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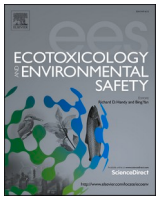

Honey bee colonies can buffer short-term stressor effects of pollen restriction and fungicide exposure on colony development and the microbiome

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ABSTRACT

Honey bees (*Apis mellifera*) have to withstand various environmental stressors alone or in combination in agriculture settings. Plant protection products are applied to achieve high crop yield, but residues of their active substances are frequently detected in bee matrices and could affect honey bee colonies. In addition, intensified agriculture could lead to resource limitation for honey bees. This study aimed to compare the response of full-sized and nucleus colonies to the combined stressors of fungicide exposure and resource limitation. A large-scale field study was conducted simultaneously at five different locations across Germany, starting in spring 2022 and continuing through spring 2023. The fungicide formulation Pictor® Active (active ingredients boscalid and pyraclostrobin) was applied according to label instructions at the maximum recommended rate on oil seed rape crops. Resource limitation was ensured by pollen restriction using a pollen trap and stressor responses were evaluated by assessing colony development, brood development, and core gut microbiome alterations. Furthermore, effects on the plant nectar microbiome were assessed since nectar inhabiting yeast are beneficial for pollination. We showed, that honey bee colonies were able to compensate for the combined stressor effects within six weeks. Nucleus colonies exposed to the combined stressors showed a short-term response with a less favorable brood to bee ratio and reduced colony development in May. No further impacts were observed in either the nucleus colonies or the full-sized colonies from July until the following spring. In addition, no fungicide-dependent differences were found in core gut and nectar microbiomes, and these differences were not distinguishable from local or environmental effects. Therefore, the provision of sufficient resources is important to increase the resilience of honey bees to a combination of stressors.

1. Introduction

Mass flowering crops, like almond orchards in the United States of America or oilseed rape (*Brassica napus,* OSR) in Germany, provide a rich source of nectar and pollen during a critical developmental stage, i.e. after overwintering of honey bee colonies. Fungal phytopathogens are

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commonly controlled for these crops using a combination of the succinate dehydrogenase inhibitor (SDHI) fungicide boscalid and a strobilurin fungicide, e.g. azoxystrobin or pyraclostrobin (Derbyshire and Denton-Giles, 2016). Both substances inhibit the mitochondrial respiration of targeted diverse taxonomic groups of fungi (Yang et al., 2011). Although fungicides are considered to be non-toxic to bees, adverse effects were found on honey bees (*Apis mellifera*) in both laboratory and field assays when using the Pristine® (25.2 % boscalid and 12.8 % pyraclostrobin) formulation. In laboratory assays, the product was found to have an impact on the physiology and behavior of adult and larval honey bees. This included a reduction in metabolic rate (Glass et al., 2021), learning performance (DesJardins et al., 2021), and survival (Fisher et al., 2021a). Additionally, field experiments have indicated that continuous worst-case exposure scenario lead to a reduction in colony development, overwintering success, and changes in foraging behavior (Fisher et al., 2017, 2021a, 2021b, 2021c, 2022, 2023; Glass et al., 2021; DeGrandi-Hoffman et al., 2013, 2015, 2017). In a large-scale monitoring project (German Bee Monitoring; DeBiMo), residues of these fungicides were frequently detected in bee bread. However, most of these concentrations were below the thresholds for acute or sub-lethal toxicity, and high detection frequencies were not associated with increased winter mortality (Genersch et al., 2010; DeBiMo, 2022). Pictor® Active is a formulation approved in the European Union that contains the same active substances in comparable total amounts as Pristine®.

It is of significant importance to understand how honey bees interact with various stressors in agricultural settings. With regard to the impact of exposure to plant protection products (PPPs), the resilience and health of honey bees depend on the availability of sufficient resources (Barascou et al., 2021; Crone and Grozinger, 2021; Wahl and Ulm, 1983). The migration of colonies to pollinate mass flowering crops, such as almonds in the US, can result in competition due to high colony densities and limited resources (DiPasquale et al., 2013; Goodrich, 2019). Furthermore, after the blooming of mass flowering crops, bees must rely on non-cropped areas for sufficient pollen provision, which may be limited (Decourtye et al., 2010). It has been shown that monofloral pollen, or a complete restriction of nutritional resources, had a negative impact on individual bee survival, immune responses, detoxification, and can increase susceptibility to other stressors (Alaux et al., 2010; Castle et al., 2023; DiPasquale et al., 2013).

Fungicides could also affect the plant nectar microbiome, which is composed of yeasts like *Metchnikowia* species (Aleklett et al., 2014). The entire spectrum of functions of the nectar-inhabiting microbiome is not entirely clear. However, it has been suggested that microorganisms in nectar can influence pollinator behavior and plant attractiveness (Herrera et al., 2013; Jacquemyn et al., 2021). Microbiome imbalances (dysbiosis) due to pesticide exposure and disruption of the microbiome have been observed in honey bee guts (reviewed by Hotchkiss et al. 2022). Dysbiosis in the core microbiome could affect honey bee physiology, since the microbiome is involved in honey bee nutrition, stimulation and maturation of the immune system, and degradation of xenobiotics (Smutin et al., 2022). The abundance of gut microbe species varies between gut segments, castes, developmental stages and colonies, but the microbiome is characterized by a core microbiome consisting mainly of five members: *Lactobacillus* Firm 4 and 5, *Bifidobacterium asteroides*, *Gilliamella apicola, Frischella perrara*, and *Snodgrassella alvi* (Kwong and Moran, 2016).

Evaluations assessing the effects of various stressors are conducted predominantly on full-sized colonies. Full-sized colonies have at least a one year old queen and have successfully overwintered. Nucleus colonies are the basis for expanding an apiary and/or replacing weak or sick colonies, thereby ensuring the maintenance of a high and consistent honey yield (Maucourt et al., 2018). They are created during the swarming period from an existing full-sized colony by taking brood frames with nurse bees, leaving them to rear a new queen or providing them a young queen. Similar to full-sized colonies, nucleus colonies have to withstand the combination of stressors, such as pesticide exposure, pathogen infection, and resource limitation in spring. Nevertheless, nucleus colonies may be less resilient due to their smaller colony size and therefore more susceptible to stressors as they will be exposed and threatened shortly after their establishment.

The aim of this study was to assess (1) the single and combined stressor effect of fungicide (Pictor® Active) exposure and resource limitation on colony health and overwintering success, (2) the resilience of nucleus colonies in comparison to full-sized colonies when exposed to these stressors, (3) the effects on the core microbiome before, during, and after interaction of combined stressors in bees, and (4) the impact of fungicide application on floral nectar yeast community.

2. Material and methods

2.1. Location setup and treatment condition

In spring 2022, our study was conducted simultaneously in five different geographical regions in Germany - Bochum, Braunschweig, Celle, Hohenheim (Stuttgart), and Veitshöchheim - to account for various environmental and climatic conditions that prevail throughout the country (for more details see Table S1). The experiment took place at one control and one treatment site in winter oilseed rape (OSR, *B. napus*) at each location. The fields were on average 6.8 ha in size and were at least 1.8 km apart or separated by geographical barriers (rivers or cities) to minimize drift and prevent bees to forage on the wrong plot. The field setup ensured homogenous climate conditions per location. To avoid contamination from other plant protection products, no systemic insecticide application was allowed before the experiment and no further application of plant protection products during the experiment. The formulation Pictor® Active with the active ingredients boscalid (150 g/L) and pyraclostrobin (250 g/L) was applied according to the maximum field recommended rate permitted in the European Union of 1 L/ha in 200–250 L water/ha (for residue detection of relevant field samples to verify application and methodological description see Suppl. Material, Table S2). Pictor® Active was provided by BASF SE, Ludwigshafen to all sites within the same batch to ensure standardized application. The spray applications were carried out during full bloom of OSR (BBCH 64–65) between 10:00 and 14:00 according to good agricultural practice. The weather conditions were sunny and temperatures were above 13°C, so that we could observe foragers on the flowers and assume that the bees were sufficiently exposed to the fungicide.

2.2. Experimental conditions

In summer 2021, 24 artificial swarms were created per location consisting of one sister queen provided with 2.5 kg of bees (i.e. 20,000–25,000 bees) in residue free apiary material. The bee colonies were treated with organic (formic and oxalic) acids against varroosis in accordance with good beekeeping practice in Germany (Schödl et al., 2022). After successful overwintering in the following year, no clinical symptoms of adult bee or brood diseases were observed during inspection. In March, colonies were divided into two groups. Half of the colonies were used to create nucleus colonies keeping their queens. These colonies were reduced to an average of 5000 (+/- 1000) bees with a brood ratio of one open cell per bee and a maximum of 5000 capped brood cells. The other half of the colonies were assigned as full-sized colonies with an average of 12,000 (+/- 3000) bees, and no further interventions were carried out affecting the brood ratio. The pollen reservoir was equalized among all colonies to leave only the brood related pollen ensuring the same nutritional conditions. Furthermore, half of all colonies were equipped with pollen traps (screen passages of 5 mm in diameter) restricting their nutritional provisioning. Pollen traps lasted for four weeks and were emptied daily. The colonies were equally migrated to the field sites (control fields without fungicide application or treatment fields with fungicide application) 10 days before

application with the beginning of OSR bloom (BBCH 59–60). They were grouped based on colony strength and alternated in line with and without pollen traps, with a distance of one meter between each colony and at least 10 m between group strengths (Fig. S1). Overall this full factorial design resulted in eight different groups labeled as: not exposed to fungicide control group (C) or fungicide exposed group (T), full-sized colony (F) or nucleus colony (N), with pollen trap (wT) or without pollen trap (woT), resulting in the following combinations: T-F-wT, T-F-woT, T-N-wT, T-N-woT, C-F-wT, C-F-woT, C-N-wT, C-N-woT. In this case control and treatment referred to the field sites colonies were migrated to, and thus the fungicide exposure. The eight groups consisted each of a sample size of n = 3 replicates (except Hohenheim, who had n = 4 replicates per group) per location (N = 5). After oil-seed rape bloom, colonies were migrated to a common monitoring site at each location at which colonies lasted until the next spring season in 2023. Treatment phase was thus defined as the time at the oilseed rape field during exposure and pollen restriction, and monitoring phase described the phase after migration to the monitoring site. Four colonies collapsed during the migration to the monitoring site not having any impact on data estimation.

