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Assessment of the ecotoxicological health status of *Apis mellifera* using a multi-tier approach based on biomarkers, proteomic analysis and quality and origin of bee products

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ABSTRACT

Pollinating insects play a role of primary importance both in agriculture, ensuring the crops productivity, and in the conservation of plant biodiversity. Among pollinators, *Apis mellifera* L., 1958 (Hymenoptera: Apidae) is the most known and widespread species and the most valuable for its pollination service. This species is disappearing globally due to different reasons, such as climate change, the massive use of Plant Protection Products (PPPs) and other environmental contaminants diffusion, habitat fragmentation and parasites infections. The sub-lethal levels of pesticide residues and other anthropic contaminants, even not leading to the death of individuals, are able to cause problems in the development, behaviour and health of animals in the short and long term. Moreover, honey bees are exposed to mixtures of contaminants in the environment, that can cause different effects. However, there is a large gap in the assessment of the sub-lethal effects of these mixtures. Another gap in the research on the ecotoxicology of these animals is the assessment of the effects of commercial formulates instead of only using the active principles. Among the sub-lethal effects that were examined in the literature relating to pesticides exposure, rarely genotoxicity and immune system biomarkers were used. Anthropic activities could also be able to modify indirectly the quality and origin of bee products, since they can alter honey bees health and consequently their productivity.

The use of an integrated approach to combine responses at different levels, could be a valid tool to evaluate the impact of contamination on these organisms.

The goal of this thesis was to assess the health of honey bee colonies using a multi-tier methodology that included biomarker responses, proteomic analysis, and bee product quality and origin. This thesis was divided in two parts:

- A laboratory study, exposing *Apis mellifera* specimens to two commercial pesticides, the fungicide Sakura® and the herbicide Elegant 2FD, alone and in combination. The effects of these compounds were assessed integrating two methodologies, consisting in a set of biomarkers and a proteomics approach. Both pesticides modulated the detoxification process. The fungicide alone had also effects on the metabolism, while the herbicide demonstrated to be neurotoxic. The results from the mixture treatments demonstrated that the effects obtained were influenced mostly by the herbicide. The proteomic approach revealed that the two pesticides were able to affect the energy

metabolism, the immune system and the protein synthesis. The proteomic approach should be improved to understand if and to what extent the above-mentioned post-translational changes happened, using specific antibodies to perform a more specific assessment.

- A two-year monitoring study, aiming to assess the ecotoxicological status of bees in natural environments. *Apis mellifera* specimens were sampled in 10 locations in Tuscany region characterised by varying contamination patterns. In this case, the used approach was made up of a set of biomarkers, used to assess the health status of honey bees, and the analyses of origin and quality of the honey, through melissopalynological and chemical-physical analyses. The biomarkers results obtained for the first year showed that the suburban area and the agricultural area were undergoing major stress but with different kinds of effects, probably because the contaminants were different in the various areas. In 2021 the specimens undergoing major stress were the ones coming from vineyards, that showed genotoxic effects, and clover field and wheat crops, showing alterations in nervous and immune systems. The comparison between the 2 years results showed that the organisms were undergoing major stress condition in 2021 compared to 2020. Bees from 2021 reported neurotoxic effects, the presence of oxidative stress and DNA damage. The different responses obtained could be due not only to contaminants but also to the changing of climatic conditions, such as differences in temperatures and rainfalls, which were also taken into consideration. The melissopalynological analysis showed that only in the clover field the pollen derived from the cultivation that we observed during the sampling. These findings suggest that the biomarker responses observed in *A. mellifera* specimens are probably not due to pollen contamination. In fact, organisms could come in contact with contaminants through other exposure routes. The carbohydrates, amino acids and humidity analysis showed that honey samples were not characterised by major differences, even if coming from different areas, except for the proportion of some amino acids, due to the presence of different pollens.

Both the studies had also the goal to start filling a research gap regarding the assessment of effects on immune system and DNA damages, obtaining promising results.

The integrated approaches that were used proved to be effective to observe the ecotoxicological health status of *Apis mellifera* from different points of view. The multi-trial approach would be a sensitive tool to measure sub-lethal effects, and not only lethal ones, of pesticide active

principles and, more important, of pesticide commercial formulations. It would be helpful to improve the current risk assessment procedure for chemical registration and use, making the agricultural environment more pollinator-friendly.

ABBREVIATIONS

1D – One-dimensional gel electrophoresis
2D-DIGE – 2D difference gel electrophoresis
2-DE – Two-dimensional gel electrophoresis
AChE – Acetylcholinesterase
ALP – Alkaline phosphatase
AQC – 6-aminoquinoly-N-hydroxysuccinimidyl carbamate
ArgK – Arginine kinase
ATCI – Acetylthiocholine
BSA – Bovine Serum Albumin
CaE – Carboxylesterase
CAT – Catalase
CBs – Carbamate insecticides
$CdSO_4$ – Cadmium sulfate
CYP450 – Cytochrome P450
DCNB – 2,4-dinitrochlorobenzene
DDA – Data Dependent Acquisition
DTNB – 2’-dinitro-5,5'-dithiodibenzoic acid
DTT – Dithiothreitol
DWV – Deformed Wing Virus
eEF-1a-f1 – Translation elongation factor eEF-1a
EFSA – European Food Safety Authority
EMS – Ethyl methane-sulfonate
GPx – Glutathione peroxidase
GSH – Glutathione
GSSG – Oxidized glutathione
GST – Glutathione S-transferase
HEL – Hen egg white lysozyme
HPLC – High-performance liquid chromatography
LC – Liquid chromatography
LC-MS/MS – Tandem mass spectrometry coupled to liquid chromatography
LPO – Lipid peroxidation
LYS – Lysozyme
MDA – Malondialdehyde
$MgCl_2$ – Magnesium chloride
MRJP3 – Major royal jelly protein 3
MS – Mass spectrometry
MW – Molecular weight
NA assay – Nuclear Abnormalities assay
NAM – N-acetylmuramic acid
NaOH – Sodium hydroxide
OPs – Organophosphorus insecticides

PAHs – Polycyclic Aromatic Hydrocarbons
PCBs – Polychlorinated biphenyls
pI – Isoelectric point
p-NPP – p-nitrophenyl phosphate
POD – Peroxidase
PPO – Prophenoloxidase
PPPs – Plants Protection Products
PSF – Profilin
RB – Rehydration Buffer
ROS – Reactive Oxigen Species
RpS8 – Ribosomal protein S8
SDS – Sodium dodecyl sulfate
SOD – Superoxide dismutase
TCA – Trichloroacetic acid
Vg – Vitellogenin

STRUCTURE OF THE THESIS

This thesis is structured in five chapters.

The first chapter is a brief introduction where are presented the main topics discussed in the dissertation. Part of this chapter was published in a review paper: Di Noi, A., Casini, S., Campani, T., Cai, G., & Caliani, I. (2021). Review on sublethal effects of environmental contaminants in honey bees (*Apis mellifera*), knowledge gaps and future perspectives. International Journal of Environmental Research and Public Health, 18(4), 1863.

The second chapter described the aims of this work.

The third chapter describes the laboratory study, in which *Apis mellifera* specimens were exposed to a fungicide and an herbicide, alone and in combination, and the effects were evaluated using a multi-biomarker and a proteomic approach.

The fourth chapter describes the monitoring study, in which Apis mellifera specimens were sampled from 10 different areas in Tuscany, characterised by various anthropic impacts. The sampling took place in 2 years, 2020 and 2021, and in the second-year honey samples were taken together with the animals. The assessment of the health status of the organisms was conducted using a multi-biomarker approach to which were added the melissopalynological analysis of honey together with chemical-physical ones.

The fifth chapter contains the final conclusions, aiming to summarize the main findings of the two experimental parts.

1. INTRODUCTION

1.1 Honey bee, *Apis mellifera*

Honey bees are Hymenoptera belonging to the Apidae family and included in the *Apis* genus. In Europe, *Apis mellifera*, a social insect that lives in families made up of thousands of individuals, is the most domesticated species. From Africa, Europe and part of Asia, it is now also widespread in the American and Australian continents. Such a wide diffusion has favoured the differentiation into many subspecies, which can be hybridized and produce fertile offspring. *A. mellifera ligustica* and *A. mellifera sicula* are the subspecies diffuse in Italy (Fontana et al., 2018).

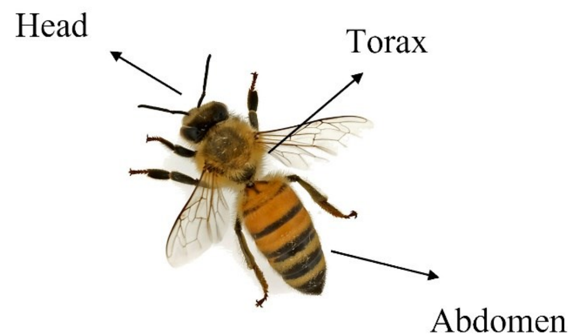

Figure 1. *Apis mellifera* morphology

A. mellifera body, protected by a chitinous exoskeleton, the cuticle, with the function of support and protection, is made up of three distinct parts: head, thorax and abdomen (Fig. 1). In the head, a rigid capsule of roughly triangular shape, the organs of vision, two large compound eyes which give the bee a mosaic vision and three simple eyes or ocelli specialized for the vision of very close objects, are present. The latter also measures the light intensity, to allow adaptation of compound eyes. In the upper part of the head, there are two mobile antennas, carrying numerous sensilla at their ends, which are tactile, olfactory, thermal and hygroscopic sense organs made up of one or more cells. In the lower part of the head there is a lapping-sucking buccal apparatus made up of a set of various organs – the upper lip, two jaws, two mandibles and the lower lip – the most important part of which is the tongue: this mobile structure is equipped with an internal channel through which saliva is emitted, essential when the bee inserts the tongue into the floral calyx and sucks up the nectar. The thorax is composed of three segments (prothorax, mesothorax and metathorax) each with a pair of legs and on the second and third segment two pairs of membranous wings are articulated. The legs are used for

walking, collecting pollen and cleaning the body of any foreign particles. The wings are made up of two thin overlapping laminae and are much more robust than they appear, being able to support the weight of the animal and the weight of the pollen and nectar they carry, sometimes for journeys exceeding 3 km. The chest is also rich in hairs and bristles, in order to harness the pollen and transport it to the hive; in addition to this, it has openings placed laterally, representing the external openings of the respiratory system. The chest also houses a part of the digestive tract, the air sacs and the wing muscles. The abdomen is made up of a fusion of ten segments, of which the first is incorporated into the final part of the mesothorax and connected to the other nine by a peduncle. Inside the abdomen there is a large part of the digestive tract, i.e. the middle and upper intestine, various air sacs, the aorta and some tracheas; as for the thorax, also the abdomen is covered with numerous hairs and bristles. The sting, an important defence weapon, is in the back of the abdomen. Only worker bees and the queen possess the sting, as it derives from the transformation of the ovipositor; consequently, the drones do not have it. It is a serrated stylet, normally found inside the body cavity and everted at necessity. It is connected directly to the venom system and, once the bee has stung, it gets stuck by way of the teeth on the sting, detaching from the rest of the body and causing the animal's death in a short time.

1.2 The beehive organization

The beehive is one of the most advanced social structures in nature. It is a society in which each individual has his own task, cooperating synergistically for the conservation of the hive and its genetic code, which is handed down from generation to generation by a single individual: the queen bee.

Three types of individuals characterise the bee colony in a hive: the queen, the only fertile female, thousands of worker bees, and drones, or males. These three different types of individuals are easily distinguished as they show considerable sexual dimorphism.

The queen, larger than workers and drones, has a body long 17-20 mm and a lifetime lasting around two years, up to a maximum of five in ideal conditions. It also has legs without pollen baskets and a sting with fewer hooks. The main distinguishing feature is that it is the only fertile female of the entire colony; this uniqueness is due to the fact that, unlike worker bees, fed with royal jelly only up to the third day of the larval stage, the queen bee is fed with royal jelly for its entire life. This particular diet allows it to become the largest bee and the only one with a reproductive system. The queen bee is born from royal cells, larger than normal cells and round in shape, which are created when the workers feel a drop in royal pheromones, usually due to

illness or old age of the previous queen. At the moment of "swarming", the old queen bee, which leaves the hive together with several worker bees, leaves a fair number of eggs from which the new queen bee will be born. After about ten days, the new queen leaves the hive for the so-called "nuptial flight", through which she reaches the drones to be fertilized. This is the only opportunity to receive the drones' spermatheca, after which the queen never leaves the hive, dealing exclusively with egg laying. The fertilization of the eggs is regulated by the needs of the hive and proceeds throughout the year, with maximum peaks of about 1500 eggs per day in spring and summer.

The drones have a body of 15-17 mm in length and are easily distinguished from the worker by the more squat and hairy body, and the larger and rounder compound eyes. The drones are characterized by the absence of the sting and by a very short tongue, not allowing the collection of nectar from flowers or fresh pollen. They are born from unfertilized eggs laid by the queen and have a very short life span of around 50 days. Their main purpose is the fertilization of the queen, immediately after which they die due to the loss of the spermatheca. The drones not engaged in the fertilization of the queen collaborate with the workers for the ventilation of the hive, heating the brood, and the manipulation of the nectar.

Worker bees carry out most of the tasks inside the hive. They have a body that varies between 10 and 15 mm in length and a very short life in summer, during the foraging season (about 30/40 days), while it is longer in winter (up to six months). A larva becomes a worker when it is fed with royal jelly for the first three days of life, and then passes on to a diet consisting of a mixture of honey and pollen. This difference in the diet is what prevents the workers from completing the development of the reproductive system. During its life, the worker bee takes on different tasks depending on its age. The youngest workers, generally in the first three days of life, clean and prepare the cells for deposition. After a few days, with working wax glands, they take care of the construction of the honeycombs. Between two and three weeks of age, workers take care of the maintenance and administration of the hive, store the honey in the cells, take care of ventilation and clean the hive. Finally, between the third and fourth week, they become foragers, becoming workers who procure nectar, pollen and propolis until they die. The foragers, dedicated to the search for new sources of raw materials, during the swarming also look for the ideal place to build the new hive. This ability to perform various tasks can be attributed to the presence of unique morphological structures: the legs, equipped with a comb, brush and foraging basket, and the tongue, long enough to suck the nectar from the flowers and accumulate it in a tract of the digestive system.

1.3 Importance and decline of pollinators

Honey bees (*Apis mellifera*) are essential organisms for the environment, in particular for their critical role in the pollination of plants including crops (Burkle et al., 2013; Kennedy et al., 2013; Potts et al., 2010). It has been estimated that honey bees are responsible for pollinating 96% of animal-pollinated crops (Klein et al., 2007; vanEngelsdorp and Meixner, 2010). Bees are also indirectly responsible for the reproduction and maintenance of wild plant communities and biodiversity (Aguilar et al., 2006; Ashman et al., 2004; de Groot et al., 2002). The value of honey bees to global food crops is estimated to be €153 billion per year (Gallai et al., 2009). In addition, honey bees provide honey, pollen, wax, propolis, and royal jelly to humans (Formato et al., 2011). Over the past decades, a decline in bees and other pollinators has been observed worldwide (Biesmeijer, 2006; Cameron et al., 2011; Ollerton et al., 2014); significant honey bee colony losses have been reported, particularly in North America and Western Europe (Jacques et al., 2016; Steinhauer et al., 2014; van der Zee et al., 2012). Beekeepers alerted the scientific community to this significant colony mortality as they monitor bee colonies worldwide and are immediately aware of any changes in the bee colony (Carnesecchi et al., 2019). This decline has raised concerns about the sustainability of the human food supply and the health of natural ecosystems (Potts et al., 2016). The causes of pollinator decline may be complex and controversial. However, the overall weakening and death of bee colonies have been observed to be mainly caused by the combined action of multiple stressors (European Food Safety Authority, 2014; Goulson et al., 2015; Potts et al., 2010; Rortais et al., 2017), such as environmental factors (Conte and Navajas, 2008; Di Pasquale et al., 2016; Goulson et al., 2015), nutritional stressors (Tong et al., 2019; Tosi et al., 2017), chemical and biological factors (Alaux et al., 2010; Klein et al., 2017; Nazzi et al., 2012; Nazzi and Pennacchio, 2014; Pettis et al., 2012; Renzi et al., 2016; Vidau et al., 2011; Williamson and Wright, 2013) and multiple chemicals (Han et al., 2019; Robinson et al., 2017; Sanchez-Bayo and Goka, 2016). In particular, the latter type of stressor is a matter of great concern because bees can be exposed to a wide range of chemical mixtures, including anthropogenic compounds, such as plant protection products (PPPs) or veterinary drugs, and those of natural origins, such as mycotoxins, flavonoids and plant toxins (European Food Safety Authority, 2014; Johnson, 2015; Tosi and Nieh, 2019). Although PPPs, such as insecticides, acaricides, herbicides, and fungicides, have many benefits on agriculture (Cooper and Dobson, 2007), there are also several potential risks associated with their use, including pest resistance, resurgence, and secondary pest outbreaks, as well as broader environmental contamination and human health

concerns (Carvalho, 2017; Silva et al., 2019; Van Bruggen et al., 2018). Although insecticides are used to target insect pests, their use in agriculture can affect non-target insects that provide beneficial services to agriculture. Among these beneficial insects, the main focus has been on social bees, with a particular interest in neonicotinoid insecticides and their lethal and sub-lethal effects at colony and population levels. However, other PPPs used in modern agriculture, such as fungicides and herbicides, have also been shown to affect honey bees' health (Caliani et al., 2021b; Lupi et al., 2020; Mullin et al., 2010; Raimets et al., 2020).

In addition, bees may be exposed to contaminants other than PPPs, such as PAHs and trace elements, which may cause adverse effects due to their presence in the environment. Studies on trace elements have highlighted that contaminants, such as aluminium, cadmium, selenium, lead, and copper may affect foraging behaviour (Hladun et al., 2012; Søvik et al., 2015) and developmental time (Hladun et al., 2016, 2013), cause histopathological changes (Dabour et al., 2019), and alter acetylcholinesterase (AChE), alkaline phosphatase (ALP), glutathione S-transferase (GST) (Badiou-Bénéteau et al., 2013; Caliani et al., 2021a), catalase (CAT) and superoxide dismutase (SOD) (Nikolić et al., 2016, 2015) activities.

The European Food Safety Authority (EFSA) pointed out that the study of the effects of mixtures of chemicals, also compared to non-chemical stressors such as *Varroa destructor* and viruses, on honey bee health is of great importance in order to support the implementation of a holistic risk assessment methodology (EFSA Panel on Animal Health and Welfare (AHAW), 2016; European Food Safety Authority, 2017).

1.4 PPPs and their effects on honey bees

The most widely studied PPPs are insecticides because they have been shown to be harmful to non-target organisms, such as honey bees. Several authors have observed that neonicotinoid insecticides, such as imidacloprid, thiamethoxam, acetamiprid, dinotefuran, thiacloprid, nitenpyram, and clothianidin, are able to impair honey bee olfactory learning performances (Decourtye et al., 2004; Imran et al., 2019; Wright et al., 2015), foraging activity (Decourtye et al., 2004; Morfin et al., 2019; Schmuck et al., 2003), and homing flight (Monchanin et al., 2019). These types of compounds can cause neurotoxicity in honey bees, by altering AChE activity, which may be induced (Boily et al., 2013) or inhibited (Badawy et al., 2015), and by modulating carboxylesterase (CaE) activity (Badawy et al., 2015; Badiou-Bénéteau et al., 2012). In addition, detoxification and antioxidant enzyme activities appear to be altered by

neonicotinoids, such as GST (Almasri et al., 2020; Badawy et al., 2015; Badiou-Bénéteau et al., 2012), CAT (Badiou-Bénéteau et al., 2012), prophenoloxidase (PPO) (Badawy et al., 2015), ALP (Badiou-Bénéteau et al., 2012) and cytochrome P450 (CYP450) (Zaworra and Nauen, 2019) activities. Moreover, these compounds can affect the immune system, for example by modulating the content of vitellogenin (Abbo et al., 2017; Christen et al., 2019b), reducing the haemocytes density, encapsulation response and antimicrobial activity (Almasri et al., 2020), and modulating the relative abundance of several key gut microbial molecules (Zhu et al., 2020). Several authors have studied the effects of pyrethroid insecticides, such as deltamethrin, bifenthrin, cypermethrin, permethrin, and λ-cyhalothrin, on honey bees; these compounds appear to cause neurotoxicity by increasing AChE activity (Badiou et al., 2008; Qi et al., 2020) and modulating CaE activity (Carvalho et al., 2013). Pyrethroids caused variations in lipids (Bounias, 1985) and carbohydrates (Bendahou et al., 1999), reduced learning and memory (Decourtye et al., 2005; Zhang et al., 2020) and foraging activity (Decourtye et al., 2004), and affected bee locomotion and social interaction (Ingram et al., 2015). This class of insecticides is also capable of altering metabolic and detoxification activities, such as increasing GST activity (Papadopoulos et al., 2004; Yu et al., 1984), modulating ALP activity (Bounias, 1985), inducing the expression of CYP450 monooxygenase (Christen et al., 2019a), and inhibiting Na+, K+-ATPase activity (Bendahou et al., 1999). In addition, they can induce immune responses, cause changes in peroxidase (POD) activity and malondialdehyde (MDA) and lipid peroxidation (LPO) levels, and induce oxidative stress (Qi et al., 2020). Authors investigating the effects of organophosphorus insecticides effects observed an inhibition of odor learning (Weick and Thorn, 2002), a modulation of AChE activity (Al Naggar et al., 2015; Christen et al., 2019a; Glavan et al., 2018; Weick and Thorn, 2002), changes in the expression of several genes related to the immune system and induction of vitellogenin transcript (Christen et al., 2019a). El-Saad et al. (2017) observed ultrastructural changes in the midgut, a decrease in glutathione (GSH) levels, inhibition of superoxide dismutase (SOD), CAT and glutathione peroxidase (GPx) activities, and an increase in MDA levels.

A recent review (Cullen et al., 2019) highlighted that other PPPs, such as fungicides and herbicides, which are not designed to target insects, may be factors influencing honey bee decline. Therefore, it would be important to increase the number of studies on their effects on these pollinators. The most frequently studied herbicide is glyphosate; it seems to cause a more indirect homing flight (Balbuena et al., 2015), reduce sensitivity to sucrose and learning performance (Herbert et al., 2014), delay the development of worker brood (Odemer et al.,

2020), have effects on the expression of CYP isoform genes (Gregorc et al., 2012), and to slightly inhibit AChE activity (Boily et al., 2013).

1.5 Gaps in research on honey bees

The members of a colony can provide toxicological indications through different signals: the mortality rate, the analysis of contaminants residues in the honey, in the pollen stored in the combs and in larvae (Porrini et al., 2002) and the alteration of biological processes at various levels of organism's organization (molecular, cellular, …) induced by the presence of contaminants. To date, almost all the studies focused on evaluating the mortality rate (LD50) or on similar endpoints, while the potential sub-lethal effects, which do not determine the death of the animal but alter important functions and structures, potentially undermining the health and well-being of bees both in the short and long term, are still poorly explored. The sub-lethal effects of these anthropogenic contaminants in *Apis mellifera* need to be investigated. As underlined, a wide range of studies has examined mortality and accumulation in honey bees to provide data on contamination that may affect these organisms (Abbo et al., 2017; Berg et al., 2018; Rabea et al., 2010; Renzi et al., 2016). In addition, studies on the general fitness of honey bees, which examined their behaviour, flight activity, and sensory ability, have been conducted over the years to observe the macroscopic effects of contaminants (Berg et al., 2018; Hladun et al., 2013; Mixson et al., 2009; Prado et al., 2019). To a lesser extent, enzymatic and molecular responses have also been studied, using biomarkers, genomic, transcriptomic, and metabolomic techniques (Badiou-Bénéteau et al., 2013, 2012; Caliani et al., 2021a, 2021b; Carvalho et al., 2013; El-Saad et al., 2017) to better understand the anthropogenic impacts on these insects.

The majority of studies on honey bees are carried out in the laboratory rather than in semi-field and field conditions, in a controlled environment and with controlled environmental exposure to the selected substances. The majority of papers on laboratory experiments focus on sub-lethal effects, mostly on foraging activity, sensory ability, neurotoxicity, detoxification, metabolism, and oxidative stress. In semi-field studies, different responses at both macroscopic and microscopic levels have been considered.

In field studies, it is more difficult to understand the effects of single contaminants, due to the presence of multiple stressors. Most of the monitoring studies investigated the accumulation of different pollutants in *Apis mellifera*. To date, only a few papers have investigated the sub-

lethal effects of environmental contamination on honeybees in their natural conditions and habitats (AL Naggar et al., 2020; Almasri et al., 2020; Badiou et al., 2008; Dabour et al., 2019; Prado et al., 2019; Renzi et al., 2016; Schmuck et al., 2003; Williamson and Wright, 2013). Badiou-Bénéteau et al. (2013) and Nikolić et al. (2015) highlighted the presence of oxidative stress and the induction of detoxification processes, in honey bees from more anthropized areas, due to the presence of neurotoxic contaminants, such as metals. Lupi et al. (2020) observed that pesticide mixtures, characterized by the combination of fungicides, insecticides, and plant regulators, could cause an increase in Reactive Oxygen Species (ROS), which can inhibit AChE and CAT activities. Inhibition of some antioxidant biomarkers (GSH, SOD, CAT, GST) has also been observed in samples collected from anthropized areas (El-Saad et al., 2017). Nicewicz et al. (2020) observed the importance of defensin and heat shock protein 70 (HSP70) levels as indicators of urban multi-stress at both individual and colony levels. All the above-mentioned studies on sub-lethal effects showed that honey bees are sensitive bioindicators of environmental pollution. Therefore, the decline of honey bees should be studied through contextual monitoring in order to understand its causes and to provide administrations with effective prevention tools.

