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Ecotoxicological Effects of the Herbicide Metribuzin on *Tenebrio molitor* Hemocytes

Maria Luigia Vommaro *, Michela Guadagnolo, Martina Lento and Anita Giglio *

Department of Biology, Ecology and Earth Science, University of Calabria, 87036 Rende, Italy; martina.lento@unical.it (M.L.)

* Correspondence: marialuigia.vommaro@unical.it (M.L.V.); anita.giglio@unical.it (A.G.); Tel.: +39-098-449-2982 (A.G.)

Abstract: Herbicides are synthetic chemicals that are extensively employed in agricultural practices with the objective of enhancing crop yield and quality. Despite their selectivity for plant systems and being generally regarded as non-toxic to animals, there is a paucity of understanding surrounding the sublethal effects on non-target organisms, including animals. This gap underscores the necessity for ecotoxicological research that prioritizes the identification of suitable models and develops reliable biomarkers for the early assessment of environmental impact. In this context, hemocytes—circulating immune cells found in invertebrates—have been identified as a crucial system for assessing sublethal toxicological effects, given their role in immune defense and overall organism health. *Tenebrio molitor*, a beetle pest of stored grain, was used as a model for the assessment of the effects of a metribuzin-based herbicide (MTB, Feinzin DF 70, 70% metribuzin, 0.25 kg ha^{-1}). Following a 96 h exposure to MTB, the males (7–10 days post-eclosion) were examined for multiple biomarkers in their hemocytes, including cell density, phagocytic activity, lysosomal membrane stability, and cytological changes. Although no mortality was observed, exposure to MTB resulted in a reduction in the phagocytic index and an increase in blast-like cells, indicating the potential for immunotoxicity. Lysosomal membrane stability was reduced, though no significant changes in hemocyte density or nuclear morphology were observed. These responses indicate potential immune system impairment, which could affect the beetle's fitness and reproductive potential. This study highlights the potential of hemocytes for assessing sublethal herbicide effects, raising concerns about the ecological impact of herbicides in agroecosystems and their potential risks to both wildlife and human health.

Keywords: agrochemicals; alternative models; apoptosis; biomarkers; cytotoxicity; hematology; immunocompetence; insects; neutral red assay

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1. Introduction

Weeds represent a significant threat to crop yield and quality, largely due to their capacity for interspecific competition. This can result in a notable reduction in production, with estimates indicating a decrease of between 20 and 50%. In response, the use of herbicides has become a widely adopted strategy to counter their detrimental impact [1]. However, their widespread and intensive use has led to an increased demand, resulting in a broad range of active ingredients and formulations on the market. While herbicides are designed to target plant systems and are expected to be non-toxic to animals, they can produce adverse effects on various organisms [2,3]. Herbicides are highly persistent in the environment, with low degradation rates, which results in their long-lasting presence [4–7].

Furthermore, their dispersion in ecosystems is facilitated through leaching and the contamination of water bodies [8]. Consequently, herbicide exposure poses risks to both human [9] and wildlife health [10], particularly through direct exposure or accidental ingestion.

Triazine herbicides are a class of chemical compounds used for the selective control of weeds on a wide range of crops, including rice, maize, sugarcane, potato, soybean, tomato, and wheat. It has been demonstrated that these herbicides are effective against weeds and thus represent a valuable tool for the control of unwanted vegetation. The economic significance of this class of herbicides can be attributed to three key factors: their efficiency, low cost, and use as mixing agents in broader-spectrum formulations [11]. Triazines are classified into symmetrical (chloro-s-triazines, thiomethyl-s-triazines, methoxy-s-triazines) and asymmetrical types (e.g., metribuzin, metamitron, hexazinone). All share a stable 1,3,5-triazine ring, with various substituents at positions 2 and 4, which influence their degradation rate and biological activity [12]. The mode of action is based on the inhibition of photosynthesis through the blockage of the Hill reaction, specifically targeting the D1 protein of photosystem II and disrupting electron transport [11,13]. They are characterized by persistence and high migratory potential, so they are considered environmental contaminants [11,14]. Among them, metribuzin [4-amino-6 t-butyl-3-methylthio)-1,2,4 triazine-5(4H) one] is a selective herbicide widely used in agriculture and when exposed, both vertebrates and invertebrates can experience a range of toxic effects. In vertebrates, this substance damages the liver, kidneys, and thyroid, and is toxic to pregnant rabbits at a concentration of 10 mg Kg^{-1} bw (body weight) per day (NOAEL) [15]. Exposure to a metribuzin-based formulation has been demonstrated to induce alterations in blood and biochemical parameters, particularly in the liver and kidneys, in fish such as carp (175.1 mg L^{-1} a.i.) and goldfish (50 mg L^{-1} a.i.) [16,17]. Immune responses in amphibians, including *Rana pipiens*, have been shown to be impaired after chronic exposure to pure metribuzin mixed with six pesticides at environmentally relevant concentrations (0.1 X, 1.0 X, and 10 X; 1 X = 0.56 mg L^{-1}) [18]. In invertebrates, metribuzin induces toxicity in crustaceans such as crayfish, disrupting hepatopancreas function (10% 96 h LC50 = 3.06 mg L^{-1}) [19], and causes mortality, genotoxicity, and metabolic disturbances in soil-dwelling arthropods exposed to recommended field rate [20–22]. Additionally, it negatively impacts the beetle *Zygogramma bicolorata*, a biocontrol agent, causing mortality and developmental delays in field conditions within 24 h [23,24]. These recent studies raise questions about the potential ecological risks associated with metribuzin. However, this crucial information is still not adequately addressed in environmental risk assessments. The estimated acceptable daily intake (ADI) was calculated by the European Commission to be 0.0013 mg Kg^{-1} bw per day [25]. The maximum residue level (MRL), the highest level of a pesticide residue that is legally permitted in fruits, vegetables, cereals, and products of animal origin, was set at 0.1 mg Kg^{-1} [26].

Ecotoxicological assessments of agrochemicals are primarily conducted using vertebrates and selected invertebrates, with a focus on acute toxicity testing [27]. However, due to the increasing regulation of vertebrate use to minimize animal suffering, it is critical to validate alternative, more sensitive models [28]. Insects are a highly abundant and diverse taxonomic class which play pivotal ecological roles in agroecosystems, contributing to nutrient cycling, pollination, and pest control [29]. Due to their availability, ease of rearing, and cost-effectiveness, insects are emerging as promising models for ecotoxicological studies [30].