2.3. Data collection

2.3.1. Colony conditions

Population estimates were conducted using the Liebefeld method (Gerig, 1983; Imdorf et al., 1987). This method involves evaluating the number of worker bees, brood cells (eggs, larvae, and capped cells), as well as nectar and pollen cells. Specifically, the estimation process involves dividing each frame into square-decimeter sections and assessing the number of bees, brood cells, nectar, and pollen cells within each section. For instance, a Zander frame, which fits exactly 8.1 dm^2 or eight Liebefeld units per side, is divided into eight parts, and the number of parts covered with bees/cells is estimated. From these observations, the respective numbers of bees, brood cells, and storage cells can be calculated. The first estimation carried out in March served as the starting point for the standardized allocation of the colonies into different groups. In addition, the colonies (n = 24, N = 5) were evaluated every 21 (+/- 2) days, starting on a fixed date according to the fungicide application. This date was defined as DAA4 (four days after application). The assessments continued until the colonies were prepared for overwintering in September or October 2022. Colonies were estimated again after successful overwintering in March or April 2023, depending on local conditions at the five locations.

2.3.2. Brood development

The evaluation of brood development was carried out at three locations (Braunschweig, Celle, and Hohenheim: n = 24, N = 3) by applying the methodology described in OECD Guideline 75 (OECD, 2007). Brood development was assessed during the first brood cycle within the exposure and followed the procedure outlined in Wernecke et al. (2023). In short, three days before application (DAA-3), one brood frame with eggs was removed from each replicate of experimental groups, henceforth referred to as brood area fixing day (BFD) 0. The development of the brood was continuously assessed and monitored until the bees emerged by selecting approximately 300 cells per comb. Photographs were taken from each comb side, following Schur et al. (2003). Briefly, the selected combs were uniquely identified at BFD0, and photographs were subsequently taken on four occasions: BFD5, 9, 15, and 22 (e.g., with a Sony Alpha 7 R III camera and a Tamron 70–300 mm at 300 mm tele lens). The cells were classified and rated according to the scheme in Wernecke et al. (2023) using the HiveAnalyzer software (Höferlin et al., 2013). From this assessment, the brood termination rate (BTR - see Wernecke et al., 2023 for details) was calculated to reveal maldevelopment or aborted brood care in the monitored brood cycle.

2.3.3. Gut microbiome assessment

Foraging and in-hive bee samples were collected from each colony at all five locations (n = 24, N = 5) before, during and after exposure to assess changes in the core microbiome of the bee gut. This was performed at DAA-4, DAA3 and DAA9 in sterile containers and stored at −20°C until processing and analysis.

Whole guts of adult bees were used for further analysis. A pool of five guts were removed from each sample type (in-hive bees and forager bees) and colony, placed in sterile lysis tubes (MN Bead Tubes Type G, Machery-Nagel GmbH&Co.KG, Düren, Germany) containing 5 mm steel beads for sample homogenization. DNA isolation and quantification was conducted as described in Steinigeweg et al. (2023). In brief, genomic DNA was isolated using the NucleoMag® VET Kit (Machery-Nagel GmbH&Co.KG, Düren, Germany), and quantified in an AriaMX Real-Time PCR System (Agilent Technologies, Santa Clara, CA, United States) performing individually per microbial target with β-actin as reference gene (Table S3). qPCRs were conducted in technical triplicates per sample, averaged using the geometric mean, and filtered for C_q values over 35 that were removed from further analysis.

2.3.4. Floral nectar microbiome

Floral (OSR) samples were collected at four different sites (Braunschweig, Celle, Hohenheim, and Veitshöchheim) at the time points DAA-2, DAA1, and DAA6. Each sample consisted of 30 flowers, which were collected in sterile 50 mL centrifuge tubes at three sampling points at each location (control and treatment site). Samples were stored at 4°C until nectar extraction, but for maximum seven days. Nectar was extracted according to a modified protocol of Bosi (1973) and Bertazzini and Forlani (2016) using centrifugation techniques (for details see Suppl. material). Nectar samples were plated on yeast extract-malt extract (YM) medium (DSMZ medium 186, https://mediadive.dsmz.de/medium/186) supplemented with 500 mg/L chloramphenicol, incubated at 16–18°C and examined every three days. Colonies were differentiated into macro-morphological types and pure cultures were prepared for Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF) MS identification. Samples were identified by sequencing of rDNA regions with amplification of the ITS and partial LSU regions performed with primers ITS1F and LR5. Sequences were compared with sequence data deposited in the NCBI GenBank (https://www.ncbi.nih. gov) and MycoBank (https://www.mycobak.org) (A more detailed description can be found in Suppl.).

2.4. Data analysis

All statistical analyses were performed in R (version 4.2.2) with the user interface RStudio (version 2022.12.0). Linear mixed effect modelling was calculated using the *glmmTMB* function from the *glmmTMB* package (version 1.1.8) (Brooks et al., 2017). The use of asterisks in models includes always the individual components and the interaction between them as it is predefined in the R code. Model fit was visually inspected using the *DHARMa* package (version 0.4.6) (Hartig, 2022). Estimated marginal means of model output were compared using the *emmean* function from *emmeans* packages (version 1.8.4 – 1) (Lenth, 2023) and a Bonferroni-Holm adjustment for multiple testing. The reported p-values correspond to the highest determined p-value of the multiple comparison described. Non-metric multidimensional scaling (NMDS), Principal Coordinate analyses (PCoA) and permutational multivariate analysis of variance (PERMANOVA) were performed using the *vegan* package (version 2.6–4) (Oksanen et al., 2022).

2.4.1. Colony conditions and brood development

The colony development was assessed by the relative increase in the number of bees and brood (total number of brood cells containing eggs, larvae and capped brood cells) compared to starting conditions. The absolute number of each parameter at each assessment date was normalized to the absolute number at the beginning of the experiment

before exposure according to the formula described in Alkassab et al. (2023). Data were logarithmically transformed to avoid heteroscedasticity (Table S4) and analyzed for differences between groups and repeated measurements using linear mixed effects models (LMMs). After a top-down model fitting using AICc values, the individual components as well as the interaction between "treatment" and "pollen trap" were used as explanatory variables, "assessment date" as an interacting covariate, and "colony number" nested in "location" as a random factor (Table S4). The brood ratio was further calculated and evaluated via a division of the total number of brood cells at the time of assessment by the corresponding number of bees. Statistical analysis for the brood ratio and brood termination rate was performed using LMMs as described above, but for brood ratio only for single measurements (Table S4).

2.4.2. Core microbiome community

The relative bacterial abundance per bacterial species and sample type was calculated according to Erler et al. (2011) with β-actin as reference gene. The microbial community was analyzed by NMDS using the *metaMDS* function. Distance matrix was calculated using chi-squared distances using relative abundances of raw data. Post-hoc analysis was performed using PERMANOVA and Bonferroni-Holm correction was applied for multiple testing where necessary. Data from all groups per time point were used for NMDS and PERMANOVA and then displayed by sample type and colony size to allow multifactorial comparison. The explanatory variables were "treatment", "pollen trap", "colony size", "sample type (in-hive or forager bees)" and "location".

2.4.3. Floral nectar microbiome

Fungal community was analyzed using PCoA and was performed separately for each time point using the presence-absence dataset. Distance matrix was calculated based on Jaccard distances with binary data. A PERMANOVA was carried out with "treatment" and "location" included as explanatory factors. Total CFU abundances, species richness and relationship between Ascomycota and Basidiomycota were analyzed using Mann Whitney U-tests, with an alpha value of 0.05.

3. Results

3.1. Colony conditions

The estimation of stored pollen showed that colonies equipped with the pollen traps had significantly fewer pollen cells during the exposure period than colonies of the same size without pollen traps (LMM, $p < 0.0007$) (Fig. S2). This confirms that the experimental design successfully restricted access to pollen resources.