Studies evaluating sub-lethal effects have focused their attention on the development and application of biomarkers assessing the exposure to and the effects of contaminants on honey bees, such as esterase activity to evaluate neurotoxic effects, mainly CAT and SOD as antioxidant enzyme activity, together with detoxification reactions and metabolic activity. However, several responses, such as genotoxicity and alteration of the immune system, remain poorly understood and require an increased interest and significant effort to ensure that research is conducted. For example, Colin et al. (2004) observed that suppression of the immune system may lead to a decrease in individual performance and, consequently, population dynamics and the level of colony disorder. In addition, Lazarov et al. (2019) observed that *Varroa destructor* infestations are responsible for the weakening of the immune system of honey bees, which may lead to a pronounced susceptibility of honey bees to contaminant exposure. To the best of our knowledge, the only two genotoxicity studies conducted on *Apis mellifera* were from Caliani et al. (2021a, 2021b); in the laboratory study, it was observed that there are not only compounds such as EMS, with known genotoxic effects; in fact, there are also Cd and fungicides that have effects on the presence of haemocytes nuclear abnormalities. In the field study, genotoxic effects were observed in samples from the orchard and the cultivated area respect to the control wood.

The majority of the above-mentioned studies have been undertaken in North America and Europe, probably stimulated by the significant colony losses reported (Jacques et al., 2016; Steinhauer et al., 2014; van der Zee et al., 2012). PPPs are most commonly used in developed countries, where they are regulated, in order to limit their environmental impact. However, this kind of studies should be also conducted in other parts of the world where these compounds are increasingly being used and where regulations and best practices may not be as stringent (Schreinemachers and Tipraqsa, 2012).

Filling the above-mentioned gaps could be useful for a better understanding of the health status of these organisms, mostly in natural conditions.

1.6 Assessment of the ecotoxicological status of honey bees

The sub-lethal effects to which honey bees are subjected are important to understand and quantify, since they could be useful to develop and apply measures vital not only for this species but also for other pollinators safety. An integrated approach could be useful to combine responses at different levels, becoming a valid tool to evaluate the impact of contamination on these organisms.

1.6.1 Biomarkers

Bees, thanks to their morphological, ecological and behavioural characteristics, have proven to be excellent bioindicators for monitoring agricultural and urban pollution (Stocker, 1980). They have been used for several years both in laboratory experiments, to test the toxicity of pesticides used in agriculture (Celli and Maccagnani, 2003) and in field studies, to monitor pollution from heavy metals, volatile organic compounds, sulphur and nitrogen oxides and ozone in urban environments (Porrini et al., 2000). Bees are easy to find and widespread in large numbers: removing a few dozen organisms has negligible weight, since they live in colonies constituted by thousands of individuals. Moreover, the colony lives permanently in a relatively small area. The foraging bees, approximately a quarter of the colony, cover an average area of about 7 km2 around the hive, concentrated on a single floral essence and therefore entirely exposed to the same contaminants (Crane, 1984). During their foraging exits, bees come into contact with particles suspended in the air, as well as those present in the nectar, pollen and water on which they feed and which they bring back to the hive. Starting from these same materials, bees produce royal jelly and secrete honey and wax, bioaccumulating any contaminants present in these samples.

In light of all this, it is of fundamental importance to evaluate the health status of these organisms and the sub-lethal effects that the contaminants in their habitats are able to cause on them, often synergistically. A sensitive methodology is the use of a battery of biomarkers.

Below we will focus on the biomarkers that were selected for the research studies of this thesis.

Acetylcholinesterase (AChE) is an enzyme responsible for the hydrolysis of the neurotransmitter acetylcholine into choline and acetic acid at the nervous synapse to allow the transmission of the nervous impulse (Augustinsson, 1971). Xenobiotics such as organophosphorus (OPs) and carbamate (CBs) insecticides are capable of inhibiting AChE reversibly (CBs) or irreversibly (OPs). Laboratory and field studies have shown that low concentrations of OPs and CBs are able to block AChE activity with an accumulation of acetylcholine at nervous synapses, causing symptoms such as tremors, motor dysfunction and death (Fulton and Key, 2001; Sancho et al., 1997). In honey bees, the activity of this enzyme has been tested after pesticide treatments. Bendahou et al. (1999) exposed *A. mellifera* to 0.2 nmol/bee of fenitrothion, an organophosphorus, and observed a strong AChE inhibition (>60%) with respect to the control. Badiou et al. (2008) observed AChE inhibition after treatments with 25 ng/bee of deltamethrin, a pyrethroid, and 5 µg/bee of pirimicarb, a carbamate, alone and in combination. Caliani et al. (2021a) evaluated AChE activity in honey bees following treatments with cadmium sulfate, the fungicide Amistar®Xtra, and ethyl methane-sulfonate (EMS) at different concentrations (0.1 g/L and 2.5 g/L CdSO4; 100 g/L and 200 g/L Amistar®Xtra; 12.4 g/L EMS). A dose-dependent inhibition was observed after each treatment: the most significant inhibition was observed after the lowest cadmium (26%) and fungicide doses (26%) and after the highest cadmium dose (36%).

Carboxylesterases (CaEs) are a group of enzymes that are part of phase I of biotransformation processes, but also act at the neuronal level. In phase I of biotransformation, they are responsible for the hydrolysis of xenobiotic molecules, increasing their solubility in water and improving their excretion (Dauterman and Hodgson, 1990). Different CaE isoforms (CaE1/ α-CaE, CaE2, CaE3) are used in ecotoxicology to monitor nervous system activity. CaE1 is involved in the resistance to OPs in a protective mechanism for AChE; CaE1 binds to OPs and hydrolyses them, protecting the AChE from inhibition and consequently protecting the nervous system (Jackson et al., 2013; Yan et al., 2009). Attencia et al. (2005) observed CaE inhibition after exposure to two OPs, malathion (0.001%; 0.01%; 0.05%; 0.1%) and methylparathion (0.02%; 0.05%; 0.5%; 1%). Carvalho et al. (2013) highlighted that CaE reacts differently

depending on the insecticide. In honey bees, sub-lethal doses of deltamethrin (5.07 ng/bee and 2.53 ng/bee) inhibit CaE activity, while fipronil (0.58 ng/bee and 0.29 ng/bee) increases its activity.

Glutathione S-transferase is a phase II biotransformation enzyme. Its main function is to catalyse the conjugation of glutathione (GSH) with electrophilic compounds derived from phase I bioactivation reactions, such as metabolites of PAHs, PCBs (Stegeman et al., 1992) and heavy metals (Pellerin-Massicotte, 1994). GST activity is associated with a decrease in the total intracellular GSH and an increase in oxidized glutathione (GSSG). This increase in GSSG is potentially cytotoxic, leading to the formation of disulphide bonds in cellular proteins; in order to maintain the GSH/GSSG ratio constant, GSSG is released from the cell and degraded in the extracellular environment or converted in GSH by the enzyme glutathione reductase (GR) (Stephensen et al., 2002). Badiou-Bénéteau et al. (2012) exposed honeybees to three doses of thiamethoxam (51.16 ng/bee. 5.12 ng/bee and 2.56 ng/bee), a neonicotinoid insecticide, and observed an increase at all treatment doses, indicating that GST metabolises this class of insecticides. Carvalho et al. (2013) exposed *Apis mellifera* to two sub-lethal doses of three different compounds: deltamethrin (5.07 ng/bee and 2.53 ng/bee), fipronil (0.58 ng/bee and 0.29 ng/bee), and spinosad (4.71 ng/bee and 2.36 ng/bee). Both deltamethrin doses and the highest spinosad dose showed an increase in GST activity.

Alkaline phosphatase (ALP) is an Mg-dependent enzyme that hydrolyses phosphoric esters under alkaline conditions, i.e. ALP removes phosphate groups from various substrates such as nucleotides, proteins, and alkaloids. ALP is mostly active at alkaline pH (pH>8) and is involved in digestive processes, metabolism and antioxidant transport (Coleman, 1992). In insects, ALP is localized in the intestinal epithelium, where its main function is to provide phosphate ions necessary for various metabolic processes; any kind of impairment of its activity has an effect on intestinal physiology. In addition, ALP also plays a role in insecticide resistance (Khan and Garnier, 2013). Bounias (1985) treated honey bees with a sub-lethal dose of deltamethrin (0.1 pmol/bee). ALP activity was monitored for two hours after insecticide administration, showing an initial inhibition followed by a strong induction of enzyme activity. Badiou-Bénéteau et al. (2012) treated honey bees with different doses of thiamethoxam (51.16 ng/bee. 5.12 ng/bee and 2.56 ng/bee) for 48 hours and observed a slight increase in ALP activity after the lowest dose of thiamethoxam. Badiou-Bénéteau et al. (2013) evaluated ALP activity in *A. mellifera* samples collected in areas with different anthropic impacts (urban and rural areas) on the island of La

Reunion. A strong induction of the enzyme was found in sample specimens from both areas. Caliani et al. (2021a) evaluated ALP activity following the treatment of honey bees with three different contaminants: $CdSO_4$ (0.1 g/L and 2.5 g/L), the fungicide Amistar®Xtra (100 g/L and 200 g/L) and EMS (12.4 g/L). Each treatment was able to inhibit ALP activity, starting at the lower doses.

Insects possess different kinds of haemocytes and in honey bees four have been identified: granulocytes, plasmatocytes, oenocytes and prohemocytes (Amaral et al., 2010; Negri et al., 2016). Among these four, only granulocytes and plasmatocytes have a clear role in the immune system (Wilson-Rich et al., 2008); granulocytes have a relevant role in phagocytosis while plasmatocytes in encapsulation (Richardson et al., 2018). Oenocytes appear to be involved in lipid metabolism and detoxification processes (Martins and Ramalho-Ortigão, 2012), while prohaemocytes do not play a role in the immune system. During a bacterial or fungal infection, phenoloxidase causes granulocytes to start producing nodules. Once activated, this enzyme stimulates the production of melanin, which is crucial for the encapsulation process (Laughton et al., 2011). The infection is then quickly neutralized by granulocytes, and plasmatocytes gather around the dead bacterial cells to enclose them in a sclerotized nodule. The number of haemocytes varies according to the age of the insect: the transition from nurse to forage bee involves a drastic reduction in the number of haemocytes, as well as a suppression of the honey bee immune system (Amdam et al., 2004b). As a result, wound healing, phagocytosis, and encapsulation abilities are drastically reduced with this transition. The number of circulating haemocytes can also fluctuate rapidly due to stress, wounds, and infections (Amdam et al., 2004b). Richardson et al. (2018) observed a difference in the haemolymph composition of larvae, with up to 67.1% granulocytes, compared to adult bees, with a range of 0.6% to 5.9% granulocytes, and queens, with 8% granulocytes in the haemolymph. Caliani et al. (2021a) observed a slight decrease in the number of plasmatocytes, with a consequent increase in the number of granulocytes in honey bees exposed to $CdSO_4$ and the fungicide Amistar®Xtra.

Lysozyme activity is often used as a biomarker of immune system efficiency because it is an enzyme secreted by phagocytes (Balfry and Iwama, 2004) and released in mucus, saliva, and plasma, can hydrolyse the bacterial cell wall, breaking the glycosidic and peptide bonds on its surface. Specifically, lysozyme creates a new polysaccharide by binding N-acetylmuramic acid (NAM) and N-acetylglucosamine through the β-1,4-glycosidic linkage. This new polysaccharide is the main component of the bacterial cell wall. After lysozyme action, the

microorganism cells rupture due to osmotic pressure imbalance, resulting in cell wall dissolution and death of the bacteria. The hydrolytic activity of lysozyme is particularly effective against Gram-positive bacteria (Chen et al., 1996). Lazarov et al. (2019) observed a decrease in the amount of lysozyme in the haemolymph of *A. mellifera carnica* populations with different levels of *Varroa destructor* infestation. Samples from the most infested population (5-20% of infected samples) had a 7% lower concentration of lysozyme than the healthiest population (5% of infected samples). Caliani et al. (2021a) observed a decrease in lysozyme activity after treatment with 0.1 g/L and 2.5 g/L $CdSO_4$, 200 g/L Amistar®Xtra and 12.4 g/L EMS.

Among the various tests used to evaluate the genotoxic damage in an organism, the NA (Nuclear Abnormalities) test is an excellent tool to observe and quantify the nuclear abnormalities in the haemocyte nucleus. The nuclear abnormalities are classified in lobed, kidney, segmented, binucleated nuclei, micronuclei (a small nucleus near the main nucleus) and apoptotic cells (cells that die in a genetically controlled process). There are very few studies on this test applied to *Apis mellifera*. Caliani et al. (2021a) observed a significant increase in nuclear abnormalities after treatment with 0.1 g/L and 2.5 g/L $CdSO_4$, and 200 g/L Amistar®Xtra, almost double the control and with a frequency of 50‰. In this cited paper, EMS was used as a positive control because of its known genotoxic capacity.

1.6.2 Proteomics

Proteomics enables the in-depth study of the proteome, which can be defined as the total protein content characterized by localization, interactions, post-translational modifications, and turnover (Wilkins et al., 1996). Along with the other "omics" technologies (genomics, transcriptomics, and metabolomics), proteomics expresses an organism's protein identity, allowing knowledge of the structure and functions of a specific protein. It is well known that an organism's protein content changes with time and environment; this is both a drawback and a benefit. It is a limitation because it necessitates examining a particular organism under various circumstances in order to get a full picture of its condition. On the other hand, this is a benefit because this technique provides a continuous view of an organism's change in protein pattern in response to external conditions (Holman et al., 2013). This science is used to achieve a variety of goals, ranging from early disease detection to disease monitoring and development via the characterization of the proteome at all stages (expression, structure, functions) to protein interaction and modification (Domon and Aebersold, 2006). Proteomics can act in three stages:

- Expression proteomics is a method for studying the quantitative and qualitative expression of proteins. Its goal is to distinguish between two conditions in terms of protein expression (Banks et al., 2000). Furthermore, it can detect disease-specific proteins as well as new proteins in signal transduction (Graves and Haystead, 2002). Two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) techniques are used to detect differences in protein expression between two or more conditions (Hinsby et al., 2003).
- Structural proteomics is the study of the three-dimensional structure and structural complexities of functional proteins. It specifies all protein interactions in the mixture, such as membranes, cell organelles, and ribosomes (Jungbauer and Hahn, 2009).
- Functional proteomics is the study of protein functions and molecular mechanisms in the cell, as well as the interactions of protein partners. It focuses on the interaction of an unknown protein with partners from a specific protein complex involved in a process. This could indicate the protein's biological function (Gavin et al., 2002)).

There are several proteomics techniques available, including one-dimensional (1D) and two-dimensional gel electrophoresis (2-DE) (Vercauteren et al., 2004), gel-free high-throughput screening technologies such as multidimensional protein identification technology (Florens and Washburn, 2006), stable isotope labelling with amino acids in cell culture (Ong et al., 2002), isotope-coded affinity tag, and isobaric tagging. Other techniques are shotgun proteomics (Wolters et al., 2001), 2D difference gel electrophoresis (2D-DIGE) (Klose et al., 2002), and protein microarrays (Cutler, 2003) which can be used in tissues, organelles, and cells. For high-throughput processing, large-scale western blot assays (Schulz et al., 2007), multiple reaction monitoring assays (Stahl-Zeng et al., 2007), and label-free quantification of high mass resolution liquid chromatography (LC)-tandem mass spectrometry (MS) are commonly used (Al-Amrani et al., 2021).

Two-dimensional gel electrophoresis is a popular technique for analyzing and separating proteins. The latter are separated by isoelectric point and molecular weight; two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is a reliable and efficient method for separating proteins based on charge (in the first dimension) and mass (in the second dimension) (Aslam et al., 2017).

Słowińska et al. (2019) used two-dimensional electrophoresis and mass spectrometry to identify a change in 44 hemolymph proteins in response to the *Varroa destructor* parasite and

one in the protein following DWV (Deforming wing virus) infection in *Apis mellifera* specimens. Following further investigation, it was found that *Varroa destructor* infestation in *Apis mellifera* compromised several functions, including carbohydrate metabolism, oxidative stress response and detoxification, nutrient storage activity, oxidoreductase, and the olfactory system. Vidau et al. (2014) found that the presence of *Nosema ceranea* had effects on the proteome of the *Apis mellifera* midgut using two-dimensional differential electrophoresis (2D-DIGE) in conjunction with mass spectrometry. According to the findings, honey bees infected with *Nosema ceranea* produced 45 proteins that differed from uninfected bees, 14 of which were identified using mass spectrometry. The microsporidian caused protein modulation in biological functions such as energy production, reactive oxygen stress, and protein regulation. Further investigation revealed that the pathogen creates a zone of "metabolic modification of the habitat" in the midgut of honey bees, thereby lowering defenses. Roat et al. (2014) investigated how sub-lethal doses of fipronil (10 g/bee per day for 5 days) affected the brains of young and old *Apis mellifera* and found that 25 proteins were regulated differently than the control. These could be caused by a connection between pathogens, neuronal chemical stress, and visual disturbance. The findings show that short-term exposure of *Apis mellifera* to low doses of fipronil causes a series of changes of neuroproteins in the brain. Wang et al. (2021) investigated the effects of agrochemical pesticides, specifically the fungicide carbendazim, on *Apis mellifera* specimens. In the study, the protein changes in the heads of adults after larval treatment were evaluated in vitro using a proteomic approach. The treatment was found to have a significant impact, inhibiting the formation of a multifunctional protein family found in royal jelly. This effect was observed in both the adult head and the worker bee hypopharyngeal gland; additionally, the fungicide caused visual and olfactory loss, impairment of immune functions and muscle activity, neuronal and cerebral development, protein synthesis and modification.

1.7 Origin and quality of bee products

The products of the hive are the result of an exhausting and coordinated work of honey bees, leading to the production of different derivatives such as honey, propolis, pollen, royal jelly, wax and poison (used in apipuncture). Anthropic activities might modify indirectly, i.e. through climate change, the quality and origin of bee products. For this reason, it is important to integrate the analysis of honey bees health status with the analysis of their products.

1.7.1 Chemical-physical properties of honey

The chemical-physical analyses of honey aim to investigate the parameters influenced by the botanical origin of the honey. Among the most relevant:

- Sugars, which serve as honey's "identity card," are measured using high-performance liquid chromatography (HPLC). It is a straightforward method for determining the sugar content of honey.
- The refractometer is used to measure humidity, which affects honey quality. This is a simple tool that yields immediate results. Humidity measurement is critical because humidity levels above 20% significantly increase the risk of triggering fermentation processes.
- Amino acids, which can be determined using HPLC, can provide valuable information about botanical and geographical origins.

1.7.2 Melissopalynological techniques

Melissopalynological analysis is a collection of techniques for determining the quality of honey, one of the most valuable bee products. The most thorough method for obtaining a nearly full characterization of honey is an integrated approach of different analyses. Melissopalynology is the study of honey's botanical and geographical origins via microscopic analysis and pollen identification. Each honey contains a certain amount of pollen derived from flowers visited by bees. This type of analysis relies on the microscopic identification of pollen grains to confirm the botanical origin of the product. The amount of pollen in honey is influenced by the size and shape of the flower, the number of stamens, and a variety of other factors. Pollen grains can be identified using a light microscope based on their size and external morphology. When combined with sensory and chemical-physical analyses, this type of assay provides a complete profile of the product, as well as information on its geographical origin. This analysis also allows for the collection of a wealth of additional information, such as the honey's processing, extraction and filtration methods, and contamination with certain components (soot and dust). There are two types of melissopalynological analyses:

- The identification and recognition of pollen grains is the focus of qualitative melissopalynological analysis. This produces a complex pollen spectrum.
- The absolute number of vegetal figurative elements per unit of honey weight (10 grams) is found through quantitative melissopalynological analysis. Furthermore, it allows the

qualitative analysis results to be correctly interpreted, allowing the origin of honey to be determined.

2. AIM

The health of bees and, more generally, of pollinating insects is increasingly at risk due to human impact. This is worrying both for the balance of natural ecosystems and for the agricultural production system. The massive use of chemicals in agriculture and in every other field is now known as one of the main causes of the global decline in bee populations: agrochemicals, heavy metals, volatile organic compounds, highly reactive chemical species and many other factors are compromising the health status of the hives, causing a weakening of these animals which are more easily attacked by pathogens and parasites. The sub-lethal levels of pesticide residues and other anthropic contaminants, even not leading to the death of individuals, are able to cause problems in the development, behaviour and health of animals in the short and long term.

Moreover, in the environment, it is likely to find not single compounds, but mixtures of pesticides, given at the same time or one right after the other, to which honey bees are exposed and that may exhibit ways of action similar or synergistic, antagonist or additive interactions (Piggott et al., 2015). However, despite the high probability of honey bees of being exposed to a blend of pesticides, there is a large gap in the assessment of the sub-lethal effects of these mixtures. Moreover, commercial pesticides are constituted not only by the active ingredients but also by co-formulants or adjuvants, also called “inerts”, specifically added to influence the absorption and stability of the active principle (Travlos et al., 2017). A pesticide compound is considered to be active when intentionally added to be toxic to target species. All others are defined as inert ingredients, although this does not exclude their own toxicity, including on non-target species (Mesnage and Antoniou, 2018). To the best of our knowledge, in literature the effects of commercial pesticides on honey bees are not studied, while a great amount of papers focused their attention only on active principles. In fact, only Caliani et al. (2021a) observed the effects of a commercial fungicide, Amistar®Xtra, on honey bees.

Most of the field studies in literature investigated the accumulation of different pollutants in *Apis mellifera*, while only a few papers focused on the sub-lethal effects of environmental contamination on honey bees in their natural conditions and habitats. Honey bees proved to be sensitive bioindicators of environmental pollution. For this reason, monitoring studies should be implemented in order to actually evaluate the health status of these organisms, and to provide effective tools to the legislation to protect this and all other insect pollinators.

Another research gap is constituted by the lack of studies evaluating genotoxicity and alteration of the immune system related to xenobiotic stressors. In fact, only two studies (Caliani et al., 2021a, 2021b) started exploring the genotoxic damages that pesticides and heavy metal could cause in honey bees. The evaluation of the immune system status, related to the exposure to pesticides was poorly studied, usually using gene expression. In this case, the use of biomarkers to diagnose the responses of certain immune system components could be useful to improve the knowledge about the effects of contaminants on honey bees immune system.

The understanding and quantification of sub-lethal toxicity mechanisms to which these animals are subjected are vital for implementing the mitigation and safeguarding measures for these and other pollinators, also supporting legislative actions. Anthropic activities could also be able to modify indirectly, i.e. through climate change, the quality and origin of bee products. An integrated approach could be useful to combine responses at different levels, becoming a valid tool to evaluate the impact of contamination on these organisms.

For these reasons, the goal of this thesis was to assess the health of honey bee colonies using a multi-tier methodology that included biomarker responses, proteomic analysis, and bee product quality and origin.

To accomplish this, the project was divided into two major phases:

- A laboratory study, aiming to fill the gaps regarding the effects of less studied pesticides, such as fungicides and herbicides, alone and in combination, using commercial formulates. For this reason, *Apis mellifera* specimens were exposed to two commercial pesticides, the fungicide Sakura® and the herbicide Elegant 2FD, alone and in combination. The effects of these compounds were assessed integrating two methodologies, consisting in a set of biomarkers and a proteomics approach.
- A two-year field study, to monitor the ecotoxicological status of bees in natural environments. The sampling of *Apis mellifera* specimens was carried out in 10 locations in Tuscany region characterised by varying contamination patterns. The used approach was constituted by the integration of different methodologies: a set of biomarkers was used to assess the health status of honey bees while the origin and quality of the main beehive product, honey, were examined through melissopalynological and chemical-physical analyses.

3. EVALUATION OF THE EFFECTS OF A COMMERCIAL FUNGICIDE AND A COMMERCIAL HERBICIDE, ALONE AND IN COMBINATION, ON *Apis mellifera*, USING AN INTEGRATED APPROACH BASED ON BIOMARKERS AND PROTEOMICS

3.1 Introduction

Agrochemical products are important components of agricultural systems worldwide. These compounds protect crops from unwanted pests and pathogens and remove weeds, allowing an increase in agricultural production (Alexandratos and Bruinsma, 2012; Carvalho, 2017). The honey bees' health status is influenced, among other causes, such as climate change, parasites, and urban pollution, by the presence of pesticides. Bees can be exposed to these compounds by contact via air particles (dust and spray droplets) to volatile compounds diluted in the air, by walking on contaminated surfaces or when they collect and use nesting materials, and by ingestion through contaminated food (nectar, pollen, water). Among pesticides, a great number of papers focused on insecticides and their lethal and sub-lethal effects at the individual (Al Naggar et al., 2015; Badawy et al., 2015; Badiou et al., 2008; Badiou-Bénéteau et al., 2012; Carvalho et al., 2013; Christen et al., 2019a; Qi et al., 2020) and colony levels (Dai et al., 2010; Decourtye et al., 2004; Imran et al., 2019; Ingram et al., 2015; Monchanin et al., 2019; Yang et al., 2012). Nonetheless, also fungicides and herbicides were demonstrated to affect honey bee's health status (Boily et al., 2013; Caliani et al., 2021a; Gregorc et al., 2012; Herbert et al., 2014; Lupi et al., 2020; Mullin et al., 2010; Odemer et al., 2020; Raimets et al., 2020). As previously mentioned, in the environment, is likely to find mixtures of pesticides to which honey bees are exposed. Various studies have been conducted to evaluate the effects of mixtures of different insecticides (Christen et al., 2017; Wang et al., 2020; Y. Wang et al., 2021; Yao et al., 2018a, 2018b; Zhu et al., 2017a) and the interactions between insecticides and fungicides (Bjergager et al., 2017; Colin and Belzunces, 1992; Iwasa et al., 2004; Meled et al., 1998; Schmuck et al., 2003; Thompson et al., 2014; Zhu et al., 2017a, 2017b).

As said before, the literature focused the attention on the effects in *Apis mellifera* of active principles instead of studying the entire commercial pesticides, made of both active principles and co-formulants. In order to start to fill this research gap, we selected two commercial pesticides, whose effects are not yet studied.

