toxics

MDPI

Article

Evaluation of the Health Status of *Apis mellifera* in Relation to the Use of Plant Protection Products in Viticulture Through a Multi-Biomarker Approach

Tommaso Campani, Agata Di Noi *, Ginevra Manieri, Ilaria Caliani and Silvia Casini

Department of Physical, Earth and Environmental Sciences, University of Siena, Via Mattioli 4, 53100 Siena, Italy; campani@unisi.it (T.C.); ginevra.manieri@student.unisi.it (G.M.); caliani4@unisi.it (I.C.); silvia.casini@unisi.it (S.C.)

* Correspondence: agata.dinoi2@unisi.it

Abstract

The global pollinator decline is linked to intensive farming and the high use of plant protection products (PPPs), necessitating risk assessment and mitigation. This study investigates the potential negative impacts of agricultural practices on pollinator health, specifically focusing on the effects of PPPs used in viticulture on the honey bee, *Apis mellifera*, despite grapevines' lack of reliance on bee pollination. The beehives sampled were from two farms with vineyards under different management regimes: one transitioning from conventional to organic practices and an organic–biodynamic site with pollinator mitigation measures. Sampling was conducted during three phases, pre-, during, and post-PPP application, to evaluate biomarkers of neurotoxicity (AChE), detoxification enzymes (CaE, GST), metabolic stress (ALP), and immune markers (Lys, PO, proPO). Comparison between the organic–biodynamic farm and the transitioning one revealed a pattern suggesting significant neurotoxic effects in the transitioning farm characterised by a trend of decreased AChE activity during treatments and the subsequent induction of GST post exposure. Crucially, both PO and proPO were induced post treatment, but with a lower PO/proPO ratio compared to previous seasons, suggesting inefficient proPO activation and potentially weakened immune competence that could favour pathogen proliferation. Bee health appeared to deteriorate most at the transitioning farm post treatment, while the biodynamic site remained relatively stable; these differences are likely associated with legacy residues and drift, exacerbated by overwintering stress and summer heat. Given the specific environmental and management characteristics of these two farms, the results provide an indicative comparison of how different agronomic approaches may influence bee health. Moreover, these results support the multi-biomarker approach for detecting potential PPP impacts, suggesting that organic transitions and mitigation strategies could play a role in pollinator conservation.

Keywords: pollinators; agroecology; pesticide; biomarkers; toxicological effects

Academic Editors: Fuliu Xu and Li Mu

Received: 19 December 2025
Revised: 10 February 2026
Accepted: 13 February 2026
Published: 17 February 2026

1. Introduction

Ecosystem services are formally defined as any ecological process that provides benefits to humans [1]. Insect pollination is one of these vital services. Regrettably, recent years have seen a marked decline in both the abundance and diversity of insect pollinators, thereby threatening their essential ecological roles. Among the most vital pollinating agents are wild insects, including bees, wasps, certain hoverfly species, butterflies, moths, and

beetles. Owing to their dependence on nectar and pollen, bees—comprising approximately 20,000 described species—are widely recognised as the most significant of these pollinators, contributing to the production of nearly one-third of global food crops [2]. Managed bees, maintained by beekeepers, are no exception, as they likewise play a crucial role in pollinating a wide range of both wild and cultivated plant species [3]. The decline in pollinators has become a subject of significant scientific and societal concern due to the critical ecological, social, and economic importance of pollination. An estimated 84% of European crops depend on pollinator activity [4], while, on a global scale, approximately 70% of crops intended for human consumption rely on insect pollinators. The global economic value of pollination services has been estimated at around €153 billion [5]. The current condition of pollinator decline is attributed to several factors: climate change, intensive agriculture, excessive pesticide use, habitat loss, parasites, and the introduction of alien species [6]. Climate change introduces various issues, such as the arrival of non-native exotic species that compete with indigenous ones. These non-native species often prevail as native species are already stressed by rising temperatures and imbalances within the terrestrial ecosystem. Habitat loss is directly linked to human activity, both directly (the conversion of natural environments into substrates for agriculture or other anthropogenic uses) and indirectly (pollution, the alteration of environmental conditions that constitute and characterise the habitat). The overuse of pesticides (insecticides, herbicides, fungicides) in agriculture, in particular through intensive farming, stands out as having the greatest impact on pollinators [7–9]. These chemicals can produce detrimental health effects on both target and non-target organisms [10]. To halt the decline in pollinators, the European Union (EU) has developed and issued directives, the most notable being the EU Pollinators Initiative, which is part of the EU Biodiversity Strategy. This strategy, enacted in 2018 and reviewed multiple times, aims by 2030 to achieve a more comprehensive global understanding of the causes and consequences of the numerical decline in pollinators [11].

From an agricultural standpoint, the protection of pollinators has been addressed through specific measures within the framework of the Common Agricultural Policy (CAP). In accordance with European directives, Italy has implemented pollinator conservation strategies under Eco-scheme 5, which promotes the adoption of mitigation practices within agroecosystems, including the maintenance of plants beneficial to apiculture, the preservation of uncultivated meadows, and the cultivation of inter-row crops. Farmers who adopt these measures receive financial incentives to support their participation [12].

Although viticulture often shows a greater propensity for sustainable practices compared to other agricultural sectors, conventional viticulture relies on materials and machinery that significantly impact the environment. The primary sources of contamination include the use of pesticides, the application of copper (Cu) and copper-based compounds, and the use of fossil fuels [13–15].

While pesticide use varies between conventional and organic farms, their dispersal causes considerable environmental effects. Copper is also a major contaminant, and its toxic effects are less widely known, yet it is often applied in larger quantities [16]. Furthermore, fuel combustion is an unavoidable factor, as traditional and intensive viticulture necessitate high volumes of diesel fuel to power agricultural machinery within a vineyard [17]. It is crucial to note that viticulture, as conventionally practised, represents an intensive monoculture. This involves exploiting agricultural land for the cultivation of a single plant species or variety for multiple years without crop rotation [18]. This practice introduces additional risks such as the weakening of the agroecosystem and reduced soil fertility, the inhibition of wild plant flowering, the loss of pollinators due to the severe decrease in adequate foraging sources and the reliance on pesticides [19]. Monocultures are characteristic of intensive agriculture, with their sole purpose being the satisfaction of human needs.

In contrast to conventional methods, certain agricultural and viticultural approaches prioritise environmental sustainability. These include organic and biodynamic farming. Organic agriculture is regulated at the European level [20]. This practice focuses on minimising environmental impact, emphasising product quality and authenticity for both producers and consumers. The key tenets of the regulation are protecting the soil ecosystem and maintaining its natural fertility (via crop rotation, cover crops, and nitrogen-fixing plants), minimising the use of non-renewable resources, limiting the use of synthetic fertilisers and plant protection products (PPPs), recycling waste and plant by-products, and safeguarding plant health through preventive techniques (e.g., selecting suitable species, maintaining resistant varieties, and fostering natural enemies of pests). Organic viticulture adheres to the same regulation, with specific rules for wine production: organic wine must be made exclusively from organically grown grapes, and certain practices common in conventional production, such as cooling condensation or the physical removal of sulphur dioxide, are forbidden [20]. Biodynamic viticulture is an advanced form of organic farming, first conceptualised by Steiner in the 1920s [21]. Its ultimate goal transcends quality production, aiming for the maintenance and restoration of the involved ecosystems, explicitly focusing on increasing biodiversity, evaluating the sustainability of every input, and minimising the use of xenobiotics, often substituting them with products of exclusively vegetal origin. The overarching objective is to maintain, implement, or reconstruct ecosystemic harmony [22].

Among pollinators, the honey bee (*Apis mellifera* Linnaeus, 1758) stands as the most studied species and a model organism for evaluating pollinator health and decline. Owing to its well-documented ecological and biological characteristics, *A. mellifera* has become a model species for studies on pollinator health and population decline [23–25]. Particularly noteworthy are studies assessing the impacts of pesticide exposure on *A. mellifera* health using biomarkers [26–28].

This work aims to evaluate the health status of *A. mellifera* in relation to the use of PPPs in viticulture. These practices conducted in vineyards could generate a substantial environmental impact capable of affecting the health of pollinating insects, including *A. mellifera*, even though the grape crop is not directly dependent on honey bee pollination. An in situ monitoring study was conducted, using beehives strategically placed within two viticultural farms with different management approaches. One farm was in conversion from conventional to organic farming, while the second was an organic–biodynamic farm that applied specific mitigation measures for pollinators. Sample collections were structured across three distinct temporal phases: before the application of PPPs to the vines, during the treatments, and after the treatments. Honey bee health status was assessed using a comprehensive multi-biomarker approach, including the analysis of acetylcholinesterase (AChE), carboxylesterase (CaE), glutathione S-transferase (GST), and alkaline phosphatase (ALP), alongside the immune-related indicators lysozyme (Lys), phenoloxidase (PO), and prophenoloxidase (proPO). The ultimate objective was to analyse and compare the health of the sampled bees based on the different sampling locations and periods. This comparison will demonstrate how varying viticultural practices and the associated use of PPPs result in differential impacts on *A. mellifera* health, specifically by contrasting the outcomes between the two farms and across the three time points.

