Manuscripts on omics research for model bee III

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PoshBee

Pan-european assessment, monitoring, and mitigation of stressors on the health of bees

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Preface

Managed and wild bees, through pollination of wildflowers and crops, are essential to maintain the biodiversity that supports the well-being of humans. In recent years, pollinators have dealt with increasing harmful events due to environmental problems arising from natural processes and anthropogenic activities. These threats may have adverse effects alone or in synergy on pollinator biodiversity and their health. Many solitary bees are facing decreases in their populations and ranges, resulting in an overall loss of pollinator diversity in different landscapes. Several deleterious factors have been implicated in this decline, for example, habitat loss, climate change, increased pesticide use, and pathogens. To support the management and defining the health status of wild bees in response to stressors, we focused on the red mason solitary bee *Osmia bicornis* and conducted a multiscale experiment (laboratory, semi-field and field). We considered that a promising way to monitor health is to track their immune status through analysing their haemolymph by biochemical analyses, as is done through a blood test in humans. Overall, through this model of solitary bee we aimed to have a better understanding of the possible harmful effects of abiotic and biotic stressors alone or in combination on the health and physiological state of *O. bicornis*.

1. Introduction

Bees are essential for the proper functioning of ecosystems and our well-being; they ensure the pollination services to more than 85% of wild flowering plants (Ollerton et al., 2011) and over 75% of cultivated crops (Klein et al., 2007). In total, about 35% of our diet is linked to their action. Nevertheless, there is a decline in these pollinating insects in different parts of the world, which is mainly due to anthropogenic activities (Potts et al., 2010). To secure pollination services and reverse the declines of bee populations, bee conservation has become a priority in many countries, and several initiatives have been undertaken at global and regional scales (Potts et al., 2016). Most of the >20,000 bee species worldwide are solitary bees (Danforth, 2019), and they are crucial for pollination of wild flowering plants and agricultural crops (Garibaldi et al., 2013; Klein et al., 2007; Schwarz et al., 2022). Until now, they have received less attention than social bees. Six solitary bee species (*O. bicornis*, *O. cornifrons*, *O. cornuta*, *O. lignaria*, *Megachile rotundata* and *Nomia melanderi*) are commercially available and could be used in risk assessment (Sgolastra et al., 2019). Solitary bee sensitivity and level of pesticide exposure can differ significantly from social bees (Arena & Sgolastra, 2014; Sgolastra et al., 2019), and this is related to the differences in their physiologies and life-history traits (Hayward et al., 2019; Schwarz et al., 2022). Among the anthropogenic chemicals used, the global use of the novel systemic insecticide sulfoxaflor, a potential replacement for neonicotinoids, is increasing (Simon-Delso et al., 2015). Sulfoxaflor, which belongs to the class of sulfoximines, is a competitive modulator of the insect nicotinic acetylcholine receptors known to play a role in central nervous system responses (Babcock et al., 2011; Cutler et al., 2013; Ulens et al., 2019; Watson et al., 2011). Sulfoxaflor has been reported to be effective against pests resistant to neonicotinoids (Sparks et al., 2013). Sulfoxaflor is an active compound indicated for use in apples, citrus, cotton, cucurbits, grapes, pear, peaches, strawberries, tomatoes and other crops (Boff et al., 2021). In 2020, sulfoxaflor was classified as posing high risks to bees when applied during flowering (European Food Safety Authority (EFSA) et al., 2020), and its use is restricted to non-flowering crop stages in the European Union (Schwarz et al., 2022). Insecticides pose the highest risk for bee populations as they are designed to kill insects (Johnson, 2015; Sanchez-Bayo & Goka, 2014). Sulfoxaflor was approved to be used in EU in 2015, but it was banned in 2020 in France while remaining authorised in 18 EU member states (Tamburini, Wintermantel, et al., 2021). Fungicides are commonly and widely used (Zhang, 2018), and while they do not target insects, there is evidence that they may negatively affect bees directly (Artz & Pitts-Singer, 2015; Bernauer et al., 2015; Mao et al., 2017) or indirectly by stimulating the toxicity of insecticides (Carnesecchi et al., 2019; Johnson et al., 2013; Sgolastra et al., 2018). Fungicides present more than 35% of the global pesticide market and their use is continuously increasing (Rondeau & Raine, 2022; Zubrod et al., 2019). Azoxystrobin is one of the most commonly used fungicides in agriculture since 1996, showing efficiency and broad-spectrum characteristics in protecting crops (Abdelraheem et al., 2015; Rodrigues et al., 2013). Its residues are the most detected due to its widespread use (Park et al., 2022). Years ago, azoxystrobin was classified as being of low toxicity to bees (European Food Safety Authority, 2010). The impact of pesticides on solitary bees could be more severe as well, because the fitness of reproductive females can be impacted directly, meanwhile, social bees have the colony protection to deal with the impairment of individual workers (Henry et al., 2015; Rundlöf et al., 2015; Sgolastra et al., 2018, 2019; Straub et al., 2015). Pesticide impact on pollinators has been widely studied in social bees (Li et al., 2021; Linguadoca et al., 2021; Siviter & Muth, 2020; Straw & Brown, 2021; Tamburini, Pereira-Peixoto, et al., 2021; Tamburini, Wintermantel, et al., 2021), but less so in solitary bees (Boff et al., 2021; Knauer et al., 2022; Schwarz et al., 2022). Consequently, potential risks to bees might be underestimated and further investigations

