Metabolomics unveils the influence of dietary phytochemicals on residual pesticide concentrations in honey bees

Ardalani, Hamidreza; Vidkjær, Nanna Hjort; Kryger, Per; Fiehn, Oliver; Fomsgaard, Inge S.

Published in:
Environment International

DOI:
10.1016/j.envint.2021.106503

Publication date:
2021

Document version
Publisher's PDF, also known as Version of record

Document license:
CC BY-NC-ND

Citation for published version (APA):
Metabolomics unveils the influence of dietary phytochemicals on residual pesticide concentrations in honey bees

Hamidreza Ardalani a, Nanna Hjort Vidkjær a,b,*, Per Kryger a, Oliver Fiehn c, Inge S. Fomsgaard a,b,**

a Department of Agroecology, Aarhus University, Forsøgsvej 1, 4200 Slagelse, Denmark
b Department of Biology, Section for Ecology and Evolution, University of Copenhagen, Universitetsparken 15, 2100 Copenhagen East, Denmark
** NIH West Coast Metabolomics Center, University of California Davis, Davis, CA, USA

ARTICLE INFO
Handling Editor: Martí Nadal

Keywords:
Honey bee
Neonicotinoid
Imidacloprid
Pesticide
Metabolomics
Flavonoid

ABSTRACT
The losses of honey bee colonies and declines of other insect pollinators have been associated with negative effects of pesticides. Honey bees as well as other pollinators are nectar and pollen foragers and thus are exposed to an extensive range of phytochemicals. Understanding the synergistic, additive, and antagonistic effects of plant secondary metabolites and pesticides in honey bees may help to protect honey bee colonies against agrochemicals. In this study, we used untargeted metabolomics to investigate the impact of dietary phytochemical composition on the residual concentration of three pesticides: imidacloprid, tau-fluvalinate and tebuconazole in exposed honey bees. Honey bees were given different diets based on pollen or nectar from four plants: Reseda odorata, Borago officinalis, Phacelia tanacetifolia, and Trifolium repens for two days. Thereafter, they were orally exposed to 10 ng/bee imidacloprid or contact-exposed to 0.9 μg/bee tau-fluvalinate or 5 μg/bee tebuconazole. After 1 h of oral exposure or 24 h of contact exposure, the honey bees were anaesthetised with CO₂, sacrificed by freezing, extracted with a validated QuEChERS method, and residual pesticide concentrations were determined by LC-QTRAP-MS/MS. The phytochemical composition in the given diets were profiled with an UHPLC-Q Exactive-MS/MS. The results revealed that the dietary phytochemical composition has a noteworthy influence on the concentration of residual pesticides in honey bees. The correlation coefficient analysis demonstrated that flavonoids have a reducing effect on the residual concentration of imidacloprid and tau-fluvalinate in honey bees. The results also highlighted that exposure to imidacloprid impaired the metabolism of sugars in honey bees. Exploiting flavonoid-rich plants may protect honey bees against pesticides and hold promise as forage plants in future beekeeping.

1. Introduction

The honey bee (Apis mellifera L.) is an important species due to its contribution to pollination services and food production (vanEngelsdorp and Meixner, 2010). Honey bees as well as other pollinators, are nectar and pollen foragers and thus are exposed to an extensive array of pesticides and phytochemicals in their diet (Irwin et al., 2014a; Crenna et al., 2010). Phytochemicals are bioactive organic compounds that occur naturally in plants and some have disease preventing or protective properties. More than 5000 chemical structures of phytochemicals belonging to different chemical classes are known. Pesticide exposure, in addition to parasites and changing floral resources, have been linked to colony losses in honey bees (Goulson et al., 2015). Flowers visited by honey bees may be contaminated with a range of pesticides and honey bees may be exposed to these chemicals during food collection (Callaway et al., 2018). Pesticide poisoning of honey bees from contaminated pollen and nectar may have severe acute and chronic consequences including depletion of hive bees and death of the whole colony (Suchail et al., 2001; Dively et al., 2015). Chemical analysis has revealed that most honey bee colonies in North America and Europe are contaminated with a range of pesticides including insecticides, acaricides, and fungicides (McMullan and Brown, 2006; Chauvat and Faucon, 2015).

https://doi.org/10.1016/j.envint.2021.106503
Received 7 November 2020; Received in revised form 1 March 2021; Accepted 2 March 2021
Available online 20 March 2021
© 2021 Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
2007; Mullin et al., 2010). According to the model proposed by Rumkee et al. (2001; David et al., 2016; Harriet et al., 2017), beekeepers use tau-fluvalinate as a moderate toxicity in-hive acaricide to control the Varroa mite (Johnson et al., 2010), an ectoparasite in honey bee colonies (Mullin et al., 2010; Francis et al., 2013; Fulton et al., 2019). Because of the moderate toxicity of tau-fluvalinate to honey bees, it has been used excessively in honey bee colonies and has been frequently observed in bee wax samples in Virginia, USA (Fulton et al., 2019), beebread in Spain (Calatayud-Vernich et al., 2019), and in honey bees in the Czech Republic (Erban et al., 2019a). Tau-fluvalinate is also used to control pests in the fruit-growing areas (Duso et al., 2014). Tebuconazole and tau-fluvalinate have low and moderate toxicity to honey bees, respectively (LD₅₀ = 200 μg/beel and >12 μg/beel, respectively). Beyond the acute effect of pesticides, chronic toxicity of agrochemicals have received attention for their negative effects on pollinator health (Johnson et al., 2010). Understanding the mechanisms contributing to insecticide, acaricide and fungicide metabolism is necessary to reveal synergistic and antagonistic interactions with phytochemicals (EFSA, 2008, 2010a; EFSA, 2015; Ardalani et al., 2021). Many factors such as nutrition, environment, and genetics have the potential to influence the responses of honey bees to pesticides (Poquet et al., 2016; Tosi et al., 2017).