2. Materials and Methods

2.1. Apiary Sites, Management Practices, and Sampling Protocol

The sampled apiaries are situated near the vineyards that employ distinct agricultural management practices and are characterised by different pressures in the surrounding environment.

The selected farms include one operating under biodynamic organic management and one under conventional management that is currently transitioning to organic certification.

One farm has been using a biodynamic approach for 25 years. Given the high priority placed on minimising the environmental impact inherent to the biodynamic method, this farm does not use metal-based or synthetic PPPs, and applies only plant-based products to defend the vineyard and the surrounding environment is a low-pesticide impact area.

Conversely, the other farm, while currently undergoing conversion to organic practices, has historically employed conventional vineyard management. In the monitored year, the conventional farm exclusively applied copper-based treatments (specifically, solutions of copper oxychloride and copper hydroxide) during the growing season, typically every two weeks between June and July. Furthermore, the area surrounding the conventional farm contains numerous other agricultural properties that continue to utilise conventional practices, raising the potential for contamination drift.

2.2. Sampling Design and Schedule

Sampling was conducted between April and July 2023 during the regional viticultural treatment schedule as reported in Table 1.

Table 1. Sampling schedule across seasons in the conventional vineyard and the organic vineyard. The table indicates the season in which the bees were sampled (✓) or not (⊗) in each vineyard.

Vineyard		**Conventional**	**Organic**
Sampling season	Early spring	✓	⊗
	Spring	✓	✓
	Summer	✓	✓

In each sampling period conducted across different seasons, we sampled three hives per vineyard from the farm-owned apiaries at each site; apiaries were fixed and site-specific, not translocated between vineyards. From every hive, approximately 50 worker bees were randomly collected across the three main castes, namely builder bees, foragers, and nurse bees, to ensure a heterogeneous and representative sample of the colony. These castes were identified both visually and by sampling from different frames within each hive.

2.3. Sample Processing and Biochemical Analysis

2.3.1. Sample Preparation

All *A. mellifera* specimens, before dissection, were placed into sealed glass jars with perforated lids and anaesthetised on ice at 4 °C for approximately 15–30 min. The head and the body were separated and stored at −80 °C until biomarker analysis.

2.3.2. Sample Homogenization

The head, body, and thorax of the bees were homogenised for the biomarker analysis. Approximately 50 honey bees were analysed for each apiary. Heads and midguts were pooled using three samples for each pool, which was weighed using an analytical balance, and 40 mM phosphate buffer (pH = 7.4) (Merck KGaA, Darmstadt, Germany) was added at a 1:10 ratio (w/v). The pools were homogenised using a Tissue Lyser (Qiagen, Hilden, Germany) for three 30 s cycles at a frequency setting of 40 Hz, with 30 s rest intervals between cycles. Head homogenates were centrifuged at 13,000× g for 20 min at 4 °C, while intestines were centrifuged at 15,000× g for 20 min at 4 °C. The resulting supernatant was stored at 80 °C. Thoraxes were homogenised individually after the removal of wings and legs, and 400 µL of phosphate buffer saline (PBS-1X) was added to each thorax before being

homogenised in the Tissue Lyser as previously described. Samples were then centrifuged at 15,000 rpm for 15 min at 4 °C. Finally, the supernatant was collected and stored at −80 °C.

2.3.3. Enzymatic Biomarker Analysis

All the reagents were purchased from Merck KGaA (Darmstadt, Germany) and Biorad (Hercules, CA, USA). Acetylcholinesterase (AChE) activity was measured in brain homogenates using a modified Ellman method [29]. This spectrophotometric assay tracks the hydrolysis of acetylthiocholine at 410 nm after 5 min. Results are expressed as nmol/min/g of tissue. Carboxylesterase (CaE) activity was assessed in brain homogenates by measuring the hydrolysis rate of two different substrates, p-nitrophenyl acetate (pNPA) and p-nitrophenyl butyrate (pNPB), following the methods of Solé et al. [30] with some modifications. The formation of p-nitrophenol was quantified at 405 nm, with results expressed in nmol/min/mg tissue. Glutathione-S-transferase (GST) activity was assessed in intestine homogenates according to Caliani et al. [27], which measures the conjugation of GSH with 1-Chloro-2,4-dinitrobenzene (CDNB) at 340 nm. Results are reported as nmol of GS-DNB conjugates/min/mg protein. Alkaline phosphatase (ALP) activity was determined in the intestine homogenates, monitoring the hydrolysis of p-NPP para-nitrophenyl phenol, at 405 nm for 5 min [27]. Results are expressed in nmol/min/mg protein. Lysozyme (Lys) activity was measured in intestine homogenates via a standard turbidity assay [27]. This assay measures the decrease in turbidity of a *Micrococcus lysodeikticus* bacterial suspension at 450 nm and is quantified using an HEL standard curve µg/mL.

Prophenoloxidase (proPO) and phenoloxidase (PO) activities were measured in thoracic homogenates using a modified L-DOPA assay [31]. ProPO requires alpha chymotrypsin activation. Instead, the PO activity is measured directly. Both kinetics are recorded at 490 nm, and activity is expressed as V/max.

Total protein concentration was measured in the intestinal homogenates using the Bradford method [32] with the BioRad Protein Assay. This is critical for normalising GST and ALP enzyme activities. Measurements were taken at 595 nm and expressed in mg/mL relative to a bovine serum albumin (BSA) standard curve.

2.3.4. Statistical Analysis

Statistical analyses were performed using RStudio (version 2023.06.0) and R (version 4.2.2). Prior to group comparisons, data normality and homogeneity of variance were assessed using the Shapiro–Wilk test and Levene's test, respectively. Since the data did not meet the assumptions for parametric analysis (non-normal distribution and/or heteroscedasticity), the non-parametric Kruskal–Wallis (KW) test was applied to each biomarker with a significance level of $\alpha = 0.05$. The null hypothesis of equal distributions was rejected when $p < 0.05$. When the null hypothesis of the KW test was rejected, Dunn's test with a Benjamini–Hochberg's stepwise adjustment was applied for pairwise multiple comparisons. All analyses and graphical representations were implemented within the R environment.

3. Results and Discussion

The results of the described biomarkers are presented in figures, where the biodynamic farm is regarded as the control and represented in the graphs by the codes C_SPRING for the April 2023 sampling and C_SUM for the post-treatment sampling in July 2023. The conventional farm is indicated by the codes E_SPRING23 for the April 2023 treatment, SPRI_23 for the sampling during treatment of June 2023 and SUM_23 for the post-treatment period of July 2023.

3.1. Acetylcholinesterase (AChE)

Figure 1 summarises AChE activities, highlighting statistically significant differences observed between the spring control (C_SPRING) and the summer control (C_SUM), as well as between the summer control and the summer sampling (SUM_23). Furthermore, the activity recorded in SUM_23 statistically differs from all other sampling at the conventional farm.

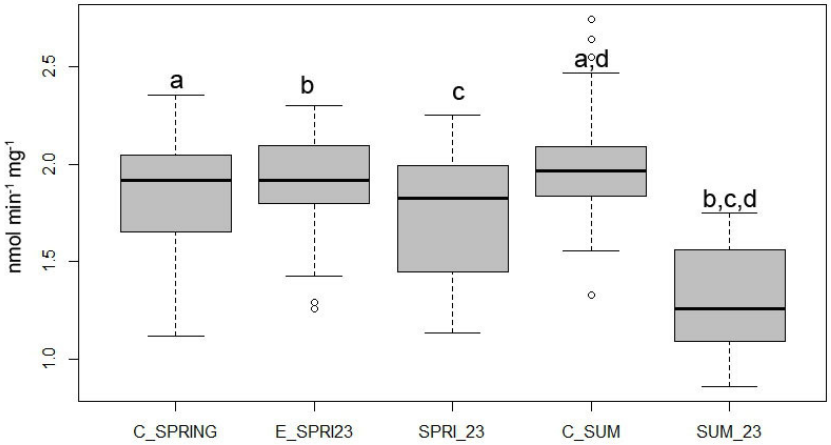

Figure 1. AChE activity expressed as nmol min^{-1} mg $tissue^{-1}$ was evaluated in brain tissue across the various sampling sites. Results are presented with boxplots and open circles represent outliers. Same letters show a statistically significant difference ($p < 0.05$).

