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A blood test to monitor bee health across a European network of agricultural sites of different land-use by MALDI BeeTyping mass spectrometry

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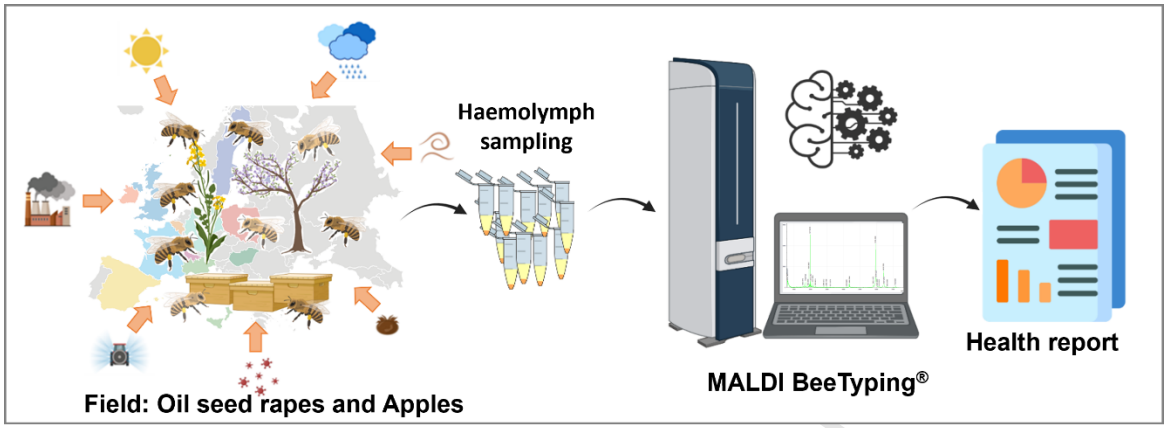
Abstract

There are substantial concerns about impaired honey bee health and colony losses due to several poorly understood factors. We used MALDI profiling (MALDI BeeTyping®) analysis to investigate how some environmental and management factors were related to the haemolymph peptidome (all peptides in the circulatory fluid), which reflects the immune status of *Apis mellifera*, under field conditions across Europe. Honey bees were exposed to varying environmental stressors across eight European countries totalling 128 agricultural sites, reflecting two different crop systems [oilseed rape (OSR) and apple (APP)]. Molecular signatures of haemolymph and the presence/absence of molecular-related ions of three immunity markers, namely the antimicrobial peptides (AMP) Apidaecin, Abaecin and Defensin-1, allowed discrimination of bee responses by country, crop type and site. However, many sites showed no significant signature related to the presence of AMP markers. Conversely, in Sweden (SWE), molecular ion intensities were very high, including those of the AMP markers. Even the lowest values were always higher than in other countries. Furthermore, all experimental sites in SWE expressed AMPs. A machine learning model was developed to discriminate the haemolymphs of bees from APP and OSR sites. The model was 90.6% accurate in identifying the crop type from the samples used to build the model. Overall, MALDI BeeTyping® on individual bee haemolymph represents an attractive and promising “blood test” for monitoring the impact of stressors on bee health at the landscape scale, thus providing policymakers with new monitoring and regulatory tools.

Key words: *Apis mellifera*; environment; immunity; MALDI profiling, field study

Graphical abstract:



1. Introduction

Honey bees are among the globally pollinators of wide range of plants (Hung et al., 2018; Klein et al., 2007; Ollerton et al., 2011). Thus, they are essential to human wellbeing. Declining bee health and losses of wild bee population and honey bee colonies is a major concern. Various factors including pesticides, pathogens (parasites and viruses), loss of adequate habitats and floral resources, climate change and beekeeping practices (hive maintenance and treatment) have been identified as potential drivers but their interplay is still poorly understood (Dicks et al., 2021). This has prompted researchers around the world to study their impact on bee health. These factors include pesticides, pathogens (parasites and viruses), the presence of toxic plants, the loss of (semi-) natural habitats and habitat diversity, climate change, and beekeeping practices (hive maintenance and treatment) (Lämsä et al., 2018; Dicks et al. 2022). It is therefore important to assess the impact of different factors on bee health at multiple scales, i.e. from the local spatial scale to landscape and country scale, and from the individual honey bee to the hive.

Monitoring bee health under specific environmental conditions could be a valuable strategy for measuring environmental impact. To assess the risk of bee exposure to stressors at the colony level, various bee products such as honey, beeswax, nectar, bee bread, propolis, royal jelly and pollen are considered to assess the environmental impact on bees (Căuia et al., 2020; Chauzat et al., 2011). Pesticides are found in bee products (Dolezal, 2022; Knapp et al., 2023; Ko et al., 2017; Sanchez-Bayo et al., 2016). The environmental DNA (eDNA) has been reported as a promising tool to monitor bee health as well (Boardman et al., 2023; Ribani et al., 2022).

In animal and human care, a blood test is usually prescribed to check how an organism is coping with, for example, an infection, medication or pathology. If the blood test results are abnormal, they may provide clues as to how to treat or prevent future disorders. Similar to blood in vertebrates, insect haemolymph is one of the indicators of the invertebrate's physiological status that could be used, for example, to monitor the immune status of an insect. This has been extensively documented for the insect model species *Drosophila melanogaster* (Huang et al., 2023; Kounatidis & Ligoxygakis, 2012; R. Xu et al., 2023; Yu et al., 2022), and subsequently in *A. mellifera* (Arafah et al., 2019).

Indeed, the insect haemolymph plays an important role in immune defence, thanks to the shield of circulating antimicrobial peptides (AMPs), among other immune effectors (Clark, 2020; Larsen et al., 2019). Several abiotic and biotic stressors can disrupt the immune system of honey bees (Brutscher et al., 2015). Downregulation of immune gene expression following infestation by the introduce mite *Varroa* sp. has been reported (Fang et al., 2022; Marche et al., 2019; Tesovnik et al., 2017; Zhang et al., 2010) as well as changes in the immune-proteome (Erban, Sopko, Kadlikova, et al., 2019; Erban, Sopko, Talacko, et al., 2019; Słowińska et al., 2019; Surlis et al., 2018).

Climate has also an impact on floral resource availability and choice of forage plants, and thus bee products. For example, a dry climate could reduce nectar and pollen production (Phillips et al., 2018), while rain could reduce the attractiveness of certain flowers to bees. Reduced pollen supply can weaken the immune system of bees, making them susceptible to pathogens, which can ultimately lead to increased winter losses on bee decline (Le Conte & Navajas, 2008). As noted by (Butolo et al., 2020), studies evaluating the effects of stressors on haemolymph are scarce due to the difficulty of extracting pure haemolymph samples that are not contaminated by other tissues or liquids.

To properly assess the impact of a stressor on the health of *A. mellifera*, Arafah and colleagues developed a mass spectrometry-based approach called MALDI BeeTyping® from an individual “blood test”/ “haemolymph test” (Arafah et al., 2019). Indeed, MALDI BeeTyping® demonstrated that individual molecular mass fingerprints (MFPs) of bee haemolymph can be analysed, and used to monitor the impact of biotic/abiotic stressors such as bacteria (Arafah et al., 2019; Bournonville et al., 2023), spores of *Nosema* (Houdelet et al, 2021; Chantaphanwattana et al, 2023), and a combination of *Crithidia* and pesticides (Askri et al., 2023).

However, to date, no study has yet assessed the impact of geographical environment with respect to different cropping systems or different land-use intensities reflecting distinct levels of pesticide use or degrees of landscape simplification, on honey bee health.

In this study, we applied MALDI BeeTyping® on bees collected in two agricultural crop types across Europe (8 countries, 2 crops, 128 sites) (Hodge et al., 2022), focusing on several potential immune markers as discriminating molecules (Askri et al., 2023; Bournonville et al., 2023). Our analyses were performed in blind conditions regardless the honey bee

exposure to the crop/orchard treatments in order to evaluate the molecular profiles of haemolymph in their environment. As part of the immune response, insects secrete a series of short antimicrobial peptides (AMPs) into their haemolymph to defend themselves against various stressors including pathogens (e.g., viruses, bacteria, fungi, and parasites) (Goulson et al., 2008, 2015). *A. mellifera* has its own arsenal of AMPs with Apidaecins, Abaecin, Defensins and Hymenoptaecin (Casteels et al., 1994; Evans et al., 2006; Kwong et al., 2017). Due to their physico-chemical properties (highly cationic), the ionisation power of such AMPs allows their detection by MALDI mass spectrometry in a linear positive detection mode. In this study, we investigated whether environmental variation can influence the profiling of *A. mellifera* haemolymph, focusing on the immune peptides Apidaecin, Abaecin and Defensin-1. Our results show that the MALDI BeeTyping® is a useful tool for distinguishing bee signatures based on their haemolymph molecular profiles and immune status across sites characterised by natural ranges environmental variation along gradients of land-use intensity across European agricultural landscapes. To our knowledge, this is the first study reporting successful application of the MALDI BeeTyping® technique to screen molecular variations including AMPs in the haemolymph of honey bees in field realistic environments.