are urgently needed (Cullen et al., 2019). Semi-field studies investigating interactive effects between fungicides and insecticides on solitary bees are rare (Knauer et al., 2022; Lehmann & Camp, 2021; Schwarz et al., 2022). As mentioned above, most of the studies done were interested on the physiological impacts of the pesticides on social bees. The multiomics studies grouping genomics, transcriptomics, metabolomics or proteomics dealing with pesticide impacts, mainly neonicotinoids, were focused on *Apis mellifera* (Almasri et al., 2020, 2022; Ardalani, Vidkjær, Kryger, et al., 2021; Ardalani, Vidkjær, Laursen, et al., 2021; Chen et al., 2021a, 2021b; Christen et al., 2018; Gao et al., 2020; Haas & Nauen, 2021; Kasiotis et al., 2021; Kim et al., 2022; Ma et al., 2018; Murawska et al., 2021; Shuai et al., 2022; Zaworra et al., 2019) and *Bombus terrestris* (Erban et al., 2019; Manjon et al., 2018; Rothman et al., 2020). Moreover, biotic stressors such as the parasites *Nosema* spp., *Crithidia* spp. and *Varroa destructor* were studied at the omics level on social bees (Erban et al., 2019; Gancarz et al., 2021; Genath et al., 2021; Houdelet et al., 2021b; Houdelet et al., 2022; Liu et al., 2019; Słowińska et al., 2019; Ward et al., 2022). To our knowledge, except for the genomic study performed by Möllmann & Colgan (2022), no other omics approaches such as proteomics explored the molecular impacts of pesticides on solitary bees. Inspired by the MALDI BioTyping routinely used in clinical microbiology, we developed the MALDI BeeTyping® (Arafah et al., 2019; Askri et al., 2023; Bournonville et al., 2023; Houdelet et al., 2021a; Houdelet et al., 2021b), which makes it possible to analyse the significant molecular alterations found in the haemolymph of bees following exposure to stressors. The immune response molecules of bees and particularly the antimicrobial peptides (AMPs) (defensin, apidaecin, abaecin and hymenoptaecin) are studied (Askri et al., 2023; Bournonville et al., 2023). This technique is used to establish a prognosis and a reliable and understandable diagnosis of the impact of stressors on the pollinators studied and therefore to define their state of health. The molecular impacts of the nutritional stress, pesticides and pathogens, individually or in combination, on the haemolymph composition of the solitary bee *O. bicornis* are not well known. To bridge this knowledge gap, we conducted three levels of studies (field, semi-field and laboratory) to understand the influence of pathogenic or nutritional stress on the susceptibility of solitary bees to pesticides. We used two complementary mass spectrometry-based approaches, MALDI BeeTyping® and Bottom-up proteomics by LC-ESI-MS/MS, to elucidate the impacts of the stressors (abiotic and biotic) on the *O. bicornis* haemoproteome.

2. Experimental section

The experimental workflow for Omics on the solitary bee model, from exposure to proteomics studies (MALDI BeeTyping® & off-gel Bottom-up proteomics), is presented in Figure 1.

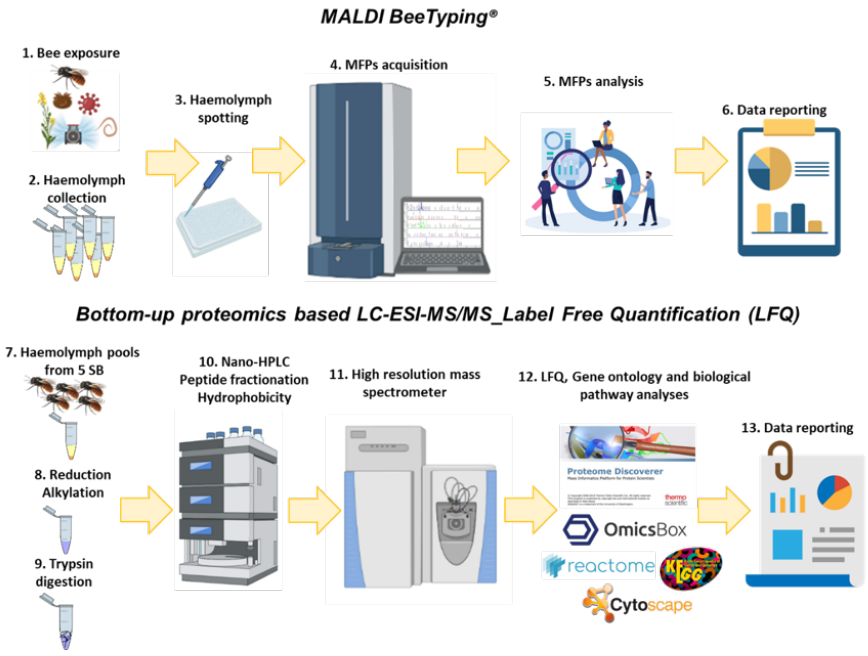

Figure 1: Omics workflow (from bee exposure to data processing) applied to *Osmia bicornis* (SB for solitary Bees), MALDI, MFPs and LC-ESI-MS/MS stands for Matrix Assisted Laser Desorption Ionization, Molecular Mass Fingerprints and Liquid Chromatography coupled on line to Electro Spray Ionization and tandem mass spectrometry (MS/MS).