The insect immune system, specifically the hemocyte system, provides a valuable tool for assessing the effects of contaminants. The role of circulating cells in insect immunity encompasses phagocytosis, melanization, nodulation, and encapsulation [30,31]. Additionally, they are involved in various other processes, including nutrient transport,

development, detoxification, and wound healing [31]. Phagocytosis, which is primarily carried out by plasmatocytes and granulocytes, involves the engulfment and subsequent degradation of pathogens [32]. The efficiency of the immune system is influenced by a number of factors, including the availability of resources, age [33], sex [34,35], neuroendocrine regulation [36,37], temperature [38], and population density [39]. Additionally, environmental fluctuations and pollutants may also affect the hemocytes in invertebrates [40–42], demonstrating a high degree of phenotypic plasticity and acclimation. Given the pivotal roles played in immune and physiological functions, circulating cells are a valuable early warning system to assess potential adverse effects in the field of ecotoxicology [43,44]. The morphological and functional variability of hemocytes may serve as an indicator of stress [45], offering a simple and effective tool for investigating cellular and molecular responses to pollutants and assessing their ecological impact, as demonstrated in aquatic invertebrates [46].

In light of the established suitability of coleopterans for immunological and ecotoxicological studies [30], *Tenebrio molitor* (Linnaeus, 1758), a species in the Tenebrionidae, was selected as an alternative model organism for this investigation. This species is a well-characterized model with known physiological and immune parameters [47–51], making it ideal for assessing the sublethal effects of herbicide exposure.

The aim of this study is to evaluate the sublethal effects of herbicide exposure on the morphological and functional properties of hemocytes. A metribuzin-based formulation (MTB) was tested on *T. molitor*. To simulate field herbicide exposure, males were exposed to an environmentally relevant concentration for 96 h. The study evaluates the effects of MTB on immunocompetence (hemocyte density and phagocytic ability), cytotoxicity and inflammation (lysosomal membrane stability), and potential genotoxicity (nuclear changes). The analysis of hemocytes was conducted to gain insight into the broader physiological effects of metribuzin and its sublethal effects on non-target organisms.

2. Materials and Methods

2.1. Animal Rearing

Specimens of *T. molitor* were obtained from a stock population housed in the Laboratory of Morpho-functional Entomology, Department of Biology, Ecology and Earth Science (University of Calabria). The beetles were reared at 60% r.h. with a natural photoperiod and room temperature (23 ± 2 °C). The stock population was fed ad libitum with wheat flour, organic fruit, and vegetables.

To control for potential side effects masked by differences in immunocompetence between sexes, only males were included in this study. Sex determination was conducted during the pupal stage using a stereomicroscope (Leica Zoom 2000, Wetzlar, Germany) to examine the developing genital papillae at the caudal portion. After eclosion, which occurred 8–12 days later, newly emerged male adults were separated from females and maintained for 7–10 days under the same conditions as the stock population to reach sexual maturity. Exposure was then carried out on virgin, sexually mature males. The beetles were housed individually in cups (30 mL volume) and provided with organic flour (0.05 ± 0.02 g per cup; Belbake, Germany) as a dietary supplement.

The species *T. molitor* is not classified as a threatened species and no license is required for its study in Europe. The beetles were maintained under optimal laboratory conditions and were freeze-killed at the conclusion of the experiment.

2.2. Experimental Set-Up

This study tested a commercial metribuzin-based (MTB) herbicide formulation (Feinzin 70 DF, 70% a.i., Adama Bergamo, Grassobbio, Italy). The MTB solution was

applied at the recommended field rate for cereals and vegetables (0.25 kg ha^{-1} in 200–500 L of water). A volume of 100 µL of the MTB solution was applied to a 3 × 3 cm filter paper, the amount being calculated on the basis of the surface area (2.25×10^{-2} mg of MTB herbicide formulation diluted in distilled water per cup, one piece of filter paper provided for each cup). The control group (CTRL) received 100 µL of distilled water in the same way. Both solutions were applied to filter paper before the animals were individually housed.

The experiment involved 150 beetles 7–10 days post-eclosion, ensuring sexual maturity and complete cuticle darkening. Males were then individually placed in cups with perforated lids for air exchange. Cups were assigned to CTRL (N = 75) or MTB-treated (N = 75) groups, each containing filter paper through which the treatment was administered, serving as the sole moisture source. Following the initial administration of the treatment solution, the filter paper was moistened with 150 µL of sterile phosphate-buffered saline (PBS, 15 mM, pH 7.2–7.6; Sigma-Aldrich, Milan, Italy) daily to maintain humidity. The beetles were observed walking on the filter paper, which led to direct contact with the solution and subsequent uptake during the exposure period.

The exposure lasted 96 h to ensure acute exposure. Survival was monitored daily and a beetle was recorded as dead when it was completely immobile. The protocol was designed in accordance with the guidelines set out by the EPA [52]. The experimental design is outlined in Figure 1.