AChE activity (K-W chi-squared = 122.3752, df = 4, p-value = 0) is inhibited if the organism comes into contact with substances that bind to the enzyme's active site, thereby preventing its lytic activity and causing neurotoxic damage [33,34]. The most substantial decrease in activity is observed in SUM_23, a period during which a greater impact on bee health was due to the treatments applied in that area between the second (SPRING_23) and third (SUM_23) sampling times. Locally applied copper-based PPPs are unlikely to directly inhibit AChE. This is confirmed by Nikolić et al. [35], who found no inhibition after exposing *A. mellifera* to various $CuCl_2$ concentrations, and by Campani et al. [26], who reported the same following exposure to a copper-based fungicide. The marked inhibition observed here therefore suggests contributions from pesticide drift of neurotoxic compounds (e.g., organophosphate or carbamate metabolites) from nearby conventional farms. Residue analysis (e.g., LC-MS for OP/carbamates vs. ICP-MS for Cu) would be essential to quantify these sources and disentangle local vs. off-site effects.

This hypothesis was confirmed considering the statistical difference found between SUM_23 and its corresponding control C_SUM, which is a farm that uses no chemical treatments and is considered reasonably isolated. Copper-specific studies indicate minimal direct neurotoxicity, supporting drift as the primary driver here, though cumulative exposure cannot be ruled out [26,35]. The results indicate a gradual decreasing trend in AChE activity in the specimens sampled at the conventional farm during the progression of treatment with PPPs in the vineyard.

It is also noted that the C_SPRING control exhibits lower activity compared to the C_SUM control. This is hypothesised to be a result of the bees sampled in spring having a generally weakened state of health due to surviving the cold winter months, which may manifest as reduced AChE enzyme activity [36,37].

The findings observed at the conventional farm diverge from those reported by Nikolić et al. [35], whose experimental exposures of *A. mellifera* to various concentrations of copper ($CuCl_2$) did not result in AChE inhibition. In contrast, their work demonstrated significant AChE inhibition following exposure to 0.1 mg/L $PbCl_2$ and 0.001–0.01 mg/L

$CdCl_2$, while treatment with 10 mg/L $PbCl_2$ led to the induction of AChE activity. Likewise, Caliani et al. [27] reported statistically significant inhibition of AChE in bees following treatments with Amistar® Xtra, cadmium, and ethyl methanesulfonate (EMS). Further supporting evidence has been provided by Benito-Murcia et al. [38] and Benchaâbane et al. [39], who both documented the enzymatic inhibition of AChE after exposure to coumaphos and tau-fluvalinate (acaricides) and thiamethoxam (insecticide), respectively.

The most vulnerable site and period appear to be the post-treatment phase at the conventional farm, which uses copper-based PPPs and is located near conventional farms that may contribute to contamination drift from other PPPs that can threaten the bees' nervous systems. Notably, neurotoxic risk is heightened by the metabolic bioactivation of certain pesticide classes present in these environments. For instance, Williamson et al. [33] demonstrated that honey bee exposure to the active metabolites of organophosphates (chlorpyrifos oxon and coumaphos oxon) and carbamates (aldicarb sulfoxide) resulted in pronounced AChE inhibition, underscoring the metabolic activation in vivo that enhances the potency of these compounds. The recent literature confirms that many classes of pesticides compromise neural function in honey bees by inactivating AChE and disrupting cholinergic signalling [36,40,41].

3.2. Carboxylesterase (CaE)

Higher CaE activity was recorded in bee specimens sampled from the conventional farm compared to the control group, as shown in Figure 2 (K-W chi-squared = 166.8248, df = 4, p-value = 0) and Figure 3 (K-W chi-squared = 160.0369, df = 4, p-value = 0). This outcome is probably linked to CaE's role in the phase I detoxification process; consequently, the contamination present in the studied environment may have triggered an induction of this enzyme, particularly evident in the SUM23 sampling, which occurred following the PPP treatment. This CaE induction aligns with copper-specific effects, as Campani et al. [26] observed increased CaE activity in *A. mellifera* following exposure to a copper-based fungicide (Ramedit®), usually used in vineyard contexts. It is important to note, however, that a statistically significant difference relative to the C_SPRING control was already observed in the initial sampling E_SPRING23 before any treatments, where CaE activity was notably higher. This is not entirely unexpected, given that the collection area transitioned to organic farming only one year prior and is situated near numerous conventional agricultural farms. Both factors suggest a higher environmental contamination load and a potential compromise in bee health, leading to the compensatory activation of CaE as a detoxification mechanism.

The observed increase in CaE activity is highly pertinent, particularly when considered alongside the corresponding reduction in AChE activity discussed previously. Unlike pure Cu effects, which consistently induce rather than inhibit CaE [26], the reciprocal low AChE and high CaE pattern in SUM_23 may implicate drifted neurotoxic compounds alongside local copper stress. The sampling location and period that showed the lowest AChE activity also registered the highest CaE activity. This reciprocal relationship lends support to the hypothesis of a substantial presence of treatment-related contaminants capable of causing both AChE inhibition and the induction of CaE's detoxifying function in *A. mellifera*.

Despite the general trend of induction, the data also displayed a statistically significant inhibition of CaE between E_SPRING23 and SPRING_23 with both substrates. As part of the B-esterase family, CaE is susceptible to inhibition by xenobiotics like organophosphate and carbamate insecticides [42]. Given its often-higher affinity for these compounds compared to AChE, CaE can serve a "scavenging" role, offering protection against AChE inhibition. The finding of reduced CaE activity in SPRING_23 is hypothesised to stem from the herbicide treatments (personal communication) applied during this time at adjacent

conventional farms. It is thus posited that contamination drift of these herbicides led to CaE inhibition in a majority of the SPRING_23 specimens.

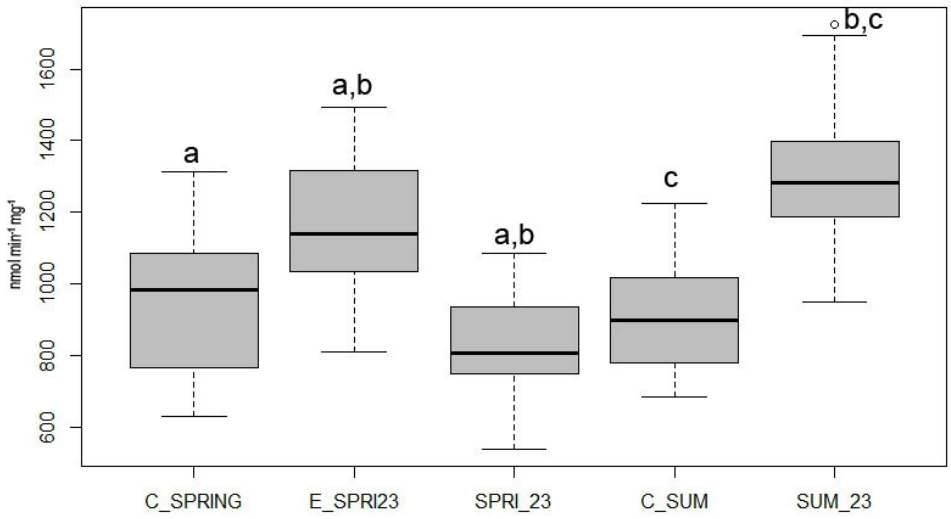

Figure 2. Carboxylesterase (CaE) activity was evaluated in brain tissue of *A. mellifera* specimens using the substrate (p-nitrophenyl acetate). The enzyme activity is expressed as nmol min^{-1} mg $tissue^{-1}$. Results are presented with boxplots and open circles represent outliers. Same letters indicate a statistically significant difference ($p < 0.05$).