2. Materials and Methods

2.1. Bee sampling across the European site network

The study was carried out as part of the PoshBee project (https://poshbee.eu/). The overall site network design and sampling scheme is described in detail by Hodge et al. (2022). The field sites were spread over eight countries, namely: Estonia (EST), Germany (GER), Great Britain (GBR), Ireland (IRL), Italy (ITA), Spain (ESP), Switzerland (CHE), and Sweden (SWE). These countries were selected to cover four major European biogeographical areas (atlantic, boreal, continental, and mediterranean). Eight sites of each of two crops, oilseed rape (OSR) and apple (APP), were selected per country (Hodge et al., 2022). Both APP and OSR flowers are valuable sources for honey bees, attractive for nectar and with protein-rich pollen, and could be considered among the main sources used by colonies in the study sites. For each site, the landscape was defined along a gradient of land-use intensity within a 1 km radius of the centre of the site and a minimum distance of 3 km between the sites (Bottero et al., 2023; Hodge et al., 2022). Three hives were introduced to the landscape one week before crop flowering at each sampling site according to PoshBee field protocols standardised for the eight countries of the study (Hodge et al., 2022). Each of the hives was placed at least 5 m apart to avoid interference. *Apis mellifera* colonies were provided by local suppliers. Colony strength was measured for the selection of the hives to ensure that all colonies had similar features (number of workers, absence of illnesses, etc). Forager honey bees were selected for haemolymph sampling.

2.2. Haemolymph collection and storage

A minimum of five foraging *Apis mellifera* individuals were sampled from each hive. A total of 2,018 individual haemolymph samples were collected and analysed (Table S1). The haemolymph collection protocol was based on the method established by (Arafah et al., 2019) and a training workshop organised for all partners prior field sampling. Briefly, haemolymph was obtained using a customised collection kit consisting of a pulled glass capillary (Sutter Instrument Corp, Model P-30, Novato, California) which was inserted dorsally under the second tergum of the abdomen of the worker honey bee, and the haemolymph collected by capillary action. The collected haemolymph was then transferred to a chilled LoBind Protein microtube (Eppendorf, Germany) precoated with phenylthiourea (PTU) and phenylmethylsulfonyl fluoride (PMSF) (both from Sigma–Aldrich, France) to

prevent melanisation and proteolysis, respectively. After collection, haemolymph samples were stored at -20°C until shipment to the analytical platform BioPark Archamps, where the samples were centrally analysed. Upon arrival, they were stored at -20°C until analysis.

2.3. Sample preparation for MALDI BeeTyping®

Each haemolymph sample was analysed using a MALDI AutoFlex III Smartbeam® instrument (Bruker Daltonics, Germany) following (Arafah et al., 2019). Molecular mass fingerprints (MFPs) were obtained according to the Bruker Biotyper recommendations (matrix, method of sample deposition and detection) with minor adjustments. Briefly, haemolymph samples were diluted 1:100 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 alpha cyano- 4-hydroxycinnamic acid MALDI matrix (4-HCCA, Sigma-Aldrich). Mass Spectrometry (MS) spectra were acquired in automatic positive linear mode using FlexControl 4.0 software (Bruker Daltonics, Germany). Each bee haemolymph sample was spotted in triplicate with three MALDI-MS readings each, totalling nine spectra per individual bee.

2.4. MALDI BeeTyping® acquisition

For MS spectra acquisition, the instrument was set up with the following parameters: 1.5 kV of electric potential difference, a dynamic range of detection of 600-18,000 in *m/z*, 40% of laser power, a global attenuator offset of 60% 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 standard mixture of peptides and proteins (Peptide Standard Calibration II and Protein Standard Calibration I, Bruker Daltonics, Germany) and APISCAL. The latter is an in-house calibration solution composed of two antimicrobial peptides (AMPs) from *A. mellifera*, namely Apidaecin (average molecular ion at *m/z* 2,109) and Abaecin (average molecular ion at *m/z* 3,879), along with Melittin (average molecular ion at *m/z* 2,847), the major venom component, and the recombinant ETD151 (average molecular ion at *m/z* 4,839). 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.

2.5. Data processing and statistical analyses

MALDI-MS datasets were imported and analysed in ClinProTools™ 2.2 Software (Bruker Daltonics) for post-processing and statistical analyses (ion distributions and modulated molecular ions). Baseline subtraction and spectral smoothing were applied to all acquired spectra. All spectra were averaged using a signal-to-noise ratio of 3 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).

In parallel, FlexAnalysis 3.4 (Bruker Daltonics, Germany) was used to extract peak lists from each MALDI-MS dataset and the molecular-related ions corresponding to the characterised immune AMPs of *A. mellifera*: Apidaecin, Abaecin and Defensin-1 (average molecular ion at *m/z* 5,520). Different comparisons were made between (i) the countries where the experiments were conducted, (ii) local geographical sites where the bees were collected and (iii) the type of crops (APP, OSR) at local sites. Using the statistical software R version 4.0.5. and the R studio extension, comparisons of peak intensities were made using Kruskal-Wallis and Dunn post-hoc tests. Contingency tables and $\chi 2$ tests of independence were used for the presence of immune peptides.

2.6. Machine Learning model development

ClinProTools™ 2.2 Software (Bruker Daltonics) was used to develop a Machine Learning-based Model. After selecting the best discriminant peaks, the software evaluates the ability of the model to discriminate the molecular signatures of the haemolymph based on the mass spectra according to the environmental conditions. In addition, a cross-validation step is performed to randomly classify the molecular signatures and to evaluate the positively classified spectra with the corresponding environmental condition. Cross-validation measures the reliability of a calculated model and can be used to predict how a model will behave in the future. Finally, the generated model was validated through an external validation step, which consisted of matching spectra that were not included in the model (for more details see Arafah et al., 2019). We selected the molecular datasets from the countries that showed the best discrimination between OSR and APP by PCA analysis, and the Genetic Algorithm (GA) was applied to determine the ion peaks' combinations relevant for sample separation. The raw mass spectra (referred to as MFPs) were baseline corrected using the Top Hat baseline algorithm (minimum baseline width of 10%) and smoothed using the Savitzky-Golay algorithm (window size 2.0 *m/z* in 5 cycles). The total

average spectra were calculated using a signal-to-noise threshold of 3 for peak selection, a picking height of 80 and baseline application. Peak lists (maximum peak number of 100) of each spectrum were extracted for data processing and statistical analyses. Comparative analyses were performed between the different experimental conditions according to the intensity of the selected peaks. The software normalised the spectra before performing statistical PCA. A Data Reduction Factor of 20 and a range of 700-18,000 *m/z* were used without Null Spectra Exclusion but with exclusion of non recalibratable spectra. The Machine Learning model was then run with the GA, with a maximum of 25 peaks and 100 generations. The other parameters were set to default values (mutation rate: 0.2; crossover rate: 0.5; number of neighbours: 5; leave out: 20%, number of iterations: 10). For external data validation, we used countries that were not clearly differentiated in the PCA analyses.

3. Results

Molecular mass fingerprint (MFP) analyses were performed by MALDI BeeTyping® on *Apis mellifera* haemolymph collected from eight different countries (Estonia, EST; Germany, GER; Great Britain, GBR; Ireland, IRL; Italy, ITA; Spain, ESP; Switzerland, CHE; and Sweden, SWE) and two crops (oilseed rape, OSR or apple, APP). Data acquired were analysed by PCA and completed on variations between countries crops and sites before building Machine Learning-based models.