Biomarkers and proteomics can be sensitive and useful tools to investigate the sub-lethal effects of contaminants on the ecotoxicological health status of honey bees. To the best of our

knowledge, the application of a set of biomarkers, integrated with a proteomic approach, was never used in previous literature.

For these reasons, the aim of this laboratory study was to evaluate the effects of a commercial fungicide and a commercial herbicide on honey bees, both separately and in combination, integrating a multi-biomarker approach with a proteomics one, enabling the evaluation of the species' toxicological status. To this aim, honey bee workers were topically exposed to the fungicide Sakura®, the herbicide Elegant 2FD, and their mixture for 4 days. Both these pesticides are widely used in wheat cultivations, and they are quite often used together during the uprooting stage of plant. Bees may be exposed to these two chemicals accidentally, since the pesticides might reach flowers near wheat cultivations, where pollinators collect pollen and nectar. In this way, forager bees could transport the pesticides to the hive, exposing the entire colony, from larvae to the queen, to the contamination risk.

Among biomarkers, we selected acetylcholinesterase (AChE) and carboxylesterase (CaE) to study neurotoxic effects, glutathione S-transferase (GST) and alkaline phosphatase (ALP) for detoxification and metabolic changes. Immune system biomarkers (lysozyme, LYS, activity, and haemocytes count) and genotoxicity (Nuclear Abnormalities, NA assay) were also assessed, in order to start filling a gap of knowledge regarding these specific responses.

3.2 Material and methods

3.2.1 Honey bees

Honey bee workers were collected from the hives of a beekeeper located near a nature reserve (Pisa, Tuscany, Italy). The bees were collected on the day before the experiment, by opening the hive and sampling home bees. The bees were then placed in seven cages, one for each experimental group (75 cm x 75 cm x 115 cm, Bug-Dorm-2400 Insect Rearing Tent, MegaView Science Co., Ltd., Taiwan), to rest overnight at 25 °C and 60% relative humidity with honey and water ad libitum. A leafless branch of *Prunus spinosa* (Rosaceae) was placed in the cage to support the bees.

3.2.2 Compounds and exposure conditions

The compounds used for the experiment were the fungicide Sakura® at two concentrations, 200 g/L (FUNG 1) and 400 g/L (FUNG 2), the herbicide Elegant 2FD at two concentrations, 225 g/L (HERB 1) and 450 g/L (HERB 2), and their mixture, Sakura® 200 g/L+ Elegant 2FD

250 g/L (MIX 1) and Sakura® 400 g/L+ Elegant 2FD 450 g/L (MIX 2), dissolved in 80% acetone. The commercial Sakura® consists mainly of bromuconazole (15,86%) and tebuconazole (10,17%), while Elegant 2FD consists mainly of 2,4D (42,3 g/100 g of product) and Florasulam (0,6 g/100 g of product). For both treatments, the lowest doses used were the recommended field concentrations for cereal crops, while the highest doses were twice the field doses.

The honey bees were lightly anaesthetized with CO_2 on the day of the experiment, and 2 µL of each contaminant solution was applied to the dorsal thorax of the bees using a Burkard hand microapplicator equipped with a 1-mL syringe (Bedini et al., 2017) (Figure 2). The control group of bees received 2 µL of 80% acetone. Each group of treated bees (50 each) was placed in a separate cage for four days, and mortality was recorded daily. At the end of the experiment bees were individually collected and sampled.

Figure 2. Cage used for the experiment and Burkard hand microapplicator equipped with a 1-mL syringe

3.2.3 Dissection and collection of biological materials

After collection, honey bees were anesthetized with ice (4°C) for at least 30 minutes. From each honey bee abdomen, 5-10 µL of haemolymph was collected and placed on a poly-lysed slide (2.5 mg/mL). The midgut was then removed from the back of the abdomen using tweezers. The head was then separated from the body with a crosscut. The biological materials were then stored at -80 °C for further analysis. The intestines were used for the analysis of lysozyme, GST and ALP, and the heads for the analysis of AChE and CaE activity.

3.2.4 Biomarkers

3.2.4.1 Preparation of samples

Homogenization of the samples was performed to extract the cellular fractions used for subsequent analyses. Pools of three honeybees each were created. The biological materials were weighed to calculate the volume of 40 mM Na-phosphate homogenization buffer (pH 7.4) to be added at a ratio of 1:10 (w/v). This procedure was repeated for each pool of samples. A metal bead was then added to each eppendorf tube for homogenization, which was performed using a Tissue Lyser; three passes were made at 30 F for 30 seconds, with a 30-second pause. Once the samples were homogenized, the beads were removed and the eppendorf tubes were centrifuged at 4 °C for 20 minutes at 13,000g for the nervous tissue pools and 15,000g for the intestinal tissue pools. At the end of both centrifugations, the supernatants were collected and stored at -80 °C.

3.2.4.2 Acetylcholinesterase (AChE)

Acetylcholinesterase activity was assessed by a spectrophotometric method according to Caliani et al. (2021a). The assay quantifies the rate of hydrolysis of acetylthiocholine (the substrate) to acetate and thiocholine. The thiocholine reacts with DTNB, which develops a colourimetric reaction that is read at 410 nm.

The reaction mixture contained sodium phosphate buffer 0.1 M (pH 7.4), 2'-dinitro-5,5'-dithiodibenzoic acid (DTNB) 10 mM, acetylthiocholine (ATCI) 41.5 mM and the homogenate. The reaction was monitored for 5 minutes using a spectrophotometer (Perkin-Elmer, Lambda EZ 201). The reading was performed in duplicate for each sample after reading the blank. The temperature was kept at 25 °C. The enzymatic activity was expressed as μmol substrate x g tissue^{-1} x min^{-1}.

3.2.4.3 Carboxylesterase (CaE)

Carboxylesterase activity was evaluated in the post-mitochondrial fraction of honey bee neural tissue (Caliani et al., 2021a). This method quantifies enzymatic activity by recording the absorbance of the reaction product between α-naphthol, resulting from the hydrolysis of the substrate by the carboxylesterase, and the diazonium salts of the dye (Fast Garnet GBC) (Dary et al., 1990). The incubation mixture contained 100 mM phosphate buffer (pH 7.4) and the homogenate. The mixture was placed in a thermostated bath at 25 °C for 5 minutes. The

substrate, α-naphthylacetate (0.4 mM), was then added to initiate the reaction, which took place for 3 minutes in the thermostated bath. Finally, 5% SDS was added to stop the reaction and used 0.4 mg/L Fast Garnet GBC, as a dye.5 mL. Reaction product was quantified using a spectrophotometer (Perkin-Elmer, Lambda EZ 201) at 538 nm. Enzyme activity was expressed as nmol α-naphthylacetate x min^{-1} x mg $prot^{-1}$, using a molar extinction coefficient of 23.59 x 10^3 mM^{-1} cm^{-1}.

3.2.4.4 Glutathione S-transferase (GST)

GST activity was assessed in the post-mitochondrial fraction of the intestine according to Caliani et al. (2021a). This assay quantifies the conjugation of reduced glutathione (GSH) to a substrate, the 2,4-dinitrochlorobenzene (DCNB). The reaction mixture consisted of 0.1 M sodium phosphate buffer (pH 7.4), 8 mM GSH, 8 mM DCNB and the extract. Reading was performed with a spectrophotometer (Perkin-Elmer, Lambda EZ 201) at 342 nm for 3 minutes at 25 °C. Enzyme activity was expressed as nmol DCNB x min^{-1} x mg $prot^{-1}$, using a molar extinction coefficient of 9.6 x 10^3 $mM^{-1}cm^{-1}$.

3.2.4.5 Alkaline phosphatase (ALP)

Alkaline phosphatase activity in the post-mitochondrial fraction of the bee intestine was evaluated using the method of Caliani et al. (2021a). The enzymatic assay quantifies the formation of p-nitrophenol, a coloured compound resulting from the hydrolysis of the substrate p-nitrophenyl phosphate (p-NPP) by alkaline phosphatase. The reaction mixture consisted of 100 mM Tris-HCl buffer (pH 8.5), 100 mM MgCl2, 100 mM p-NPP and the homogenate. Reading was performed using a spectrophotometer (Perkin-Elmer, Lambda EZ 201) at 25 °C for 5 minutes at 405 nm. Enzyme activity was expressed as nmol p-nitrophenylphosphate x min^{-1} x mg $prot^{-1}$, using a molar extinction coefficient of 18.81 x 10^3 $mM^{-1}cm^{-1}$.

3.2.4.6 Lysozyme (LYS)

Lysozyme activity was measured in the post-mitochondrial fraction of intestinal cells, using a standard turbidity assay according to the method of Caliani et al. (2021a). A stock solution of 1 mg/mL of hen egg white lysozyme (HEL, Sigma, St. Louis, MO) in 0.1 M phosphate buffer (pH 5.9) and a solution of 0.3 mg/mL *Micrococcus lysodeikticus* (Sigma, St. Louis, MO) were prepared. HEL was serially diluted in 0.1 M phosphate buffer (pH 5.9) to generate a standard curve of 0, 0.3, 0.6, 1.25, 2.5, 5, 10, 15, and 20 µg/mL. Aliquots of each concentration were added to a 96-well plate, in triplicate. For each sample, the homogenate was added to the plate

in triplicate. The *M. lysodeikticus* solution was rapidly added to the three standard curve wells and three sample wells. The blank was prepared by adding 0.1 M phosphate buffer to each sample. Absorbance was measured at 450 nm using a multiplate reader (Multiskan Skyhigh Thermo Scientific). Readings were taken immediately (T0) and after 5 minutes (T5). The activity was expressed as HEL concentration (μg/mL) by linear regression of the standard curve.

3.2.4.7 Differential haemocytes count and Nuclear Abnormalities (NA) assay

The differential haemocytes count was carried out using the method proposed by Şapcaliu et al. (2009), while the NA assay was carried out using the method proposed by Pacheco and Santos (1997). Both biomarkers were evaluated on the same slide, which has previously been stained with Diff-Quick dye. For each of the two assays, 1000 sample cells were read at 100x magnification using an optical immersion microscope (Olympus BX41), and only mature haemocytes (those with a well-defined nucleus) were considered. The result of the NA assay (Figure 3) was expressed as the number of nuclear abnormalities per 1000 cells. The number of haemocytes/1000 cells was calculated using a differential haemocyte count that included the two most common types of haemocytes, plasmatocytes and granulocytes.

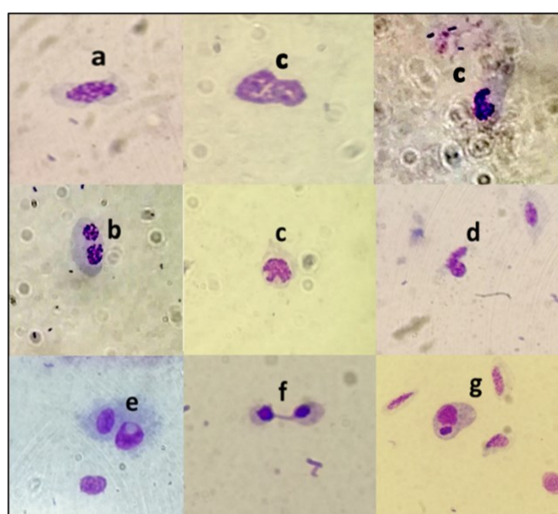

Figure 3. Haemocytes nuclear abnormalities considered in the NA assay: a) mature haemocyte; b) binucleated; c) lobed; d) kidney; e) apoptotic cell; f) segmented; g) micronucleus

3.2.4.8 Total protein content

Protein concentration was determined using the Bradford (1976) spectrophotometric method. A calibration standard curve was created using bovine serum albumin (BSA) at the following concentrations: 0.1 mg/mL; 0.2 mg/mL; 0.3 mg/mL; 0.4 mg/mL; and 0.5 mg/mL. The samples

were diluted in 0.02% TRITON. 20 μL of each diluted sample were taken and mixed with 1 mL of BioRad Protein dye (1:5). The absorbance was measured at 595 nm using a spectrophotometer (Perkin-Elmer, Lambda EZ 201). The blank (BioRad Protein solution) was read first, followed by duplicate samples. The protein concentration was calculated using the BSA standard curve and expressed in mg/mL.

3.2.4.9 Statistical Analysis

We tested for significant differences in each biomarker between ACETONE, the fungicide Sakura®, the herbicide Elegant 2FD and the mixture samples using the Kruskal-Wallis (KW) test (Kruskal and Wallis, 1952). This non-parametric test is used when the data do not satisfy the normality property and contain outliers. In addition, Dunn's test with a Benjamini–Hochberg stepwise adjustment (Benjamini and Hochberg, 1995) was used for pairwise multiple-comparison when the null hypothesis of the KW test was rejected. These tests were implemented using STATA 17- software (StataCorp. 2021).

3.2.5 Proteomic analysis

3.2.5.1 Protein extraction

Intestines collected from bees were resuspended in 600 μL of 15% trichloroacetic acid (TCA) and 1% dithiothreitol (DTT) diluted in cold acetone. The beads were placed in each eppendorf tube to allow solubilization of the tissue using a Tissue Lyser; two passes were made at 30 F for 2 minutes, with a 30-second break between each pass. The samples were left on ice for 30 minutes and then centrifuged at 16,000g for 15 minutes at 4°C. The supernatant was removed, and the pellet was washed three times with 600 μL of cold acetone, centrifuged as before, and allowed to dry for 30 minutes at room temperature. Subsequently, 50 μL of 0.2 M NaOH was added to each eppendorf to neutralize TCA residues, and the samples were allowed to stand for 2-3 minutes. Then, 200 μL of Rehydration Buffer (RB) was added and the samples were sonicated five times for 30 seconds with 1 minute on ice in between. Finally, the samples were centrifuged at 16,000g for 15 minutes at room temperature and the supernatant was collected.

3.2.5.2 Protein concentration of the extracts

To load the same amount of protein into the gel for each sample, the protein concentration of each pool was calculated using the 2-D Quant Kit (GE General Electric Company by G-

Biosciences, USA) according to the protocol provided. Proteins are precipitated and resuspended in a copper solution in this method. Some copper ions bind to the proteins, while the free ions are measured using a colorimetric method. The color intensity, or absorbance, measured by the spectrophotometer at 480 nm is inversely proportional to the protein concentration and is compared to a standard curve prepared with known amounts of bovine serum albumin (BSA). The kit provides a linear response in the range of 0 - 50 μg of protein.

3.2.5.3 Bidimensional electrophoresis

First dimension: 10 μL of 1 M DTT per 200 μL of sample and 4 μL of IPG Buffer per 200 μL of sample were added to the samples. Readystrip IPG strips (Bio-Rad) were used at two different pH values, 4-7 and 7-10, in order to better visualize the acidic and basic proteins. The sample was loaded into the center of the IPG Readystrips positioned on the Immobiline DryStrip Reswelling Tray support (Pharmacia Biotech). The sample self-expanded and adhered to the strip along the entire length of the gel. After 1 hour, 2 mL of mineral oil (Bio-Rad) were added to prevent oxidation of the proteins and to keep the strips sufficiently hydrated during the 24 hours required to fully absorb the sample. Then, strips were placed on the Focusing Tray (Bio-Rad) and anointed again with mineral oil; the support was placed on the Protean IEF Cell (Bio-Rad). Isoelectric focusing was then performed, an electrophoretic run designed to separate proteins based on their isoelectric point, using the following protocol: from 0 to 500 V in 1h, to 500 V for 1 h, from 500 to 4000 V in 2 h, to 4000 V for 2 h, from 4000 to 8000 V in 2 h, to 8000V until 15000 V was reached and then back to 500 V for 30 minutes. At the end of the run, the strips were collected, placed in special trays and washed with 4 mL Equilibration Buffer 1 (130 mM DTT; 6 M urea; 2% SDS; 0.375 M Tris-HCl, pH 8.8; 20 % glycerol), for 10 minutes, then discarded. A second wash was performed with 4 mL Equilibration Buffer 2 (135 mM Iodoacetamide; 6 M urea; 2% SDS; 0.375 M Tris-HCl, pH 8.8; 20% glycerol), for 10 minutes.

Second dimension: at the end of the incubation, the strips were placed in the well of the Criterion gel (Bio-Rad) and immobilized with agarose gel previously heated to 95°C on a plate. A molecular weight standard was loaded and the electrophoretic run was started, separating the proteins on the basis of their molecular weight orthogonal to the order assumed on the basis of the first separation. XT MOPS Running Buffer (Bio-Rad) was used as the running buffer. The run took approximately 55 minutes at 200V.

Coomassie staining: the gels were placed in a staining solution consisting of 0.04% Coomassie Brilliant Blue R-250, 25% methanol and 20% acetic acid for 1 hour. They were then placed in a decolorizing solution composed of 25% methanol and 10% acetic acid overnight.

3.2.5.4 Image analysis

The gels were stained and then placed in a digital imaging system, the Fluor-S Multimager (Bio-Rad), with a Tamron 80 mm objective. Images of the gels were acquired and analyzed using Quantity One software (Bio-Rad) to locate the spots. Spots were automatically detected and manually re-evaluated before the software generated a "master gel," which is a virtual gel that contains all of the spots present in the gels examined. Following that, matching was performed, which involved pairing each gel spot with the corresponding virtual spot in the master gel. This enabled the alignment and comparison of all of the spots, both qualitatively and quantitatively.

3.2.5.5 Statistical Analysis

The relative volume of each spot, expressed as a percentage (Vol%), was measured for each gel using PDQuest software (Bio-Rad). This value corresponded to the ratio, expressed as a percentage, between the volume of the individual spot and the total volume of the spots present in the gel being analyzed. Variations in the average Vol% (average Vol% = arithmetic mean of the Vol% values of the corresponding individual spots, by matching, in different gels) between the two conditions studied were considered significant when satisfied Tukey's test with a p-value ≤ 0.05.

3.2.5.6 Mass spectrometry (MS)

Statistically significant differential spots were cut out manually from MS-preparative 2-DE gels. Spots were destained in a solution of 40% acetonitrile and 0.05 M ammonium bicarbonate, until complete destaining. Then, spots were reduced for 20 min with reducing solution (0.01 M DTE and 0.1 M ammonium bicarbonate). The gel spots were then alkylated with alkylation solution (10 mg/mL iodoacetamide and 0.1 M ammonium bicarbonate) for 20 min. After washinf the spots with 0.1 M ammonium bicarbonate for 20 min, with destain solution for 20 min and with ultrapure water for 20 min, the samples were dried in a vacuum centrifuge. The samples were then digested overnight in a trypsin solution (10 ng/µL trypsin and 0.01 M ammonium bicarbonate, pH 8.5). The clean peptide solution was dried in a vacuum centrifuge. The resuspension of the dried peptides was performed prior the LC-MS/MS analysis in peptide

loading buffer, constituted by 0.1 v/v formic acid, pH 3.5. LC-MS/MS analysis was performed on aDionex Ultimate 3000 UHPLC nano flow system coupled to a Thermo Q Exactive mass spectrometer (Thermo Fisher Scientific,Waltham, Massachusetts, United States). Prior to the analysis, each sample was reconstituted in 12 µL mobile phase A (0.1% Formic Acid, pH 3.5) and 6 µL loaded on the column. A Dionex 0.1 × 20 mm, 5 µm, 100 Å C18 nano trap column with a flow rate of 5 µL/min was used. The analytical column was a PepMap C18 nano column 75 µm × 50 cm, 2 µm 100 Å. Gradient elution of analytes was carried out with aqueous 0.1% (v/v) formic acid (A) and 0.1% (v/v) formic acid in acetonitrile (B). The gradient elution was: T=0min, 2%B; T=10 min, 2% B, T=200 min, 33% B, T=210 min, 80% B, T=230 min, 2% B; T = 240 min, 2% B. The flow rate was 300 nL/min. The samples were analyzed using a Data Dependent Acquisition (DDA) methodology. More precisely, the analysis of the samples was performed in the full scan ion mode, applying a resolution of 70,000 with a mass range of 200 to 2000 m/z while the acquisition of the mass spectra was performed in the centroid mode. The maximum injection time was set at 100 ms and the AGC target at 3E6. The top 10 most abundant precursor ions were forwarded to HCD (higher energy collisional dissociation) fragmentation. The normalized collision energy was set to 33, the maximum injection time was set at 50 ms and the AGC target at 1E5. Raw files were processed with Thermo Proteome Discoverer 1.4 software.

3.3 Results and discussion

3.3.1 Biomarkers

After exposing honey bees to Sakura®, Elegant 2FD, and a combination of the two, for 4 days, biomarkers of neurotoxicity (AChE and CaE), metabolism (ALP and GST), immune system (LYS and hemocyte count), and genotoxicity (NA assay) were measured. The mortality registered during the experiment was never higher than 10%, the contaminant doses used were therefore confirmed to be sub-lethal.

In Table 1 the statistically significant differences between the acetone control, the fungicide Sakura®, the herbicide Elegant 2FD and the mixture samples were reported.

Table 1. p values of the multiple pairwise comparison tests of the seven biomarkers

	Acetone	FUNG 1	FUNG 2	HERB 1	HERB 2	MIX 1
AChE						
FUNG 1	< 0.01					
FUNG 2	< 0.01	n.s.				
HERB 1	n.s.					
HERB 2	< 0.01			< 0.01		
MIX 1	n.s.	< 0.01				
MIX 2	< 0.05		< 0.01			n.s.
CaE						
FUNG 1	< 0.01					
FUNG 2	< 0.01	n.s.				
HERB 1	n.s.					
HERB 2	n.s.			n.s.		
MIX 1	n.s.	< 0.01		n.s.		
MIX 2	< 0.01		< 0.05		< 0.05	< 0.01
GST						
FUNG 1	n.s.					
FUNG 2	< 0.01	< 0.01				
HERB 1	n.s.					
HERB 2	< 0.01			< 0.05		
MIX 1	< 0.01	< 0.05		n.s.		
MIX 2	< 0.01		< 0.01		n.s.	< 0.05
ALP						
FUNG 1	< 0.01					
FUNG 2	< 0.01	n.s.				
HERB 1	n.s.					
HERB 2	n.s.			n.s.		
MIX 1	n.s.	< 0.01		n.s.		
MIX 2	n.s.		< 0.01		n.s.	n.s.
LYS						
FUNG 1	n.s.					
FUNG 2	n.s.	n.s.				
HERB 1	n.s.					
HERB 2	n.s.			n.s.		
MIX 1	n.s.	n.s.		n.s.		
MIX 2	n.s.		n.s.		n.s.	n.s.
PLASM						
FUNG 1	< 0.05					
FUNG 2	n.s.	n.s.				
HERB 1	n.s.					
HERB 2	n.s.			< 0.05		
MIX 1	n.s.	n.s.		< 0.05		
MIX 2	n.s.		n.s.		n.s.	n.s.
NA assay						
FUNG 1	n.s.					
FUNG 2	n.s.	n.s.				
HERB 1	n.s.					
HERB 2	n.s.			n.s.		
MIX 1	n.s.	n.s.		n.s.		
MIX 2	n.s.		n.s.		n.s.	n.s.

AChE is a key enzyme controlling and modulating neural transmission (Badiou et al., 2008) and can be inhibited not only by organophosphorus and carbamate insecticides, but also by other contaminants (Boily et al., 2013; Carvalho et al., 2013; Frasco et al., 2005; Fukuto, 1990), representing a sensitive biomarker of neurotoxicity. AChE activity (Figure 4) was found to be statistically different in bees exposed to both doses of the fungicide. This result is different from those of Caliani et al. (2021a), which demonstrated that the commercial fungicide Amistar®Xtra, mainly composed by azoxystrobin, which is a strobilurin, in co-formulation with cyproconazole, inhibited AChE activity. On the contrary, no variations in AChE activity were observed after the 10 days treatment with difenoconazole, a curative and preventive fungicide that belongs to the class of triazoles (Almasri et al., 2020). The results of our work suggest that the triazoles, unlike the strobilurins, are not able to cause variations in AChE activity. Differently from the fungicide, HERB 2 displayed significant AChE inhibition compared to the control and to HERB 1. To the best of our knowledge, the sub-lethal effects of Elegant 2FD, as well as the active principle 2,4 D, were never investigated in *Apis mellifera* before. Recently Almasri et al. (2020) exposed *A. mellifera* specimens to the herbicide glyphosate (0.1 and 1 μg/L) for 10 and 20 days, observing an AChE inhibition at the highest dose. The results obtained in our study let us hypothesize that the herbicide is able to cause neurotoxicity. MIX 2 was significantly inhibited compared to the control and to FUNG 2, and MIX 1 showed a significant inhibition compared to FUNG 1. Since the fungicide showed no variations while the herbicide was able to inhibit this enzyme activity, the neurotoxic effects after both mix treatments could be attributed to the herbicide.