It has recently been established that dietary phytochemicals are taken up from the guts of honey bees (Vidkjær et al., 2021) and positive effects of dietary phytochemicals on longevity, pathogen tolerance, detoxification system, and behavioural responses of honey bees have already been demonstrated (Mao et al., 2013; Liao et al., 2017a; Bernklau et al., 2019). Moreover, the beneficial effects of different classes of secondary metabolites in plants, such as phenols, flavonoids, and alkaloids, have been shown in honey bees (Wright et al., 2013; Liao et al., 2017b; Ardalani et al., 2021). However, dietary phytochemicals can also influence honey bees negatively (Wong et al., 2018). Indeed, recent studies have indicated that phytochemical profiles in plants play key roles in biochemistry, physiological, and behavioral responses of pollinators (Irwin et al., 2014b).

Understanding the interaction between dietary phytochemicals and pesticides is vital to enhance honey bee development, productivity, and colony growth, which will help maintaining healthy and sustainable colonies in future beekeeping. The aim of this study was to examine the effects of dietary pollen and nectar from various plants on the residual concentrations of three pesticides to which honey bees had been exposed: imidacloprid, tau-fluvalinate and tebuconazole. Mass spectrometry-based untargeted metabolomics was used to investigate the phytochemical profile of pollen from Reseda odorata, Borage officinalis, Phacelia tanacetifolia, and Trifolium repens, and of fresh collected nectar from Reseda odorata and Borage officinalis. The relation of the phytochemical profiles with residual pesticide concentration in honey bees was determined.

2. Materials and methods

2.1. Chemicals and reagents

Imidacloprid (98% purity), tebuconazole (99.9% purity), tau-fluvalinate (91.2% purity), ammonium formate (99% purity), and sucrose (99.5% purity) were purchased from Sigma-Aldrich (Steinheim, Germany). Acetic acid was purchased from Fisher Scientific (Leicester-shire, UK). Formic acid was purchased from Merck (Darmstadt, Germany). Methanol, acetonitrile (HPLC grade), and acetone were purchased from Rathburn (Walkerburn, UK). Bond Elut QuEChERS dispersive kits (2 ml) were procured from Agilent (Glostrup, Denmark). MgSO₄ (99% purity) and CH₃COONa (100% purity) were obtained from VWR Chemicals (Herlev, Denmark). A Milli-Q system (Millipore, MA, USA) was used to deliver water for the LC-MS/MS analyses. HPLC-MS grade water was purchased from Fisher Scientific for the UHPLC-Q Exactive-MS/MS analyses.

2.2. Honey bees specimens

A colony of honey bees was maintained at the Department of Agroecology, Aarhus University, Flakkebjerg Research Centre (55° 19’ 32” N, 11° 23’ 24” E), Denmark. The colony strength was considered normal for the season of late summer. Eleven frames with bee bread (approximately 1 kg pollen each) were obtained from an isolated organic oilseed rape farm to avoid pesticide influence (Asnæs, Sealand, Denmark: N 55°39’45, E 11°00’38) and stored in a freezer until usage (~20 °C). A new bee bread frame was introduced into the brood producing hive each Monday, Wednesday and Friday. In order to further minimize the risk of pesticide contamination, the hive entrance was closed. After two weeks, frames with capped brood were taken from the hive, moved into a dark incubator, kept at 35 °C and 30–50% humidity. After one day, all newly hatched bees (0–24 h old) were collected from the frames in the incubator and ~ 600 honey bees transferred to a nucleus (smaller version of a normal beehive) colony (24 × 15 × 15 cm) (Apidea, Switzerland) for feeding (2.4). In order to obtain the sufficient number of newly hatched bees, the collection of frames with capped brood was repeated seven times.

2.3. Pollen and nectar collection

Seeds of R. odorata (Hornum, Denmark) and B. officinalis (UAB Agrofirma, Lithuania) were sowed in 450 two-liter pots, which after two–three weeks were transferred to a greenhouse (50 m²) constructed with a plastic roof and fine mesh on all sides. Once the plants started blooming, a nucleus colony consisting of three combs (10 × 10 cm) plus a few capped brood cells, sucrose paste, and worker bees (~500) were placed in the greenhouse. The colony was inspected every two days, and combs, filled with either pollen or nectar, were replaced with an empty waxcomb. After harvest, the combs were immersed in liquid nitrogen, and pollen and nectar were extracted separately. The pollen of P. tanacetifolia and T. repens were grown organic (without use of pesticides) on free land and was collected from a pollen trap mounted under a beehive placed in the field. The collected pollen was subsequently sorted based on the colours, dark blue (P. tanacetifolia) and brown (T. repens), respectively. The plants were selected based on two criteria: known to attract honey bees (Kryger et al., 2011), and representing various phytochemical profiles. Pollen and nectar were kept at ~ 20 °C until use for feeding to a new set of bees (2.4).