Colony development, expressed by the relative increase in bees, showed a typical development within the season: a continuous growth in spring with a peak at the beginning of June and then the proportion remains constant or decreases slightly after summer solstice (Fig. 1). The nucleus colonies equipped with pollen traps and exposed to Pictor® Active showed a lower growth rate over time compared to the other nucleus colonies (Fig. 1A). In early May, the number of bees in these nucleus colonies exposed to both stressors (pollen trap and fungicide exposure) remained almost constant, while the other treatment groups showed a 100 % increase in bee numbers. This trend was significant compared to the fungicide-exposed group without pollen trap at the first three assessment dates (LMM, $p < 0.0229$), and compared to the control groups only at the monitoring site in June (LMM, $p < 0.0248$). No significant differences were detected among groups at the last assessment in July (LMM, $p > 0.42$), where colonies became smaller. There were no major differences in the full-sized colonies throughout the season, albeit the control colonies with pollen trap tended to show the slowest

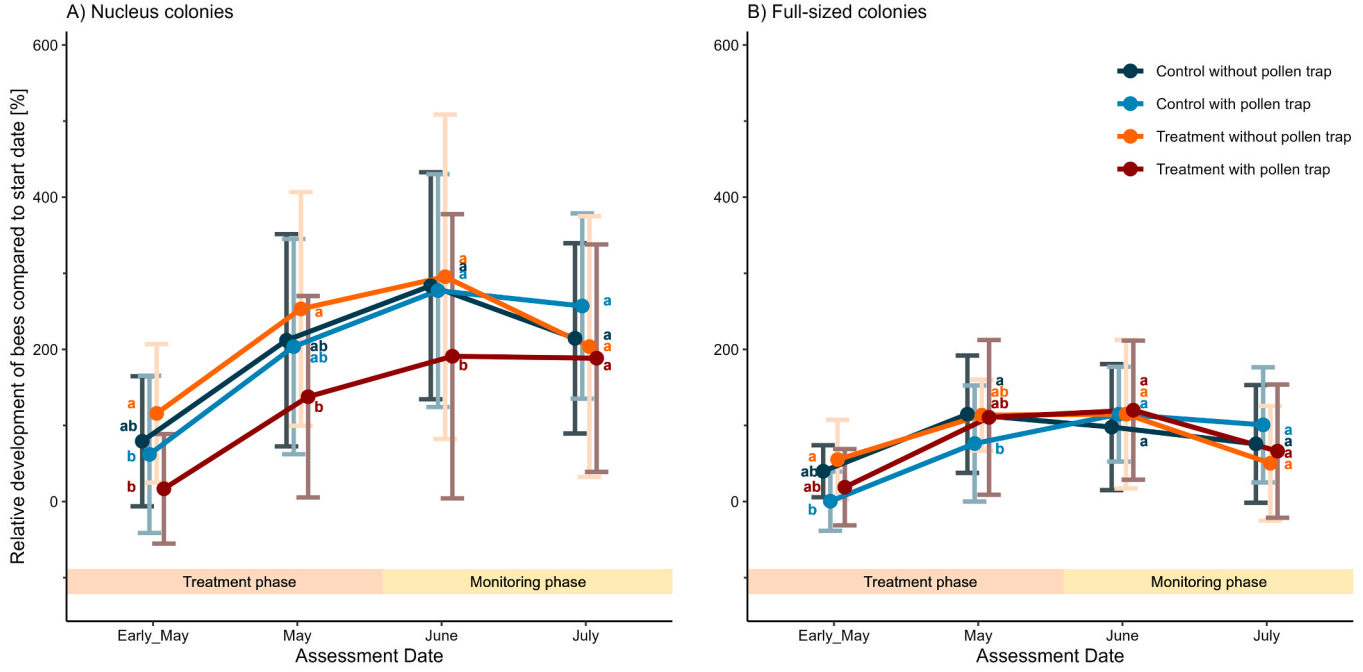

Fig. 1. Population growth expressed in bee mass development. Colony development was assessed via population estimations (Liebefeld method) in a three-week interval during exposure at OSR field and after exposure at a monitoring site. The field experiment was conducted with colonies assigned to eight groups comprising of n = 3 colonies per group at N = 5 locations. At the treatment site Pictor® Active (active ingredient (a. i.) boscalid and pyraclostrobin) was sprayed on OSR during flowering according to label instructions with 1 L/ha product. No treatment was applied to the control field. To ensure pollen restriction, half of the colonies at each field site were equipped with a pollen trap for four weeks. A) Colony development of nucleus colonies, B) colony development of full-sized colonies. The number of bees at each assessment date were normalized to the starting conditions, and are expressed as a percentage growth rate (with 0 = zero net growth, 100 % = doubling of the number of bees). Points represent the mean value among all locations per group at each time point. Error bars show the standard deviation. Different letters indicate significant differences of population growth between groups considered individually for each time point (LMM: treatment * pollen trap * time point + (1 | location / colony number), α = 0.05).

population growth in May (LMM, $p < 0.006$). The relative increase in the number of brood cells did not differ over the course of the season, neither for open nor for capped brood cells (LMM, $p > 0.05$) (Fig. S3, S4).

Brood ratio (number of brood cells per bee) of nucleus colonies exposed to the combined stressors was significantly higher on the first assessment day within the exposure compared to the control group without pollen traps (LMM, $p = 0.0029$) (Fig. 2A). The brood ratio of unexposed full-sized colonies with pollen traps was significantly higher compared to colonies without pollen trap on both assessment dates throughout the pollen restriction period (LMM, $p < 0.048$) (Fig. 2B). Nucleus colonies had on average a brood ratio of 2.54 (SD = 1.19) while full-sized colonies had a ratio of 1.94 (SD = 0.94) (Fig. 2). The lower brood ratio of full-sized colonies compared to nucleus colonies did not affect colony development, as expressed by bee mass (Fig. 1B) or brood (Fig. S3B, S4B). The combined stressor of pollen trap and fungicide exposure resulted in a peak in brood ratio of 3.54 (SD = 0.97) in the nucleus colonies (Fig. 2A). Furthermore, high brood ratios did not lead to an increased brood termination rate (Fig. 3A).

The overwinter success rate in the following year was 94 %, with 110 out of 117 colonies overwintering successfully. Four colonies collapsed during migration to the monitoring site. These losses were not attributed to any specific group but were randomly distributed across all groups. Overwintering did not lead to a reduction in the number of bees in colonies (Fig. S5). However, full-sized colonies in the control group with the pollen trap had the highest number of bees in autumn and spring compared to all other full-sized colonies (LMM, $p = 0.014$) (Fig. S5B).

3.2. Brood development

The maximum mean brood termination rate after 22 days was 19 % and was observed in the full-sized control group with pollen trap. (Fig. 3). Overall, brood termination rate remained relatively constant within the groups with on average of 12.3 % in the nucleus colonies (Figs. 3A) and 10.9 % in the full-sized colonies (Fig. 3B). The nucleus colonies of the fungicide-treatment group without pollen traps showed a significantly lower termination rate compared to the treatment group with pollen trap group. (LMM; $p = 0.0079$).

3.3. Core microbiome community

The results were split by sample type and colony size to enable a multifactorial comparison after individual NMDS analysis by time point. Stress value of NMDS was 0.123, 0.101, and 0.108 before, during and after exposure. The variance within the forager bees was higher than in the in-hive bees. The core microbiome differed significantly between sample types (in-hive and forager bees) (PERMANOVA, $p < 0.001$) and locations (PERMANOVA, $p < 0.001$) at each time point (Fig. 4). No effects of pollen restriction, fungicide-treatment, or colony size were observed between groups.

3.4. Floral nectar microbiome

The PCoA showed no differences in species composition between the untreated control site and fungicide-treatment site at any analyzed time point (Fig. 5). This was also represented in the total abundances and species richness of fungi ($p > 0.05$) (Fig. S6). The slight decline in abundances and richness one day after treatment was not statistically significant (Fig. S6). Total abundances of yeasts increased over time, while the species richness and abundance-ratio between Basidiomycota and Ascomycota decreased (Fig. S6). However, the effect of the sampling location was significant at every time point ($p < 0.05$).

4. Discussion

Pollinators are exposed to various biotic (e.g., parasites, pathogens, and natural enemies) and abiotic (e.g., agrochemicals, environmental change, and pollution) stressors, which can interact with each other additively or synergistically (Gaubert et al., 2023; Lin et al., 2023). This study aimed to assess the impact of two major stressors - fungicide exposure and resource limitation - on the health of honey bees in agriculture under realistic field conditions.