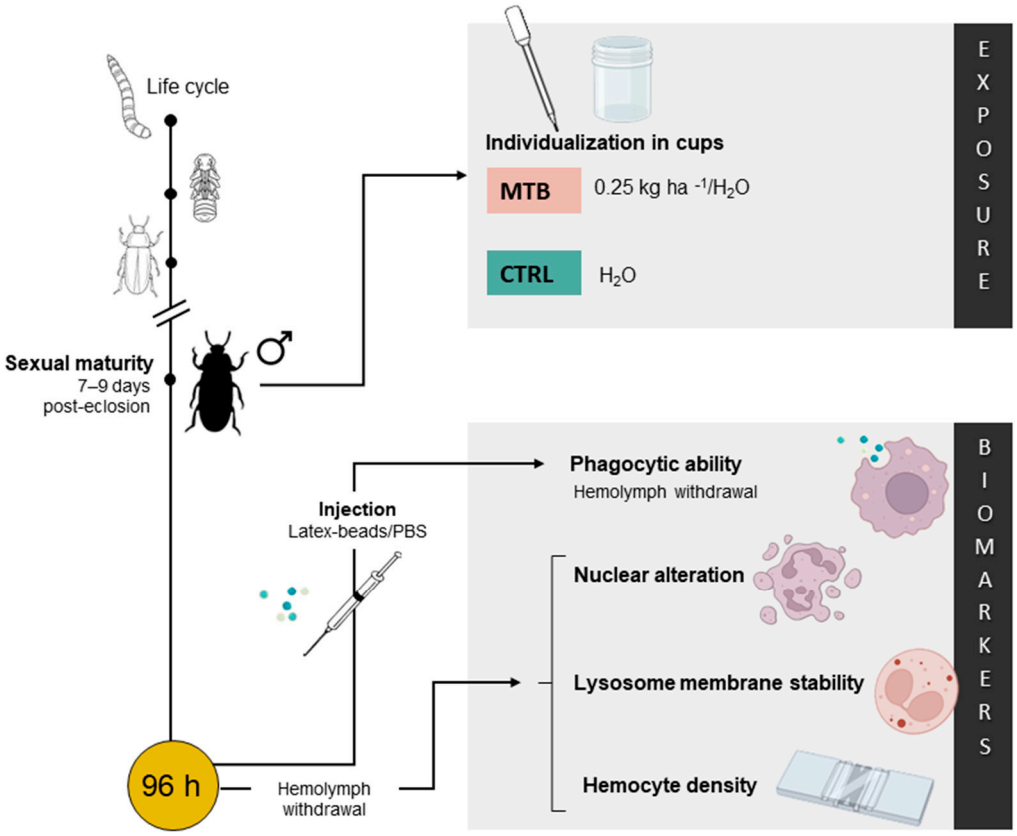

Figure 1. Experimental design. Adult, mature, virgin males of *Tenebrio molitor* (N = 150) were exposed to a metribuzin-based herbicide (MTB; N = 75) for a period of 96 h at the field rate, while a control group (CTRL; N = 75) was not exposed to the chemical. At the conclusion of the exposure period, the hemolymph was extracted and the hemocyte was evaluated for density, lysosome membrane stability, and nuclear alterations. The phagocytic index was assessed in vivo by injecting a solution of latex beads into the hemocoel and subsequently quantifying the number of engulfing cells two hours post non-self immune challenge.

2.3. Hemolymph Withdrawal and Assays

After 96 h of exposure, beetles were processed for analysis as described in Vommaro et al. [47]. A volume of 3 µL hemolymph was collected using a micropipette from each beetle at the ventral promesothorax junction after puncture with a 29-gauge needle. It was then transferred to Eppendorf tubes containing 9 µL of cold saline buffer with anticoagulant (PBS 10 mM, pH 7.4 + EDTA 17 mM), resulting in a 1:4 dilution. A distinct set of beetles was employed for each assay, with each beetle undergoing a single withdrawal event.

2.3.1. Total Hemocyte Counts (THC)

A total of 54 males (27 per each group: MTB-exposed and CTRL) were individually tested for hemocyte density. For THC analysis, a volume of 10 µL of hemolymph-buffer solution (dilution 1:4), collected as described in section above, was placed in a Bürker's hemocytometer (Carlo Erba, Cornaredo, Italy) and analyzed under a Zeiss Primo Star microscope at 100× magnification. Images were captured using an OptikamB3 Digital Camera (Optika Microscopes, Bergamo, Italy), and cell counts were performed using ImageJ software (version 1.54i). THCs are expressed as the mean ± standard error of cells per mL of hemolymph.

2.3.2. In Vivo Phagocytosis Assay

To assess in vivo phagocytosis, males were divided in CTRL and MTB-exposed groups. At the end of the exposure period, each group was subdivided into two subsets. The first subset included animals injected with phosphate-buffered saline (PBS) only (CTRL-PBS = 5; MTB-PBS = 5), serving as a negative control. The second subset included animals injected with polystyrene latex beads (CTRL-Latex beads = 15; MTB-Latex beads = 12).

The in vivo non-self immune challenge was performed injecting 3 µL of carboxylate-modified polystyrene latex beads (0.9 µm diameter, 10% solids, Sigma-Aldrich) into the hemocoel. The beads were diluted 1:10 in sterile PBS 0.15 M and administered with a 10 µL Hamilton syringe. Two hours post-injection, 3 µL of hemolymph was collected as described previously from both the control and MTB groups, mixed with 9 µL of cold saline buffer (PBS 10 mM + EDTA 17 mM) to prevent coagulation, and smeared onto poly-L-lysine-coated slides. After air-drying, slides were stained with May–Grünwald–Giemsa (Merck KGaA, Darmstadt, Germany) [47], they were incubated with undiluted May–Grünwald for 3 min, followed by a 5 min staining in a 1:1 dilution with water. Giemsa staining (1:20 in distilled water) was applied for 5 min, and the slides were washed with tap water. After mounting with Eukitt (Merck KGaA, Darmstadt, Germany), slides were examined under a Zeiss Primo Star microscope at 1000× magnification to detect engulfed beads in hemocytes. Images were captured using an OptikamB3 digital camera (Optika Microscopes, Bergamo, Italy). The phagocytic index (PI) was expressed as the percentage of cells containing at least one bead, a minimum of 150 cells were counted for each sample.

2.3.3. Nuclear Alterations (NA) and Morphological Evaluation

Hemolymph smears stained with May–Grünwald–Giemsa (described above) were used to assess nuclear damage by the nuclear alteration (NA) assay. NA evaluation categorized cells based on nuclear regularity (normal shape) or abnormal nuclear morphology. Abnormalities were classified into micronucleated (MN), binucleated (BN), lobed and polymorphic (LN, PN), kidney-shaped (KN), segmented (SN), pyknotic and karyorrhectic (KN) nuclei, apoptotic (A, BLN), vacuolated (VC), blast-like (BLAST), and mitotic cells (MITO) following Vommaro et al. [53]. The NA is expressed as the relative percentage of each cellular category, with a minimum of 150 cells counted for each sample. The assay was performed on 27 individuals (CTRL = 15; MTB = 12). The smears were also

subjected to qualitative morphological evaluation according to the characterization of Vommaro et al. [47], and signs of cytotoxicity were also reported.

2.3.4. Neutral Red Assay

A stock solution was prepared by dissolving 20 mg of neutral red powder (Sigma-Aldrich) in 1 mL of dimethyl sulfoxide (DMSO; Merck Life Science, Milan, Italy) and stored at −20 °C. The working solution was made by diluting 10 µL of stock solution in 2.5 mL of PBS 10 mM at 4 °C immediately before use and protected from light. The assay was performed on 29 individuals (CTRL = 14; MTB = 15). After 96 h of exposure, a 4 µL of hemolymph was collected and diluted 1:3 in buffer solution (PBS 10 mM + EDTA 17 mM at 4 °C). The mixture was loaded onto poly-L-lysine-coated slides and incubated for 2 min. Then, the working solution was added (1:1 ratio) and incubated in a dark, humid chamber to prevent photooxidation. Slides were observed at six time points (10, 40, 70, 110, 155, 215 min) under a Zeiss Primo Star microscope at 400× magnification. Images per sample were captured using an OptikamB3 digital camera (Optika Microscopes, Bergamo, Italy). Images were evaluated to determine the neutral red retention time (NRRT), defined as the time at which 50% of cells showed no lysosomal alterations.

