

Article

# Continuous Agrochemical Treatments in Agroecosystems Can Modify the Effects of Pendimethalin-Based Herbicide Exposure on Immunocompetence of a Beneficial Ground Beetle

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**Abstract:** Herbicide application for pest control can negatively affect soil biodiversity, mainly acting on species that are involved in ecosystem service. In this study, field and laboratory trials were designed to assay herbicide exposure effects on the constitutive immunity of *Harpalus (Pseudoophonus) rufipes* (De Geer, 1774), a beneficial carabid species that inhabits croplands. The circulating hemocytes (THCs) and plasmatic levels of basal and total phenoloxidase (PO), as well as lysozyme-like enzyme activities, were measured as markers of exposure. In laboratory tests, the exposure to realistic field doses of pendimethalin-based herbicides for two, seven and 21 days caused a reduction in enzyme activities in beetles from organic crops. In beetles from conventional fields, the THCs and total PO activity decreased significantly at two and seven days after the initial exposure, though no effects were recorded on basal PO and lysozyme like-enzyme activities. These differences in enzyme activities and THCs indicate that the interference of pendimethalin with immune parameters clearly depends on both the different field conditions from which the population comes and the cumulative effects of repeated applications over the time.

**Keywords:** carabid beetles; cellular response; dinitroaniline; phenoloxidase; ecological immunology; sub-lethal effect

## 1. Introduction

Herbicides are widely used for selective weed control to increase the yield and quality of crops. Though they were designed to only have effects on targeted weed plants, recent studies have highlighted that herbicides applied in agroecosystems also have negative effects on the biodiversity of soil macrofauna [1,2]. Detrimental effects related to application rate and residual persistence in the soil have been recorded in soil invertebrates including nematodes [3], earthworms [4], collembolans and isopods [5], and spiders [6,7]. In insects, lethal and sublethal effects of herbicides have been recorded at different levels of biological organization in species with relevant ecological roles in agroecosystems [1,8]. However, information on direct and indirect effects is fragmentary and, in particular, concerns a low number of species [1].

Pendimethalin (PDM) is a dinitroaniline herbicide used in pre-emergence for the control of grasses in farmlands. It acts on tubulin and interrupts the polymerization of microtubules, arresting cell

division (mitosis) and elongation, resulting in plant death [9–11]. Its half-life is from 24–39 to 76–98 days in aerobic soil (depending on weather conditions and soil pH). and 10–15% of residues remain 300 days after initial application [11,12]. Thus, its potential impact on non-target species may be related to its long dissipation time under cold and dry conditions [12], the residual dose of which remains in the soil after physical, chemical or microbiological transformations of the active ingredient and repeated applications over time [10,13]. Because of its low affinity for animal tubulins, the effects of pendimethalin have mainly been investigated in soil microbiota [12]. Very few studies have addressed potential toxicity following the application of sublethal doses of pendimethalin on a low number of aquatic organisms [11,14,15], as well as on non-target soil invertebrates, including insects [16,17]. This raises the question of whether exposure to pendimethalin may be harmful and can cause potential direct (feeding on contaminated prey or absorption by contact) and indirect (depletion of food) effects on non-target species.

A large number of carabid species are very active predators on soil surfaces in agroecosystem [18,19], and these species are recognized to provide critical ecosystem service by controlling pest populations in crops [20–22]; they are very suitable biological indicators of environmental change [23,24]. Thus, in this study, we used *Harpalus (Pseudoophonus) rufipes* (De Geer, 1774) as a model to measure the sublethal effects of exposure to a pendimethalin-based herbicide (hereafter referred to as PDN, using the commercial formulation Activus). This ground beetle is one of the most frequent epigeal species in agricultural ecosystems [18,21,25–32]. Moreover, it is well known to be involved in pest control as predators of seeds [33–38]. Its phenology and high dispersal activity [39] make *H. rufipes* able to survive in conventional crops [21,40] and also makes it useful to provide early warnings of sublethal effects in field exposure to pesticides [41].