3.1. MFPs variation by country, crop and site of haemolymph composition

Using the software ClinProTools™, we observed variations in honey bee haemolymph composition between the two crops, among the eight countries, and among sites within countries (Fig. 1). In most countries, individual variability was observed in haemolymph samples collected from bees at OSR or APP sites. Conversely, there was no strong variability within individuals foraging on APP or OSR in the samples collected in Italy. In addition, the MALDI BeeTyping® analyses of the haemolymph samples revealed MFPs harbouring similar variabilities within individuals following PCA. This result suggests that no measurable impact was recorded in Italy based on OSR and APP factors. Interestingly, the different haemolymph spectra recorded on the Swedish samples allowed to distinguish bees from APP compared to those from OSR sites, although more individual variations were observed within a single crop than in Italy.

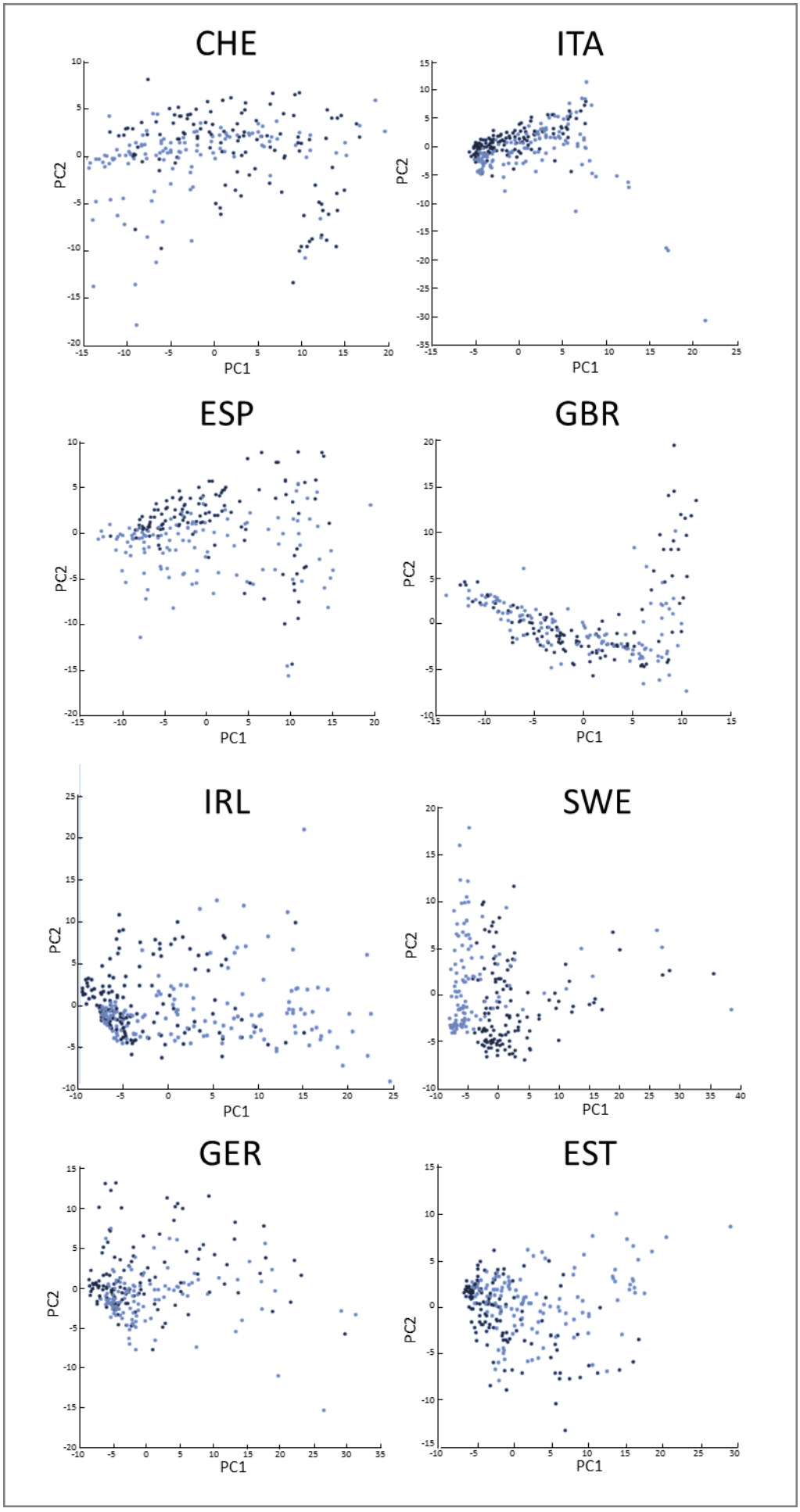
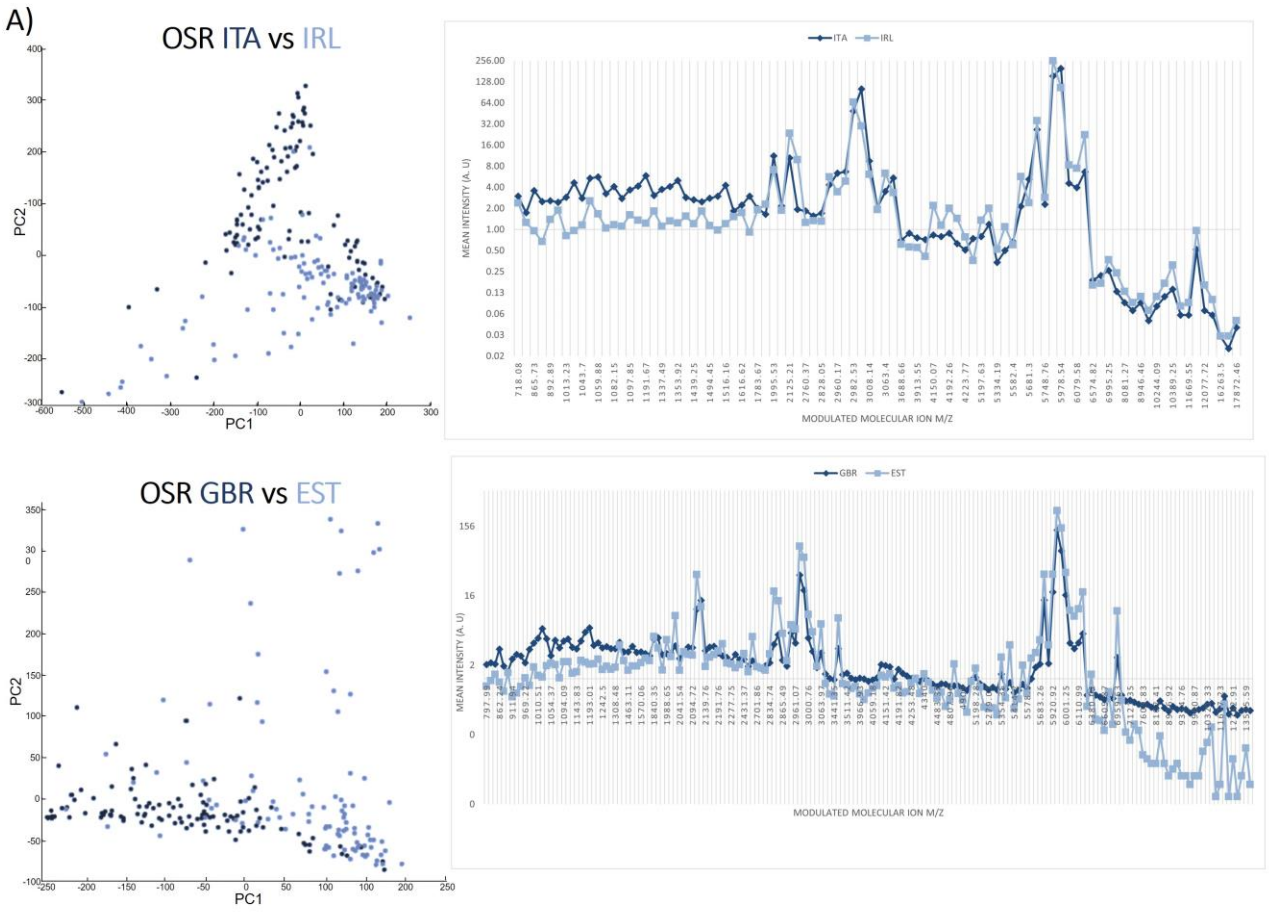

CHE
ITA
ESP
GBR
IRL
SWE
GER
EST
PC1
PC2

Fig. 1. Principal component analysis presenting the crop system impact on haemolymph molecular mass fingerprints (MFPs) signatures (spectral repartition) between oilseed rape (OSR in dark blue) and apple (APP in light blue) in each of the eight European countries studied. PC1 and PC2 explained cumulatively about 40% of the variance. CHE Switzerland, ITA Italy, ESP Spain, GBR Great Britain, IRL Ireland, SWE Sweden, GER Germany, and EST Estonia. Each dot represents one MFP spectrum recorded from one individual haemolymph sample.