2.4. Feeding of honey bees

Pollen paste was prepared by grinding the defrosted pollen samples (Reseda pollen, Borage pollen, Phacelia pollen, Trifolium pollen) to a fine powder with pestle and mortar, and mixing (1:1 w/w) with sucrose-water solution (50% w/v). The collected nectar samples (Reseda
nectar and *Borago* nectar) were defrosted at room temperature. Each nectar or pollen paste sample was distributed on 96-well plates, attached to a comb, and hung vertically in the nucleus colonies. Next, ~ 600 newly hatched honey bees were transferred to each nucleus colony and kept at room temperature for 48 h. The control group received sucrose paste consisting of a mixture of (1:1 w/w) sucrose powder and sucrose-water solution (50% w/v). The entire feeding experiment for all diets was executed in 7 days.

2.5. Pesticide exposure

The pesticide exposure was performed according to Ardalan et al., (2021) with minor modifications. In short, after 48 h of feeding, each nucleus colony of honey bees was transferred to a fume hood and anesthetized with CO₂. Twenty subgroups, each with 30 bees, were prepared by taking paralyzed honey bees gently with soft tweezers and placing them in 20 wooden cages. Four subgroups of bees were orally exposed to 10 ng/bee imidacloprid, four subgroups were contact-exposed to 5 µg/bee tebuconazole and four subgroups were contact-exposed to 0.9 µg/bee tau-fluvalinate. Four subgroups served as oral-control, receiving only sucrose-water solution (50% w/v), and the last four subgroups (contact-control) were contact exposed to 1 µl acetone and thus served as contact exposure control groups (Fig. S1).

For the oral-exposure of honey bees to imidacloprid: after starving for 1 h, small caps with 150 µl of sucrose-water solution (50% w/v) spiked with 2 µg/ml of imidacloprid were placed into the wooden cages. Honey bees ingested all of the spiked solution immediately after its presentation.

For contact-exposure of honey bees to tebuconazole and tau-fluvalinate, paralyzed honey bees were taken gently with soft tweezers and exposed to 1 µl of either tebuconazole (5000 µg/ml) or tau-fluvalinate (900 µg/ml) with a syringe dispenser (Hamilton, USA) directly on their thorax.

Afterward, orally-exposed honey bees were incubated for 1 h and honey bees which were contact-exposed to either tebuconazole, tau-fluvalinate or acetone, were incubated for 24 h. The same food that each subgroup had consumed during the feeding was provided during incubation. After incubation, the honey bees were anesthetized with CO₂ and immediately sacrificed by freezing at −20 °C.

2.6. Extraction of pesticides from honey bees and phytochemicals from pollen and nectar samples

The frozen honey bees were lyophilized for 72 h in a freeze dryer (Heto Drywinner, Birkerød, Denmark). From each subgroup (30 honey bees), 15 dried honey bees were chosen, weighed individually with four decimal accuracy, and placed in three small containers with five honey bees in each (each subgroup thus generating 12 replicates) (Fig. S1).

After adding three metal balls to each container, the honey bees were ground to a fine powder using a mechanical grinder (Spee Certiprep-2000, Metuchen, USA) at 1500 S/min for 1 min. The 4.5 ml of extraction solvent (water:acetonitrile:acetic acid, 44:55.1: v/v) was then added. The mixtures were shaken again (1500 S/min for 30 s) before and after adding 1 g of MgSO₄ and 0.25 g of CH₂COONa. The homogenized extracts were transferred into 15 ml Falcon tubes and centrifuged at 4500 g and 21 °C for 10 min (Heraeus Multifuge 35-R, Thermo Fisher Scientific, USA). The supernatants (−2.5 ml) were carefully transferred to 5 ml brown vials, and 1 ml from each was transferred to 2 ml Bond Elut QuEChERS dispersive SPE tubes (50 µg of PSA, 50 µg of C18EC, 150 µg of MgSO₄), vortexed for 1 min, and centrifuged at 16,602 g and 21 °C for 10 min (Sigma 1–14 K, Oстро, Germany). Afterward, the supernatants (−0.5 ml) were collected, filtered through 0.22 µm KX PTFE syringe filters (Mikrolab, Aarhus, Denmark), and diluted with methanol:water (1:1 v/v) before analysis (Ardalan et al., 2021).

Three replicates of two grams of pollen and nectar samples representing the pollen and nectar that were fed to the honey bees initially were transferred to small containers and freeze-dried for 72 h. Ten milligrams of each sample was transferred to a new 1.5 ml Eppendorf tube, and 1.5 ml of extraction solvent (chloroform:methanol:water, 2:5:2) was added. The mixtures were vortexed for 20 s, sonicated for 5 min, shaken for 20 min in 4 °C, and centrifuged at 16,602 g and 21 °C for 10 min. Subsequently, 0.6 ml of the supernatant was collected in a new Eppendorf tube and dried in a SpeedVac (SPD212P, Thermo Scientific, USA) for 8 h. Dried samples were resuspended in 0.5 ml of acetonitrile:water (1:1 v/v) before analysis. Method blanks, consisting of empty Eppendorf tubes and pooled extracts of pollen and nectar samples, were included as quality control samples.