Despite sublethal doses of insecticides having adverse effects on immunity in insects [42], effects of exposure to herbicides have been poorly investigated [43]. To study sublethal effects of the direct PDN exposure on the immune functions of *H. rufipes*, we measured their total number of circulating hemocytes, plasmatic phenoloxidase, and lysozyme-like enzyme activities. The amount of hemocytes in a hemolymph is a direct parameter of cellular immune responses [44]. When pathogens break the barrier of the cuticle, epidermis, or gut epithelium, a set of cellular immune responses, such as phagocytosis, nodulation and encapsulation involving hemocytes [45–48], are activated to block and remove pathogens from the haemocoel. Additionally, pathogen defense also includes the activation of different antimicrobial peptides (AMPs), including cytotoxic molecules, lysozymes and the phenoloxidase (PO) cascade [49,50]. The inactive PO zymogen is constitutively synthesized in hemocytes and released into the hemolymph. Upon infection, pattern recognition receptors activate serine protease cascades, a process that culminates in the activation of prophenoloxidase (PPO), which drives the production of melanin [51]. PPO's main role in melanogenesis is to catalyze the oxidation of phenols to quinones, which polymerize to form melanin [52,53]. Melanin is involved in cellular immune responses including nodulation, melanotic encapsulation, and wound healing [49,54]. AMPs such as lysozymes are constitutively present at a very low level in the hemolymph. Lysozymes perform a hydrolytic action against the peptidoglycan of Gram-positive cell walls [49,55–57] and Gram-negative bacteria [58], and they also have fungistatic [59] and antiviral [60] properties.

In this study, we performed two types of laboratory tests, with the hypothesis that the susceptibility of beetles to herbicide exposure depends on previous contact with pollutants in the natural habitat from which the population comes. The first experiment was designed by using beetles from the organic field to assess the effects of direct exposure to contaminated soil on beetles that had never been exposed before. The second experiment involved beetles from a conventionally treated field to test the cumulative effects of repeated applications over the time.

## 2. Material and Methods

### 2.1. Insect Sampling and Maintenance

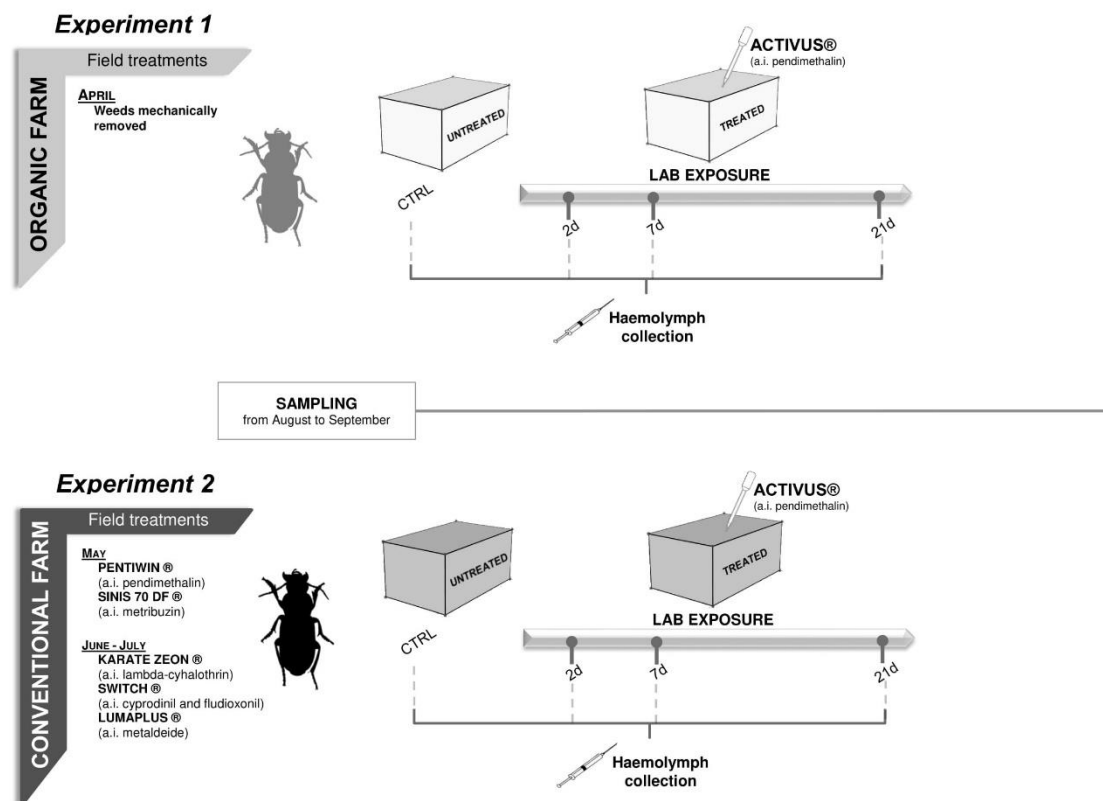
Adults of *H. rufipes* were collected from two sites in the Sila Mountain (Calabria, Southern Italy) by using in vivo pitfall traps (a plastic jar that is 9 cm in diameter) containing fruit as an attractant from August until mid-September, 2018. We selected this sampling period because it is the major period of activity of this species in the sampled area. The first group of tested beetles was collected from a non-treated wheat field located in an organic farm (organic; 39°17'10.28" N, 16°42'28.33" E, 1150 m.a.s.l.; Macchia di Tuono Farm, San Giovanni in Fiore, Calabria, Southern Italy). This field was ploughed in April to avoid weeds. The second group was from a lettuce field in a conventional farm (conventional; 39°16'58.05" N, 16°38'43.26" E, 1240 m.a.s.l., Società Cooperativa Orti dei Monti, Torre Garga Farm, San Giovanni in Fiore, Calabria, Southern Italy), located 5.5 km south-west of the sampled organic field. The typical crop rotation was spring potatoes, wheat, and lettuce in three years. In mid-May, before lettuce was planted, the field was ploughed up, and the soil was sprayed with the pre-emergence herbicides Pentiwin (4 L per ha of active ingredient pendimethalin from 400 g L<sup>-1</sup> of commercial formulation) and Sinis 70 Df (0.25 Kg per ha of active ingredient (a.i.) metribuzin from 700 g Kg<sup>-1</sup> of commercial formulation) to avoid weeds. Moreover, treatments were performed in June and July during the vegetable growth phase with the insecticides Karate Zeon (a.i. lambda-cyhalothrin 100 g L<sup>-1</sup>) and Altacor (a.i. chlorantraniliprole 350 g Kg<sup>-1</sup>) against aphids and Noctuidae. Fungicides were applied against *Phytophthora* spp. and *Peronospora* spp. (Idrorame 193; a.i. copper sulfate tribasic 193.04 g L<sup>-1</sup>) and against the grey mold *Botrytis cinerea* (Switch; a.i. cyprodinil 375 g Kg<sup>-1</sup> and fludioxonil 250 g Kg<sup>-1</sup>) and molluscicide (Lumaplus; a.i. metaldehyde 49 g Kg<sup>-1</sup>) against snails.