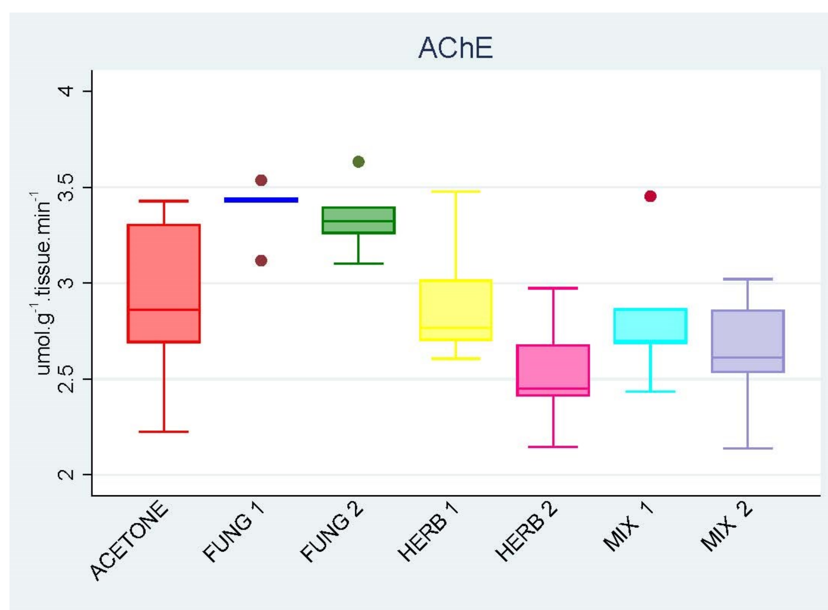

Figure 4. AChE activity measured in the head of honeybees (*Apis mellifera*) exposed to the fungicide Sakura® (200 and 400 g/L), the herbicide Elegant 2FD (225 and 450 g/L), and their mixtures

CaE activity (Figure 5) was significantly inhibited in a dose-dependent way compared to the control in both fungicide treatment groups and in MIX 2. MIX 1 showed a significant induction with respect to FUNG 1 treatment group while MIX 2 showed a statistically significant inhibition with respect to HERB 2 and MIX 1 and induction with respect to FUNG 2. CaEs are hydrolases catalysing the reactions of a wide range of aliphatic/aromatic esters and choline esters, as well as some xenobiotics (Dauterman and Hodgson, 1990). CaEs are enzymes involved in phase I of detoxification processes but also have a defence function, protecting AChE from its inactivation (Jackson et al., 2013; Yan et al., 2009). An induction in CaE activity was observed by various authors after the exposure of *A. mellifera* specimens to different classes of pesticides (Badiou-Bénéteau et al., 2012; Carvalho et al., 2013; Hashimoto et al. 2003; Roat et al., 2017). In agreement with our data Caliani et al. (2021a) and Almasri et al. (2020) observed a decrease in CaE activity after exposure to the fungicide Amistar®Xtra (200 g/L) and the difenoconazole (0.1 and 1 µg/L), respectively. The absent reduction of AChE and simultaneous decrease in CaE activity after exposure to the fungicide could indicate that CaE act as detoxification enzyme and not for the protection of AChE. The only author that evaluated sublethal effects in honey bees after exposure to the herbicide glyphosate (0.1 and 1 µg/L) found a decrease in CaE (Almasri et al., 2020), while no variations was found after Elegant 2FD treatments. The observed AChE reduction and no increase in CaE activity after the herbicide and mix treatments could suggest that CaE did not perform a protection on AChE activity demonstrating that these compounds could affect bees. The highest dose of each

compound caused a decrease in CaE values compared to those found at the lowest dose; in particular, the fungicide showed the most evident inhibition at both doses. Carvalho et al. (2013) observed CaE1 inhibition in honey bees at the lowest deltamethrin dose (5.07 ng/bee); similarly, the lowest CaE levels were observed in our study at the highest pesticide doses. These results could indicate that high concentrations of these compounds can modulate this phase I biotransformation enzyme.

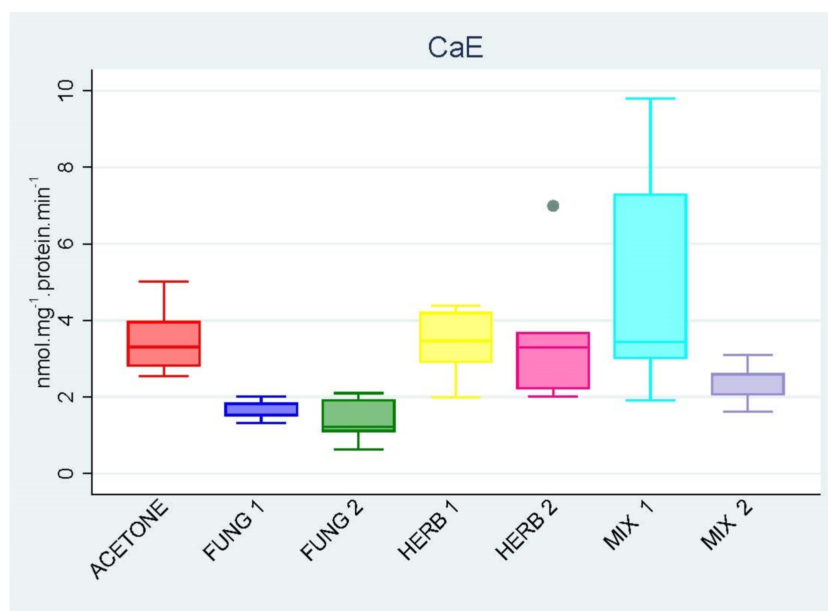

Figure 5. CaE activity measured in the head of honeybees (*Apis mellifera*) exposed to the fungicide Sakura® (200 and 400 g/L), the herbicide Elegant 2FD (225 and 450 g/L), and their mixtures

The enzymatic assay results also showed that GST followed different patterns depending on the exposure to fungicide and herbicide. In fact, GST activity was inhibited after FUNG 2 treatment, with a statistically significant difference compared to the control while HERB 2, MIX 1 and MIX 2 specimens showed significant induction (Figure 6). There was a statistically significant difference observed between the two herbicide doses, with HERB 2 showing higher values compared to HERB 1. Similarly, there was a significant difference between the two fungicide doses, with GST activity decreasing from FUNG 1 to FUNG 2. Furthermore, GST activity increased significantly from MIX 1 to MIX 2. The two mix doses showed activities similar to the herbicide ones, and each of them showed a statistically significant difference with respect to the corresponding fungicide dose, as already observed in AChE results. This suggest that the herbicide is the main responsible for the GST increase observed in the mix treatments. GST is an enzyme involved in phase II biotransformation processes, in fact it is responsible for detoxifying various contaminants and most likely contributes to cellular protection against oxidative damage (Babczyńska et al., 2006; Barata et al., 2005). The lipophilic compounds are

able to induce GST activity, since GST participate in the conjugation of a wide variety of drugs and metabolites with GSH (Letelier et al., 2010). GST induction is a well-known process in vertebrates species exposed to lipophilic compounds (Topić Popović et al., 2023), while in pollinator species few papers are available about the modulation of GST activity after the exposure to chemical compounds (Koirala et al., 2022) and in particular to pesticides (Araújo et al., 2023; Caliani et al., 2021a; Martins et al., 2022). Caliani et al. (2021a) observed an increase in GST activity in honey bees exposed to a fungicide containing cyproconazole and azoxystrobine. This type of response has also been observed in other taxa exposed to fungicides, such as the earthworm *Eisenia fetida* (Han et al., 2014) and the moth *Mamestra brassicae* (Johansen et al., 2007). On the contrary, Martins et al. (2022) observed, in agreement with our results, a decrease in this activity in *Osmia bicornis* specimens exposed to an insecticide, the Confidor® (the main active principle is imidacloprid), and a fungicide, the Folicur® SE (constituted by tebuconazole). The decreased GST activity in bees exposed to fungicides could be related or the manifestation of an adaptation mechanism to an oxidative stress condition. In fact, triazole fungicides demonstrated to cause oxidative stress in zebrafish specimens (Huang et al., 2022; Zhu et al., 2021). This kind of pesticide could, in fact, be able to exceed the ability of the enzyme to detoxify, resulting in a decrease in the GST values. On the other hand, our results suggest that the herbicide and consequently the mix treatments are able to induce and activate the detoxification processes.

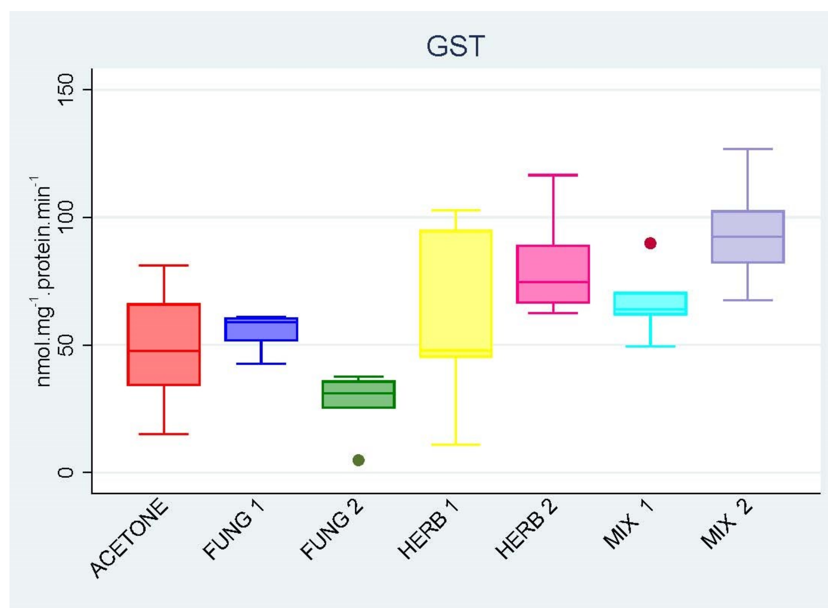

Figure 6. GST activity measured in the midgut of honeybees (*Apis mellifera*) exposed to the fungicide Sakura® (200 and 400 g/L), the herbicide Elegant 2FD (225 and 450 g/L), and their mixtures

ALP is part of an enzyme family involved in digestive processes, cell signaling, and the transport of metabolites and antioxidants through the hydrolysis of phosphate groups (Bounias et al., 1996). Both fungicide treatments significantly inhibited ALP activity in a dose-dependent manner (Figure 7). The decreased response obtained after Sakura® treatments was also observed by Caliani et al. (2021a) after the treatment of honey bees with the fungicide Amistar®Xtra. Although it is known that ALP is not involved in pesticide detoxification (Caliani et al., 2021a), the fungicide Sakura® could affect the ALP activity in honey bees. This could potentially cause irregularities in nutrients transport and absorption at the intestinal level, ultimately weakening the animals. As with AChE and GST activities, the two different mix doses showed values that were similar to the herbicide values and statistically different from the corresponding fungicide dose (Figure 7). In this case, the herbicide appears to counteract the effects of the fungicide in response to the combination treatments.

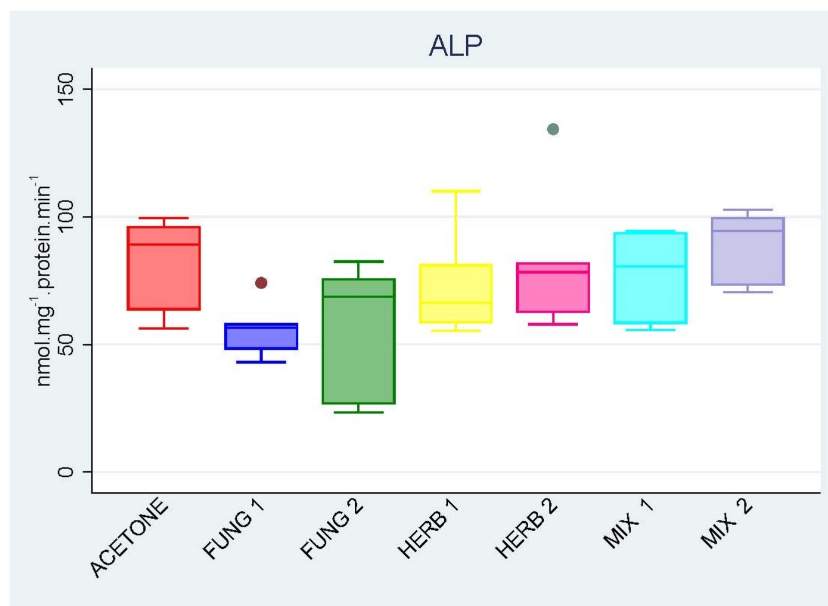

Figure 7. ALP activity measured in the midgut of honeybees (*Apis mellifera*) exposed to the fungicide Sakura® (200 and 400 g/L), the herbicide Elegant 2FD (225 and 450 g/L), and their mixtures

Lysozyme activity did not show differences among the different treatments (Figure 8). Lysozyme, along with other proteins and haemocytes, is an important enzyme in honey bee immune responses; it can degrade the bacterial cell wall and its synthesis occurs throughout development (Lazarov et al., 2016). Only a few papers have examined lysozyme activity in honey bees; Caliani et al. (2021a) found a decrease in lysozyme activity after exposing honey bees to a fungicide. Our findings indicate that the compounds studied had no effect on this type of immune response.

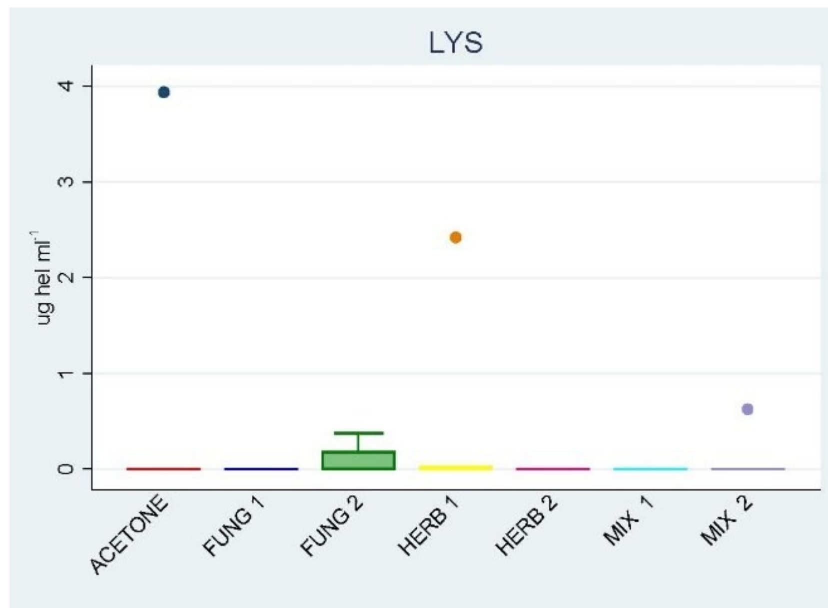

Figure 8. LYS activity measured in the midgut of honeybees (*Apis mellifera*) exposed to the fungicide Sakura® (200 and 400 g/L), the herbicide Elegant 2FD (225 and 450 g/L), and their mixtures

Plasmatocytes (Figure 9) showed a statistically significant decrease in HERB 1 group with respect to the control and MIX 1. The bees' immune system is divided into a humoral, with enzyme like lysozyme and phenoloxidase, and a cellular component, characterised by haemocytes. While lysozyme act against bacteria, the haemocytes (prohemocytes, plasmatocytes, granulocytes, oenocytoids and macrophage-like cells) have several important immunological functions in phagocytosis, encapsulation, nodulation, and wound repair (Amdam et al., 2005). Plasmatocytes and granulocytes are the most abundant circulating cell types (Giglio et al., 2015); granulocytes have a relevant role in phagocytosis while plasmatocytes in encapsulation response (Richardson et al., 2018). Granulocytes are the first cells to come into contact with a foreign body at the beginning of nodule formation. When in contact with the foreign body, they release their granular content. This exocytosis of the granulocytes content serves to attract plasmatocytes or help them to build the capsule (Ribeiro and Brehélin, 2006). The number of circulating haemocytes reflects the organism's capacity to face immunogenic challenges (Doums et al., 2002; Kraaijeveld et al., 2001). Caliani et al. (2021a) reported a similar decrease in the number of plasmatocytes after the treatment with the fungicide Amistar®Xtra, as in our herbicide lowest dose. To the best of our knowledge, no other ecotoxicological studies examined the haemocytes count related to contaminants exposure in *A. mellifera*. The haemocytes results confirm the hypothesis that the compounds studied had no effect on the immune system, as observed after lysozyme results.

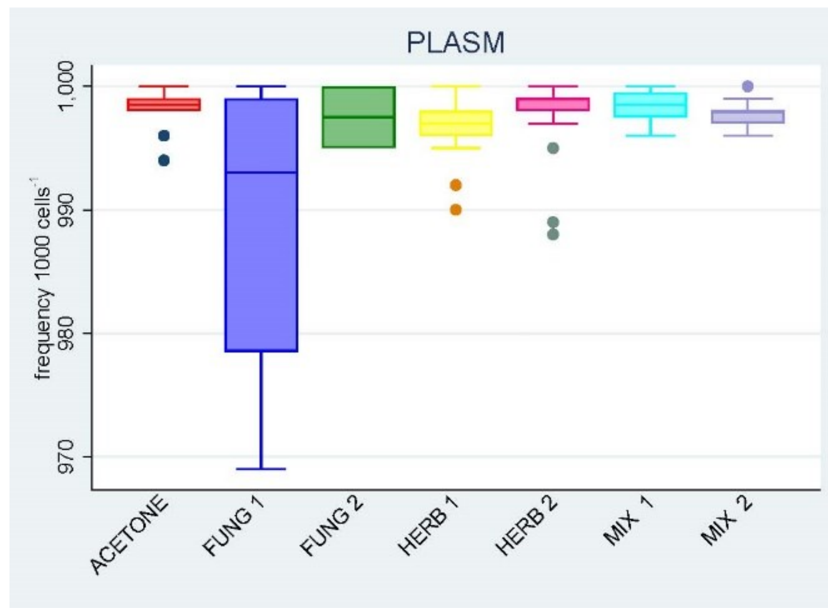

Figure 9. Differential haemocytes count measured in the haemolymph of honeybees (*Apis mellifera*) exposed to the fungicide Sakura® (200 and 400 g/L), the herbicide Elegant 2FD (225 and 450 g/L), and their mixtures

NA assay values didn't show differences among the different treatments (Figure 10). Exposure to genotoxic compounds can alter the DNA integrity of an organism. The Nuclear Abnormalities (NA) assay is a valid instrument to evaluate the presence of genetic damage, counting the number and types of abnormalities of haemocyte nuclei. Our results let us hypothesize that both fungicide and herbicide, alone and in combination, did not cause genotoxic effects in the specimens.

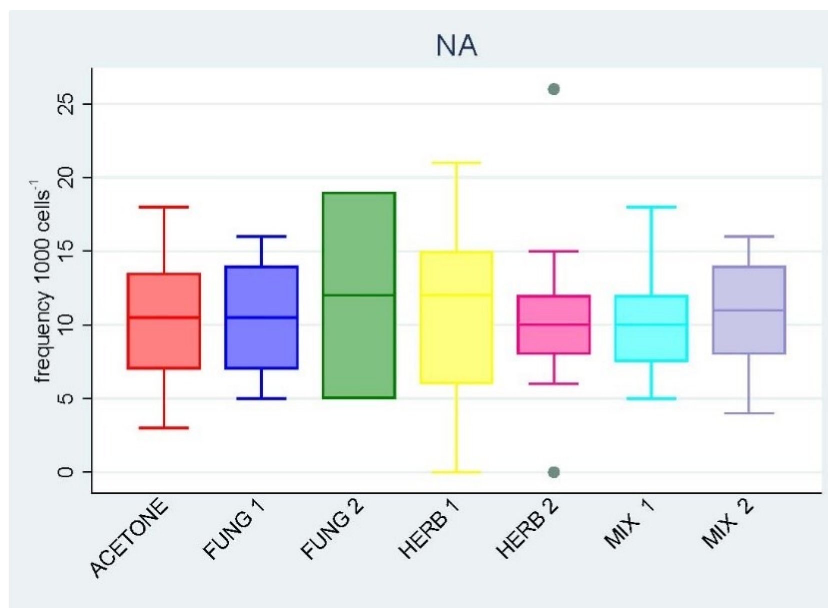

Figure 10. NA assay measured in the haemolymph of honeybees (*Apis mellifera*) exposed to the fungicide Sakura® (200 and 400 g/L), the herbicide Elegant 2FD (225 and 450 g/L), and their mixtures

Table 2. Statistically significant differences in the seven analysed biomarkers in each treatment compared to ACETONE. Red ticks are for differences where the values are higher than the control ones; green ticks are for values lower compared to the control; no ticks are for the absence of statistically significant differences

	FUNG 1	HERB 1	MIX 1	FUNG 2	HERB 2	MIX 2
AChE	✓ (red)			✓ (red)	✓ (green)	✓ (green)
CaE	✓ (green)			✓ (green)		✓ (green)
GST			✓ (red)	✓ (green)	✓ (red)	✓ (red)
ALP	✓ (green)			✓ (green)		
LYS						
PLASM		✓ (green)				
NA assay						

Table 3. Statistically significant differences in the seven analysed biomarkers compared to MIX. FUNG 1 and HERB 1 are compared to MIX 1; FUNG 2 and HERB 2 are compared to MIX 2. Red ticks are for differences where the values are higher than the control ones; green ticks are for values lower compared to the control; no ticks are for the absence of statistically significant differences

	FUNG 1	HERB 1	FUNG 2	HERB 2
AChE	✓ (red)		✓ (red)	
CaE	✓ (green)		✓ (green)	✓ (red)
GST	✓ (green)		✓ (green)	
ALP	✓ (green)		✓ (green)	
LYS				
PLASM		✓ (green)		
NA assay				

The selected fungicide, Sakura®, was demonstrated to affect the metabolism and the detoxification system of *Apis mellifera*, mostly at the highest dose. Elegant 2FD, the herbicide used in this experiment, at the highest dose caused neurotoxic damage and also activated the detoxification system. The obtained results highlighted that the herbicide and the mix had an effect on the examined cellular immune response, causing an increase in the number of granulocytes. Furthermore, the herbicide appeared to play a significant role in the responses observed following the combination treatments.

3.3.2 Proteomics

The proteomic analysis was carried out using two-dimensional electrophoresis on three pools for each treatment, which consisted of intestines from five *Apis mellifera* specimens. The method for extracting proteins from the intestines was found to be effective, yielding an adequate amount of protein for electrophoretic runs. Two electrophoretic runs were thus

performed for each sample, as we chose two different pH ranges to achieve better resolution. The comparison of gels with pH 4-7 revealed 15 statistically significant differences between the control and treatments, as well as between the different treatments. A comparison of the gels obtained with pH 7-10 revealed 24 statistically significant differences between the control and treatments, as well as between the different treatments. In total, 39 statistically significant differences were found. Figure 10 shows the representative Master gel of the samples at pH 4-7, while Figure 11 shows the representative Master gel of the samples at pH 7-10. In both cases, the qualitative differences were highlighted in red, the quantitative differences between the control and the Sakura® at different concentrations (200 g/L and 400 g/L) in green, the quantitative differences between the control and Elegant 2fd at different concentrations (225 g/L and 450 g/L) in blue and in yellow the quantitative differences between the control and the mix at different concentrations (mix Elegant 2fd 225 g/L + Sakura® 200 g /L and Elegant 2fd 450 g/L + Sakura® 400 g/L).

- pH 4-7: Figure 11 shows the Master gel obtained from the image analysis performed on each gel. The proteins are distributed uniformly across the pH range under consideration. Spots in the image that differ statistically significantly from one condition to the next were highlighted. Significant differences are classified as either qualitative (a spot can only be found in one condition) or quantitative (a spot varies in abundance but is present in multiple conditions). Table 4 displays the relative abundance of each spot in the pH 4-7 range. As shown in Table 4, 10 spots (spots 1-3-4-7-8-10-11-12-13) were more abundant in the control than in the treatments. Spot 6 was found only in the FUNG 1 treatment. Furthermore, 5 spots (spots 2-5-9-14-15) showed a quantitative difference, with spot 2 being present in both the control and the mix doses, and being more abundant in MIX 1. Spot 5 is more abundant in the control than in the FUNG 1 treatment and is absent from the other treatments; similarly, spots 14 and 15 are more abundant in the control than in the FUNG 2 treatment and are absent from the other treatments. Spots 2 and 9 are more abundant in the control than in MIX 2, but not in the other treatments.

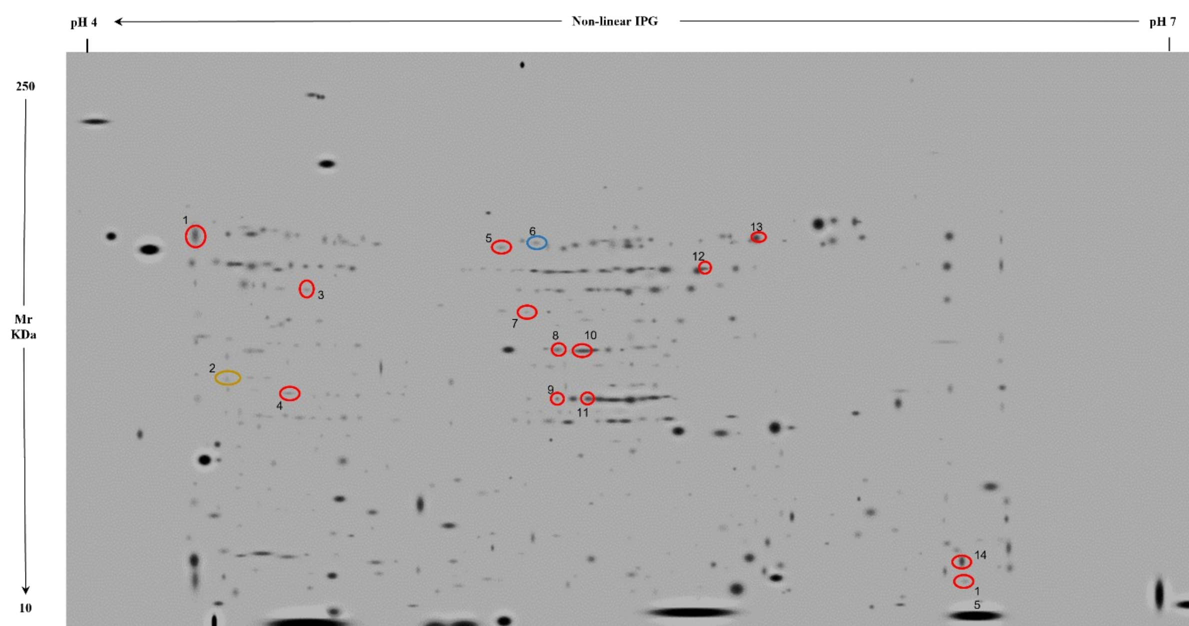

Figure 11. Representative Mastergel of the samples at pH 4-7. Statistically significant differences ($p<0.05$) are highlighted by circles of different colors

Table 4. Relative abundance of each spot in the pH 4-7 gel

Spot ID	ACETONE	FUNG 1	FUNG 2	HERB 1	HERB 2	MIX 1	MIX 2
1	0,5	0	0	0	0	0	0
2	0,4	0	0	0	0	0,6	0,1
3	2,2	0	0	0	0	0	0
4	0,5	0	0	0	0	0	0
5	4,2	0,8	0	0	0	0	0
6	0	2,2	0	0	0	0	0
7	1	0	0	0	0	0	0
8	1,2	0	0	0	0	0	0
9	0,5	0	0	0	0	0	0,1
10	0,9	0	0	0	0	0	0
11	1,5	0	0	0	0	0	0
12	0,8	0	0	0	0	0	0
13	2,9	0	0	0	0	0	0
14	8,8	0	2,4	0	0	0	0
15	53,3	0	7	0	0	0	0

- pH 7-10: Figure 12 shows the Master gel obtained after image analysis on each gel. Because the majority of the proteins are detectable near the anode, they have an isoelectric point close to 7-8, which is a fairly common datum. The image highlights the spots that differ from one condition to another in a statistically significant way. Table 5 displays the average relative abundance of each spot at pH 7-10. 21 spots (16-

17-18-19-20-21-22-23-25-26-27-28-29-30-31-32-33-34-35-36) were more abundant in the control than in the treatments, while only two spots (24 and 38) showed a quantitative difference. Spot 37 appears to be present only in the two mix treatments, and spot 39 appears to be present only in HERB 1 and MIX 1; the latter is more abundant. As far as quantitative differences are concerned, spot 24 is present only in the control, where is more abundant and in HERB 2, and spot 38 is present in the control, in which it is most abundant and in MIX 1.