2.7. Pesticide quantification

The chromatographic separation of the three pesticides was performed on an Agilent 1200 HPLC system (Santa Clara, CA, USA) coupled to an AB Sciex (Forest City, CA, USA) 3200 QTRAP mass spectrometer. The entire system was controlled by Analyst software (version 1.6.2). The mass spectrometer was equipped with electrospray ionization (ESI), operated in multiple reaction monitoring mode (MRM), and data were recorded in positive mode. Nitrogen was used as both the source and collision gas. The instrument source operating parameters for the QTRAP mass spectrometer were set as follows: curtain gas (psi), 20; ion spray voltage (V), 4500; temperature (°C), 450; gas 1 (psi), 60; and gas 2 (psi), 50. The optimized compound-dependent parameters were set according to Ardalan et al. (2021) as shown in the Supplementary Material (Table S1). The two most intense ion transitions were chosen for each compound: the most intense peak was used as the quantifier, and the other was used as the qualifier. Stock solutions were serially diluted to concentrations of 800 to 0.09 ng/ml and used for the external calibration curves. The calibration curves were fitted by least-squares regression using 1/y as the weighting factor of the peak area. To calculate the pesticide concentrations in ng/bee, the weight of the freeze-dried honey bees was taken into consideration.

All three pesticides were separated on a Kinetex biphenyl column (100 mm × 2.1 mm × 2.6-µm particle size; Phenomenex, Macclesfield, UK) with a flow rate of 200 µl/min and an injection volume of 20 µl. The temperature of the column oven was set at 40 °C, and the autosampler temperature was set at 10 °C. The mobile phases were (A): 1% methanol with 0.2% formic acid, and (B): 99% methanol with 0.2% formic acid. The gradient was as follows: 0 min, 10% B; 2 min, 10% B; 6 min, 95% B; 15 min, 100% B; 20 min, 100% B; 20.1 min, 10% B; 25 min, 10% B. Before the next injection, the column was re-equilibrated with the initial gradient condition for 10 min. The validation of the method previously reported (Ardalan et al. 2021) and the recoveries for the three pesticides were within the acceptable range 60–140% (SANTE/11813/2017, 2017).

2.8. Phytochemical profiling of pollen and nectar samples

The phytochemical profiling of pollen and nectar was carried out via a Thermo Vanquish UHPLC coupled to a high-resolution Thermo Q Exactive HF mass spectrometer. The whole system was controlled by Xcalibur software (version 4.2.28). Spectra were acquired in full scan ESI(-) modes with a mass range of m/z 70–1000 Da. Full scan MS spectra were acquired with a Thermo Vanquish UHPLC coupled to a high-resolution Thermo Q Exactive HF mass spectrometer. The entire system was controlled by Analyst software (version 1.6.2). The mass spectrometer was equipped with electrospray ionization (ESI), operated in multiple reaction monitoring mode (MRM), and data were recorded in positive mode. Nitrogen was used as both the source and collision gas. The instrument source operating parameters for the QTRAP mass spectrometer were set as follows: curtain gas (psi), 20; ion spray voltage (V), 4500; temperature (°C), 450; gas 1 (psi), 60; and gas 2 (psi), 50. The optimized compound-dependent parameters were set according to Ardalan et al. (2021) as shown in the Supplementary Material (Table S1). The two most intense ion transitions were chosen for each compound: the most intense peak was used as the quantifier, and the other was used as the qualifier. Stock solutions were serially diluted to concentrations of 800 to 0.09 ng/ml and used for the external calibration curves. The calibration curves were fitted by least-squares regression using 1/y as the weighting factor of the peak area. To calculate the pesticide concentrations in ng/bee, the weight of the freeze-dried honey bees was taken into consideration.
and probe heater temperature at 370 °C. Nitrogen was used as both the source and collision gas. A UPLC Waters Acquity BEH C18 column (2.1 mm × 50 mm, 1.7-µm particle size; Milford, MA, USA) was used for chromatographic separation of pollen and nectar compounds with a flow rate of the 400 µL/min and an injection volume of 5 µL. The temperature of the column oven was set at 45 °C. The mobile phases were (A): 100% LC-MS grade water with 0.1% acetic acid and (B): acetonitrile with 0.1% acetic acid. The gradient was as follows: 0 min, 4% B; 2 min, 4% B; 10 min, 96% B; 12 min, 96% B; 12.5 min, 4% B; 15 min, 4% B. To check the performance of the instrument, a quality control sample consisting of pooled extracts of pollen and nectar samples was injected every ten runs.

2.9. Data processing, statistics, and visualization

A one-way ANOVA with a post-hoc Tukey’s test was applied to compare the concentrations of pesticides between groups and the P values lower than 0.05 were considered significant. The data were checked for a normal distribution. Comparisons were performed using GraphPad Prism version 8.4.2 (La Jolla California, USA). The metabolization ratio of pesticides were calculated as shown:

\[ x = \frac{(\alpha - \beta)}{\alpha} \times 100 \]

where \( x \) is the metabolization ratio, \( \alpha \) is the amount of exposed pesticide to each bee (ng/bee), and \( \beta \) is the amount of pesticide (ng/bee) detected after 1 h for orally exposed pesticide (imidacloprid) and 24 h for contact-exposed pesticides (tau-fluvalinate and tebuconazole).

After recording phytochemical profiles of the pollen and nectar samples, the raw data files (.RAW) were converted into ABF files using the freely available converter (https://www.refsys.com/AbfConverter/index.html) (Lai et al., 2018). MS-DIAL software (ver. 3.50) was used for spectra deconvolution, peak alignment, gap filling, and compound annotations were performed by MS/MS spectral similarity matching using the NIST17 (https://chemdata.nist.gov) and MoNA (https://mona.fiehnlab.ucdavis.edu) MS/MS libraries. Representative MS/MS spectra, MS² isotopic spectrum, compound annotation, adducts, reverse-dot score, tentative formula, InChIKey, and peak heights were extracted from MS-DIAL in an MSP format for further analysis (Tsuwawa et al., 2015). After initial processing, MS-FLO was used to improve the quality of feature lists to expedite the process of data curation by removing duplicate peaks, isotopes and adducts (Defelice et al., 2017). The parameters used in MS-DIAL and MS-FLO can be found in Table S2. The entire dataset was inspected manually and only the phytochemicals which had a considerable similarity to the MS/MS spectra in libraries were kept and considered for the statistical analysis.