In the laboratory, the beetles were kept in groups separately for each sampled site in 10 L plastic boxes that were filled to a depth of 6 cm with soil from the capture site, held at 60% relative humidity (rh), had a natural photoperiod, and were at room temperature. They were fed homogenized meat and fruit ad libitum. The experimental design was conducted in accordance with all applicable government and institution laws and rules.

### 2.2. Laboratory Toxicity Test Set up

Toxicity tests were performed in plastic boxes (40 × 25 cm) containing a 6 cm layer of clean soil from control untreated site (sandy soil, pH 5). Before the beetles were housed in the boxes, the soil was wetted with deionized water for the control group and with the working solution of the tested herbicide for the treated group. The pendimethalin working solution was created with the commercial formulation Activus (a.i. pendimethalin 330 g L<sup>-1</sup>). The formulation was diluted in distilled water by using a recommended field rate (4 L per ha) for cereal and vegetable cultures, and it was sprayed with a pipette onto the soil surface of each box. The beetles were introduced in the box 15 min after the herbicide has been sprayed.

Two different experiments were designed to measure the sublethal effects of pendimethalin (Figure 1). Experiment 1 was conducted with beetles from a control site that had never been exposed to chemicals; these beetles were acclimated in the laboratory for a minimum of 1 month prior the experiment. Experiment 2 was performed with beetles from a treated lettuce field that were collected at the beginning of September, approximately three months after the first exposure to field herbicide treatments and one month after the last field treatment with insecticides and fungicides. Specimens were kept in the laboratory for two months on soil from the capture site to ensure exposure to the residual amount of agrochemical residues from the field prior the laboratory exposure to the PDM-based herbicide.



**Figure 1.** Experimental model. Two experiments were designed to measure the effects of exposure to PDM-based commercial formulation Activus at different time points (2, 7, and 21 d) on *Harpalus (Pseudoophonus) rufipes* adults. To test differences in the immune parameters depending on the different field conditions, laboratory exposure trials were carried out with beetles from organic (Experiment 1) and conventional (Experiment 2) fields. Clean soil from the organic field was used in both the control (ctrl) and exposure boxes.