2.1. Experimental designs of laboratory, semi-field and field *Osmia bicornis* experiments

The laboratory experiments were carried out within Work Packages (WP) 3, 5, and 6 (Table 1).

Table 1: Laboratory experiments performed by 14-MLU and 32-WBF-Agroscope within Work WP 3, 5 and 6.

Country	Partner (WPs)	Haemolymph	Experimental condition
GER*	14-MLU (WP3 + WP6)	253	- Control - Sulfoxaflor (3.125 ng/bee) - Amistar®, Azoxystrobin active ingredient 40 µg/bee - Roundup®, Glyphosate active ingredient 100 µg/bee - *Crithidia* - Flupyradifurone (FPF)+ *Crithidia*
CHE*	32-WBF-Agroscope (WP5)	349	- Sulfoxaflor, active ingredient (high-3 ppm; low-0.3 ppm; AC; C) - Azoxystrobin, active ingredient (high-1.9 ppm; low-0.19 ppm; AC; C) - *Prunus* - *Cistus* - Pollen Mix

*GER, Germany and CHE, Switzerland

The semi-field WP7 experiments were carried out in Switzerland (32--WBF-Agroscope) in 2019 and 2020 (Figure 2). The details of the implementation designs were reported in Schwarz et al. (2022) and Knauer et al. (2022), respectively.

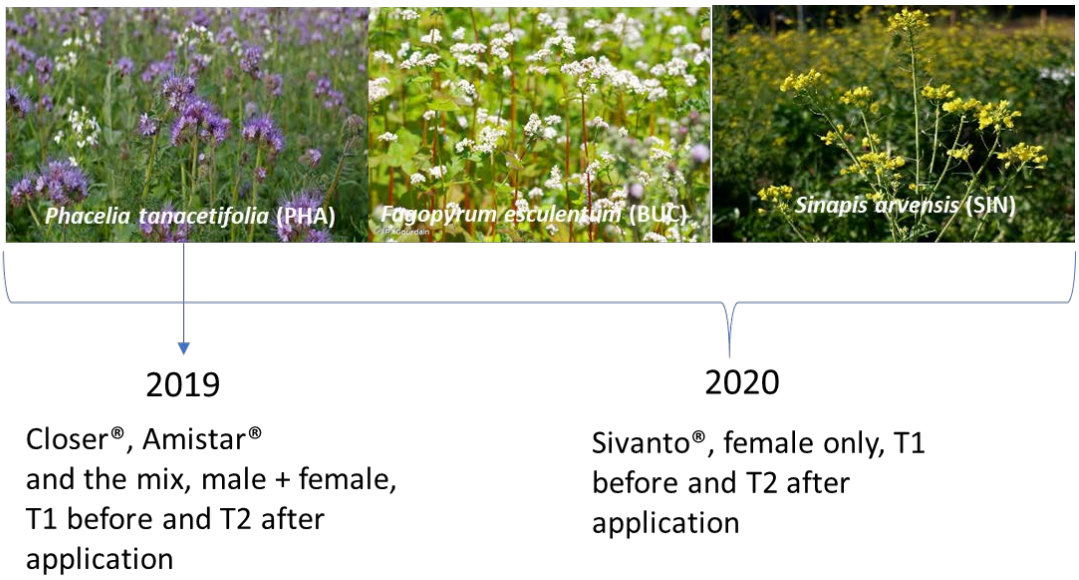

Figure 2: WP7 semi-field experiments (2019 and 2020) carried out by 32-WBF-Agroscope (CHE).

The field experiments were conducted in Germany (GER) and Italy (ITA), on oilseed rape (OSR) and apple (AAP). The design of these experiments was reported in Hodge et al. (2022).

2.2. Haemolymph collection

The haemolymph collection protocol applied for field, semi-field and laboratory experiments is based on the method established by Arafah et al., (2019); Askri et al., (2023); Bournonville et al. (2023) and adjusted for *O. bicornis* samples. Briefly, the haemolymph was collected using a homemade collection kit consisting of a pulled glass capillary (Sutter Instrument Corp, Model P-30, Novato, California). The glass capillary was inserted dorsally under the second tergum of the abdomen and the haemolymph rose by capillary action. Then, the collected haemolymph was transferred to a chilled LoBind Protein microtube (Eppendorf, Germany) precoated with a solution of phenylthiourea (PTU) and phenylmethylsulfonyl fluoride (PMSF) to prevent melanisation and proteolysis, respectively. After collection, haemolymph samples were stored at -20°C until shipment.

