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Facing multiple threats: Diet–pesticide interactions in bumblebees

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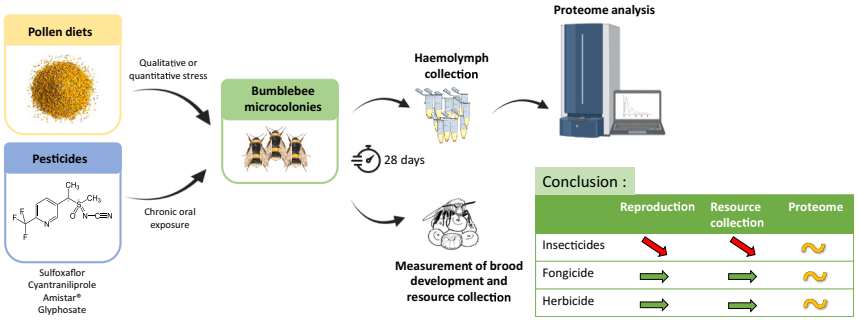
HIGHLIGHTS

- Both brood development and proteome was affected by insecticides.
- Exposure to Amistar® or glyphosate did not impact brood development but induced proteome modification.
- Proteome was impacted by pesticides when colonies are nutritionally stressed.
- Colonies that were strongly impacted by pesticides did not benefit from a good pollen diet.
- Agrochemical effects on bumblebees can differ depending on diet and can be better detected based on proteome analyses.

GRAPHICAL ABSTRACT



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ABSTRACT

Global change presents a threat to pollinators, with declining populations trends jeopardising the delivery of pollination services. In agroecosystems, the reduction of floral resources and exposure to pesticides have been identified as stressors of bee pollinators. The present study investigated the interaction between nutritional and agrochemical stresses on sublethal endpoints in the bumblebee *Bombus terrestris*. We used two pollen diets differing in quality and quantity and exposed micro-colonies to various concentrations of two insecticides (sulfoxaflor and cyantraniliprole), a fungicide (Amistar®; active ingredient azoxystrobin), or an herbicide (glyphosate) in a crossed experiment comprising 29 experimental conditions. Regardless of the pollen regime, there were negative impacts of the insecticides on both brood development and proteome; however, there was no

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significant impact on brood development following exposure to Amistar® or glyphosate. The proteome was affected by all pesticides when the colonies were nutritionally stressed, indicating that pesticides induced a molecular differentiation between groups fed on different diets. Nutritionally stressed colonies were less developed compared to control colonies, but this effect was not synergistically increased by exposure to sulfoxaflor, Amistar®, or glyphosate. The development of colonies exposed to cyantraniliprole was affected similarly, regardless of the nutritional condition and therefore did not benefit from a quality pollen diet. Overall, our results highlight that the effects of agrochemicals on bumblebees can vary depending on diet and can be detected via proteome analyses. We therefore urge for conservation action supporting pollinators with suitable and diverse floral resources alongside improved risk assessment regarding pesticides.

1. Introduction

More than 85 % of flowering plants rely at least partly on animal pollination for their sexual reproduction (Ollerton et al., 2011). Among pollinators, bees are one of the most diverse and abundant groups and play an important role in ecosystem functioning, especially for agro-ecosystems (e.g., Kleijn et al., 2015). Preserving bee populations is crucial for maintaining pollination service, (Potts et al., 2016). Many bee species are threatened (Brodschneider et al., 2018; Potts et al., 2010) due to multiple factors associated with human-induced global changes (Carvalheiro et al., 2013; Duchenne et al., 2020; Goulson et al., 2015) including the use of pesticides (LeBuhn and Luna, 2021; Brittain et al., 2010; Scott-Dupree et al., 2009), habitat loss (Persson et al., 2015; Vray et al., 2019), global warming (Ghisbain et al., 2023; Kerr et al., 2015), the spread of invasive species (Stout and Morales, 2009), and infections by parasites (Williams et al., 2008) and pathogens (Ravoet et al., 2014).

Intensification of agricultural practices is associated with the loss of pollinator habitat (Persson et al., 2015). Habitat loss can prevent bees from obtaining a balanced diet (i.e., balanced profiles of amino acids, sterols and protein:lipid (P:L) ratios). However, what constitutes a proper balanced diet is difficult to determine, as each of the ca. 20,000 bee species have specific nutritional constraints (Barraud et al., 2022; Vaudo et al., 2020). For example, honeybees are known to be positively impacted by a higher content of protein in their pollen diet, but this positive effect may turn negative if the protein content exceeds 40 % (Herbert et al., 1977; Human et al., 2007). Alternatively, bumblebees and mason bees develop normally on >40 % protein diets but struggle on diets with a low total amino acid and sterol content, specifically with low concentrations of 24-methylenecholesterol and beta-sitosterol (Barraud et al., 2022). Overall, a poor-quality pollen diet will affect the development of bumblebee offspring, leading to reduced colony size, higher mortality, and compromised immunity (Moerman et al., 2017; Roger et al., 2017; Taseï and Aupinel, 2008; Vanderplanck et al., 2014, 2018; Vaudo et al., 2016). Nutritional stress can also be induced through starvation. Pollen and nectar starvation negatively impact honeybee brood development (Brodschneider and Crailsheim, 2010), honeybee behaviour (Schulz et al., 1998), bumblebee lipid storage (Woodard et al., 2019), and bumblebee immune response to parasite infection (Brunner et al., 2014).

The intensification of agriculture has increased the use of various agrochemicals (Matson et al., 1997). More than 1000 pesticides have been registered for use during the past decade (World Health Organisation, 2020). Although some pesticides (e.g., neonicotinoids) have been banned or are strictly controlled in Europe due to high risks or potential harm to non-target organisms, new molecules have emerged (e.g., Sulfoxaflor). For many of them, especially fungicides and herbicides, their potential harm to bees is not well understood (Franklin and Raine, 2019; Sanchez-Bayo and Goka, 2014; Stoner, 2016; Thompson and Maus, 2007). Numerous studies have demonstrated the lethal and sublethal effects of various pesticides on bees (Artz and Pitts-Singer, 2015; Balbuena et al., 2015; Mao et al., 2017; Motta et al., 2018; Mussen et al., 2004), as well as the existence of synergistic effects (Azpiazu et al., 2021; Iverson et al., 2019; Johnson et al., 2013; Zhu et al., 2014). Pesticides can impact both domesticated and wild bees at field-realistic concentrations, with effects on foraging abilities (Barascou et al., 2022; Stanley et al., 2016), reproduction (Baron et al., 2014; Mussen et al., 2004; Whitehorn et al., 2012) and immunity (Al Naggar and Baer, 2019).

Such molecules, once in the bee's body, can be metabolised by the detoxification system. Xenobiotic detoxification generally involves the conversion of lipid-soluble substances to water-soluble, excretable metabolites. The proteins involved in the detoxification of xenobiotics are diverse and intervene at different stages of the process (Berenbaum and Johnson, 2015). During phase I of detoxification (functionalization), Cytochrome P450 monooxygenases (P450) and carboxylesterases (CCE) alter the structure of the toxin, making it more water-soluble and less likely to interact with lipophilic target sites (Sehlmeyer et al., 2010). In phase II (conjugation), Glutathione S-transferases (GST) conjugate the Phase I products for solubilization and transport (Xu et al., 2013). Phase III of detoxification involves the transport of Phase II conjugates out of cells for excretion. The proteins involved in this process include multidrug resistance proteins (MRPs) that are members of the C family, a group of proteins named ATP-binding cassette (ABC) transporters, and other ABC transporters (Dermauw and Van Leeuwen, 2014).

Bees can be exposed to pesticides while foraging, notably through the consumption of contaminated pollen and nectar (Botías et al., 2015). Nutritional stress (i.e., low quality and/or quantity of food) can be exacerbated when bees are exposed to pesticides (e.g. Knauer et al., 2022), as the upregulation of detoxification genes may trigger a down-regulation of genes associated with the production of energy (Derecka et al., 2013) needed for the detoxification system (Berenbaum and Johnson, 2015). Previous studies have shown that the pesticide sensitivity of adult honeybees can depend on the quality of pollen consumed during early life stages, with bees consuming high-quality pollen displaying greater pesticide resistance (Wahl and Ulm, 1983). Pollen quality can also influence the capacity for detoxification (Knauer et al., 2022), with different pollen species being associated with variation in gut GST in honeybees (Archer et al., 2014). These results are concerning, given the fact that bees do not necessarily have many choices in large monocultures and that some studies have shown that bees do not shift their intake towards a higher-quality diet under such stressful conditions (Archer et al., 2014). While research on interactive diet-pesticide effects on bee survival concluded that pollen-based diets reduce pesticide sensitivity or that diet effects are overshadowed by the effects of pesticides (Barascou et al., 2021; Barraud et al., 2020; Leza et al., 2018; Schmehl et al., 2014), very few studies have been conducted on their effects on bee development or physiology (Schwarz et al., 2024). Regarding physiology, the link between diet and the proteome, the set of proteins the organism produces in a specific tissue/cell type under various conditions (e.g. developmental stage, abiotic or biotic stress conditions) remains poorly understood. The manipulation of sterols can alter the head protein profiles of honeybees, with important nutritional marker proteins being upregulated (Chakrabarti et al., 2020).

To address this knowledge gap on diet-pesticide interactions, we conducted a laboratory experiment with the common crop pollinator *Bombus terrestris* as a model species. Based on Barraud et al. (2022), we used two pollen diets demonstrating different nutritional quality: a high-quality pollen diet (*Salix*-based diet), and a low-quality pollen diet

(*Cistus*-based diet). The diets comprised either a full or half optimal ration (i.e., starvation treatment). The following pesticides were tested: glyphosate (an herbicide), Amistar® (a fungicide; active ingredient azoxystrobin), and two insecticides (sulfoxaflor and cyantraniliprole). For each bumblebee micro-colony, we monitored the food intake (i.e., the quantity of pollen and nectar collected) and the brood development (i.e., the mass of larvae) to calculate the pollen efficacy (defined as larval mass developed per gram of consumed pollen). We extracted haemolymph as an index of the health status of workers for proteomic analyses to explore sublethal effects on the bumblebee physiology and immunity. Regarding the importance of nutrition for bee development and its impact on the molecular responses and defence tools, we hypothesised that a poor diet would enhance the negative effect of pesticides and negatively impact brood development.

2. Materials and methods

2.1. Bees

Bumblebee colonies were provided by Biobest NV (Westerlo, Belgium). A total of 25 queen-right colonies, each comprising ~100 *Bombus terrestris* workers, were used to establish 480 queenless micro-colonies of five workers in plastic boxes (8 × 16 × 16 cm). The number of individuals per micro-colony had been optimised during previous bioassays (Moerman et al., 2016; Roger et al., 2017; Vanderplanck et al., 2018) and has been shown to be the most favourable for the production of male offspring (Gradish et al., 2013). Using more workers can dilute the brood-tending responsibilities across more individuals and increase the temperature of the microclimate (Klinger et al., 2019). Due to the high number of tested conditions, the experiment ran over five months; five different queen-right colonies were used for each month. For each experimental condition, a total of 50 workers divided into 10 individual micro-colonies were used, with 10 workers (i.e., two micro-colonies) established from one of the five queen-right colonies to avoid colony bias.