Lysosomal membrane stability (LMS%) was calculated using the formula:

$$\text{LMS\%} = [1 - (\text{sum of weighted scores}/75)] \times 100$$

where the weighted score was obtained by multiplying the assigned score (1–5) by the corresponding weighting factor for each time point. The sum of the weighted scores was divided by 75, representing the maximum severity and complete loss of lysosomal membrane stability. Scores and signs followed Martínez-Gómez et al.'s methodology [54], whereby scores range from 1 to 5, with 0 indicating no effect, 1 representing enlargement without leakage of lysosomes, 2 signifying the presence of lysosomes with leakage but no enlargement, 3 denoting leakage and enlargement of lysosomes, 4 indicating leakage and enlargement with colorless lysosomes, and 5 referring to rounded fragmenting cells.

2.4. Statistics

Statistical analyses were conducted using R version 3.0.1 [55]. Data from THCs, in vivo phagocytosis, nuclear alterations, and neutral red assays were tested for normality with the Shapiro–Wilk test, which indicated non-normal distributions ($p < 0.05$). Consequently, the non-parametric Mann–Whitney U test was applied. The distribution of data between the CTRL and MTB-exposed groups was represented using violin plots, while integrated biological response was represented using star plot. Daily survival data were recorded through direct observation of individual beetles and represented with Kaplan–Meier curves to estimate survival over time. Survival analysis was performed using a Cox proportional hazards model with the coxme function from the "survival" package. Results are presented as mean ± standard error. The integrated biological response (IBR) was calculated according to Sanchez et al. [56] with the updated version referred to as the IBRv2 mean and sum and as the Integrative Biomarker Response–Threshold (IBR-T) according to [57]. In the star plot, the control group was used as the reference deviation for each biomarker investigated. Areas extending upwards from 0 indicate biomarker enhancement, while areas extending downwards from 0 on the *x*-axis represent biomarker suppression.

3. Results

3.1. Survival, Hemocyte Density, and In Vivo Non-Self Immune Challenge

Statistical analysis showed no significant differences in mortality, suggesting that the herbicide did not have a lethal effect. The survival rate for the control group was

100%, while the survival rate for the MTB group was 98.67 ± 0.08%. Kaplan–Meier curves (Figure 2a) showed considerable overlap between the CTRL and MTB groups.

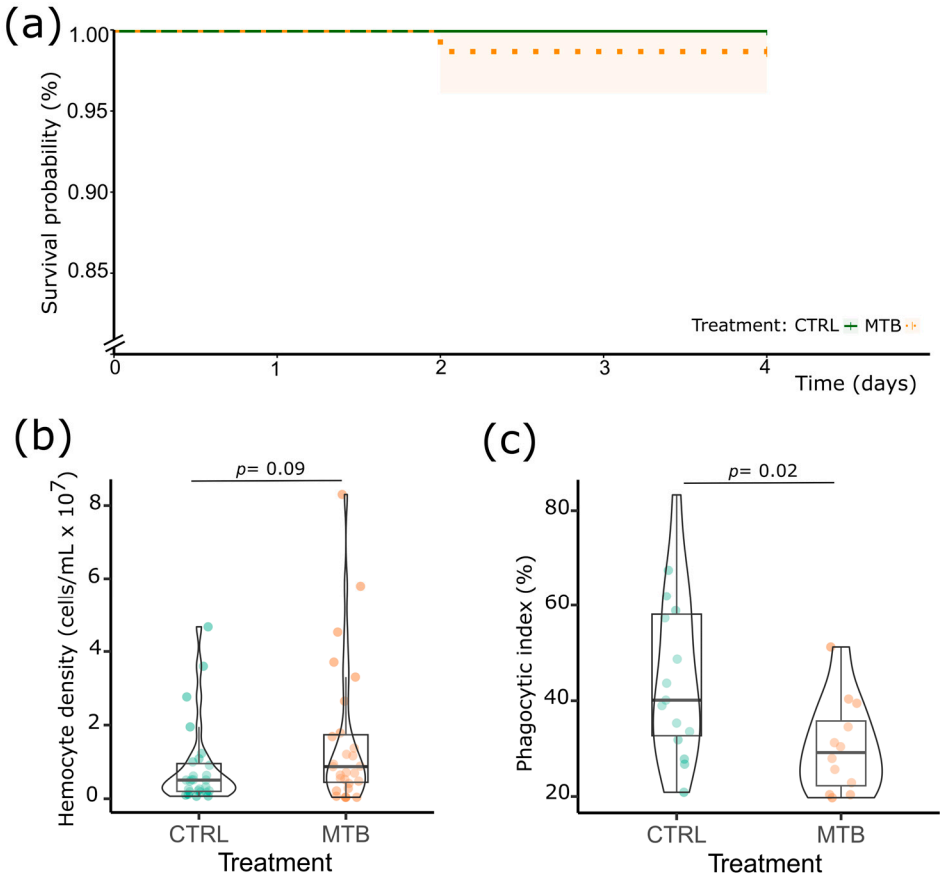

Figure 2. (**a**) Kaplan–Meier survival curves for *T. molitor* exposed to MTB-based herbicide for 96 h (N = 75) and control (N = 75). (**b**,**c**) Violin plot of total hemocyte counts (**b**): CTRL, N = 27; MTB, N = 27) and phagocytic index following in vivo non-self immune challenge with latex beads (**c**): CTRL, N = 15; MTB, N = 12) at 96 h post-treatment.

The analysis of hemocyte density (Figure 2b) revealed an average of (8.58 ± 2.18) × 10^6 cells mL^{-1} in the control group, while the MTB-treated group reached (15.89 ± 3.82) × 10^6 cells mL^{-1}. Although non-parametric analysis revealed no significant differences ($p = 0.09$, Figure 2b), an increase in hemocyte density was recorded in the MTB-exposed beetles, showing greater individual variation compared to the control group.