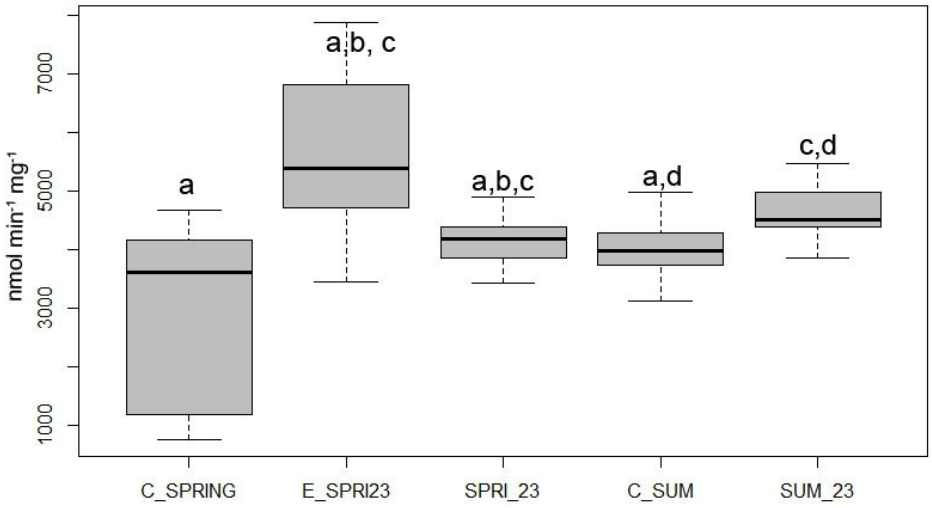

Figure 3. Carboxylesterase (CaE) activity was evaluated in brain tissue of *A. mellifera* specimens using the substrate p-NPB (p-nitrophenyl butyrate). The enzyme activity is reported as nmol min^{-1} mg $tissue^{-1}$. Results are presented with boxplots and open circles represent outliers. Same letters signify a statistically significant difference ($p < 0.05$).

The findings confirm the biphasic response modality of CaE observed in previous investigations. Benito-Murcia et al. [38] assessed CaE activity in *A. mellifera* following exposure to the acaricides coumaphos and tau-fluvalinate. Coumaphos exposure resulted in a dose-dependent inhibition of the enzyme in vivo and in vitro, whereas tau-fluvalinate exposure led to CaE induction in in vitro samples. The authors suggested that the induction data support a specific detoxification pathway for such compounds. Caliani et al. [27] observed CaE induction following treatment with EMS, cadmium (g/L), and AmistarXtra (100 and 200 g/L). Conversely, older studies, [42,43] documented a decrease in CaE activity in *A. mellifera* after exposure to thiamethoxam or fipronil.

3.3. Glutathione-S-Transferase (GST)

Glutathione-S-transferase (GST) is a crucial enzymatic component of the phase II detoxification pathway in many species and also in insects [44]. The induction of this enzyme serves as a biomarker of exposure to pesticides and other xenobiotics, reflecting the organism's activation of detoxification pathways required to manage these compounds [45,46].

The analyses conducted in this study (Figure 4) revealed that the highest GST values (K-W chi-squared = 143.3785, df = 4, *p*-value = 0) were found in SUM_23 with a statistically significant difference with respect to the C_SUM control. In addition, a statistically significant increase in GST activity was observed between the SPRING_23 and SUM_23 samplings. Furthermore, SUM_23 showed a statistically significant difference when compared to both E_SPRING23 and SPRING_23 (which exhibited similar values to each other).

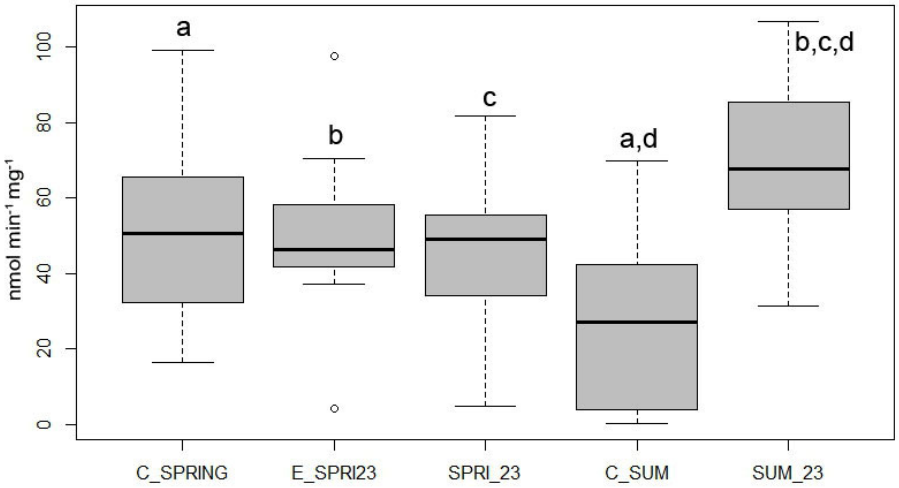

Figure 4. Glutathione S-transferase (GST) activity was evaluated in midgut of *A. mellifera* specimens. The enzyme activity is reported as nmol min^{-1} mg $protein^{-1}$. Results are presented with boxplots and open circles represent outliers. Same letters indicate a statistically significant difference ($p < 0.05$).

GST levels were significantly higher in SUM_23, providing additional evidence of impaired health status in the specimens sampled during this period. The statistically significant increase between SPRING_23 and SUM_23 supports the hypothesis that the cumulative treatments applied between these two periods resulted in a greater impact on bee health. The observed difference between C_SUM (control) and SUM_23 indicates that the type of agricultural management strongly influences bee health outcomes. Although both groups were sampled following the main treatment season, GST values were substantially lower in the control, underscoring a greater degree of environmental exposure in SUM_23. Given that the application of PPPs constituted the main documented change at the sampling site, the pronounced increase in GST activity observed in SUM_23 relative to E_SPRING23 and SPRING_23 is interpreted as strong evidence that the applied chemicals act as potent inducers of detoxification enzymes.

The GST induction observed aligns with findings from Caliani et al. [27], where GST induction was reported in *A. mellifera* exposed to AmistarXtra, cadmium, and EMS. Similarly, Benito-Murcia and collaborators [38] observed GST induction following exposure to tau-fluvalinate. However, the literature presents mixed findings: the same study by Benito-Murcia et al. [38] reported GST inhibition due to coumaphos, and Nikolic et al. [35] documented GST inhibition in *A. mellifera* following exposure to 1 g/L copper solution.

3.4. Alkaline Phosphatase (ALP)

Alkaline phosphatase (ALP) is an enzyme with a primary function of the hydrolysis of phosphate compounds in a basic pH environment [47]. In insects, including honey bees, ALP is widely used as a biomarker of metabolic alteration because of its involvement in key processes such as digestion and nutrient transport, cell maintenance and growth, immune function, and other essential physiological activities [26,46,48]. Consequently, the presence of contaminants can disrupt its activity.

Figure 5 shows the ALP activity (K-W chi-squared = 147.5838, df = 4, p-value = 0) evaluated in the intestinal tissues of honey bees sampled at the two farms at different times. A statistically significant decrease was observed between the C_SPRING control and all other sampling groups. The differences observed between the C_SPRING group with respect to the other sampling periods could be attributable to the action of stressors such as suboptimal temperatures [49], nutritional restriction [50,51], or PPP exposure that reduced metabolic activity in the bees sampled. These reductions are commonly associated with physiological stress and impaired condition, and are therefore interpreted as indicative of a suboptimal health status [46,52]. Our findings partially align with Caliani and collaborators [27], where an inhibition was observed following treatments with AmistarXtra, cadmium, and EMS. Conversely, research by Migdal et al. [53], involving *A. mellifera* exposure to single solutions and mixtures of acetamiprid, glyphosate, and tebuconazole, generally showed an induction of ALP activity, likely reflecting its detoxification role.

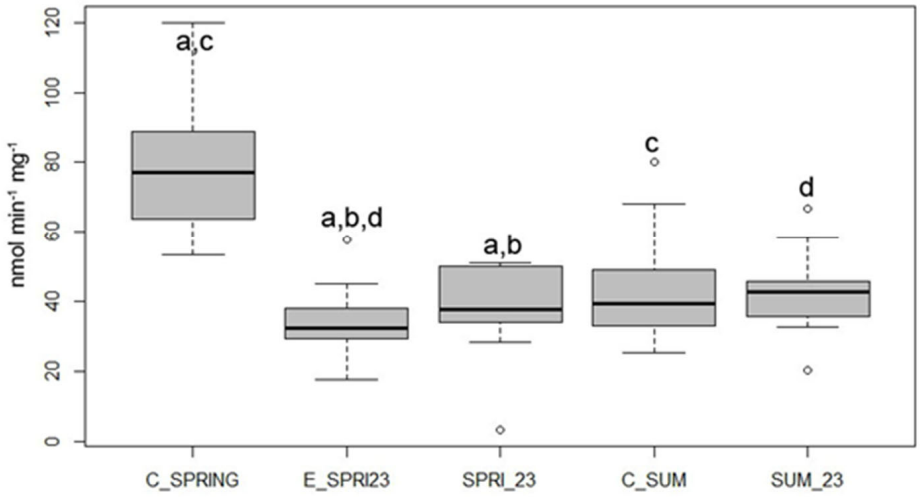

Figure 5. Alkaline phosphatase (ALP) activity was evaluated in midgut of *A. mellifera* specimens. The enzyme activity is reported as nmol min^{-1} mg protein^{-1}. Results are presented with boxplots and open circles represent outliers. Same letters indicate a statistically significant difference ($p < 0.05$).

