Environmental Research 305 (2026) 125038

Contents lists available at ScienceDirect

Environmental Research

journal homepage: www.elsevier.com/locate/envres

Multi-biomarker assessment of *Apis mellifera* as a sentinel of environmental stress: A two-year field study across diverse land-use gradients in Tuscany (Italy)

Agata Di Noi [a], Ilaria Caliani [a,*], Matteo Vitale [a], Tommaso Campani [a], Antonella D'Agostino [b], Giampiero Cai [c], Marco Romi [c], Silvia Casini [a]

[a] Department of Physical, Earth and Environmental Sciences, University of Siena, via Mattioli, 4, Siena, 53100, Italy
[b] Department of Economics and Statistics, University of Siena, Piazza S. Francesco 7, Siena, 53100, Italy
[c] Department of Life Sciences, University of Siena, via Mattioli, 4, Siena, 53100, Italy

ARTICLE INFO

Keywords:
Honey bee
Sublethal effects
Biomarkers
Climatic variations

ABSTRACT

Honey bees (*Apis mellifera*) are essential pollinators currently facing global declines driven by the interaction of multiple stressors, including habitat fragmentation, climate change, diseases, invasive species acting as predators, and exposure to anthropogenic contaminants. While laboratory studies have extensively explored these factors, field-based assessments of cumulative sublethal effects remain limited. Biomarkers represent sensitive methodologies for identifying early-warning signals of physiological impairment before population-level declines occur. In this study, we evaluated the ecotoxicological health status of honey bee adults across ten locations in Tuscany (Italy) with varying degrees of anthropogenic impact, including high-naturality sites (wood and wildflower field), agricultural areas (orchard, vineyards, olive grove, berries field, wheat crop and clover field), and urban sites. Sampling was conducted over two consecutive years (2020–2021) to account for inter-annual climatic variations. A multi-biomarker approach was applied to assess neurotoxicity (acethylcolinesterase and carboxylesterase activities), detoxification and metabolism (glutathione S-transferase and alkaline phosphatase), immune response (lysozyme activity and haemocyte counts), and genotoxicity (Nuclear Abnormalities assay). The results demonstrated that biomarker responses are significantly modulated by land use ($p < 0.05$). Clearer signs of physiological disturbance consistently emerged in anthropized sites, whereas the areas with a high degree of naturality exhibited comparatively lower biomarker values. These findings highlight that climatic conditions influenced the sublethal responses observed in organisms. However, multiple stressors, besides climate-related ones, should be taken into account for a more complete evaluation of honey bees' ecotoxicological status in a field scenario.

1. Introduction

Pollinating insects are essential organisms, playing a primary role both in agriculture, ensuring the productivity of crops, and in the preservation of habitats, natural resources, and plant biodiversity (Burkle et al., 2013; Kennedy et al., 2013; Potts et al., 2010). Among pollinators, *Apis mellifera* is the most well-known and widespread species and plays a vital role for its pollination service, contributing to 35% of crop production and to the conservation of biodiversity (Chen et al., 2026; Scheper et al., 2023). Despite the ability of honey bee colonies to be more resilient than solitary insects (Cunningham et al., 2022), these valuable insects are declining worldwide (Mackei et al., 2026). This phenomenon is driven by several causes, including climate change, habitat fragmentation, atmospheric pollution, and the intensification of land use through monoculture plantations. Furthermore, the decrease in floral diversity, combined with diseases, invasive species acting as predators, parasites, and the widespread use of pesticides exacerbates the situation (Askri et al., 2024; Ghazoul, 2005; Potts et al., 2010). As generalist pollinators with extensive foraging ranges, honey bees (*Apis mellifera*) serve as a critical model for understanding vulnerability to the diverse environmental pressures. Crucially, the primary concern lies in the possible interaction of the above-mentioned stressors, rather than

* Corresponding author.
E-mail address: caliani4@unisi.it (I. Caliani).

https://doi.org/10.1016/j.envres.2026.125038
Received 1 April 2026; Received in revised form 11 June 2026; Accepted 11 June 2026
Available online 15 June 2026
0013-9351/© 2026 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

their isolated impact, accelerating population-level declines (Askri et al., 2024; Goulson et al., 2015; Potts et al., 2010), although the mechanisms are still poorly understood. Therefore, it is of great importance to investigate how these various stressors interact with each other and how they can cause honey bee mortality (Askri et al., 2024).

Among these stressors, nutritional stress represents a critical driver of the global decline in honey bee colonies (Goulson et al., 2015). Since pollen constitutes the sole source of essential proteins, vitamins, lipids, and amino acids, any reduction in floral diversity severely constrains the bees' ability to satisfy their physiological requirements (Brodschneider and Crailsheim, 2010; Crailsheim, 1990). Consequently, such nutritional deficiencies can impair immunocompetence, larval development, and the stability of the gut microbiota, thereby undermining the colony's overall resilience to pathogens and environmental pressures (Alaux et al., 2010; Branchiccela et al., 2019; Castelli et al., 2020; Pasquale et al., 2013).

In addition to nutritional challenges, honey bees (*Apis mellifera*) are frequently exposed to different chemical compounds, including hydrocarbons and metals (Caliani et al., 2021a; Monchanin et al., 2021). Among the chemical compounds, pesticides are the main threats for honey bees (Murawska et al., 2025; Porrini et al., 2003; Raeymaekers, 2006). Bees can come into contact with these toxic compounds through multiple routes, such as contaminated food and water sources. The bioaccumulation of these substances within honey bees can severely compromise both individual health and overall colony development (Decourtye et al., 2005; Kairo et al., 2016; Teeters et al., 2012; Williamson and Wright, 2013), acting as chronic stressors that threaten the stability of the entire hive (Pohorecka et al., 2017).

However, the stability of the colony is threatened not only by these anthropogenic pressures but also by the escalating frequency of climatic extremes. In this context, despite their innate ability to regulate hive temperature (Fahrenholz et al., 1989), rising global temperatures may adversely affect thermoregulation and disrupt colony homeostasis (Cunningham et al., 2022). Parallel to these environmental factors, biological stressors remain a significant cause of mortality. Among these, the ectoparasitic mite *Varroa destructor* and the microsporidian *Nosema ceranae* represent primary threats, as they compromise individual immunity and facilitate the transmission of viral diseases, ultimately leading to large-scale colony losses (Alarcón et al., 2026).

In this context, assessing sublethal effects is essential to identify the risks posed by multiple stressors. Integrated and sensitive methodologies, such as the application of biomarkers, are useful tools to assess possible alterations at the molecular and cellular levels, serving as critical early-warning signals, potentially preventing long-term impacts at the population level (Pal et al., 2022).

Biomarkers, in fact, encompass a range of specific investigations, including physiological alterations that can be observed at cellular and enzymatic levels, being signals for neurotoxicity, detoxification, digestive processes, immune functions, and genotoxic alterations. The integration of all these endpoints would give a more complex and yet elucidating answer to comprehend the toxicological health status of bees inhabiting areas with different environmental impacts.

Acetylcholinesterase (AChE) is a neural enzyme involved in the transmittance of the nervous impulse in the cholinergic synapses by hydrolyzing the neurotransmitter acetylcholine. The alteration of its activity has been demonstrated to cause neurotoxic effects in pollinators, such as sustained neuronal stimulation, paralysis, leading even to the death of the organism (Badiou et al., 2008; Dorigo et al., 2026). Carboxylesterases (CaE) are a class of hydrolytic enzymes widely distributed in insects, where they play multiple physiological roles. These include participating in fundamental processes such as growth, development, and nutrient metabolism, mediating adaptation to environmental stress (Ran et al., 2026), and metabolizing exogenous toxins (Jackson et al., 2013; Ran et al., 2026). In fact, CaE participates in a protective mechanism that prevents the inhibition of AChE by organophosphates and carbamates (Jackson et al., 2013; Yan et al., 2009) and belong to the phase I of detoxification process with very broad substrate specificities (Hatfield et al., 2016). Glutathione S-transferase (GST) is a phase-II detoxifying enzyme that catalyzes the conjugation of reduced glutathione to a large number of xenobiotics, increasing the polarity of the compounds and thus their excretion or further transformation (Badiou-Bénéteau et al., 2012; Decio et al., 2021; Stone et al., 2002; Weirich et al., 2002). In bees, GST activity changes have been documented following exposure to pesticides (Almasri et al., 2020; Balieira et al., 2018; Caliani et al., 2021a; Decio et al., 2021; Renzi et al., 2016), emphasizing the fundamental role in detoxification (Murawska et al., 2025) and oxidative stress defense (Briscoe et al., 2026; Bryś, 2025; Strachecka et al., 2016), although not acting directly on reactive oxygen species (ROS) (Dorigo et al., 2026), but neutralizing oxidative intermediates (Cang et al., 2023). Alkaline phosphatase (ALP) is a digestive enzyme involved in adsorption and transport mechanisms through the hydrolysis of phosphate groups from many types of molecules, including nucleotides, proteins, and alkaloids in alkaline conditions (Zibaee et al., 2011). Also, ALP has an additional role in the final steps of lipid digestion in insects and general metabolic regulation (Dorigo et al., 2026) and physiological status (Bryś, 2025). The exposure to various xenobiotics proved to affect ALP activity in insects (Murawska et al., 2025; Zibaee et al., 2011). The lysozyme (LYS) is one of the main proteins involved in the humoral immune response (Kunat-Budzyńska et al., 2025) and a factor of resistance that determines the protective functions of the bee organism (Lazarov et al., 2016). The main function of lysozyme is to destroy the cell wall peptidoglycan mainly of Gram-positive bacteria and is an indicator of immunity, being often used to assess the condition of honey bee colonies (Kunat-Budzyńska et al., 2025). Its activity is induced upon systemic infection and also after injection of cell wall components of bacteria and fungi (da Luz et al., 2022; Doublet et al., 2017). Some pesticides were demonstrated to affect honey bee's immune response (Castelli et al., 2021; Tesovnik et al., 2020) thus facilitating the multiplication of pathogens. Similarly, plasmatocytes and granulocytes carry out important immunological functions, contributing to the honey bee's immune system. Granulocytes have a relevant role in phagocytosis (Richardson et al., 2018). Nuclear Abnormalities (NA) assay represents a test widely used to evaluate the DNA damage in cells from various organisms (Cherednichenko et al., 2024; Marangi et al., 2024) exposed to genotoxic contaminants. Recently, this test was performed for the first time on bees (Caliani et al., 2021a; Di Noi et al., 2024), demonstrating its efficacy as a genotoxicity biomarker in pollinators.

To date, biomarker responses in bees have been predominantly evaluated in laboratory studies (Caliani et al., 2021a; Castelli et al., 2021; Chen et al., 2026; Choi et al., 2024; Di Noi et al., 2024; Dorigo et al., 2026; Fellows et al., 2022; Paloschi et al., 2026; Roat et al., 2017; Sonter et al., 2026; Strachecka et al., 2016) while only a limited number of studies applied this approach in field scenarios (Briscoe et al., 2026; Caliani et al., 2021b; Campani et al., 2026; de Souza et al., 2026; Lupi et al., 2020; Wegener, 2016). Furthermore, most monitoring programs have focused on the bioaccumulation of pollutants rather than their biological effects, which remain challenging to decipher due to the complex nature of multiple stressors. Furthermore, previous studies have focused on limited geographical areas (Caliani et al., 2021b; Campani et al., 2026; Glavan et al., 2026; Lupi et al., 2020). To address this gap, our study presents a comprehensive approach to environmental biomonitoring, carried out in two different years (2020-2021). Specifically, this investigation integrates a wide number of biomarkers to assess simultaneously neurotoxicity (AChE and CaE), detoxification processes (GST), metabolic and nutritional alterations (ALP), genotoxicity (NA assay) and immune system status (LYS and differential haemocyte count) in honey bees sampled in 10 locations at different anthropic impact. Moreover, several environmental factors that can be influenced by climate change, such as rain, high temperature and drought, may affect honey bee's health status. To address this contingency, we performed a monitoring extended through two years to

provide a broader temporal perspective and account for potential inter-annual climatic variations that influence bee health and colony conditions. This methodology applied to an ecologically highly relevant species, such as *Apis mellifera*, aims to provide a global overview of the honey bee's ability to serve as a sensitive sentinel of environmental stress.