2.3. MALDI BeeTyping® analysis

Each individual haemolymph sample was analysed using a MALDI AutoFlex III Smartbeam® instrument (Bruker Daltonics, Germany) according to Arafah et al., (2019); Askri et al., (2023); and Bournonville et al., (2023). Molecular mass fingerprints (MFPs) were acquired according to Bruker Biotyper recommendations with minor changes. Briefly, the haemolymph was diluted 1:10 in water acidified with 1% trifluoroacetic acid (TFA, Sigma–Aldrich, France). A volume of 1 µL from each sample was spotted onto a MALDI MTP 384 polished ground steel plate (Bruker Daltonics, Germany), dried under gentle vacuum for 15 min and then mixed with 1 µL of the α-cyano- 4-hydroxycinnamic acid matrix (4-HCCA, Sigma-Aldrich). MFPs were recorded in an automatic positive linear mode using FlexControl 4.0 software (Bruker Daltonics). Each bee haemolymph sample was spotted in triplicate with one reading

for each. For MFPs acquisition, the instrument was set up with the following parameters: 1.5 kV of electric potential difference, a dynamic range of detection of 700-18,000 in *m/z*, 30% of laser power, a global attenuator offset of 70% with 200 Hz laser frequency, and 1,000 laser shots were summed per spectrum. The linear detector gain was set at 1.762 kV with a suppression mass gate up to *m/z* 600. Calibration was performed using a combination of a standard mixture of peptides and proteins (Peptide Standard Calibration II and Protein Standard Calibration I, Bruker Daltonics, Germany) and an in-house calibration solution referred to as APISCAL. After drying under vacuum, the calibrants (0.5 µL each) were covered with 1 µL of matrix. The plate was dried again before MALDI-TOF analysis. Data were previewed using the FlexAnalysis 3.4 software.

2.4. Data post-processing and statistical analyses

ClinProTools™ 2.2 Software (Bruker Daltonics) was used to analyse the MALDI-MS datasets for post-processing and statistical analyses (ion distributions and modulated molecular ions (MMIs)). Baseline subtraction and spectral smoothing were applied to all acquired spectra. All spectra were averaged using a signal-to-noise ratio of three and a resolution threshold of 800 for peak-picking and area calculations. A post-processing step involving spectral normalisation of all calculated peak areas was performed before the analysis of the variances using Principal Component Analysis (PCA).

2.5. Off-gel Bottom-up proteomic by Nano-LC-MS/MS and Label free quantification (LFQ)

Based on the MFPs spectra generated by MALDI BeeTyping®, individual bees were selected to form pools for LFQ b Off-gel Bottom-up proteomics analyses by Liquid Chromatography coupled to Electrospray Ionisation Tandem Mass Spectrometry (LC-ESI-MS/MS). This approach was used for semi-field and laboratory experiments as they were carried out under controlled conditions to generate the differential ratios. Three pools of five individual haemolymphs were prepared for each condition of an experimental set. For the semi-field experiment performed in 2019, only three individuals (male or female separately) were considered to form the pools, because of the limited sampling. The pools were dried under vacuum centrifugation (Labconco, Kansas City, MO) and analysed according to (Askri et al., 2023; Bournonville et al., 2023). Briefly, 20 µL of 0.1% RapiGest surfactant (Waters, Milford) in 50 mM ammonium bicarbonate buffer (NH4HCO3, ABC) were added to the samples. After adding 2 µL of 280 mM dithiothreitol (DTT, disulfide bond reducing agent), the tubes were incubated at 56°C for 45 min in the dark, centrifuged briefly and then allowed to cool down. 4 µL of 4-vinylpyridine (4-VP, alkylating agent to block cysteine residues) were added, followed by a 30 min incubation in the dark at room temperature. 2 µL of 0.2 µg/µL a trypsin solution (sequencing grade modified, Promega, Madison, WI) were used for protein digestion. The samples were incubated overnight at 37°C under gentle shaking and the digested samples were acidified with 5µL of 20% acetonitrile (CAN) - 10% trifluoroacetic acid (TFA, LC-MS grade, Carlo-Erba Reagents, Val de Reuil, France) to stop enzymatic reaction and neutralise the buffer. Samples were then incubated for 45 min at 37°C and were centrifuged for 10 min at 15,000 g and analysed by LC/ESI-MS/MS. The digested haemolymph pooled samples were loaded onto an U3000 nano-HPLC connected to a high-resolution Q-Exactive Orbitrap (all instruments Thermo Fisher Scientific) equipped with an Acclaim C_{18} PepMap 100 nanocolumn (75µm x 150mm, 3 µm and 100 Å) on-line with a concentration micro-precolumn C_{18} PepMap 100 (3 µm and 100Å). The tryptic-digested peptides were separated at a flow rate of 300nL/min using a biphasic linear gradient of ACN in 0.1% formic acid in water (FA, v/v, LC-MS grade, Carlo-Erba Reagents, Val de Reuil, France). A multistep gradient of 155 min started at 2% B for 6 min, reaching 35% B in 120 min; then from 35% to 70% B in 5 min, followed by a plateau for 5 min was used. NanoLC-

MS/MS datasets were acquired in a positive and data-dependent mode, using the *m/z* range from 600 to 18,000. The acquired MS/MS datafiles were processed by Proteome Discoverer v3.0 (Thermo Fisher Scientific) for identification and LFQ Protein identification was done against a protein database totalling 4,352,898 entries from Hymenoptera and the relevant pathogens downloaded from Uniprot and NCBI. The protein quantification was calculated using the summed abundance with subsequent ANOVA tests. The minimum trace length value was set to 5 and the maximum retention time shift of isotope pattern was equal to 0.2 min. Proteins with a ratio < 0.5 (down regulation) and > 2 (up regulation) were considered as significant along with a p-value < 0.05.