3.5. Lysozyme (Lys)

The results obtained for lysozyme activity (Figure 6) showed a generally low activity (K-W chi-squared = 14.1714, df = 4, p-value = 0.01), but the values are consistent with those published in the limited existing literature which analyses this enzyme's activity [54]. The activity of Lys was investigated as a biomarker of immune defence in honey bees. Its specific function is the lytic activity directed against the cell wall of Gram-positive bacteria, achieved by cleaving peptidoglycan components. Its action is therefore specialised against bacterial infections [55].

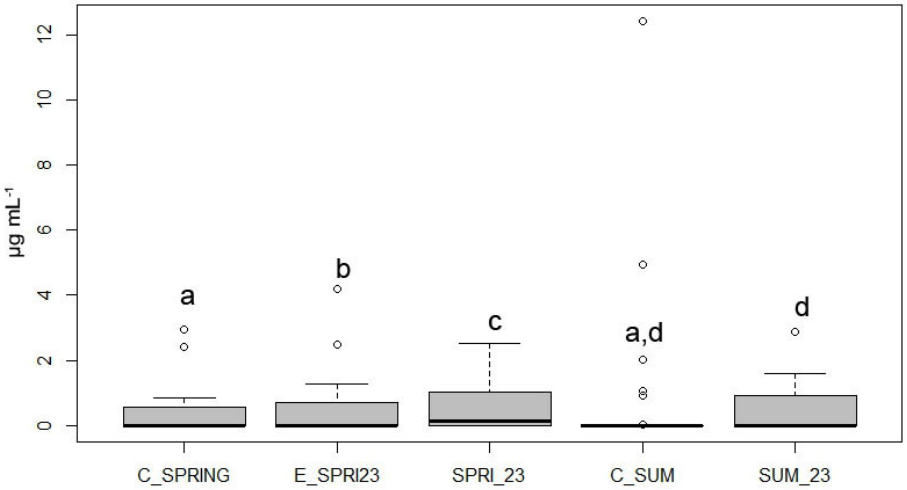

Figure 6. Lysozyme (Lys) activity, measured in µg mL^{-1}, was evaluated in midgut of *A. mellifera*. Results are presented with boxplots and open circles represent outliers. Same letters indicate a statistically significant difference ($p < 0.05$).

Lytic activity decreased from the C_SPRING control to the C_SUM control. This change likely reflects the resolution of a bacterial infection over that period, restoring Lys activity to a lower baseline. The study by Caliani et al. [27] reported a decrease in lysozyme activity following the exposure of *A. mellifera* to AmistarXtra, cadmium, and EMS. This suggests a possible direct correlation between decreases in lysozyme activity and exposure to PPP treatments. While this specific hypothesis is not statistically confirmed in the present thesis, a slight decrease in enzyme activity is observable between SPRING_23 and SUM_23, following the period of treatments.

3.6. Prophenoloxidase (proPO) and Phenoloxidase (PO)

Figure 7 shows the proPO activity measured in VMAX (K-W chi-squared = 67.2908, df = 4, *p*-value = 0), representing the amount of proPO potentially activatable by naturally present pathogens, with SUM_23 recording the highest activity values with statistically significant differences with respect to the control C_SUM. The activities of PO (K-W chi-squared = 76.9786, df = 4, *p*-value = 0) (Figure 8) were lower than proPO, indicating only the partial expression of the defence potential. Similar to proPO, SUM_23 exhibited statistically significant differences with respect to the control C_SUM, registering the highest activity values of PO.

The activities of phenoloxidase and prophenoloxidase were investigated as biomarkers of the immune system in honey bees. As integral parts of the cellular innate immune system, they are specifically involved in pathogen nodulation and encapsulation [56]. Their function is to catalyse melanin synthesis, which forms the protective envelope surrounding foreign materials. ProPO is the inactive form of PO, often referred to as a zymogen [57]. Its activation is triggered by contact with substances like LPS, peptidoglycan, or 1,3-glucan, initiating a cascade that includes serine-proteinase to catalyse the transformation of proPO into its active form, PO [31,58–60].

The PO/proPO ratio (Figure 9), indicating transformation efficiency, reaches its maximum as it approaches unity. Although values generally trended toward unity, most did not attain it. The SUM_23 sampling notably exhibited a lower median and deviated further from unity than other samplings.

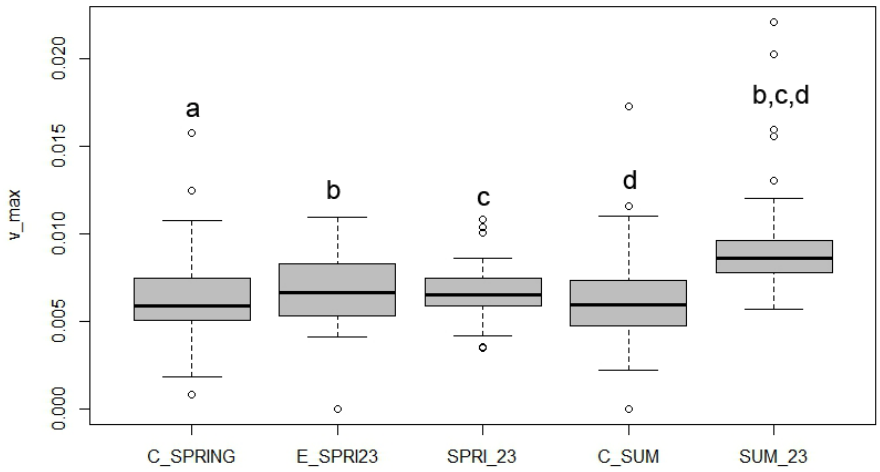

Figure 7. Prophenoloxidase (proPO) activity evaluated in thorax of *A. mellifera* samples, measured as the maximal reaction velocity (V_MAX). Results are presented with boxplots and open circles represent outliers. Same letters indicate a statistically significant difference ($p < 0.05$).

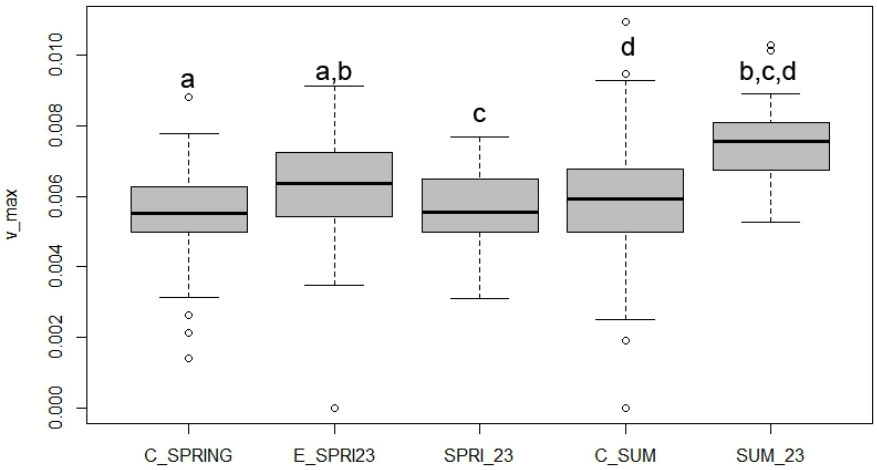

Figure 8. Phenoloxidase (PO) activity evaluated in thorax of *A. mellifera* samples, measured as the maximal reaction velocity (V_MAX). Results are presented with boxplots and open circles represent outliers. Same letters indicate a statistically significant difference ($p < 0.05$).