Boxes were maintained for each experiment at 60% rh under a natural photoperiod and room temperature. The beetles checked every day and fed on homogenized meat and fruit ad libitum. The immune parameters were measured in the beetles before the treatments (hereafter referred to as the control group) and at three different time points (2, 7 and 21 days) after a single application of pendimethalin for both experiments. The exposure time was chosen by considering the degradation time of pendimethalin [12,61] in sandy acid soil. A different set of individuals was used for each time point.

### 2.3. Measured Parameters

#### 2.3.1. Total Hemocyte Counts (THCs)

Three  $\mu\text{L}$  of hemolymphs were collected from the control ( $n = 12$  for each field) and treated animals (Experiment 1:  $n_{2d} \text{ and } 7d = 16$   $n_{21d} = 10$ ; Experiment 2:  $n_{2d} = 16$   $n_{7d} = 13$   $n_{21d} = 12$ ) by puncturing cold anaesthetized adults at the ventral level of the pro-mesothorax articulation with a 29-gauge needle. Hemocytes were counted in a Bürker's chamber (Carlo Erba, Milano, Italy) without dilution and observed via light microscopy (LM) at 400 $\times$  magnification (Zeiss Primo Star). THCs were expressed as the number of cells (mean  $\pm$  SE (standard error)) per mL of hemolymphs.

#### 2.3.2. Enzymatic Assays

A pool of 10  $\mu\text{L}$  of hemolymphs was collected from three specimens, immediately transferred into 90  $\mu\text{L}$  ice-cold sterile phosphate-buffered saline (PBS, 10 mM; Sigma-Aldrich, Milano, Italy), and

centrifuged at 1700 g for 5 min at 4 °C. The cell-free hemolymphs obtained as supernatant was collected and stored at −20 °C for a maximum of 7 days prior to measure phenoloxidase and lysozyme-like enzyme activities.

Plasmatic phenoloxidase (PO) enzyme activity was measured in both the control (organic field:  $n_{\text{basal PO}} = 15$ ,  $n_{\text{total PO}} = 14$ ; conventional field:  $n_{\text{total and basal PO}} = 25$ ) and treated animals exposed to herbicide (Experiment 1: basal PO  $n_{2d} = 13$ ,  $n_{7d \text{ and } 21d} = 11$ ; total PO  $n_{2d} = 11$ ,  $n_{7d} = 12$ ,  $n_{21d} = 10$ ; Experiment 2: basal and total PO  $n_{2d \text{ and } 21d} = 11$ ,  $n_{7d} = 14$ ), as described above. PO activity was assayed spectrophotometrically as the formation of dopachrome from 3,4-dihydroxy-L-phenylalanine (L-DOPA, Sigma-Aldrich). To determine the basal PO, 10  $\mu\text{L}$  of hemolymph-buffer solution were mixed with 90  $\mu\text{L}$  of L-DOPA solution (3 mg/mL in PBS) in a microtiter plate. To measure the total PO enzyme activity, 10  $\mu\text{L}$  of the hemolymph–PBS mixture were added to 5  $\mu\text{L}$  of bovine pancreas alpha-chymotrypsin (5 mg/mL PBS, Sigma) and incubated for 5 min at room temperature to activate PO from its inactive zymogen. Subsequently, 85  $\mu\text{L}$  of L-DOPA were added to the solution. The change in absorbance was recorded at 492 nm and 25 °C for 30 min in 1 min intervals by using a plate reader (Sirio S, SEAC, Firenze, Italy). All samples were assayed in duplicate. The enzyme activity was measured as the slope (absorbance vs time) of the reaction curve during the linear phase of the reaction ( $V_{\text{max}}$  value; between 0 and 30 min after the reaction began). The slope of the reaction curve at  $V_{\text{max}}$  was plotted as absorbance per  $\mu\text{L}$  of hemolymph per min for specimens from each group.