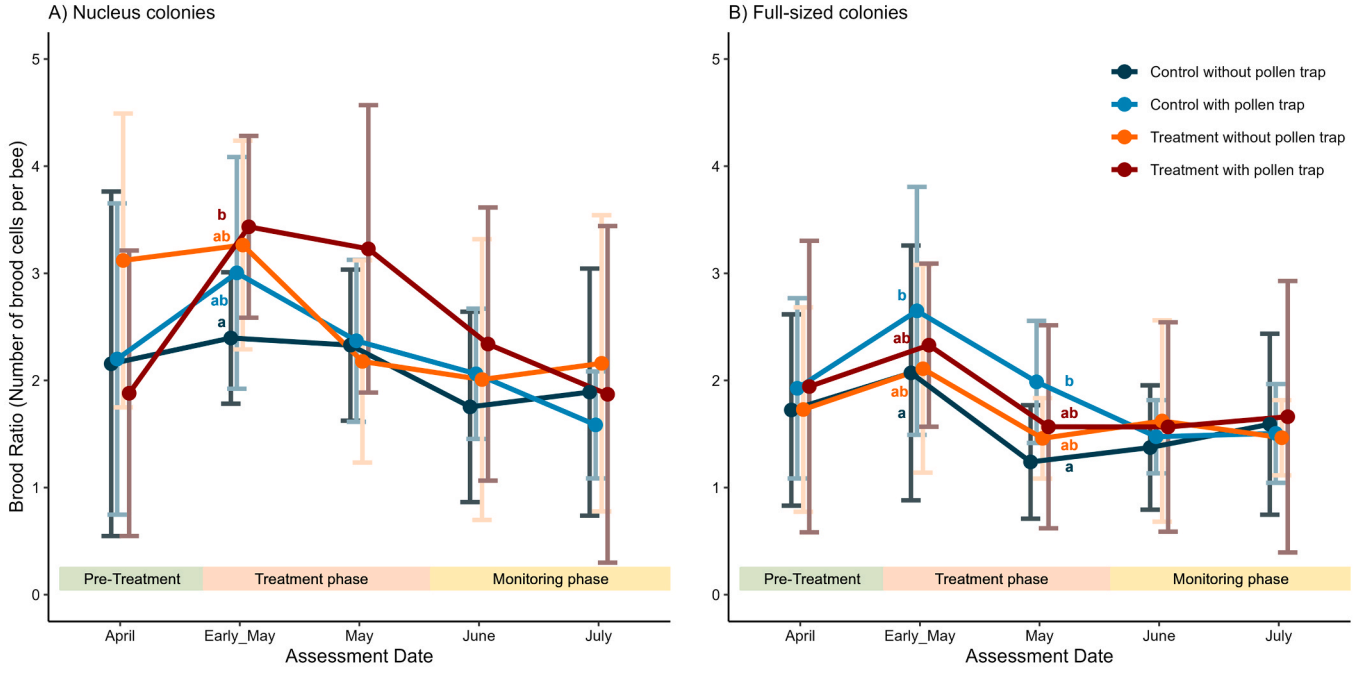

Fig. 2. Colony development. Brood ratio given as the number of brood cells per bee at each time point, before, during and after the exposure for A) nucleus colonies and B) full-sized colonies. Experimental details see description Fig. 1. Points represent the mean value among all locations per group at each time point. Error bars show the standard deviation. Different letters indicate significant differences between groups considered individually for each time point (LMM: treatment * pollen trap + (1 | location / colony number), $\alpha = 0.05$).

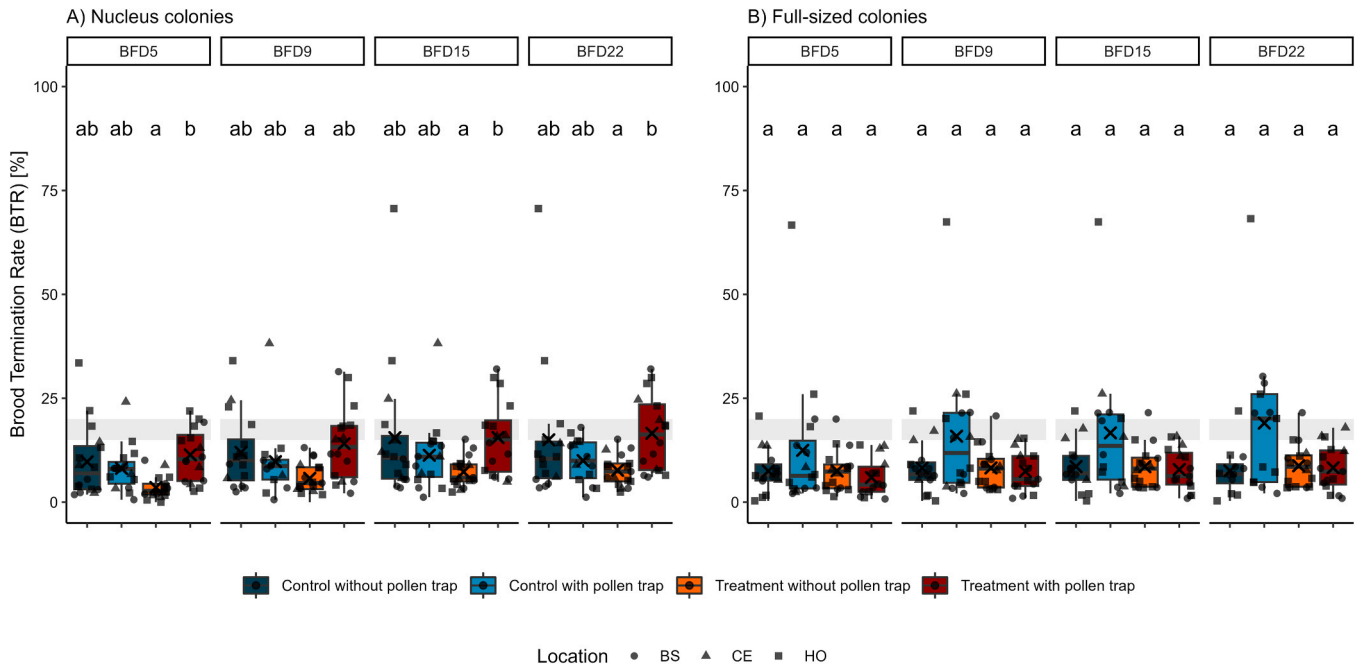

Fig. 3. Brood Termination Rate. Brood termination rate (BTR) in percent cumulative at each assessment date of one brood cycle during exposure for A) nucleus colonies and B) full-sized colonies (n = 3 colonies per group at N = 3 locations). For experimental details see description of Fig. 1. Assessment days were abbreviated as BFD = Brood area Fixing Day. Data points represent determined BTR per colony and boxes represent the first and third quantile of data and the median, whiskers extend the hinge by values with a maximum of the 1.5-fold inter quantile range. Black X in boxes represents the mean value. Different letters indicate significant differences between groups considered individually for each time point (LMM: treatment * pollen trap + time point + (1 | location/colony number), α = 0.05). Horizontal grey bars indicate the expected brood termination rate of 15–20 % of colonies under field realistic conditions without any artificial stressor (Lückmann and Tänzler, 2020).

4.1. Colony development under combined stressors

The combined effects of the stressors were observed only when examining specific time points during the exposure period. A four-week pollen restriction combined with fungicide exposure impacted the nucleus colonies in the field, as shown by a reduced colony development, measured by the number of bees (Fig. 1A), and a peak in brood ratio (Fig. 2A). However, these effects were compensated within six weeks and the combined stressor effect was mitigated when considering the entire bee season.

There is ongoing research to assess combined effects of various stressors on honey bee health and development. The influence of pollen as a vital nutritional resource has been shown to enhance bees' resilience against pesticides (Arathi and Bernklau, 2021; Barascou et al., 2021; Crone and Grozinger, 2021; Hýbl et al., 2021; Tosi et al., 2017), pathogens (Bernklau et al., 2019; Dolezal et al., 2019), or parasites (Dolezal and Toth, 2018). Conversely, malnutrition may contribute to the stressor effects and increase susceptibility to pathogen or parasite infections. Most studies focus on laboratory assays under controlled conditions, consequently extrapolations to field conditions should be made cautiously. In our experiment, we found that the combined stressors of fungicide exposure and resource limitation delayed the development of nucleus colonies. Colonies exhibited a short-term stressor dependent response characterized by a reduced bee mass development and a higher brood ratio following in-field spray application (Figs. 1A, 2A).

It has been shown, that colonies with an initial strength of approximately 4500 bees produce the most brood per bee in spring with a brood ratio of 2.8–3.04 (Harbo, 1986). Furthermore, the number of brood cells per bee correlates negatively with the total number of bees (Free and Racey, 1968; Westerhoff and Büchler, 1994a). A high brood ratio can be a sign of colony growth and resource abundances, but it might also be a signal of potential stress. Conversely, a low brood bee ratio may reflect resource limitations or seasonal adjustments, but could also indicate colony decline if persistent or occurring out of season (Groeneveld et al., 2024). In this study, the brood ratio was on average of 2.54 with a peak of 3.54 in nucleus colonies (Fig. 2A). Higher brood ratios did not lead to a high brood termination rate (Fig. 3A). Overall, the brood termination rate was relatively low among all colonies with a maximum of 19 % (Fig. 3), which corresponds to the expected termination rate for colonies under field conditions of approximately 15–20 % (Lückmann and Tänzler, 2020). Thus, nucleus colonies are able to compensate for the stress factors of pollen restriction and fungicide exposure despite their lower colony strength at the beginning of the season. The results showed that one bee had to maintain more brood cells compared to the untreated nucleus colonies, which led to a recovery in colony growth. This may come at the expense of individual bees' longevity (Westerhoff and Büchler, 1994b). Others showed that a product mixture with the same active ingredients resulted in a reduced worker population but not to a lower brood volume during an artificial four-week exposure period in summer (Fisher et al., 2022). However, colonies were still able to compensate for this effect after the exposure period, which is consistent with our findings.