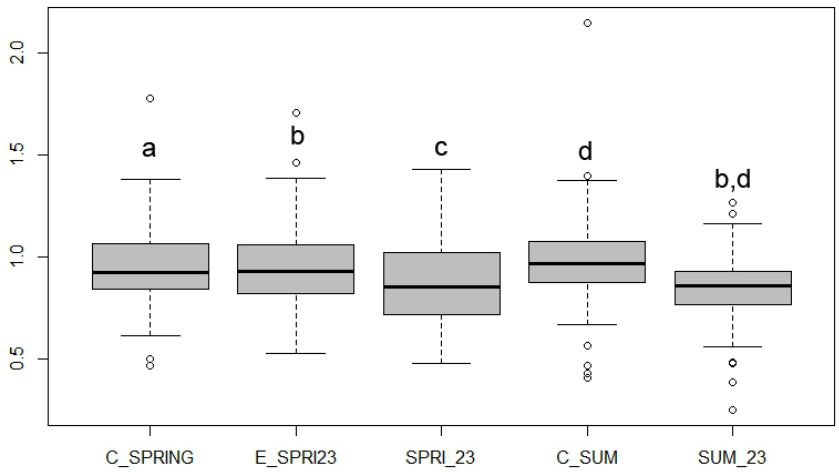

Figure 9. PO/proPO ratio, evaluated in *A. mellifera* samples, indicating transformation efficiency, reaches its maximum as it approaches unity. Results are presented with boxplots and open circles represent outliers. Same letters indicate a statistically significant difference ($p < 0.05$).

The higher proPO activity in SUM_23 may indicate that the samples already had a baseline induction due to pathogens present at the time of collection, which was amplified by the artificial activator (alpha-chymotrypsin).

The fact that PO activity, measured without artificial activation, is higher in SUM_23 confirms the hypothesis that these *A. mellifera* specimens are actively engaging defence mechanisms against naturally occurring external agents [61]. This pathogen exposure, facilitated by PPP treatments applied between spring and summer as demonstrated in prior studies [62,63], likely suppressed general immune defences, leading to later pathogen proliferation and a marked activation of proPO and PO following the treatment period.

The low efficiency (ratio distant from unity) observed in SUM_23, despite having the highest individual PO and proPO activities, suggests an impairment of the proPO-PO system (K-W chi-squared = 22.664, df = 4, p-value = 0). Although the system is activated for defence, its operational efficiency is reduced. This weakening aligns with the hypothesis that pre-SUM_23 PPP treatments compromise the immune system, including the proPO-PO cascade capabilities.

The finding is partially consistent with Millanta et al. [64], who confirmed increased PO production following artificial infection with DWV (Deformed Wing Virus), suggesting that infection causes continuous PO synthesis activation. Conversely, Motta et al. [65] revealed a correlation between herbicide use and melanisation, demonstrating that high glyphosate concentrations interrupt the oxidation–reduction steps catalysed by PO that are necessary for melanin production in *A. mellifera* ex vivo samples.

4. Conclusions

This work investigates how different viticulture management approaches and the use of PPPs affect the health of honey bees. This work compared bees from a biodynamic control farm with those from a conventional-to-organic transition farm located near conventional vineyards. However, it is important to acknowledge that the evidence is limited to a comparison between only two farms, each characterised by specific environmental and management features. This specificity implies that the reported scientific evidence can not be assumed as absolute; nevertheless, these results effectively highlight how different agronomic management practices and the reduction in PPP use in viticulture may significantly influence the health of pollinating insects. Neurotoxic damage (AChE) inhibition increased gradually at the transition farm, peaking post treatment, suggesting a link to pesticide exposure. Both phase I (CaE) and phase II (GST) detoxification enzymes showed significant induction in the SUM_23 samples, indicating a high contaminant burden; CaE also showed some inhibition, which may be associated with neurotoxic side effects. Metabolic stress (ALP) activity was significantly decreased in spring and summer samplings, suggesting widespread metabolic compromise. While lysozyme activity was mostly stable, PO and proPO activity were highly activated in SUM_23, potentially in response to a pathogen whose proliferation might have been facilitated by treatments that had weakened the bees' immune defences. This was further suggested by the low efficiency of the proPO-to-PO conversion in SUM_23.

In conclusion, bee health status appeared most significantly altered at the farm undergoing conversion, particularly after the treatment period. The results validate the multi-biomarker methodology for assessing the potential negative influence of PPPs on bee health in vineyard environments, highlighting the likely role of both the farm's historical conventional usage and contamination drift from nearby operations. While this short-term study effectively captured acute biomarker responses, the inherent limitations in geographic and temporal scale mean that potential long-term cumulative impacts warrant further investigation across full annual cycles and multiple locations.

Author Contributions: Conceptualization, T.C.; methodology, T.C. and S.C.; formal analysis, T.C., A.D.N. and G.M.; investigation, T.C. and G.M.; resources, S.C.; data curation, T.C.; writing—original draft preparation, T.C., A.D.N., G.M. and I.C.; writing—review and editing, T.C., A.D.N. and I.C.; supervision, S.C.; funding acquisition, S.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by FSE-REACT EU for the PON RICERCA E INNOVAZIONE 2014–2020, action IV.6 GREEN: Counteracting the decline of pollinators: Evaluating the environmental sustainability of pesticides and developing bee-friendly agroecological strategies to improve pollinator health.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors on request.

Acknowledgments: We would like to express our sincere gratitude to the farms for their cooperation in this study. We trust that the information presented in this research will be of great assistance in the transition toward sustainable agriculture. During the preparation of this manuscript, the authors used perplexity.ai for the purposes of improving the English language. The authors have reviewed and edited the output and take full responsibility for the content of this publication.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

PPPs	Plant protection products
AChE	Acetylcholinesterase
CaE	Carboxylcholinesterase
GST	Glutathione S-transferase
GSH	Glutathione
ALP	Alkaline phosphatase
LYS	Lysozime
PO	Phenoloxidase
proPO	Prophenoloxidase

References

1. Fisher, B.; Turner, R.K.; Morling, P. Defining and Classifying Ecosystem Services for Decision Making. *Ecol. Econ.* **2009**, *68*, 643–653. [CrossRef]
2. Pires, C.; Maués, M. Insect Pollinators, Major Threats and Mitigation Measures. *Neotrop. Entomol.* **2020**, *49*, 469–471. [CrossRef] [PubMed]
3. Hristov, P.; Shumkova, R.; Palova, N.; Neov, B. Factors Associated with Honey Bee Colony Losses: A Mini-Review. *Vet. Sci.* **2020**, *7*, 166. [CrossRef] [PubMed]
4. Klein, A.-M.; Vaissière, B.E.; Cane, J.H.; Steffan-Dewenter, I.; Cunningham, S.A.; Kremen, C.; Tscharntke, T. Importance of Pollinators in Changing Landscapes for World Crops. *Proc. R. Soc. B Biol. Sci.* **2007**, *274*, 303–313. [CrossRef]
5. Gallai, N.; Salles, J.-M.; Settele, J.; Vaissière, B.E. Economic Valuation of the Vulnerability of World Agriculture Confronted with Pollinator Decline. *Ecol. Econ.* **2009**, *68*, 810–821. [CrossRef]
6. Lin, Z.; Shen, S.; Wang, K.; Ji, T. Biotic and Abiotic Stresses on Honeybee Health. *Integr. Zool.* **2024**, *19*, 442–457. [CrossRef]
7. Guzman, L.M.; Elle, E.; Morandin, L.A.; Cobb, N.S.; Chesshire, P.R.; McCabe, L.M.; Hughes, A.; Orr, M.; M'Gonigle, L.K. Impact of Pesticide Use on Wild Bee Distributions across the United States. *Nat. Sustain.* **2024**, *7*, 1324–1334. [CrossRef]
8. Brunet, J.; Fragoso, F.P. What Are the Main Reasons for the Worldwide Decline in Pollinator Populations? *CABI Rev.* **2024**, *19*, 1. [CrossRef]
9. Raine, N.E.; Rundlöf, M. Pesticide Exposure and Effects on Non-Apis Bees. *Annu. Rev. Entomol.* **2024**, *69*, 551–576. [CrossRef]
10. Dar, S.A.; Ansari, M.J.; Al Naggar, Y.; Hassan, S.; Nighat, S.; Zehra, S.B.; Rashid, R.; Hassan, M.; Hussain, B. Causes and Reasons of Insect Decline and the Way Forward. In *Global Decline of Insects*; IntechOpen: London, UK, 2021.