Lysozyme-like activity was turbidometrically assayed in the hemocyte-free hemolymph. The decrease in absorbance over time indicated that lysozyme degraded the cell wall in the lysozyme-sensitive Gram-positive bacterium *Micrococcus lysodeikticus*. Ten  $\mu\text{L}$  of the hemolymph–PBS mixture (collection described above) from the untreated and treated insects were loaded into the well of a 96-well microplate and mixed with 190  $\mu\text{L}$  of a *M. lysodeikticus* (strain ATCC 7468, DSMZ) cell wall suspension (approximately  $1.6 \times 10^8$  cell/mL of cold PBS). The turbidity reduction in the wells was read on a plate reader (Sirio S, SEAC, Firenze, Italy) at 450 nm and 25 °C for 45 min in 5 min intervals. All samples were assayed in duplicate (conventional field  $n = 18$ , organic field  $n = 12$ . Experiment 1  $n_{2d \text{ and } 7d} = 13$ ,  $n_{21d} = 11$ ; Experiment 2:  $n_{2d} = 13$ ,  $n_{7d} = 11$ ,  $n_{21d} = 7$ ). The enzyme activity was reported as the change in absorbance (absorbance vs. time) of the reaction curve during the linear phase of the reaction ( $V_{\text{max}}$  value; between 5 and 15 min after the reaction began). The slope of the reaction curve at  $V_{\text{max}}$  was plotted as absorbance per  $\mu\text{L}$  of hemolymph per min for both the treated and control adults. Standards of enzyme activity were made by using the lysozyme from chicken egg whites (Sigma) and a suspension of *M. lysodeikticus* as substrate. Standards were incubated and simultaneously recorded with hemolymph samples to confirm that the assay had progressed as expected (i.e., absorbance values were decreasing).

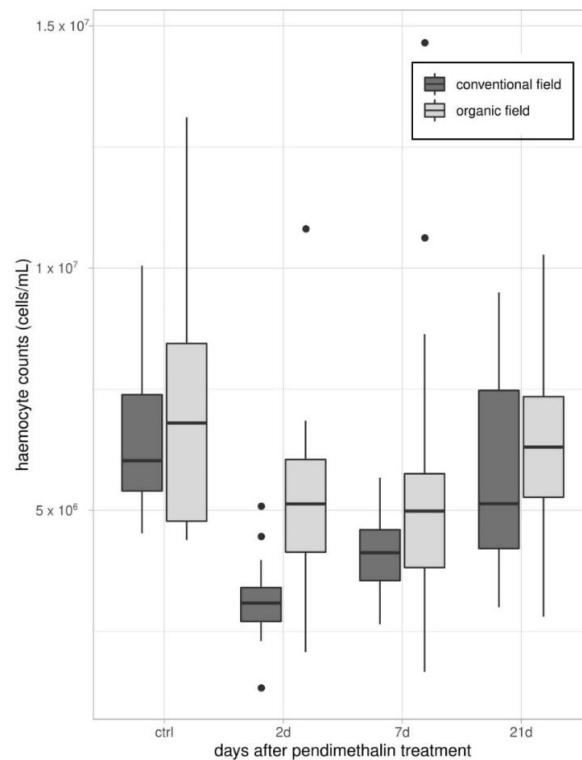
### 2.3.3. Statistical Analyses

Statistical analyses were performed with R version 3.0.1 software [62]. Data were checked for normality and homoscedasticity prior to analysis. As data did not show normal distribution and homogeneity of variance, a Kruskal–Wallis test followed by pairwise comparisons using the Wilcoxon rank sum test and the Bonferroni correction were made to compare differences in baseline THC and enzyme activities of the beetles from the organic and treated fields in both experiments at different times of exposure. The differences of the THC and the enzymatic activities between the beetles from the organic and treated fields at different times were compared via the Wilcoxon rank sum test. Results are indicated as mean  $\pm$  SE (standard error).