All micro-colonies were maintained in the same room in constant darkness with a relative humidity of 60–65 % and were provided ad libitum with a 65 % sugar solution. The colonies were manipulated under red light to minimise disturbance (Sadd, 2011) for a period of 28 days, time for the first male offspring to emerge.

2.2. Pollen diet

Two organic pollen blends of different quality were purchased from "Abeille Heureuse" (France): *Salix* pollen blend as a high-quality diet and *Cistus* pollen blend as a low-quality diet. Assessments of their chemical composition (i.e., protein and lipid contents, amino acids, and sterols), their botanical origins, and the absence of pesticides were detailed in a previous study on bee nutritional stress (Barraud et al., 2022). Prior to the experiment, the pollen was treated with gamma rays to eliminate potential pathogens (Álvarez Hidalgo et al., 2020) and stored at -80 °C. The micro-colonies were fed with pollen candies composed of either *Salix* or *Citrus* pollen mixed with sugar water. New pollen candy was provided every three days, while the previous pollen was removed before drying or decaying and weighed to assess pollen consumption (Fig. 1). In the treatment "ad libitum", large amounts of pollen were provided (~3 g). A starvation treatment using *Salix* pollen blend was also tested by providing only half the quantity of the pollen a micro-colony usually consumed (Barraud et al., 2022). However, this starvation was not coupled to each pollen treatment because of the large number of experimental treatments this would have generated.

2.3. Pesticides

Four different widely-used pesticides were used in this study: sulfoxaflor (an insecticide: 20; 300; 1000 and 2000 ppb, for treatments designated S1, S2, S3 and S4), Cyantraniliprole (an insecticide: 6200 ppb, treatment CY), Amistar® (a fungicide, active ingredient Azoxystrobin: 200 and 1900 ppb, for treatments A1 and A2) and Glyphosate (herbicide: 3800 and 7600 ppb, treatments G1 and G2). One mixture (treatment AS) of sulfoxaflor (1000 ppb) and Amistar® (1900 ppb) was additionally tested; other mixtures were not considered due to time and resource constraints. These pesticides were selected based on a combination of complementary criteria to ensure both ecological relevance and broader applicability of our findings: (i) widespread use; (ii) future relevance; (iii) mode of action; (iv) broad exposure potential, and (v)

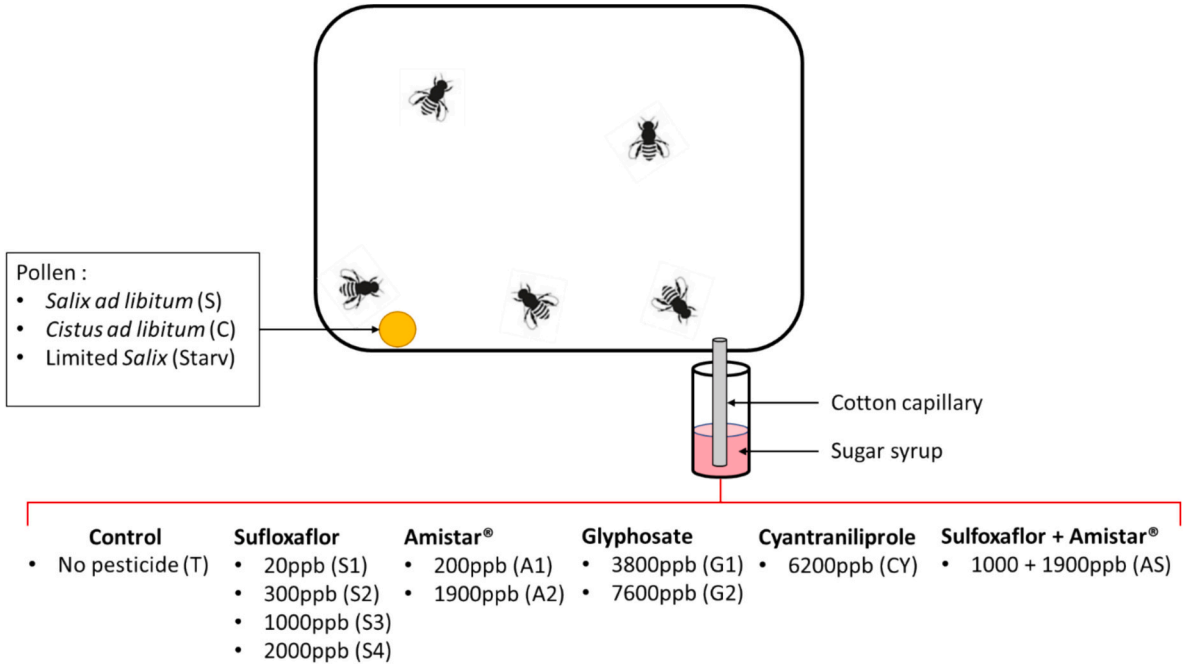

Fig. 1. Experimental set-up. Bumblebees were fed with sugar syrup (treated or not) and pollen (*Salix*/*Cistus* ad libitum or limited *Salix*) for 28 days and maintained in constant darkness at 26 ± 2 °C with a relative humidity of 60–65 %. S3, S4, CY and AS treatments were not tested with Starv condition.

known or suspected toxicity. The pesticides were administered for 28 days using tube feeders (Fig. 1), while the sugar solution used to prepare pollen candy remained pesticide-free. The chosen concentrations were considered field-realistic based on pesticide residue data in pollen and nectar found in the literature and databases: sulfoxaflor residues ranged from 6.6 to 13.8 ppb in nectar and 7.7–39.2 ppb in the pollen of cotton flowers (Jiang et al., 2020) while another study reported higher levels of sulfoxaflor, ranging from 0.05 to 1 ppm in nectar and from 220 to 2780 ppb in pollen collected by honeybees during crop flowering periods (EPA, 2019). The highest reported concentration of residues was 3000 ppb shortly after sulfoxaflor application in flowering apples (EFSA, 2020). Azoxystrobin has been found at levels ranging from 30 to 110 ppb in pollen collected by honeybees in North America (Mullin et al., 2010). Another study in the US recorded 4.6 to 1870 ppb in honeybee pollen loads (Rennich et al., 2013). The concentrations of glyphosate were chosen based on studies that detected sub-lethal effects on honeybees at concentrations ranging from 500 to 10,000 ppb (Balbuena et al., 2015; Herbert et al., 2014), amounts that are in the range of values measured in natural environments (1400 to 7600 ppb) (Feng and Thompson, 1990; Giesy et al., 2000). Finally, the concentrations of cyantraniliprole used in our study were based on the range of detected residues in both pollen and nectar (400 to 32,000 ppb, Dinter and Samel, 2015; Kyriakopoulou et al., 2017; Lee et al., 2019) and sub-lethal effects on food intake (Barraud et al., unpublished data).

To reach these concentrations, the pesticides were first diluted in acetone, then in water and in nectar (sugar solution, 65 % sugar, 35 % water). The solutions were given ad libitum to the bees in 100 mL containers with a capillary tube. Control and contaminated nectar were replaced every four days and weighed to assess sugar and pesticide consumption. To avoid bias, a similar quantity of acetone used to dilute the pesticides was added to the control nectar.

2.4. Experimental set-up and colony parameters

Each of the three nutritional conditions described above (i.e., *Salix* ad libitum, *Cistus* ad libitum, and limited *Salix*) was crossed with six pesticides treatments (S1, S2, A1, A2, G1, and G2) and the control (i.e., pesticide-free sugar water), whereas four pesticide treatments (S3, S4, CY and AS) were crossed with only two nutritional conditions (*Salix* and *Cistus* ad libitum) due to a lack of time and resources. The design comprised a total of 29 experimental conditions.

Several parameters were assessed to evaluate the performance and development of bumblebee micro-colonies: total pollen and nectar collection, which can impact mortality; brood production and development (Plowright et al., 2008; Sutcliffe and Plowright, 1990), and colony growth after 28 days of development (i.e., the mass of individuals from all brood stages: eggs, larvae, pupae, and non-emerged and emerged adults) (Vanderplanck et al., 2014, 2018). For each micro-colony, each of the parameters was divided by the total mass of the five workers to standardise the results and avoid potential effects of worker activity related to size (i.e., consumption and brood care) (Cnaani and Hefetz, 1994). Additionally, we calculated the pollen efficacy as the mass of total offspring divided by the total pollen collected to estimate the colony performance (Vanderplanck et al., 2014, 2018).

2.5. Haemolymph collection

Haemolymph samples from 30 workers of each experimental condition were collected after 28 days using the protocol developed by (Arafah et al., 2019). Briefly, haemolymph was collected using pulled glass capillaries (Sutter Instrument Corp, Model P-30, Novato, California). The glass capillary was inserted dorsally between the second and third terga of the abdomen. The collected haemolymph was transferred to a chilled LoBind Protein microtube (Eppendorf, Germany) precoated with 1-phenyl-2-thiourea (PTU) and phenylmethylsulfonyl fluoride (PMSF) to prevent melanisation and proteolysis, respectively. After collection, the haemolymph samples were stored at -20 °C until use.

2.5.1. Haemolymph preparation for MALDI molecular mass fingerprinting (MFP) and MALDI BeeTyping® acquisition

To acquire MFPs by Matrix Assisted Laser Desorption Ionization mass spectrometry (MALDI MS, referred to MALDI BeeTyping®), haemolymph samples were handled according to Bournonville et al. (2023) with minor adjustments. Each individual haemolymph sample was analysed with an AutoFlex III Smartbeam® MALDI MS (Bruker Daltonics, Germany). MFPs were acquired following the Bruker BioTyper® recommendations (matrix, method of sample deposition, and detection) with modifications. The haemolymph samples were diluted 1:10 in acidified water (1 % trifluoroacetic acid, TFA). One microliter from each diluted sample was spotted on a MALDI MTP 384 polished ground steel plate (Bruker Daltonics), dried under gentle vacuum, and then mixed with 1 μL of the α-Cyano-4-hydroxycinnamic acid (4-HCCA) matrix. Following co-crystallisation of the haemolymph spots with the matrix droplet, MALDI MFPs were recorded in a linear positive mode via automatic data acquisition using FlexControl 3.4 software (Bruker Daltonics). The samples were deposited in triplicate (three spots) and read once.

The following instrument settings were used to perform the MALDI analysis: 1.5 kV of electric potential difference, dynamic range of detection of 600–18,000 *m/z*, a global attenuator offset of 70 % with 200 Hz laser frequency, and 1000 accumulated laser shots per haemolymph spectrum. The linear detector gain was set at 1.762 kV with a suppression mass gate up to m/z 600 to prevent detector saturation by clusters of the 4-HCCA matrix. A standard mixture of peptides and proteins (Peptide Standard Calibration II and Protein Standard Calibration I, Bruker Daltonics) covering the dynamic range of analysis was used to calibrate the mass spectrometer.