2. Materials and methods

2.1. Experimental design

The sampling of bees was carried out in 10 different locations in Tuscany (Italy) (Fig. 1), characterised by different kinds of impact. A wooded environment, wildflower field, vineyards, an orchard, olive grove, clover field, wheat crop field, a berries field and two urban areas were used to collect honey bee foragers. In Table 1, the selected areas are described with their geographic coordinates and the different types of cultivations. The wood is located in the province of Pisa, more than 7 km in a direct distance from the towns of Pontedera and Ponsacco. It is situated within a vast forested area of over 130 km^2, characterized by chestnut and holm oak woods. The wildflower area is uncultivated and the beekeeper annually sows a mix of pollinator-friendly flower seeds to facilitate the pollination process. The area is surrounded by an extensive hilly woodland of broad-leaved trees, consisting mainly of holm oaks, oaks, chestnuts, and hornbeams. Furthermore, the investigated area is surrounded by small residential towns, with no industrial complexes present. The Urban I and Urban II areas are a few kilometers away from the city of Siena. These sampling areas are surrounded by small, densely populated villages. Medium-traffic roads are located at short distance from the investigated areas. The clover site is located approximately 2 km in a direct distance from the city of Pisa, and it is an area rich in cereal crops and olive groves. About 5 km in a direct distance from the clover area there is Monte Faeta, an 831-m-high mountain that is part of the Monti Pisani system in Tuscany, marking the natural border between the provinces of Pisa and Lucca. The native vegetation of Monte Faeta is typical of the Mediterranean maquis (downy oak, chestnut trees, holm oaks, and garrigue). The orchard is part of a system of experimental orchards and fields managed by the University of Pisa, which includes apple, plum, peach, and grape trees. Similar to the clover area, the surrounding environment includes the city of Pisa, intensive cultivation, and Monte Faeta. The bees sampled in the area named “olive grove” are located in the province of Siena, in the Chianti Classico region. The hives are situated in a small wooded area bordering large agricultural estates that produce wine and olive oil. The wheat crop area is located in the province of Siena. It is characterized by extensive fields of wheat or other cereals. Surrounding the area, in addition to the cultivated fields, are the “Crete Senesi”, a landscape characterized by clay hills, solitary

Table 1
Detail of the sampled areas with geographical coordinates and cultivations observed near the area studied.

Type of area	Sampled area	Geographical coordinates	Cultivations
Natural	Wood	43.64525 N 10.67579 E 34 m asl	Wood
	Wildflower field	43.573681 N 10.593210 E 106 m asl	Wildflower
Agricultural	Orchard	43.72879 N 10.46283 E 4 m asl	Apple, plum, peach, grapes
	Vineyards	43.514077 N 11.895551 E 179 m asl	Vineyards
	Berries field	42.859081 N 11.679691 E 822 m asl	Berries, chestnut
	Olive grove	43.391424 N 11.350889 E 450 m asl	Olive trees, vineyards, wood
	Clover field	43.727434 N 10.462937 E 4 m asl	Clover
	Wheat crop	43.302539 N 11.449288 E 207 m asl	Wheat, olive trees
Urban	Urban area I	43.298849 N 11.382147 E 304 m asl	-------
	Urban area II	43.290623 N 11.396943 E 260 m asl	-------

Fig. 1. Location of the different sampling areas in Tuscany (Italy).

oaks, and cypresses. The vineyard area is located in the province of Arezzo, featuring extensive wine-producing grape cultivation. Numerous olive groves and cereal-cultivated fields are also present in the area. All the agricultural areas selected for the study use conventional farming practices, with no organic cultivation in any of the studied sites. About 50 adult honey bees were sampled from each study area during the summers of 2020 and 2021.

2.2. Biomarker analysis

2.2.1. Bee's dissection

Once sampling was performed, honeybees were anaesthetized in the laboratory with ice (4 °C) for 30 min prior to haemolymph collection from the abdomen. 50 honey bees per sampling location were tested, for a total of 900 samples analysed. For each slide, haemolymph from two specimens were placed on a poly-lysate slide (2.5 mg/mL) to perform the differential haemocyte count and NA assay. The intestine was pulled out from the back of the abdomen and then used to assess LYS, GST and ALP activities. The head was separated from the body by transverse cutting for the assessment of esterase activities (AChE and CaE). Biological materials were then stored at −80 °C for subsequent analysis.

2.2.2. Enzyme assays

For each extract, pools were prepared using biological tissue from three animals. Tissue samples were pooled, weighed, and the extraction medium (40 mM Na-phosphate buffer pH 7.4, a mixture of protease inhibitor enzymes, 1% Triton X-100) was added in a volume corresponding to 10% (w/v) of the tissue. After homogenization by a Tissue Lyser (Qiagen) for three periods of 30 s at 30 s intervals, the homogenates were centrifuged at 4 °C for 20 min at 13,000g and 15,000g for heads and guts samples, respectively. The resulting supernatants were used for the analysis.

The assay for the determination of AChE, according to the technique described by Caliani et al. (2021a), was measured for 5 min at 412 nm in a medium containing 0.1 M sodium phosphate buffer (pH 7.4), 10 mM DTNB and 41.5 mM acetylthiocoline. CaE activity was quantified using a spectrophotometer at 538 nm, adding 100 mM sodium phosphate buffer (pH 7.4) to the head extract and incubating at 25 °C for 5 min. The reaction was started by adding 0.4 mM α-NA as a substrate and stopped by adding 1.5% SDS and 0.4 mg/L Fast Garnet GBC (Caliani et al., 2021a). GST activity was assessed in the post-mitochondrial fraction of the intestine, according to the technique described by Caliani et al. (2021a). A reaction mixture was prepared: 0.1 M Na-phosphate buffer (pH = 7.4), 8 mM CDNB (1-chloro-2,4-dinitrobenzene), 8 mM GSH (reduced glutathione) and the reading was performed for 3 min at 340 nm. ALP was monitored at 405 nm in a medium containing 100 mM $MgCl_2$, 6 mM p-NPP (p-nitrophenyl phosphate) and 100 mM Tris-HCl (pH = 8.5) (Caliani et al., 2021a). LYS activity was measured using a turbidity test according to the method by Caliani et al. (2021a), and the absorbance was evaluated at 450 nm with a Microplate Reader (Model 550, BioRad, Germany). Protein concentrations were estimated using the method described by Bradford (1976), with bovine serum albumin as the standard.

2.2.3. Differential haemocytes count and NA assay

Differential haemocytes count was performed according to Șapcaliu et al., 2009, while the NA assay followed the procedure by Caliani et al. (2021a). Both biomarkers were evaluated on the same slide, and the slides were stained with Diff-Quick stain (Bio-Optica) following the standard three-step protocol. Briefly, slides were first immersed in the methanol-based fixative (Reagent A), then stained with the eosin-containing solution (Reagent B), followed by immersion in the thiazine-based solution (Reagent C). After each step, excess reagent was allowed to drain, and the slides were finally rinsed under distilled water until the run-off was clear, then air-dried at room temperature before microscopic examination. For each assay, 1000 cells were counted on a slide, using an immersion light microscope (Olympus BX41), at 100× magnification, and only mature haemocytes with a well-defined nucleus were considered. The abnormalities evaluated were categorized in: lobed nuclei, kidney, segmented, binucleated, apoptotic cells and micronuclei (Fig. S1). The results of the NA assay are expressed as the number of nuclear abnormalities/1000 cells; differential haemocytes count was performed by considering the two most frequent classes of haemocytes, plasmatocytes and granulocytes, and the result was expressed as the number of haemocytes/1000 cells.

2.3. Statistical analysis

We tested for significant differences in each biomarker between the different areas' samples using the Kruskal-Wallis (KW) test (Kruskal and Wallis, 1952). This non-parametric test is used when the data does not satisfy the normality property and contains outliers. Furthermore, Dunn's test with a Benjamini–Hochberg stepwise adjustment (Benjamini and Hochberg, 1995) was applied for pairwise multiple-comparisons when the null hypothesis of the KW test was rejected, controlling for Type I error inflation via the False Discovery Rate (Dinno, 2015). Dunn's *post-hoc* test makes multiple pairwise comparisons based on Dunn's z-test-statistic approximations to the actual rank statistics. The Mann-Whitney test (Mann and Whitney, 1947) was used for the comparison between 2020 and 2021 for each area. The significance level for all tests was set at $\alpha = 0.05$. These tests have been implemented by STATA 17 software (StataCorp, 2021).

3. Results

Neurotoxicity (AChE and CaE), metabolism (ALP and GST), immune system (LYS and differential haemocytes count), and genotoxicity (NA assay) biomarkers were measured in honey bees from areas with different anthropogenic impacts in two sampling years (2020-2021). The suburban area was only sampled in 2020, while the beehives in the berries field were only sampled in 2021.

3.1. Biomarker responses across sampling sites

Figs. 2 and 3 show the different biomarker responses measured in the bees collected in the sites sampled in 2020 and 2021, respectively. More details are reported in Tables S1 and S2 (Supplementary material).

3.1.1. Results for the 2020 sampling year

In 2020, AChE activity (K-W $\chi^2_8 = 81.803$, $p = 0.0001$) (Fig. 2A) had lower values in honey bees from every area compared to the ones sampled in vineyards and orchard. Urban area II specimens showed the lowest values and statistically significant compared to wood ($z = -3.17$, $p = 0.0183$), orchard ($z = 5.39$, $p < 0.0001$), vineyards ($z = -7.14$, $p < 0.0001$) and urban area I ($z = -4.05$, $p = 0.0008$). Vineyards showed differences compared to wood ($z = 3.81$, $p = 0.0019$), wildflower field ($z = 6.70$, $p < 0.0001$), clover field ($z = -4.94$, $p < 0.0001$), wheat crop ($z = 4.54$, $p = 0.0001$), olive grove ($z = -3.13$, $p = 0.0198$) and urban area I ($z = -3.09$, $p = 0.0221$) Orchard was found to be different from wildflower ($z = 4.99$, $p < 0.0001$) and clover field ($z = -3.19$, $p = 0.0176$). Honey bees sampled in the wildflower field were found to be different from wood ($z = -2.82$, $p = 0.0489$), olive grove ($z = 3.49$, $p = 0.0062$) and urban area I animals ($z = 3.68$, $p = 0.0032$).

Urban area II showed the highest values of CaE activity (K-W $\chi^2_8 = 101.720$, $p = 0.0001$) (Fig. 2B), and statistically significant differences compared to wood ($z = 5.30$, $p < 0.0001$), wildflower field ($z = 5.09$, $p < 0.0001$), orchard ($z = -4.71$, $p < 0.0001$), clover field ($z = -6.63$, $p < 0.0001$), wheat crop ($z = 6.77$, $p < 0.0001$) and urban area I ($z = 4.26$, $p = 0.0003$). Vineyards samples showed statistically significant differences when compared to wood ($z = 4.52$, $p = 0.0001$),

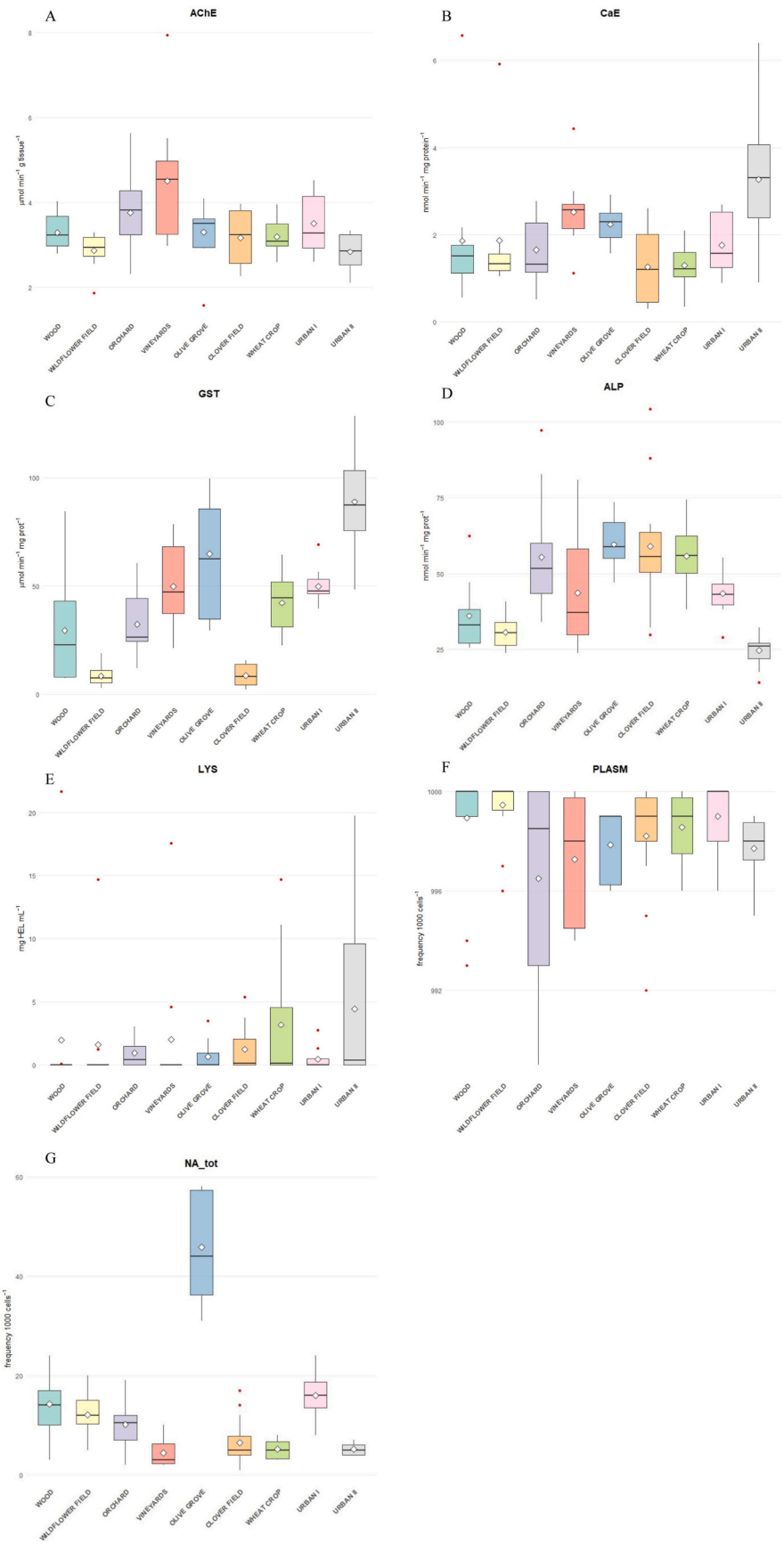

Fig. 2. Boxplots of the biomarkers measured in *Apis mellifera* sampled in 2020 across nine sites with varying degrees of anthropogenic impact, including high-naturality sites (wood and wildflower field), agricultural areas (orchard, vineyards, olive grove, wheat crop and clover field), and urban sites: AChE (A); CaE (B); GST (C); ALP (D); LYS (E); differential haemocytes count (F) and NA assay (G).