2.6. Gene ontology annotation and biological pathway analysis

For functional annotation of the sequences generated from the LC-ESI-MS/MS analyses, the bioinformatic solution OmicBox software v2.2.4, functional analysis module Blast2Go (https://www.biobam.com) was used based on our method published in (Askri et al., 2023; Bournonville et al., 2023). Briefly, to get the most complete annotation labels, the analyses were performed using the four cloud-powered algorithms (Blast, InterProScan, GO Mapping, GO slim). Separate lists of dysregulated proteins in each of the experiments (semi-field and laboratory) were loaded to investigate the biological pathways and the protein functions following bee exposure to the different stressors. Combined pathway analysis was performed on the annotated sequences (proteins) joining Reactome and KEGG to identify enriched pathways with expression profiles.

3. Multiscale analysis by MALDI BeeTyping® and LFQ Off-gel Bottom-up proteomics of *Osmia bicornis* haemolymph samples

The data obtained in this holistic experiment from laboratory-controlled conditions, through a proof of concept in semi-field experiments, and finally on sets of samples collected in the field will be illustrated through the examples discussed below.

3.1. MALDI BeeTyping® outcomes of the *Osmia bicornis* haemolymph analysis

3.1.1. Pesticide exposure and pathogen impact *O. bicornis* in laboratory experiments

The first level of the investigations carried out on the impact of stressors on *O. bicornis* haemolymph and therefore their health status was laboratory experiments-based. Three pesticides (active ingredients (sulfoxaflor, azoxystrobin, and glyphosate) were tested in these experiments. We observed a difference of impact on the bee haemolymph composition between sulfoxaflor (3.125 ng/bee) or azoxystrobin (40 µg/bee) and the controls when applied separately. In our study, no discrimination was visible between the haemolymph of bees exposed to glyphosate (100 µg/bee) versus non-exposed bees (Figure 3).

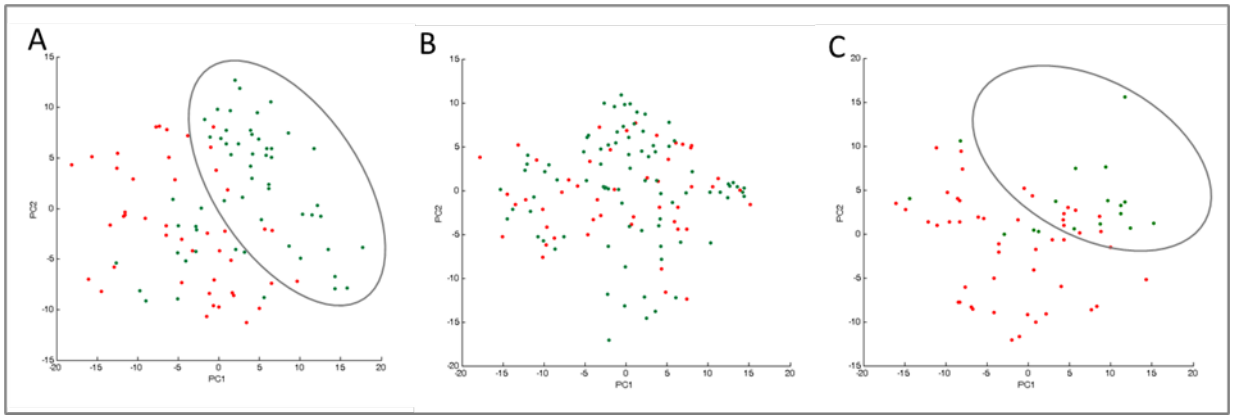

Figure 3: Impact of the pesticides azoxystrobin (A), glyphosate (B) and sulfoxaflor (C) on *Osmia bicornis* haemolymph MFPs. Each dot represents a spectrum recorded from an individual haemolymph sample. PCAs were generated using ClinProTools™.