Phagocytic activity (Figure 2c), assessed by injecting latex beads into the hemocoel, showed that the control group exhibited a mean phagocytic index of 45.11 ± 4.52 whereas the MTB-treated group demonstrated a significantly diminished index of 30.36 ± 2.80% ($p < 0.05$; Figure 2c), indicative of a 15% reduction in phagocytic activity. Phagocytosis was observed to be primarily carried out by plasmatocytes and granulocytes.

3.2. Cytotoxicity and Genotoxicity

The genotoxicity of the herbicide was assessed via the nuclear alteration index. The total percentage of altered cells at 96 h of exposure was similar between the control (34.00 ± 2.99%) and the MTB-treated groups (34.01 ± 2.48%) ($p > 0.05$; Figure 3). A significant increase ($p < 0.05$; Figure 3a) in blast-like cells was observed in the MTB group (4.46 ± 1.16%), compared to the control (2.02 ± 0.78%), which exhibited a high cytoplasm-to-nucleus ratio, a folded nucleus, visible euchromatin, and prominent nucleoli (Figures 3b and 4f). No significant differences were observed in the incidence of other nu-

clear alterations ($p > 0.05$; Figure 3b). The proportion of lobed and polymorphic (Figure 4j,k) nuclei was 21.77 ± 2.00% in the control and 24.21 ± 2.35% in the treated group ($p > 0.05$). The frequency of kidney-shaped nuclei was higher in the control group (0.83 ± 0.26%) than in the treated group (0.64 ± 0.26%), although this difference was not statistically significant ($p > 0.05$). The occurrence of segmented nuclei was observed in 6.32 ± 1.55% of the control group and 2.61 ± 0.54% of the treated beetle group ($p > 0.05$). Pyknotic and karyorrhectic nuclei were rare in both groups (0.40 ± 0.19% in control, 0.81 ± 0.37% in treated), with no significant differences ($p > 0.05$). However, MTB-exposed individuals showed slightly higher values (Figure 3b; PK-NR).

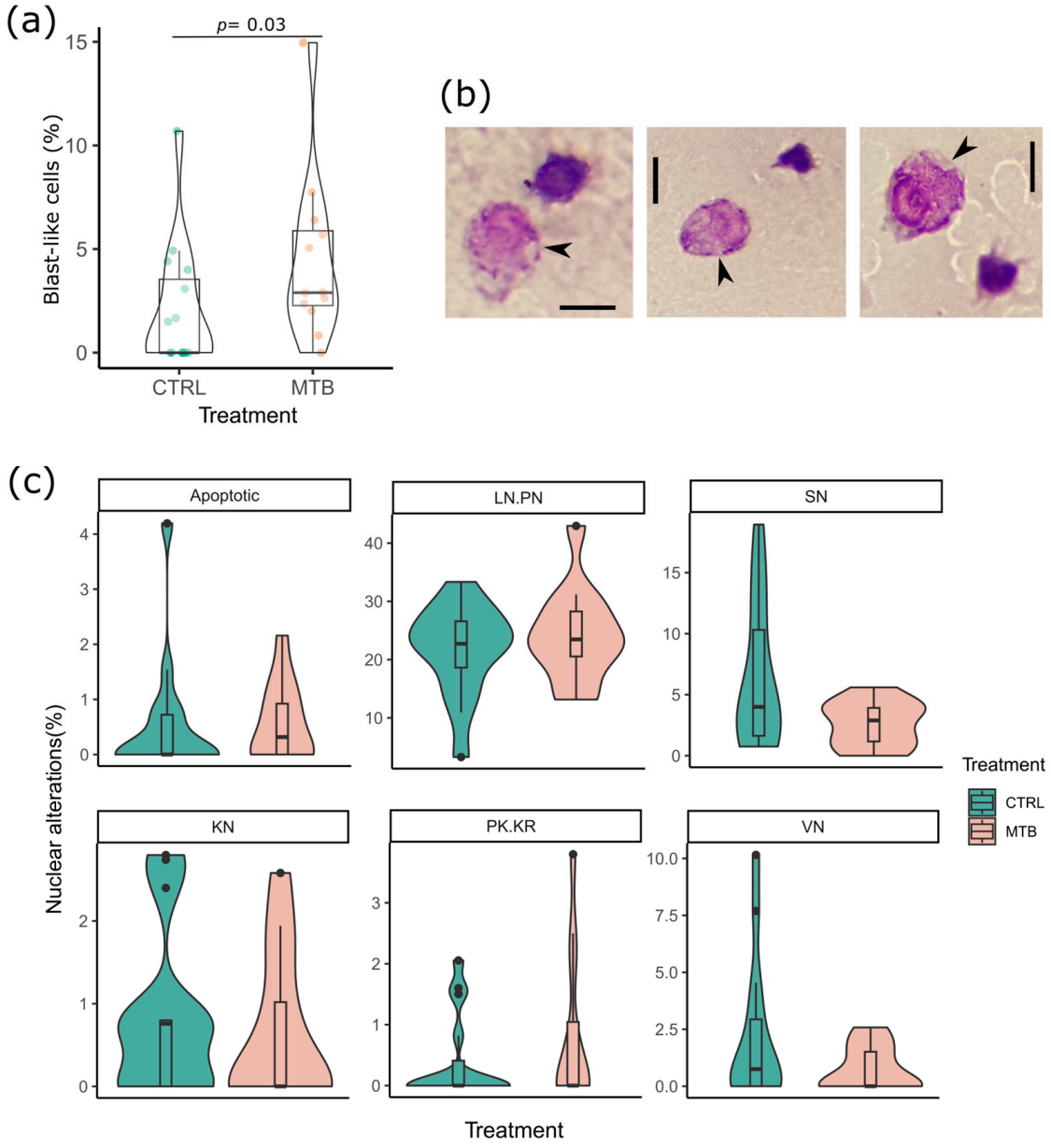

Figure 3. Nuclear alterations in *Tenebrio molitor* hemocyte smears at 96 h post-treatment. Experimental groups include CTRL (control, N = 15) and MTB (metribuzin-treated beetles, N = 12). (**a**) Violin plot shows the percentage of blast-like cells. (**b**) Blast-like cells (black arrowhead) in May–Grunwald–Giemsa smears of MTB-exposed beetles. (**c**) Violin plots depicting the percentage of apoptotic, kidney-shaped (KN), lobed and polymorphic (LN, PN), pyknotic and karyorrhectic (PK, KR), segmented (SN), and vacuolated (VN) nuclei (CTRL). Bars: 10 µm.