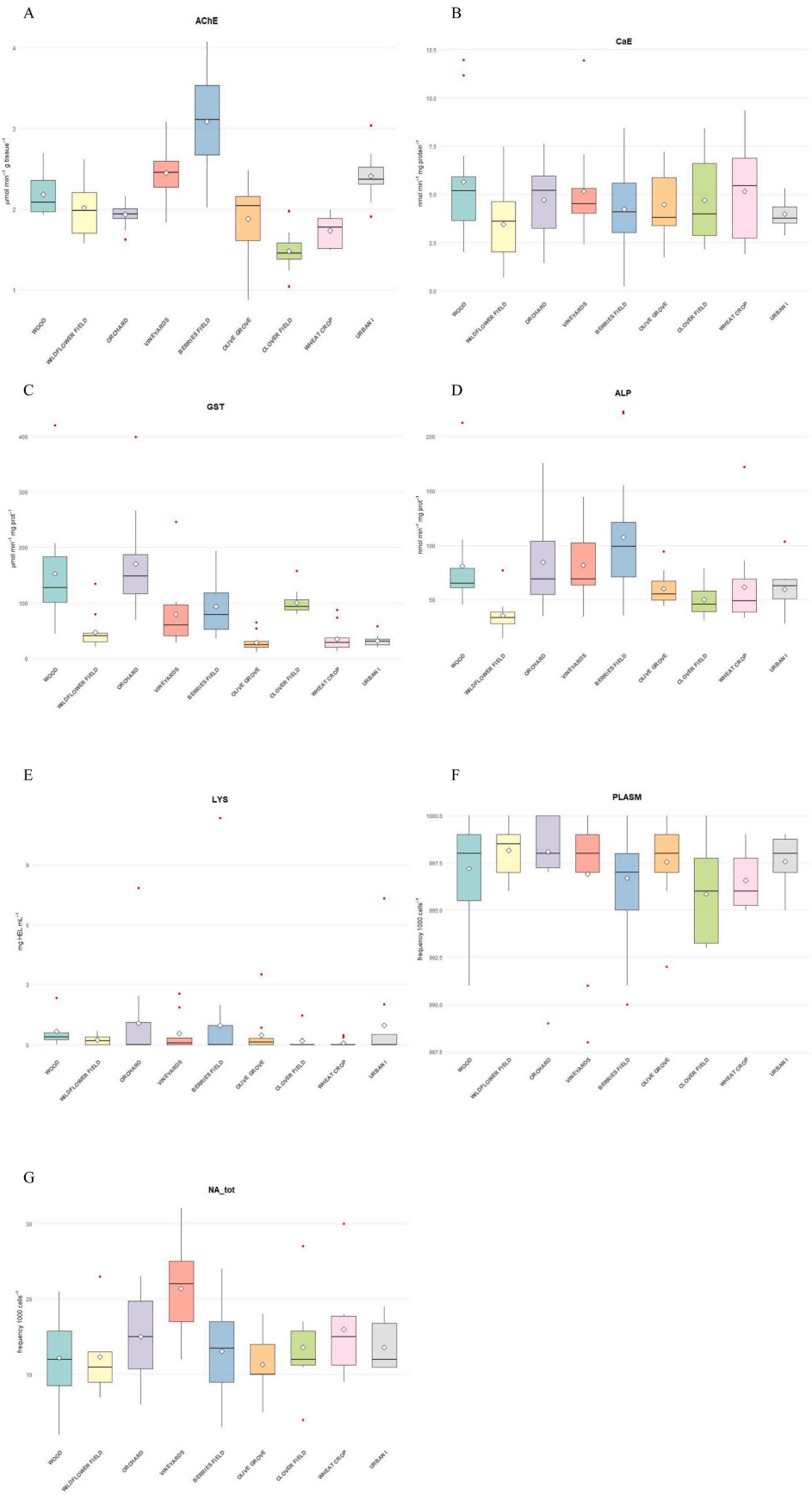

Fig. 3. Boxplots of the biomarkers measured in *Apis mellifera* sampled in 2021 across nine sites with varying degrees of anthropogenic impact, including high-naturality sites (wood and wildflower field), agricultural areas (orchard, vineyards, olive grove, berries field, wheat crop, clover field), and urban site: AChE (A); CaE (B); GST (C); ALP (D); LYS (E); differential haemocytes count (F) and NA assay (G).

wildflower field (z = −4.35, p = 0.0002), orchard (z = −3.93, p = 0.001), wheat crop (z = 5.97, p < 0.0001), clover field (z = −5.85, p < 0.0001) and urban area I (z = −3.46, p = 0.0056). Olive grove specimens exhibited statistically significant higher CaE activity with respect to wood (z = 3.76, p = 0.002), wildflower field (z = 3.63, p = 0.0031), orchard (z = −3.19, p = 0.0143) and wheat crop (z = 5.16, p < 0.0001).

GST (K-W χ^2_8 = 221.209, p = 0.0001) (Fig. 2C) mean values were significantly higher in the urban area II compared to wood (z = 7.41, p < 0.0001), wildflower (z = 11.03, p < 0.0001), clover field (z = −11.1, p < 0.0001) and orchard (z = −6.84, p < 0.0001), vineyards (z = 4.07, p = 0.0004), wheat crop (z = 5.16, p < 0.0001) and urban area I (z = 3.86, p = 0.001). The second highest activity was observed in olive grove being significantly different from wood (z = 4.97, p < 0.0001), wildflower field (z = 8.52, p < 0.0001), clover field (z = −8.53, p < 0.0001), wheat crop (z = 2.72, p = 0.0382) and orchard (z = −4.37, p = 0.0001). Vineyards samples showed statistically significant higher values compared to wood (z = 3.27, p = 0.0076), wildflower (z = 6.82, p < 0.0001), orchard (z = −2.62, p = 0.0469) and clover field (z = −6.79, p < 0.0001). Orchard specimens showed significant differences compared to wildflower field (z = 4.34, p = 0.0001), clover (z = −4.26, p = 0.0002) and urban area I ones (z = −2.99, p = 0.0182). Urban area I showed statistically significant higher values when compared to wood (z = 3.63, p = 0.0024), wildflower (z = 7.26, p < 0.0001) and clover field (z = −7.24, p < 0.0001). Wheat crop animals showed higher activity with respect to wildflower field specimens (z = 5.98, p < 0.0001) and clover ones (z = −5.94, p < 0.0001). Honey bees from the wood area showed higher activity compared to wildflower (z = −3.55, p = 0.0031) and clover field bees (z = −3.45, p = 0.0042).

Honey bees sampled in the olive grove showed the highest ALP activity (K-W χ^2_8 = 179.787, p = 0.0001) (Fig. 2D) that was found to be statistically significant with respect to wood (z = 6.06, p < 0.0001), wildflower field (z = 7.70, p < 0.0001), vineyards (z = 4.37, p = 0.0001), urban area I (z = 3.93, p = 0.0009) and urban area II (z = 9.49, p < 0.0001). Specimens from wheat crop showed differences compared to wood (z = 5.31, p < 0.0001), wildflower field animals (z = 6.98, p < 0.0001), and also to vineyards (z = −3.60, p = 0.0030), urban area I (z = −3.11, p = 0.0139) and urban area II ones (z = −8.80, p < 0.0001). Urban area I bees showed differences compared to wildflower (z = −1.62, p = 0.0009), clover field (z = 2.81, p = 0.0337) and urban area II (z = −5.69, p < 0.0001). Vineyard samples were found to be different from wildflower field (z = 3.14, p = 0.0134), clover field (z = 3.32, p = 0.0082), orchard (z = 2.77, p = 0.0355) and urban area II (z = −4.78, p < 0.0001). Orchard bees showed statistically significant differences compared to wood (z = 4.46, p = 0.0001), wildflower field (z = 6.13, p < 0.0001) and urban area II (z = 7.93, p < 0.0001). Clover field exhibited differences compared to wood (z = 5.02, p < 0.0001), wildflower (z = 6.69, p < 0.0001) and urban area II (z = 8.50, p < 0.0001). At last, the urban area II showed a difference also compared to wood (z = −3.30, p = 0.0083).

LYS activity (K-W χ^2_8 = 31.969, p = 0.0001) (Fig. 2E) was significantly higher in the wheat crop and urban area II honey bees. Urban area II samples showed differences compared to wood (z = 3.46, p = 0.0095), wildflower field (z = 3.06, p = 0.0354) and vineyards (z = 3.06, p = 0.0355). This enzyme activity was also found to be different in wheat crop with respect to wood (z = 3.29, p = 0.0169), and in clover field compared to wood (z = 2.96, p = 0.0437). Orchard showed differences compared to wood (z = 3.38, p = 0.0125), wildflower (z = 2.96, p = 0.0447) and vineyards (z = 2.97, p = 0.0450).

Urban area II showed a significant decrease in plasmatocyte numbers (K-W χ^2_8 = 43.779, p = 0.0001) (Fig. 2F), with a consequent increase in granulocytes presence, compared to wood (z = −3.66, p = 0.0041), wildflower field (z = −4.48, p = 0.0001), urban area I (z = −3.06, p = 0.0320). Olive grove showed lower numbers of plasmatocytes with differences compared to wood (z = −3.25, p = 0.0177) and wildflower field (z = −4.06, p = 0.0009). Vineyards honey bees were found to have differences compared to wildflower field ones (z = −3.58, p = 0.0054), while the clover ones were different from wildflower (z = −3.90, p = 0.0017).

The highest values of nuclear abnormalities (K-W χ^2_8 = 129.037, p = 0.0001) (Fig. 2G) were found in samples from olive grove compared to both wood (z = 3.61, p = 0.0027) and wildflower field (z = 4.41, p = 0.0001), clover field (z = −7.46, p < 0.0001), orchard (z = −4.43, p = 0.0001), vineyards (z = 7.31, p < 0.0001), wheat crop (z = 6.80, p < 0.0001) and urban area II (z = 6.77, p < 0.0001). Honey bees from urban area I exhibited differences compared to clover field (z = −6.25, p < 0.0001), vineyards (z = 6.04, p < 0.0001), wheat crop (z = 5.45, p < 0.0001) and urban area II (z = −5.41, p < 0.0001). Wood bees showed differences compared to clover field (z = −5.02, p < 0.0001), vineyards (z = −5.08, p < 0.0001), wheat crop (z = −4.47, p < 0.0001), urban area II (z = −4.44, p = 0.0001). Wildflower samples showed differences in NA assay compared to clover field (z = −4.15, p = 0.0003), vineyards (z = −4.42, p = 0.0001), wheat crop (z = −3.80, p = 0.0014) and urban area II (z = −3.76, p = 0.0016).

3.1.2. Results for the 2021 sampling year

AChE activity (K-W χ^2_9 = 245.235, p = 0.0001) (Fig. 3A) was significantly the lowest in samples coming from clover field, compared to wood (z = −6.15, p < 0.0001), wildflower field (z = −4.63, p = 0.0001), orchard (z = −3.63, p = 0.0028), vineyards (z = −8.28, p < 0.0001), berries field (z = 10.17, p < 0.0001), olive grove (z = −3.92, p = 0.0010), urban area I (z = −8.18, p < 0.0001). The second lowest activity was observed in bees from wheat crop compared to wood (z = −4.51, p = 0.0001), wildflower field (z = −3.00, p = 0.0217), vineyards (z = 6.64, p < 0.0001), berries field (z = 8.61, p < 0.0001) and urban area I (z = 6.44, p < 0.0001). Olive grove showed differences compared to vineyards (z = −4.35, p = 0.0002), berries field (z = 6.43, p < 0.0001) and urban area I (z = −4.16, p = 0.0004). Orchard samples were found to be different from vineyards (z = −4.65, p < 0.0001), berries field (z = 6.70, p < 0.0001) and urban area I (z = −4.45, p = 0.0001). Samples from wildflower field were different from vineyards (z = 3.65, p = 0.0028), berries field (z = 5.75, p < 0.0001) and urban area I (z = 3.45, p = 0.0054). Wood bees were found to be different from the ones coming from berries field (z = 4.31, p = 0.0002).