11. European Commission. *COM/2023/35 Final*; Communication from the Commission to the European Parliament, the Council, the European Economic and Social Committee and the Committee of the Regions Revision of the EU Pollinators Initiative a New Deal for Pollinators; European Commission: Brussels, Belgium, 2023.
12. Decreto Legislativo 23 Dicembre 2022, n. 200 Disposizioni Nazionali di Applicazione del Regolamento (UE) 2021/2115 del Parlamento Europeo e del Consiglio del 2 Dicembre 2021, per Quanto Concerne i Pagamenti Diretti. 2022. Available online: https://www.gazzettaufficiale.it/eli/id/2023/02/24/23A01082/sg (accessed on 10 February 2026).
13. Rouault, A.; Beauchet, S.; Renaud-Gentie, C.; Jourjon, F. Life Cycle Assessment of Viticultural Technical Management Routes (TMRs): Comparison between an Organic and an Integrated Management Route. *OENO One* **2016**, *50*. [CrossRef]
14. Foerster, F.C.; Döring, J.; Koch, M.; Kauer, R.; Stoll, M.; Wohlfahrt, Y.; Wagner, M. Comparative Life Cycle Assessment of Integrated and Organic Viticulture Based on a Long-Term Field Trial in Germany. *Sustain. Prod. Consum.* **2024**, *52*, 487–497. [CrossRef]
15. De Bernardi, A.; Marini, E.; Casucci, C.; Tiano, L.; Marcheggiani, F.; Vischetti, C. Copper Monitoring in Vineyard Soils of Central Italy Subjected to Three Antifungal Treatments, and Effects of Sub-Lethal Copper Doses on the Earthworm Eisenia Fetida. *Toxics* **2022**, *10*, 310. [CrossRef]
16. Droz, B.; Payraudeau, S.; Rodríguez Martín, J.A.; Tóth, G.; Panagos, P.; Montanarella, L.; Borrelli, P.; Imfeld, G. Copper Content and Export in European Vineyard Soils Influenced by Climate and Soil Properties. *Environ. Sci. Technol.* **2021**, *55*, 7327–7334. [CrossRef]
17. Foerster, F.C.; Wagner, M. A Simplified LCA Model to Facilitate Viticulture Sustainability Assessment. *Clean. Environ. Syst.* **2025**, *19*, 100357. [CrossRef]
18. Winter, S.; Paredes, D.; Beaumelle, L.; Alcalá Herrera, R.; Chen, Y.; Hoffmann, C.; Sander, M.; Dumitraţa, D.; Batáry, P.; Rusch, A. Extensive Vineyard Management and Semi-Natural Habitats Increase Biodiversity and Ecosystem Services: Insights from a Global Meta-Analysis. *J. Environ. Manag.* **2025**, *395*, 128029. [CrossRef]
19. Griffiths-Lee, J.; Davenport, B.; Foster, B.; Nicholls, E.; Goulson, D. Sown Wildflowers between Vines Increase Beneficial Insect Abundance and Richness in a British Vineyard. *Agric. For. Entomol.* **2023**, *25*, 139–151. [CrossRef]
20. Schmidt, H. Regulation (EU) 2018/848—The New EU Organic Food Law. *Eur. Food Feed Law Rev.* **2019**, *14*, 15–28.
21. Cravero, M.C. Organic and Biodynamic Wines Quality and Characteristics: A Review. *Food Chem.* **2019**, *295*, 334–340. [CrossRef]
22. Villanueva-Rey, P.; Vázquez-Rowe, I.; Moreira, M.T.; Feijoo, G. Comparative Life Cycle Assessment in the Wine Sector: Biodynamic vs. Conventional Viticulture Activities in NW Spain. *J. Clean. Prod.* **2014**, *65*, 330–341. [CrossRef]
23. Halvorson, K.; Baumung, R.; Leroy, G.; Chen, C.; Boettcher, P. Protection of Honeybees and Other Pollinators: One Global Study. *Apidologie* **2021**, *52*, 535–547. [CrossRef]
24. Barmaz, S.; Potts, S.G.; Vighi, M. A Novel Method for Assessing Risks to Pollinators from Plant Protection Products Using Honeybees as a Model Species. *Ecotoxicology* **2010**, *19*, 1347–1359. [CrossRef] [PubMed]
25. Goulson, D.; Nicholls, E.; Botías, C.; Rotheray, E.L. Bee Declines Driven by Combined Stress from Parasites, Pesticides, and Lack of Flowers. *Science* **2015**, *347*, 1255957. [CrossRef] [PubMed]
26. Campani, T.; Manieri, G.; Caliani, I.; Di Noi, A.; Casini, S. *Apis mellifera* as a Model Species to Evaluate Toxicological Effects of Fungicides Used in Vineyard Agroecosystems. *J. Xenobiotics* **2025**, *15*, 18. [CrossRef]
27. Caliani, I.; Campani, T.; Conti, B.; Cosci, F.; Bedini, S.; D'Agostino, A.; Ammendola, A.; Di Noi, A.; Gori, A.; Casini, S. Multi-Biomarker Approach and IBR Index to Evaluate the Effects of Different Contaminants on the Ecotoxicological Status of *Apis mellifera*. *Ecotoxicol. Environ. Saf.* **2021**, *208*, 111486. [CrossRef]
28. Pisa, L.W.; Amaral-Rogers, V.; Belzunces, L.P.; Bonmatin, J.-M.; Downs, C.A.; Goulson, D.; Kreutzweiser, D.P.; Krupke, C.; Liess, M.; McField, M. Effects of Neonicotinoids and Fipronil on Non-Target Invertebrates. *Environ. Sci. Pollut. Res.* **2015**, *22*, 68–102. [CrossRef]
29. Ellman, G.L.; Courtney, K.D.; Andres, V.; Featherstone, R.M. A New and Rapid Colorimetric Determination of Acetylcholinesterase Activity. *Biochem. Pharmacol.* **1961**, *7*, 88–95. [CrossRef]
30. Solé, M.; Sanchez-Hernandez, J.C. Elucidating the Importance of Mussel Carboxylesterase Activity as Exposure Biomarker of Environmental Contaminants of Current Concern: An in Vitro Study. *Ecol. Indic.* **2018**, *85*, 432–439. [CrossRef]
31. Burciaga, R.A.; Ruiz-Guzmán, G.; Lanz-Mendoza, H.; Krams, I.; Contreras-Garduño, J. The Honey Bees Immune Memory. *Dev. Comp. Immunol.* **2023**, *138*, 104528. [CrossRef]
32. Bradford, M.M. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Anal. Biochem.* **1976**, *72*, 248–254. [CrossRef]
33. Williamson, S.M.; Moffat, C.; Gomersall, M.; Saranzewa, N.; Connolly, C.; Wright, G.A. Exposure to Acetylcholinesterase Inhibitors Alters the Physiology and Motor Function of Honeybees. *Front. Physiol.* **2013**, *4*, 13. [CrossRef] [PubMed]
34. Ayoub, L.; Yaqoob, M.; Kanth, R.H.; Wani, F.J.; Shah, Z.A.; Dar, E.A.; Wani, F.F.; Mir, M.S.; Naikoo, N.B.; Gull, A.; et al. Exposure to Organophosphate Insecticides Induces Behavioral Changes and Acetylcholinesterase Inhibition in *Apis mellifera*. *Ecotoxicol. Environ. Saf.* **2024**, *287*, 117279. [CrossRef]