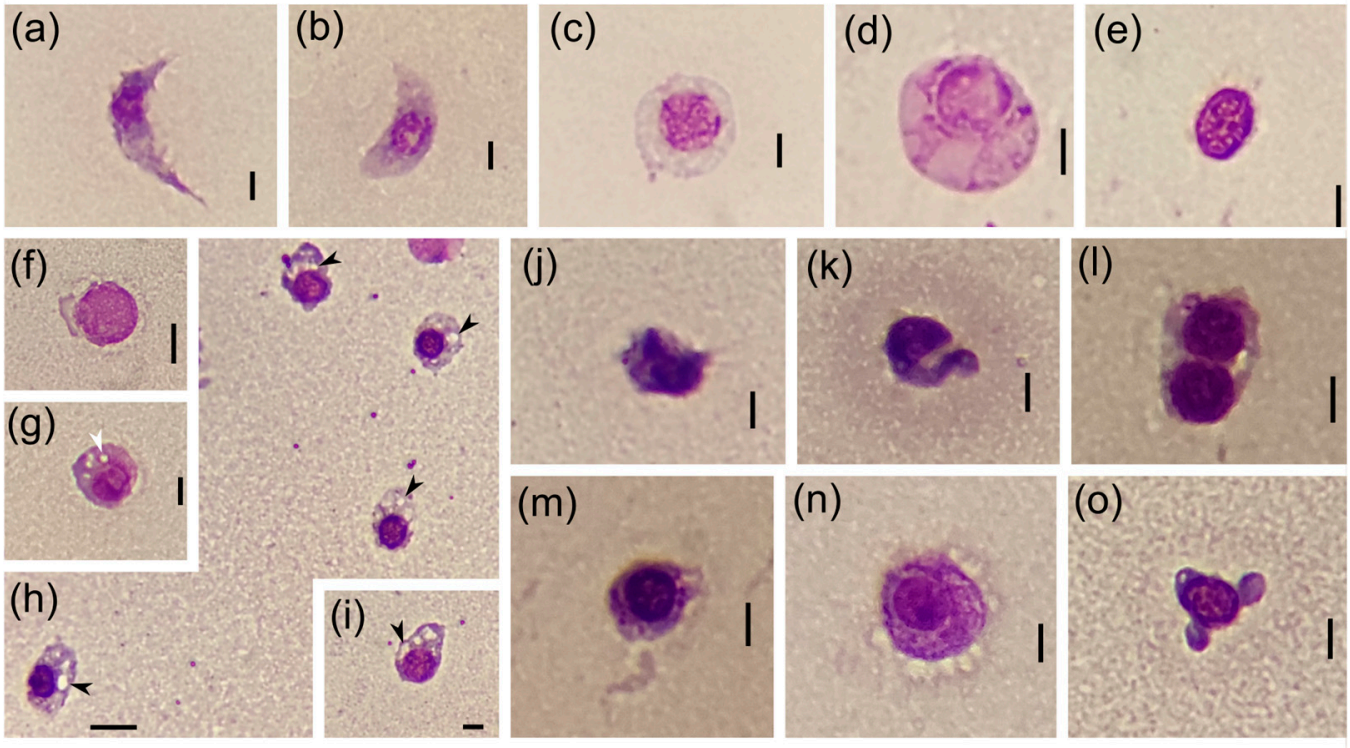

Figure 4. Hemocytes from *T. molitor* control (**a**–**e**) and exposed to MTB (**f**–**o**), May–Grünwald–Giemsa staining. (**a**,**b**) Plasmatocytes. (**c**) Early plasmatocytes. (**d**) Granulocytes. (**e**) Prohemocytes. (**f**) Blast-like cell. (**g**) Phagocytic granulocytes (white arrowhead: latex beads). (**h**,**i**) Vacuolated cells (black arrowheads). (**j**,**k**) Cells with lobed and polymorphic nuclei. (**l**) Mitotic cell. (**m**) Cell with cytoplasmic basophilic granules. (**n**) Granulocytes. (**o**) Apoptotic cell. Bars: 5 µm (**a**–**e**,**g**,**i**,**j**,**l**–**o**) 10 µm (**f**,**h**,**k**).

The comparative analysis of smears (Figure 4a–o), conduced to assess the presence of exposure effects on cell morphology, also showed cytoplasmic vesicles (Figure 4h,i) and apoptotic bodies and blebs (Figure 4o) in hemocytes from MTB-exposed beetles. Furthermore, early plasmatocytes and granulocytes (Figure 4m,n), along with blast-like cells (Figure 4f) and mitotic cells (Figures 3b and 4l) were found in the MTB-exposed group compared to control ones (Figure 4a–e).

3.3. Lysosomal Membrane Stability

The neutral red assay provided two key insights: lysosomal retention time (Figure 5a), reflecting organelle stability, and lysosomal membrane integrity (Figure 5b), assessed through morphological analysis (Figure 5c). The control group exhibited a neutral red retention time (NRRT) of 51.76 ± 12.89 min, while the MTB-treated beetles had a reduced NRRT of 31.00 ± 12.04 min. However, this difference was not statistically significant ($p > 0.05$; Figure 5a).

Lysosomal membrane stability (LMS) was lower in the MTB-treated group (66.58 ± 5.22%) compared to the control (79.80 ± 3.41%), with a trend toward reduced stability in the treated group ($p = 0.08$; Figure 5b). The morphological analysis of lysosomal abnormalities (Figure 5c) revealed comparable patterns in both groups, with alterations in frequency and severity increasing over time. At the 10 min mark, both cell groups exhibited the presence of small red lysosomes, with the incidence and severity of abnormalities increasing over time. Notable alterations were observed as early as 40 min post-incubation. By 70 min, the lysosomes had undergone enlargement, and some cells exhibited leaky lysosomes,

resulting in red-stained cytoplasm. The aforementioned alterations were more pronounced in smears derived from MTB-exposed beetles.