The data were transformed by log transformation followed by Pareto scaling. Principle component analysis (PCA) was performed for all the annotated compounds (reverse-dot score < 750) in positive and negative ionization modes with SIMCA version 15.0 (Umea, Sweden). The number of annotated compounds in each individual diet was visualized by a Venn diagram (Heberle et al., 2015). A one-way ANOVA with a post-hoc Tukey’s test was applied to find the significant phytochemicals among the diet samples and the P values lower than 0.001 were considered significant (please see the list of P values in the Supplementary Material). A hierarchical clustering heatmap of the normalized phytochemicals (peak heights were log-transformed and Pareto scaled) was created using MetaboAnalyst version 4.0 (Chong et al., 2018). MetaboAnalyst was also used to analyze the Pearson correlation between the concentration of pesticides and annotated compounds (Xia and Wishart, 2011) (please see the list of correlation P values in the Supplementary Material).

The annotated compounds were classified based on their InChIKeys algorithm by ClassyFire (Feunang et al., 2016) (The chemical names and corresponding InChIKeys can be found in the Supplementary Material).

3. Results and discussion

3.1. Phytochemical profiling of pollen and nectar samples

Data processing with MS-DIAL resulted in the detection of 12,461 peaks in positive and negative ionization modes of which 315 phytochemicals were annotated by MS/MS spectral similarity matching using public and licensed MS/MS libraries (list of annotated phytochemicals in the Supplementary Material). Phytochemicals were classified at super-class and class levels in the ClassyFire system and 38% were grouped as lipids and lipid-like molecules, 27% were grouped as phenylpropanoids plus polyketides, and 9% were grouped as organic acids at the superclass level. At the class level, the most abundantly annotated compounds in all pollen and nectar samples were flavonoids with 22%, fatty acids with 20%, and glycerophospholipids with 11% (Fig. 1).

The Venn diagram revealed the chemical differences and similarities among the six diets, of which Phacelia pollen (284) and Borago nectar (195) had the highest and lowest number of annotated phytochemicals, respectively. There were 115 shared phytochemicals distributed in all six diets (Fig. 2).

The PCA score plots (PC1: 31%, PC2: 22%) displayed clear and separate clustering of the four pollen diets in terms of annotated phytochemicals. The two nectar diets did not separate distinctly; this result can be explained by the fact that both nectar diets mainly contain carbohydrate molecules (Fig. 3A).

The PCA biplot (Fig. 3B) as well as the hierarchical clustering heatmap (Fig. 3B) revealed information on discriminating compounds, among which quercetin 3-O-glucose-6'-acetate, malvidin 3,5-digluco-side, 1-dilinolenoyl lecithin, luteolin 7-glucoside, and isouqueritin were the most significant phytochemicals among the diets.

The one-way ANOVA test with 315 annotated phytochemicals unveiled 282 significantly different phytochemicals (peak intensity) among the diets (P ≤ 0.001) (Fig. S3) (list of P values in the Supplementary Material).

The hierarchical clustering heatmap of the 25 most significant peak intensities (one-way ANOVA, P ≤ 0.05) of the phytochemicals among the diets were shown in Fig. 3B. The concentration of almost all phytochemicals in nectar samples (Borago and Reseda) was lower than in the pollen samples (Borago, Reseda, Phacelia, and Trifolium). The phytochemicals with the highest peak intensity in Borago pollen were the fatty acid cis-4,10,13,16-docosatetraenoic acid, the sugar maltose, and a diterpenoid isosteviol, whereas the flavonoids malvidin-3,5-diglucoside, luteolin-7-glucoside, and cyanidin-3-glucoside were the dominant compounds in Phacelia pollen. Quercetin 3-O-glucose-6'-acetate and kaempferitin were the most dominant compounds in Trifolium pollen and Reseda pollen, respectively. Overall, 9 of the 25 most significant phytochemicals among all the diets were flavonoids which were mainly found in the pollen samples from Phacelia, Reseda, and Trifolium (Fig. 3B).

The PCA score plots (PC1: 36%, PC2: 17%) confirmed an accurate instrumental performance on the basis of the close clustering of the QC and blank samples (Fig. S4).

3.2. Influence of diets on the concentration of imidacloprid in honey bees

The concentration of imidacloprid in honey bees fed Reseda pollen and Phacelia pollen was significantly lower than in honey bees in the control group fed only sugar (P < 0.05) (Fig. 4). However, there was no significant difference in the results between Borago pollen, Trifolium pollen, Reseda nectar, Borago nectar and Control. The highest and lowest concentration of imidacloprid was observed in the control group (4.9 ± 0.7 ng/bee) and in the Phacelia pollen group (2.0 ± 0.4 ng/bee), respectively. The bees fed Phacelia pollen were able to metabolize 79 ± 4% of the ingested imidacloprid after 1 h, whereas the metabolism ratio in the control group was 50 ± 7% (Table 1). On the basis of an overall comparison, the correlation coefficient analysis (Fig. 5A)
Fig. 1. The ClassyFire classification of annotated phytochemicals in pollen and nectar diets. Inner circle: superclass level, outer circle: class level.