### 3. Results

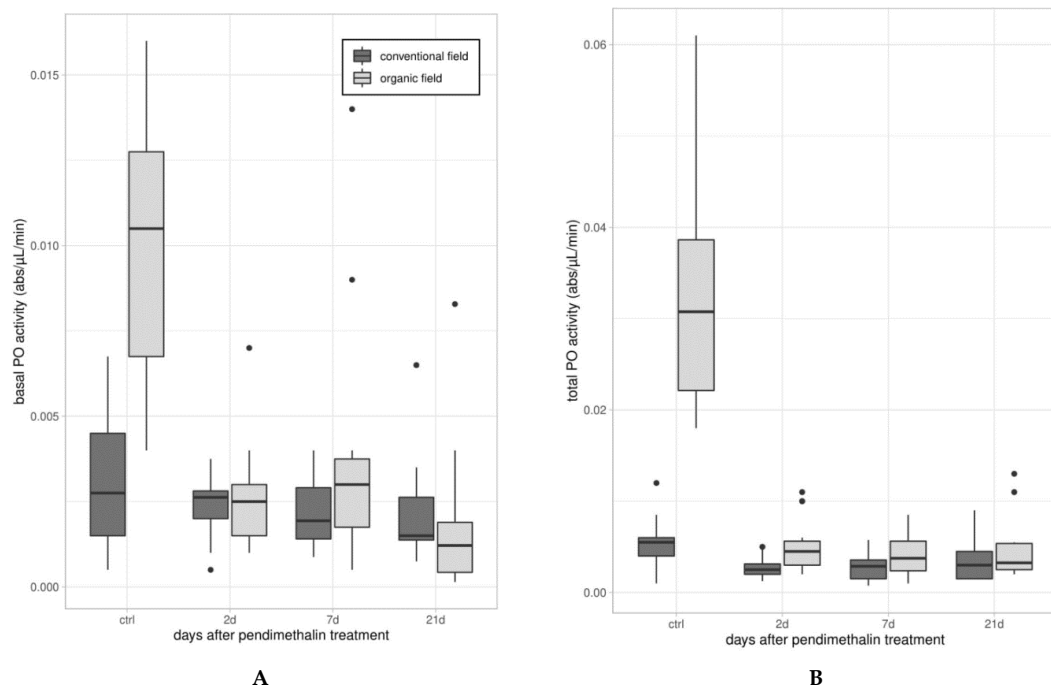
#### 3.1. Immune Parameters in Beetles from Organic and Conventional Fields

The total hemocyte counts (THCs) performed on the beetles from the fields before the laboratory treatments (ctrl) showed that there were no significant differences between beetles from the conventional and the organic field (Figure 2;  $p = 0.7551$ ).

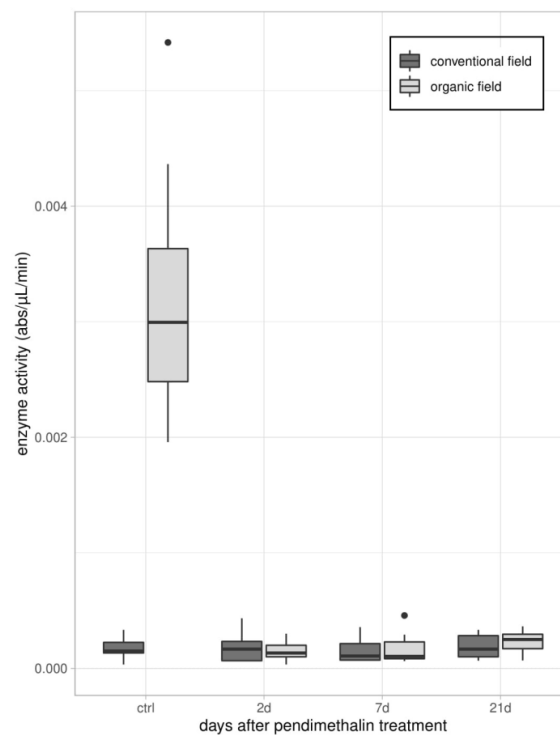


**Figure 2.** Total hemocytes counts (THCs) in *H. rufipes* adults. In both experimental groups, THCs were measured in the beetles (ctrl) from the organic and conventional fields before the laboratory treatment, as well as in animals exposed to the PDM-based commercial formulation at different time points (2, 7, and 21 d). THCs are expressed as the number of cells per mL of hemolymph.

The plasmatic basal and total PO activities (Figure 3A,B) measured in the beetles from the organic field were significantly higher than the field-exposed beetles from the conventional site (basal PO  $p = 1.87 \times 10^{-6}$ ; total PO  $p = 3.12 \times 10^{-7}$ ). Pesticide field-exposure significantly affected the lytic activity of the hemolymph (Figure 4). Thus, lysozyme-like enzyme activity recorded in the beetles from the conventional field was significantly lower than in the specimens from the organic field ( $p = 3.84 \times 10^{-7}$ ).



**Figure 3.** Total (A) and the basal (B) phenoloxidase activities (PO) assessed in cell-free hemolymph of adults. Plasmatic PO was measured in the beetles (ctrl) from the organic and conventional fields before the laboratory treatment, as well as in animals exposed to PDM-based commercial formulation at different time points (2, 7, and 21 d). The PO activity was recorded as the absorbance units per  $\mu\text{L}$  of hemolymph per min.