CaE (K-W χ^2_9 = 38.021, p = 0.0001) (Fig. 3B) values exhibited statistically significant differences in berries field samples compared to wildflower field (z = 4.00, p = 0.0014) and urban area I (z = 3.12, p = 0.0341). Vineyards specimens showed higher CaE activity compared to wildflower field (z = 3.08, p = 0.0037), as well as wheat crops with statistically significant higher activity compared to wildflower field (z = 3.08, p = 0.0365). Wood showed a significantly higher activity when compared to wildflower field (z = −3.36, p = 0.0155).

The highest GST (K-W χ^2_9 = 236.384, p = 0.0001) (Fig. 3C) activity was observed in the orchard that showed statistically significant differences when compared to wildflower (z = 7.24, p < 0.0001), berries field (z = −3.81, p = 0.0014), vineyards (z = 4.59, p = 0.0001), olive grove (z = 9.69, p < 0.0001), wheat crop (z = 8.88, p < 0.0001) and urban area I (z = 9.01, p < 0.0001). Vineyards specimens showed statistically lower activity compared to wood (z = −3.98, p = 0.0008), while higher activity compared to wheat crop (z = 4.29, p = 0.0002), olive grove (z = −5.10, p < 0.0001) and urban area I (z = −4.42, p = 0.0001). Berries field showed higher activity compared to wildflower field (z = 3.09, p = 0.0186), wheat crop (z = 4.65, p < 0.0001), olive grove (z = 5.43, p < 0.0001) and urban area I (z = 4.77, p < 0.0001), while a lower activity when compared to wood (z = −3.23, p = 0.012). Clover field possessed higher activity compared to olive grove (z = 7.49, p < 0.0001) and wheat crop (z = 6.68, p < 0.0001), to wildflower field (z = 5.05, p < 0.0001) and to urban area I (z = 6.81, p < 0.0001). Wood was found to be different from the olive grove

($z = -9.08$, $p < 0.0001$), wheat crop ($z = -8.27$, $p < 0.0001$) and urban area I specimens ($z = -8.40$, $p < 0.0001$). Wood was also significantly higher that wildflower field ($z = -6.63$, $p < 0.0001$).

The lowest ALP (K-W $\chi^2_9 = 141.835$, $p = 0.0001$) (Fig. 3D) activity was observed in samples from wildflower field compared to wood ($z = -6.44$, $p < 0.0001$), orchard ($z = 6.53$, $p < 0.0001$), vineyards ($z = 7.05$, $p < 0.0001$), berries field ($z = 7.49$, $p < 0.0001$), olive grove ($z = 4.38$, $p = 0.0002$), wheat crop ($z = 3.52$, $p = 0.0059$) and urban area I ($z = 4.34$, $p = 0.0002$). Honey bees from wood were found to be different from clover field ($z = -3.74$, $p = 0.0027$) and wheat crop ($z = -2.91$, $p = 0.0387$). Samples from berries field exhibited different ALP activity compared to clover field ($z = 4.92$, $p < 0.0001$), olive grove ($z = 3.30$, $p = 0.0118$), wheat crop ($z = 4.12$, $p = 0.0006$) and urban area I ($z = 3.34$, $p = 0.0111$). Orchard specimens showed differences compared to clover field ($z = -3.84$, $p = 0.0019$) and wheat crop ($z = 3.01$, $p = 0.0300$). Vineyards showed differences compared to clover field ($z = -4.35$, $p = 0.0002$) and wheat crop ($z = 3.52$, $p = 0.0061$).

The LYS (K-W $\chi^2_9 = 26.980$, $p = 0.0014$) (Fig. 3E) activity resulted in significantly lower levels in clover field ($z = -3.64$, $p = 0.0059$) and wheat crop ($z = -4.15$, $p = 0.0008$) specimens compared to the wood.

Significant differences in haemocytes differential count (K-W $\chi^2_9 = 25.894$, $p = 0.0021$) (Fig. 3F) from specimens in orchard were found compared to wheat crop ($z = 3.16$, $p = 0.0337$) and clover field ($z = -3.42$, $p = 0.0137$).

NA assay (K-W $\chi^2_9 = 52.874$, $p = 0.0001$) (Fig. 3G) exhibited the highest values in vineyards honey bees compared to wood ($z = 5.43$, $p < 0.0001$), wildflower field ($z = 4.38$, $p = 0.0003$), orchard ($z = -3.73$, $p = 0.0039$), berries field ($z = -6.00$, $p < 0.0001$), clover field ($z = -3.79$, $p = 0.0031$), olive grove ($z = -5.41$, $p < 0.0001$) and urban area I ($z = -3.55$, $p = 0.0072$).

3.2. Inter-annual comparison (2020 vs. 2021)

A comparison between the biomarker results obtained in the two years for each area was performed (Fig. 4). The berries field and the urban area II are not included in this comparison since the sampling in these areas was conducted only for one year. A significant decrease in AChE activity was observed in all sampled areas from 2020 to 2021 (Fig. 4A). CaE showed a significant increase in all samples from 2020 compared to the ones from 2021 (Fig. 4B). GST values, from 2020 to 2021, showed a significant increase in all areas except for the olive grove, the wheat crop and the urban area I, whose samples revealed a significant decrease in this enzyme's activity (Fig. 4C). A significant increase in ALP activity was observed in wood, orchard, vineyards and urban area I from 2020 to 2021, while a significant decrease was observed in clover field samples (Fig. 4D). Lysozyme activity decreased significantly in clover and wheat crop fields from 2020 to 2021 (Fig. 4E). On the contrary, from 2020 to 2021, a slight but significant increase in LYS activity was observed in wood, wildflower field and vineyards. Regarding plasmatocytes frequency, a significant decrease from 2020 to 2021, with a consequent increase in granulocytes, was observed in wood, wildflower field, clover field, wheat crop and urban area I (Fig. 4F). From 2020 to 2021, the NA assay revealed a significant increase in the frequency of nuclear abnormalities in clover field, orchard, vineyards, and wheat crop, while a significant decrease was observed in olive grove and urban area I (Fig. 4G).

The different responses obtained from the analysis between the two sampling years could be due also to climatic conditions, such as temperature and rain. In Tables 2 and 3, we reported the average temperatures (min and max) and the precipitation, respectively, for the month interested by the sampling (June and July 2020-2021). In these two years, there was a difference mainly in the rainfalls, even if the average temperatures of June were slightly higher in 2021 compared to 2020. Moreover, in 2021 rainfalls were less abundant with respect to the ones observed in 2020.

4. Discussion

The results of this study help fill a research gap regarding the field scenario and the impact that the multiple stressors have on the health of honey bees, a species facing global population decline. Overall, the biomarker responses indicated clear signs of physiological disturbance in honey bees from anthropized contexts whereas high-naturality areas exhibited comparatively lower stress levels. The two-years monitoring showed neurotoxic effects across all sites, while other biomarkers indicated more heterogeneous differences that aligned with land-use characteristics and the presence of multiple stressors.

In both years (2020 and 2021), the sites with a high degree of naturality, namely wood and wildflower field, showed lower biomarker values than the more anthropized areas, suggesting a lower degree of physiological disturbance than in urban and managed agricultural sites. These results are in line with previous studies showing that bees from less anthropized environments often exhibited a reduced activation of detoxification processes and lower immune alterations than bees from urban or intensively cultivated landscapes (Caliani et al., 2021b; Nicewicz et al., 2021). Moreover, bees from wildflower field exhibited the lowest ALP activity in 2021, which may indicate a different digestive or metabolic status compared with more agriculturally influenced sites, reflecting lower digestive demand or altered physiological balance under certain environmental conditions (Caliani et al., 2021a; Carvalho et al., 2013). At the same time, the relatively slight changes in GST and NA assay levels in the natural sites confirm that these areas remained less exposed to toxicological stress than vineyards or other cultivated areas (El-Seedi et al., 2022).

Observing the biomarker responses in two years, a progressive intensification of physiological stress can be observed. The simultaneous AChE decrease and CaE increase in wood and wildflower field indicate that even high-naturality areas are not completely free from environmental pressure, likely because bees forage over broad areas and can encounter diffuse contamination beyond the immediate site (Nicewicz et al., 2021), affecting cholinergic function and phase-I detoxification. The significant increase for ALP and lysozyme values may indicate an enhanced digestive and antimicrobial activity, potentially reflecting adaptive responses to shifting resource quality or slight contaminant presence. Most notably, the marked decline in plasmatocytes with granulocyte compensation suggests an immune defence mechanism, consistent with observations that high-naturality areas can still impose immune challenges due to pathogens or nutritional variability. All these data suggest that even in areas with higher floral diversity and lower anthropogenic disturbance, bees may still experience enzymatic modulation linked to local floral resource availability or landscape integrity.

Regarding urban areas, in 2020 organisms showed a lower AChE activity than in the other sites, which still fell within the expected physiological range of variation for this enzyme (around 15%), suggesting a moderate modulation rather than a clear neurotoxic effect. Notably, the observed levels are also consistent with those reported by Caliani et al. (2021b) in which no neural alterations were observed in urban areas. More interestingly, a marked induction of CaE and GST activities, as well as an impairment in haemocyte count and lysozyme activity were observed in the urban bees compared to the ones from wood and wildflower areas. Urban landscapes are increasingly recognized as environments that can impose substantial multistress pressure on colonies of pollinators: on the one hand, they may offer floral resources and on the other hand, they can also impose chronic pressure through high temperature conditions, habitat fragmentation and contamination (Ayers and Rehan, 2021). In urban environments, such changes as we observed have been linked to contamination by metals and other anthropogenic compounds, which may induce detoxification pathways and impair immune balance and leave bees more vulnerable to pathogens and secondary stressors (Caliani et al., 2021a, 2021b; Garner and Di Giulio, 2012; Mdaini et al., 2019; Nicewicz et al., 2021; Wu et al., 2007; Yu et al., 2012). In the study by Nicewicz et al. (2021)

Fig. 4. Comparison of biomarkers measured in *Apis mellifera* AChE (A); CaE (B); GST (C); ALP (D); LYS (E); differential haemocytes count (F) and NA assay (G) in samples from 2020 to 2021. ** indicate statistically significant differences with $p < 0.01$; * indicates statistically significant differences with $p < 0.05$.

Table 2
Average temperatures (min and max) expressed in °C for June and July 2020 and 2021 for the provinces of the sampling locations.

		2020		2021	
		June (min-max)	July (min-max)	June (min-max)	July (min-max)
Siena province	Rural area Wheat crop Urban area I	15.2-26.2	18.5-31.6	17.3-29.8	18.8-30.7
Pisa province	Clover field Orchard Wood Wildflower field	14.9-25.6	17.6-28.9	15.4-28.2	18.6-30
Arezzo province	Vineyards	13.6-25.4	16.2-30.3	15.5-30.6	16.9-32.1

Table 3
Rainfall expressed in mm for June and July 2020 and 2021 for the provinces of the sampling locations.

		2020		2021	
		June	July	June	July
Siena province	Rural area Wheat crop Urban area I	57	9	45	22
Pisa province	Clover field Orchard Wood Wildflower field	124	4	11	7
Arezzo province	Vineyards	96	9	23	3

bees from an urban rooftop apiary showed higher stress-related responses in gut and fat body tissues, supporting the hypothesis that colonies can be subjected to substantial multipressure that can be present in urban environments. Similarly, Caliani et al. (2021b) reported that urban bees exhibited biomarker responses consistent with immunosuppression and exposure to lipophilic compounds and metals. In 2021, although urban area still showed differences in some biomarkers (ALP and lysozyme) in comparison with other sites, the obtained responses were lower than in 2020, probably indicating that urban pressure was lower. In particular, the genotoxic pressure between the two years decreased, as shown by haemocytes count and NA assay values. COVID-19 public health restrictions (Summan and Nandi, 2022) drastically cut transport and industrial emissions (Zambrano-Monserrate et al., 2020), resulting in cleaner air across many urban centers (Gkatzelis et al., 2021; Kashibadze et al., 2026). PM2.5 declined by nearly 50%, NO_2 dropped by one-third, and land surfaces cooled by more than 5 °C (Gao and Yang, 2026). The COVID-19 lockdown measures implemented from late 2020 through much of 2021 could explain the milder biomarker responses and the relative improvement in honey bee health in 2021.

