EFFECTS OF MIXED NEONICOTINOID AND FUNGICIDE EXPOSURE ON THE POLLEN COLLECTION AND COLONY DEVELOPMENT OF BOMBUS TERRESTRIS

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ABSTRACT

Bumblebees are essential pollinators for a wide range of plant species and thus essential for global ecosystems and food security. However, recent declines in their populations have made understanding their underlying causes necessary. One of those causes is the application of pesticides in agricultural landscapes. The toxicity of these pesticides is commonly assessed in isolation, which oftentimes fails to account for synergisms between pesticide mixtures that pollinators are often exposed to. In honeybees one of those synergisms that has been observed is between ergosterol biosynthesis inhibiting (EBI) fungicides like tebuconazole and neonicotinoids like acetamiprid and imidacloprid. Acetamiprid is the last remaining pesticide approved for outdoor use in the EU, since it is commonly shown to be less toxic to pollinators that other neonicotinoids like imidacloprid. However, the basis of research for that assumption is mostly focused on honeybees (*Apis mellifera*) and without taking into account synergisms. Therefore, this study aimed at characterizing effects of acetamiprid, tebuconazole and a possible combined effect on the buff-tailed bumblebee *Bombus terrestris*, with the well-studied neonicotinoid imidacloprid as a baseline for effects. Specifically, effects on colony development, pollen collection, and colony performance were evaluated. However, due to an insufficient degree of exposure to and possibly a short period of observation, no effect of acetamiprid and tebuconazole could be documented. The only effect seen was imidacloprid decreasing the amount of pollen collected by foragers on a foraging bout. Other covariates like the initial weight of the colonies, their batch and site, were found to affect colony development and colony performance. This being said, future research addressing those methodological problems is promising to understand the complex relationship between combined pesticide effects, foraging behaviour, and colony development.

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1 INTRODUCTION

Social bees are essential providers of pollination services, with around 78% of flowering plant species in the temperate zone being pollinated by animals (Ollerton et al., 2011). Apart from their key role in ecosystems, they are essential for humans and global food security, since pollination by bees benefits crop quality as well as yields (Khalifa et al., 2021). However, in a world where food security is threatened by other factors, such as climate change (Myers et al., 2017), their decline is especially worrying. Species richness of wild bees has declined globally (Zattara & Aizen, 2021), and although insect biodiversity overall is threatened, Hymenoptera seems to be one of the worst affected orders of insects on land(Sánchez-Bayo & Wyckhuys, 2019). This order is declining in species richness as well as abundance, to such an extent that forty percent of the species within the order are at risk of extinction within the next decades (Sánchez-Bayo & Wyckhuys, 2019). There are many factors responsible for this decline, according Sánchez-Bayo and Wyckhuys (2019) the most important threats in descending order are habitat loss and land-use conversion associated with agricultural intensification, the use of fertilizers and pesticides, biotic factors like invasive species and parasites, and climate change. To contribute to the understanding of this decline in Hymenoptera populations, the aim with this project was to explore the effects of pesticides on bumblebees.

Pesticides are used worldwide in agriculture, as they protect crops from pests and thus contribute to food security. However, they also have impacts on many non-target organisms, threatening the integrity of global ecosystems (Sharma et al., 2019). One well-known class of pesticides are the neonicotinoids. They are toxic to insects by specifically targeting the postsynaptic nicotinic acetylcholine receptors (nACHR) in insects. Because this receptor differs strongly from the nAChR in mammals, neonicotinoids have very low toxicity against humans and other mammals (Sheets, 2014; van der Sluijs et al., 2013; Wallace, 2014). They act systemically, found in all parts of the exposed plants (van der Sluijs et al., 2013) and have a wide range of uses, for example in agriculture and to control ticks and flees in pets (Sheets, 2014). The first neonicotinoid was approved in the EU in 2005 (EU, 2024a). However, due to growing concerns about their safety, especially for bees, clothianidin, imidacloprid, thiamethoxam were restricted in 2013 and ultimately - except for emergency authorization cases - are no longer approved in the EUfor outdoor plant protection since 2020. However, acetamiprid's approval for use was renewed until 2033 (EU, 2024b) as a result of its lower toxicity (Varga-Szilay & Toth, 2022).