**Figure 4.** The lysozyme-like enzyme activity measured in cell-free hemolymph. Lytic activity was measured in the beetles (ctrl) from the organic and conventional fields that were untreated in the laboratory, as well as in animals exposed to the PDM-based commercial formulation at different time points (2, 7, and 21 d). This activity was recorded as absorbance units per  $\mu\text{L}$  of hemolymph per min.



### 3.2. Immune Parameters Following the Laboratory Exposure to Sub-Lethal Dose of PDN

#### 3.2.1. Experiment 1—Treated Beetles from Organic Untreated Field

In the beetles from the organic field, exposure to PDN resulted in a decrease of the total number of circulating hemocytes at 2 and 7 d with a successive gradual return to the levels of the beginning of the treatment at 21 d. However, these values of THCs were not significantly different than the control group (Figure 2;  $p \geq 0.05$ ).

Exposure to PDN had a significant impact on the plasmatic PO and lysozyme-like enzyme activities at all the time points. Basal (Figure 3A) and total (Figure 3B) PO activities significantly decreased at 2 d (basal PO  $p = 1 \times 10^{-4}$ ; total PO  $p = 4.3 \times 10^{-5}$ ), 7 d (basal PO  $p = 4.8 \times 10^{-3}$ ; total PO  $p = 1 \times 10^{-4}$ ), and 21 d (basal PO  $p = 1 \times 10^{-4}$ ; total PO  $p = 2.8 \times 10^{-4}$ ) compared to the control group.

After the initial exposure to PDN, significantly lower values of lytic activity were recorded at 2 d ( $p = 1.5 \times 10^{-4}$ ), 7 d ( $p = 1.5 \times 10^{-4}$ ) and 21 d ( $p = 3.3 \times 10^{-4}$ ) compared to the control group (Figure 4).

#### 3.2.2. Experiment 2—Treated Beetles from Conventional Field

In the beetles from the conventional field, significantly lower THCs were recorded at 2 d ( $p = 2.5 \times 10^{-5}$ ) and 7 d ( $p = 1.6 \times 10^{-4}$ , Figure 1). At 21 d after the initial exposure, THCs went back to the levels recorded in the control group with a significant increase at 7 d ( $p = 4.7 \times 10^{-2}$ ) and at 21 d ( $p = 2.5 \times 10^{-3}$ ) compared to the value recorded 2 d after the initial exposure. Moreover, THCs were significantly lower in the beetles from the conventional field 2 d after the initial exposure than the beetles from the organic field at the same time point ( $p = 7.4 \times 10^{-4}$ ).

Statistical analyses showed that exposure to PDM had no effect on the basal PO activity (Figure 3A) at any tested time point compared to the control. A significantly lower plasmatic total PO activity (Figure 3B) was recorded in the beetles at 2 d ( $p = 0.018$ ) and 7 d ( $p = 0.007$ ) compared to the control group. After 21 d, the plasmatic total PO ( $p = 0.318$ ) tended to go back to the level of the enzymatic activity recorded in the control group.

No significant differences were recorded in the baseline lytic activity of the hemolymph (Figure 3) at the different time points, as compared to the control.

## 4. Discussion

Herbicides such as atrazine, simazine, paraquat and glyphosate are well known to have effects on carabid population dynamics [63–68] or to cause mortality [8,69,70]. However, the studies on these subjects have mainly focused on indirect effects from the organism to community level related to food depletion or habitat modification [1]. In contrast, the direct sublethal effects of herbicides are very rarely reported in the literature and only concern effects on locomotion and mating behavior [6,71]. The results of our study provide the first evidence of the direct effects on the immunity of *H. rufipes* exposed to a sublethal dose of PDM.

Thus far, there have been few data on the toxicity of dinitroaniline and, especially, pendimethalin. Earlier studies have reported on the latter's genotoxicity and cytotoxicity effects, as related to its capability to bind DNA [72], in the non-target freshwater fish *Channa punctatus* [14,15]. Moreover, an increase of the incidence in chromosomal aberration and micronuclei has been recorded at the cellular level in Chinese hamster ovaries [73], lung fibroblast cells [74], and mouse bone marrow [75]. Its clastogenic potential has also been tested on human lymphocytes and rat bone-marrow cells [76]. In invertebrates, laboratory tests have shown that exposure to pendimethalin causes mortality in male wasps [17], disrupts reproducing capability in springtails, and reduces growth in earthworms [16]. In our study, we observed a depletion of circulating hemocytes at two days after initial exposure. This effect was more evident in the beetles from the conventional field (Experiment 2) than the group (organic field) that had never been exposed (Experiment 1), even though 21 days after the initial exposure, the number of circulating hemocytes went back to the initial level in both experiments. We assess that the temporal trend observed in the THCs of *H. rufipes* followed the dissipation time of the



active ingredient in the acid sandy soil. This observation is in accordance with previous studies on the lymphocytes of rainbow trout [77] and the hemocytes of blue dasher dragonfly [43], thus indicating that the effect of herbicides on circulating cells is dose-dependent.