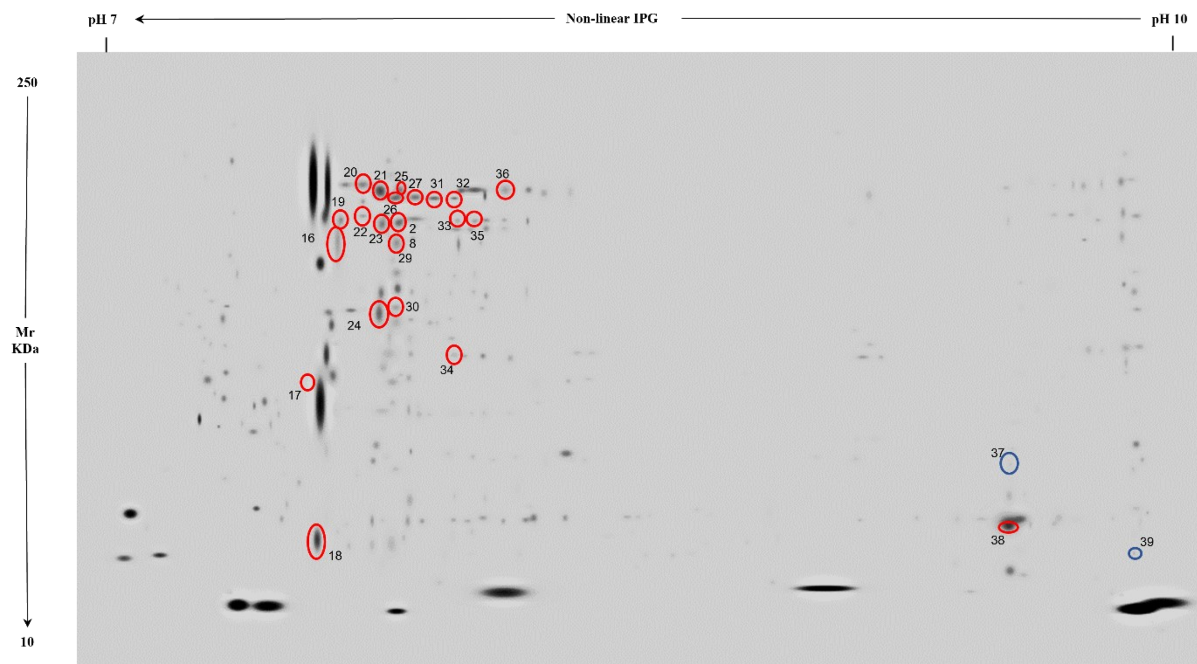

Figure 12. Representative Mastergel of the samples at pH 7-10. Statistically significant differences ($p<0.05$) are highlighted by circles of different colors

Table 5. Relative abundance of each spot in the pH 7-10 gel

Spot ID	ACETONE	FUNG 1	FUNG 2	HERB 1	HERB 2	MIX 1	MIX 2
16	1,2	0	0	0	0	0	0
17	0,6	0	0	0	0	0	0
18	2,4	0	0	0	0	0	0
19	7,1	0	0	0	0	0	0
20	5,4	0	0	0	0	0	0
21	9,8	0	0	0	0	0	0
22	2	0	0	0	0	0	0
23	5,9	0	0	0	0	0	0
24	9,1	0	0	0	1,2	0	0
25	5,2	0	0	0	0	0	0
26	0,7	0	0	0	0	0	0
27	2	0	0	0	0	0	0
28	0,5	0	0	0	0	0	0
29	1,8	0	0	0	0	0	0
30	0,7	0	0	0	0	0	0
31	1,6	0	0	0	0	0	0
32	1,2	0	0	0	0	0	0
33	1,2	0	0	0	0	0	0
34	0,8	0	0	0	0	0	0
35	1	0	0	0	0	0	0
36	0,8	0	0	0	0	0	0
37	0	0	0	0	0	1,8	2,1
38	4,8	0	0	0	0	0,9	0
39	0	0	0	1,1	0	16,7	0

Overall, 32 spots were found in only one of the exposure conditions, showing the presence of qualitative differences. Conversely, 7 spots are characterized by a quantitative difference, as the same spot, present in several conditions, has a variation in abundance between treatments.

In order to evaluate the biological relevance they may have in the exposed samples, we then attempted the identification of protein differences by using mass spectrometry. 11 protein spots were identified, as shown in Table 6.

Table 6. Significant protein-spot differences, identified by mass spectrometry

Spot N	Protein Identification							
	Protein ID	UniProt Accession Number	Gene Name	MW	pI	Sum PEP Score	Identified Peptides	Seq Coverage %
2	Transaltion elongation factor eEF1-alpha chain (eEF-1a-f1) Apis mellifera	P19039	EF1A	50.5	9.09	1.80	3	6
11	Arginine kinase (ArgK) Apis mellifera	O61367	ARGK	40	5.99	1.98	1	4
12	Major royal jelly protein 3 (Mrjp3) Apis mellifera	Q17060	MRJP3	65.5	5.24	6.32	3	6
15	Profilin (PRF) Apis mellifera	Q6QEJ7	PROF	13.7	5.92	3.21	2	17
26	Vitellogenin (Vg) Apis mellifera	Q868N5	Vg	200.9	6.73	54.25	25	17
27	Vitellogenin (Vg) Apis mellifera	Q868N5	Vg	200.9	6.73	18.262	10	6
28	Vitellogenin (Vg) Apis mellifera	Q868N5	Vg	200.9	6.73	47.143	36	23
31	Vitellogenin (Vg) Apis mellifera	Q868N5	Vg	200.9	6.73	32.559	11	7
32	Vitellogenin (Vg) Apis mellifera	Q868N5	Vg	200.9	6.73	16.552	7	4
38	Ribosomal protein s8 (Rps8) Apis mellifera	O76756	RpS8	24	10.58	2.279	1	6

The protein arginine kinase (ArgK) catalyzes the reversible transfer of phosphate from MgATP to arginine, resulting in phosphoarginine and MgADP. It is an important protein for energy metabolism and helps invertebrates to grow and develop. Our findings revealed that arginine kinase was abundant in the control group but not in the treatments. In a proteomic study of *Artemia sinica* larvae exposed to copper sulfate ($CuSO_4$), ArgK was found to be down-regulated (Zhou et al., 2010). ArgK was found to be downregulated in the anterior gills of the Chinese mitten crab, *Eriocheir sinensis*, after chronic cadmium exposure, by Silvestre et al. (2006) using a proteomic approach. ArgK expression was found to be significantly lower in the plasma of *Fenneropenaeus chinensis* 45 minutes after injection of an immunostimulant, laminarin, but it recovered after 3 hours (Yao et al., 2005). Furthermore, Roat et al. (2020) found changes in a molecular pathway for a visual process, including a higher content of ArgK, after exposing *Apis mellifera* specimens to the insecticide thiamethoxam, compared to the control. These studies provided preliminary evidence that ArgK plays a role in the response to abiotic stress and in innate immune responses.

The major royal jelly protein 3 (MRJP3) was also identified in this study. This protein, along with eight other similar proteins, is part of the MRJP family and contains a high concentration of amino acids needed to nourish both the queen bee and the larvae. The ten essential amino acids most commonly found in MRJPs are arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine (Ramanathan et al., 2018). MRJP3 has been found to be involved in T-cell immune responses, downregulating the production of IL-4, IL-2, and IFN-γ and suppressing the production of IgE and IgG1, effectively acting as an anti-allergic agent (Kohno et al., 2004; Okamoto et al., 2003; Tamura et al., 2009). According to Kohno et al. (2004) and Qu et al. (2008), MRJP3 acts as an anti-inflammatory agent in activated mouse macrophages both in vitro and in vivo by inhibiting the production of pro-inflammatory cytokines such as TNF-, IL-6, and IL-1. Wang et al. (2021) observed an inhibition in the formation of a MRJPs after the treatment with the fungicide carbendazim, on *Apis mellifera* specimens. Our results showed that this protein was present only in the control with respect to all the treatments. The absence of this protein in the honey bees exposed could imply the incapacity of the organism to react to inflammatory processes, causing stress to the immune system.

Profilin (PRF) is a key regulator of actin polymerisation and is critically important to cellular function (Ding et al., 2012). The actin cytoskeleton is involved in almost all cellular processes

including motility, endocytosis, metabolism, signal transduction and gene transcription (Olson and Nordheim, 2010). There is growing evidence that profilins, in addition to actin binding, act as hubs that control a complex network of molecular interactions. Profilins interact with a wide range of proteins, and the significance of this aspect of their function is only now becoming clear. For example, profilins have a key role in cellular processes such as membrane trafficking, small-GTPase signalling, and nuclear activities, as well as neurological diseases and tumor formation (Witke, 2004). Our results showed a great abundance of this protein in the control and a down-regulation in the highest fungicide dose, while in all the other treatments the protein was not present. Zaluski et al. (2020) observed a down-regulation in profilin expression in bees exposed to pyraclostrobin and fipronil. Lewis et al. (2009) exposed *Caenorhabditis elegans* specimens to dichlorvos and observed induction in profilin expression after the treatment.

RpS8 (ribosomal protein S8) is a medium-sized ribosomal protein that functions as a primary RNA-binding protein in the 30S subunit (Held et al., 1974; Zimmermann and Singh-Bergmann, 1979). It is required for the proper folding of the central domain of 16S rRNA (Gregory et al., 1984; Svensson et al., 1988), and mutations within the protein have been shown to cause defective ribosome assembly (Geyl et al., 1977). Our results showed this protein to be abundant in the control and down-regulated in the lowest mix dose, while in all the other treatments the protein was not present. Wu et al. (2017) found an increase in the expression of ribosomal protein genes after exposing honey bees to sub-lethal doses (ranging from 0.1 to 10 ppb) of the insecticide imidacloprid. Given the ribosomal and extra-ribosomal functions of these proteins, imidacloprid could cause cell dysfunction. The mRNA of the RpS8 gene was found to be up-regulated in the liver of eels (*Anguilla anguilla* L.) after exposure to 7,12-dimethylbenz[a]anthracene (DMBA) (Nogueira et al., 2009).

The translation elongation factor eEF-1A catalyses the first step of the elongation cycle (Kaziro et al., 1991; Moldave, 1985). It is a member of the GTP binding protein class (Dever et al., 1987), which is distinguished by a molecular switch from an active conformation bound to GTP to an inactive conformation bound to GDP. Translation elongation factor eEF-1A, for example, promotes GTP-dependent binding of aminoacyl-tRNA to the ribosome and participates in proof-reading of the codon-anticodon match (Lamberti et al., 2004). Furthermore, it is thought that EF1A binds to cytoskeletal proteins such as actin (Yang et al., 1990) and tubulins (Negrutskii and El'skaya, 1998). It is also thought to play roles in tumorigenesis, signal transduction, and apoptosis (Lamberti et al., 2004), as well as oxidative stress protection (Chen et al., 2000; Kim et al., 2009). Our results showed an abundance of this

protein in the control and a down-regulation in the highest mix dose, while in all the other treatments the protein was not present. Chen et al. (2000) demonstrated that increased protein expression of the peptide elongation factor EF-1a is an immediate early event of prooxidant-induced apoptosis in rat heart cell culture after H_2O_2 treatment. Wang et al. (2011) discovered that shrimp (*Litopenaeus vannamei*) exposed to acidic pH and cadmium had higher levels of EF-1a expression. To the best of our knowledge, a decrease of eEF-1A abundance was never observed in literature. A decrease in this protein, fundamental for the elongation process, could directly or indirectly influence the regulation of the cell growth and cell transformation, as well as be a response to oxidative stress.

Vitellogenin (Vg) is an egg-yolk precursor protein synthesized in the insect fat body that is used to deliver nutrients to developing eggs in most oviparous animals (Pan et al., 1969; Raikhel and Lea, 1983). In honey bees, Vg also performs numerous immunological functions, including binding to and eliminating pathogenic bacterial and fungal cells by recognizing pathogen-associated molecular patterns (PAMPs) (Li et al., 2009, 2008; Salmela et al., 2016; Zhang et al., 2015) and protecting host cells from oxidative stress by binding to and neutralizing ROS (Havukainen et al., 2013; Salmela et al., 2016). It also binds to damaged host cells, protecting them from further damage (Havukainen et al., 2013), and transports zinc, which is required to maintain innate immune cells (haemocytes) (Amdam et al., 2004a). In our study, we identified isoforms with different isoelectric points that were found to be abundant only in the control. Vitellogenin gene expression and protein levels increased after honey bees were exposed to neonicotinoids such as thiamethoxam, acetamiprid, clothianidin, and imidacloprid, both alone and in combination (Christen et al., 2017, 2016). Christen et al. (2019a) observed a strong induction of the full-length vitellogenin protein in bees exposed to 2 ng/bee/day dimethoate over a period of 72 h, while deltamethrin did not alter its expression. Tarek et al. (2018) observed an increase in vitellogenin expression after treating honey bees with imidacloprid and clothianidin after 24 hours, while after 72 hours the expression of this gene was significantly down-regulated in clothianidin and imidacloprid treated bees. The authors also observed an increase in Vg expression after 72 hours of the carbaryl treatment. Another study showed that Vg was also down-regulated in the long term, between 25 and 33% when honey bees were exposed to acaricides such as thymol and coumaphos (Boncristiani et al., 2012). The exposure of honey bees to LD50 of tau-fluvalinate, coumaphos, amitraz, and formic acid relative caused an increase after 48 hours in Vg expression (Gashout et al., 2018).

The pesticides used for this experiment seemed to be able to affect the energy metabolism of the organisms, as the arginine kinase absence in the midgut of treated animals suggested. The immune system was affected by chemical stress, leading to a suppression of the MRJP3 and vitellogenin. Moreover, proteins involved in protein synthesis were down-regulated in the midgut of treated animals. Based on the results, we cannot rule out the possibility that Sakura®, Elegant 2FD, or a combination of them may induce post-translational changes that result in a pI and/or MW shift of the corresponding proteoform(s), which could also represent a tissue-specific response to short-term exposure to these compounds.

3.4 Conclusions

Honey bees are constantly exposed to pesticides, whose active principles were demonstrated to cause sub-lethal and lethal effects. This work aimed to fill multiple gaps in the assessment of the sub-lethal effects of xenobiotics in *Apis mellifera* specimens. Two commercial pesticides were used to understand the effects of the complete product, and not only of the active principles. Moreover, they were used alone and in combination, since honey bees in the environment are usually exposed to mixtures of contaminants that can cause synergistic, additive or antagonistic effects.

The evaluation of the effects of the selected pesticides on honey bees, was performed integrating a multi-biomarker approach with a proteomics one.

The fungicide Sakura® demonstrated to affect the metabolism and the detoxification system of *Apis mellifera*. The herbicide Elegant 2FD was able to cause neurotoxic effects and also to activate the detoxification system. Both the herbicide and the mixture of the pesticides resulted to have an effect on the cellular immune response, demonstrated by the increase in the number of granulocytes. The mixture of the two pesticides demonstrated that the effects obtained are probably due to the herbicide.

The proteomic approach revealed that the two pesticides were able to affect the energy metabolism, as the absence of ArgK suggested, and to alter the immune system of the organisms by the suppression of MRJP3 and vitellogenin. Proteins involved in the protein synthesis were down-regulated in the treated animals. We must also consider that Sakura® and Elegant 2FD, or a combination of them might have induced post-translational changes resulting in a pI and/or MW shift of the corresponding proteoform(s). The proteomic approach can be deepened, for example using specific antibodies to perform a more specific assessment.

4. ECOTOXICOLOGICAL HEALTH STATUS OF HONEY BEES SAMPLED IN AREAS WITH DIFFERENT ANTHROPIC IMPACT: BIOMARKERS AND HONEY ANALYSIS

4.1 Introduction

Honey bees forage in a wide range, and therefore they come in contact with food and water contaminated by pesticides (Porrini et al., 2003; Raeymaekers, 2006); the routes of exposure are pollen and nectar collected and stored in the hive (Chauzat and Faucon, 2007), exudation from plants (Girolami et al., 2009), surface water (van Dijk, 2010), air contamination (Greatti et al., 2003), and extra floral secretion from some plants (Mizell, 2001). The uptake and accumulation of pesticides in honey bees can influence their health and the colony's population development, acting as stressors for the entire colony.

As mentioned in the introduction, the majority of studies on honey bees are carried out in the laboratory rather than in semi-field and field conditions. In these latter conditions, it is more difficult to understand the effects of contaminants, since multiple stressors could affect honey bees health. Most of the monitoring studies investigated the accumulation of different pollutants in *Apis mellifera*, while sub-lethal effects were less studied.

The aim of this study was to assess the ecotoxicological health status of honey bees and the origin and quality of the beehive's products from areas with different anthropic impact. Honey bees and honey were collected from 10 different locations in Tuscany over the course of two years (2020-2021). A set of biomarkers that evaluate neurotoxic effects (AChE and CaEs activities) and biotransformation and metabolic changes (GST and ALP activities) were performed, as well as immune system biomarkers (lysozyme activity, and haemocytes count) and genotoxicity (NA assay). This multi-biomarker approach was selected to observe different responses, in order to obtain a more complete picture of honey bees health status. As previously said, some of these biomarkers were already used in similar contexts, such as neurotoxicity, biotransformation and metabolic biomarkers. The evaluation of genotoxicity and immune system alterations were, instead, poorly used in monitoring studies. However, DNA damages are very important endpoints to evaluate, since various xenobiotics and mixtures of contaminants could cause them. In the same way, immune system alterations, that could be caused by contaminants, are extremely important to assess, since a weaking of the immune system of honey bees could make them more vulnerable to pathogens and parasites, and it is well known that even small concentrations of contaminants can imbalance this system.

Furthermore, a characterisation of honey floral origin and of their chemical-physical properties was carried on by analysing respectively the melissopalynological profile and humidity, carbohydrates, and amino acids contents. This honey characterisation is useful to understand where honey bees forage, leading to a comprehension of the locations for possible contaminants intake. Moreover, combining the observations about the honey composition and quality and the honey bees' physiological modifications, it could be possible to understand if honey bees' health status can modify the characteristics of this important product. To the best of our knowledge, this is the first time that a multi-biomarker approach and the honey analyses were integrated.

4.2 Materials and methods

4.2.1 Sampling sites

The sampling was carried out in 10 different locations in Tuscany (Figure 13). A suburban site, a rural area, a sunflower field, a wheat crop field, a wildflower field, a clover field, an orchard, a vineyard, a berries field, a wooded environment were used to collect honey bee foragers. The suburban area was in the province of Siena, with the beehives located just below a beltway. The beehives in the rural area, the wheat crops, and the sunflower field were all located in the province of Siena. The beehive in the berries field was also located near the mount Amiata (Siena, Italy). The bees collected from the wildflower field came from a beehive near Pisa. The orchard and the clover field were located in Colignola (Pisa, Italy). The orchard was located in the Agricultural Faculty of the University of Pisa and used for experimental and teaching activity and productive purposes. The clover field was also located in Colignola (Pisa, Italy). The beehive from the wood was placed in Le Castelline, near Pontedera (Pisa, Italy), far from direct sources of urban or intensive agriculture contamination. The vineyards area was located near Arezzo.

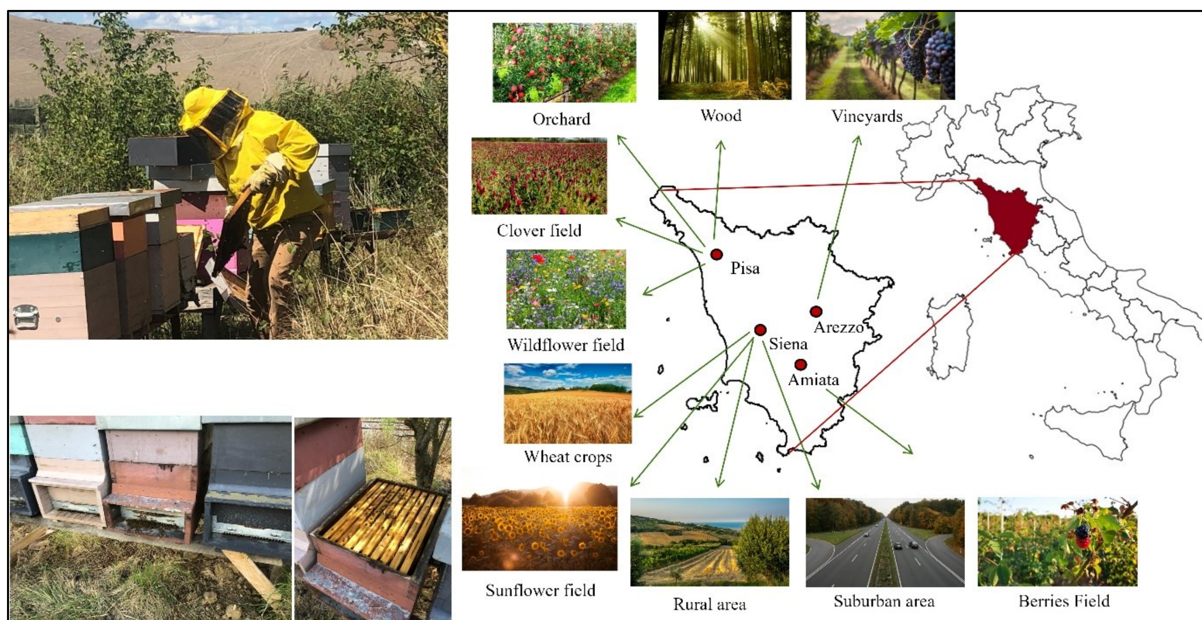

Figure 13. Map of the 10 different sampling locations in Tuscany.

Table 7. Name of the sampled area, geographical coordinates, cultivations observed near the areas, pesticides that could be used in each area.

Sampled area	Geographical coordinates	Cultivations	Possible pesticides
Wood	43.64525 N 10.67579 E 34 m asl	Wood	
Wildflower field	43.573681 N 10.593210 E 106 m asl	Wildflower	
Rural area	43.391424 N 11.350889 E 450 m asl	Wood, olive trees, vineyard	Pyrethroids, OPs, neonicotinoids, fungicides
Wheat crops	43.302539 N 11.449288 E 207 m asl	Wheat, alfalfa, olive trees	Pyrethroids, OPs, neonicotinoids, fungicides
Sunflower field	43.298849 N 11.382147 E 304 m asl	Sunflower, cherry-tree	Herbicides
Clover field	43.727434 N 10.462937 E 4 m asl	Clover	
Vineyards	43.514077 N 11.895551 E 179 m asl	Vineyards	Fungicides
Orchard	43.72879 N 10.46283 E 4 m asl	Apple, plum, peach, and grapes	Insecticides
Suburban area	43.290623 N 11.396943 E 260 m asl	------	--------------
Berries field	42.859081 N 11.679691 E 822 m asl	Berries, chestnut	Insecticides

4.2.2 Honey bees and honey sampling

Honey bee workers were collected from each beehive by beekeepers during the summers of 2020 and 2021. The specimens were sampled at least one month after the acaricide treatments for *Varroa destructor* infestations. Each hive was randomly sampled for 50 bees and 40 g of honey. The suburban area was only sampled in 2020, while the beehives in the berries field were only sampled in 2021. Following the sampling, the biological materials were processed as described in the 3.1.3 section.

4.2.3 Biomarkers analysis

The methods for biomarkers analysis are already reported shown in 3.1.4 section.

4.2.4 Analyses of honey origin and quality

4.2.4.1 Melissopalynological analysis

To make the slides, 10 g of honey was weighed into a 50 mL conical bottom test tube, followed by 20 mL of distilled water (previously heated in a 40°C water bath). To remove the sugars, the solution was centrifuged for 10 minutes at 2500 rpm and the supernatant was removed. A second washing was performed by adding 10 mL of distilled water and centrifuging at 2500 rpm for 10 minutes to remove the supernatant. The slide was prepared using a Pasteur pipette, which was used to aspirate the sediment and then disperse it on the slide, attempting to distribute it uniformly before covering it with the coverslip. The sediment was studied under the microscope using the Louveaux et al. (1978) method, with modifications from Von Der Ohe et al. (2004). The pollen was identified using the bibliography and reference materials. The analysis can take two paths, orientative or complete. In the first instance, we limit ourselves to identifying the most prevalent elements in the sediment and looking for those that are especially important for the analysis. The thorough identification of all pollen and other elements found in the sediment, on the other hand, is required for the complete analysis. The slides were examined using an optical microscope (Axiophot Zeiss) and the AxioCam MRc 5 image capture system. An analysis of the honey was performed with the identification of the most common elements (Figure 14) in a count of 200-300 elements.