The effects of neonicotinoids on bees, in particular honeybees, has gained a lot of research attention (Dirilgen et al., 2023) to such an extent that imidacloprid is the world's most studied insecticide when it comes to effects on this organism group (Tosi et al. 2022). They have been shown to negatively affect wild bee populations in species richness, density, and colony growth (Rundlöf et al., 2015; Woodcock et al., 2016).

Two neonicotinoid pesticides were selected as a focus for this study: acetamiprid and imidacloprid. With imidacloprid as the most widely studied insecticide (Tosi et al., 2022) being used as a benchmark/positive control. Imidacloprid has, apart from lethal effects due to its toxicity, shown to have a range of sublethal effects at field realistic exposures. Among those are impairment of foraging performance, altered floral preferences, increased worker

mortality, lower colony growth rate, and lower production of queens (Gill & Raine, 2014; Gill et al., 2012; Whitehorn et al., 2012). Impairment of colony thermoregulation and larval nursing have also been observed (Crall et al., 2018).

In contrast to the well-studied imidacloprid, less is known about acetamiprid (Tosi et al., 2022). Acetamiprid has a short half-life, about 95% of acetamiprid is degraded after just 15 days (Wallace, 2014), therefore it has a low persistence in the soil (Wallace, 2014). Although there is only limited research on acetamiprid's effects on bumblebees, the studies that exist, largely suggest that acetamiprid has low acute and chronic toxicity and few sublethal effects at field realistic levels (Varga-Szilay & Toth, 2022). However, at very high concentrations, effects on colony development could be observed (Camp et al., 2020). In addition to that, possible synergies with other pesticides (Migdał et al., 2024; Schmuck et al., 2003) make acetamiprid an important substance for study in complex mixtures of pesticides that pollinators are often exposed to in the field (David et al., 2016).

One group of these pesticides with which synergisms have been observed are ergosterol biosynthesis inhibiting (EBI) fungicides, like tebuconazole, which has been approved in the European Union since 2009 (EU, 2024c). It is a systemic triazole fungicide, that acts by inhibiting cytochrome P450 mediated ergosterol biosynthesis, which affects the cell walls of fungi. In honeybees *(Apis mellifera)* EBI fungicides have been shown to disrupt the cytochrome P450 monooxygenases mediated detoxification, increasing the toxicity of other compounds such as neonicotinoids (Schuhmann et al., 2022). This synergism between EBI fungicides and neonicotinoids has been further confirmed in Migdał et al. (2024) and Schmuck et al. (2003). At higher doses, tebuconazole can be toxic to a wide range of animals, especially to aquatic life (Dong, 2024), however its effects on bumblebees are under research. In bumblebees it was shown to have a negative effect on colony growth, but only as a combined effect of multiple stressors (pesticides and parasites) (Botias et al., 2021). Tebuconazole, as well as acetamiprid, are highly abundant in the environment and bees are likely to become exposed to a combination of these(Knapp et al., 2023), which highlights the importance of understanding potential cocktail effects.

A review by Tosi et al. (2022) found that the vast majority of studies on the effects of pesticides on Hymenopterans are done on *Apis* species, mostly *A. mellifera.* Only 12% of studies were conducted on non-*Apis* species, highlighting necessity for further research on those species. The need to expand our research focus and reduce the bias towards honeybees was also highlighted by Dirilgen et al. (2023). We chose *Bombus terrestris* as our model organism in this study, since they are important pollinators for many species, their ecology is fairly well understood (Goulson, 2010) and commercial availability makes them easily accessible. *B. terrestris* colonies have a yearly life cycle starting with a single queen emerging from hibernation in spring. She selects an appropriate nesting site for her colony, for which she then starts foraging for pollen and nectar. The pollen is formed into a clump, into which she deposits her first batch of eggs. During this time, she both incubates her brood and forages for nectar and pollen to ensure the survival of herself and her offspring. After the first batch of workers hatch, which typically takes 4-5 weeks, they take over the brood care and foraging, while the queen remains in the hive instead of undertaking the risky foraging herself and continues to produce further batches of offspring. Workers are typically divided into foragers and nest workers who take over nest maintenance and brood care, although their occupations can change throughout their lifespan, with younger workers being more likely to stay in the nest and only becoming foragers later in life. Bumblebee workers forage for both nectar and