The agricultural sites (orchard, vineyards, olive grove, clover field, wheat crop, berries field) in 2020 and 2021 showed variable biomarker responses, indicating that agricultural land use impose highly site-specific stress patterns. GST has different roles in the organism; it has a detoxification function and also protects from oxidative stress (van Bladeren, 2000). Several studies reported changes in GST activity after the exposure to various pesticides, such as imidacloprid (Balieira et al., 2018; Dussaubat et al., 2016; Li et al., 2017; Paleolog et al., 2020), acetamiprid (Han et al., 2019), fipronil (Renzi et al., 2016), spinosad (Carvalho et al., 2013), bromfenvinphos (Strachecka et al., 2016), clothianidin (Abdelkader et al., 2018, 2019; Li et al., 2017; Tavares et al., 2019; Yanhua Wang et al., 2020; Yao et al., 2018; Zhu et al., 2017), glyphosate (Almasri et al., 2020), 2,4-Dichlorophenoxyacetic acid (Di Noi et al., 2024), alachlor (Fellows et al., 2022), azoxystrobin (Caliani et al., 2021a), atrazine (Fellows et al., 2022), uniconazole (Chen et al., 2026). Other stressors, such as pathogens (Jovanovic et al., 2023; Kunat-Budzyńska et al., 2025) and monofloral diet (Bryś et al., 2024), were demonstrated to increase detoxification processes mediated by GST and CYP450. The elevated GST values found in orchard, vineyard and berries field compared to the natural areas, in 2020, may indicate that GST is responding to mitigate the oxidative stress and to detoxify the organism from harmful stressors. In the same areas, increased ALP activity was found both in 2020 and 2021 indicating that metabolic functions were also affected in the sampled animals, compared to natural areas. ALP activity can be modulated by the presence of different compounds, like plant protection products alone and in combination (Murawska et al., 2025). Some pesticides may reduce ALP activity (Caliani et al., 2021a; Di Noi et al., 2024; Murawska et al., 2025; Paleolog et al., 2020, 2021; Wilde et al., 2016), while others increase it (Almasri et al., 2020; Badiou-Bénéteau et al., 2012; Migdal et al., 2022; Wang et al., 2020a,b). For instance, glyphosate in mixture with acetamiprid is able to increase ALP activity, while glyphosate combined with tebuconazole can decrease it (Murawska et al., 2025). Other environmental pressures can influence ALP activity, such as metals or persistent lipophilic compounds (Badawy et al., 2015; Badiou-Bénéteau et al., 2012); pathogens such as *Varroa destructor* can decrease its activity (Strachecka et al., 2016), conversely an increase can derive from high temperatures (Şapcaliu et al., 2010). ALP differences observed in this work point to possible effects on metabolic processes and physiological state of insects (Zibaee et al., 2011). While in 2020 no marked differences were found for AChE activity among the agricultural sites, the most notable effect in 2021 was the AChE inhibition in bees from clover field and wheat crop, which points to a neurotoxic burden in these areas (Boily et al., 2013; Frasco et al., 2005; Lupi et al., 2020). Since AChE is a sensitive biomarker of neurotoxic exposure, its reduced values in agricultural sites may indicate increased contact with compounds able to interfere with the neural transmission. Besides cholinesterase-inhibiting specific compounds such as organophosphate and carbamates (Ayoub et al., 2024; Čolović et al., 2013; Yao et al., 2018; Zhu et al., 2017), other classes of pesticides, like insecticides (Almasri et al., 2020; Dorigo et al., 2026; Yufei Wang et al., 2020; Zhu et al., 2017), herbicides (Almasri et al., 2020; Di Noi et al., 2024) and fungicides (Caliani et al., 2021a; Cang et al., 2023) were found to affect AChE activity causing its inhibition. Moreover, the olive grove area in 2020 appeared to be particularly relevant for DNA alterations, even when compared with the urban sites, while the strongest genotoxic damages in 2021 were observed in clover field, vineyards and orchard. In both years, agricultural areas with higher NA values also showed an increase in GST activity. Chemicals such as pesticides, metals and PAHs are able to cause oxidative stress due to the presence of Reactive Oxygen Species (ROS) (Chakrabarti et al., 2020; Olgun et al., 2020). ROS are able to attack DNA and produce various DNA lesions such as oxidized DNA bases, abasic regions, and DNA strand breaks, leading to genomic instability (Čolović et al., 2013; Olgun et al., 2020). Therefore, the simultaneous increase in both GST activity and NA assay values led to the hypothesis that bees were subjected to oxidative stress, which can result in DNA damage, consistently caused by cumulative effects due to repeated agricultural treatments.

The inter-annual comparison for the agricultural areas showed a general weakening of the organisms in 2021, as highlighted by the response of nervous, detoxification and immune systems and by the presence of DNA alterations. Environmental stress, due to pesticides and other contaminants, pathogens, malnutrition and temperatures can impair detoxification functions and immune and metabolic systems (Al Naggar et al., 2026). Pesticides are able to compromise the health status of organisms causing not only neurotoxicity and oxidative stress, but also impairing the microbial community in bees and reducing immunity abilities (Al Naggar et al., 2026; Daisley et al., 2020). Even other contaminants, including heavy metals, may affect bees immunity and metabolism (Rothman et al., 2019), thus making them more susceptible

environmental conditions. Pathogens can seriously arm honey bees health not only by enhancing the immune response but also causing an impairment in nutrient assimilation with a consequent general weakening (Al Naggar and Baer, 2019; Mahmoud et al., 2024; Paris et al., 2018). Environmental factors such as temperature, relative humidity, precipitation, wind speed, photoperiod, solar radiation and clouds are demonstrated to have a severe impact on honey bees (Zapata-Hernández et al., 2024). Temperature increase beyond bees' optimal 20-30 °C foraging range, make bees more vulnerable, impacting their gut microbiome and infection severity (Palmer-Young et al., 2018, 2019), food reserves (Gajardo-Rojas et al., 2022), plant-pollinator interaction (Maluf et al., 2022), survival (Aldea-Sánchez et al., 2021), thermal biology (Lima et al., 2019) and productivity (Vincze et al., 2025). A decrease in rainfall can affect the behaviour (Gordo et al., 2010), reduce honey production and plant-pollinator interactions (Gounari et al., 2022; Maluf et al., 2022). Additionally, the propagation of pests and diseases can be influenced by lower precipitations (Giliba et al., 2020; Hosni et al., 2022). We can hypothesize that the observed differences in analytical responses between 2020 and 2021 may partly derive from varying climatic conditions, particularly rainfall patterns and June temperatures, which were slightly higher in 2021 despite markedly lower precipitation compared to 2020. The review by Zapata-Hernández et al. (2024) observed that 45% of the analysed papers highlight that temperature increase could exert negative effects in *A. mellifera*. Water scarcity and elevated temperatures in 2021 likely stressed colonies, impairing foraging efficiency and triggering the observed biochemical and cellular alterations. Some studies have also demonstrated that temperature fluctuations could impair the gut microbiome composition by facilitating the vulnerability of bees to parasites (Palmer-Young et al., 2018, 2019). Our results let us hypothesize that bees were undergoing major stress during 2021, with an impairment of physiological parameters leading to higher vulnerability to different stressors. The analysis performed in this study do not give a certain cause-effect reason to the observed alterations, since no pesticides residues and pathogens were analysed; however, the interaction of the above-mentioned kind of stressors can be pictured as a reasonable explanation for the resulted differences in bees health status.

5. Conclusion

Our monitoring study, which was based on a wide set of biomarkers, proved to be an effective tool for detecting sublethal effects of environmental stressors before colony-level damage becomes visible, supporting its utility for ecotoxicological risk assessment on *Apis mellifera*. This two-year field approach demonstrated that honey bees from anthropized environments exhibit clear signs of physiological disturbance, while bees from areas with a high degree of naturality show comparatively lower stress levels. The inter-annual comparison also highlighted that honey bee toxicological health can be influenced not just by a single pressure, but by multiple stressors, including rising temperatures, rainfall, and chemical compounds, underscoring the value of multi-year monitoring to effectively evaluate their ecotoxicological status. Finally, the study revealed a negative impact of diverse stressors on *A. mellifera*, and emphasized the urgent need to integrate biomarker analyses with pesticide residue monitoring and microbiome assessments to establish definitive cause-effect relationships and develop more comprehensive ecotoxicological risk assessment frameworks for pollinator conservation.

Declaration of generative AI and AI-assisted technologies in the manuscript preparation process

During the preparation of this work, the authors used “Gemini” as AI for English revision. The authors reviewed and edited the output as needed and take full responsibility for the content of the published article.

CRediT authorship contribution statement

Agata Di Noi: Conceptualization, Data curation, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing. **Ilaria Caliani:** Conceptualization, Data curation, Methodology, Supervision, Writing – original draft, Writing – review & editing. **Matteo Vitale:** Investigation, Methodology. **Tommaso Campani:** Investigation, Methodology, Writing – review & editing. **Antonella D'Agostino:** Formal analysis, Writing – review & editing. **Giampiero Cai:** Writing – review & editing. **Marco Romi:** Writing – review & editing. **Silvia Casini:** Conceptualization, Data curation, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

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

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2026.125038.

Data availability

Data will be made available on request.

References

Abdelkader, B.F., Kairo, G., Tchamitchian, S., Bonnet, M., Cousin, M., Barbouche, N., Belzunces, L.P., Brunet, J.-L., 2018. Effects of clothianidin exposure on semen parameters of honey bee drones. J. New Sci. 59, 791–3798.

Abdelkader, F.B., Kairo, G., Bonnet, M., Barbouche, N., Belzunces, L.P., Brunet, J.L., 2019. Effects of clothianidin on antioxidant enzyme activities and malondialdehyde level in honey bee drone semen. J. Apicult. Res. 58, 740–745. https://doi.org/10.1080/00218839.2019.1655182.

Al Naggar, Y., Ghramh, H.A., Elfarnawany, A., Mohamed, A., 2026. How environmental and ecological stressors reprogram honey bee chemistry through the microbiome–metabolome axis. Insects 17, 336. https://doi.org/10.3390/insects17030336.

Al Naggar, Y., Baer, B., 2019. Consequences of a short time exposure to a sublethal dose of flupyradifurone (Sivanto) pesticide early in life on survival and immunity in the honeybee (Apis mellifera). Sci. Rep. 9, 19753. https://doi.org/10.1038/s41598-019-56224-1.

Alarcón, M., Castelli, L., Branchiccela, B., Invernizzi, C., Antúnez, K., 2026. Mitigating the impacts of nutritional stress on honey bee colonies located in monoculture eucalyptus plantations. Apidologie 57, 7. https://doi.org/10.1007/s13592-025-01242-4.

Alaux, C., Brunet, J.-L., Dussaubat, C., Mondet, F., Tchamitchan, S., Cousin, M., Brillard, J., Baldy, A., Belzunces, L.P., Le Conte, Y., 2010. Interactions between Nosema microspores and a neonicotinoid weaken honeybees (Apis mellifera). Environ. Microbiol. 12, 774–782. https://doi.org/10.1111/j.1462-2920.2009.02123.x.

Aldea-Sánchez, P., Ramírez-Cáceres, G.E., Rezende, E.L., Bozinovic, F., 2021. Heat tolerance, energetics, and thermal treatments of honeybees parasitized with varroa. Front. Ecol. Evol. 9. https://doi.org/10.3389/fevo.2021.656504.

Almasri, H., Tavares, D.A., Pioz, M., Sené, D., Tchamitchian, S., Cousin, M., Brunet, J.-L., Belzunces, L.P., 2020. Mixtures of an insecticide, a fungicide and a herbicide induce high toxicities and systemic physiological disturbances in winter Apis mellifera honey bees. Ecotoxicol. Environ. Saf. 203, 111013. https://doi.org/10.1016/j.ecoenv.2020.111013.

Şapcaliu, A., Pavel, C., Savu, V., Căuia, E., Matei, M., Rădoi, I., 2010. Biochemical and cytological investigations on Haemolymph of Apis Mellifera Carpathica Bee in stressful conditions. Bull. Univ. Agric. Sci. Vet. Med. Cluj-Napoca - Anim. Sci. Biotechnol. 67, 313.

Șapcaliu, A., Pavel, C., Căuia, E., Rădoi, I., Tudor, N., et al., 2009. Morpho-cytometric investigations on haemolymph collected from honeybees originated from south of Romania. Bulletin UASVM Animal Science and Biotechnologies 66, 270–275.

Askri, D., Pottier, M., Arafah, K., Voisin, S.N., Simon, H., Stout, J.C., Dominik, C., Schweiger, O., Tamburini, G., Pereira-Peixoto, H., Klein, A.-M., Martinéz Lopez, V., De la Rùa, P., Cini, E., Potts, S.G., Schwarz, J.M., Knauer, A.C., Albrecht, M., Raimets, R., Karise, R., Di Prisco, G., Ivarsson, K., Svensson, G.P., Ronsevych, O., Knapp, J.L., Rundlöf, M., Onorati, P., de Miranda, J.R., Bocquet, M., Bulet, P., 2024. A blood test to monitor bee health across a European network of agricultural sites of different land-use by MALDI BeeTyping mass spectrometry. Sci. Total Environ. 929, 172239. https://doi.org/10.1016/j.scitotenv.2024.172239.