Figure 14. Examples of pollens identified in the analysed honey, through melissopalynological analysis: A) *Rubus* s.p.; B) *Prunus* sp.; C) *Eucalyptus* sp.; D) *Tilia* s.p.; E) *Acacia* s.p.; F) *Castanea sativa;* G) *Hedysarum coronarium;* H) *Trifolium* s.p

4.2.4.2 Chemical-physical properties

Humidity

The determination of honey humidity was carried out with an ATAGO HHR-2N refractometer pre-calibrated on the honey on a water concentration scale ranging from 12 to 30%. The calibration of the humidity scale was carried out at 20 °C.

Carbohydrates

The samples were prepared by weighing 0.4 to 0.6 g of honey and dissolved in 10 mL of milliQ. The sample was directly inoculated into HPLC, using a WATERS system, consisting of a 600E pump and a refractive index detector model 2410. The sample was inoculated with a 50 µl syringe, through a RHEODYNE valve equipped with a 20 µL loop. The column used was a SUGAR-PAK I WATERS, 300 mm x 6.5 mm in size, packed with cation exchange gel microparticles in the form of calcium. The column was kept at a constant temperature of 90°C, and the mobile phase was MilliQ water. The flow rate was kept constant at 0.5 mL per minute. The carbohydrate analysis took a total of 20 minutes to complete. The detector signal was sent to a computer via an A/D converter and managed by Clarity CSW-32 Software. Carbohydrate concentrations were calculated using reference curves built with certified standards ranging from 0.1 to 10 mg/mL.

Amino acids

The samples were prepared by weighing 0.4 to 0.6 g of honey and dissolved in 10 mL of milliQ. Amino acids were determined by HPLC using the WATERS AccQ.Tag method. The HPLC

system consists of WATERS LCMODULE1 and a fluorometric detector 2475. The samples and reference standards were treated according to the derivatization protocol contained in the relative manual. 70 µL of borate buffer and 20 µL of 6-aminoquinoly-N-hydroxysuccinimidyl carbamate (AQC) fluorescent reagent were added to 10 µL of sample for a final volume of 100 µL. The standards were processed as follows: 10 µL of protein amino acid standards plus 10 µL of non-protein amino acid standards were added to 60 µL of borate buffer and 20 µL of fluorescent reagent. Samples and standards were incubated in an oven at 55°C for 15 minutes. A C18 250 x 4.6 mm, 5µm column was used, thermostated at 40°C. For the mobile phase, a gradient formed by two eluents, A and B, was used. Eluent A was a buffer based on phosphates and triethylamine at pH 5, eluent B was a 60% solution of acetonitrile in MilliQ water. The flow was set at 1.5 mL/min and, 5 µL of standard and 10 µL of the sample were injected. The total duration of each analysis was 70 minutes according to the gradient showed in Table 8.

Table 8. HPLC parameters: time (min), flow (mL/min), the two eluents (A and B) and the curve

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	1.5	100	0	*
0.84	1.5	98	2	6
25	1.5	93	7	6
31.7	1.5	90	10	6
53.4	1.5	67	33	6
55.1	1.5	67	33	6
56.8	1.5	67	33	6
61.8	1.5	75	25	6
63.5	1.5	100	0	6
70	1.5	100	0	6

The excitation wavelength is 250 nm, and the emission wavelength is 395 nm. The detector signal is sent to a computer via an A/D converter and managed by Clarity CSW-32 Software. The amino acid concentrations were calculated using reference curves built from certified standards with variable concentrations starting at 50 nmol/mL.

4.2.5 Statistical analysis

We tested for significant differences in each biomarker between the different areas samples using 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-comparison when the null hypothesis of the KW test was

rejected. Mann-Whitney test (Mann and Whitney, 1947) was used for the comparison between 2020 and 2021 for each area. These tests have been implemented by STATA 17- software (StataCorp., 2021).

4.3 Results and discussion

4.3.1 Biomarkers

Biomarkers of neurotoxicity (AChE and CaE), metabolism (ALP and GST), immune system (LYS and hemocyte count), and genotoxicity (NA assay) were measured in honey bees from areas with different anthropic impacts. The results obtained for biomarkers let us hypothesize that the wood and the wildflower field can be considered as control areas. Tables 9 and 10 show the statistically significant differences between the different sites sampled in 2020 and 2021, respectively.

Table 9. p values of the multiple pairwise comparison tests of the seven biomarkers in 2020 sampling

	Clover field	Orchard	Rural area	Suburban area	Sunflower field	Vineyards	Wheat crops	Wildflower field
AChE								
Orchard	< 0.05							
Rural area	n.s.	n.s.						
Suburban area	n.s.	< 0.01	n.s.					
Sunflower field	n.s.	n.s.	n.s.	< 0.01				
Vineyards	< 0.01	n.s.	< 0.05	< 0.01	< 0.05			
Wheat crops	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		
Wildflower field	n.s.	< 0.01	< 0.01	n.s.	< 0.01	< 0.01	n.s.	
Wood	n.s.	n.s.	n.s.	< 0.05	n.s.	< 0.01	n.s.	< 0.05
CaE								
Orchard	n.s.							
Rural area	< 0.01	< 0.05						
Suburban area	< 0.01	< 0.01	n.s.					
Sunflower field	n.s.	n.s.	n.s.	< 0.01				
Vineyards	< 0.01	< 0.01	n.s.	n.s.	< 0.01			
Wheat crops	n.s.	n.s.	< 0.01	< 0.01	n.s.	< 0.01		
Wildflower field	n.s.	n.s.	< 0.01	< 0.01	n.s.	< 0.01	n.s.	
Wood	n.s.	n.s.	< 0.01	< 0.01	n.s.	< 0.01	n.s.	n.s.
GST								
Orchard	< 0.01							
Rural area	< 0.01	<0.01						
Suburban area	< 0.01	<0.01	n.s.					
Sunflower field	< 0.1	< 0.05	n.s.	< 0.01				
Vineyards	< 0.01	< 0.05	n.s.	< 0.01	n.s.			
Wheat crops	< 0.01	n.s.	< 0.05	< 0.01	n.s.	n.s.		
Wildflower field	n.s.	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	
Wood	< 0.01	n.s.	< 0.01	< 0.01	< 0.01	< 0.01	n.s.	< 0.01
ALP								
Orchard	n.s.							
Rural area	n.s.	n.s.						
Suburban area	< 0.01	< 0.01	< 0.01					
Sunflower field	< 0.05	n.s.	< 0.01	< 0.01				
Vineyards	< 0.01	< 0.05	< 0.01	< 0.01	n.s.			
Wheat crops	n.s.	n.s.	n.s.	< 0.01	< 0.05	< 0.01		
Wildflower field	< 0.01	< 0.01	< 0.01	n.s.	< 0.01	< 0.05	< 0.01	
Wood	< 0.01	< 0.01	< 0.01	< 0.01	n.s.	n.s.	< 0.01	n.s.
LYS								
Orchard	n.s.							
Rural area	n.s.	n.s.						
Suburban area	n.s.	n.s.	n.s.					
Sunflower field	n.s.	n.s.	n.s.	n.s.				
Vineyards	n.s.	< 0.05	n.s.	< 0.05	n.s.			
Wheat crops	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		
Wildflower field	n.s.	< 0.05	n.s.	< 0.05	n.s.	n.s.	n.s.	
Wood	< 0.05	< 0.05	n.s.	< 0.01	n.s.	n.s.	< 0.05	n.s.
PLASM								
Orchard	n.s.							
Rural area	n.s.	n.s.						
Suburban area	n.s.	n.s.	n.s.					
Sunflower field	n.s.	n.s.	n.s.	< 0.05				
Vineyards	n.s.	n.s.	n.s.	n.s.	n.s.			
Wheat crops	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		
Wildflower field	< 0.01	n.s.	< 0.01	< 0.01	n.s.	< 0.01	n.s.	
Wood	n.s.	n.s.	< 0.05	< 0.01	n.s.	n.s.	n.s.	n.s.
NA assay								
Orchard	n.s.							
Rural area	< 0.01	< 0.01						
Suburban area	n.s.	n.s.	< 0.01					
Sunflower field	< 0.01	n.s.	n.s.	< 0.01				
Vineyards	n.s.	n.s.	< 0.01	n.s.	< 0.01			
Wheat crops	n.s.	n.s.	< 0.01	n.s.	< 0.01	n.s.		
Wildflower field	< 0.01	n.s.	< 0.01	< 0.01	n.s.	< 0.01	< 0.01	
Wood	< 0.01	n.s.	< 0.01	< 0.01	n.s.	<0.01	< 0.01	n.s.

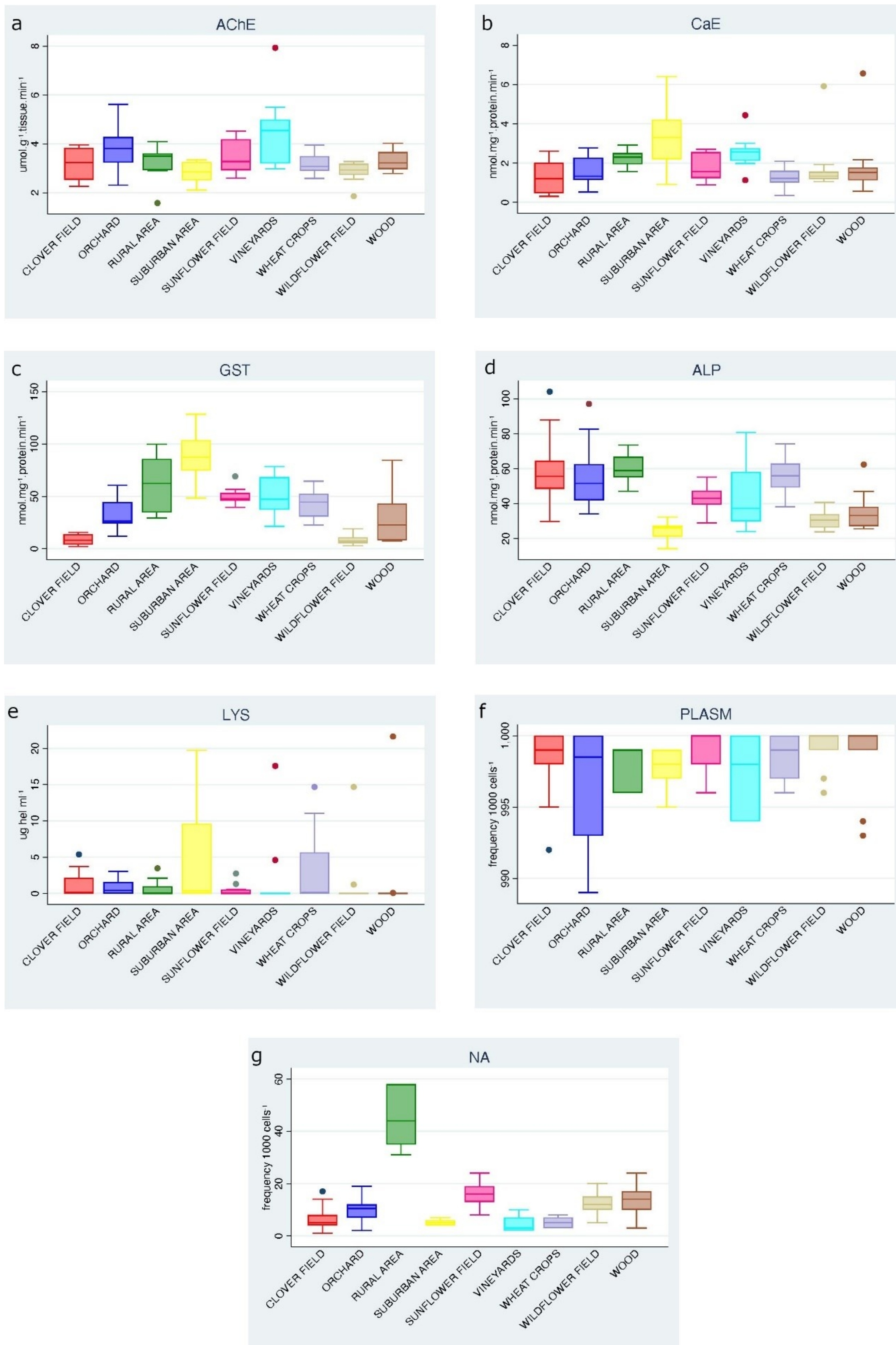

Figure 15. Boxplots of the seven biomarkers (activity of AChE (a); CaE (b); GST (c); ALP (d); LYS (e); differential haemocytes count (f) and NA assay (g)) measured in honeybees, *Apis mellifera* by the nine experimental sites sampled in 2020

Figure 15 shows the results obtained for biomarkers analyses in 2020.

AChE in 2020 showed significant induction in the orchard, with respect to the wildflower field, and in vineyards, compared to wood and wildflower field. An inhibition was observed in the suburban area samples, compared to the wood ones. The values obtained for this enzyme's activity are in line with the ones by Badiou-Bénéteau et al. (2013). Moreover, Badiou-Bénéteau et al. (2013) observed a result similar to those we observed in the suburban. In fact, AChE activity showed 26%, 15%, 4% and 9% of inhibition in bees collected from August and November 2009, February and May 2010 in area with high anthropic impact. AChE activity can undergo inhibition following exposure to organophosphorus and carbamate insecticides (Fukuto, 1990), but also to heavy metals (Frasco et al., 2005) and to herbicides such as glyphosate (Boily et al., 2013). The induction observed in the orchard and in vineyards is a result observed also by Al Naggar et al. (2015) in honey bees exposed to OPs insecticides. Badiou et al. (2008) after deltamethrin treatment in surviving bees observed and induction in AChE activity, while dead bees presented a decrease of this enzyme activity. Boily et al. (2013) also observed an increase in AChE activity both in field, near a corn crop, and in laboratory experiments; the latter consisted in exposing bees to neonicotinoid insecticides.

CaE was significantly induced in the rural, suburban and vineyards areas compared to the controls, in organisms sampled in 2020. Heavy metals and insecticides such as fipronil and thiamethoxam are able to induce CaE activity, as shown by the data obtained in the work of Caliani et al. (2021a), Roat et al. (2017) and Badiou-Bénéteau et al. (2012), respectively. Besides being a phase I enzyme (Carvalho et al., 2013), CaE is also responsible for a defence mechanism that protects AChE from the inactivation caused by organophosphates and carbamates (Jackson et al., 2013; Yan et al., 2009). In the case of the suburban area, the CaE increase was probably not sufficient to prevent the inhibition of AChE, as previously observed.

In 2020, GST was significantly induced in all areas compared to the wood except for the orchard, while significantly inhibited in the clover field. Moreover, each area showed significantly higher GST activity with respect to the wildflower field, except for the clover field. As several authors reported, toxic compounds such as heavy metals, PAHs and PCBs are able to induce biotransformation processes; the GST enzyme is part of this process, and an induction of GST activity is attributed to these compounds (Garner and Di Giulio, 2012; Yu et al., 2012). The strong induction observed in the suburban area are in line with those obtained by Caliani et al. (2021b), showing a GST induction in the cultivated and urban areas. The

responsible contaminants could be PAHs and heavy metals, originating from vehicular traffic and other urban sources. The GST induction observed in our agricultural areas are similar to Badawy et al. (2015) data after bees exposure to acetamiprid, pymetrozine and pyridalyl. Badiou-Bénéteau et al. (2012) reported that the insecticide thiamethoxam cause a significant increase in GST activity. On the other hand, a marked inhibition of GST activity is observed in the clover field in 2020. Some authors reported that GST is modulated by some insecticides which are able to cause a decrease in its activity (Lupi et al., 2020; Yao et al., 2018a). These findings let us hypothesize that in clover field were used insecticides different from the ones used in all the other agricultural areas.

In 2020 samples, ALP resulted significantly induced in each area with respect to wildflower field. In the suburban area a significantly inhibition compared to the wood, was observed, while clover field, orchard, wheat crops and rural area were significantly induced. The increase in this enzyme activity found in all cultivated areas agreed with the findings of Caliani et al. (2021b), who evaluated the ecotoxicological status of *Apis mellifera* specimens from four sites subjected to varying anthropic pressure. This would imply that pesticides are responsible for the induction of this enzyme in cultivated areas. However, the results obtained in the suburban area do not agree with the work of Badiou-Bénéteau et al. (2013), who evaluated the ALP activity in specimens of *A. mellifera* sampled in two areas, urban and rural, revealing that ALP activity was more induced in specimens from urban areas than in specimens from rural ones. Our findings could permit to hypothesize that the chronic exposure to environmental contaminants, produced by vehicular traffic and other anthropic sources, such as home heating, can cause significant inhibition of this enzyme in suburban areas.

In specimens from 2020 sampling, LYS activity was significantly induced in the orchard and suburban area compared to the wildflower field, and in the clover field, orchard, suburban area and wheat crops compared to the wood. To date, few studies evaluated immune system alterations related to possible contamination in *A. mellifera*. Insecticides are able to influence the immune system by altering the expression of several related genes (Christen et al., 2019a; Zhu et al., 2020), haemocyte density and antimicrobial activity (Brandt et al., 2016). Some studies conducted on different taxa (Mdaini et al., 2019; Wu et al., 2007) showed that some pesticides, PAHs, PCBs, and heavy metals can also modulate the response of the immune system. In insects, lysozyme gene expression can be modulated by exposure to insecticides (Tesovnik et al., 2017). In this study, in agreement with what was observed from the results of the previous biomarkers, we can be hypothesized that the samples from the suburban and the

wheat crops areas are exposed to contaminants, such as lipophilic compounds, heavy metals and pesticides, which lead to a marked induction of lysozyme activity, altering the function of the bee immune system.

In 2020, suburban and rural areas showed a significant decrease in plasmatocytes number, with a consequent increase in granulocytes presence, compared to both wood area and wildflower field. Clover field and vineyards specimens showed a decrease in plasmatocytes with respect only to wildflower field. Granulocytes have a relevant role in phagocytosis (Richardson et al., 2018). Since the lysozyme results showed significant induction in the suburban area, it is possible to hypothesize that the presence of PAHs and heavy metals can alter the immune system response of bees, involving both the examined responses. These findings would be supported by the findings of Caliani et al. (2021b), who found an increase in the number of granulocytes and lysozyme activity in the urban area. The increase in granulocytes number corresponded to an increase in LYS also for the clover field samples, meaning that also in this case both the immune responses were necessary to protect the organisms from external stress. Moreover, we can hypothesize that a chemical stress could be able to weaken the organism that is more susceptible to infections and pathogens. On the contrary, the increase in granulocytes in rural area and vineyards samples did not correspond to a modification of LYS activity. This could probably be due to the fact that the cellular response was enough to protect the organism, not involving an enzymatic activity.

Figure 25 shows, in samples from 2020, a significant increase in nuclear abnormalities in rural area compared to both wood and wildflower field, while a significant decrease was observed in clover field, suburban area, vineyards and wheat crops compared to the controls. The results obtained in this work are not consistent when compared to Caliani et al. (2021b), which showed statistically higher values of total nuclear abnormalities in bees sampled in the orchard and cultivated areas, compared to the wood control area. An increase in the frequency of nuclear abnormalities was found in different organisms exposed to heavy metals (De Flora et al., 1994, 1990), pesticides (Bolognesi, 2003) and PAHs (Pacheco and Santos, 1997). An increase in the frequency of nuclear abnormalities was found in *A. mellifera* when exposed to EMS, CdSO4 and the fungicide Amistar®Xtra Caliani et al. (2021a). It is possible that genotoxic compounds able of causing DNA damage to organisms are present in rural areas. The higher values observed in the control areas could be due to the presence of some contaminants able to increase the number of nuclear abnormalities of haemocytes. All other sampling sites, on the other hand, could not be affected by compounds able to cause genotoxic damage.

The results obtained fort this first year of monitoring highlighted that the honey bees sampled in the suburban area were the most affected animals, showing effects on the nervous and immune systems, on metabolism and on biotransformation process. All the agricultural areas showed an induction in GST activity, probably due to the exposure to pesticides that the bees tried to eliminate operating a detoxification process. In the same way, for all the agricultural areas there was an increased activity of the metabolism, underlined by the ALP response. The bees from the rural area showed also the presence of DNA damages.

Table 10. p values of the multiple pairwise comparison tests of the seven biomarkers in 2021 sampling

	Berries field	Clover field	Orchard	Rural area	Sunflower field	Vineyards	Wheat crops	Wildflower field
AChE								
Clover field	< 0.01							
Orchard	< 0.01	< 0.01						
Rural area	< 0.01	< 0.01	n.s.					
Sunflower field	n.s.	< 0.01	< 0.01	< 0.01				
Vineyards	n.s.	< 0.01	< 0.01	< 0.01	n.s.			
Wheat crops	< 0.01	n.s.	n.s.	n.s.	< 0.01	< 0.0.1		
Wildflower field	< 0.01	< 0.01	n.s.	n.s.	< 0.01	< 0.01	< 0.05	
Wood	< 0.01	< 0.01	n.s.	n.s.	n.s.	n.s.	< 0.01	n.s.
CaE								
Clover field	n.s.							
Orchard	n.s.	n.s.						
Rural area	n.s.	n.s.	n.s.					
Sunflower field	< 0.05	n.s.	n.s.	n.s.				
Vineyards	n.s.	n.s.	n.s.	n.s.	n.s.			
Wheat crops	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		
Wildflower field	< 0.01	n.s.	n.s.	n.s.	n.s.	< 0.05	< 0.05	
Wood	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	< 0.05
GST								
Clover field	n.s.							
Orchard	< 0.01	n.s.						
Rural area	< 0.01	< 0.01	< 0.01					
Sunflower field	< 0.01	< 0.01	< 0.01	n.s.				
Vineyards	n.s.	n.s.	< 0.01	< 0.01	< 0.01			
Wheat crops	< 0.01	< 0.01	< 0.01	n.s.	n.s.	< 0.01		
Wildflower field	< 0.01	< 0.01	< 0.01	n.s.	n.s.	n.s.	n.s.	
Wood	< 0.05	n.s.	n.s.	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
ALP								
Clover field	< 0.01							
Orchard	n.s.	< 0.01						
Rural area	< 0.05	n.s.	n.s.					
Sunflower field	< 0.05	n.s.	n.s.	n.s.				
Vineyards	n.s.	< 0.01	n.s.	n.s.	n.s.			
Wheat crops	< 0.01	n.s.	< 0.05	n.s.	n.s.	< 0.01		
Wildflower field	< 0.01	n.s.	< 0.01	< 0.01	< 0.01	< 0.01	n.s.	
Wood	n.s.	< 0.01	n.s.	n.s.	n.s.	n.s.	< 0.05	< 0.01
LYS								
Clover field	n.s.							
Orchard	n.s.	n.s.						
Rural area	n.s.	n.s.	n.s.					
Sunflower field	n.s.	n.s.	n.s.	n.s.				
Vineyards	n.s.	n.s.	n.s.	n.s.	n.s.			
Wheat crops	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		
Wildflower field	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	
Wood	n.s.	< 0.01	n.s.	n.s.	n.s.	n.s.	< 0.01	n.s.
PLASM								
Clover field	n.s.							
Orchard	n.s.	< 0.05						
Rural area	n.s.	n.s.	n.s.					
Sunflower field	n.s.	n.s.	n.s.	n.s.				
Vineyards	n.s.	n.s.	n.s.	n.s.	n.s.			
Wheat crops	n.s.	n.s.	< 0.05	n.s.	n.s.	n.s.		
Wildflower field	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	
Wood	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
NA assay								
Clover field	n.s.							
Orchard	n.s.	n.s.						
Rural area	n.s.	n.s.	n.s.					
Sunflower field	n.s.	n.s.	n.s.	n.s.				
Vineyards	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01			
Wheat crops	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		
Wildflower field	n.s.	n.s.	n.s.	n.s.	n.s.	< 0.01	n.s.	
Wood	n.s.	n.s.	n.s.	n.s.	n.s.	< 0.01	n.s.	n.s.