pollen, with the sugar rich nectar satisfying energetic needs of the colony, while pollen is supplying most of the protein and lipids (Goulson, 2010). The makeup of the pollen diet is of high importance for colony development (Moerman et al., 2017), and bees are very selective in their pollen diets and floral preferences under normal conditions, choosing plants with optimal ratios of protein, carbohydrates, and lipids, with differing preferences depending on brood presence and absence (Kraus et al., 2019; Ruedenauer et al., 2016; Vaudo et al., 2016). Interestingly, a few studies suggest that neonicotinoids can alter bees' floral preferences (Gill & Raine, 2014; Stanley & Raine, 2016), but the reason for this is unknown. Because the nutritive value of pollen varies among plant species, it is possible that altered floral preferences due to pesticide exposure, influences the nutritive value of the pollen collected by bumblebee workers, but this has never been studied. The yearly cycle of a colony concludes with males and/or new queens being produced. Those then leave to mate, and the fertilized new queens hibernate to start their own colony in the next season, while the males and the old colony die (Goulson, 2010).

The specificity of floral preferences and the importance of pollen for the colony in terms of development and performance are thus key to understanding colony level implications of individual effects of pesticides. Therefore, contributing to the understanding of the decline in Hymenoptera populations, this study aimed to investigate the effects of the neonicotinoid acetamiprid and the fungicide tebuconazole, and their possible synergism on *B. terrestris.* Imidacloprid was used as a benchmark. Specifically, I was interested in understanding how exposure to the selected pesticides affected floral preference and pollen protein content of collected pollen, and consequently colony health and development, estimated as weight gain, worker survival and larval production. Based on the results from previous studies, I expected imidacloprid to affect all the studied variables. I also expected no, or little effect of single pesticide exposure to tebuconazole or acetamiprid. Because tebuconazole is expected to inhibit detoxification from neonicotinoids, I did, however, expect a stronger effect of their combined exposure.

2 MATERIALS AND METHODS

2.1 TIMELINE

Table 1 Timeline of the field experiment. Including arrival, tagging, start of exposure, and period of data collection. The hives were killed the day after or on the last day of data collection

DATE	
24.5.	Arrival of the 1st batch
24.5. – 1.6.	Tagging of the 1st batch
2.6.	Preparing sites on the outside, starting exposure of 1st batch hives,
5.6 – 26.6.	Collecting data from first batch: pollen and weighing twice per week
5.6.	Arrival of the 2nd batch of hives
6.6 – 8.6	Tagging of the 2nd batch
9.6.	Start of exposure of the 2nd batch
12.6. – 4.7.	Collecting data from the 2nd batch
28.6.	Arrival of the 3rd batch of hives
29.6 – 6.7.	Tagging workers from the 3rd batch of hives
7.7.	Start of exposure of the 3rd batch
10.7 – 30.7.	Collecting data from 3rd batch of hives
30.7.	Freezing the last batch, end of field experiment

2.2 STUDY DESIGN

The experimental part of this study consisted of two parts: a field study carried out during the summer months (May-July), where pollen samples, video recordings, and hive weights were collected, and a laboratory analysis of in the field collected samples and a dissection of frozen hives.

2.2.1 Hive arrival and initial treatment

Early-stage colonies (20 workers) were ordered from Biobest. Our initial plan was to release one batch of 15 hives early June and another batch by the end of June. Because several queens had died during the transport of the first batch, Biobest replaced these colonies and we adjusted the time plan slightly, resulting in 3 batches, with 10-15 colonies per batch. All of these were treated equally in the lab, but they were placed at different positions and throughout different times of the season.

On, or shortly after their arrival, the hives were checked for an alive queen and overall health, weighed, and assigned to a treatment. The first 5 hives were assigned to treatment groups at random. Afterwards the leftover hives were assigned to the treatment groups in a way to keep the average weight constant between treatments.