In addition, we performed pairwise comparisons for all possible country combinations for OSR and for APP to study the country-crop impact on the molecular signatures (MFPs) of bee haemolymph. In these comparisons, we focused on the modulated molecular ions (MMIs) showed by the discriminating MFPs of haemolymph spectra (Tables S2, S3). For OSR, a minimal percentage of MMIs (42.33 %) was found between OSR ITA and OSR IRL. The maximum percentage (96.24%) discriminated OSR GBR from OSR EST. For APP, the lowest percentage was 35.59% between APP CHE vs ESP and the highest 95.83% between APP IRL vs SWE. The corresponding PCAs for these four comparisons and the distributions of MMIs were shown in Fig.2 (Fig.2A OSR and Fig. 2B APP) (see also Tables S2, S3).



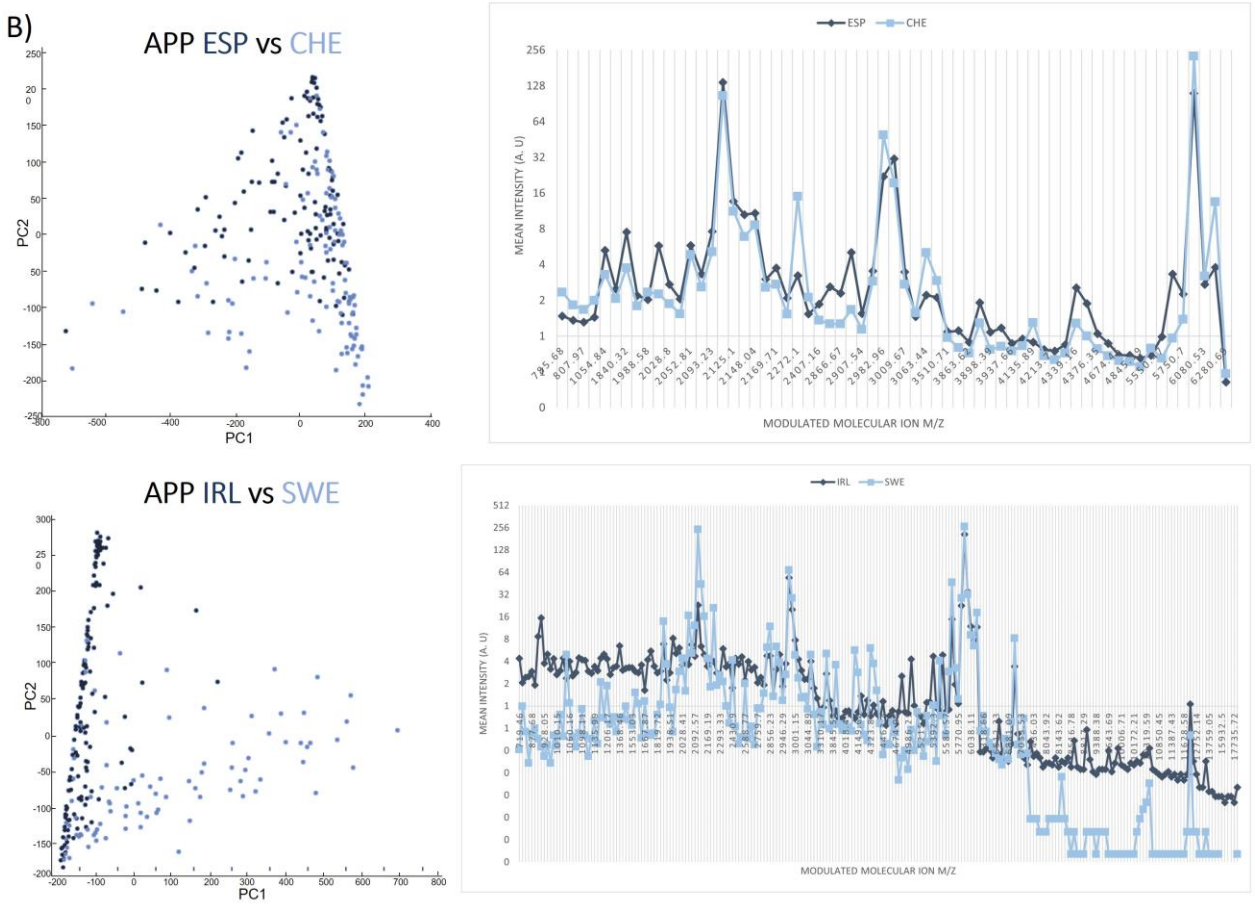


Fig. 2. Principal component analysis of individuals (left graphs) with the lowest and highest modulated molecular ions (MMIs, right graphs) and their corresponding distribution (Log2-transformed) in OSR (A) and APP (B). ITA Italy, IRL Ireland, GBR Great Britain, EST Estonia, ESP Spain, CHE Switzerland, and SWE Sweden. Each point represents one haemolymph MFP from an individual bee.

3.2. Machine Learning-based models to differentiate MFPs from OSR and APP

3.2.1. List of ions selected for model building

In this section, we developed a machine learning-based model to test whether we were able to discriminate the MFPs bee spectra according to the floral conditions in the landscape around the sampled honey bee colonies. The ML-based model selected the following list of ions (Table S4).

3.2.2. Results with internal data

In the model generation set, the global recognition capability of the model reached 90.6% with 94.04% for APP and 89.15% for OSR. For the data set test, the cross-validation process left out 20% of the spectra and performed 10 iterations. The overall recognition

was 76.78%, 79.92% for APP and 73.65% for OSR. As the foraging area of each site may contain both APP and OSR crops, we observed a large variability in the percentage of bees classified in each of the ML-model categories, from less than 10% to 100% of bees recognised in the correct model category.

3.2.3. Results with external data

For external data validation, we used the countries that did not show a clear distinction between APP and OSR sites in the previous PCA analyses: Estonia (EST), Italy (ITA), Germany (GER), Spain (ESP) and Great Britain (GBR). In terms of APP recognition, ESP and GBR showed high levels of success, while ITA differed greatly between sites, and EST and GER showed a rather low model efficiency. For OSR, the results varied greatly between sites, the best results were found in GER and ITA (Fig. 3).