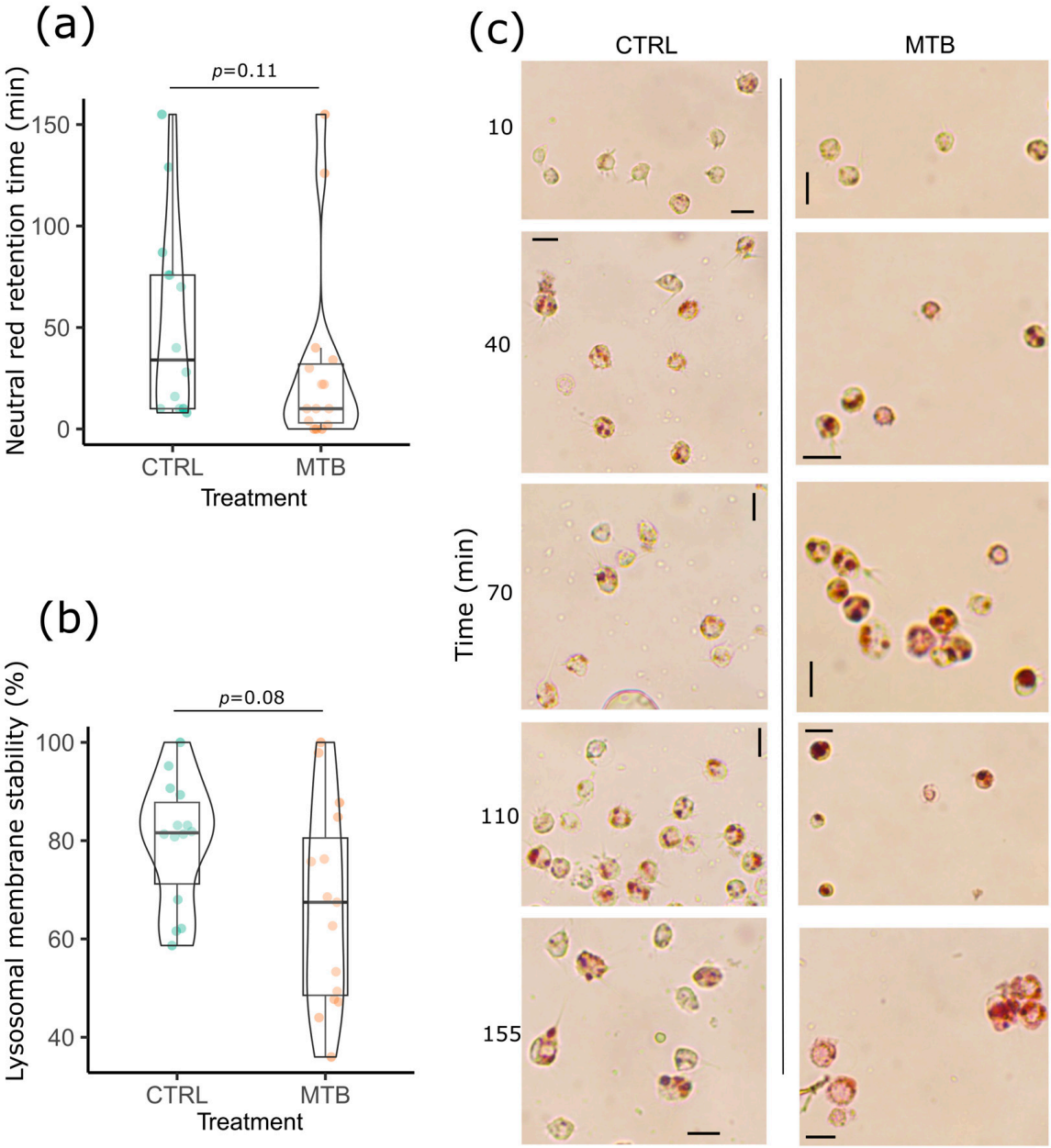

Figure 5. Violin plots shows neutral red retention time (**a**) and lysosomal membrane stability (**b**) of circulating hemocytes in *Tenebrio molitor* at 96 h post-treatment for the control (CTRL) and metribuzin-treated (MTB) groups. (**c**) Neutral red-stained hemocytes at 10, 40, 70, 110, and 155 min, showing increased lysosomal dilation over time and the presence of enlarged, leaky lysosomes and red cells (CTRL, N = 14; MTB, N = 15). Bars: 5 µm.

3.4. Integrated Biological Response

The integrated biological response of the tested parameters, revealed that exposure to MTB (IBR-T: 1.41; IBRv2 sum: 8.49; Figure 6) resulted in a suppression in the capacity of the beetle to perform phagocytosis following non-self immune challenge, compared to the control group as a reference. This was associated with an increase in the percentage of blast-like cells and a slight decrease in the stability of lysosomal membranes and THC.

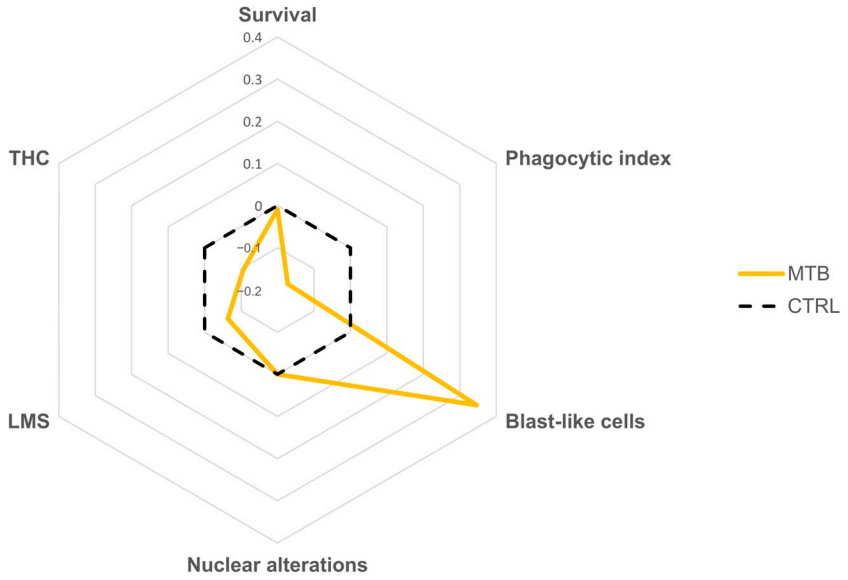

Figure 6. Star plot of integrated biological response of the morpho-functional characteristics in *Tenebrio molitor* circulating hemocytes at 96 h post-treatment for the metribuzin-treated (MTB) groups (solid yellow curve). The reference condition is represented by the black dotted curve (CTRL). LMS: lysosomal membrane stability; THC: total hemocyte counts.

4. Discussion

The present study demonstrates that a field rate of metribuzin (MTB) causes sublethal effects on *T. molitor* as early as 96 h after the initial exposure. Although it is expected to be non-toxic to animals, the application of MTB results in notable alterations to the morphological and functional characteristics of circulating cells in a non-target organism, according with detrimental effects previously observed on a range of organisms, including mammals, arthropods, and soil microorganisms [15,20,22].