The overwintering success is, in addition to the colony development, a crucial factor in determining long-term stressor effects. Overall, 94 % of all colonies overwintered successfully and only seven out of 117 colonies were lost (Fig. S5A, B), which correspond to the winter losses recorded in recent years (Gray et al., 2023). Furthermore, the colony size in spring depends on the initial colony size in the previous autumn (Free and Racey, 1968). The full-sized colonies with pollen restriction and without fungicide exposure had the highest estimated number of bees in autumn and spring compared to the other full-sized colonies (Fig. S5B). However, the colony size of all colonies with pollen restriction was not significantly different from those without pollen restriction. The high number of bees in the full-sized unexposed colonies with pollen traps in autumn presumably resulted from their strong population growth during the season (Fig. 1B, S5B). Hence, this study was unable to reproduce the fungicide induced reduction in worker population or higher winter

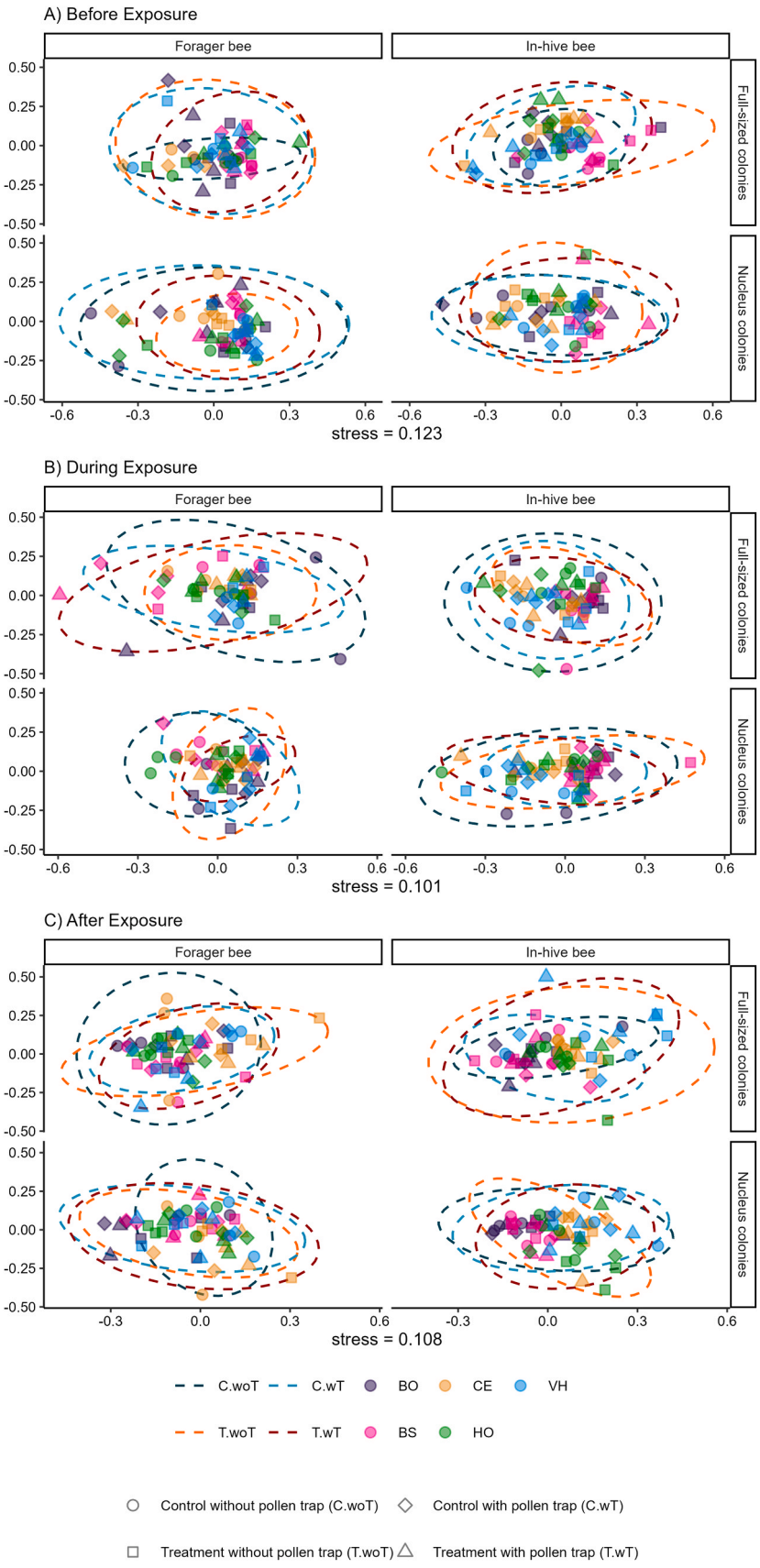

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Fig. 4. NMDS analysis of honey bee core microbiome community. Five core species (*Bifidobacterium asteroides*, *Frischella perrara*, *Gilliamella apicola*, *Lactobacillus* Firm-4&5, and *Snodgrassella alvi*) were analyzed in guts of honey bee foragers and in-hive bees at three time points of the experiment (see Fig. 1). NMDS analysis was performed with relative abundance data and chi-squared distances individually by sampling time point and was divided afterwards according to sample type (in-hive bee and forager bee) and colony size (nucleus and full-sized colony). Microbial core community of bees before exposure (A), during exposure (B), and after exposure (C). Each data point represents one colony of the experiment and dotted lines the 0.95 confidence intervals of the experimental groups. Locations were abbreviated as: BO = Bochum, BS = Braunschweig, CE = Celle, HO = Hohenheim, and VH = Veitshöchheim.

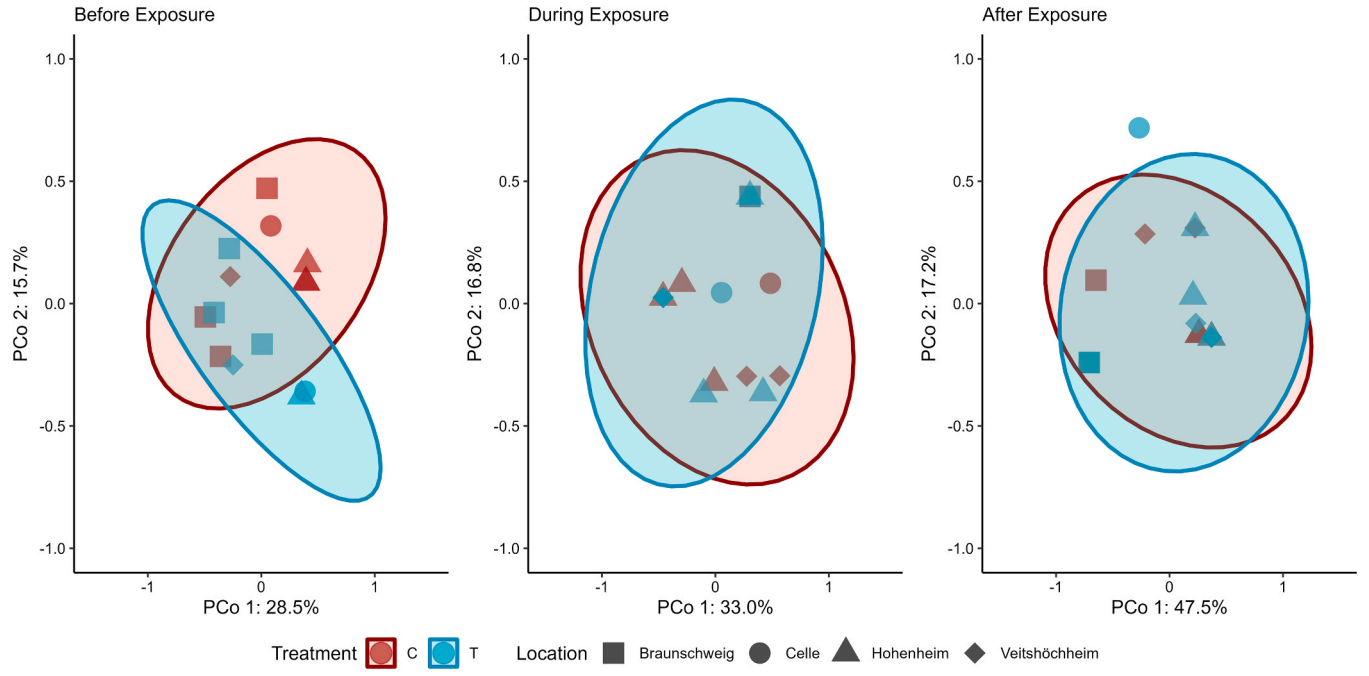

Fig. 5. Floral nectar yeast community analysis. Floral nectar samples were collected before (DAA-2), during (DAA1), and after (DAA6) exposure at control and fungicide-treated OSR fields at all locations (n = 3 per field, N = 4 locations). For experimental details see description of Fig. 1. Principal Coordinate Analysis (PCoA) was performed using presence-absence data and Jaccard distances. Missing data points resulted from no possible nectar extraction from collected floral sample.

losses reported by Fisher et al. (2021b). The authors highlighted that they exposed colonies over an unlikely long and continuous period of six months, which led to the adverse effects observed in colonies. They reported a significant reduction in worker population only after a continuous exposure of two months (Fisher et al., 2021b), so a field-realistic exposure with a single application may not affect colony size. Our results showed that the colony can compensate for possible negative effects at the individual bee level that do not necessarily result in colony losses.

The capability of honey bee colonies to compensate during a period of malnutrition was recently shown by Castaños et al. (2023). They showed that colonies had less brood and fewer emerging bees during a period of malnutrition, but afterwards colonies returned to natural levels. We showed, that colonies equipped with the pollen traps stored significantly less pollen and had a lower pollen-to-larvae ratio compared to colonies without pollen traps (Fig. S2A, S7). However, the number of pollen cells was the same in all colonies after the pollen restriction. The pollen restriction did not influence the colony development in terms of their proportion of open or capped brood cells (Fig. S3, S4) or brood termination rate during the first brood cycle of pollen restriction (Fig. 3). Colonies with pollen traps had significantly less pollen cells during the pollen trap treatment than colonies without pollen traps, and also a slightly reduced number of nectar cells (Fig. S2B). Consequently, foragers presumably turned to collect predominantly pollen with smaller pollen baskets, instead of nectar, to compensate for pollen restriction. In this context, full-sized colonies might be more resilient to the effects of the pollen trap than nucleus colonies due to their larger capacity of foragers. Nonetheless, this observation was not significant. Additionally, pollen restricted full-sized colonies without fungicide exposure had a significantly higher brood ratio than the unexposed group, but still within the expected brood ratio (2.8–3.04) for full-sized colonies (Harbo, 1986). Nursing frequency of young larvae is positively correlated with the amount of stored pollen, and a deficit in pollen leads to a nursing preference for older larvae, thus affecting colony development (Schmickl and Crailsheim, 2002, 2004). Consequently, care should be taken when setting up honey bee colonies in spring to ensure a sufficient nutritional resource availability.