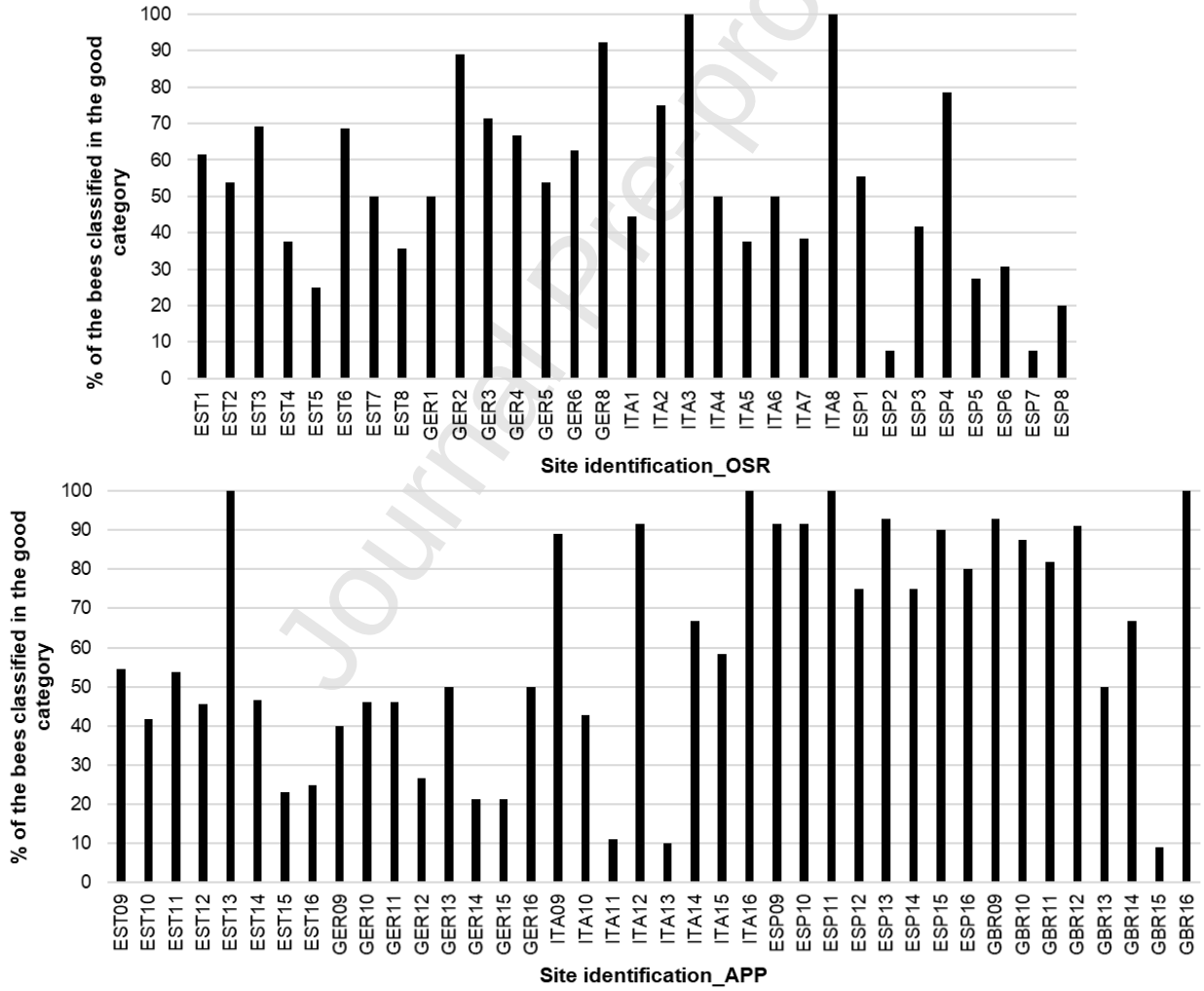


Fig. 3. Percentage of bees classified in the correct category at each site for OSR (top) and APP (bottom). Estonia (EST), Italy (ITA), Germany (GER), Spain (ESP) and Great Britain (GBR)

To explain the variability of the results, we crossed our results with the surface of each crop collected at the different sites (1km radius sectors). No clear information could be obtained for the APP results in this cross, as the orchard area of APP was generally limited to a few hectares. For OSR, however, we found a different country profile in terms of cultivated areas in the sites with lower values in ITA and ESP, and with a larger gradient in GER and EST. In all cases, there was no clear correlation between the crop area and the proportion of correctly classified bees, except for a weak positive correlation in ESP, but with a low recognition rate (Fig. 4).

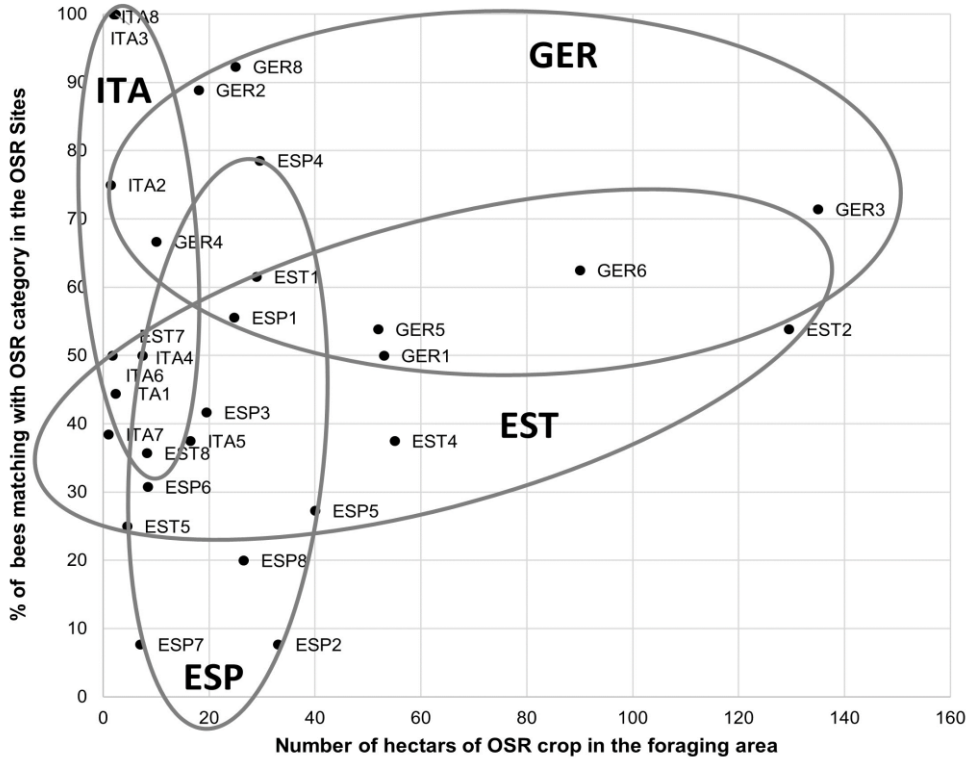


Fig. 4. Percentage of bees matching with the OSR category in the different OSR sites, identified by the specific country codes (Estonia, EST; Italy, ITA; Germany, GER) and site ID (i.e. ITA2, GER6, EST4).

The date of collection of the haemolymph sample during the flowering period influenced the profile, in line with the fact that the number of flowers generally varies considerably during the flowering period at a study site (Fig. 5).

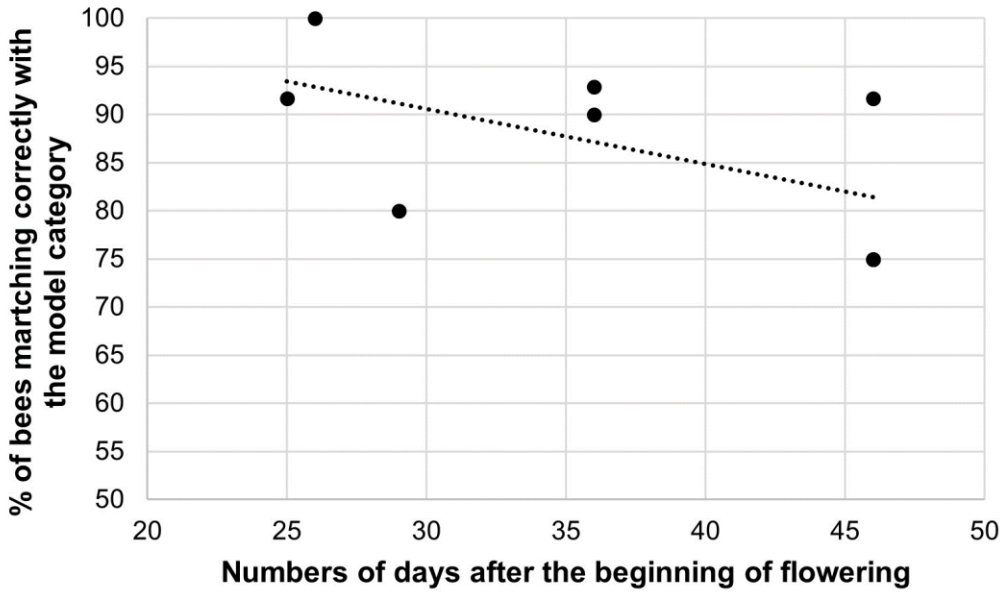


Fig. 5. Percentage of correct recognition of the site condition depending on the date after the start of flowering, in the case of Spain with Apple crop. Each point represents the average percentage of recognition for each site.

3.3. Impact of the country/crop/site on the expression of AMPs-based immunity in Apis mellifera

Using the MALDI BeeTyping® approach, we were able to distinguish between countries, crops and sites based on the MFP analyses. The detected differences could be related to the presence/absence of the immune peptides of interest in the haemolymph of bees from these sites and/or their mean peak intensities.

3.3.1. Specific AMP variations by country

The Apidaecin antimicrobial peptide was present in more than 50% of bees in each of the eight countries. The percentages ranged from 57.4% of bees in CHE to 97.1% in SWE (Fig. 6). Furthermore, the peak intensities of Apidaecin (max 50,000 arbitrary units) were much higher than those of Abaecin (maximum of 1,200 a.u.). This difference was found significant (p-value <0.001) and observed as well in CHE and SWE with intensities of 959.7 and 26,883.6 respectively (p-value <0.001) (Fig. 6).