The findings of our study reveal that, while metribuzin (MTB) does not induce lethal effects in adult *T. molitor*, it does cause sublethal effects on immune function. Notably, MTB exposure resulted in a significant reduction in phagocytic activity in hemocytes, thereby impairing the beetle's immune response. This observation aligns with findings from other studies showing similar immune impairments induced by herbicides. For example, the inhibition of phagocytosis was observed in laboratory studies conducted on the pond snail *Lymnaeid stagnalis* exposed to the herbicide firesafe (nitrophenyl ethers) [58], while even atrazine led to a decrease in hemocyte density [59]. Likewise, exposure to a field dose of the pendimethalin-based herbicide resulted in the diminished phagocytic capacity of the ground beetle *Pseudoophonus rufipes* and alterations in the hemogram following an immune challenge [60]. In contrast with the outcomes observed in other species, the effect of MTB on hemocyte density in *T. molitor* males was found to be relatively modest after 96 h of exposure, failing to elicit the same variations in density observed in previous studies upon atrazine and pendimethalin exposure [59,60]. In a previous study, the same species was tested during sexual maturity seven days after exposure to MTB (field dose), demonstrating a significant increase in hemocyte density in males but not in females [61], highlighting a differential effect related to age and sex.

The MTB exposure resulted in an increase in blast-like cells, which may suggest a higher turnover of circulating cells. This may represent a mechanism by which damaged hemocytes are replaced. Indeed, the increase in the number of circulating cells showing vesicles in the cytoplasm upon MTB exposure could be indicative of a role for circulating cells in the detoxification process [45]. It is recognized that hemocytes possess the ability to sequester and accumulate toxicants, such as heavy metals, within vesicles of the

endocytic–lysosomal system [62]. An alternative hypothesis is that the toxicant exerts a direct effect on cell metabolism and structural components, resulting in damage to circulating cells. Indeed, an increase in blast-like cells has been observed even in *P. rufipes* exposed to pendimethalin [60]. The herbicides clethodim and haloxyfop-P-methyl have been demonstrated to induce alterations in the nucleocytoplasmic ratio of hemocytes in *Apis mellifera* [63], indicating a potential impact on the maturation status of circulating cells. In fish, metribuzin has been demonstrated to result in a notable reduction in lymphocyte and a considerable increase in neutrophil granulocyte levels. This phenomenon has been observed in both *Oncorhynchus mykiss* (96 h LC50 = 89.3 mg L^{-1}) [64] and *Danio rerio* (LOEC = 33 mg L^{-1}) [65], where an increased prevalence of developmental forms of the myeloid sequence has also been documented. This confirms the effect on differentiation or hemopoiesis found in invertebrates.

The considerable number of immature cells observed in MTB-exposed beetles may be a contributing factor to the observed reduction in their phagocytic capacity. Immature cells that have not undergone differentiation are typically unable to perform the functions typically associated with differentiated granulocytes or plasmatocytes, such as phagocytosis [66]. We can therefore assume that the organism adopts a strategy to cope with the presence of a toxicant, which results in the reallocation of resources from the ability of hemocytes to engulf and neutralize pathogens to detoxification mechanisms. A reduced ability of hemocytes to fight pathogens can lead to an impaired immune response and increased susceptibility to infections. Altered susceptibility to pathogens in terms of immune response or survival has been observed in species of tenebrionid beetles exposed to pendimethalin and infected with natural fungal [67] or bacterial [68] entomopathogens. Conversely, enhanced resilience against the combined stress of the herbicide glyphosate and the natural pathogen in the pest *Plagiodera versicolora* [69] has been observed. This shows that the exposure to herbicides has the potential to alter fundamental interspecific relationships. Further research is required to investigate the differentiation of hemocytes and the composition of the hemogram to determine whether specific populations are subject to variations that could compromise immune defenses and survival from infections.

Genotoxicity analysis showed no significant DNA damage in *T. molitor* hemocytes, unlike previous findings on other herbicide classes, such as pendimethalin-based formulations on *Harpalus rufipes* [21], *Apis mellifera* [53], and glyphosate-based herbicide on *Lycaena dispar* [70]. This may be due to differences in herbicide formulation, exposure conditions, or mode of actions.

The observed effects on the lysosomal membrane stability in MTB-treated beetles suggest mild cellular stress, and the possibility of the herbicide influencing the stability of internal cell membrane systems. This phenomenon may be attributed to the herbicide's ability to generate reactive oxygen species (ROS), as evidenced by the effects observed in insects and vertebrates exposed to herbicide 2,4-D [71]. This resulted in a reduction in the enzymatic activities of the antioxidant system, as well as an age-related decline in antioxidant enzyme activity in glyphosate-exposed crickets (*Gryllus assimilis*) [72]. This was accompanied by an increase in GST levels, which is part of a cellular detoxification strategy. The administration of metribuzin (1.3 mg kg^{-1}, 3 months, contaminated diet) resulted in an increase in oxidative stress in rats [73], and the chronic exposure disrupts the activity of antioxidative enzymes in crayfish (10% 96 h LC50 = 3.06 mg L^{-1}) [19]. An increased lysosomal fragility was detected in the pond snail *Lymnaea stagnalis* exposed to the herbicide fomesafen [58].

The findings indicate that, even herbicide field concentrations that do not directly kill *T. molitor* may significantly affect their physiological parameters, particularly immune

functions such as phagocytosis, and overall organism health. Further research is required to elucidate the mechanistic framework underlying the toxicity of MTB.

5. Conclusions

At the cellular level, metribuzin-based herbicides significantly impair the phagocytic function of hemocytes, leading to an increase in the number of immature cells and slight alterations in lysosomal membrane stability. At the organismal level, reduced phagocytic activity may affect immunocompetence, potentially compromising the organism's ability to cope with pathogens, which could have a negative impact on fitness and reproduction. The reduced phagocytosis may be due to the increased presence of blast-like cells, which, being immature and undifferentiated, are incapable of performing phagocytosis or other functions typical of differentiated granulocytes and plasmatocytes. The observed decrease in lysosomal membrane stability suggests an elevation in pro-inflammatory mediators, such as reactive oxygen species, which can lead to self-damage.

Further research is required to comprehensively assess the long-term effects of herbicide exposure and its impact across all developmental stages of beetles. This study makes a valuable contribution to the field of herbicide ecotoxicity by highlighting the potential of sensitive biomarkers as early warning indicators for even transient changes in non-target organisms exposed to field-relevant doses.

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