The MFP dataset was analysed in two steps. First, the data were pooled into three sub-datasets based on the pollen diet (i.e., *Salix* ad libitum, *Cistus*, and *Salix* starvation) to estimate the molecular impact of pesticide exposure. Second, the data were pooled according to the pesticide exposure (i.e., 10 sub-datasets: A1, A2, G1, G2, S1, S2, S3, S4, AS, CY) to estimate the impact of the diet on the MFPs of the haemolymph.

2.5.2. Bottom-up proteomics: off-gel digestion and nano-LC-ESI-MS/MS

Based on the MFP profiles of the different conditions, individual haemolymph samples were selected to form pools for off-gel digestion and bottom-up proteomics analyses using online nano-Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (nano-LC-ESI-MS/MS).

For each experimental condition, three groups of five individual haemolymph samples were prepared based on the MFP profiles. The pooled samples were dried by vacuum centrifugation (Labconco, Kansas City, MO) and subjected to proteomic analysis according to our previous studies (Askri et al., 2023; Bournonville et al., 2023; Houdelet et al., 2021). Briefly, 20 μL of 0.1 % RapiGest surfactant in 50 mM ammonium bicarbonate (ABC) buffer was added to the samples. After adding 2 μL of 280 mM Dithiothreitol (DTT, a disulfide bond reducing agent), the tubes were incubated at 56 °C for 45 min in the dark, briefly centrifuged, and then cooled to room temperature. Four microliters of 4-vinyl-pyridine (an alkylating agent used to block cysteine residues) was added, followed by incubation in the dark at room temperature for 30 min. Then, 2 μL of a 0.2 μg/μL sequencing grade trypsin solution (Promega) was used for protein digestion. The samples were incubated overnight at 37 °C under gentle agitation. To stop the enzymatic reaction and inactivate RapiGest, samples were acidified by 5 μL of acetonitrile (ACN) 20 %-10 % TFA and incubated for 45 min at 37 °C. The tryptic digests were centrifuged for 10 min at 15,000 ×g, and 10 μL of the samples were analysed by nanoLC-ESI-MS/MS using a U3000 nano-HPLC connected to a high-resolution Q-Exactive Orbitrap (all instruments from Thermo Scientific). The tryptic digests were separated by reverse phase

chromatography on an Acclaim PepMap 100 C_{18} nanocolumn (75 μm internal diameter, 150 mm length, 3 μm granulometry, and 100 Å porosity, Thermo Fisher Scientific) online with a concentration micro-precolumn C_{18} PepMap 100 (300 μm internal diameter, 3 μm granulometry, and 100 Å porosity; Thermo Fisher Scientific). The flow rate was set to 300 nL/min using a diphasic linear gradient of ACN in 0.1 % formic acid (FA) from 2 % to 35 % in 120 min; then from 35 % to 70 % B in 5 min, followed by washing and column equilibration between each injection. The eluted peptides were injected on-line into a high-resolution Q-Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific) and analysed in a positive and data-dependent acquisition mode, using the *m/z* range 380–2000 as previously described in Houdelet et al. (2021). The acquired MS/MS data files were processed using Proteome Discoverer v2.5 (Thermo Fisher Scientific) for identification. The following parameters were used: N-terminal protein acetylation, methionine and tryptophan oxidation were set as variable modifications; trypsin digest with two maximum missed cleavages; six and 144 amino acids as minimum and maximum peptide lengths, respectively; a tolerance of 10 ppm/0.02 Da for precursors and fragment ions, respectively; cysteine pyridyl-ethylation was set as a fixed modification. The identification confidence was set at a false discovery rate of 1 %. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD043718.

2.5.3. Database searching, annotation, and label-free quantification (LFQ)

The data sets obtained by nano-LC-ESI-MS/MS were analysed as described by Houdelet et al. (2021) and Askri et al. (2023). The Proteome Discoverer software v2.5 (ThermoFisher Scientific) was used for sequencing and to quantify the proteins' relative abundance using label-free quantification. The ion-based quantification utilised unique and razor peptides, and the calculation of peptide abundance was based on intensity following a normalisation of the datasets comprising all peptides characterised in the LC-MS/MS runs. The protein quantification was performed using the summed abundance with subsequent ANOVA tests. Protein fold-changes <0.5 or >2 and a *P*-value less than 0.05 compared to the control were considered significant.

2.5.4. Functional annotation: gene ontology and pathway analysis

OmicBox software (v2.1.10, functional analysis module Blast2Go https://www.biobam.com) was used to perform the functional annotation of the dysregulated proteins. To obtain the most complete annotation labels, the analyses were performed using the four cloud-powered algorithms (Blast, InterProScan, GO Mapping, and GO Slims). A list of all quantified proteins was loaded to investigate biological pathways and protein functions following bee exposure to chemicals. A combined pathway analysis was performed on the annotated sequences (proteins) joining Reactome and KEGG to identify enriched pathways with expression profiles.

2.6. Statistical analysis

We performed comparative analyses of micro-colony performance and feeding behaviour using R version 3.6.0 (R Core Team, 2023).

As the experiment was carried out over several months using different colonies, differences in observed results could have been due to genetic variation between colonies. To avoid any such bias, statistical analyses were performed on reduced-centred data standardised on non-exposed micro-colonies fed with the optimal pollen diet (*Salix*), and the results were represented in terms of difference from the optimal diet of the corresponding month. Since data were not normally distributed, the effects of pesticide and pollen treatments on development and resource consumption were analysed using Kruskal-Wallis tests. Multiple pairwise comparisons between all experimental conditions were conducted using Dunn's tests with the Benjamini–Hochberg correction when Kruskal-Wallis detected a significant difference among treatments ($P < 0.05$).

All the MALDI-MS datasets were imported into ClinProTools™ 2.2 Software (Bruker Daltonics) for post-processing and statistical analyses. Baseline subtraction and spectral smoothing were applied to the acquired spectra. The total average spectra were calculated based on a signal-to-noise ratio equal to 3 for peak-picking and area calculations. The null spectra were excluded from the analysis. A post-processing step involving spectral normalisation of all calculated peak areas was performed with ClinProTools™ software prior to the application of a Principal Component Analysis (PCA). The Wilcoxon-Kruskal-Wallis test was used for intensity comparisons. PAD (*P*-value from the Anderson-Darling) test was used to check for normality. Comparison peak lists were generated from ClinProTools™, listing all of the ions considered for the analysis.

3. Results

3.1. Colony development

There was no significant effect on the development (i.e., relative brood mass) of bumblebee colonies exposed to Amistar® (200 ppb A1 and 1900 ppb A2), glyphosate (3800 ppb G1 and 7600 ppb G2), the low concentrations of sulfoxaflor (20 ppb S1 and 300 ppb S2), or the mixture (1000 + 1900 ppb AS) (Figures SI1, SI2, 1000 ppb S3; $P = 0.452$; 0.426, $P = 0.798$; 0.747 and $P = 0.190$, respectively). The total brood mass of micro-colonies fed with either low-quality pollen (*Cistus*) or low-quantity pollen (starvation/Starv) was negatively impacted by these diets compared to bumblebees fed ad libitum on *Salix* pollen (Figs. SI1-SI3, all $P < 0.001$). There was no significant synergistic effect on brood mass between the nutritional stress (i.e., *Cistus* diet and starvation) and an additional pesticide stress (all $P < 0.3$, Figs. SI1- SI3). However, the negative impact of qualitative nutritional stress (*Cistus*) on brood mass was no longer significant when the micro-colonies were exposed to cyantraniliprole (Fig. SI4, $P = 0.946$). Regardless of pollen diet, the insecticides sulfoxaflor at the highest concentrations and cyantraniliprole significantly impacted brood development compared to the control (1000 ppb S3 and 2000 ppb S4, $P = 0.013$ and $P < 0.0001$, respectively; CY, $P < 0.001$) (Fig. SI4).

Regarding resource consumption, there were decreases in micro-colonies exposed to the highest concentration of sulfoxaflor (SI4, $P = 0.008$) and cyantraniliprole (CY, $P < 0.001$) (Fig. SI4). For nectar consumption, the impact was significant in micro-colonies exposed to the highest concentrations of sulfoxaflor (S3 and S4, $P = 0.011$ and $P < 0.001$, respectively), the azoxystrobin/sulfoxaflor mixture (AS, $P = 0.018$), and to cyantraniliprole (CY, $P = 0.008$) (Fig. SI4). No significant effects were observed for bumblebees exposed to glyphosate or azoxystrobin alone (Figs. SI1, SI2, all $P > 0.1$). While pollen consumption was clearly impacted when bumblebees were maintained under starvation ($P < 0.001$), there were no significant differences among qualitative stress treatments, except for colonies exposed to cyantraniliprole that consumed more pollen when fed with *Cistus* compared to those fed with *Salix* (Fig. SI4, $P = 0.043$). Diet conditions affected nectar consumption, being greater when the micro-colonies were fed with *Salix* compared to those under a qualitative (*Cistus*, $P = 0.011$) or quantitative (starvation, $P = 0.009$) stress, except for microcolonies from AS ($P = 0.668$) and CY ($P = 0.549$) treatments (Figs. SI1-SI4).