Fig. 2. Venn diagram of the number of annotated phytochemicals in pollen and nectar samples. Each number inside the figure indicates the number of annotated phytochemicals shared with other diets. The numbers next to the diets are the numbers of annotated phytochemicals in each diet.
between the concentration of imidacloprid and phytochemicals in the diets showed that datiscin (flavonoid), kaempferol-3-O-β-glucopyranosyl-7-O-α-rhamnopyranoside (flavonoid), 3-hydroxybutyric acid (hydroxy acid), 3,4-dihydroxyphenylethanol (phenolic compound) and undecanedioic acid (fatty acid) were negatively correlated with the concentration of imidacloprid, whereas maltotriose (sugar), palatinose (sugar), acacetin (flavonoid), abscisic acid (sesquiterpenoid), and glucose-1-phosphate (sugar) were positively correlated with the concentration of imidacloprid in honey bees (Fig. 5A). The dietary phenolic compound p-coumaric acid and the flavonoid quercetin have been demonstrated to up-regulate the bee detoxification system (Mao et al., 2013), and enhance the longevity of honey bees after uptake of imidacloprid (Wong et al., 2018). Dietary quercetin resulted in a concentration reduction of absorbed imidacloprid in honey bees (Ardalani et al., 2021). To confirm the results from these earlier investigations, we compared the peak intensity of quercetin among the diets. The results revealed that the peak intensity of quercetin in *Phacelia* pollen was significantly higher than in the other diets (one-way ANOVA, *P* ≤ 0.05) (Fig. 3A). The PCA score plot of the pollen and nectar samples (PC1: 31%, PC2: 22%). B: Hierarchical clustering heatmap of the top 25 significant annotated phytochemicals in diets (one-way ANOVA, *P* ≤ 0.05). The Ward and Euclidean were used for distance measure and clustering algorithm, respectively. Dark red indicates higher peak intensity, while dark blue indicates lower peak intensity. Diet averages are shown (n = 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 4. Concentration of imidacloprid (A), tau-fluvalinate (B), and tebuconazole (C) in the groups of bees fed different diets (n = 12). Bars with the same letter are not significantly different (one-way ANOVA, *P* ≤ 0.05). Error bars denote standard errors (SEs).

Table 1

<table>
<thead>
<tr>
<th>Pesticide</th>
<th>Feeding groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Reseda pollen</td>
</tr>
<tr>
<td>Imidacloprid</td>
<td>76 ± 4</td>
</tr>
<tr>
<td>Tau-fluvalinate</td>
<td>76 ± 3</td>
</tr>
<tr>
<td>Tebuconazole</td>
<td>71 ± 1</td>
</tr>
</tbody>
</table>

The concentration of imidacloprid, tau-fluvalinate, and tebuconazole in the groups of bees fed different diets (n = 12). Bars with the same letter are not significantly different (one-way ANOVA, *P* ≤ 0.05). Error bars denote standard errors (SEs).
This result is consistent with findings from previous studies reporting that dietary quercetin altered the concentration of imidacloprid in honey bees by upregulating their detoxification system (Mao et al., 2013; Ardalani et al., 2021). In our study datiscin, kaempferol-3-O-β-glucopyranosyl-7-O-α-rhamnopyranoside, and 3-hydroxybutyric acid which were present primarily in Phacelia pollen and Reseda pollen (Fig. 5D), were negatively correlated with the concentration of imidacloprid. The aglyconic forms of datiscin (datiscein) and kaempferol-3-O-β-glucopyranosyl-7-O-α-rhamnopyranoside (kaempferol) are hydroxyflavones similar to quercetin; all of them having hydroxy groups in the 3,5 and 7 positions (in the A and C rings). However, their structures in the B-ring differ; datiscin has a hydroxy group in the 2’ position, kaempferol has a hydroxy group in the 4’ position and quercetin has two hydroxy groups in the 3’ and 4’ positions (Fig. S6). These two flavonoids likely act similarly to quercetin increasing the metabolization of imidacloprid, resulting in the reduction of its concentration (Ardalani et al., 2021). Erban et al. (2019b) reported that exposure to imidacloprid suppressed the entire mevalonate pathway and fatty acid synthesis in bumblebees. The suppression of the mevalonate pathway in bumblebees is directly associated with the ketone bodies’ synthesis, including 3-hydroxybutyric acid (Erban et al., 2019b). Thus our observed negative correlation between 3-hydroxybutyric acid and imidacloprid concentration is probably due to the mevalonate pathway suppression.

A positive correlation between the concentration of imidacloprid and the peak intensity of maltotriose, palatinose, and glucose-1-phosphate which were present primarily in Borago nectar and Reseda nectar, was...
observed (Fig. 5A). These results are supported by Derecka et al. (2013) who found that imidacloprid exposure impaired the metabolism of sugars (maltotriose, palatinose, and glucose-1-phosphate) in honey bees. Our results may also suggest that due to the lower abundance of bioactive constituents in the nectar diets, honey bees fed on nectar diets were not able to increase their metabolism ratio of imidacloprid.

### 3.3. Influence of diets on the concentration of tau-fluvalinate in honey bees

The highest concentration of tau-fluvalinate was observed in honey bees in the control group fed only sucrose (300 ± 11 ng/bee) and was significantly higher than in bees fed Reseda pollen, Borago pollen, Trifolium pollen and Reseda nectar (P ≤ 0.05) (Fig. 4). However, there was no significant difference in the tau-fluvalinate concentrations among bees fed the four pollen diets (Reseda, Phacelia, Borago, Trifolium).