During this process we collected data on the initial weight and the initial number of individuals.

2.2.2 Tagging

We tagged bumblebees with individual number tags, ranging from 1-100. One tag color per treatment was used: Red, yellow, green, lime, and white. If more than 100 individuals were tagged in a single hive, or if no tags of the corresponding color were available anymore (for batch 3 of the hives), a unique combination of white tags were used together with colored markings to make a distinction of every individual and treatment possible.

Before the tagging, we trapped the bumblebees from the hives, in honeybee queen marking tubes. We did this in a dark room using red headlights, because red light cannot be seen by bumblebees. In another room, these caught bumblebees were then tagged just behind the head on the thorax using super glue (brand: Loctite) and if applicable marked with Posca pens, used for honeybee queen marking. The bees were then released back into their hives. The number of tagged individuals was then written down.

2.2.3 Preparing field sites

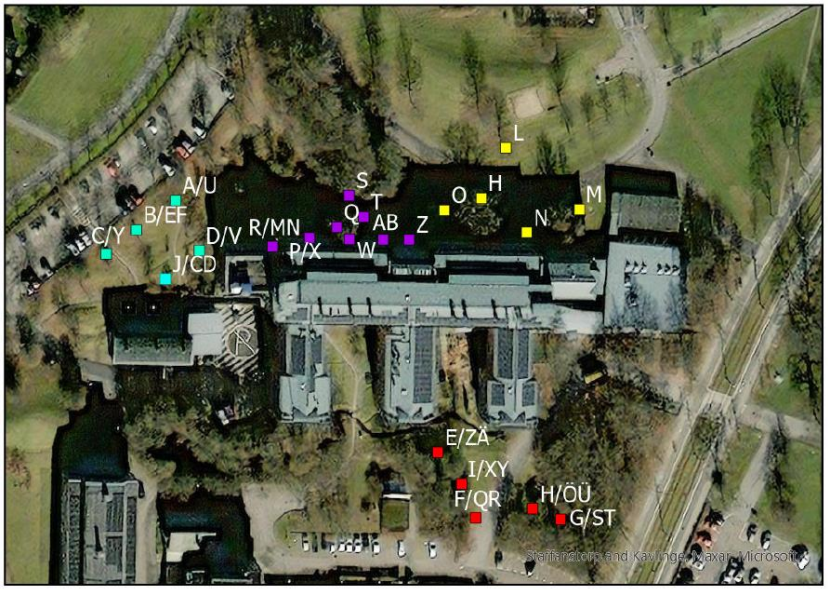

Figure 1 A map depicting the positions of the hives around the building. With each site being distinguished by colour: A (blue), B (yellow), C (red), and D (purple).

Sites for the hives were selected in a way to ensure access to flowering resources while also giving protection from wind and weather in the shade.

Each hive was placed in its own wooden nest box with the only entrance and exit being a about 7cm long white plastic tunnel with a removable see-through acrylic lid. Mounts for the cameras were placed on top of the nestboxes' lids, facing the upper side of the tunnels.

The entrances of the wooden nestboxes in which the individual hives were placed were facing northwards.

2.2.4 Initial exposure

On the day of the initial exposure, all hives were weighed, and the percentage of tagged bees to untagged bees estimated, so that the number of individuals could later be extrapolated, from knowing the total number of tagged individuals. Afterwards, the hives were put into their respective nest boxes, in a way that equally distributes treatments among sites (Fig 1). The exposure method was adapted from Gill and Raine (2014). They were exposed to the pesticides by feeding them sucrose solution through a gravity feeder. For the first exposure in the closed nest boxes, the bees were given 50 ml solution. Afterwards, in the first two weeks, the hives were given 15 ml of sucrose-pesticide solution per exposure, and in the last week 20 ml. The sucrose was prepared by Natalia Davila.