Micro-colony efficacy was only impacted when the bumblebees were exposed to high concentrations of sulfoxaflor (S3, $P = 0.014$ and S4, $P = 0.019$) or to cyantraniliprole ($P < 0.001$) (Fig. 2C). No significant differences were observed for bumblebees exposed to glyphosate, Amistar®, or low concentrations of sulfoxaflor (all $P > 0.4$) (Fig. 2). Micro-colonies fed ad libitum with *Salix* pollen were more efficient compared to those fed with limited amounts of *Salix* pollen ($P = 0.038$) or with *Cistus* pollen ($P < 0.001$). Even though brood development was lower, bumblebees under starvation were more efficient than those under qualitative stress (*Cistus*) ($P = 0.028$). Diet effects did not mitigate

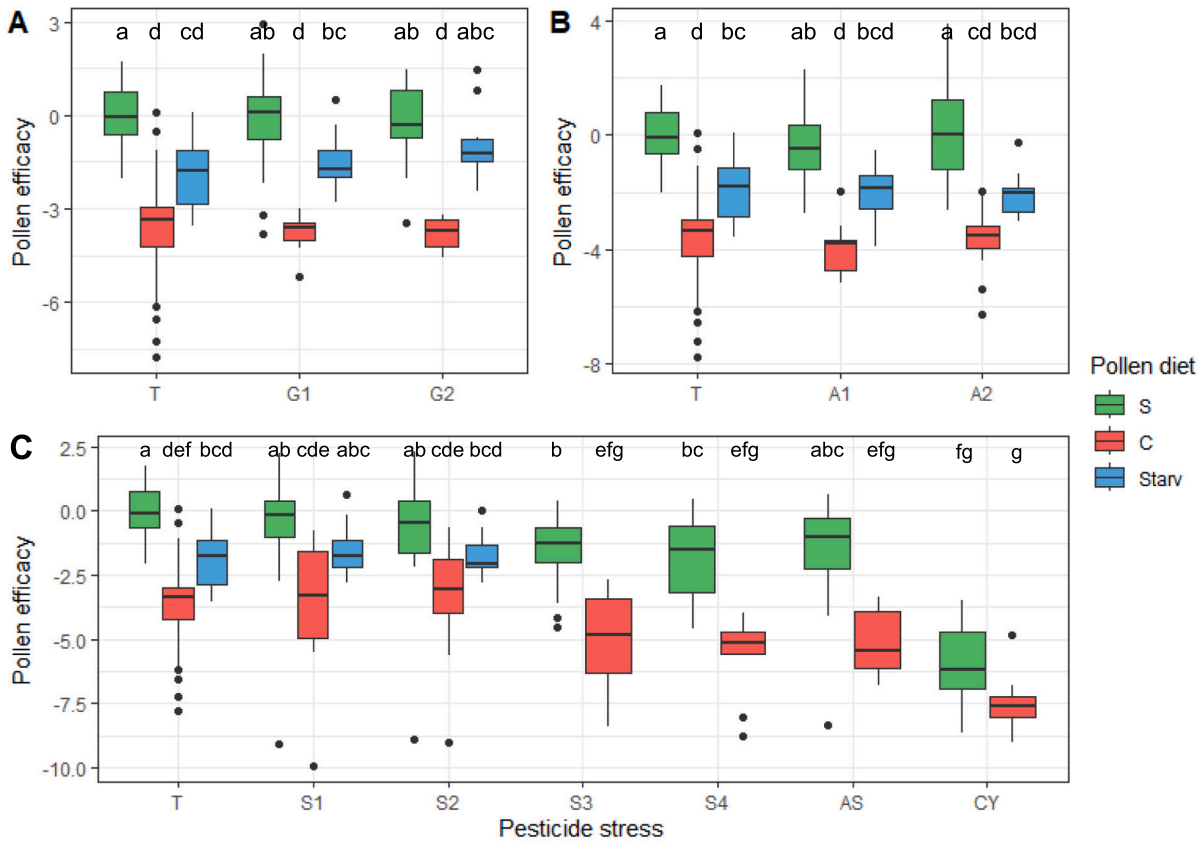

Fig. 2. Relative pollen efficacy on bumblebee colonies exposed to Amistar®, glyphosate, sulfloxaflor, Amistar® + sulfloxaflor or cyantraniliprole. T = control; G1 = glyphosate 3800 ppb; G2 = glyphosate 7600 ppb; A1 = Amistar® 200 ppb; A2 = Amistar® 1900 ppb; S1 = sulfoxaflor 20 ppb; S2 = sulfoxaflor 300 ppb; S3 = sulfoxaflor 1000 ppb; S4 = sulfoxaflor 2000 ppb; AS = Amistar® 1900 ppb + sulfoxaflor 1000 ppb; CY = cyantraniliprole 6200 ppb; C = *Cistus* diet, S = *Salix* diet, Starv = Starvation. Each treatment has 10 replicates. Stars indicate statistical significance compared to the control of the same nutritional condition (* = $P < 0.05$; ** = $P < 0.01$; n.s. = non-significant).

the pesticide treatments, except in micro-colonies exposed to cyantraniliprole, for which the efficiency did not differ significantly, regardless of the diet ($P = 0.79$) (Fig. 2).

3.2. Impact of the diet and pesticides on haemolymph molecular mass fingerprints (MFPs): Global analysis by MALDI BeeTyping® analysis

A total of 1140 individual bumblebee haemolymph samples were collected over three sampling sessions corresponding to our 29 experimental conditions. Table SI1 presents a summary of the number of samples collected per experimental condition. Each haemolymph sample was analysed by MALDI-BeeTyping® to visualise the potential impact of the treatments (diet and pesticides) on the haemolymph peptidome profile through the MFPs as a readout of the health status of bees. To this end, the MFPs recorded from the various experimental conditions were compared.

The Principal Component Analysis indicated no impact of the pesticides on the MFPs of the haemolymph in bumblebees fed with *Salix* ad libitum (Fig. SI5). However, the PCA showed that bumblebees fed with *Cistus* pollen and exposed to glyphosate had different MFPs compared to the *Cistus* control individuals. This was not observed with Amistar® or sulfoxaflor at the lowest doses tested (Fig. 3). When analysing the groups under starvation and those exposed to sulfoxaflor, Amistar® or glyphosate at high or low doses, there was a clear discrimination between each of the treatments compared to the controls (Figure SI6). Amistar® and glyphosate induced greater changes in the bumblebees' haemolymph than sulfoxaflor (percentage of significant modulated ions ≈ 90-95 % vs. ≈ 40-47 % following sulfoxaflor) compared to the control condition. There was no significant difference between the high and low doses of the chemicals. Table SI2 lists summary data for the ion distributions among the conditions cited above and the percentages of significantly modulated ions detailed in Table SI3.

Comparison of the molecular impact of the diet in workers exposed to the same pesticide at the same concentration revealed a discrimination between *Cistus* and *Salix* starvation diets for the bumblebees exposed to sulfoxaflor, Amistar®, and glyphosate regardless of the dose, while there was no difference between the two control groups, i.e., the *Cistus* control and StarvT (reduced quantity of *Salix* pollen control) (Fig. 4). Regarding the cyantraniliprole (CY), we observed a difference between bumblebees exposed to cyantraniliprole (CY) at 6200 ppb when they were fed with *Cistus* or *Salix* compared to the controls (Figure SI7). However, there was no impact of the diet under sulfoxaflor at 1000 ppb or 2000 ppb (the two highest doses, S3 and S4) (Figure SI7).

3.3. Effectors of the systemic immune response of Bombus terrestris illustrate the impact of diets and pesticides

The ClinProTools™ software was used to analyse peptide markers of the bumblebee humoral immune response, namely Apidaecin (molecular ion at *m/z* 1978), Abaecin (*m/z* 4397), Defensin (*m/z* 5529) (Choi et al., 2008), and the Chymotrypsin inhibitor (*m/z* 5938, Bournonville et al., 2023). In our experiments, the measured average molecular-related ions for Apidaecin, Defensin, and Chymotrypsin inhibitor identified by MALDI BeeTyping® were at *m/z* values between 1978 and 1979, 5530-5536, and 5940-5941, respectively. Abaecin was not detectable under our experimental conditions. By analysing the pairwise

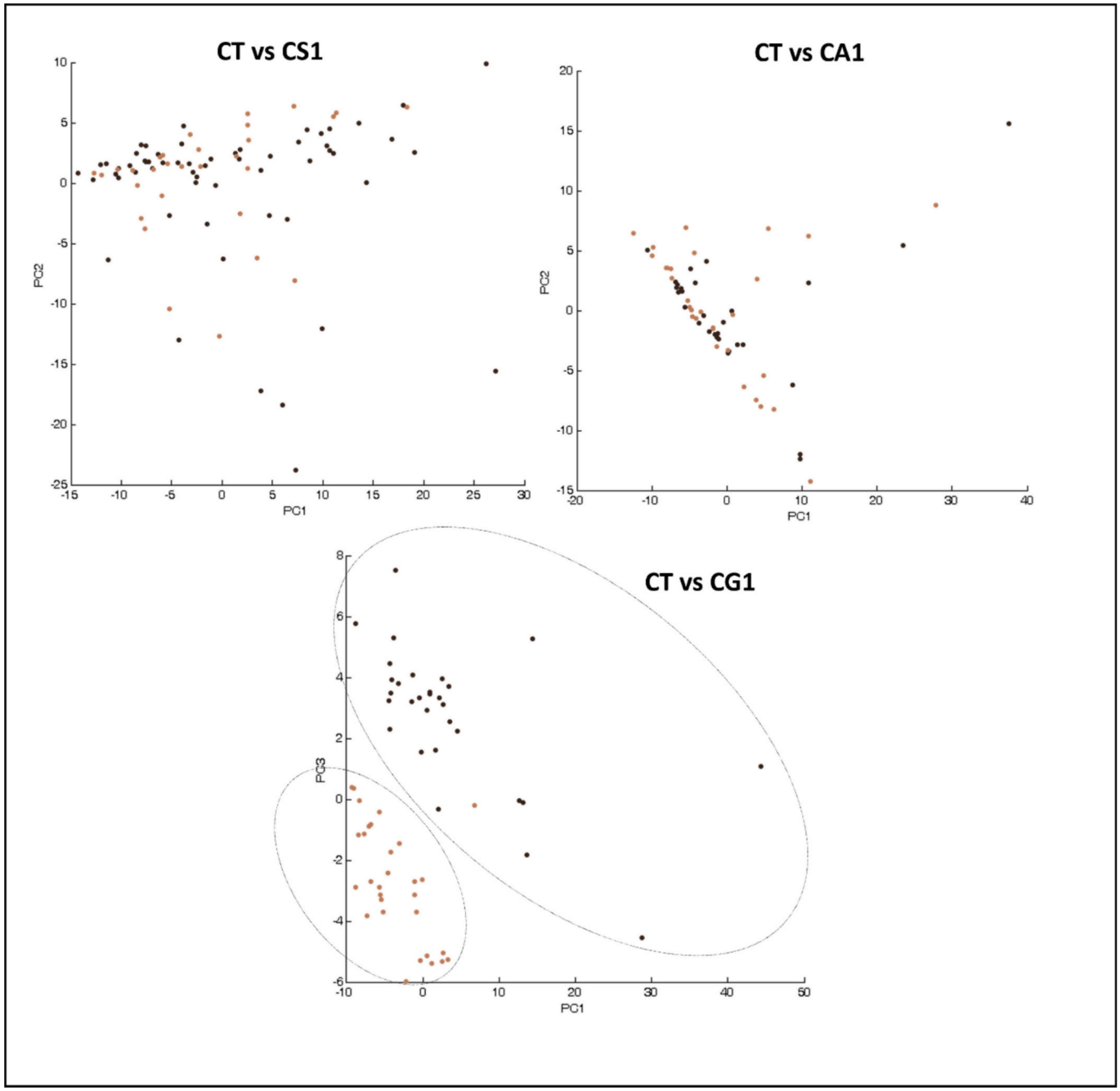

Fig. 3. PCA analyses of the molecular profiles of *Bombus terrestris* fed with *Cistus* pollen diet and exposed to pesticides. CT (*Cistus* control without pesticide) vs CS1 (Cistus pollen + sulfoxaflor 20 ppb) (A), CT vs CA1 (Cistus pollen + Amistar® 200 ppb) (B); CT vs CG1 (Cistus pollen + glyphosate 3800 ppb) (C). The CT is represented by dark orange, while the other conditions are in light orange. Each point represents the haemolymph MFP from an individual bee. PCAs are generated from ClinProTools™.

comparisons between diverse experimental conditions, we found that Apidaecin was significantly impacted by starvation (Starv) ($P < 0.01$) in the presence of each of the three chemicals (sulfoxaflor, Amistar®, and glyphosate) compared to the *Cistus* diet or Starv controls (Fig. 5). In almost all conditions mentioned above, Apidaecin was decreased compared to the controls. The serine-type endopeptidase chymotrypsin inhibitor (accession number K7WRE1_BOMTE) was decreased in almost the same conditions as Apidaecin, with additional variation following exposure to glyphosate at 3800 ppb (CG1) in the haemolymph of bumblebees fed with *Cistus*. There was no significant variation in bumblebees exposed to sulfoxaflor. The level of Defensin was also decreased by exposure to Amistar when the bumblebees were fed a reduced quantity of *Salix* pollen or following exposure to glyphosate when the bees were fed *Cistus* compared to the control conditions.