Following azoxystrobin exposure, the bees expressed the highest number of modulated molecular ions (MMIs) (53.30%) compared to glyphosate (14.65%) and sulfoxaflor (9.87%) compared to controls. Even between the pesticides, azoxystrobin showed high percentages of MMIs close to 50% when compared to sulfoxaflor or glyphosate. Regarding the Apidaecin or AMCI variations, only the azoxystrobin induced the up-regulation of Apidaecin compared to controls (*m/z* 1936.71, p=0.0156) and to sulfoxaflor (*m/z* 1936.82, p=0.0416). However, no variation of AMCI (*Apis mellifera* chymotrypsin inhibitor) was observed when we compared each of the pesticides to the control. Interestingly, the AMCI varied significantly between the haemolymph collected from treated solitary wild bees (i.e, when comparing pesticides to each other, $p < 0.05$). Sulfoxaflor and azoxystrobin have been shown to disrupt bee health in various studies (Al Naggar et al., 2022; Christen et al., 2019; Linguadoca et al., 2022; Serra et al., 2023; Tamburini, Pereira-Peixoto, et al., 2021). In contrast, Tamburini et al 2021a reported no major impacts on bees as Benbrook reported for glyphosate (Benbrook, 2016). However, glyphosate has been shown to affect bee behaviour and physiology (Vázquez, Balbuena, et al., 2020; Vázquez, Latorre-Estivalis, et al., 2020).

3.1.2. Pesticide exposure impact *O. bicornis* in semi-field experiments

The aim of the study in semi-field carried out by 32-WBF-Agroscope partner was to study the impact of the pesticides Closer®, Amistar® and the mix on *O. bicornis* on purple tansy (*Phacelia tanacetifolia*, PHA). The results showed that only azoxystrobin-based formulation has an impact on *Osmia* haemolymph compared to controls when the bees are foraging on PHA. This effect was observed when we compared the control samples to the set of samples without considering the sex of the bees and for the females. No impact of Amistar® was observed on the bee's haemolymph from males (Figure 4).

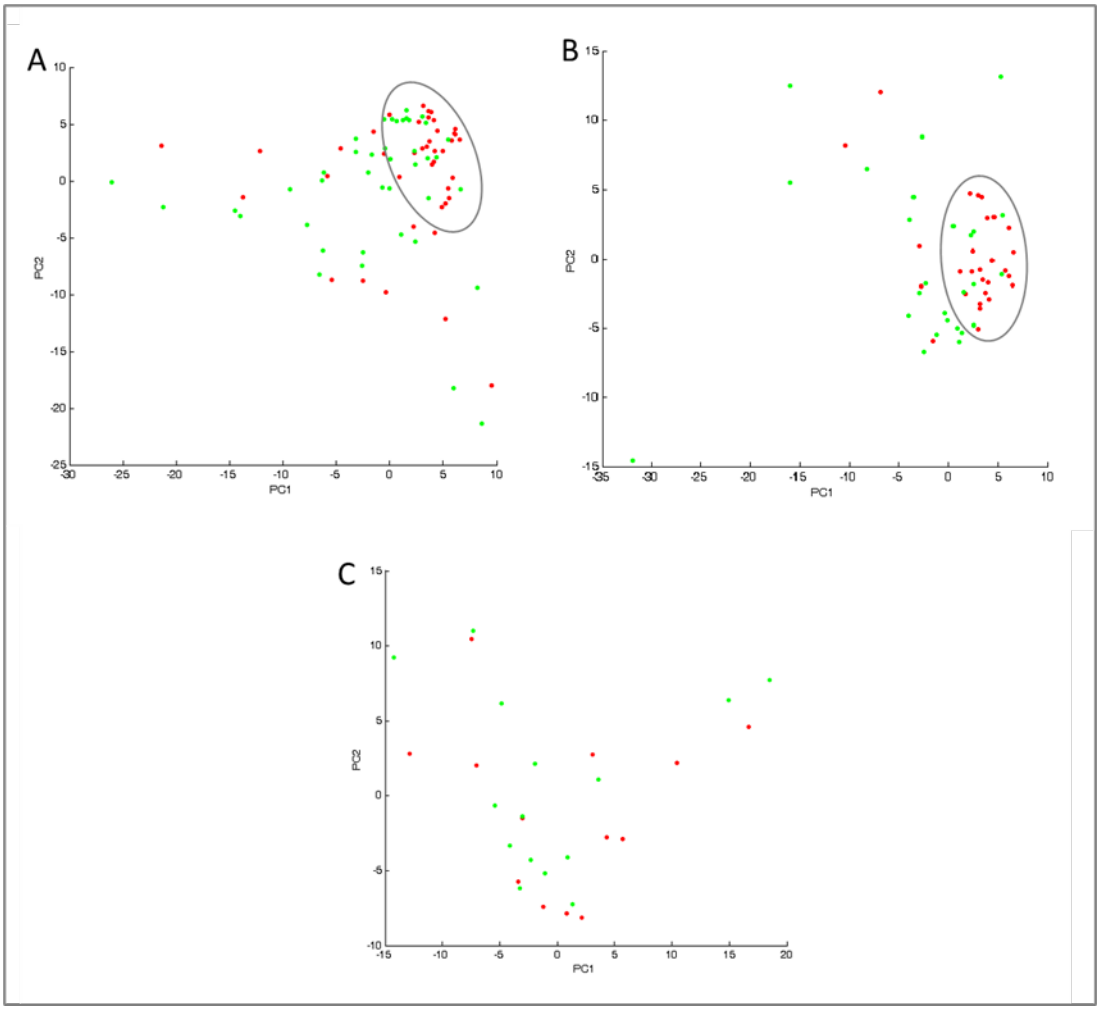

Figure 4: Impact of Amistar® on the haemolymph composition of *O. bicornis* foraging on *Phacelia tanacetifolia*. Males and females (A), females (B), and males (C). Each dot represents a spectrum recorded from an individual haemolymph sample. PCAs were generated using ClinProTools™.