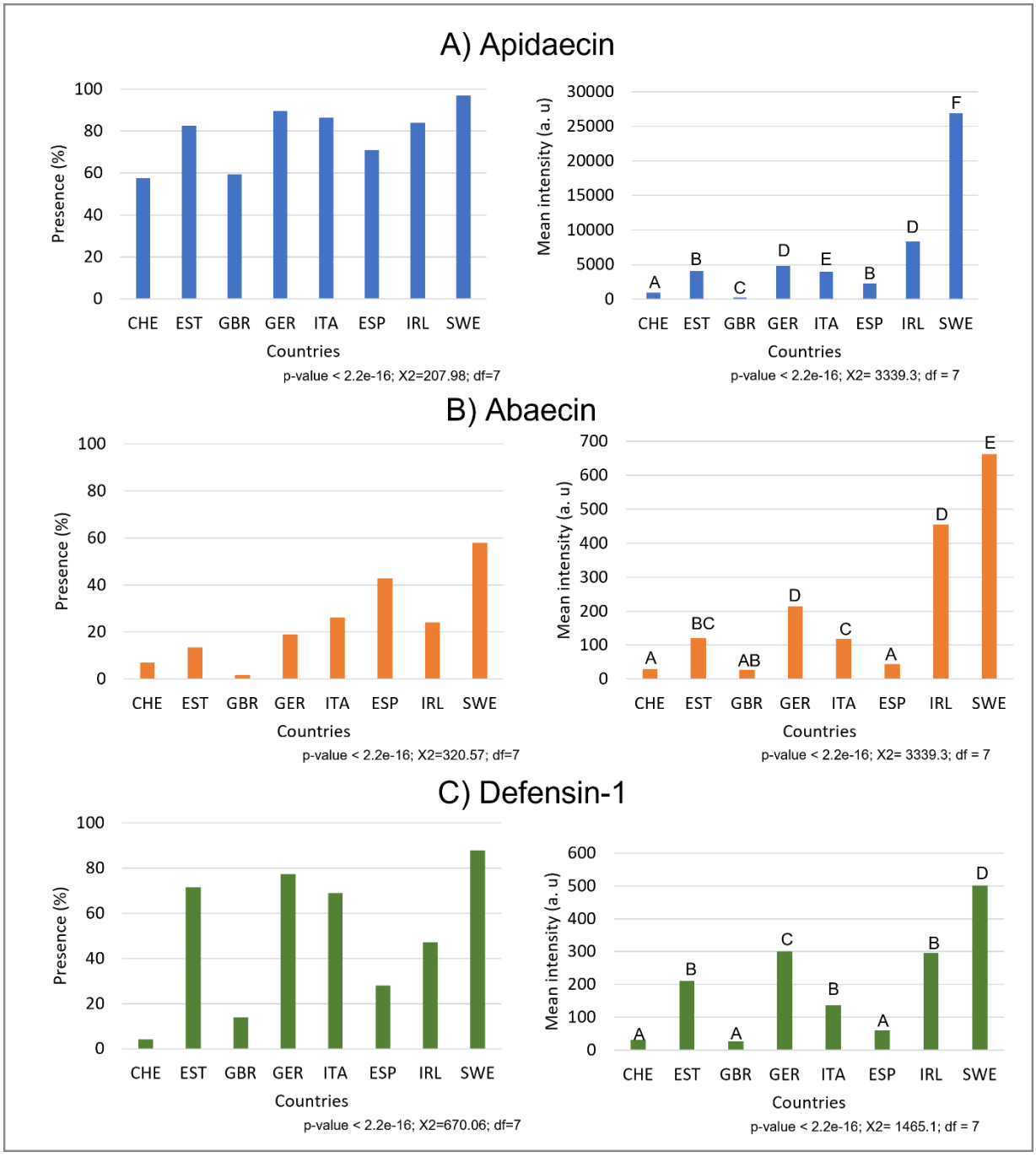


Fig. 6. AMP variations in the eight European countries studied. A) Apidaecin, B) Abaecin and C) Defensin-1. CHE Switzerland, EST Estonia, GBR Great Britain, GER Germany, ITA Italy, ESP Spain, IRL Ireland and SWE Sweden. The different alphabetic letters show statistical differences between the countries.

Regarding the Abaecin peptide, at the country level, the percentage of *A. mellifera* bees expressing Abaecin varied between countries and was below 50% in most countries, which was lower than for Apidaecin. In GBR only 1.7% of bees expressed Abaecin (mean peak intensity of 27.74) and almost 60% in SWE. However, compared to Apidaecin, the presence

of Abaecin was detected between 1.7 (for SWE) and 30 times less frequently (for GBR). In terms of intensities, the mean intensity peak of Abaecin in SWE or IRL was significantly different from ESP, CHE, GBR or EST. Defensin-1, the last immune peptide examined in our study, was poorly detected in CHE (4% of bees) but highly expressed in SWE (almost 90% of bees). Presence and intensity levels varied widely between countries (Kruskal-Wallis p-value <0.001; χ^2= p-value <0.001). We observed that less than 20% of individual bees expressed Defensin-1 in CHE and GBR, as opposed to more than 70% in EST, GER, and SWE. Although this peptide was poorly expressed by bees raised in CHE with only 4% (lowest), it was highly expressed by bees from SWE with almost 90% (highest). In contrast to Abaecin, the presence of Defensin-1 could be correlated with the mean peak intensity of this peptide in CHE (presence 4.1% and mean intensity 31.53) and in SWE (presence 87.9% and mean intensity 501.83). The mean peak intensities of Defensin-1 were low (31-136) in CHE, GBR, ITA and ESP as opposed to EST, GER, IRL and SWE (211-502), and correlated with the presence of the peptide except in IRL. This was particularly evident in the three intermediate countries (EST, GER and ITA), where the intensities were slightly different yet in accordance with the percentages of presence (EST 71.5% and 211.50; GER 77.3% and 300.73; and ITA 69% and 136.3).

3.3.2. Effect of crop variation on AMP expression

Interestingly, variations in Apidaecin intensity (Fig. 7A) were observed in CHE crops. Indeed, a clear separation was observed between CHE_OSR (23.1%) and CHE_APP (91.7%) (χ^2= 113.66, p-value <0.001; Kruskal-Wallis test: p-value = 0.002) based on the percentage of bees expressing Apidaecin. The mean intensity of Apidaecin was also significantly different between the two crops, 316.82 *versus* 1,014.65 a.u for OSR and APP, respectively (Kruskal-Wallis test: p-value = 0.002). When analysing the effect of the crop on Abaecin (Fig. 7B), we observed that four countries (ESP, CHE, GBR and EST) had low mean intensities for both OSR and APP crops compared to the others; but there are no significant differences between crops. Although the mean intensity was not found to be significantly different between the two crops (p-value = 0.09), a significant difference related to the percentage of Abaecin was found in both crops 30.1% *versus* 19% in OSR and APP respectively (p-value = 0.034). For Defensin-1, EST was considered particularly relevant for analysing the presence of this AMP in relation to crop type. Although a significant difference (p-value<0.001) was detected between OSR and APP in EST with mean intensities of

223.05 and 197.5, respectively, no significant difference in Defensin-1 expression (p-value = 0.20) could be found (Fig. 7C).