3.4. Proteomics analysis of the bumblebee haemolymph in response to diet and pesticide exposure

To evaluate the effects of the stressors on the bumblebee proteome of the haemolymph under the different treatments, we used a combination of high-resolution mass spectrometry coupled to nano liquid chromatography (nano-LC-ESI-MS/MS) and Label Free Quantification (LFQ) on the pooled haemolymph samples. The pools were organised according to the MFPs obtained by MALDI BeeTyping®.

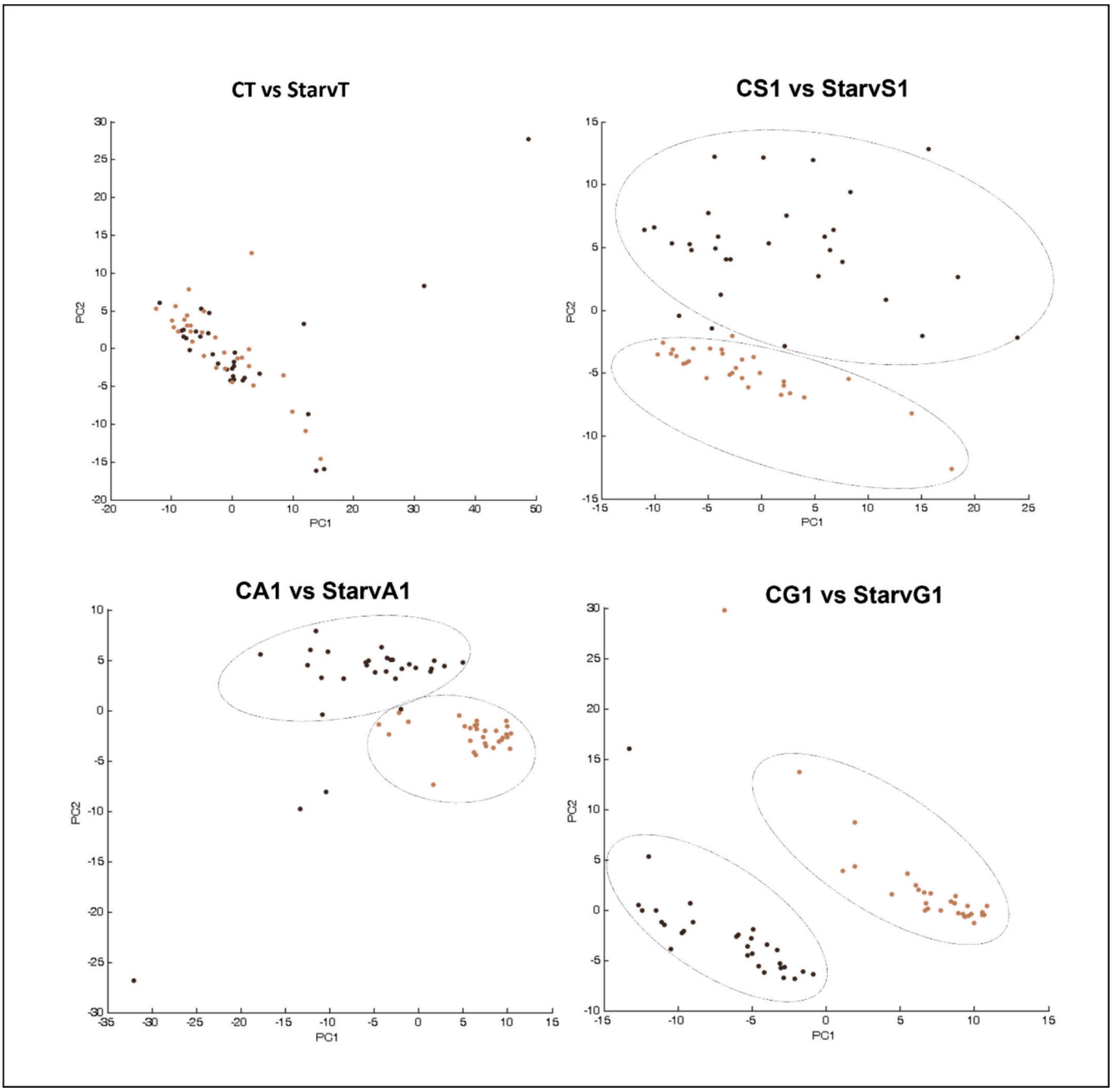

Fig. 4. PCA analyses of the molecular profiles of *Bombus terrestris* fed with *Cistus* diet vs reduced quantity of *Salix* (Starv), and exposed to the same pesticide at the same concentration. CT (*Cistus* control) vs StarvT (reduced quantity of *Salix* diet control) (A); CS1: *Cistus* diet + sulfoxaflor 20 ppb vs StarvS1: reduced quantity of *Salix* diet + sulfoxaflor 20 ppb (B); CA1: *Cistus* diet + Amistar® 200 ppb vs StarvA1: reduced quantity of *Salix* diet + Amistar® 200 ppb (C). CG1: *Cistus* diet + glyphosate 3800 ppb vs StarvG1: reduced quantity of *Salix* diet + glyphosate 3800 ppb (D). The *Cistus* conditions are represented in dark orange and the Starv conditions are in light orange. Each point represents a haemolymph MFP from an individual bee. PCAs are generated from ClinProTools™.

Fold-change ratios were calculated as a measure of peptide/protein regulation in the exposure vs control groups or in comparisons of the type of exposure. In the bumblebee haemolymph samples, 173 proteins (Table SI4) were quantified following exposure to chemicals under starvation conditions. These proteins were involved in 39 biological processes and had a total of 25 molecular functions (Table SI5). The top 15 biological processes (A) and molecular functions (B), and the corresponding number of quantified proteins are presented in Fig. 6.

Among these proteins, three were up-regulated and three were down-regulated (Table 1). Among the up-regulated proteins, hemocytin isoform X1 (A0A6P3UD80_BOMIN) was modulated following treatments with Amistar® at high (ratio 3.11) and low (ratio 5.60) concentrations, or with glyphosate at the lowest concentration tested (3800 ppb; ratio 8.06). The chymotrypsin inhibitor (A0A6P3URW3_BOMIM) and phospholipase A1 (PLA1, A0A6P3U5Q8_BOMTE) were upregulated only after exposure to sulfoxaflor at 300 ppb and to Amistar® at 200 ppb, respectively. Following exposure to Amistar® at a high concentration (1900 ppb), fibrillin-2 (A0A6P5I445_BOMTE) and DE-cadherin (A0A6P3UBB1_BOMTE) were both down-regulated at ratios of 0.01 and 0.03, respectively.

In addition to the most significantly dysregulated proteins, we looked for proteins that are involved in the immune response and the