The bees in the control group metabolized 10% less tau-fluvalinate than bees fed Reseda pollen, 9% less than bees fed Trifolium pollen and Reseda nectar, and 6% less than bees fed Borago pollen (Table 1). The correlation coefficient analysis between the concentration of tau-fluvalinate and phytochemicals in the diets revealed that d-gluconomic acid (sugar) and four flavonoids delphinidin 3-sambubioside, luteolin 7-methyl ether, acacetin, and quercetin 3-O-(6′-acetyl-glucoside) were negatively correlated with the concentration of tau-fluvalinate in honey bees (Fig. 5B). In contrast, 3-hydroxybutyric acid (hydroxy acid), 3-deazaneplanocin (imidazopyridine), uridine (pyrimidine nucleoside), 3,5-dihydroxybenzyl alcohol (phenol) and NGCG00384983 (flavonoid) were positively correlated with the concentration of tau-fluvalinate in honey bees (Fig. 5B). The distribution of the negatively correlated phytochemicals indicated that d-gluconomic acid was present primarily in Reseda nectar, delphinidin 3-sambubioside and quercetin 3-O-(6′-acetyl-glucoside) were present in Trifolium pollen, luteolin 7-methyl ether was present in Borago pollen, and acacetin was present in Borago nectar (Fig. 5E). Other studies have shown that honey constituents may influence the tau-fluvalinate detoxification ability of honey bees (Mao et al., 2011). D-gluconomic acid is the predominant organic acid in honey (Mato et al., 1997; Kalaycıoğlu et al., 2017). According to our results, d-gluconomic acid may up-regulate the detoxification process of tau-fluvalinate in honey bees. Honey bees, similarly to most other insects, rely on their detoxification enzymes, particularly within the CYP6 and CYP9 families among the P450s, to metabolize pesticides. Honey bees use CYP9Q1-3 to convert tau-fluvalinate to a less toxic compound in their detoxification system and the expression of CYP9Q1-3 was up-regulated in quercetin fed honey bee larvae (Berenbaum and Johnson, 2015; Mao et al., 2017). Ardalani et al. (2021) found that dietary quercetin did not alter the concentration of tau-fluvalinate in adult honey bees; however, Johnson et al. (2012) showed that dietary quercetin at the same concentration reduced the toxicity of tau-fluvalinate in honey bees through upregulated transcription of P450 genes.

Together, the present findings confirm that quercetin 3-O-(6′-acetyl-glucoside) may act similarly to the aglyconic form of this flavonoid, and, due to the detoxification system activation in honey bees, may decrease the concentration of tau-fluvalinate. Honey bees might possibly also metabolize quercetin 3-O-(6′-acetyl-glucoside) to the aglyconic form of quercetin. The positive correlation between the concentration of tau-fluvalinate and 3,5-dihydroxybenzyl alcohol might be due to the fact that tau-fluvalinate diminished the metabolism of 3,5-dihydroxybenzyl alcohol in honey bees.

### 3.4. Influence of diets on the concentration of tebuconazole in honey bees

The highest concentration of tebuconazole was observed in honey bees fed Borago nectar (2132 ± 55 ng/bee) and Borago pollen (2040 ± 70 ng/bee), respectively. The tebuconazole concentration in honey bees fed only sugar (control diet) was 1876 ± 105 ng/bee; a value significantly different from that in honey bees given Reseda pollen (1422 ± 54 ng/bee) (P ≤ 0.05) (Fig. 4).

Honey bees fed Reseda pollen were able to metabolize 71 ± 1% tebuconazole after 24 h while the metabolization ratio in honey bees in the Borago nectar and control group was 58 ± 1% and 62 ± 2%, respectively (Table 1). The tebuconazole concentration in the bees fed Reseda pollen was significantly lower (P ≤ 0.05) than in bees fed the other diets, except for bees fed Reseda nectar (Fig. 4C).

The correlation coefficient analysis between the concentration of tebuconazole and the peak areas of phytochemicals in the diets revealed that d-desityrbiotin, 2-hydroxyymristic acid (fatty acids), quinolin-3-ol (quinoline), 3-hydroxydodecanoic acid (hydroxy acid) and pantothenic acid (vitamin B5) were negatively correlated with the tebuconazole concentration (Fig. 5C). The phytochemicals with positive correlation included: linalool oxide (tetrahydrofuran), glucose-1-phosphate (sugar), luteolin 7-methyl ether (flavonoid), acacetin (flavonoid), and d-gluconomic acid (sugar) (Fig. 5C). The five phytochemicals that were negatively correlated with the concentration of tebuconazole were present predominantly in the Reseda pollen (Fig. 5F).

Although tebuconazole is considered a pesticide with low toxicity to honey bees, earlier investigations have shown that the toxicity of insecticides to honey bees intensifies in the presence of EBI triazole fungicides, owing to the inhibition of oxidative detoxification genes (primarily P450s) and thus can negatively affect honey bees (Thompson et al., 2014; Mao et al., 2017).