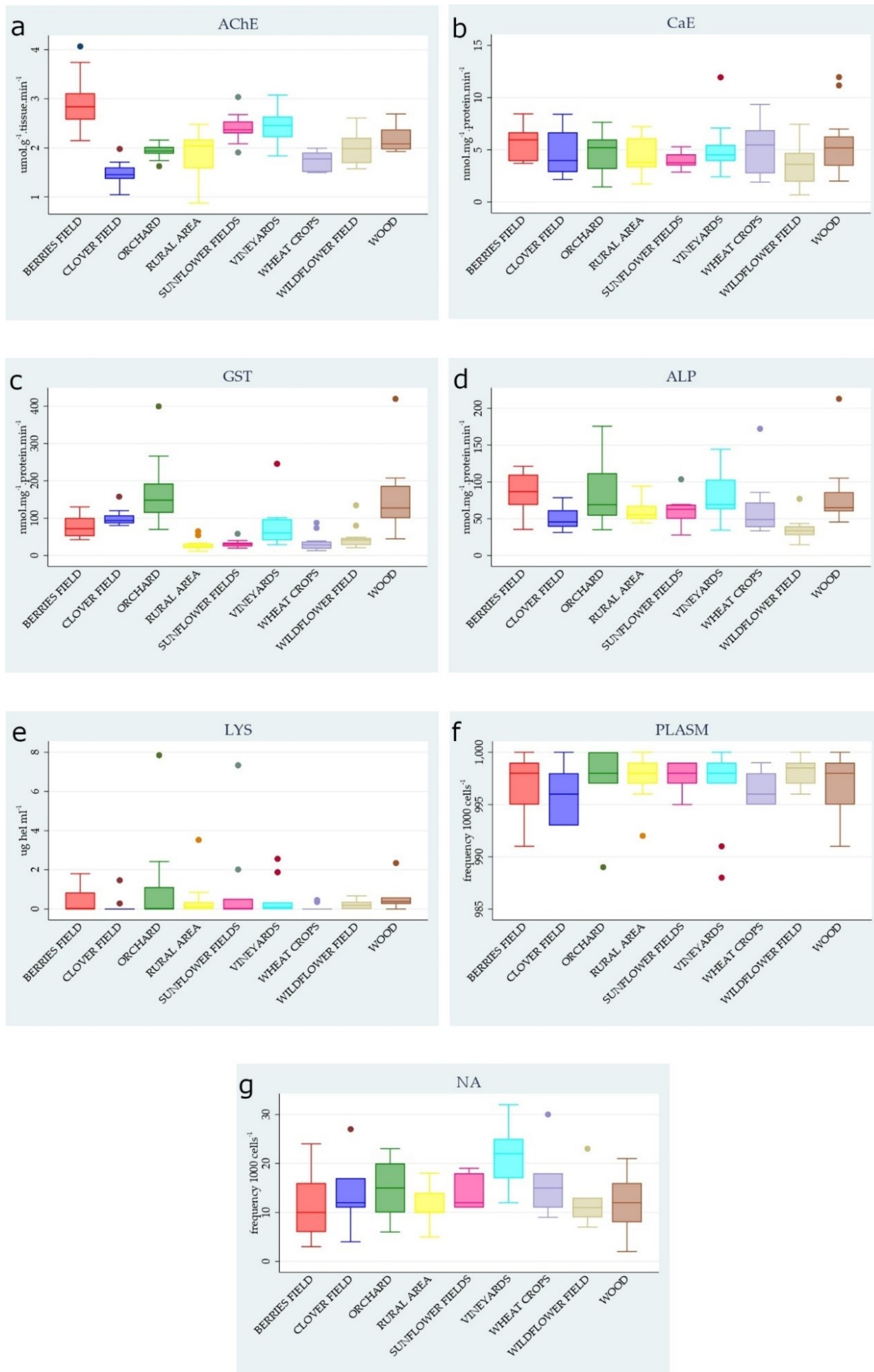

Figure 16. Boxplots of the seven biomarkers (activity of AChE (a); CaE (b); GST (c); ALP (d); LYS (e); differential haemocytes count (f) and NA assay (g)) measured in honeybees, *Apis mellifera* by the nine experimental sites sampled in 2020

Figure 16 shows the results obtained for biomarkers analyses in 2021.

In 2021, AChE was significantly induced in samples coming from berries field, compared to both controls, and in sunflower field and vineyards, compared to the wildflower field. Different authors observed an induction in this enzyme activity after the exposure to OPs and other insecticides, as also mentioned before. Specimens from the clover field showed inhibition compared to the wood and wildflower field. As previously mentioned, insecticides, herbicides and heavy metals are able to inhibit AChE activity. Lupi et al. (2020), reported an inhibition in AChE in honey bee specimens from an area near an orchard and cereal crops; treatments with commercial formulations of a wide range of insecticides and fungicides were carried out in this area. The results led us to hypothesize that compounds capable of decreasing the AChE activity were present in the clover field and wheat crops.

In 2021, CaE was significantly induced in berries field, vineyards and wheat crops compared to wildflower field. As said in 2020 monitoring, heavy metals and insecticides are able to induce CaE activity. In this case, an induction in CaE did not correspond to the inhibition of AChE, leading to the hypothesis that CaE was able to detoxify from xenobiotics actually protecting AChE.

In 2021, induction in GST activity was observed in berries field, clover field and orchard compared to the wildflower samples. Berries field, sunflower field, rural area, vineyards and wheat crops showed significantly lower values of GST activity compared to the wood. As mentioned before, the induction of this enzyme activity could be due to most of the xenobiotics to which these organisms are exposed. The higher values observed in wood in 2021 let us hypothesize that also in that area a kind of stress caused the activation of this biotransformation process.

ALP activity in 2021 samples was significantly induced in all areas with respect to wildflower field organisms, except for clover field and wheat crops. Clover field and wheat crops samples were inhibited compared to the wood. ALP may be modulated in the honeybee by insecticides (Bounias, 1985), fungicides, or acaricides (Bounias et al., 1996). As mentioned for 2020 ALP results, the induction in this enzyme activity agrees with the observations of Caliani et al. (2021b) in cultivated areas, where bees are mostly exposed to pesticides. A decrease in ALP activity was observed by Carvalho et al. (2013) after exposing honey bees to the insecticide fipronil, and by Caliani et al. (2021a) that exposed bees to cadmium.

In 2021 LYS was significantly inhibited in clover field and wheat crops specimens compared to the wood. Caliani et al. (2021a) observed a decrease in lysozyme levels in the laboratory in *A. mellifera* exposed to cadmium (0.1 and 2.5 g/L), and to the fungicide Amistar®Xtra (100 and 200 g/L). The results obtained in this thesis, as regards the area with wheat crops, agree with the monitoring carried out in the study by Caliani et al. (2021b), in which the same trend was observed in bees from an agricultural area.

Specimens sampled in 2021 did not show any significant difference in haemocytes differential count, meaning that this kind of response was not diversely influenced in any of the examined areas.

In 2021, only vineyards showed a significant increase in haemocytes nuclear abnormalities compared to both controls. It is possible that genotoxic compounds capable of causing DNA damage to organisms were present in this area.

The results of this multi-biomarker approach, obtained from 2021 sampling, showed that probably the specimens undergoing major stress were the ones coming from vineyards, clover field and wheat crops. Bees from vineyards were most affected regarding the presence of DNA damage, while clover field and wheat crops animals showed alterations in neurotoxicity biomarkers but also in lysozyme activity.

4.3.2 Comparison between 2020 and 2021 biomarkers

A comparison between the results obtained in the two years for each area was performed. The berries field and the suburban area are not included in this comparison since the sampling in these areas was conducted only for one year. A significant decrease in AChE activity (Figure 17) was observed in all sampled areas from 2020 to 2021. As previously mentioned, AChE activity can be inhibited by organophosphorus and carbamate insecticides, and herbicides. Also pyrethroids insecticides are known to have neurotoxic effects (Soderlund et al., 2002; Zhou et al., 2011). Beside pesticides, the decrease found in AChE activity in 2021 could be due to other environmental contaminants, including heavy metals. Khalifa et al. (2020) found a significant correlation between AChE inhibition and zinc concentration in honey bees sampled in Egypt. Caliani et al. (2021a) observed a dose-dependent inhibition in AChE after exposing bees to 2 different cadmium concentrations. CaE showed a significant decrease in all samples from 2021 compared to the ones from 2020 (Figure 18). We can hypothesize that CaE was activated to

protect the organism from neurotoxic damage, but this kind of response was not enough, leading to the inhibition of AChE.

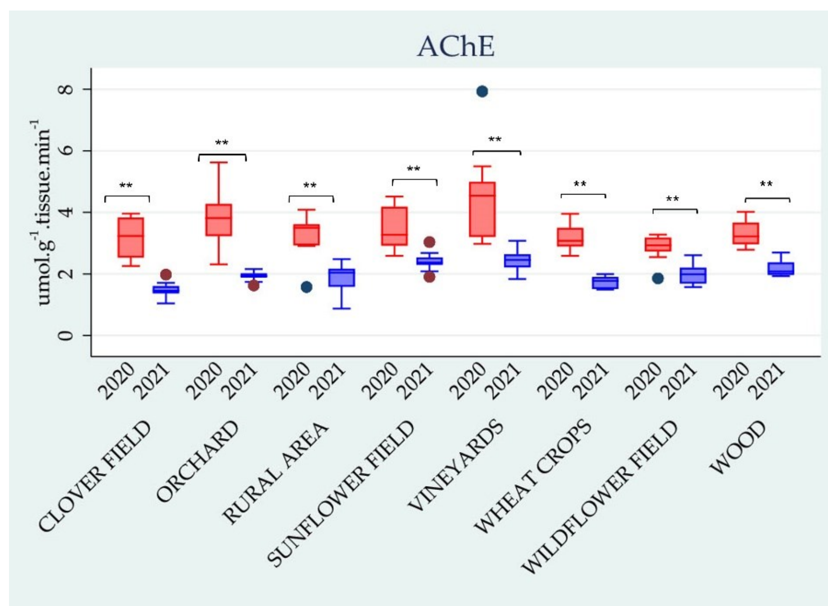

Figure 17. Comparison of AChE activity measured in the head of honeybees (*Apis mellifera*) in samples from 2020 and 2021. ** indicate statistically significant differences with p<0.01; * indicates statistically significant differences with p<0.05

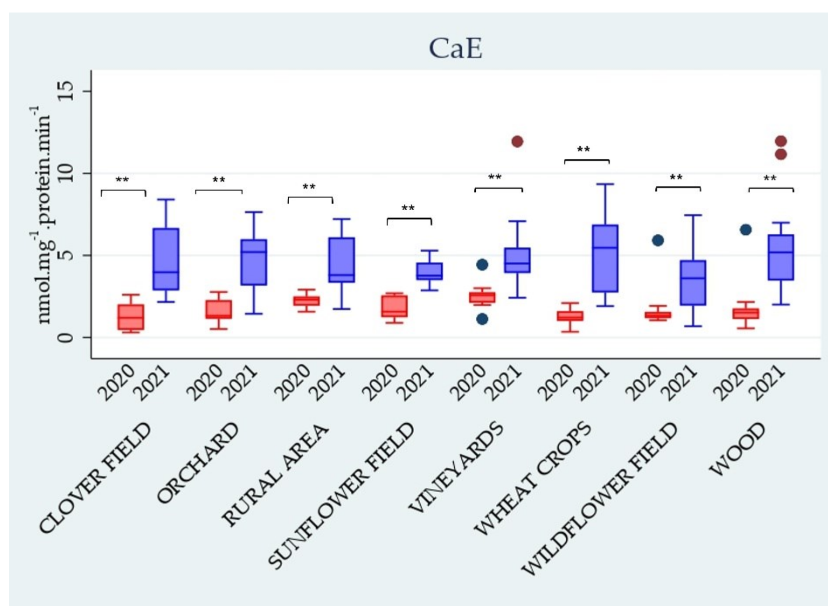

Figure 18. Comparison of CaE activity measured in the head of honeybees (*Apis mellifera*) in samples from 2020 and 2021. ** indicate statistically significant differences with p<0.01; * indicates statistically significant differences with p<0.05

GST results (Figure 19), from 2020 to 2021, showed a significant increase in all areas except for the rural, the sunflower and the wheat crops ones, whose samples revealed a decrease in this enzyme's activity. Several authors demonstrated a high degree of GST activity induction following exposure to various contaminants such as metals, pesticides, PAH and PCB (Garner and Di Giulio, 2012; Papadopoulos et al., 2004; Yu et al., 2012). It has been also demonstrated

that insecticides can cause a decrease in GST activity (Badiou-Bénéteau et al., 2012; Lupi et al., 2020; Yao et al., 2018b). The rural, the sunflower and wheat crops areas could be affected by the presence of insecticides that caused the observed decrease in GST activity from one year to the other, while bees from all other areas could be influenced by the presence not only of different pesticides but also from heavy metals.

From 2020 to 2021, the NA assay revealed a significant increase in the frequency of nuclear abnormalities in clover fields, orchards, vineyards, and wheat crops, while a significant decrease was observed in sunflower and rural areas (Figure 20). Caliani et al. (2021a) observed a genotoxic effect of cadmium and fungicide in *A. mellifera* specimens. Caliani et al. (2021b) observed an increase in nuclear abnormalities in the haemolymph of bees sampled from an orchard and a cultivated area. Chemicals such as pesticides, metals, PAHs are able to cause oxidative stress, due to the presence of ROS (Chakrabarti et al., 2020, 2015; Olgun et al., 2020). These molecules are also able to cause DNA damage. Therefore, the increase in both GST activity and NA assay values observed in 2021 in clover field, orchard and vineyards let us hypothesize that the specimens were subjected to oxidative stress, which can cause DNA damage.

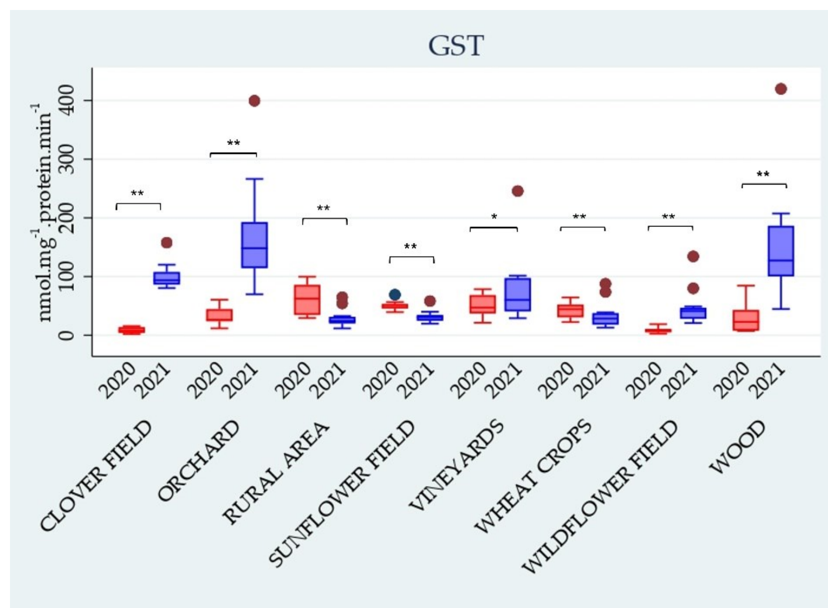

Figure 19. Comparison of GST activity measured in the midgut of honeybees (*Apis mellifera*) in samples from 2020 and 2021. ** indicate statistically significant differences with $p<0.01$; * indicates statistically significant differences with $p<0.05$

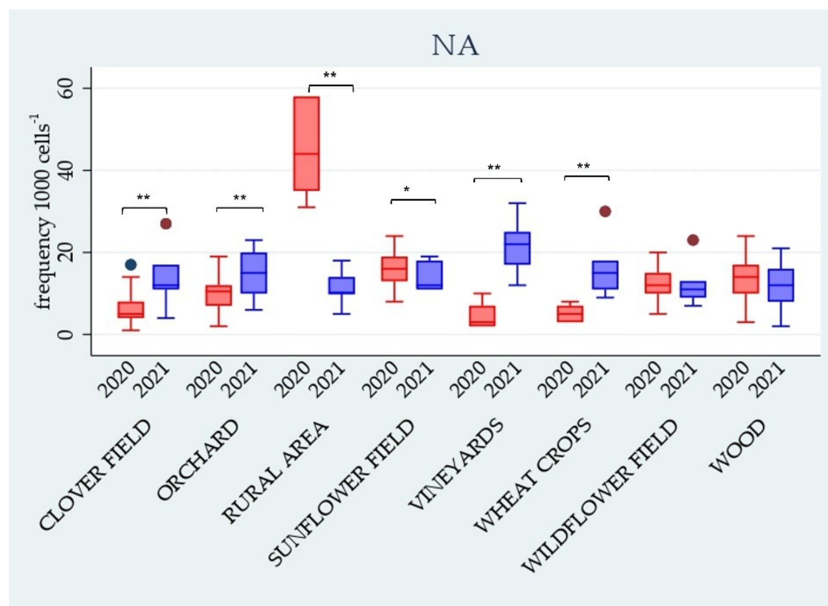

Figure 20. Comparison of NA assay values measured in the haemolymph of honeybees (*Apis mellifera*) in samples from 2020 and 2021. ** indicate statistically significant differences with $p<0.01$; * indicates statistically significant differences with $p<0.05$

A significant increase in ALP activity was observed in the orchard, sunflower field, vineyards and wood areas from 2020 to 2021, while a significant decrease was observed in clover field samples (Figure 21). The increase in enzyme activity agrees with the findings of Caliani et al. (2021b), who assessed the ecotoxicological status of *Apis mellifera* in four different locations under different anthropic pressure. This would imply that pesticides are responsible for the induction of this enzyme in cultivated areas. The inhibition observed in the clover field, however, could be due to pesticide effects, as we observed in this study and as Caliani et al. (2021a) observed after fungicide treatments, or to metals, such as cadmium. A decrease in ALP activity was also found by Carvalho et al. (2013) in *A. mellifera* specimens exposed to the insecticide fipronil.

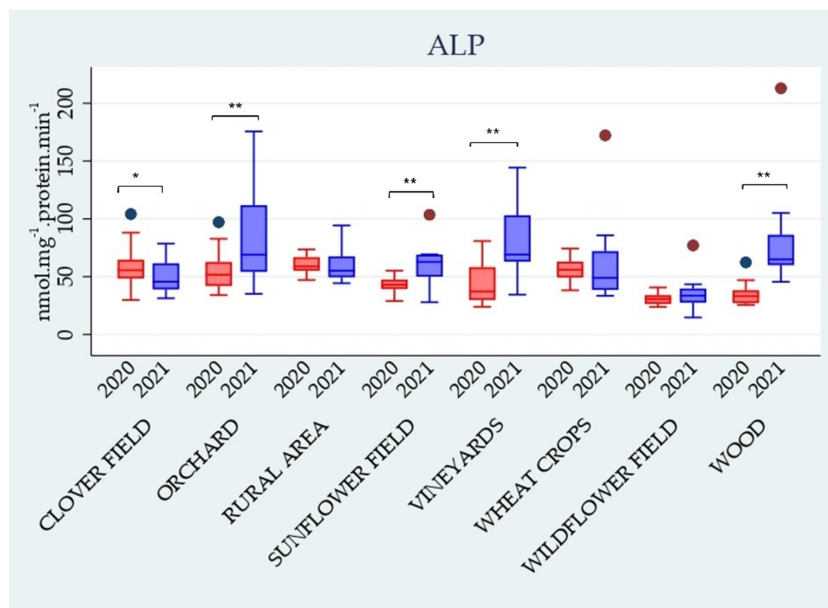

Figure 21. Comparison of ALP activity measured in the midgut of honeybees (*Apis mellifera*) in samples from 2020 and 2021. ** indicate statistically significant differences with p<0.01; * indicates statistically significant differences with p<0.05

Lysozyme activity (Figure 22) decreased significantly in clover and wheat crop fields from 2020 to 2021, while granulocyte frequency increased (Figure 23); this led us to hypothesize that xenobiotic compounds perturbed the immune system and that a cellular response was more involved than an enzymatic one to defend the organisms. On the contrary, from 2020 to 2021, a slight but significant increase in LYS activity was observed in vineyards, wood, and wildflower field (Figure 22), corresponding to an increase in granulocyte frequency in wheat crops, wildflower field, and wood (Figure 23). In this case, both immune defences were most likely required to keep the immune system functioning properly. Insecticides can affect the immune system by influencing gene expression (Abbo et al., 2017; Christen et al., 2019a; Tesovnik et al., 2017; Zhu et al., 2020), haemocyte density, and antimicrobial activity (Brandt et al., 2016). Chemical compounds and heavy metals, on the other hand, have been shown in studies on different taxa to modify bees' immune system response (Mdaini et al., 2019; Wu et al., 2007).

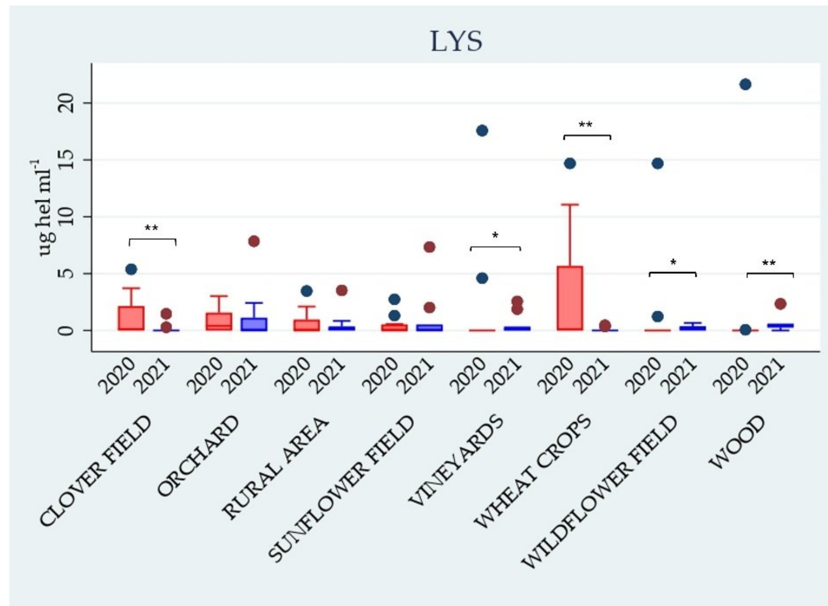

Figure 22. Comparison of LYS activity measured in the midgut of honeybees (*Apis mellifera*) in samples from 2020 and 2021. ** indicate statistically significant differences with p<0.01; * indicates statistically significant differences with p<0.05

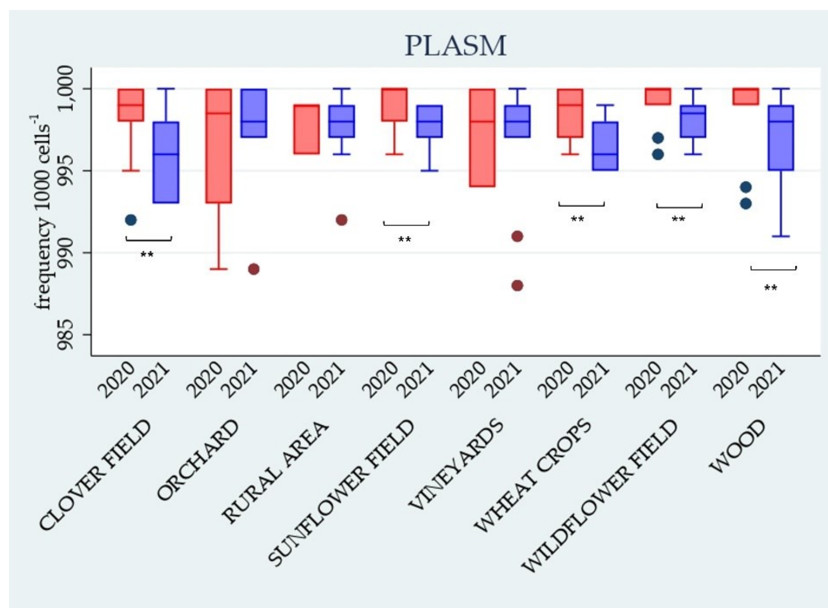

Figure 23. Comparison of differential haemocytes count values measured in the haemolymph of honeybees (*Apis mellifera*) in samples from 2020 and 2021. ** indicate statistically significant differences with p<0.01; * indicates statistically significant differences with p<0.05

Table 11. Statistically significant differences for the seven biomarkers analysed in each area for 2021 compared to 2020. Red ticks are for differences where the values are higher in 2021 compared to 2020; green ticks are for values lower in 2021 compared to 2020; no ticks are for the absence of statistically significant differences

	Clover field	Orchard	Rural area	Sunflower field	Vineyards	Wheat crops	Widflower field	Wood
AChE	✓ (green)	✓ (green)	✓ (green)	✓ (green)	✓ (green)	✓ (green)	✓ (green)	✓ (green)
CaE	✓ (red)	✓ (red)	✓ (red)	✓ (red)	✓ (red)	✓ (red)	✓ (red)	✓ (red)
GST	✓ (red)	✓ (red)	✓ (green)	✓ (green)	✓ (red)	✓ (green)	✓ (red)	✓ (red)
ALP	✓ (green)	✓ (red)		✓ (red)	✓ (red)			✓ (red)
LYS	✓ (green)				✓ (red)	✓ (green)	✓ (red)	✓ (red)
PLASM	✓ (green)			✓ (green)		✓ (green)	✓ (green)	✓ (green)
NA assay	✓ (red)	✓ (red)	✓ (green)	✓ (red)	✓ (red)	✓ (red)		

The results obtained for the 2 years show that in 2021 the organisms were undergoing major stress condition compared to 2020. In fact, bees from 2021 reported neurotoxic effects, observing both AChE and CaE results, as well as the presence of oxidative stress, causing a modification in detoxification enzyme activity and the presence of DNA damage. The different responses obtained from the analysis between the 2 sampling years could be due not only to the presence of contaminants from different anthropic sources but also to climatic conditions, such as temperature and rain. In Tables 12 and 13 we reported the medium temperatures (min and max) and the precipitations respectively, for June and July of 2020 and 2021. In these two years, there was a difference mainly in the rainfalls, even if the medium temperatures of June were slightly higher in 2021 compared to 2020. Moreover, in 2021 precipitations were less abundant with respect to the ones observed in 2020. Bees adjust their behaviour to weather conditions. In fact, they do not leave the hive when it rains; in extremely hot weather, they gather water to keep the colony cool (Conte and Navajas, 2008). In 2021, the scarcity of water and the high temperatures could have caused a weakening of the colonies, probably due to difficulties in foraging, with consequences on the biochemical and cellular responses observed in the specimens. Another hypothesis could be that the less abundant rains could have caused a concentration in the presence of contaminants, that were not washed away from the plants were bees went to forage, leading to a major exposure of the specimens.

Table 12. Average temperatures (min and max) expressed in °C for the months of June and July in 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 Sunflower field Wheat crops	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 13. Rainfalls expressed in mm for the months of June and July in 2020 and 2021 for the provinces of the sampling locations

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

4.3.3 Honey origin and quality

In Table 14 humidity, the three most important amino acids, carbohydrates, melissopalynological results and physical state of the 9 sampled honey were summarized.