PDM was found to affect the level of plasmatic PO and lysozyme-like enzyme activities, but significant differences were recorded between the experimental groups. Indeed, a more massive reduction of PO and lytic baseline enzymatic activities mainly occurred in adults of *H. rufipes* from the organic field. The laboratory exposure to a sublethal dose of PDM had no effects on enzymatic levels in the beetles that inhabited the conventional field—except for a significant reduction of plasmatic total PO two days after the first laboratory exposure, which went back to the control level at 21 days. This finding is in accordance with a recent study that showed a variation of tolerance to herbicide exposure in Colorado potato beetles from a population field-exposed to pesticide [78,79]. In *H. rufipes*, the significantly lower enzyme activities recorded in the control group from the conventionally-treated field suggest that field exposure to a combination of herbicide, insecticide and fungicide made the adults less responsive to the PDM laboratory treatment. Indeed, the levels of enzymatic activities, measured before the laboratory trial in the beetles exposed to field treatments, were comparable to those recorded in specimens from the organic untreated field two days after the first laboratory exposure. The reduction of constitutive PO and lytic activities in the exposed individuals may have limited their capability to mount a rapid nonspecific response, which is a key component of the insect immune response. Thus, exposure to sublethal dose of PDM may produce a delayed toxic effect over time that involves other immune functions. Further long-term studies are needed to clarify the effects of exposure on pathogen resistance and fitness. It is very likely that the lower levels of PO and lysozyme like-enzyme activities, involved in pathogen clearance in the hemolymph, may have an effect on related immune traits such as phagocytosis, nodulation, encapsulation, melanization, and clotting, thus causing an increase in organism pathogen susceptibility with potential consequences at the population level.

Ecological immunology studies have provided evidence that the constitutive immune response is closely tied to different life history traits in insects and plays a crucial role in the maintenance of homeostasis and the prevention of diseases and infections [80]. On the other hand, maintaining and using an immune system has a physiological cost [81] that results from a resource-based trade-off between the immune defense and other life traits including mating, molting, and cuticle sclerotization, all of which are heavily dependent on endocrine regulation [82–85]. In particular, the costs for the activation of the PO cascade require protein investment and are dietary-dependent [52]. To recover the physiological levels of plasmatic enzymes that are lost after treatments with herbicide, a high amount of resources is needed—an larger amount than that spent for maintenance. That leads to a long time to restore the initial levels of PO and lytic activities, resulting in a chronic malfunctioning of the humoral response. In summation, our findings indicate that continuous contact with residual amounts of PDM in soil might be harmful for insects such as *H. rufipes* that perform metamorphosis, foraging activities, reproduction and breeding on treated soils. Further studies must clarify how pendimethalin affects hormone regulation and resource allocation from immunity to other functions.

## 5. Conclusions

Exposure to PDM can have sublethal effects on non-target soil organisms such as *H. rufipes*. Our preliminary study demonstrates that PDM is able to interfere with some key components of the immune response in insects. Though the specific binding site and the biochemical involvement in the physiological system on insects are unknown, we assume that this highly lipophilic herbicide, which is applied in early spring when adults start to active forage in the field, may be quickly absorbed through the cuticle or through the direct consumption of contaminated food. Additional studies will clarify its accumulation in the hemolymph, the mechanism of interference with cellular and humoral effectors of the immune system, and the persistence of the residual activity in soil as critical variables to evaluate the risk for biodiversity conservation in agroecosystems. The increased tolerance to PDN

exposure under laboratory conditions in the beetles from a conventional field highlights the importance of considering the background of environmental conditions in field studies of risk assessment in environmental management.

**Author Contributions:** Conceptualization, A.G.; methodology, A.G., F.C. and M.L.V.; data analysis, P.G.G. and F.C.; writing—original draft preparation, A.G.; writing—review and editing, A.G., P.G.G., M.L.V.; J.K.; project administration and funding acquisition, P.B.

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