Establishment of nucleus colonies is of great importance for beekeepers to ensure an efficient and healthy apiary (Maucourt et al., 2018). Although the nucleus colonies have been shown to compensate for the combined stressor effects over time, caution is still required and the interaction of multiple factors should be considered. Residues of other pesticides may remain in stored bee products and the resulting effects are not yet fully understood. The threat of pollen restriction or pathogen infections in addition to exposure risks should also be the focus of future research.

The variance resulting from the different locations could further be minimized by increasing sample sizes and field sites at each location. Here, regional effects were taken into account through five different locations in Germany, each with two field sites. Increasing the number of field sites, replications, and the number of colonies at each site would confirm the results by reducing the variance.

4.2. *Core microbiome community*

The honey bee microbiome was analyzed to assess the effects on individual bees in addition to the colony level assessments. The similarity comparison and post-hoc PERMANOVA analysis showed a

significant difference between abundance of the five core members tested (*Bifidobacterium asteroides*, *Frischella perrara*, *Gilliamella apicola*, *Snodgrassella alvi*, and *Lactobacillus* Firm-4 and Firm-5) in the microbial community of in-hive and forager bees at each sampling time point (Fig. 4). Neither pollen restriction, nor exposure to the fungicide led to dysbiosis in the core microbial community. The results are consistent with the expected changes in the core gut resulting from honey bee development (Kwong and Moran, 2016). Honey bee workers perform different tasks associated with different diet preferences during their post-emergence life cycle. This shift and exposure to environmental factors are related to changes in the gut microbiome rather than age effects. Although the species richness of the core members of the microbiome is not affected, the relative abundances and overall diversity of the microbial community change (Copeland et al., 2022; Corby-Harris et al., 2014; Jones et al., 2018; Kapheim et al., 2015).

We showed that field realistic exposure to Pictor® Active did not affect the core microbiome of honey bees and only obvious task and location dependent differences were observed. Nevertheless, due to the mode of action of the active ingredients, boscalid and pyraclostrobin, on the mitochondria, alterations in histology and potential sub-lethal effects should not be neglected. The focus here was on the core members of the entire honey bee gut microbiome. Species abundances of core members differ between gut segments, and bacteria from other families and species as well as fungi can also be found, which may affect the microbiome community and community assembly (Kwong and Moran, 2016). Further research should additionally focus on changes within separated gut segments of the whole microbiome community, e. g. using next-generation sequencing methods.

4.3. Floral nectar microbiome

Nectar inhabiting yeasts, such as *Metschnikowia* and related *Candida* species, can improve pollinator visitation rates and flower attractiveness, probably by influencing floral scent through modifications of nectar chemistry. Consequently, changes in yeast abundances might impact pollinator preferences (Herrera et al., 2013; Martin et al., 2022; Schaeffer et al., 2019). Both, succinate-dehydrogenase inhibitor boscalid and strobilurin pyraclostrobin act on the mitochondrial respiratory chain of fungi, and the effect is not restricted to any particular taxonomic group of fungi. Thus, the compounds could inhibit the growth of nectar inhabiting yeasts in the same way as they affect plant pathogenic fungi. However, the application of the product containing boscalid and pyraclostrobin showed no treatment-dependent effect on nectar yeast communities (Fig. 5). Neither species richness nor total abundances were significantly affected by the fungicide exposure (Fig. S6). Six days after the treatment, a decrease in species richness was observed in combination with a strong increase in total yeast abundances, suggesting that the communities can quickly overcome the fungicide stress (Fig. S6). Despite the large variation among species found in nectar, one species, *Metschnikowia pulcherrima*, accounted for the greatest abundance at the last time point. The observed changes in the yeast community were presumably related to regional (geographic) variation rather than the effects of fungicide exposure. The application of the fungicides had a stronger impact on basidiomycetous (phyllosphere-related) than ascomycetous yeasts, which are more common in flowers. Thus, flower attractiveness or foraging behavior may not be affected by the fungicide treatment. The question of whether honey bees respond to nectar alterations by yeasts and bacteria requires further investigations. For example, Rutkowski et al. (2023) summarized that honey bees, unlike bumble bees, are not attracted to nectar yeasts; and the effects of yeast community in nectar on honey bee behavior might be negligible.

5. Conclusion

To evaluate the effects of combined stressors on colony health and development, it is important to design experiments under realistic field conditions where plant protection products are applied according to permitted label instructions. Worst-case scenarios with artificial feeding treatments are leading to worst-case results and may not reflect a realistic exposure. In addition, worst-case scenarios may not take into account certain factors that influence exposure, like repellency factors of formulations, degradation after application, or the composition of formulations that influence their persistence in the environment. Here, we showed that both nucleus colonies and full-sized colonies were able to compensate for the short-time stressor effects of pollen restriction and fungicide exposure in spring when plant protection products were applied according to the label instructions. The core bacteria of the honey bee gut and the microbiome in the floral nectar of OSR were not affected by the fungicide treatment. Differences in community composition could not be distinguished from local environmental differences. This study provides the first comparative assay to assess effects of full-sized and nucleus colonies under realistic field conditions. We showed that there are obvious differences in colony growth and resilience between the two colony sizes. However, since the establishment of nucleus colonies serves the multiplication of colonies in beekeeping, special attention should be paid to their health under environmental stressors. Therefore, ensuring sufficient nutrient resources for bee colonies in spring is crucial to benefit their resilience against additional stressor factors.

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CRediT authorship contribution statement

Karoline Wueppenhorst: Conceptualization, Formal analysis, Methodology - Field experiment & Microbiome analysis & Nectar yeasts, Writing - Original draft, Review & Editing; **Abdulrahim T Alkassab:** Conceptualization, Formal analysis, Funding acquisition, Methodology - Field experiment, Writing - Review & Editing; **Hannes Beims:** Conceptualization, Funding acquisition, Methodology - Field experiment & Microbiome analysis, Writing - Review & Editing; **Ulrich Ernst:** Methodology - Field experiment, Writing - Review & Editing; **Elsa Friedrich:** Methodology - Field experiment, Writing - Review & Editing; **Ingrid Illies:** Conceptualization, Funding acquisition, Methodology - Field experiment, Writing - Review & Editing; **Martina Janke:** Conceptualization, Funding acquisition, Methodology - Field experiment, Writing - Review & Editing; **Wolfgang H Kirchner:** Conceptualization, Funding acquisition, Methodology - Field experiment, Writing - Review & Editing; **Kim Seidel:** Methodology - Microbiome analysis, Writing - Review & Editing; **Michael Steinert:** Conceptualization, Writing - Review & Editing; **Andrey Yurkov:** Methodology - Nectar yeasts, Writing - Review & Editing; **Silvio Erler:** Conceptualization, Formal analysis, Funding acquisition, Methodology - Field experiment & Microbiome analysis & Nectar yeasts, Project administration, Writing - Review & Editing; **Richard Odemer:** Conceptualization, Formal analysis, Funding acquisition, Methodology - Field experiment, Writing - Review & Editing.

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.

Data Availability

The data will be deposited at Open Agrar doi.org/10.5073/20240715-163626-0.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2024.116723.

References

Alaux, C., Ducloz, F., Crauser, D., Le Conte, Y., 2010. Diet effects on honeybee immunocompetence. Biol. Lett. 6 (4), 562–565. https://doi.org/10.1098/rsbl.2009.0986.

Aleklett, K., Hart, M., Shade, A., 2014. The microbial ecology of flowers: an emerging frontier in phyllosphere research. Botany 92 (4), 253–266. https://doi.org/10.1139/cjb-2013-0166.

Alkassab, A.T., Kunz, N., Bischoff, G., Lüken, D., Janke, M., Wallner, K., et al., 2023. Large-scale study investigating the effects of a tank mixture containing thiacloprid-prochloraz on honey bees (*Apis mellifera*). Chemosphere, 137396. https://doi.org/10.1016/j.chemosphere.2022.137396.

Arathi, H.S., Bernklau, E., 2021. Context-Dependent Effect of dietary phytochemicals on honey bees exposed to a pesticide, thiamethoxam. J. Insect Sci. 21 (4) https://doi.org/10.1093/jisesa/ieab053.

Barascou, L., Sene, D., Barraud, A., Michez, D., Lefebvre, V., Medrzycki, P., et al., 2021. Pollen nutrition fosters honeybee tolerance to pesticides. R. Soc. Open Sci. 8 (9) https://doi.org/10.1098/rsos.210818.

Bernklau, E., Bjostad, L., Hogeboom, A., Carlisle, A., H S A, 2019. Dietary phytochemicals, honey bee longevity and pathogen tolerance. Insects 10 (1). https://doi.org/10.3390/insects10010014.

