C. albiceps,* we tested two dosages. Although this number is insufficient to demonstrate a clear dose-dependent response, it represents the minimum required under current protocols (Hartmann *et al.,* 2003).

The ecotoxicological consequences of genomic instability and its correlation with DNA breaks measured by the Comet Assay are greatly overlooked (Jha, 2008). DNA strand breaks can be repaired by a series of mechanisms, so that the damage represented by the comet does not necessarily result in cell death. To gain ecological relevance, a mechanistic association between genotoxic stress and effects at higher biological levels should be confirmed. DNA damage in insect populations can lead to reduced survival, impaired reproduction, and developmental abnormalities, which may ultimately affect population growth and stability (Lapuente *et al.,* 2015).

A recent review by Collins *et al.* (2023) lists only *D. melanogaster* as the insect species targeted for CE.

Table 2. Assessment of the Comet assay for detection of genotoxic effects caused by IVM and IMI in cells of *Chrysomya albiceps* larvae.

Criteria	Evaluation
High sensitivity to detect genotoxic damage	🪰🪰🪰
Low quantity of specimens (insects) needed	🪰🪰🪰
Low volume of biological sample (haemolymph) needed	🪰🪰🪰
Standardization of visual damage	🪰🪰🪰
Ease of detecting dose-dependent responses	🪰
Access to standard molecular biology equipment	🪰🪰🪰
Reproducibility and accuracy of data	🪰🪰
Possibility of testing several chemical compounds	🪰🪰
Reliability of comparison with established positive and negative controls	🪰🪰
Suitability to statistical analysis	🪰🪰🪰
Integration with other methods (*e.g.,* image processing)	🪰🪰🪰

🪰🪰🪰 = Meets the requirements of common laboratory practices.
🪰🪰 = Needs minor adjustments on the reagents or equipment.
🪰 = Demands significant modifications in the protocol, such as reagents, equipment, etc.

We expand this spectrum by testing *C. albiceps* larvae. The species can be used as a sentinel to detect the fate of xenobiotics in terrestrial environments. They can respond to drugs, toxicants, hormones and biocides by altering their development and behaviour (Chophi *et al.,* 2019). We add a novel approach for the use of *C. albiceps* in *forensic entomology,* which can be applied to other necrophagous species.

CONCLUSION

This innovative study combines a well-established method for detecting DNA strand breaks with a novel application strategy using the necrophagous fly *C. albiceps*. The approach stands out not only for its sensitivity and laboratory applicability but also for paving the way towards new experimental models for insects. Although widely validated in other organisms, this work proposes *C. albiceps* larvae as a promising sentinel species for the genotoxic assessment of xenobiotics in overlooked terrestrial ecosystems.

AUTHORS' CONTRIBUTIONS: CR: Data Curation; CR, SDV: Conceptualization, Project Administration, Resources, Supervision, Validation, Writing – original draft; CR, EMA: Formal Analysis; CR, WKMV, EMA, AAS, SDV: Investigation; CR, WKMV, EMA, AAS, HRPF, SDV: Methodology; CR, WKMV: Visualization; HRPF, SDV: Writing – review and editing. All authors actively participated in the discussion of the results; they reviewed and approved the final version of the paper.

CONFLICT OF INTEREST: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

DATA AVAILABILITY: All data generated or analysed during this study are included in this published article.

FUNDING: Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco (FACEPE, APQ1263.201-21).

ACKNOWLEDGMENTS: We thank Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco (FACEPE), Conselho Nacional de Desenvolvimento Científico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for scholarships, the colleagues at the Laboratory of Insects of Forensic Importance (UFPE) for technical support, and Dr. Trevor Williams for revising the English version. SDV is a recipient of a productivity grant from CNPq.

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Published with the financial support of the "Programa de Apoio às Publicações Científicas Periódicas da Universidade de São Paulo".