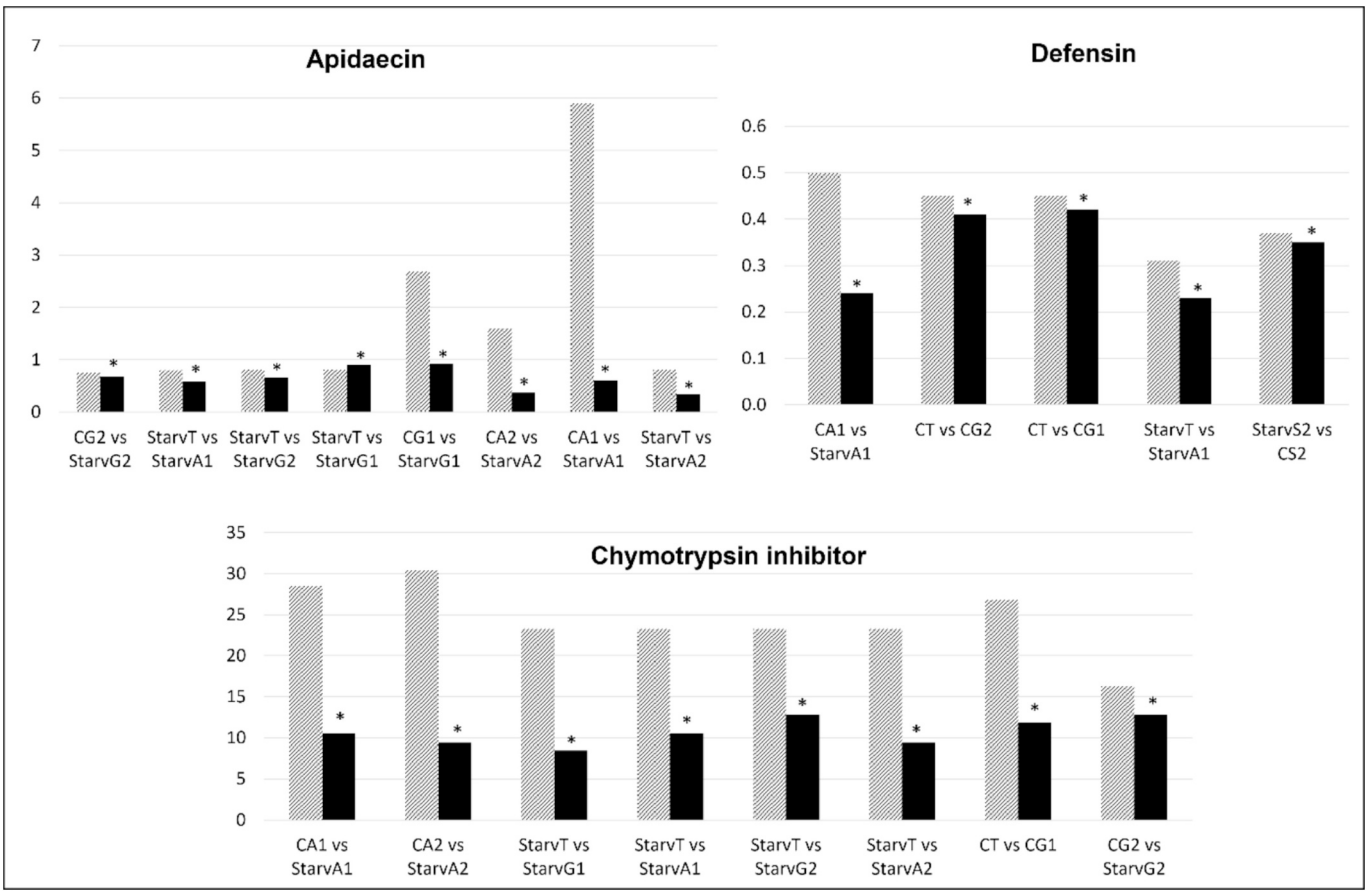

Fig. 5. Immune response profiles of Apidaecin, Defensin and Chymotrypsin inhibitor of *Bombus terrestris* fed with *Cistus* diet (C) or reduced quantity of *Salix* (Starv) following chemical exposure. CT: *Cistus* pollen control; CS2: *Cistus* pollen + sulfoxaflor 300 ppb; CA1: *Cistus* pollen + Amistar® 200 ppb; CA2: *Cistus* pollen + Amistar® 1900 ppb; CG1: *Cistus* pollen + glyphosate 3800 ppb; CG2: *Cistus* pollen + glyphosate 7600 ppb. StarvT: reduced quantity of *Salix* pollen control; StarvS1: reduced quantity of *Salix* pollen + sulfoxaflor 20 ppb; StarvS2: reduced quantity of *Salix* pollen + sulfoxaflor 300 ppb; StarvA1: reduced quantity of *Salix* pollen + Amistar® 200 ppb; StarvA2: reduced quantity of *Salix* pollen + Amistar® 1900 ppb; StarvG1: reduced quantity of *Salix* pollen + glyphosate 3800 ppb; StarvG2: reduced quantity of *Salix* pollen + glyphosate 7600 ppb (G). *The figure shows only the significant differences from the full crossed analysis ($P < 0.05$).

detoxification processes. We identified several proteins known to be involved and to play major roles in such defence reactions. For example, vitellogenin (C4PB33_BOMIG; A0A9B0BV41_BOMTE) and vitellogenin-like precursor (A0A6P9FR62_BOMTE), two proteins involved in melanisation namely phenoloxidase 2 (A0A6P3DHW1_BOMTE) and phenoloxidase subunit A3 (A0A195CM22_9HYME), immune responsive protein (A0A650C291_9HYME), apidaecin +A (C0HKX3, APD_BOMTE), defensin 1 (D2XR05_BOMTE), chymotrypsin inhibitor (K7WRE1_-BOMTE), heat shock 70 kDa protein cognate 4 (A0A154NZW5_DUFNO), abaecin (D2XR04_BOMTE/Q6TEK2_BOMIG), and bumblebee peptidoglycan recognition protein SA (A0A452CSL6_6HYME). The detoxification proteins associated with reactive oxygen species identified were thioredoxin (A0A6P3TZ43_BOMTE), superoxide dismutase [Cu—Zn] (A0A6P3DBJ9_BOMTE, A0A1B1JIC8_BOMIG), Peroxiredoxin 1 (A0A6P3DJW0_BOMIG), and dipeptidase (A0A6P3DLE5_BOMTE). They were not impacted by any of the stressors used in this study. Other proteins involved in detoxification mechanisms such as the Cytochrome P450 superfamily (CYP450s) of heme-thiolate proteins, proteins that play a pivotal role in the clearance of various toxic compounds (Sigel et al., 2007) and glutathione transferases (GSTs; also known as Glutathione S-transferases) that are major detoxification enzymes (Sheehan et al., 2001), were not characterised in our experimental conditions.

4. Discussion

4.1. Colony development

4.1.1. The impact of diet

Our micro-colonies were affected differently depending on their pollen diet. Resource collection and pollen efficacy of bumblebees fed with *Cistus* pollen or a limited amount of *Salix* pollen were negatively impacted compared to those fed ad libitum with *Salix* pollen. This indicates that diet quality and quantity affected micro-colony performance.

Qualitative stress (the *Cistus* diet) had no impact on relative pollen consumption. There was therefore no modification of pollen collection behaviour from the bumblebee workers to compensate for the low-quality diet. Even if this result is in accord with previous studies (Barraud et al., 2022; Roger et al., 2017; Taseï and Aupinel, 2008; Vanderplanck et al., 2014), it is important to note that this might not necessarily be the case with other less favourable diets. Pollen from Asteraceae (e.g., *Taraxacum* sp. or *Cirsium* sp.) is considered to be a low-quality diet and is less consumed by micro-colonies compared to pollen from other plant families (Roger et al., 2017; Vanderplanck et al., 2018).

As expected, developmental parameters of bumblebees fed with *Cistus* pollen were negatively impacted (i.e., lower brood mass and pollen efficacy compared to the bumblebees fed with *Salix* pollen). This has been observed in previous studies showing that an unbalanced supply of nutrients can lead to delayed development, reduced body

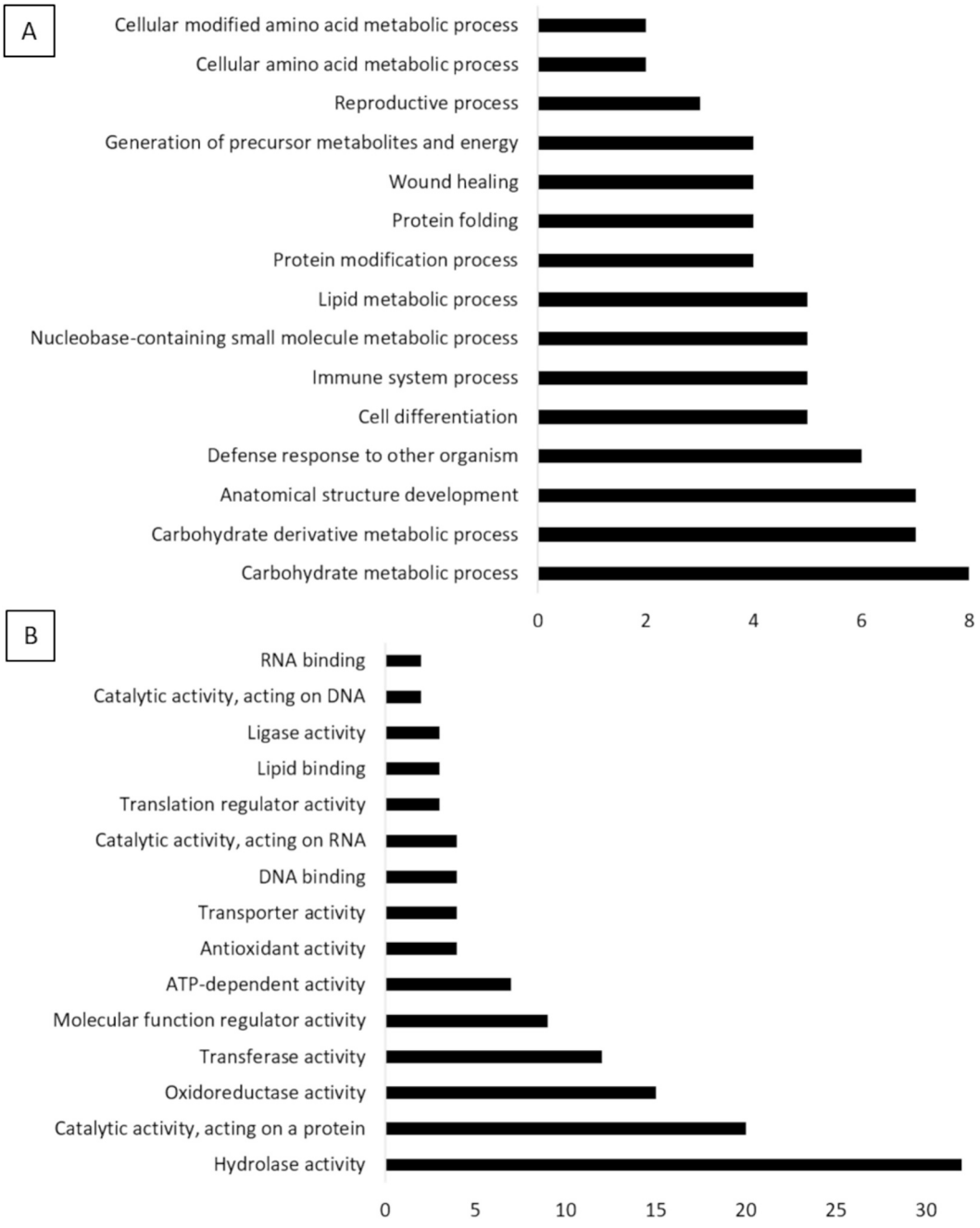

Fig. 6. Top 15 biological processes (A) and molecular functions (B) and the corresponding number of quantified proteins under starvation condition. The X axis represents the number of proteins.

weight, or even colony mortality (Archer et al., 2021; Moerman et al., 2016, 2017; Sutcliffe and Plowright, 1990). There is a common assumption that proteins or the protein/lipid ratio are positively related to colony performance in bumblebees and honeybees (Nicolson, 2011; Roulston and Cane, 2002; Stabler et al., 2015). Other studies have shown that amino acids and sterols play a crucial role in diet quality. In this regard, previous studies have found that amino acid intake was positively correlated with bumblebee body mass, and that the effects of total amino acid intake may depend on the composition of individual amino acids (Archer et al., 2021; Barraud et al., 2022). Furthermore, some sterol compounds have been reported to be positively correlated with larval growth or brood development (Barraud et al., 2022; Vanderplanck et al., 2014). For example, 24-methylenecholesterol is known to influence moulting and ovary development (Human et al., 2007; Regali, 1996; Svoboda et al., 1983), while sitosterol and D5-avenasterol are apparently involved in metabolic pathways of *B. terrestris* (Regali, 1996). Here, the *Cistus* pollen had lower total amino acid and sterol contents (125.04 mg/g and 4.54 mg/g, respectively) compared to *Salix* pollen (181.35 mg/g and 8.22 mg/g, respectively; see (Barraud et al., 2022), with also a low quantity of 24-methylenecholesterol (1.43 mg/g for *Cistus*, 2.48 mg/g for *Salix*) and b-sitosterol (0.73 mg/g for *Cistus*, 2.42 mg/g for *Salix*). However, it is important to realize that such nutritional requirements are not necessarily the same among bee species (Barascou et al., 2022; Barraud et al., 2022; Schwarz et al., 2024).

Limited access to diet (the starvation diet) impacted pollen consumption and had a negative impact on total brood mass compared to the *Salix* ad libitum diet and led to greater efficacy compared to *Cistus* but lesser efficacy compared to *Salix*. While we expected to observe a reduction in brood mass under this treatment, it was interesting to observe a difference between the ad libitum and limited *Salix* diets.

Table 1
List of significantly dysregulated proteins following chemical exposure under starvation conditions. The protein quantification was calculated using the summed abundance with subsequent ANOVA tests. Protein fold-changes <0.5 or >2 in the different conditions compared to the control, along with a *P*-value less than 0.05, were considered significant.