Ayers, A.C., Rehan, S.M., 2021. Supporting bees in cities: how bees are influenced by local and landscape features. Insects 12. https://doi.org/10.3390/insects12020128.
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., Arifie, U., Alwasel, Y.A., Alwahibi, M.S., 2024. Exposure to organophosphate insecticides induces behavioral changes and acetylcholinesterase inhibition in Apis mellifera. Ecotoxicol. Environ. Saf. 287, 117279. https://doi.org/10.1016/j.ecoenv.2024.117279.
Badawy, M.E.I., Nasr, H.M., Rabea, E.I., 2015. Toxicity and biochemical changes in the honey bee Apis mellifera exposed to four insecticides under laboratory conditions. Apidologie 46, 177–193. https://doi.org/10.1007/s13592-014-0315-0.
Badiou, A., Meled, M., Belzunces, L.P., 2008. Honeybee Apis mellifera acetylcholinesterase—A biomarker to detect deltamethrin exposure. Ecotoxicol. Environ. Saf. 69, 246–253. https://doi.org/10.1016/j.ecoenv.2006.11.020.
Badiou-Bénéteau, A., Carvalho, S.M., Brunet, J.-L., Carvalho, G.A., Buleté, A., Giroud, B., Belzunces, L.P., 2012. Development of biomarkers of exposure to xenobiotics in the honey bee Apis mellifera: application to the systemic insecticide thiamethoxam. Ecotoxicol. Environ. Saf. 82, 22–31. https://doi.org/10.1016/j.ecoenv.2012.05.005.
Balieira, K.V.B., Mazzo, M., Bizerra, P.F.V., Guimarães, A.R., de, J.S., Nicodemo, D., Mingatto, F.E., 2018. Imidacloprid-induced oxidative stress in honey bees and the antioxidant action of caffeine. Apidologie 49, 562–572. https://doi.org/10.1007/s13592-018-0583-1.
Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. Roy. Stat. Soc. B 57, 289–300. https://doi.org/10.1111/j.2517-6161.1995.tb02031.x.
Boily, M., Sarrasin, B., DeBlois, C., Aras, P., Chagnon, M., 2013. Acetylcholinesterase in honey bees (Apis mellifera) exposed to neonicotinoids, atrazine and glyphosate: laboratory and field experiments. Environ. Sci. Pollut. Res. 20, 5603–5614. https://doi.org/10.1007/s11356-013-1568-2.
Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
Branchiccela, B., Castelli, L., Corona, M., Díaz-Cetti, S., Invernizzi, C., Martínez de la Escalera, G., Mendoza, Y., Santos, E., Silva, C., Zunino, P., Antúnez, K., 2019. Impact of nutritional stress on the honeybee colony health. Sci. Rep. 9, 10156. https://doi.org/10.1038/s41598-019-46453-9.
Briscoe, K., Byarlay, M., Pudasaini, R., Johnson, R., Rehan, S., Li-Byarlay, H., 2026. Oxidative stress in honey bees (Apis mellifera) and small carpenter bees (Ceratina calcarata) across different landscapes. Physiol. Entomol. 1–11. https://doi.org/10.1111/phen.70046.
Brodschneider, R., Crailsheim, K., 2010. Nutrition and health in honey bees. Apidologie 41, 278–294. https://doi.org/10.1051/apido/2010012.
Bryś, M.S., 2025. Analysis of panels of chemical biomarkers in the honeybee in hemolymph and fat body in response to physiological and environmental factors. Metabolites 15, 743. https://doi.org/10.3390/metabo15110743.
Bryś, M.S., Staniec, B., Strachecka, A., 2024. The effect of pollen monodiets on fat body morphology parameters and energy substrate levels in the fat body and hemolymph of Apis mellifera L. workers. Sci. Rep. 14, 15177. https://doi.org/10.1038/s41598-024-64598-0.
Burkle, L.A., Marlin, J.C., Knight, T.M., 2013. Plant-Pollinator interactions over 120 years: loss of species, co-occurrence, and function. Science 339, 1611–1615. https://doi.org/10.1126/science.1232728.
Caliani, I., Campani, T., Conti, B., Cosci, F., Bedini, S., D'Agostino, A., Ammendola, A., Di Noi, A., Gori, A., Casini, S., 2021a. Multi-biomarker approach and IBR index to evaluate the effects of different contaminants on the ecotoxicological status of apis mellifera. Ecotoxicol. Environ. Saf. 208, 111486. https://doi.org/10.1016/j.ecoenv.2020.111486.
Caliani, I., Campani, T., Conti, B., Cosci, F., Bedini, S., D'Agostino, A., Giovanetti, L., Di Noi, A., Casini, S., 2021b. First application of an integrated biological response index to assess the ecotoxicological status of honeybees from rural and urban areas. Environ. Sci. Pollut. Res. 28, 47418–47428. https://doi.org/10.1007/s11356-021-14037-8.
Campani, T., Di Noi, A., Manieri, G., Caliani, I., Casini, S., 2026. Evaluation of the health status of apis mellifera in relation to the use of plant protection products in viticulture through a multi-biomarker approach. Toxics 14, 176. https://doi.org/10.3390/toxics14020176.
Cang, T., Lou, Y., Zhu, Y.-C., Li, W., Weng, H., Lv, L., Wang, Y., 2023. Mixture toxicities of tetrachlorantraniliprole and tebuconazole to honey bees (Apis mellifera L.) and the potential mechanism. Environ. Int. 172, 107764. https://doi.org/10.1016/j.envint.2023.107764.
Carvalho, S.M., Belzunces, L.P., Carvalho, G.A., Brunet, J.-L., Badiou-Beneteau, A., 2013. Enzymatic biomarkers as tools to assess environmental quality: a case study of exposure of the honeybee Apis mellifera to insecticides: biomarker responses in honeybees exposed to pesticides. Environ. Toxicol. Chem. 32, 2117–2124. https://doi.org/10.1002/etc.2288.
Castelli, L., Balbuena, S., Branchiccela, B., Zunino, P., Liberti, J., Engel, P., Antúnez, K., 2021. Impact of chronic exposure to sublethal doses of glyphosate on honey bee immunity, gut microbiota and infection by pathogens. Microorganisms 9, 845. https://doi.org/10.3390/microorganisms9040845.
Castelli, L., Branchiccela, B., Garrido, M., Invernizzi, C., Porrini, M., Romero, H., Santos, E., Zunino, P., Antúnez, K., 2020. Impact of nutritional stress on honeybee gut microbiota, immunity, and Nosema ceranae infection. Microb. Ecol. 80, 908–919. https://doi.org/10.1007/s00248-020-01538-1.
Chakrabarti, P., Carlson, E.A., Lucas, H.M., Melathopoulos, A.P., Sagili, R.R., 2020. Field rates of SivantoTM (flupyradifurone) and transform® (sulfoxaflor) increase oxidative stress and induce apoptosis in honey bees (Apis mellifera L.). PLoS One 15, e0233033. https://doi.org/10.1371/journal.pone.0233033.
Chen, L., Liu, Q., Xu, M., Mao, L., Liu, X., Wang, D., Wu, C., Wang, Y., 2026. Synergistic impacts of flupyradifurone and uniconazole on oxidative stress biomarkers and immune-related gene expression in honey bees. Environ. Sci. Eur. 38, 6. https://doi.org/10.1186/s12302-025-01280-w.
Cherednichenko, O., Magda, I., Nuraliyev, S., Pilyugina, A., Azizbekova, D., 2024. Cytome analysis (micronuclei and nuclear anomalies) in bioindication of environmental pollution in animals with nuclear erythrocytes. Heliyon 10, e37643. https://doi.org/10.1016/j.heliyon.2024.e37643.
Choi, J.-Y., Chon, K., Kim, J., Vasamsetti, B.M.K., Kim, B.-S., Yoon, C.-Y., Hwang, S., Park, K.-H., Lee, J.-H., 2024. Assessment of lambda-cyhalothrin and Spinetoram toxicity and their effects on the activities of antioxidant enzymes and acetylcholinesterase in honey bee (Apis mellifera) larvae. Insects 15. https://doi.org/10.3390/insects15080587.
Crailsheim, K., 1990. The protein balance of the honey bee worker. Apidologie 21, 417–429. https://doi.org/10.1051/apido:19900504.
Cunningham, M.M., Tran, L., McKee, C.G., Ortega Polo, R., Newman, T., Lansing, L., Griffiths, J.S., Bilodeau, G.J., Rott, M., Marta Guarna, M., 2022. Honey bees as biomonitors of environmental contaminants, pathogens, and climate change. Ecol. Indic. 134, 108457. https://doi.org/10.1016/j.ecolind.2021.108457.
da Luz, G.F., Santana, W.C., Santos, C.G., Medeiros Santana, L., Serrão, J.E., 2022. Cuticle melanization and the expression of immune-related genes in the honeybee Apis mellifera (Hymenoptera: Apidae) adult workers. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 257, 110679. https://doi.org/10.1016/j.cbpb.2021.110679.
Daisley, B.A., Chmiel, J.A., Pitek, A.P., Thompson, G.J., Reid, G., 2020. Missing microbes in bees: how systematic depletion of key symbionts erodes immunity. Trends Microbiol. 28, 1010–1021. https://doi.org/10.1016/j.tim.2020.06.006.
de Souza, I.L.B., Macarini, L.C., de Oliveira, C.M.R., Ferreira, N.G.C., Bittencourt Guimarães, A.T., 2026. Effects of anthropogenic stress on stingless bees Melipona mandacaia (Hymenoptera: Apidae) inhabiting urban and natural environments-dataset. Data Brief 66, 112657. https://doi.org/10.1016/j.dib.2026.112657.
Decio, P., Miotelo, L., Pereira, F.D.C., Roat, T.C., Marin-Morales, M.A., Malaspina, O., 2021. Enzymatic responses in the head and midgut of Africanized Apis mellifera contaminated with a sublethal concentration of thiamethoxam. Ecotoxicol. Environ. Saf. 223, 112581. https://doi.org/10.1016/j.ecoenv.2021.112581.
Decourtye, A., Devillers, J., Genecque, E., Menach, K.L., Budzinski, H., Cluzeau, S., Pham-Delgue, M.H., 2005. Comparative sublethal toxicity of nine pesticides on olfactory learning performances of the honeybee Apis mellifera. Arch. Environ. Contam. Toxicol. 48, 242–250. https://doi.org/10.1007/s00244-003-0262-7.
Di Noi, A., Caliani, I., D'Agostino, A., Cai, G., Romi, M., Campani, T., Ferrante, F., Baracchi, D., Casini, S., 2024. Assessing the effects of a commercial fungicide and an herbicide, alone and in combination, on Apis mellifera: insights from biomarkers and cognitive analysis. Chemosphere 359, 142307. https://doi.org/10.1016/j.chemosphere.2024.142307.
Dinno, A., 2015. Nonparametric pairwise multiple comparisons in independent groups using Dunn's test. STATA J. 15 (1), 292–300.
Dorigo, A.S., Miotelo, L., Grella, T.C., Antonia Tavares, D., Nocelli, R.C.F., Malaspina, O., De Souza Rosa-Fontana, A., 2026. Species-specific responses of bees to flupyradifurone: enzymatic stress biomarkers reveal higher sensitivity in Neotropical larvae compared to Apis mellifera. Environ. Toxicol. Pharmacol. 124, 105035. https://doi.org/10.1016/j.etap.2026.105035.
Doublet, V., Poeschl, Y., Gogol-Döring, A., Alaux, C., Annoscia, D., Aurori, C., Barribeau, S.M., Bedoya-Reina, O.C., Brown, M.J.F., Bull, J.C., Flenniken, M.L., Galbraith, D.A., Genersch, E., Gisder, S., Grosse, I., Holt, H.L., Hultmark, D., Lattorff, H.M.G., Le Conte, Y., Manfredini, F., McMahon, D.P., Moritz, R.F.A., Nazzi, F., Niño, E.L., Nowick, K., Van Rij, R.P., Paxton, R.J., Grozinger, C.M., 2017. Unity in defence: honeybee workers exhibit conserved molecular responses to diverse pathogens. BMC Genom. 18, 207. https://doi.org/10.1186/s12864-017-3597-6.
Dussaubat, C., Maisonnasse, A., Crauser, D., Tchamitchian, S., Bonnet, M., Cousin, M., Kretzschmar, A., Brunet, J.-L., Le Conte, Y., 2016. Combined neonicotinoid pesticide and parasite stress alter honeybee queens' physiology and survival. Sci. Rep. 6, 31430. https://doi.org/10.1038/srep31430.
El-Seedi, H.R., Ahmed, H.R., El-Wahed, A.A.A., Saeed, A., Algethami, A.F., Attia, N.F., Guo, Z., Musharraf, S.G., Khatib, A., Alsharif, S.M., Naggar, Y.A., Khalifa, S.A.M., Wang, K., 2022. Bee stressors from an immunological perspective and strategies to improve bee health. Vet. Sci. 9. https://doi.org/10.3390/vetsci9050199.
Fahrenholz, L., Lamprecht, I., Schricker, B., 1989. Thermal investigations of a honey bee colony: thermoregulation of the hive during summer and winter and heat production of members of different bee castes. J. Comp. Physiol. B 159, 551–560. https://doi.org/10.1007/BF00694379.
Fellows, C.J., Anderson, T.D., Swale, D.R., 2022. Acute toxicity of atrazine, alachlor, and chlorpyrifos mixtures to honey bees. Pestic. Biochem. Physiol. 188, 105271. https://doi.org/10.1016/j.pestbp.2022.105271.
Frasco, M.F., Fournier, D., Carvalho, F., Guilhermino, L., 2005. Do metals inhibit acetylcholinesterase (AChE)? Implementation of assay conditions for the use of AChE activity as a biomarker of metal toxicity. Biomarkers 10, 360–375. https://doi.org/10.1080/13547500500264660.
Gajardo-Rojas, M., Muñoz, A.A., Barichivich, J., Klock-Barría, K., Gayo, E.M., Fontúrbel, F.E., Olea, M., Lucas, C.M., Veas, C., 2022. Declining honey production and beekeeper adaptation to climate change in Chile. Prog. Phys. Geogr. Earth Environ. 46, 737–756. https://doi.org/10.1177/03091333221093757.
Gao, Y., Yang, X., 2026. Urban ecosystem responses to human activity shifts: multi-year evidence from New York City before, during, and after the COVID-19 pandemic. Ecosystems 29, 30. https://doi.org/10.1007/s10021-026-01051-5.
Garner, L.V.T., Di Giulio, R.T., 2012. Glutathione transferase pi class 2 (GSTp2) protects against the cardiac deformities caused by exposure to PAHs but not PCB-126 in