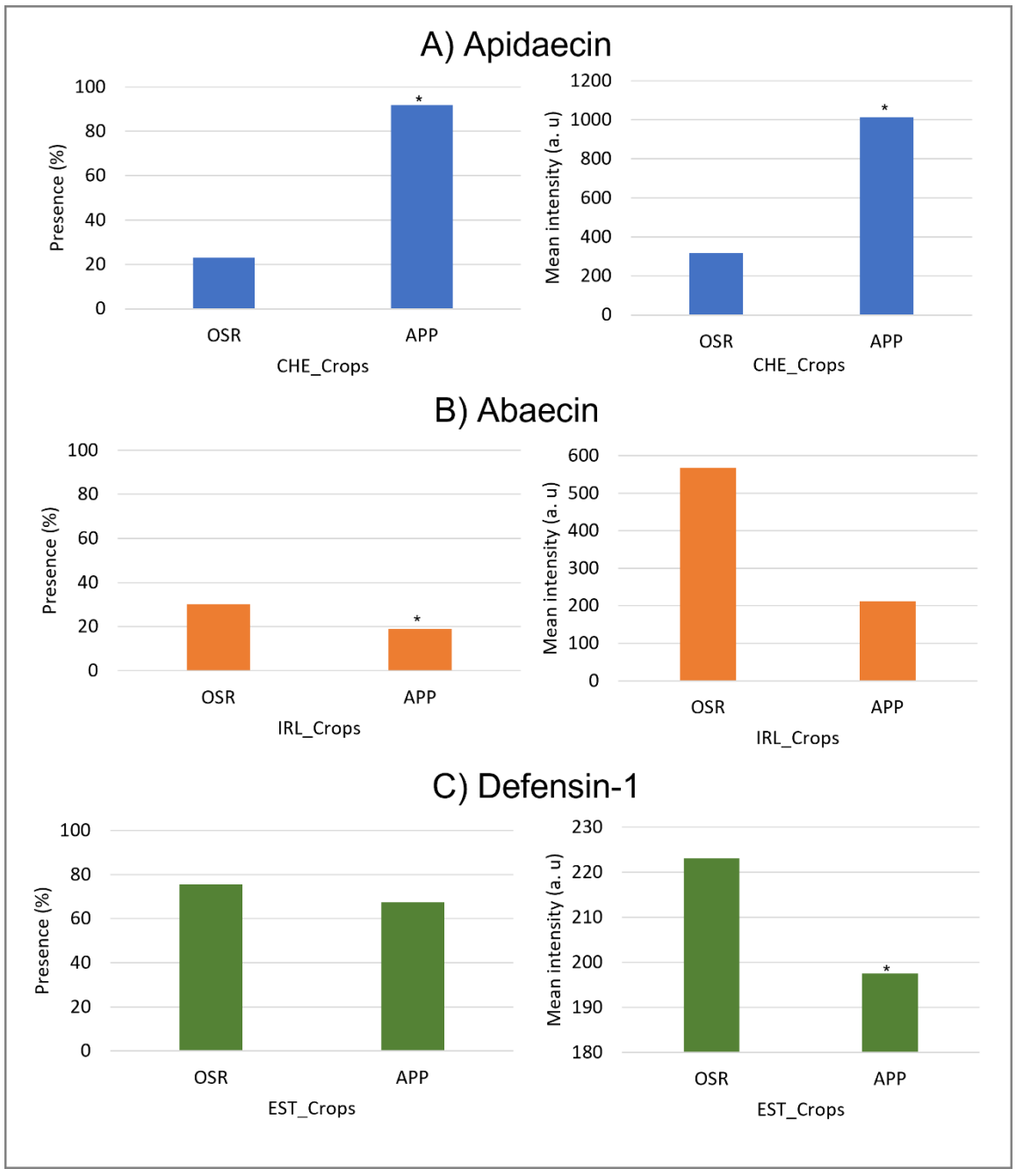


Fig. 7. Variations of the antimicrobial peptides in CHE Switzerland, IRL Ireland and EST Estonia in the studied crops oilseed rape (OSR) and apple (APP). A) Apidaecin, B) Abaecin and C) Defensin-1. * p-value < 0.05.

3.3.3. Site specificities in selected countries

The impact of location was investigated in all countries. In this section, we present the most important variations (see also Fig. 8).

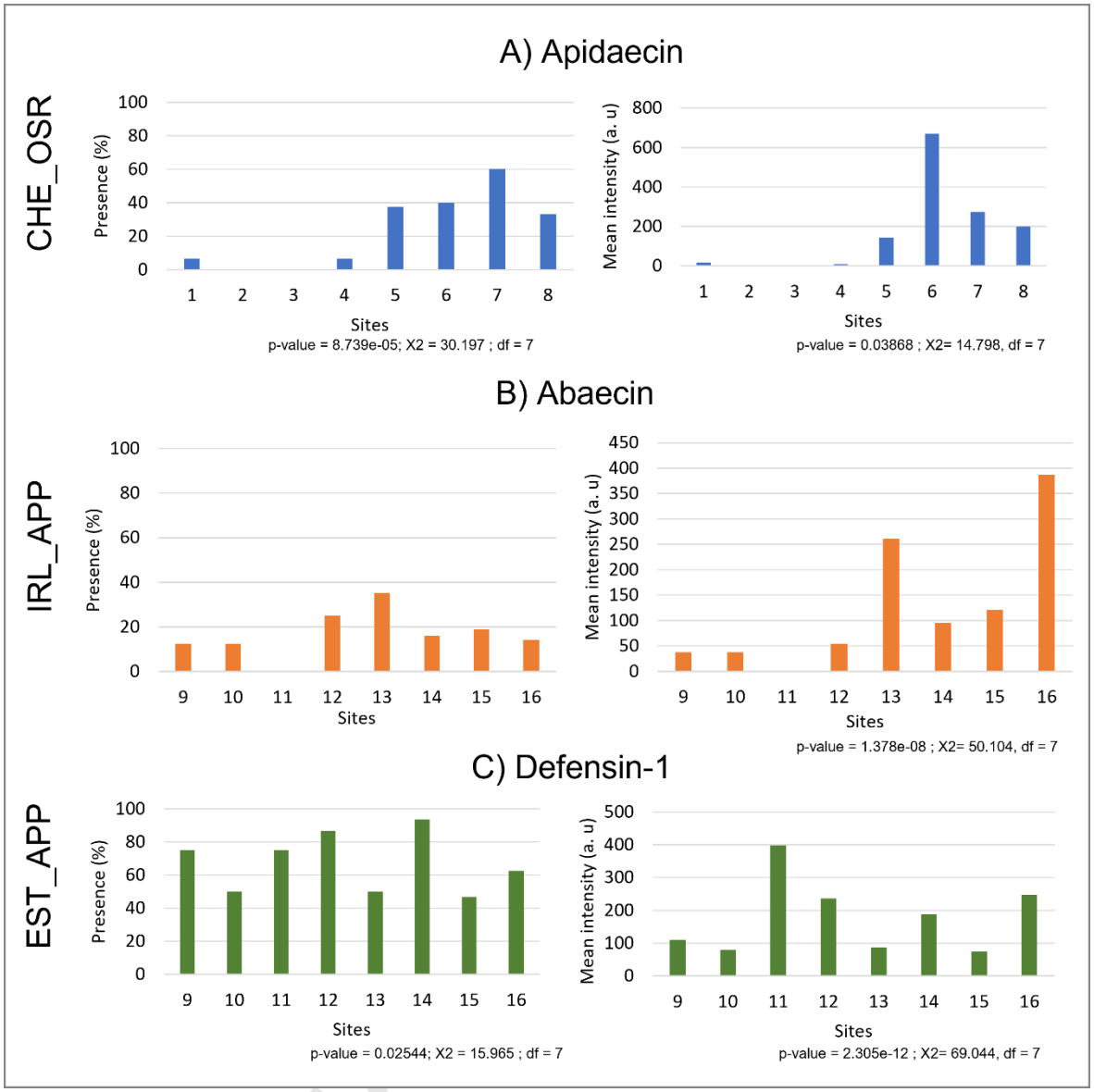


Fig. 8. Variations of the antimicrobial peptides in CHE Switzerland (Apidaecin), IRL Ireland (Abaecin) and EST Estonia (Defensin-1) in specific sites (1-8) for oilseed rape (OSR) and (9-16) for apple (APP). No bars indicate null values.

For Apidaecin, within the crops, a few sites have been highlighted to show specific profiles compared to others. In Switzerland, sites CHE_OSR_02 and CHE_OSR_03 had no bees expressing Apidaecin, whereas site CHE_OSR_07 showed that 60% of bees expressed Apidaecin+ (χ^2=8.739e-05). Similarly, differences between sites were also highlighted based on mean peak intensity with 670.6 for CHE_OSR_06 and 17.30 for CHE_OSR_01 (p-value=0.034).

To analyse the impact of site on Abaecin, we focused on IRL as a country of interest. Although significant differences in intensity in the IRL sites were found (p-value=1.378e-8),

no site dependence was observed (p-value=0.13). We found a maximum presence in the site IRL_APP_13 with 35.3% of Abaecin-positive bees, and a minimum in IRL_APP_09 and IRL_APP_10 at 12.5%. No Abaecin-positive bees were found at the IRL_APP_11 site. The mean peak intensity showed variability between the sites, with a highest intensity detected in IRL_APP_16 (387.18) and the lowest one in IRL_APP_10 (37.30). In the Estonian sites, we observed differences in the percentage of bees expressing Defensin-1 (p-value=0.0254) and intensity (p-value=2.305e-12). For example, EST_APP_15 with 46.7% peptide presence and a mean peak intensity of 73.47 and EST_APP_11 with a percentage of presence reaching 75% and a mean peak intensity of 398.11.