Mao et al. (2017) showed that myclobutanil, an EBI triazole fungicide that is non-lethal to honey bees (LD50 39.6 µg/bee) (EFSA, 2010b), disrupted the quercetin metabolism and affected honey bee energy production (Mao et al., 2017). Ardalani et al. (2021) demonstrated that dietary quercetin did not alter the concentration of tebuconazole in honey bees and the metabolization rate of quercetin was increased in quercetin fed bees contact-exposed to tebuconazole. The present study shows that two flavonoids, acacetin and luteolin 7-methyl ether, which are structurally similar to quercetin, are positively correlated with the concentration of tebuconazole (Fig. 5C). Our results support the findings by Mao et al. (2017) that dietary flavonoids (in this case acacetin and luteolin 7-methyl ether) interfere with the metabolism of EBI triazole fungicide tebuconazole in honey bees.

### 4. Conclusion

Feeding honey bees pollen/nectar from different plants reduced the residual concentration of the pesticides imidacloprid, tau-fluvalinate and tebuconazole in honey bees exposed to these pesticides. The reduction in residual pesticide concentration correlated with the peak height of a range of phytochemicals. None of the diets, compared with honey bees fed sugar (control diet), resulted in significantly higher concentration of any residual pesticides in honey bees. The metabolization ratio of the tested pesticides in honey bees fluctuated when bees consumed diets based on sugar and additionally were fed plant rewards in the form of pollen and nectar, with varying phytochemical profiles. Dietary Reseda pollen and Phacelia pollen, which were diets rich in flavonoids, significantly increased the metabolization ratio of imidacloprid in honey bees relative to the sugar fed control bees. Consumption of Reseda pollen, Borago pollen, Trifolium pollen and Reseda nectar reduced the concentration of tau-fluvalinate in comparison with tau-fluvalinate concentrations in bees given only sugar (control diet). These observations support earlier findings suggesting that flavonoids up-regulate the honey bee detoxification system which results in the decrement in tau-fluvalinate concentration in honey bees. Our results also demonstrated that dietary pollen and nectar from R. odorata enable honey bees to increase the metabolism of tebuconazole. Complementary studies are needed to validate the findings of our study by testing the effects of the phytochemicals with reported significant associations with residual pesticide concentrations in honey bees individually using authentic
compounds. Future investigations should also consider the mechanisms of interaction between phytochemicals and pesticides in honey bees.

Funding

This study was funded by a project (27060) at the Graduate School of Science and Technology, Aarhus University (GSST, AU), Denmark.

CRediT authorship contribution statement

Hamidreza Ardalani: Data curation, Formal analysis, Investigation, Validation, Visualization, Writing - original draft. Nanna Hjort Vidkjaer: Methodology, Conceptualization, Supervision, Funding acquisition. Per Kryger: Methodology, Conceptualization, Supervision. Oliver Fiehn: Supervision. Inge S. Fomsgaard: Methodology, Conceptualization, Supervision, Funding acquisition.

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.

Acknowledgements

We would like to thank Bente B. Laursen, Aarhus University, Denmark; Arpana Vaniya, Tong Shen and Jacob Polz, University of California, Davis, CA, USA, for their valuable support.

Funding

This study was funded by a project (27060) at the Graduate School of Science and Technology, Aarhus University (GSST, AU), Denmark.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2021.106503.

References


McMullan, J.B., Brown, M.J., 2006. The influence of small-cell brood combs on the
morphometry of honeybees (Apis mellifera). Apidologie 37, 665–672.
levels of miticides and agrochemicals in North American apiaries: implications for
Acetylcholine receptors as sites for developing neonicotinoid insecticides. In:
Honeybee Behaviors on the Distribution of Pesticide in Nectar within a Hive and
Sánchez-Bayo, F., Goulson, D., Pennacchio, F., Nazzi, F., Goka, K., Debrauwer, L.,
Stoner, K.A., Eitzer, B.D., 2012. Movement of soil-applied imidacloprid and
toxicity induced by imidacloprid and its metabolites in Apis mellifera. Environ.
Toxicol. Chem. 20, 2482–2486.
Thompson, H.M., Fryday, S.L., Harkin, S., Milner, S., 2014. Potential impacts of
synergism in honey bees (Apis mellifera) of exposure to neonicotinoids and sprayed
fungicides in crops. Apidologie 45, 545–553.
Tosi, S., Nieh, J.C., Sgolastra, F., Cabbri, R., Medrzycki, P., 2017. Neonicotinoid
pesticides and nutritional stress synergistically reduce survival in honey bees. Proc.
Tsugawa, H., Caja, T., Kind, T., Ma, Y., Higgins, B., Ikeda, K., Kanazawa, M.,
Vanderghynst, J., Fiehn, O., Arita, M., 2015. MS-DIAL: data-independent MS/MS
Van Der Sluijs, J.P., Simon-Delso, N., Goulson, D., Maxim, I., Bonmatin, J.-M.,
Belzunces, L.P., 2013. Neonicotinoids, bee disorders and the sustainability of
pollinator services. Curr. Opin. Environ. Sustain. 5, 293–305.
VanEngelsdorp, D., Meixner, M.D., 2010. A historical review of managed honey bee
populations in Europe and the United States and the factors that may affect them.
J. Invertebr. Pathol. 103, S80–S95.
Vidkjær, N.H., Fomsgaard, L.S., Kryger, P., 2021. LC-MS/MS Quantification Reveals
Ample Gut Uptake and Metabolization of Dietary Phytochemicals in Honey Bees
interaction between imidacloprid and dietary phytochemicals in honey bees (Apis
Borland, A.M., Stevenson, P.C., 2013. Caffeine in Floral Nectar Enhances a
Xia, J., Wishart, D.S., 2011. Web-based inference of biological patterns, functions and
pathways from metabolomic data using MetaboAnalyst. Nat. Protoc. 6, 743–760.