Accession number	A0A6P3UD80	A0A6P3U5Q8	A0A6P3URW3	A0A6P3DEU3	A0A6P5I445	A0A6P3UBB1
Protein name	Hemocytin isoform X1	Phospholipase A(1)	Chymotrypsin inhibitor	Serine protease inhibitor 88Ea	Fibrillin-2	DE-cadherin
Abundance Ratio: (StarvA2) / (StarvT)	3.11				0.01	0.03
Abundance Ratio P-Value: (StarvA2) / (StarvT)	0.04				0.01	0.01
Abundance Ratio: (StarvA1) / (StarvT)	5.6	100				
Abundance Ratio P-Value: (StarvA1) / (StarvT)	0.02	<0.01				
Abundance Ratio: (StarvG1) / (StarvT)	8.06					
Abundance Ratio P-Value: (StarvG1) / (StarvT)	0.03					
Abundance Ratio: (StarvS3) / (StarvT)			100	0.03		
Abundance Ratio P-Value: (StarvS3) / (StarvT)			<0.01	0.03		

Since it was the same pollen, we could have expected a similar effect. While we do not know the reasons for this difference, we suggest that, as workers need to consume pollen for their own health, they may have used relatively less pollen to feed the larvae compared to the bees fed ad libitum. Overall, this negative impact of starvation was in agreement with previous studies (Treanore et al., 2023).

4.1.2. The impact of pesticides

Impacts on the resource consumption and development of micro-colonies were related to the specific agrochemicals. Regarding fungicides, we found that Amistar® alone did not impact any of the colony parameters. This result was in line with recent studies on *Apis mellifera* (Barascou et al., 2021) and on the solitary bee *Osmia bicornis* (Schwarz et al., 2024) under laboratory conditions. Other studies have found effects of this agrochemical at realistic field levels under semi-field conditions, using *B. terrestris* as a model (Tamburini et al., 2021a; Wintermantel et al., 2022). In these studies, various parameters, including foraging performance, body mass, colony growth, or pollen deposition, were negatively impacted by Amistar® when the maximum application rate (1 L/ha of formulated product) was applied to crops. The mechanisms underlying the observed patterns are not evident, as only a few studies have explored the effects of the active compound azoxystrobin on bees. However, azoxystrobin has been shown to impact the hormone system of honeybees, which could affect the development of worker bees into foragers (Christen et al., 2019). These differences between laboratory and semi-field experiments are of interest. We suggest that semi-field conditions might be more energetically demanding and complex (e.g., foraging) and that other stresses could be involved (e.g., temperature), making the bees more sensitive to agrochemical stress. Another reason for this lack of sensitivity could be that bees may efficiently detoxify azoxystrobin (the active compound of Amistar®) compared to other pesticides. In a recent study, Barascou et al. (2021) showed that azoxystrobin was eliminated 100-fold faster than sulfoxaflor. Although the mechanisms underlying this rapid metabolization of azoxystrobin are unknown, the authors suggested a potentially higher substrate specificity of the cytochrome P450 monooxygenases, as is known to be the case for the metabolization of tau-fluvalinate that is driven by the CYPQ9 family of cytochrome P450s (Mao et al., 2011).

Glyphosate had no impact on either feeding or colony development parameters. Studies exploring the impact of glyphosate on bees, especially on bumblebees, are relatively scarce, but a recent meta-analysis (Battisti et al., 2021) synthesised the current state of knowledge. Observed effects found in the literature at the concentrations used in this experiment (3.6 and 7.8 mg/L) are variable and at times contradictory. Vázquez et al. (2018) showed that *A. mellifera* larvae fed with 1.25 mg/L and 5 mg/L of glyphosate exhibited delayed ecdysis as well as reductions in weight and survival. Conversely, another study showed that the mortality of adult workers of *A. mellifera* orally administered two concentrations of GLY, 2.5 mg/L and 5 mg/L, for 15 days were not impacted (Herbert et al., 2014). To our knowledge, the only demonstrated effects of glyphosate on bumblebees are on social thermoregulation after chronic exposure (Weidenmüller et al., 2022), and on fine-colour discrimination after a field-realistic acute exposure experiment (Helander et al., 2023). Various studies have shown that newly emerged larvae and adults of *A. mellifera* exposed to glyphosate showed reduced expression of several detoxification-related genes (Gregorc et al., 2012; Tomé et al., 2020; Vázquez et al., 2020). This suggests that although glyphosate is not directly harmful, it can make bees more sensitive to other agrochemicals. Finally, glyphosate, being an herbicide, it is important to consider that it can diminish food resources by decreasing the diversity of plants around the crop, thereby reducing pollen and nectar accessibility (Battisti et al., 2021).

In contrast to herbicide and fungicide exposure, insecticides at high concentrations (S3, (sulfoxaflor 1000 ppb), S4 (sulfoxaflor 2000 ppb) and CY (cyantraniliprole 6200 ppb) impacted each of the evaluated parameters (i.e., resource consumption and brood development). Studies focusing on sulfoximine insecticides are currently limited (Siviter and Muth, 2020). However, this class of chemicals shares the same mode of action to neonicotinoids, i.e., interfering with nerve impulse transmission of the central nervous system via modulation of the nicotinic acetylcholine receptors. These molecules cause tremors, followed by paralysis and death of the exposed targets (Cutler et al., 2013; Matsuda et al., 2001). In addition to such lethal effects, neonicotinoids are also known to induce various sub-lethal effects that can lead to a loss of fitness, including locomotion, reproduction, cognition, olfaction, and nutritional intake (Barraud et al., 2020; Hesselbach and Scheiner, 2018; Kessler et al., 2015; Thompson et al., 2015; Tosi et al., 2017a; Tosi et al., 2017b; Tomé et al., 2012). Reduction in resource consumption following sulfoxaflor exposure has been reported in other laboratory experiments with different bee species (Barascou et al., 2021; Linguadoca et al., 2021; Schwarz et al., 2024). The reductions in nectar and pollen collection observed in this study may imply that we are not observing a simple repellent effect, given that pollen did not contain any pesticides in our experiments, and that exposure to sulfoxaflor leads directly to a

reduction in nutritional intake, as has been suggested in other studies using neonicotinoids (Kessler et al., 2015; Thompson et al., 2015; Tosi et al., 2017b). The observed effects on brood development and pollen efficacy were in line with the results of other studies (Linguadoca et al., 2021; Schwarz et al., 2024). However, the low concentrations used did not affect the bumblebees in our experiments, while Linguadoca et al. (2021) found lethal effects at 1.37 mg/kg and sublethal effects at 0.16 mg/kg. Here, under field realistic concentrations (20 ppb, S1 and 300 ppb, S2), we did not observe any effect on our measured parameters. Nonetheless, (Tamburini et al., 2021a) observed reductions in foraging performance and colony growth in a semi-field experiment using regulatory application rates of sulfoxaflor. While this reinforces the idea that bees are less sensitive under laboratory conditions (see the previous discussion of fungicides), it also suggests that further studies are needed to better assess the risks associated with this pesticide.

Bumblebees exposed to the insecticide cyantraniliprole were also highly impacted, with a reduction in resource consumption and a higher reduction of both brood development and pollen efficacy compared to the other treatments. While some studies have investigated the effects of acute and chronic exposure to this compound on adult and larval mortality (Kim et al., 2022; Mundy-Heisz et al., 2022), to our knowledge, there are no studies that have examined sub-lethal parameters using this pesticide.

Finally, we did not find a significant interaction between sulfoxaflor and Amistar®. Fungicides can indirectly impact bees by increasing insecticide toxicity (Sgolastra et al., 2018; Tosi et al., 2017b; Tosi and Nieh, 2019). To date, the results of various studies conducted using different species in the laboratory or under semi-field conditions have been mixed. While some studies have established an interaction between sulfoxaflor and a fungicide (Linguadoca et al., 2021; Tamburini et al., 2021a), others have not (Azpiazu et al., 2021; Schwarz et al., 2022; Tamburini et al., 2021b; Wintermantel et al., 2022). More studies should be conducted to better understand the situations in which an interaction can occur.

4.1.3. Interactions between diet and pesticides

As there were no significant impacts of herbicide or fungicide exposure under our experimental conditions, we focus on the interaction between diet and insecticides (i.e., sulfoxaflor and cyantraniliprole) regarding colony development. It has been hypothesised that adequate nutrition could mitigate the negative effects of pesticides on bees (Ardalani et al., 2021; Barascou et al., 2021; Crone and Grozinger, 2021; Wahl and Ulm, 1983; Wong et al., 2018), through increased expression of detoxification genes (Johnson et al., 2012; Schmehl et al., 2014). Proteins, lipids, and carbohydrates serve as important energy sources for the detoxification of toxic compounds (Even et al., 2012), and adequate intake of these nutrients could therefore compensate for the increased energy demand during the detoxification process (du Rand et al., 2015).

In this study, we only observed additive negative effects of these two stresses following exposure to the insecticide sulfoxaflor. We confirmed the results of a recent meta-analysis reviewing the interactive effects of pesticides with further factors such as food stress, where the authors concluded that food stress and pesticide exposure generally act additively (Siviter et al., 2021). However, other studies have demonstrated synergistic interactions between pesticide exposure and nutritional stress in honeybees, bumblebees, and *Osmia* (Kopit et al., 2022; Linguadoca et al., 2021; Tosi et al., 2017b).

Nonetheless, other nutritional stresses may affect the bees in alternate ways that could lead to variability in the sensitivity to pesticides. In our study, the low quality of the pollen diet was linked to a lack of total amino acids and specific sterols (Barraud et al., 2022). Given the importance of proteins in the stress response, it would be interesting to test a pollen diet with a low protein content, or use pollen from some plants (e.g., *Asteraceae*) that contain toxic compounds that, coupled with exposure to a pesticide, could overwhelm the detoxification system already weakened by this pollen diet. In addition, several studies have shown that the pollen phytochemicals quercetin and p-coumaric acid can significantly enhance the tolerance to several pesticides (Liao et al., 2020; Wong et al., 2018). It would therefore be interesting to examine pollen diets with various quantities of these phytochemicals. Finally, we must keep in mind that nutritional stresses can also be caused by the quality/quantity of the consumed nectar. Linguadoca et al. (2021) have explored this question and showed that sulfoxaflor, coupled with a nutritional deficiency using different sugar solutions, synergistically reduced the survival and fecundity of bumblebee microcolonies.

Interestingly, no additive effects were observed with cyantraniliprole. Instead, there was no difference between the measured parameters of exposed bees fed the various pollen diets. This result was observed in a previous study in which brood development of bumblebees exposed to imidacloprid was more negatively impacted compared to colonies exposed to sulfoxaflor in the present study (Barraud et al., 2020). It seems that the bumblebees affected by pesticides could no longer benefit from a high-quality diet. Additionally, while pollen consumption was similar regardless of the diet quality for every other pesticide treatment, this was not the case when the bees were exposed to cyantraniliprole, with an observed increase in *Cistus* pollen consumption compared to *Salix* pollen. While this effect was observed in a previous study (Barraud et al., 2020), the reasons remain unknown. We suggest that when highly stressed, bumblebees will consume the minimum quantity necessary for their survival and for basic maintenance of the brood. *Cistus* pollen being of lesser quality, the bees must consume more to achieve the same result.