35. Nikolić, T.V.; Kojić, D.; Orčić, S.; Vukašinović, E.L.; Blagojević, D.P.; Purać, J. Laboratory Bioassays on the Response of Honey Bee (*Apis mellifera* L.) Glutathione S-Transferase and Acetylcholinesterase to the Oral Exposure to Copper, Cadmium, and Lead. *Environ. Sci. Pollut. Res.* **2019**, *26*, 6890–6897. [CrossRef]
36. Kim, Y.H.; Kim, J.H.; Kim, K.; Lee, S.H. Expression of Acetylcholinesterase 1 Is Associated with Brood Rearing Status in the Honey Bee, *Apis mellifera*. *Sci. Rep.* **2017**, *7*, 39864. [CrossRef]
37. Minaud, É.; Rebaudo, F.; Davidson, P.; Hatjina, F.; Hotho, A.; Mainardi, G.; Steffan-Dewenter, I.; Vardakas, P.; Verrier, E.; Requier, F. How Stressors Disrupt Honey Bee Biological Traits and Overwintering Mechanisms. *Heliyon* **2024**, *10*, e34390. [CrossRef]
38. Benito-Murcia, M.; Botías, C.; Martín-Hernández, R.; Higes, M.; Soler, F.; Pérez-López, M.; Míguez-Santiyán, M.P.; Martínez-Morcillo, S. Biomarker Responses and Lethal Dietary Doses of Tau-Fluvalinate and Coumaphos in Honey Bees: Implications for Chronic Acaricide Toxicity. *Environ. Toxicol. Pharmacol.* **2024**, *105*, 104330. [CrossRef]
39. Benchaâbane, S.; Ayad, A.; Loucif-Ayad, W.; Soltani, N. Multibiomarker Responses after Exposure to a Sublethal Concentration of Thiamethoxam in the African Honeybee (*Apis mellifera intermissa*). *Comp. Biochem. Physiol. Part C Toxicol. Pharmacol.* **2022**, *257*, 109334. [CrossRef] [PubMed]
40. Li, W.; Gao, K.; Lu, L. Environmental Pollutant-Induced Cholinergic Disruption: Advances and Perspectives in Mechanistic Insights, Target Heterogeneity, and Neurotoxic Synergy. *Ecotoxicol. Environ. Saf.* **2025**, *308*, 119470. [CrossRef]
41. Paoli, M.; Giurfa, M. Pesticides and Pollinator Brain: How Do Neonicotinoids Affect the Central Nervous System of Bees? *Eur. J. Neurosci.* **2024**, *60*, 5927–5948. [CrossRef]
42. Badiou-Bénéteau, A.; Carvalho, S.M.; Brunet, J.-L.; Carvalho, G.A.; Buleté, A.; Giroud, B.; Belzunces, L.P. Development of Biomarkers of Exposure to Xenobiotics in the Honey Bee *Apis mellifera*: Application to the Systemic Insecticide Thiamethoxam. *Ecotoxicol. Environ. Saf.* **2012**, *82*, 22–31. [CrossRef] [PubMed]
43. Carvalho, S.M.; Belzunces, L.P.; Carvalho, G.A.; Brunet, J.; Badiou-Beneteau, A. Enzymatic Biomarkers as Tools to Assess Environmental Quality: A Case Study of Exposure of the Honeybee *Apis mellifera* to Insecticides. *Environ. Toxicol. Chem.* **2013**, *32*, 2117–2124. [CrossRef] [PubMed]
44. BK, S.K.; Moural, T.; Zhu, F. Functional and Structural Diversity of Insect Glutathione S-Transferases in Xenobiotic Adaptation. *Int. J. Biol. Sci.* **2022**, *18*, 5713. [CrossRef]
45. Caliani, I.; Campani, T.; Conti, B.; Cosci, F.; Bedini, S.; D'Agostino, A.; Giovanetti, L.; Di Noi, A.; Casini, S. First Application of an Integrated Biological Response Index to Assess the Ecotoxicological Status of Honeybees from Rural and Urban Areas. *Environ. Sci. Pollut. Res.* **2021**, *28*, 47418–47428. [CrossRef] [PubMed]
46. Badiou-Bénéteau, A.; Benneveau, A.; Géret, F.; Delatte, H.; Becker, N.; Brunet, J.; Reynaud, B.; Belzunces, L. Honeybee Biomarkers as Promising Tools to Monitor Environmental Quality. *Environ. Int.* **2013**, *60*, 31–41. [CrossRef]
47. Benito-Murcia, M.; Riva, C.; García-Vicente, E.J.; Pérez, A.; Domínguez, M.M.; Hermosilla, N.; Cochard, P.; Charistos, L.; Hatjina, F.; Holmiere, M.; et al. Reducing Honey Bee Winter Mortality with Molybdenum Supplementation: Field Evidence across Europe. *Res. Vet. Sci.* **2025**, *197*, 105932. [CrossRef]
48. Migdał, P.; Murawska, A.; Bieńkowski, P.; Strachecka, A.; Roman, A. Effect of the Electric Field at 50 Hz and Variable Intensities on Biochemical Markers in the Honey Bee's Hemolymph. *PLoS ONE* **2021**, *16*, e0252858. [CrossRef]
49. Alburaki, M.; Madella, S.; Cook, S.C. Non-Optimal Ambient Temperatures Aggravate Insecticide Toxicity and Affect Honey Bees *Apis mellifera* L. Gene Regulation. *Sci. Rep.* **2023**, *13*, 3931. [CrossRef] [PubMed]
50. Rudelli, C.; Galuppi, R.; Cabbri, R.; Dalmonte, T.; Fontanesi, L.; Andreani, G.; Isani, G. Field Application of an Innovative Approach to Assess Honeybee Health and Nutritional Status. *Animals* **2024**, *14*, 2183. [CrossRef] [PubMed]
51. Tosi, S.; Nieh, J.C.; Sgolastra, F.; Cabbri, R.; Medrzycki, P. Neonicotinoid Pesticides and Nutritional Stress Synergistically Reduce Survival in Honey Bees. *Proc. R. Soc. B Biol. Sci.* **2017**, *284*, 20171711. [CrossRef]
52. Paleolog, J.; Wilde, J.; Gancarz, M.; Strachecka, A. Imidacloprid Decreases Energy Production in the Hemolymph and Fat Body of Western Honeybees Even Though, in Sublethal Doses, It Increased the Values of Six of the Nine Compounds in the Respiratory and Citric Cycle. *PLoS ONE* **2025**, *20*, e0320168. [CrossRef]
53. Migdal, P.; Murawska, A.; Berbeć, E.; Plotnik, M.; Skorus, A.; Latarowski, K. Selected Biochemical Markers Change after Oral Administration of Pesticide Mixtures in Honey Bees. *Toxics* **2022**, *10*, 590. [CrossRef]
54. Di Noi, A.; Casini, S.; Campani, T.; Cai, G.; Caliani, I. Review on Sublethal Effects of Environmental Contaminants in Honey Bees (*Apis mellifera*), Knowledge Gaps and Future Perspectives. *Int. J. Environ. Res. Public Health* **2021**, *18*, 1863. [CrossRef]
55. Ferraboschi, P.; Ciceri, S.; Grisenti, P. Applications of Lysozyme, an Innate Immune Defense Factor, as an Alternative Antibiotic. *Antibiotics* **2021**, *10*, 1534. [CrossRef]
56. Evans, J.D.; Aronstein, K.; Chen, Y.P.; Hetru, C.; Imler, J.; Jiang, H.; Kanost, M.; Thompson, G.J.; Zou, Z.; Hultmark, D. Immune Pathways and Defence Mechanisms in Honey Bees *Apis mellifera*. *Insect Mol. Biol.* **2006**, *15*, 645–656. [CrossRef]
57. Lavine, M.; Strand, M. Insect Hemocytes and Their Role in Immunity. *Insect Biochem. Mol. Biol.* **2002**, *32*, 1295–1309. [CrossRef]
58. Nicoletti, M.; Gilles, F.; Galicia-Mendoza, I.; Rendón-Salinas, E.; Alonso, A.; Contreras-Garduño, J. Physiological Costs in Monarch Butterflies Due to Forest Cover and Visitors. *Ecol. Indic.* **2020**, *117*, 106592. [CrossRef]

59. Laughton, A.M.; Siva-Jothy, M.T. A Standardised Protocol for Measuring Phenoloxidase and Prophenoloxidase in the Honey Bee, *Apis mellifera*. *Apidologie* **2011**, *42*, 140–149. [CrossRef]
60. Schmid, M.R.; Brockmann, A.; Pirk, C.W.; Stanley, D.W.; Tautz, J. Adult Honeybees (*Apis mellifera* L.) Abandon Hemocytic, but Not Phenoloxidase-Based Immunity. *J. Insect Physiol.* **2008**, *54*, 439–444. [CrossRef] [PubMed]
61. González-Santoyo, I.; Córdoba-Aguilar, A. Phenoloxidase: A Key Component of the Insect Immune System. *Entomol. Exp. Appl.* **2012**, *142*, 1–16. [CrossRef]
62. Pettis, J.S.; Vanengelsdorp, D.; Johnson, J.; Dively, G. Pesticide Exposure in Honey Bees Results in Increased Levels of the Gut Pathogen Nosema. *Naturwissenschaften* **2012**, *99*, 153–158. [CrossRef]
63. Vidau, C.; Diogon, M.; Aufauvre, J.; Fontbonne, R.; Viguès, B.; Brunet, J.-L.; Texier, C.; Biron, D.G.; Blot, N.; El Alaoui, H. Exposure to Sublethal Doses of Fipronil and Thiacloprid Highly Increases Mortality of Honeybees Previously Infected by Nosema Ceranae. *PLoS ONE* **2011**, *6*, e21550. [CrossRef]
64. Millanta, F.; Sagona, S.; Mazzei, M.; Forzan, M.; Poli, A.; Felicioli, A. Phenoloxidase Activity and Haemolymph Cytology in Honeybees Challenged with a Virus Suspension (Deformed Wings Virus DWV) or Phosphate Buffered Suspension (PBS). *Ciênc. Rural* **2019**, *49*, e20180726. [CrossRef]
65. Motta, E.V.; Powell, J.E.; Moran, N.A. Glyphosate Induces Immune Dysregulation in Honey Bees. *Anim. Microbiome* **2022**, *4*, 16. [CrossRef] [PubMed]