There were 5 groups with the following concentrations of pesticide in 40% sucrose w/v:

Control:	no pesticide
Imidacloprid:	10 ppb
Acetamiprid:	92 ppb
Tebuconazole:	56 ppb
Combined:	56 ppb tebuconazole and 92 ppb acetamiprid

The concentration of imidacloprid was taken from Gill and Raine (2014) and the concentration of acetamiprid and tebuconazole were based on to date unpublished field realistic data by Maj Rundlöf. They correspond to the 90th percentile of concentrations of these pesticides from bumblebee (*B. terrestris*) and honeybee (*A. mellifera*) nectar from 8 European countries.

On the first day of exposure, after the hives were put into their respective nest boxes in the field, they were supplemented with the filled gravity feeders and closed for the next 2 days. After that, the nest boxes were opened, and new sucrose solution was given every 2-3 days.

2.3 DATA COLLECTION (FIELD)

2.3.1 Pollen sampling

To collect the pollen, the hives were observed for one hour. If there was little activity, the time could be prolonged to up to two hours. This pollen collection was then repeated multiple times throughout the 3-week exposure period. We aimed at spending equal time with all hives, however some hives had very low activity and had to be prioritized to get enough samples from each hive and treatment. During the observation time, every incoming

bumblebee was caught and checked for pollen, which was put into Eppendorf tubes. A piece of paper with the date, hive, number of the individual, and number of the observation was included with the pollen sample.

If untagged bumblebees were encountered, they were also caught and tagged with the appropriate hive and tag combination for each hive.

2.3.2 Weight

We weighed the entire next boxes twice a week. The weighing was done at night after dark to ensure that all bees had returned to the hive after foraging throughout the day.

2.4 FREEZING HIVES

On the last day after collecting all necessary data and weighing the hives one last time, the entire nest boxes were transferred to a freezer at -18°C and left to freeze overnight. Afterwards the nest boxes were cleaned up, all outside hive material photographed and transferred into the plastic inner nest boxes and stored at -18°C for later dissection.

2.5 DATA COLLECTION (LAB)

2.5.1 Hive dissection

The first step of the hive dissection was to check the hive condition. Focuse was on the amount of moth damage, parasites, and mold coverage. Afterwards the number of empty and cocooned cells and wax cells were estimated. To simplify the counting, bigger pieces were broken down into smaller ones. Queen and worker cells were differentiated based on size. During this process, numbers of old, young larvae/eggs and dead larvae were also estimated. The different categories were based on level of development and age, whereas dead larvae can be identified due to their brown discoloration.

In addition to that, all bees in the hive were counted. I distinguished between tag colours/no tags, workers, males, queens. Queens can be identified based on their much bigger size. Males were distinguished from workers by looking at their abdomen tips: whereas workers have a pointy tip, with a stinger visible in some cases, males have a rounded tip, and the penis can sometimes be seen when applying pressure on the abdomen.

At the end all bees were weighed in groups based on sex and tag color and if a visible pollen clump was present the pollen clump was weighed. After the dissection, the hives were returned to the freezer for storage.

Figure 2 photograph of different stages of larvae. With two late stage/old larvae to the left, and a young larva and an egg to the centre right and right respectively.

Figure 3 photographs of bumblebee cells. With opened worker cells to the left and closed worker cells to the right (indicated by the red arrow).