Importantly, no MMIs were detected in the other pairwise comparisons except for those mentioned above. Regarding the peptides Apidaecin and AMCI, only the AMCI was found to be up-regulated following azoxystrobin treatment (*m/z* 6,169.05, p= 0.025).

The Agroscope team published the biological impacts on the *O. bicornis* experiments in relationship with our data (Schwarz et al., 2022). The researchers reported no significant negative effects of the single and combined exposure to sulfoxaflor and azoxystrobin on *O. bicornis* survival, reproduction, offspring mortality, size and sex ratio in the semi-field experiment. Similarly, semi-field experiments on bumble bees (Tamburini, Wintermantel, et al., 2021) reported no significant effects of azoxystrobin on bee colony development or foraging activity. However, the proteomic studies showed significant impact of azoxystrobin on females' MFPs and more precisely on the AMCI. This supports the need to combine different metrics such as behaviour, physiological parameters and molecular data to understand the full impact of stressors.

3.1.3. MALDI BeeTyping® showed differences between the *Osmia* sample sets collected from the field experiments

The results obtained on the haemolymph samples collected from field experiments showed a discrimination between bees reared in Germany (GER) and those in Italy (ITA) (Figure 5). Moreover, when we compared the two crops (OSR vs APP) in each of the countries, we observed a discrimination between OSR and APP in GER and not in ITA. We also compared the same crop between both countries, and only the APP crop was distinct from the others.

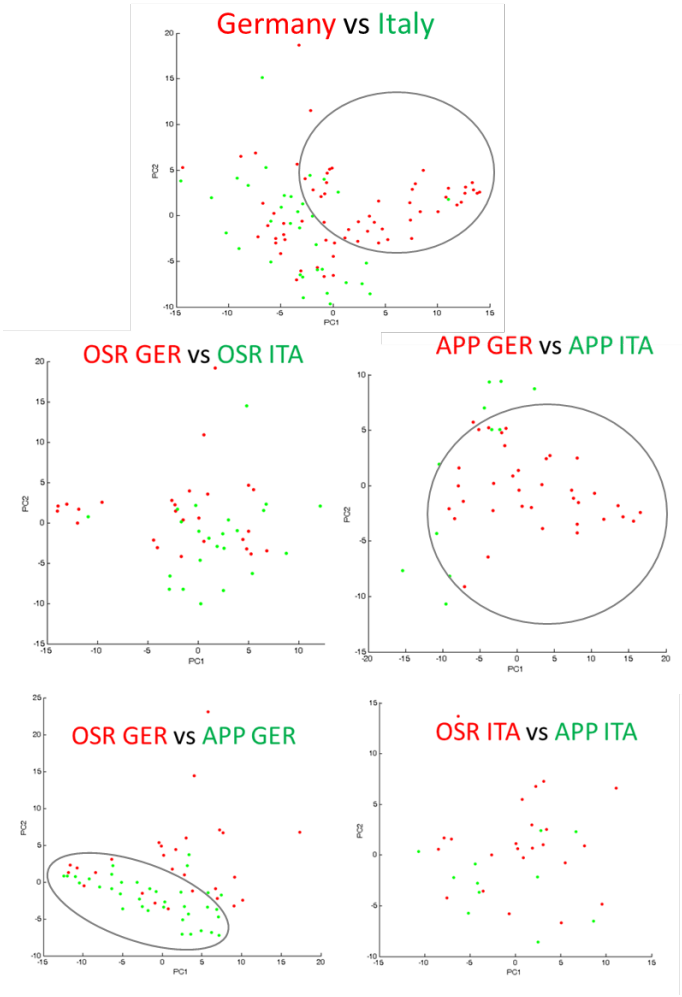

Figure 5: PCAs presenting the country or crop impact on haemolymph molecular mass fingerprints (MFPs) signatures (spectral distribution) between GER Germany and ITA Italy or oilseed rapes (OSR) and apples (APP). Each dot represents a spectrum recorded from an individual haemolymph sample. PCAs were generated using ClinProTools™.

3.2. Proteomics outcomes of the *O. bicornis* haemolymph analysis in the context of semi-field experiments

These MALDI-MS analyses revealed the impact of pesticide alone or in combination with a second stressor such as nutrition or pathogen on the molecular composition of solitary bee haemolymph, particularly on certain immune peptides and proteins associated to immunity (e.g., vitellogenin, major royal jelly, defensin). Unfortunately, the use of this technique limits the analysis and interpretation of

such results. As a matter of fact, in our parameter settings, MALDI BeeTyping® is not the most appropriate method for detecting proteins over 12-15 kDa. This is why we performed an off-gel bottom-up proteomic analysis by high-performance liquid chromatography coupled to high-resolution tandem electrospray mass spectrometry (LC-ESI-MS/MS).