zebrafish embryos. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 155, 573–579. https://doi.org/10.1016/j.cbpc.2012.01.007.
Ghazoul, J., 2005. Buzziness as usual? Questioning the global pollination crisis. Trends Ecol. Evol. 20, 367–373. https://doi.org/10.1016/j.tree.2005.04.026.
Giliba, R.A., Mpinga, I.H., Ndimuligo, S.A., Mpanda, M.M., 2020. Changing climate patterns risk the spread of Varroa destructor infestation of African honey bees in Tanzania. Ecol. Process. 9, 48. https://doi.org/10.1186/s13717-020-00247-4.
Gkatzelis, G.I., Gilman, J.B., Brown, S.S., Eskes, H., Gomes, A.R., Lange, A.C., McDonald, B.C., Peischl, J., Petzold, A., Thompson, C.R., Kiendler-Scharr, A., 2021. The global impacts of COVID-19 lockdowns on urban air pollutionA critical review and recommendations. Elem. Sci. Anth. 9. https://doi.org/10.1525/elementa.2021.00176.
Glavan, G., Lazarus, M., Bituh, T., Sekovanić, A., Petrinec, B., Haneklaus, N., Filipi, J., 2026. Physiological markers and trace elements in honey bees residing phosphogypsum deposition site: field and laboratory scenario. Ecotoxicol. Environ. Saf. 315, 120112. https://doi.org/10.1016/j.ecoenv.2026.120112.
Gordo, O., Sanz, J.J., Lobo, J.M., 2010. Determining the environmental factors underlying the spatial variability of insect appearance phenology for the honey bee, Apis mellifera, and the small white, Pieris rapae. J. Insect Sci. 10, 34. https://doi.org/10.1673/031.010.3401.
Goulson, D., Nicholls, E., Botias, C., Rotheray, E.L., 2015. Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. Science 347. https://doi.org/10.1126/science.1255957, 1255957–1255957.
Gounari, S., Proutsos, N., Goras, G., 2022. How does weather impact on beehive productivity in a mediterranean island? Italian. J. Agrometeorol. 65–81. https://doi.org/10.36253/ijam-1195.
Han, W., Yang, Y., Gao, J., Zhao, D., Ren, C., Wang, S., Zhao, S., Zhong, Y., 2019. Chronic toxicity and biochemical response of Apis cerana cerana (Hymenoptera: Apidae) exposed to acetamiprid and propiconazole alone or combined. Ecotoxicology 28, 399–411. https://doi.org/10.1007/s10646-019-02030-4.
Hatfield, M.J., Umans, R.A., Hyatt, J.L., Edwards, C.C., Wierdl, M., Tsurkan, L., Taylor, M.R., Potter, P.M., 2016. Carboxylesterases: general detoxifying enzymes. Chem. Biol. Interact. 259, 327–331. https://doi.org/10.1016/j.cbi.2016.02.011.
Hosni, E.M., Al-Khalaf, A.A., Nasser, M.G., Abou-Shaara, H.F., Radwan, M.H., 2022. Modeling the potential global distribution of honeybee Pest, Galleria mellonella under changing climate. Insects 13, 484. https://doi.org/10.3390/insects13050484.
Jackson, C.J., Liu, J.-W., Carr, P.D., Younus, F., Coppin, C., Meirelles, T., Lethier, M., Pandey, G., Ollis, D.L., Russell, R.J., Weik, M., Oakeshott, J.G., 2013. Structure and function of an insect α-carboxylesterase (α Esterase 7) associated with insecticide resistance. Proc. Natl. Acad. Sci. USA. 110, 10177–10182. https://doi.org/10.1073/pnas.1304097110.
Jovanovic, N.M., Glavinic, U., Ristanic, M., Vejnovic, B., Ilic, T., Stevanovic, J., Stanimirovic, Z., 2023. Effects of plant-based supplement on oxidative stress of honey bees (Apis mellifera) infected with Nosema ceranae. Animals 13, 3543. https://doi.org/10.3390/ani13223543.
Kairo, G., Provost, B., Tchamitchian, S., Ben Abdelkader, F., Bonnet, M., Cousin, M., Sénéchal, J., Benet, P., Kretzschmar, A., Belzunces, L.P., Brunet, J.-L., 2016. Drone exposure to the systemic insecticide Fipronil indirectly impairs queen reproductive potential. Sci. Rep. 6, 31904. https://doi.org/10.1038/srep31904.
Kashibadze, T., Kiladze, N., Ruadze, E., 2026. Impact of COVID-19 lockdowns on air pollution in Tbilisi. Environ. Monit. Assess. 198, 7. https://doi.org/10.1007/s10661-025-14883-w.
Kennedy, C.M., Lonsdorf, E., Neel, M.C., Williams, N.M., Ricketts, T.H., Winfree, R., Bommarco, R., Brittain, C., Burley, A.L., Cariveau, D., Carvalheiro, L.G., Chacoff, N. P., Cunningham, S.A., Danforth, B.N., Dudenhöffer, J.-H., Elle, E., Gaines, H.R., Garibaldi, L.A., Gratton, C., Holzschuh, A., Isaacs, R., Javorek, S.K., Jha, S., Klein, A. M., Krewenka, K., Mandelik, Y., Mayfield, M.M., Morandin, L., Neame, L.A., Otieno, M., Park, M., Potts, S.G., Rundlöf, M., Saez, A., Steffan-Dewenter, I., Taki, H., Viana, B.F., Westphal, C., Wilson, J.K., Greenleaf, S.S., Kremen, C., 2013. A global quantitative synthesis of local and landscape effects on wild bee pollinators in agroecosystems. Ecol. Lett. 16, 584–599. https://doi.org/10.1111/ele.12082.
Kruskal, W.H., Wallis, W.A., 1952. Use of ranks in one-criterion variance analysis. J. Am. Stat. Assoc. 47, 583–621.
Kunat-Budzyńska, M., Łabuć, E., Ptaszyńska, A.A., 2025. Changes in enzymatic activity and oxidative stress in honeybees kept in the apiary and laboratory conditions during the course of nosemosis. PLoS One 20, e0317384. https://doi.org/10.1371/journal.pone.0317384.
Lazarov, S., Zhelyazkova, I., Salkova, D., Shumkova, R., Takova, S., 2016. Lysozyme levels in haemolymph of worker bees (Apis mellifera L.) from bee colonies with different degree of expression of hygienic behaviour. Agricultural Science and Technology 8 (3), 201–204. https://doi.org/10.15547/ast.2016.03.037, 2016.
Li, Z., Li, M., He, J., Zhao, X., Chaimanee, V., Huang, W.-F., Nie, H., Zhao, Y., Su, S., 2017. Differential physiological effects of neonicotinoid insecticides on honey bees: a comparison between Apis mellifera and Apis cerana. Pestic. Biochem. Physiol. 140, 1–8. https://doi.org/10.1016/j.pestbp.2017.06.010.
Lima, M.V., Pascoal, L.A.F., Saraiva, E.P., Soares, K.O., Queiroz, J.P.A.F. de, Evangelista-Rodrigues, A., 2019. Individual acclimatization of Apis mellifera L. to the thermal homeostasis of the colony. Sociobiology 66, 81–87. https://doi.org/10.13102/sociobiology.v66i1.3378.
Lupi, D., Tremolada, P., Colombo, M., Giacchini, R., Benocci, R., Parenti, P., Parolini, M., Zambon, G., Vighi, M., 2020. Effects of pesticides and electromagnetic fields on honeybees: a field study using biomarkers. Int. J. Environ. Res. 14, 107–122. https://doi.org/10.1007/s41742-019-00242-4.
Mackei, M., Kámán-Tóth, E., Mátis, G., Neogrády, Z., Fébel, H., Huber, F., 2026. Sublethal deltamethrin exposure dysregulates brain fatty acid homeostasis in honey bee (Apis mellifera). Environ. Pollut. 399, 128204. https://doi.org/10.1016/j.envpol.2026.128204.
Mahmoud, S.H., Kandel, M., El-Seedi, H., Al Naggar, Y., 2024. Honey bee venom promotes the immune system and reduces Vairimorpha (Nosema) ceranae infection in honey bees (Apis mellifera L.). Apidologie 55, 8. https://doi.org/10.1007/s13592-023-01048-2.
Maluf, R.P., Alzate-Marin, A.L., Silva, C.C., Pansarin, L.M., Bonifácio-Anacleto, F., Schuster, I., de Mello Prado, R., Martinez, C.A., 2022. Warming and soil water availability affect plant–flower visitor interactions for Stylosanthes capitata, a tropical forage legume. Sci. Total Environ. 817, 152982. https://doi.org/10.1016/j.scitotenv.2022.152982.
Mann, H.B., Whitney, D.R., 1947. On a test of whether one of two random variables is stochastically larger than the other. Ann. Math. Statist. 18, 50–60. https://doi.org/10.1214/aoms/1177730491.
Marangi, M., Papini, R.A., Furii, G., Gordigiani, L., Di Noi, A., Fossi, M.C., Casini, S., Caliani, I., 2024. Parasitological investigation and potential effects of parasitism on hematological, genotoxic and immune parameters in free ranging sea turtles rescued along the Southern Adriatic coast (Eastern Mediterranean Sea). Mar. Pollut. Bull. 209, 117274. https://doi.org/10.1016/j.marpolbul.2024.117274.
Mdaini, Z., El Cafsi, M., Tremblay, R., Pharand, P., Gagné, J.-P., 2019. Spatio-temporal variability of biomarker responses and lipid composition of Marphysa sanguinea, Montagu (1813) in the anthropic impacted lagoon of Tunis. Mar. Pollut. Bull. 144, 275–286. https://doi.org/10.1016/j.marpolbul.2019.04.065.
Migdal, P., Murawska, A., Berbeć, E., Plotnik, M., Skorus, A., Latarowski, K., 2022. Selected biochemical markers change after oral administration of pesticide mixtures in honey bees. Toxics 10, 590. https://doi.org/10.3390/toxics10100590.
Monchanin, C., Drujont, E., Devaud, J.-M., Lihoreau, M., Barron, A.B., 2021. Metal pollutants have additive negative effects on honey bee cognition. J. Exp. Biol. 224. https://doi.org/10.1242/jeb.241869.
Murawska, A., Berbeć, E., Latarowski, K., Roman, A., Migdał, P., 2025. Semi-field studies on biochemical markers of honey bee workers (Apis mellifera) after exposure to pesticides and their mixtures. PLoS One 20, e0309567. https://doi.org/10.1371/journal.pone.0309567.
Nicewicz, Ł., Nicewicz, A.W., Kafel, A., Nakonieczny, M., 2021. Set of stress biomarkers as a practical tool in the assessment of multistress effect using honeybees from urban and rural areas as a model organism: a pilot study. Environ. Sci. Pollut. Res. https://doi.org/10.1007/s11356-020-11338-2.
Olgun, T., Dayioğlu, M., Taşkiran, N.Ö., 2020. Pesticide and pathogen induced oxidative stress in honey bees (Apis mellifera L.). Mellifera 20 (2), 32–52.
Čolović, M.B., Krstić, D.Z., Lazarević-Pašti, T.D., Bondžić, A.M., Vasić, V.M., 2013. Acetylcholinesterase inhibitors: pharmacology and toxicology. Curr. Neuropharmacol. 11, 315. https://doi.org/10.2174/1570159X11311030006.
Pal, E., Almasri, H., Paris, L., Diogon, M., Pioz, M., Cousin, M., Sené, D., Tchamitchian, S., Tavares, D.A., Delbac, F., Blot, N., Brunet, J.-L., Belzunces, L.P., 2022. Toxicity of the pesticides imidacloprid, difenoconazole and glyphosate alone and in binary and ternary mixtures to Winter Honey bees: effects on survival and antioxidative defenses. Toxics 10, 104. https://doi.org/10.3390/toxics10030104.
Paleolog, J., Wilde, J., Miszczak, A., Gancarz, M., Strachecka, A., 2021. Antioxidation defenses of Apis mellifera queens and workers respond to imidacloprid in different age-dependent ways: old queens are resistant, foragers are not. Animals 11, 1246. https://doi.org/10.3390/ani11051246.
Paleolog, J., Wilde, J., Siuda, M., Bąk, B., Wójcik, Ł., Strachecka, A., 2020. Imidacloprid markedly affects hemolymph proteolysis, biomarkers, DNA global methylation, and the cuticle proteolytic layer in western honeybees. Apidologie 51, 620–630. https://doi.org/10.1007/s13592-020-00747-4.
Palmer-Young, E.C., Ngor, L., Burciaga Nevarez, R., Rothman, J.A., Raffel, T.R., McFrederick, Q.S., 2019. Temperature dependence of parasitic infection and gut bacterial communities in bumble bees. Environ. Microbiol. 21, 4706–4723. https://doi.org/10.1111/1462-2920.14805.
Palmer-Young, E.C., Raffel, T.R., McFrederick, Q.S., 2018. Temperature-mediated inhibition of a bumblebee parasite by an intestinal symbiont. Proc. Biol. Sci. 285, 20182041. https://doi.org/10.1098/rspb.2018.2041.
Paloschi, C.L., Tavares, M.H.F., Berte, E.A., Model, K.J., Rosa, K.M., Da Conceição, F.G., Domanski, F.R., De Souza Vismara, E., Maciel, R.M.A., Da Silva Ribeiro, L., Mertz, N. R., Lopes, J.M.H., Lozano, E.R., Battisti, L., Potrich, M., 2026. Impact of the pesticide fipronil on honey quality and the survival of Apis mellifera bees. Environ. Sci. Pollut. Res. 33, 2744–2758. https://doi.org/10.1007/s11356-026-37404-9.
Paris, L., El Alaoui, H., Delbac, F., Diogon, M., 2018. Effects of the gut parasite Nosema ceranae on honey bee physiology and behavior. Curr. Opin. Insect Science 26, 149–154. https://doi.org/10.1016/j.cois.2018.02.017. Ecology • Parasites/Parasitoids/Biological control.
Pasquale, G.D., Salignon, M., Conte, Y.L., Belzunces, L.P., Decourtye, A., Kretzschmar, A., Suchail, S., Brunet, J.-L., Alaux, C., 2013. Influence of pollen nutrition on Honey Bee health: do pollen quality and diversity matter? PLoS One 8, e72016. https://doi.org/10.1371/journal.pone.0072016.
Pohorecka, K., Szczęsna, T., Witek, M., Miszczak, A., Sikorski, P., 2017. The exposure of honey bees to pesticide residues in the hive environment with regard to winter colony losses. J. Apicult. Sci. 61, 105–125. https://doi.org/10.1515/jas-2017-0013.
Porrini, C., Sabatini, A.G., Girotti, S., Ghini, S., Medrzycki, P., Grillenzoni, F., Bortolotti, L., Gattavecchia, E., Celli, G., 2003. Honey Bees and Bee Products as Monitors of the Environmental Contamination.
Potts, S.G., Biesmeijer, J.C., Kremen, C., Neumann, P., Schweiger, O., Kunin, W.E., 2010. Global pollinator declines: trends, impacts and drivers. Trends Ecol. Evol. 25, 345–353. https://doi.org/10.1016/j.tree.2010.01.007.