4. Discussion:

As shown, honey bee haemolymphs were discriminated by MALDI BeeTyping® based on proteomic signatures including the AMPs pattern of expression, altogether at three levels of investigation: country, crop and site. Deciphering the overall humoral immune responses of the honey bee *A. mellifera* at the molecular level is essential for a comprehensive understanding of how the bee is impacted by its environment. We collected honey bee haemolymph in the field from different sites and from different countries with two different crop cultures to evaluate by mass spectrometry (MALDI BeeTyping®) the molecular changes occurring in the bee haemolymph and focused on the well-known antimicrobial peptides (namely Apidaecin, Abaecin and Defensin-1(Casteels et al., 1990, 1993; Casteels-Josson et al., 1994; Danihlík et al., 2015; Ishii et al., 2014).

In CHE, the differentiation between OSR and APP was strongly marked as fewer bees in OSR had AMPs with low intensities. In general, in many sites, bees expressed no AMPs and intensities were extremely low. Conversely, in SWE the intensities were high and consistent with a high presence of AMPs; even the lowest values were always higher than those in other countries. In addition, AMPs were expressed in bees at all experimental sites in SWE.

For GER, the presence of AMPs was rather important, displaying intermediate intensities for each of the peptides, and no significant differences were observed between the OSR and APP cultures, though bees in most of the sites expressed the peptides. Finally, for IRL, we found a strong differentiation between OSR and APP, although neither presence nor intensity was very high, and bees in all sites presented AMPs. OSR had more bees

presenting AMPs and higher intensities than APP. For CHE, we found the lowest peptide intensities with the alpine climate. Hypothetically, this could be associated, with bad weather and some diseases like *Varroa*. This can hinder the movement of bees, preventing them from foraging and bringing pollen and nectar to the hive (Le Conte & Navajas, 2008). In that case, bees cannot develop their immune responses because they are not exposed to external environmental factors. Apart from metrological conditions, if the bees' environment is good enough, they can choose their resources (Aronne et al., 2012). Therefore, we can suggest that there is a strong influence of weather and possibly pathogens in CHE. The synergy of both may lead to higher bee mortality, especially in winter, leading to colony losses (Beaurepaire et al., 2017). Indeed, an overall reduced metabolic activity is associated with a decrease in immune function and increased susceptibility to DWV infection (Steinmann et al., 2015).

For GER, with average intensities, the continental climate is characterised by warm minimum and maximum temperatures and average precipitation. These high temperatures could lead to a strong expression of the immune response signalling pathways (J. Xu & James, 2012). The landscape is quite diverse. There are agricultural, urban, natural and wetland areas. For GER, relatively high prevalence of *Varroa* was found during the field study (Babin et al., 2024). Christen et al. (2019) reported a high use of pesticides with more than 40,000 tones, which in combination with a high prevalence of Varroa leads to higher bee mortality (Christen et al., 2019). Hence, we suggest that GER seems to be an intermediate country with respect to environmental stressors, with pesticides and *Varroa* likely being combined important factors, which alone do not seem to have a major impact on bee immunity. In addition, we observed a low variation between OSR and APP, although they are located in very distant regions.

SWE has a boreal climate with average temperatures and low precipitation. The strongest peptide intensities were observed in SWE for either OSR or APP, and these high intensities were also present for IRL_OSR. IRL has an atlantic climate and the OSR and APP cultures are in the same geographical regions of the country. However, IRL_APP and IRL_OSR exhibited very different peptide intensities, indeed IRL_OSR had a similar profile to SWE with high peptide intensities. Those differences observed in APP in IRL *vs* WE and ESP *vs* CHE could be explained by the differences of the honey bee subspecies in these countries. For instance, in IRL, the naturally distributed subspecies is *Apis. m. mellifera with a high level of genetic integrity* (Browne et al., 2021; Hassett et al., 2018). In SWE, the naturally

distributed should be *A. m. mellifera* (Jensen et al., 2005), however, analyses from other work packages in the PoshBee project evidenced the presence of *Apis. m. ligustica* or *Apis. m. carnica*. For ESP and CHE, the subspecies are completely different: *Apis. m*. *iberiensis* and *Apis. m. mellifera* respectively (Henriques et al., 2020; Parejo et al., 2016)(Henriques 2020 and Parejo 2016). Besides, *A. mellifera* is sensitive to temperature, so workers will raise the temperature of the hive to protect the larvae (Zhao et al., 2021).This is called social fever. This social fever is a form of social immunity involving behavioural, organisational and physiological mechanisms that social organisms use to defend themselves against parasites and agents responsible for maintaining the health of the group (Goblirsch et al., 2020).This group reaction is associated with an increase in Abaecin and Hymenoptaecin (Goblirsch et al., 2020), these two AMPs being secreted and released into the bee haemolymph as a consequence of the activation of the Toll pathway by pathogen recognition receptors that bind fungal pathogen associated molecular patterns such as fungal β-glucans (Brutscher et al., 2015).

Overall, looking at each peptide variation, we found that Apidaecin is expressed in more than 60% of individuals except in CHE_OSR. Its intensities seem to vary according to the previous causes, very high for SWE and IRL_OSR, intermediate for GER and IRL_APP and low for CHE. Defensin-1 seems to follow the same pattern overall, except for CHE where it is silent with less than 5% of individuals presenting this AMP, but we have more important variations between sites which would imply stressors at smaller scales either at site or hive level. For Abaecin, we observed a similar profile with lower intensities and variations between sites and hives.

To obtain a computational model, we also built a Machine Learning model to discriminate protein signatures from bee haemolymph profiles under APP and OSR conditions (Table S4). The model selects 25 ions that discriminate APP/OSR. A promising result was obtained with 90% recognition of spectra in the correct category. The cross-validation showed a lower value, 76%. We then tried to apply the model to external data, involving countries where PCA statistics were not able to discriminate the APP and OSR profiles. We observed significant variability in the results obtained at the honey bee scale, and the % of bees correctly classified in APP and OSR was taken as the main parameter.

We generated different profiles for each crop and country. A good level of recognition was obtained for APP in GBR and ESP and for OSR in GER. In the remaining countries, however, the results were highly variable and did not show a clear correlation with the surface of each crop in the foraging area. This may reflect the individual behaviour of foragers in the presence of a choice of different lipid/protein ratios (Vaudo et al., 2020). Studying haemolymph molecular profiles can provide a global view of how honey bees explore the environment, the complexity of which is beginning to emerge. For example, honey bees were drawn away from APP orchards by a mass co-flowering crop such as OSR, even when APP pollination was provided by wild bees. This may also occur with other flowers in the landscape, as suggested by the lower results in ESP at the end of the flowering period (Osterman et al., 2021).

5. Conclusions

In this field-scale study, we have demonstrated the feasibility to correlate the expression of MMIs and the three AMPs Apidaecin, Abaecin and Defensin-1 with countries, crops system and local sites. Hence, we were able to collect these field-related molecular datasets from honey bees and build the first proteomics field-realistic computational model to investigate the potential biotic and abiotic stressor impacts on honey bees, to date. Using foraging bees, the recognition of such impacts showed an accuracy of 90% roughly with a subsequent quite well recognition for some crop/country combinations, whereas poorly in some others, when individual bee profiles greatly varied withing the same location. The interest of such model relies mainly on its relationship with other models such as for studying pesticides impacts with nutritional stress. Developing monitoring tools to follow the impact of stressors (biotic and abiotic) on the health of living organisms is essential for prognosis and diagnosis, and MALDI BeeTyping® is one possible tool to assist beekeepers to follow the honey bee health status. We evidenced that AMPs are pertinent markers to be followed by this method to visualise the impact of different stressors. This tool based on a simple blood test has the capacity to be a non-supervised approach compared to tools based on ELISA tests or PCR analysis, both of latter approaches focusing on what you are looking for. As an innovative molecular tool, MALDI BeeTyping® could be used to monitor pollinator health in multiple scenarios by generating computational models to monitor

impacts on bee health in addition to global field information such as climate or the presence of diseases.

Data linking

The MALDI-MS raw files have been deposited in the Figshare repository and made available to the reviewers through the following private weblink
https://figshare.com/s/355b69c98a0686139e5c

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Highlights

The field environment impacts the molecular profile/composition of honey bee haemolymph

- Country, crop and site modify the honey bee haemolymph content as evidenced by measuring its molecular diversity by mass spectrometry
- Large variability of immune bee response is evidenced in different environmental conditions studied across eight European countries
- MALDI BeeTyping® approach is able to discriminate honey bees in different environments across Europe and therefore to monitor bee health