From these experiments, herein, we report the results on the *Osmia* samples generated from the semi-field experiments testing the impact of sulfoxaflor, azoxystrobin formulations (Closer® and Amistar® respectively) and the mix on the purple tansy plant, performed by partner 32-WBF-Agroscope. Herein, we studied the pesticide-nutrition interaction on the *O. bicornis* haemoproteome. The number of the identified, quantified and dysregulated proteins is presented in figure 6.

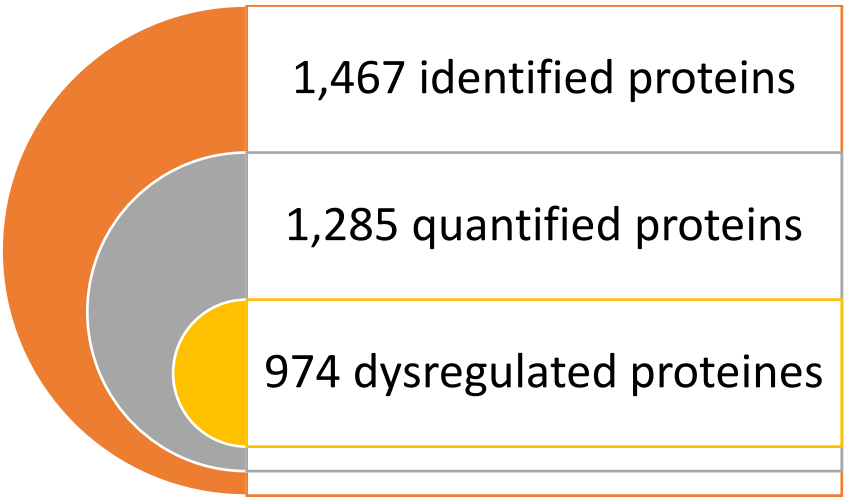

Figure 6: Number of proteins detected from the label-free quantification (LFQ) by LC-ESI-MS/MS.

The details in the protein variation are provided in table 2.

Table 2: Number of significant, up and down-regulated proteins in the semi-field experiment on purple tansy testing pesticide and sex impact.

Experimental conditions	**Type of regulation**		
	Significant	**Up**	**Down**
Female Amistar® / Female control	401	172	229
Female Closer® / Female control	306	148	114
Female mix / Female control	307	196	158
Male Amistar® / Male control	276	90	186
Male Closer® / Male control	257	116	141
Male mix / Male control	242	86	116
Female control / Male control	569	253	316
Female Amistar® / Male Amistar®	543	226	317
Female Closer® / Male sulfoxaflor	399	187	212
Female mix / Male Mix	556	291	265

From these data, we observed that *O. bicornis* females are more impacted than males. Indeed, the number of significantly dysregulated proteins is higher in females compared to males in the three pesticide exposures azoxystrobin, sulfoxaflor and the mix. For both, the azoxystrobin showed the highest impact.

The comparison between males and females in each condition identified various impacted proteins.

The 1,285 dysregulated proteins were interrogated using OmicsBox software to study their biological processes and molecular functions to identify altered pathways and potential markers that could be impacted by bee exposure to the stressors.

4. Conclusions and Perspectives

The assessment of the effects of different stressors (alone or in combination) was evaluated on the solitary bee *Osmia bicornis*, by two complementary mass spectrometry methods, MALDI BeeTyping® and Off-gel bottom-up proteomics. Aiming to validate the applicability of these two techniques at different levels, we collected haemolymph samples from solitary bees treated by different stressors in controlled-laboratory conditions, before collecting information from haemolymph samples from a semi-field environment and finally from field experiments where bees are existing in their habitat. Through the data presented in this manuscript, our results established that MALDI BeeTyping® and Off-gel bottom-up proteomics present powerful tools to monitor stressor impacts on *O. bicornis* and specially to identify impacts that were not detected by classic biological tests such as flower visiting, behaviour or survival rates. Moreover, effects of pesticides such as azoxystrobin were varied between bee sex.

5. Associated information

A manuscript presenting this work and including all *O. bicornis* experiments will be submitted shortly. The title is "**Mass spectrometry reveals stressor impact on *Osmia bicornis* haemolymph composition under laboratory, semi-field and field conditions**" with the proposed authors: Dalel Askri, Karim Arafah, Sébastien N. Voisin, Janine M. Schwarz, Anina Knauer, Matthias Albrecht, Sara Hellström, Robert J. Paxton, WP1 partners from Germany and Italy, Michel Bocquet, and Philippe Bulet. In addition, the list of proteins identified through this work have been included in the OSMDBase-1.0 which lists *Osmia* proteins identified during the proteomics analysis of the bee haemolymph samples. OSMDBase-1.0 will be made public by end of May 2023. OSMDBase-1.0 is intended to be mined by any researcher looking for specific proteins or interested in cross-referencing their findings with ours. OSMDBase-1.0 will be further implemented and updated as more sample analyses are still ongoing by 10-BIOP.

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