Raeymaekers, B., 2006. A prospective biomonitoring campaign with honey bees in a district of upper-bavaria (Germany). Environ. Monit. Assess. 116, 233–243. https://doi.org/10.1007/s10661-006-7389-8.

Ran, X., Zheng, Z., Zhao, Z., Liu, Y., 2026. Cloning and expression pattern of the carboxylesterase gene GmCarE4a in Galleria mellonella (Lepidoptera: Pyralidae) under chemical stress. J. Entomol. Res. Soc. 1. https://doi.org/10.51963/jers.v28i1.3033.

Renzi, M.T., Amichot, M., Pauron, D., Tchamitchian, S., Brunet, J.-L., Kretzschmar, A., Maini, S., Belzunces, L.P., 2016. Chronic toxicity and physiological changes induced in the honey bee by the exposure to fipronil and Bacillus thuringiensis spores alone or combined. Ecotoxicol. Environ. Saf. 127, 205–213. https://doi.org/10.1016/j.ecoenv.2016.01.028.

Richardson, R.T., Ballinger, M.N., Qian, F., Christman, J.W., Johnson, R.M., 2018. Morphological and functional characterization of honey bee, Apis mellifera, hemocyte cell communities. Apidologie 49, 397–410. https://doi.org/10.1007/s13592-018-0566-2.

Roat, T.C., Carvalho, S.M., Palma, M.S., Malaspina, O., 2017. Biochemical response of the Africanized honeybee exposed to fipronil: enzymatic assessment of honeybees exposed to fipronil. Environ. Toxicol. Chem. 36, 1652–1660. https://doi.org/10.1002/etc.3699.

Rothman, J.A., Leger, L., Kirkwood, J.S., McFrederick, Q.S., 2019. Cadmium and selenate exposure affects the honey microbiome and metabolome, and bee-associated bacteria show potential for bioaccumulation. Appl. Environ. Microbiol. 85, e01411. https://doi.org/10.1128/AEM.01411-19, 19.

Scheper, J., Badenhausser, I., Kantelhardt, J., Kirchweger, S., Bartomeus, I., Bretagnolle, V., Clough, Y., Gross, N., Raemakers, I., Vilà, M., Zaragoza-Trello, C., Kleijn, D., 2023. Biodiversity and pollination benefits trade off against profit in an intensive farming system. Proc. Natl. Acad. Sci. 120, e2212124120. https://doi.org/10.1073/pnas.2212124120.

Sonter, C.A., Shakya, M., Tighe, M., Stevenson, G., Datta, K., Lowe, R.G.T., Holland, A., Rader, R., Wilson, S.C., 2026. Intergenerational and biochemical responses of the Western honey bee (Apis mellifera) to Sublethal Perfluorooctanesulfonate (PFOS) exposures. Environ. Sci. Technol. 60, 5991–6001. https://doi.org/10.1021/acs.est.5c08902.

StataCorp, 2021. Stata Statistical Software: Release 17. StataCorp LLC, College Station, TX.

Stone, D., Jepson, P., Laskowski, R., 2002. Trends in detoxification enzymes and heavy metal accumulation in ground beetles (Coleoptera: Carabidae) inhabiting a gradient of pollution. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 132, 105–112. https://doi.org/10.1016/S1532-0456(02)00052-2.

Strachecka, A., Olszewski, K., Paleolog, J., 2016. Varroa treatment with bromfenvinphos markedly suppresses honeybee biochemical defence levels. Entomol. Exp. Appl. 160, 57–71. https://doi.org/10.1111/eea.12451.

Summan, A., Nandi, A., 2022. Timing of non-pharmaceutical interventions to mitigate COVID-19 transmission and their effects on mobility: a cross-country analysis. Eur. J. Health Econ. 23, 105–117. https://doi.org/10.1007/s10198-021-01355-4.

Tavares, D.A., Roat, T.C., Silva-Zacarin, E.C.M., Nocelli, R.C.F., Malaspina, O., 2019. Exposure to thiamethoxam during the larval phase affects synapsin levels in the brain of the honey bee. Ecotoxicol. Environ. Saf. 169, 523–528. https://doi.org/10.1016/j.ecoenv.2018.11.048.

Teeters, B.S., Johnson, R.M., Ellis, M.D., Siegfried, B.D., 2012. Using video-tracking to assess sublethal effects of pesticides on honey bees (Apis mellifera L.). Environ. Toxicol. Chem. 31, 1349–1354. https://doi.org/10.1002/etc.1830.

Tesovnik, T., Zorc, M., Ristanić, M., Glavinić, U., Stevanović, J., Narat, M., Stanimirović, Z., 2020. Exposure of honey bee larvae to thiamethoxam and its interaction with Nosema ceranae infection in adult honey bees. Environ. Pollut. 256, 113443. https://doi.org/10.1016/j.envpol.2019.113443.

van Bladeren, P.J., 2000. Glutathione conjugation as a bioactivation reaction. Chem. Biol. Interact. 129, 61–76. https://doi.org/10.1016/S0009-2797(00)00214-3.

Vincze, C., Leelőssy, Á., Zajácz, E., Mészáros, R., 2025. A review of short-term weather impacts on honey production. Int. J. Biometeorol. 69, 303–317. https://doi.org/10.1007/s00484-024-02824-0.

Wang, Yanhua, Zhu, Y.C., Li, W., 2020b. Comparative examination on synergistic toxicities of chlorpyrifos, acephate, or tetraconazole mixed with pyrethroid insecticides to honey bees (Apis mellifera L.). Environ. Sci. Pollut. Res. 27, 6971–6980. https://doi.org/10.1007/s11356-019-07214-3.

Wang, Yufei, Zhang, W., Shi, T., Xu, S., Lu, B., Qin, H., Yu, L., 2020a. Synergistic toxicity and physiological impact of thiamethoxam alone or in binary mixtures with three commonly used insecticides on honeybee. Apidologie 51, 395–405. https://doi.org/10.1007/s13592-019-00726-4.

Wegener, J., 2016. Secondary biomarkers of insecticide-induced stress of honey bee colonies and their relevance for overwintering strength. Ecotoxicol. Environ. Saf. 11.

Weirich, G.F., Collins, A.M., Williams, V.P., 2002. Antioxidant enzymes in the honey bee, Apis mellifera. Apidologie 33, 3–14. https://doi.org/10.1051/apido:2001001.

Wilde, J., Frączek, R.J., Siuda, M., Bąk, B., Hatjina, F., Miszczak, A., 2016. The influence of sublethal doses of imidacloprid on protein content and proteolytic activity in honey bees (Apis mellifera L.). J. Apicult. Res. 55, 212–220. https://doi.org/10.1080/00218839.2016.1211394.

Williamson, S.M., Wright, G.A., 2013. Exposure to multiple cholinergic pesticides impairs olfactory learning and memory in honeybees. J. Exp. Biol. 216, 1799–1807. https://doi.org/10.1242/jeb.083931.

Wu, S.M., Shih, M.-J., Ho, Y.-C., 2007. Toxicological stress response and cadmium distribution in hybrid tilapia (Oreochromis sp.) upon cadmium exposure. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 145, 218–226. https://doi.org/10.1016/j.cbpc.2006.12.003.

Yan, S., Cui, F., Qiao, C., 2009. Structure, function and applications of carboxylesterases from insects for insecticide resistance. PPL 16, 1181–1188. https://doi.org/10.2174/092986609789071243.

Yao, J., Zhu, Y.C., Adamczyk, J., Luttrell, R., 2018. Influences of acephate and mixtures with other commonly used pesticides on honey bee (Apis mellifera) survival and detoxification enzyme activities. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 209, 9–17. https://doi.org/10.1016/j.cbpc.2018.03.005.

Yu, X., Sun, R., Yan, H., Guo, X., Xu, B., 2012. Characterization of a sigma class glutathione S-transferase gene in the larvae of the honeybee (Apis cerana cerana) on exposure to mercury. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 161, 356–364. https://doi.org/10.1016/j.cbpb.2011.12.009.

Zambrano-Monserrate, M.A., Ruano, M.A., Sanchez-Alcalde, L., 2020. Indirect effects of COVID-19 on the environment. Sci. Total Environ. 728, 138813. https://doi.org/10.1016/j.scitotenv.2020.138813.

Zapata-Hernández, G., Gajardo-Rojas, M., Calderón-Seguel, M., Muñoz, A.A., Yáñez, K.P., Requier, F., Fontúrbel, F.E., Ormeño-Arriagada, P.I., Arrieta, H., 2024. Advances and knowledge gaps on climate change impacts on honey bees and beekeeping: a systematic review. Glob. Change Biol. 30, e17219. https://doi.org/10.1111/gcb.17219.

Zhu, Y.C., Yao, J., Adamczyk, J., Luttrell, R., 2017. Feeding toxicity and impact of imidacloprid formulation and mixtures with six representative pesticides at residue concentrations on honey bee physiology (Apis mellifera). PLoS One 12, e0178421. https://doi.org/10.1371/journal.pone.0178421.

Zibaee, A., Zibaee, I., Jalali Sendi, J., 2011. A juvenile hormone analog, pyriproxifen, affects some biochemical components in the hemolymph and fat bodies of Eurygaster integriceps Puton (Hemiptera: Scutelleridae). Pestic. Biochem. Physiol. 100, 289–298. https://doi.org/10.1016/j.pestbp.2011.05.002.