Bertazzini, M., Forlani, G., 2016. Intraspecific variability of floral nectar volume and composition in rapeseed (*Brassica napus* L. var. *oleifera*). Front Plant Sci. 7, 288. https://doi.org/10.3389/fpls.2016.00288.

Bosi, G., 1973. Methode rapide pour la determination par chromatographie en phase gazeuse des glucides du nectar: technique de prelevement du nectar et de preparation des ethers trimethylsilyles en presence d'eau. Apidologie 4 (1), 57–64. https://doi.org/10.1051/apido:19730105.

Brooks, M., Kristensen, K., Benthem, K., Magnusson, A., Berg, C., Nielsen, A., et al., 2017. *glmmTMB* balances speed and flexibility among packages for zero-inflated generalized linear mixed modeling. R. J. 9 (2), 378.

Castaños, C.E., Boyce, M.C., Bates, T., Millar, A.H., Flematti, G., Lawler, N.G., et al., 2023. Lipidomic features of honey bee and colony health during limited supplementary feeding. Insect Mol. Biol. https://doi.org/10.1111/imb.12864.

Castle, D., Alkassab, A.T., Steffan-Dewenter, I., Pistorius, J., 2023. Nutritional resources modulate the responses of three bee species to pesticide exposure. J. Hazard Mater. 443 (Pt B), 130304 https://doi.org/10.1016/j.jhazmat.2022.130304.

Copeland, D.C., Maes, P.W., Mott, B.M., Anderson, K.E., 2022. Changes in gut microbiota and metabolism associated with phenotypic plasticity in the honey bee *Apis mellifera*. Front Microbiol 13, 1059001. https://doi.org/10.3389/fmicb.2022.1059001.

Corby-Harris, V., Maes, P., Anderson, K.E., 2014. The bacterial communities associated with honey bee (*Apis mellifera*) foragers. PLoS One 9 (4), e95056. https://doi.org/10.1371/journal.pone.0095056.

Crone, M.K., Grozinger, C.M., 2021. Pollen protein and lipid content influence resilience to insecticides in honey bees (*Apis mellifera*). J. Exp. Biol. https://doi.org/10.1242/jeb.242040.

Decourtye, A., Mader, E., Desneux, N., 2010. Landscape enhancement of floral resources for honey bees in agro-ecosystems. Apidologie (41), 264–277. https://doi.org/10.1051/apido/2010024.

DeGrandi-Hoffman, G., Chen, Y., Simonds, R., 2013. The effects of pesticides on queen rearing and virus titers in honey bees (*Apis mellifera* L.). Insects 4 (1), 71–89. https://doi.org/10.3390/insects4010071.

DeGrandi-Hoffman, G., Chen, Y., Watkins Dejong, E., Chambers, M.L., Hidalgo, G., 2015. Effects of oral exposure to fungicides on honey bee nutrition and virus levels. J. Econ. Entomol. 108 (6), 2518–2528. https://doi.org/10.1093/jee/tov251.

DeGrandi-Hoffman, G., Corby-Harris, V., DeJong, E.W., Chambers, M., Hidalgo, G., 2017. Honey bee gut microbial communities are robust to the fungicide Pristine® consumed in pollen. Apidologie 48 (3), 340–352. https://doi.org/10.1007/s13592-016-0478-y.

Derbyshire, M.C., Denton-Giles, M., 2016. The control of sclerotinia stem rot on oilseed rape (*Brassica napus*): current practices and future opportunities. Plant Pathol. 65 (6), 859–877. https://doi.org/10.1111/ppa.12517.

DesJardins, N.S., Fisher, A., Ozturk, C., Fewell, J.H., DeGrandi-Hoffman, G., Harrison, J. F., et al., 2021. A common fungicide, Pristine®, impairs olfactory associative learning performance in honey bees (*Apis mellifera*). Environ. Pollut. 288 https://doi.org/10.1016/j.envpol.2021.117720.

Deutsches Bienen Monitoring (DeBiMo). Zwischenbericht 2022. ⟨https://static1.squarespace.com/static/62fe5049548fce26fd503807/t/64536971a98d8a08bad54115/1683188101564/DeBiMo_Zwischenbericht_2022.pdf.2022⟩.

Di Pasquale, G., Salignon, M., Le Conte, Y., Belzunces, L.P., Decourtye, A., Kretzschmar, A., et al., 2013. Influence of pollen nutrition on honey bee health: do pollen quality and diversity matter? PLoS One 8 (8), e72016. https://doi.org/10.1371/journal.pone.0072016.

Dolezal, A.G., Toth, A.L., 2018. Feedbacks between nutrition and disease in honey bee health. Curr. Opin. Insect Sci. 26, 114–119. https://doi.org/10.1016/j.cois.2018.02.006.

Dolezal, A.G., Carrillo-Tripp, J., Judd, T.M., Allen Miller, W., Bonning, B.C., Toth, A.L., 2019. Interacting stressors matter: diet quality and virus infection in honeybee health. R. Soc. Open Sci. 6 (2), 181803 https://doi.org/10.1098/rsos.181803.

Erler, S., Popp, M., Lattorff, H.M.G., 2011. Dynamics of immune system gene expression upon bacterial challenge and wounding in a social insect (*Bombus terrestris*). PLoS One 6 (3), e18126. https://doi.org/10.1371/journal.pone.0018126.

Fisher, A., Coleman, C., Hoffmann, C., Fritz, B., Rangel, J., 2017. The synergistic effects of almond protection fungicides on honey bee (Hymenoptera: Apidae) forager survival. J. Econ. Entomol. 110 (3), 802–808. https://doi.org/10.1093/jee/tox031.

Fisher, A., Cogley, T., Ozturk, C., DeGrandi-Hoffman, G., Smith, B.H., Kaftanoglu, O., et al., 2021a. The active ingredients of a mitotoxic fungicide negatively affect pollen consumption and worker survival in laboratory-reared honey bees (*Apis mellifera*). Ecotoxicol. Environ. Saf. 226, 112841 https://doi.org/10.1016/j.ecoenv.2021.112841.

Fisher, A., DeGrandi-Hoffman, G., Smith, B.H., Johnson, M., Kaftanoglu, O., Cogley, T., et al., 2021b. Colony field test reveals dramatically higher toxicity of a widely-used mito-toxic fungicide on honey bees (*Apis mellifera*). Environ. Pollut. 269, 115964 https://doi.org/10.1016/j.envpol.2020.115964.

Fisher, A., DeGrandi-Hoffman, G., Smith, B.H., Ozturk, C., Kaftanoglu, O., Fewell, J.H., et al., 2021c. Field cross-fostering and in vitro rearing demonstrate negative effects of both larval and adult exposure to a widely used fungicide in honey bees (*Apis mellifera*). Ecotoxicol. Environ. Saf. 217, 112251 https://doi.org/10.1016/j.ecoenv.2021.112251.

Fisher, A., Glass, J.R., Ozturk, C., DesJardins, N., Raka, Y., DeGrandi-Hoffman, G., et al., 2022. Seasonal variability in physiology and behavior affect the impact of fungicide exposure on honey bee (*Apis mellifera*) health. Environ. Pollut. 311, 120010 https://doi.org/10.1016/j.envpol.2022.120010.

Fisher, A., DeGrandi-Hoffman, G., Liao, L.-H., Tadei, R., Harrison, J.F., 2023. The challenge of balancing fungicide use and pollinator health. Environmental threats to pollinator health and fitness. Advances in Insect Physiology. Elsevier, pp. 117–190. https://doi.org/10.1016/bs.aiip.2023.01.002.

Free, J.B., Racey, P.A., 1968. The effect of the size of honeybee colonies on food consumption, brood rearing and the longevity of bees during winter. Ent Exp. Appl. 11, 241–249. https://doi.org/10.1111/j.1570-7458.1968.tb02048.x.

Gaubert, J., Giovenazzo, P., Derome, N., 2023. Individual and social defenses in *Apis mellifera*: a playground to fight against synergistic stressor interactions. Front Physiol. 14, 1172859 https://doi.org/10.3389/fphys.2023.1172859.

Genersch, E., Ohe, W. von der, Kaatz, H., Schroeder, A., Otten, C., Büchler, R., et al., 2010. The German bee monitoring project: a long term study to understand periodically high winter losses of honey bee colonies. Apidologie 41 (3), 332–352. https://doi.org/10.1051/apido/2010014.

Gerig, L., 1983. Lehrgang zur Erfassung der Volksstärke. Schweiz. Biene -Ztg. 106 (4), 199–204.

Glass, J.R., Fisher, A., Fewell, J.H., DeGrandi-Hoffman, G., Ozturk, C., Harrison, J.F., 2021. Consumption of field-realistic doses of a widely used mito-toxic fungicide reduces thorax mass but does not negatively impact flight capacities of the honey bee (*Apis mellifera*). Environ. Pollut. 274 https://doi.org/10.1016/j.envpol.2021.116533.

Goodrich, B.K., 2019. Do more bees imply higher fees? Honey bee colony strength as a determinant of almond pollination fees. Food Policy 83, 150–160. https://doi.org/10.1016/j.foodpol.2018.12.008.

Gray, A., Adjlane, N., Arab, A., Ballis, A., Brusbardis, V., Bugeja Douglas, A., et al., 2023. Honey bee colony loss rates in 37 countries using the COLOSS survey for winter 2019–2020: the combined effects of operation size, migration and queen replacement. J. Apic. Res. 62 (2), 204–210. https://doi.org/10.1080/00218839.2022.2113329.