Table 14. Summary of all the chemical-physical and melissopalynological parameters of the analysed samples

SAMPLES	Humidity	Amino acids			Carbohydrates						Melissopalynological analysis			Physical state
	Water	*Proline (PRO)*	Taurine (TAU)	γ amino butyric acid (GABA)	Sucrose	Glucose	Fructose	Fructose + Glucose + Sucrose	Fructose/ Glucose	Glucose/ Water	Dominant pollens (x > 45%)	Secondary pollens (45% > x > 16%)	Isolated important pollens (16% > x>3%)	
	g/100 g	*mg/100 g*	*mg/100 g*	*mg/100 g*	g/100 g	g/100 g	g/100 g				% of pollen	% of pollen	% of pollen	
Wood	16,9	*699,72*	2,60	1,26	8,62	27,33	47,11	83,06	1,72	1,62		*Tilia* (21,47%), *Robus ulmifolius* (31,94%)	*Trifolium* (7,62%), *Medicago sativa* (7,47%), *Acacia* (15,58%), Unknown (15,91%)	Crystallized
Clover field	15,2	*1135,21*	12,10	7,26	9,76	34,61	44,01	88,39	1,27	2,28	*Trifolium* (79,3%)		Hedysarum coronarium (7,08%), Unknown (13,61%)	Crystallized
Orchard	18,8	*1667,45*	60,13	10,47	5,27	31,70	47,13	84,11	1,49	1,69	*Castanea* (56,74%)	*Oleace* (10,37%), *Eucaliptus* (10,25%)	*Hedysarum coronarium* (4,6%), *Heliantus annuus* (4,76%), *Tilia* (7,25%), *Prunus* (6,28%),*Oleace* (10,37%), *Eucaliptus* (10,25%), Unknown (2,02%)	Crystallized
Sunflower field	18,1	*1604,62*	19,51	12,29	6,47	34,18	38,68	79,34	1,13	1,89	*Hedysarum coronarium* (46,05%)	*Trifolium* (25,57%), *Castanea* (25,30%)	Unknown (3,06%)	Crystallized
Rural area	16,4	*1635,69*	12,68		8,09	32,15	39,55	79,79	1,23	1,96	*Castanea* (58,4%)	*Trifolium* (20%), *Quercus* (19,50%)	Unknown (2,07%)	Crystallized
Vineyard	18,2	*1458,13*	13,56	7,58	7,75	31,18	43,84	82,77	1,41	1,71	*Castanea* (83,63%)		*Hedysarum coronarium* (6,25%), Unknown (10,11%)	Crystallized
Wheat crops	17,6	*1432,40*	13,69		6,97	33,62	42,29	82,88	1,26	1,91	*Castanea* (46,84%)	*Hedysarum coronarium* (43,78%)	Unknown (9,37%)	Crystallized
Wildflower field	16,6	*2057,63*	21,94	50,68	9,33	34,76	41,84	85,92	1,20	2,09		*Begonia* (26,55%), *Prunus* (29,20 %), *Castanea* (16,27%), *Hedysarum coronarium* (21,49%)	Unknown (6,47%)	Crystallized
Berries field	18,1	*877,59*	5,92		11,15	30,39	46,29	87,83	1,52	1,68	*Castanea* 96,64%)		*Hedysarum coronarium* (4,18%), Unknown (2,31%)	**Not** crystallized

4.3.3.1 Melissopalynological analysis

Melissopalynological analysis is classified into two types: quantitative and qualitative and is usually carried out in accordance with the rules of the International Commission of Apistical Botany (Louveaux et al., 1978; Von Der Ohe et al., 2004). The melissopalynological analysis we performed was qualitative, in that it allowed us to identify and count the pollen types and other figurative elements in the various honey samples. The qualitative analysis also allowed the floral origin of the honey to be determined. To obtain statistically valid percentages, 1200 pollen grains/honey were counted. Counts were taken on at least two slides for each honey sample. Pollens that exceed 45% are considered dominant; pollens between 45 and 16% are considered secondary or accompanying, between 15 and 3% are considered important isolates, and less than 3% are considered rare. In general, honey is considered unifloral if the pollen normally represented by one species exceeds 45%. However, in the case of under-represented pollen (scarcely present) a lower percentage is sufficient (for example 15% for Robinia), while with over-represented pollen (mostly present), a higher percentage is required (over 90% for Chestnut). Among the 9 analyzed honey samples, clover and berries field ones are monofloral honey while the remaining are polyfloral honey (Table 14). In particular, among the monofloral, the berries field one was a chestnut honey (*Castanea sativa* Miller), while the clover field sample was a clover honey (*Trifolium*). Chestnut and Spanish esparcet were the most present pollens; specifically, the chestnut in five samples (rural area, wheat crops, vineyard, orchard, and berries field) had a frequency greater than 45%, thus defining itself as dominant pollen. Spanish esparcet pollen was found in only one sample (sunflower field) in a dominant form, twice as secondary pollen, and four times as important isolated pollen. Clover pollen was in 4 samples, as dominant pollen in only one sample, in two samples as secondary pollen and in one sample (clover field) as important isolated pollen. The frequency of the remaining pollen species varied between secondary pollen and important isolated pollen. Through qualitative melissopalynological analysis, a total of 13 different pollen species were identified; this result fully expressed the different pollen species present in the sampling sites and the ecosystems that surrounded them. Chestnut pollen was the most abundant in the honey, followed by Spanish esparcet and clover (Figure 24).

The melissopalynological analysis highlighted that only in the clover field the pollen was actually derived from the cultivation that we observed during the sampling. The pollen from chestnut, Spanish esparcet, and clover made up most of the honey. These findings suggest that

the biomarker responses observed in *A. mellifera* specimens collected in areas with varying anthropogenic impacts are probably not due to pollen contamination. This could be attributed to organisms being exposed to xenobiotic compounds via plant guttation water or topic contact with the contaminants. However, it is possible that the pollen collected by bees could be exposed to pesticides accidentally, leading to the observed effects in the specimens.

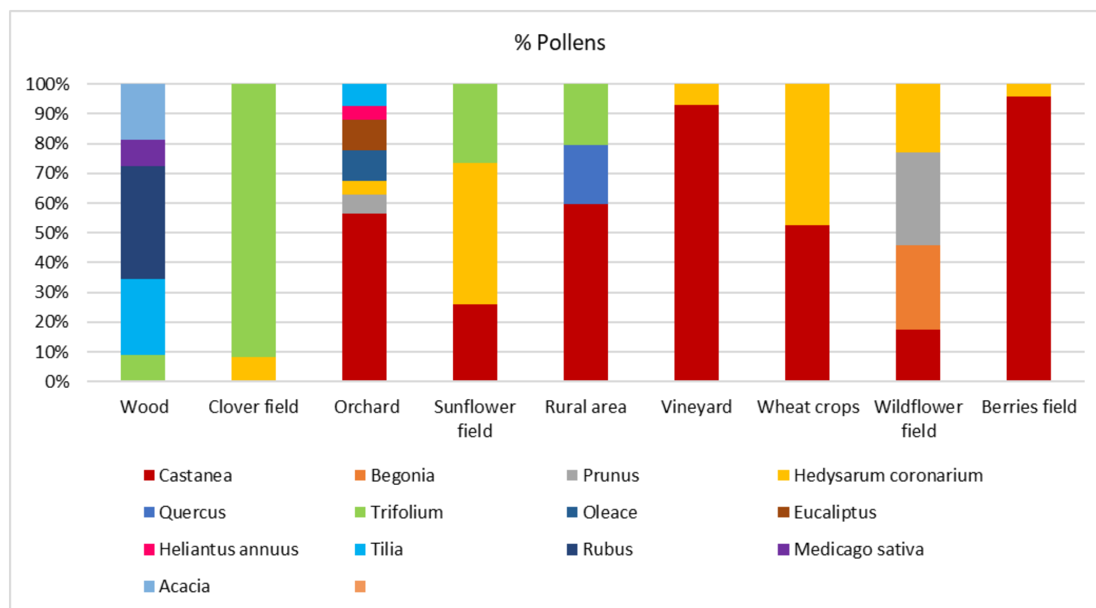

Figure 24. Presence of each pollen species in the analysed samples

4.3.4 Humidity

The humidity of the examined samples starting from a minimum of 15.2% and arriving at a maximum of 18.8%, showed an average value of 15.6% (Figure 25). The minimum value was observed in the monofloral honey from clover field while the maximum value was recorded in the polyfloral honey from the orchard site. Overall, the samples appeared to have values consistent with those reported by da Silva et al. (2016), who reported numerous chemical-physical data, including humidity, for European and non-European countries. The water content of honey appeared to be within legal limits, allowing for good honey conservation.

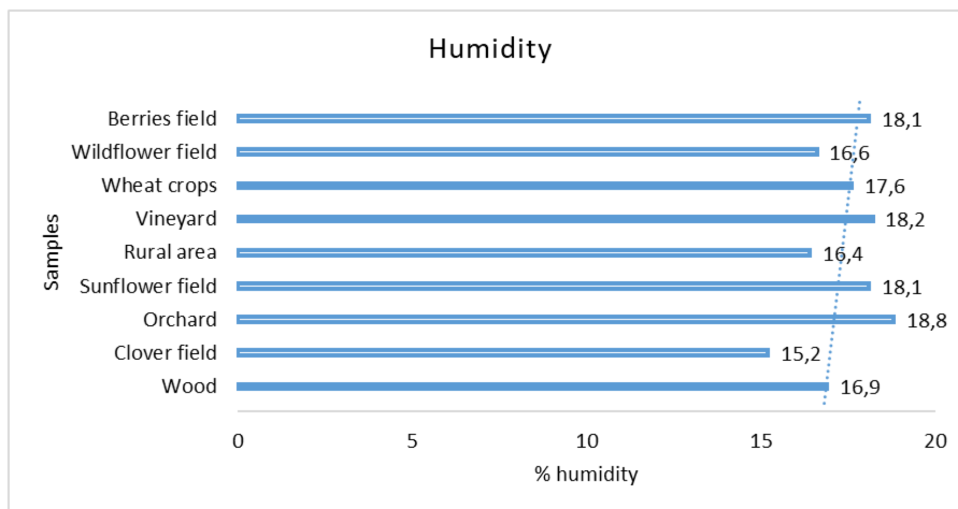

Figure 25. % humidity values of the analysed samples

4.3.5 Carbohydrates

The carbohydrate content of the nine different honey was determined using HPLC analysis. The most prevalent carbohydrate in each sample was fructose, followed by glucose, sucrose, melezitose, and maltohexaose. Other minor compounds included galacturonic acid and ethanol. Glucose and fructose showed homogeneous values, whereas sucrose ranged from 5.27% (orchard) to 11.15% (berries field). This variability and lack of consistency in sucrose content could be attributed to the invertase enzyme, which converts sucrose to glucose and fructose. Maltohexaose was found in six samples with a maximum value of 0.29%. Ethanol was found in two samples, vineyards (0.33%) and berries field (2.5%) (Figure 26). The presence of a high concentration of ethanol indicates that the honey is particularly humid, which could result in fermentation processes that favour yeast activity. The higher value was found in honey from berries field, confirming it as a honey with a high humidity rate; this process irreversibly modifies the organoleptic characteristics of the honey. The results revealed that the most prevalent carbohydrates were fructose and glucose, confirming the findings of Escuredo et al. (2014). The percentage of the three main carbohydrates (fructose, glucose, and sucrose) is 84.11%, ranging from 79% to 87% (Table 14); these values are consistent with the literature. Because glucose is less soluble in water than fructose, the fructose/glucose ratio is an indicator of the physical state of honey (granulation), making it an important parameter to predict honey crystallization (low values indicate more crystallized honey) (Laos et al., 2011). According to the National Honey Board, another parameter that provides information on crystallization is the glucose/humidity ratio (G/W), because humidity affects the physical properties of honey (viscosity, crystallization, etc...). According to some researchers (Dobre et al., 2012), the G/W

ratio may provide a better indication of honey crystallization. According to published data, honey with a G/W ratio less than 1.7 will crystallize slowly or not at all; on the other hand, honey with a G/W ratio greater than 2 crystallizes much faster and more completely (Escuredo et al., 2014). The physical state of the honey is therefore confirmed, since generally honey with a higher G/W value is in crystallized form (wildflower and clover field) unlike honey with lower G/W values which is in liquid form (berries field).

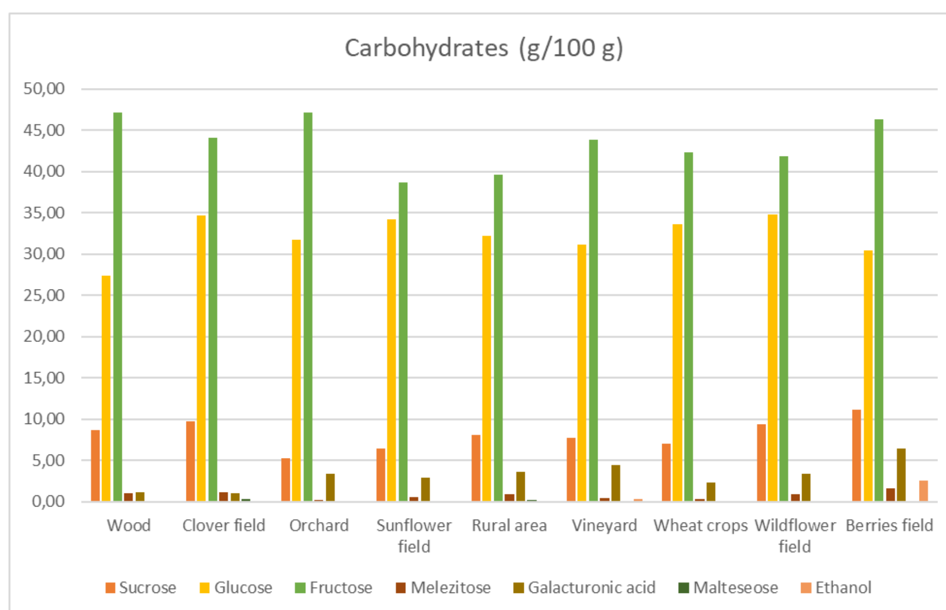

Figure 26. Carbohydrates and other compounds concentrations in the analysed honey

4.3.6 Amino acids

The separation and quantification of 21 amino acids reported in Table 15 were performed by HPLC. In the honey, 15 proteinogenic amino acids and 6 non-proteinogenic amino acids (Table 15 – blu amino acids) were found. The most abundant amino acid was proline (699.72-2057.63 mg/kg). This value is in line with what was reported in the work of Meda et al. (2005), relating to numerous varieties of honey with different botanical origins. Glutamic acid was the second most frequent amino acid (56.23-497.68 mg/kg). Lower values were observed for lysine (4.11-77.84 mg/kg), ornithine (16.57-85.92 mg/kg), aspartic acid (7.37-119.25 mg/Kg), serine (7.09-135.97 mg/kg), tyrosine (2.08-71.70 mg/kg), phenylalanine (3.99-50.49 mg/Kg), taurine (2.60-60.13 mg/kg), γ amino butyric acid (1.26-50.68 mg/kg), valine (4.24-68.45 mg/kg) and β-alanine (13.33-49.63 mg/kg). All the other amino acids showed concentrations between 2 and 30 mg/kg. As shown in Table 15, polyfloral honey generally had higher amino acid values; specifically, wildflower and orchard honey had the highest values in 6 (proline, aspartic acid, glutamic acid, tyrosine, lysine and taurine) of the 21 amino acids detected. Two samples

previously mentioned showed a high variability of pollen species, some of which are in common (chestnut, Spanish esparcet and prunus). This finding could be partly in agreement with the work by Rebane and Herodes (2008) where average values of the main amino acids of Estonian polyfloral honey were compared with monofloral honey, which showed lower values of proline, glutamic acid and lysine than wildflowers. On the contrary, the monofloral honey from berries field often had lower values in 4 of the 21 amino acids (threonine, leucine, isoleucine and phenylalanine) than all other honey.

Table 15. Amino acids concentrations in the analysed honey

Amino acids (mg/kg)	Acronym	SAMPLES								
		Wood	Clover field	Orchard	Sunflower field	Rural area	Vineyards	Wheat crops	Wildflower	Berries field
Aspartic acid	ASP	8,81		119,25	22,38	13,48	17,84	7,37	52,19	50,87
Serine	SER	7,09	16,50	135,97	38,28	24,43	17,12	19,05	30,70	10,03
Glutamic acid	GLU	141,85		497,68	61,89	70,07	205,98	56,23	268,53	210,25
Arginine	ARG	4,34	10,39	16,72	27,37	13,27	1,63	8,07	17,85	2,62
Threonine	THR	9,50		14,18	25,35	8,09	4,43	25,48	23,99	0,60
Alanine	ALA			26,90	1,81	4,94	3,71	0,76		
Proline	*PRO*	*699,72*	*1135,21*	*1667,45*	*1604,62*	*1635,69*	*1458,13*	*1432,40*	*2057,63*	*877,59*
Cysteine	CYS			0,42						
Tyrosine	TYR	2,27	2,08	39,09	38,27	9,05	3,41	25,69	71,70	
Valine	VAL	9,72	10,57	68,45	24,84	29,11	18,26	20,70	24,96	4,24
Methionine	MET			2,08						
Lysine	LYS	26,75	37,86	64,65	65,78	37,24	11,94	54,61	77,84	4,11
Isoleucine	ILE	5,04	3,57	27,18	11,50	13,82	6,10	10,00		3,81
Leucine	LEU	2,58	3,51	23,95	10,33	11,06	3,48	8,70		2,42
Phenylalanine	PHE	7,37	11,98	50,49	36,86	29,43	37,25	34,75		3,99
Taurine	TAU	2,60	12,10	60,13	19,51	12,68	13,56	13,69	21,94	5,92
β alanine	BALA	49,63	13,33							
γ amino butyric acid	GABA	1,26	7,26	10,47	12,29		7,58		50,68	
α amino butyric acid	AABA		4,50							
Ornithine	ORN	16,57	34,61	85,92	39,50	25,50	24,58	28,79		16,70
β amino butyric acid	BABA		14,47							

The humidity, proline, glucose, and fructose values of the monofloral chestnut honey, as shown in Table 14, were consistent with those found in the work of Kolayli et al. (2016), confirming the botanical origin. Sucrose was found in all honey, but in higher concentrations in monofloral honey from berries field. Sucrose was not found by Kolayli et al. (2016), but its presence in monofloral samples could be attributed to the invertase enzyme, which does not hydrolyse all of the sucrose (low invertase activity), thus justifying the presence of this carbohydrate in honey. The above statement is supported by Kumar and Kesavapillai (2012), who reported the optimal conditions for invertase activity (40°C). The conditions under which the analyses were carried out were differed from those described in the previously cited paper. Reduced invertase activity results in less sucrose hydrolysis in its two derivatives, justifying a higher sucrose concentration in honey. The sunflower field and wheat crop samples showed a percentage of Spanish esparcet (*Hedysarum coronarium* L.) pollen close to the monofloral values of the same pollen (% of Spanish esparcet > 50%). In confirmation of the botanical origin, very similar carbohydrate values (sucrose: 6.97 g/100g wheat crops and 6.47 g/100g sunflower field; glucose: 33.62 g/100g wheat crops and 34.18 g/100g sunflower field; acid galacturonic acid: 2.32 g/100g wheat crops and 2.95 g/100g sunflower field) were found. Table 14 shows all the parameters previously mentioned for each honey. The values obtained fall within the ranges indicated by da Silva et al. (2016), in which chemical-physical parameters of honey from different European countries were taken into consideration. Sucrose also appears to have a value similar to that reported for European, Asian and Turkish countries.

The combined results of honey composition and biomarkers analysis let us hypothesize that in this field study the health status of honey bees did not affect the quality of honey. Nonetheless, we cannot exclude that some physiological change in the organism could influence the quality of honey bees products. For this reason, it could be interesting to deepen these aspects, to better understand the effects of contamination and climatic change on bees colonies.

4.4 Conclusions

Field studies are particularly useful to understand the effects of multiple stressors on honey bees. In fact, honey bees come in contact with contaminated food and water in their foraging activity. The contaminants can also accumulate in the hives and its products exposing not only foragers but also workers with other functions, drones, larvae and queen bees.

The aim of this study was to assess the ecotoxicological health status of honey bees sampled in areas with different anthropic impact, with a two-years sampling (2020-2021). To do so, we integrated a multi-biomarker approach with the analyses of the floral origin and quality of beehives products. Some of the selected biomarkers have already been used for this kind of study, while genotoxicity and immune system biomarkers were poorly adopted. To the best of our knowledge, this is also the first time that the sub-lethal effects, the melissopalynological and chemical-physical analysis were used together.

The results obtained for the monitoring in 2020 showed that in the suburban area the animals were undergoing nervous and immune systems stress, as well as effects on metabolism and biotransformation process. All the agricultural areas showed an induction in detoxification enzyme activity, probably due to the exposure to pesticides that the bees tried to eliminate. In the same way, for all the agricultural areas there was an increased activity of the metabolism. The bees from the rural area showed also the presence of DNA damage.

In 2021 the specimens undergoing major stress were the ones coming from vineyards, clover field and wheat crops. Bees from vineyards were most affected regarding genotoxicity, while clover field and wheat crops animals showed alterations in nervous and immune systems responses.

The comparison between the 2 years results showed that the organisms were undergoing major stress condition in 2021 compared to 2020. Bees from 2021 reported neurotoxic effects, the presence of oxidative stress and DNA damage. The different responses obtained could be due not only to contaminants but also to the changing of climatic conditions, such differences in temperatures and rainfalls, which were taken into consideration. In fact, the scarcity of water and the high temperatures in 2021 could have caused a weakening of the colonies, with consequences on the biochemical and cellular responses observed. Another hypothesis could be that the less abundant rains could have caused a concentration in the presence of contaminants, that were not washed away from the plants where bees went to forage, leading to a major exposure of the specimens.

The melissopalynological analysis showed that only in the clover field the pollen was taken from the cultivation that we observed during the sampling. The most abundant pollens in the samples were from chestnut, Spanish esparcet, and clover. These findings suggest that the biomarker responses observed in *A. mellifera* specimens are probably not due to pollen contamination. In fact, the exposure of organisms to contaminants could probably happened through plant guttation water or topic contact during the treatments of the fields. However, it is possible that the pollen collected by bees could be exposed to pesticides accidentally, leading to the observed effects in the specimens.

The carbohydrates, amino acids and humidity analysis showed that honey samples were not characterised by major differences, even if coming from different areas, except for the proportion of some amino acids. In fact, the polyfloral honey showed higher content of proline, aspartic acid, glutamic acid, tyrosine, lysine and taurine compared to the two monofloral honey.

The multi-biomarker approach proved to be effective in order to evaluate the physiological changes due to contamination and other factors, such as climatic conditions, on *Apis mellifera* colonies. The integration of the multi-biomarker approach with the analyses if floral origin and chemical-physical properties of honey was useful to understand the potential routes of exposure to xenobiotics and to observe the absence of major differences in chemical-physical properties of different honeys.

5. FINAL CONCLUSIONS

Pollinating insects, bees included, are continuously exposed to risks for their health and survival due to anthropic activities. Agricultural activities are one of the major stress factors, due to the use of agrochemical products, followed by the exposure to heavy metals, PAHs, PCBs, and other anthropogenic contaminants.

This work of thesis gave a contribution to fill different gaps in the assessment of the health status of *Apis mellifera*, in particular concerning:

- The study of the sub-lethal effects of pesticides mixture.
- The assessment of the effects of commercial pesticides, instead of only active principles.
- The implementation of field studies, to evaluate the status of honey bees exposed to multiple stressors in the environment.

A multi-tier methodology including biomarker responses, proteomic analysis, and bee product quality and origin, was used.

The main findings obtained from this thesis can be summarized as follow:

- The laboratory study contributed to evaluate the effects of less studied commercial compounds, a fungicide and an herbicide, alone and in combination, using an approach made up of different techniques, biomarkers and proteomics. Both the pesticides alone were found to influence the detoxification process. The fungicide alone had also effects on the metabolism, while the herbicide demonstrated to be neurotoxic. The results from the mixture exposure demonstrated that the effects obtained were influenced mostly by the herbicide. The proteomic approach revealed that the two pesticides were able to affect the energy metabolism, the immune system and the protein synthesis.

- The field monitoring study assessed the ecotoxicological status of bees from 10 different natural environments. The used approach integrated different methodologies: a set of biomarkers to assess the health status of honey bees while the origin and quality of honey were examined through melissopalynological and chemical-physical analyses. The results obtained for the monitoring in 2020 showed that the suburban area and the agricultural area were undergoing biochemical stress but in different ways, probably because the contamination was different among the areas. In 2021 the specimens

undergoing major stress were the ones coming from vineyards, clover field and wheat crops. The comparison between the 2 years results showed that the organisms were undergoing major stress condition in 2021 compared to 2020. Bees from 2021 reported neurotoxic effects, the presence of oxidative stress and DNA damage. The different responses obtained could be due not only to contaminants but also to the changing of climatic conditions. The melissopalynological analysis showed that only in the clover field the pollen derived from the cultivation that we observed during the sampling. These findings suggest that the biomarker responses observed in *A. mellifera* specimens are probably not due to pollen contamination. In fact, organisms could be exposed to contaminants through other exposure routes. The carbohydrates, amino acids and humidity analysis showed that honey samples were not characterised by major differences, even if coming from different areas, except for the proportion of some amino acids.

Both studies had also the goal to start filling a research gap regarding the assessment of effects on immune system and DNA damages, obtaining promising results.

The used integrated approaches proved to be effective to observe the ecotoxicological health status of *Apis mellifera* from different points of view. The integrated methodology proposed in this thesis would be a useful tool to help in guiding decisions and informing policies.

The multi-trial approach would be in fact a sensitive tool to measure sub-lethal effects, and not only lethal ones, of pesticide active principles and, more important, of pesticide commercial formulations. It would be helpful to improve the current risk assessment procedure for chemical registration and use and, consequently, make the agricultural environment more pollinator-friendly.

The integrated approach, applied in the field, would also be able to guide the selection of specific mitigation measures to be put in place to improve pollinators wellness. Furthermore, the approach can be used to monitor if the pollinator mitigation measures already applied and foreseen by the EU CAP for agricultural practices, are effective in halting and reversing the decline of pollinators and in particular of honey bees.

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