4.2. Effects on the peptidome/proteome of Bombus terrestris haemolymph (haemoproteome)

Mass spectrometry analyses indicated that diet quality (*Cistus*) and quantity (starvation) affected the peptide molecular mass fingerprints (MFPs) of bumblebee haemolymph. To our knowledge, this is the first study reporting such results on the peptidome of haemolymph from *Bombus terrestris*. For the same pesticide concentration, the MFPs discriminated between bumblebees fed with *Cistus* and those fed a reduced quantity of *Salix* as the diet. This suggests that when the diet is poor (quality or quantity), there is an impact on the composition of the bumblebee haemolymph (mass range covered from 600 Da to 18 kDa). This was not observed when bumblebees exposed to pesticides were fed a high-quality pollen diet. Moreover, we observed that under quantitative nutritional stress, the bumblebees were more sensitive to Amistar® and glyphosate than to sulfoxaflor. In a previous study (Roger et al., 2017), we reported that the constitutive immunity of *B. terrestris* increased when individuals were given unrestricted access to a high-quality diet.

In a complementary approach using off-gel bottom-up proteomics, we evaluated the impact of the pesticides in association with starvation on the *B. terrestris* haemoproteome. Among the 173 proteins quantified following exposure to pesticides under starvation conditions, several were observed to be modulated. Upon treatment with pesticides, the most up-regulated proteins were Hemocytin, Phospholipase A1 (PLA1), and the Chymotrypsin inhibitor, all of which are involved in multiple biological pathways. The variation of the lectin protein Hemocytin (the von Willebrand factor in humans), following exposure to Amistar® and glyphosate, could impact the pathways of O-glycosylation of TSR domain-containing proteins, termination O-glycan biosynthesis, platelet activation and degranulation, MAP2K and MAPK activation, integrin signalling, complement and coagulation cascades, Neutrophil extracellular trap formation, PI3K-Akt signalling, Focal adhesion, and ECM-receptor interaction. In insects, hemocytin is closely associated with systemic immunity; it is synthesised by haemocytes before maturation and secretion in the haemolymph following stimulation by pathogens. The protein facilitates coagulation, melanisation, nodulation, and encapsulation (reviewed in Hu et al., 2024). Hemocytin gene expression was confirmed to occur upon bacterial infection and before pupation

under naive conditions in the silkworm *Bombyx mori*, suggesting that Hemocytin plays an important role in immunity and also in metamorphosis (Yamakawa and Tanaka, 1999). Ni et al. (2020) reported that hemocytin facilitates the immune response of *B. mori* against *Nosema bombycis* infections and promotes hemocyte-mediated cellular immune responses.

Additionally, it has been reported that poor nutrition associated with parasites impacts immune gene expression. Brunner et al. (2014) reported that when *B. terrestris* was infected with the parasite *Crithidia bombi* in association with starvation, immune responses to infection were reduced (Brunner et al., 2014; Houdelet et al., 2021). Indeed, many genes failed to be up-regulated, including the humoral immune antimicrobial peptides (AMPs), in response to infection. The bumblebees also showed less specific immune expression patterns across individuals and colonies. In the present study, upon pesticide exposure, we observed a decrease of the expression of the AMPs Apidaecin and Defensin and of the Chymotrypsin inhibitor, a protease inhibitor involved in the immune response. This ties in with previous studies that examined bee exposure to *Nosema ceranae* (Chaimanee et al., 2012; Houdelet et al., 2021).

Phospholipase A1 (PLA1), one of the two highly abundant phospholipases in ants and bees (reviewed in Perez-Riverol et al., 2019), is involved in phospholipid hydrolysis. PLA1 was up-regulated in *B. terrestris* haemolymph upon exposure to Amistar® at the low dose of 200 ppb when bees were fed a reduced quantity of *Salix* pollen. A PLA1 was first isolated from the haemolymph of the tobacco hornworm *Manduca sexta* (Nieder and Law, 1983). PLA1 is involved in innate immunity through lipid peroxidation mechanisms (Weismann and Binder, 2012); in human-embryonic kidney cells and liver cancer cells, PLA1 facilitates the recruitment of tank-binding kinase 1 to mitochondria to prevent alterations in morphology (Gao et al., 2018).

Interestingly, following exposure to sulfoxaflor, there was no impact on the variation of the immune peptides investigated in this study. However, the off-gel bottom-up haemoproteome analyses revealed that sulfoxaflor at low concentrations increased the Chymotrypsin inhibitor (serine protease inhibitor/serpins) with a fold-change of 100. This Chymotrypsin inhibitor is important for immune manipulation and pathology mediation (Patel, 2017). However, the protein serine protease inhibitor 88Ea (Spn 88Ea or Spn5), which belongs to the serpin family, was down-regulated following exposure to sulfoxaflor. It is well documented that serine protease inhibitors, as is the serine protease itself, are ubiquitous in all living organisms. Functionally, both components are interdependent and are essential for blood coagulation, melanisation, and the production of AMPs (Patel, 2017; Shakeel et al., 2019). In the fruit fly *Drosophila melanogaster*, Spn88Ea negatively regulates the Toll signalling pathway and suppresses the expression of the antifungal peptide drosomycin and the melanisation response through the phenoloxidase (PPO1) cascade (Khan and Graze, 2024). This protein possesses functions related to the immune response in *D. melanogaster* in addition to the four *Drosophila* serpins Spn43Ac, Spn27A, Spn28Dc, and Spn77Ba (Reichhart et al., 2011). In insects, as in all living organisms, the innate immune system plays a major role in the defence reactions against stressors. Here, serine protease was identified as essential in the initiation of innate immune responses, including melanisation and the production of AMPs, and is precisely regulated by Serpins (Shakeel et al., 2019). Serpin 88Ea identified in the present study is involved in Common Pathway of Fibrin Clot Formation, Glucocorticoid biosynthesis, COPII-mediated vesicle transport, Regulation of Insulin-like Growth Factor (IGF) transport and uptake by Insulin-like Growth Factor Binding Proteins (IGFBPs), Intrinsic Pathway of Fibrin Clot Formation, ECM proteoglycans, Platelet degranulation, Cargo concentration in the ER, Neutrophil degranulation, post-translational protein phosphorylation, and Amoebiasis.

Two other proteins were down-regulated following exposure to high doses of Amistar®: Fibrillin-2 and DE-cadherin. As for Spn88Ea, Fibrillin-2 is involved in the regulation of IGF transport and uptake by IGFBPs. In addition, Fibrillin-2 functions in Integrin cell surface interactions, post-translational protein phosphorylation, and elastic fibre formation. Zeyer and Reinhardt (2015), reported that Fibrillins strongly regulate pathways of the immune response, inflammation, and tissue homeostasis. In *B. terrestris* infected by the trypanosome *Crithidia bombi*, the gene coding for fibrillin was down-regulated (Riddell et al., 2014). Thus, the down-regulation of this protein in our study may reflect the disturbance of *B. terrestris* physiology upon chemical exposure. The last down-regulated protein, referred to as the *Drosophila* E-cadherin (DE-cadherin), is a calcium-dependent cell adhesion protein. Cell adhesion is essential in the cellular immune responses of encapsulation and nodule formation in invertebrate immunity (Johansson, 1999). In vertebrates, E-cadherin is a central protein in epithelial cell adhesion (Van den Bossche and Van Ginderachter, 2013) and an immunological modulator following environmental challenge in airway epithelia (Nawijn et al., 2011). A more recent study in humans by Devaux et al. (2019) suggested that the reduction of the surface expression of E-cadherin on epithelia could be accompanied by alteration of the antibacterial and anti-tumoral immune responses.

Under our experimental conditions, the major enzyme superfamilies responsible for the metabolism or detoxification of xenobiotics (Cytochrome P450, Glutathione transferases, and Carboxylesterases) (Gong and Diao, 2017) were not detected. However, the abundances of the identified ROS detoxification proteins, such as Thioredoxin, Superoxide dismutase [Cu—Zn], and Peroxiredoxin 1, were unaffected by pesticides and nutritional stresses under our experimental parameters (concentration and duration). On the one hand, this could be explained by specific responses indirectly linked to ROS detoxification systems without down- or up-regulation of the proteins. On the other hand, this absence of variation may be linked to the pesticide concentration not being high enough to induce ROS and hence their detoxification.

5. Conclusions

Contrary to expectation, this study has shown that under laboratory conditions, a poor diet does not necessarily impact the sensitivity of bumblebees to pesticides and does not amplify their effects with respect to development and resource consumption parameters. However, when strongly affected by pesticides, bumblebees fail to benefit from a high-quality diet. This may be important for bee conservation measures: enhancing the accessibility and diversity of floral resources may fail to be effective if the use of pesticides is not monitored (i.e., by suitable regulation, reduction, or banning of unsafe compounds). Moreover, proteomic analyses showed significantly different profiles upon exposure to pesticides, even when the colony parameters were unaffected by the pesticide. In fact, we observed no difference in the haemolymph profiles between samples collected from bumblebees fed with *Cistus* and those from starved bees under normal conditions (Controls). In contrast, when the bees were exposed to pesticides, there were differences in the haemolymph profiles between the *Cistus* and the starvation treatments. This could indicate that other unmeasured parameters may have been affected, or that the effects could be observed under less favourable natural conditions where floral resources are more difficult to collect or other stresses (e.g., unfavourable temperatures, infection by parasites and pathogens, fragmentation of forage and nesting habitats). Overall, our work provides new insights into how diet and pesticides could impact bumblebee physiology. We urge conservation action to support pollinators with suitable and diverse floral resources, along with improved risk assessment regarding pesticides.

CRediT authorship contribution statement

Alexandre Barraud: Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Dalel Askri:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Lena**

Barascou: Writing – review & editing, Methodology. **Janine M. Schwarz:** Writing – review & editing, Resources, Methodology. **Yusuf Toktas:** Writing – review & editing, Methodology, Investigation. **Louisane Nicodème:** Writing – review & editing, Methodology, Investigation. **Cedric Alaux:** Writing – review & editing, Methodology, Funding acquisition. **Matthias Albrecht:** Writing – review & editing, Methodology, Funding acquisition. **Karim Arafah:** Writing – review & editing, Methodology. **Michel Bocquet:** Writing – review & editing, Methodology. **Maryse Vanderplanck:** Writing – review & editing, Supervision, Methodology. **Philippe Bulet:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. **Denis Michez:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization.

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Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2025.180716.

Data availability

The mass spectrometry proteomics data generated in this study have been deposited in the Proteome Xchange Consortium via the PRIDE partner repository with the dataset identifier PXD043718. The MALDI-MS data are available through the following weblink https://figshare.com/s/c59f54ae15695a0a8c51

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