Groeneveld, J., Odemer, R., Requier, F., 2024. Brood indicators are an early warning signal of honey bee colony loss—a simulation-based study. PLoS ONE 19 (5), e0302907. https://doi.org/10.1371/journal.pone.0302907.

Harbo, J.R., 1986. Effect of population size on brood production, worker survival and honey gain in colonies of honeybees. J. Apic. Res. 25 (1), 22–29. https://doi.org/10.1080/00218839.1986.11100687.

Hartig F. DHARMa: Residual diagnostics for hierarchical (multi-level / mixed) regression model. R package version 0.4.6. 2022: <⟨https://CRAN.R-project.org/package=DHARMa⟩>.

Herrera, C.M., Pozo, M.I., Medrano, M., 2013. Yeasts in nectar of an early-blooming herb: sought by bumble bees, detrimental to plant fecundity. Ecology 94 (2), 273–279. https://doi.org/10.1890/12-0595.1.

Höferlin, B., Höferlin, M., Kleinhenz, M., Bargen, H., 2013. Automatic analysis of *Apis mellifera* comb photos and brood development. In: Association of institutes for bee research report of the 60th seminar in Würzburg. Apidologie Vol. 44, 19.

Hotchkiss, M.Z., Poulain, A.J., Forrest, J.R.K., 2022. Pesticide-induced disturbances of bee gut microbiotas. FEMS Microbiol Rev. 46 (2) https://doi.org/10.1093/femsre/fuab056.

Hýbl, M., Mráz, P., Šipoš, J., Hoštičková, I., Bohatá, A., Čurn, V., et al., 2021. Polyphenols as food supplement improved food consumption and longevity of honey bees (*Apis mellifera*) intoxicated by pesticide thiacloprid. Insects 12 (7). https://doi.org/10.3390/insects12070572.

Imdorf, A., Bühlmann, G., Gerig, L., Kilchenmann, V., Wille, H., 1987. Überprüfung der Schätzmethode zur Ermittlung der Brutfläche und der Anzahl Arbeiterinnen in freifliegenden Bienenvölkern. Apidologie 18 (2), 137–146.

Jacquemyn, H., Pozo, M.I., Álvarez-Pérez, S., Lievens, B., Fukami, T., 2021. Yeast-nectar interactions: metacommunities and effects on pollinators. Curr. Opin. Insect Sci. 44, 35–40. https://doi.org/10.1016/j.cois.2020.09.014.

Jones, J.C., Fruciano, C., Marchant, J., Hildebrand, F., Forslund, S., Bork, P., et al., 2018. The gut microbiome is associated with behavioural task in honey bees. Insect Soc. 65 (3), 419–429. https://doi.org/10.1007/s00040-018-0624-9.

Kapheim, K.M., Rao, V.D., Yeoman, C.J., Wilson, B.A., White, B.A., Goldenfeld, N., et al., 2015. Caste-specific differences in hindgut microbial communities of honey bees (*Apis mellifera*). PLoS One 10 (4), e0123911. https://doi.org/10.1371/journal.pone.0123911.

Kwong, W.K., Moran, N.A., 2016. Gut microbial communities of social bees. Nat. Rev. Microbiol 14 (6), 374–384. https://doi.org/10.1038/nrmicro.2016.43.

Lenth R. emmeans: Estimated marginal means, aka least-squares means. 2023: R package version 1.8.7, <⟨https://CRAN.R-project.org/package=emmeans⟩>.

Lin, Z., Shen, S., Wang, K., Ji, T., 2023. Biotic and abiotic stresses on honeybee health. Integr. Zool. https://doi.org/10.1111/1749-4877.12752.

Lückmann, J., Tänzler, V., 2020. Honeybee brood testing under semi-field and field conditions according to Oomen and OECD GD 75: is there a difference of the brood termination rate? Jul. -K. ühn-Arch. 465 https://doi.org/10.5073/JKA.2020.465.043.

Martin, V.N., Schaeffer, R.N., Fukami, T., 2022. Potential effects of nectar microbes on pollinator health. Philos. Trans. R. Soc. Lond. B Biol. Sci. 377 (1853), 20210155 https://doi.org/10.1098/rstb.2021.0155.

Maucourt, S., Fournier, V., Giovenazzo, P., 2018. Comparison of three methods to multiply honey bee (*Apis mellifera*) colonies. Apidologie 49 (3), 314–324. https://doi.org/10.1007/s13592-017-0556-9.

OECD, 2007. No. 75: Guidance document of the honeybee (Apis mellifera L.) brood test under semi-field conditions. OECD Publishing, Paris, p. 27. ⟨https://www.oecd-ilibrary.org/docserver/9789264085510-en.pdf?expires=1634199345&id=id&accname=oid018224&checksum=F6AE8D28F840DB3243A9CCAC6966FD03⟩.

Oksanen J., Simpson G., Blanchet F., Kindt R., Legendre P., Minchin P., O'Hara R., Solymos P., Stevens M., Szoecs E., Wagner H., Barbour M., Bedward M., Bolker B., Borcard D., Carvalho G., Chirico M., De Caceres M., Durand S., Evangelista H., FitzJohn R., Friendly M., Furneaux B., Hannigan G., Hill M., Lahti L., McGlinn D., Ouellette M., Ribeiro Cunha E., Smith T., Stier A., Ter Braak C., Weedon J. vegan: Community ecology package, R package version 2.6-4. 2022 ⟨<https://CRAN.R-project.org/package=vegan>⟩.

Rutkowski, D., Weston, M., Vannette, R.L., 2023. Bees just wanna have fungi: a review of bee associations with non-pathogenic fungi. FEMS Microbiol Ecol. https://doi.org/10.1093/femsec/fiad077.

Schaeffer, R.N., Rering, C.C., Maalouf, I., Beck, J.J., Vannette, R.L., 2019. Microbial metabolites elicit distinct olfactory and gustatory preferences in bumblebees. Biol. Lett. 15 (7), 20190132. https://doi.org/10.1098/rsbl.2019.0132.

Schmickl, T., Crailsheim, K., 2002. How honeybees (*Apis mellifera* L.) change their broodcare behaviour in response to non-foraging conditions and poor pollen conditions. Behav. Ecol. Socio 51 (5), 415–425. https://doi.org/10.1007/s00265-002-0457-3.

Schmickl, T., Crailsheim, K., 2004. Inner nest homeostasis in a changing environment with special emphasis on honey bee brood nursing and pollen supply. Apidologie 35 (3), 249–263. https://doi.org/10.1051/apido:2004019.

Schödl, I., Odemer, R., Becher, M.A., Berg, S., Otten, C., Grimm, V., Groeneveld, J., 2022. Simulation of varroa mite control in honey bee colonies without synthetic acaricides: Demonstration of good beekeeping practice for Germany in the BEEHAVE model. Ecol. Evol. 12 (11), e9456 https://doi.org/10.1002/ece3.9456.

Schur, Tornier, A., Brasse, I., Muehlen, D., Ohne, W., von der, W., Wallner, K., et al., 2003. Honey bee brood ring-test in 2002: method for the assessment of side effects of plant protection products on the honey bee brood under semi-field conditions. Bull. Insect 56 (1), 91–96.

Smutin, D., Lebedev, E., Selitskiy, M., Panyushev, N., Adonin, L., 2022. Micro"bee"ota: Honey bee normal microbiota as a part of superorganism. Microorganisms 10 (12). https://doi.org/10.3390/microorganisms10122359.

Steinigeweg, C., Alkassab, A.T., Erler, S., Beims, H., Wirtz, I.P., Richter, D., et al., 2023. Impact of a microbial pest control product containing *Bacillus thuringiensis* on brood development and gut microbiota of *Apis mellifera* worker honey bees. Micro Ecol. 85, 1300–1307. https://doi.org/10.1007/s00248-022-02004-w.

Tosi, S., Nieh, J.C., Sgolastra, F., Cabbri, R., Medrzycki, P., 2017. Neonicotinoid pesticides and nutritional stress synergistically reduce survival in honey bees. Proc. Roal Soc. B 284 (1869). https://doi.org/10.1098/rspb.2017.1711.

Wahl, O., Ulm, K., 1983. Influence of pollen feeding and physiological condition on pesticide sensitivity of the honey bee *Apis mellifera carnica*. Oecologia 59 (1), 106–128. https://doi.org/10.1007/BF00388082.

Wernecke, A., Eckert, J.H., Bischoff, G., Forster, R., Pistorius, J., Odemer, R., 2023. A selected organosilicone spray adjuvant does not enhance lethal effects of a pyrethroid and carbamate insecticide on honey bees. Front Physiol. 14, 1171817 https://doi.org/10.3389/fphys.2023.1171817.

Westerhoff, A., Büchler, R., 1994b. Die Entwicklung kleiner Überwinterungsableger im Vergleich zu Vollvölkern. Allg. Dtsch. Imkerztg. 9, 12–15.

Westerhoff, A., Büchler, R., 1994a. Zusammenhänge zwischen Volksstärke, Brutpflege, Lebenserwartung und Honigleistung. Allg. Dtsch. Imkerztg. 10, 30–33.

Yang, C., Hamel, C., Vujanovic, V., Gan, Y., 2011. Fungicide: modes of action and possible impact on nontarget microorganisms. ISRN Ecol. 2011, 1–8. https://doi.org/10.5402/2011/130289.