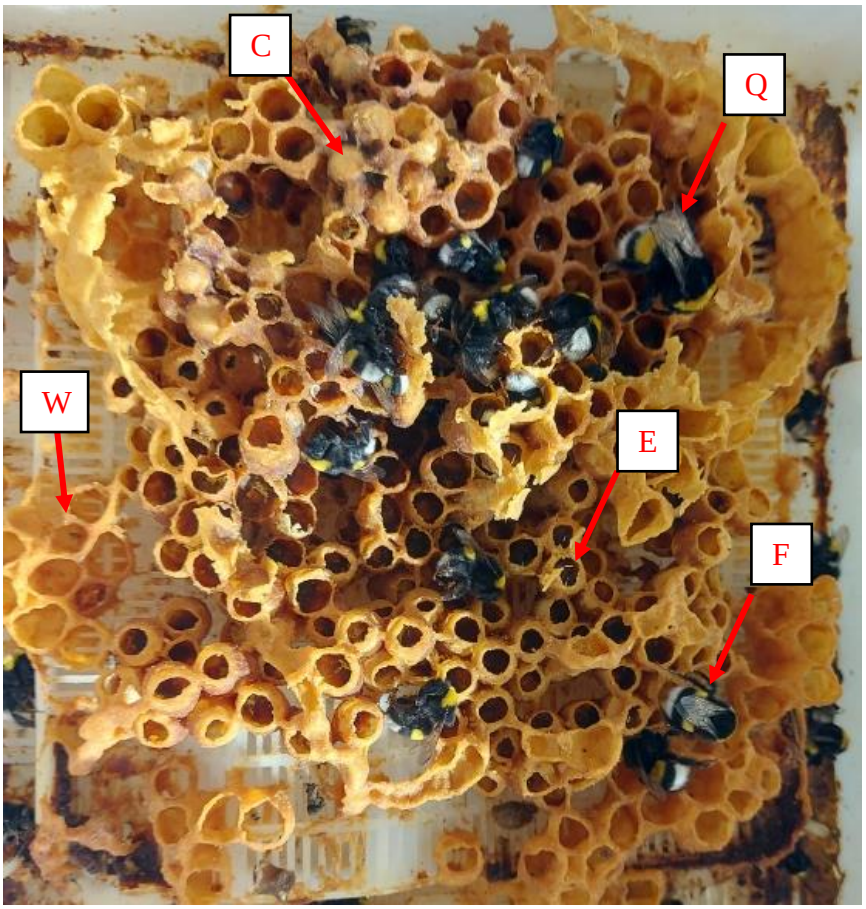

Figure 4 Overview of a healthy bumblebee colony, with a queen (Q), empty worker cells (E), cocooned worker cells (C) , female workers (F), and wax cells (W)

2.5.2 Pollen analysis

The analysis of the pollen samples was carried out using the method outlined in Olsson et al. (2021) and in collaboration with Johan Aune and Natalia Davila. The pollen samples were weighed individually, solved in ethanol to have a similar concentration of pollen grains in each sample, and then fixated and stained on microscopy slides with fuchsin gel. These pollen grains were then scanned and cross referenced with the existing pollen image database in Olsson et al. (2021), to obtain information the species/family of origin of pollen and their relative abundance in the pollen samples.

2.6 STATISTICS

The statistical analysis was carried out in R studio version 2023.09.1. The first step of the statistical analysis was an exploratory analysis of the initial conditions, to ensure that there was no inherent difference between treatments. To do this, I performed two separate one-way ANOVAs with pesticide treatment as the independent variable and initial weight, or number of workers on the first day of exposure, respectively, as the dependent variable. Moreover, I expected larger colonies to have advantages at the start of the experiment, which is why I wanted to account for variation in initial colony size. Therefore, I aimed to use the initial number of workers as a predictor variable in the following analyses. However, since that specific data was not complete for all hives, I analyzed the relationship between the initial weight and the initial number of workers to confirm initial weight as a suitable variable instead. To do that, a linear regression model with initial weight as the predictor and initial number of workers as the response was fitted.

To reduce unexplained variation and improve model fit, possible covariates that were unrelated to treatment were selected. Those were site, batch and initial weight. These covariates were then individually modelled as predictors for each response variable, i.e. weight gain, pollen protein, larval survival, larval count, worker survival, worker count. Only the factors that significantly impacted the respective response variable were then included in the final (see sections 2.6.1-2.6.3) models as a covariate alongside pesticide treatment as a predictor. Additionally, all final models were checked for collinearity. In cases where the collinearity was high (VIF above 5), the variable with the highest p-value and if applicable lowest R^2 was removed.

2.6.1 Hive weights

To analyze the effects of treatments on the hive weights over the entire time period, I calculated weight gain from the day of the opening of the hives until the final day. This weight difference was then used as a response variable in a linear model dependent on treatment with batch and site as covariates. Level of significance were determined by performing a one-way ANOVA.

2.6.2 Pollen analysis

To determine possible effects on the amount of pollen collected by the workers per foraging bout, the weight of the pollen load was used. The data was gamma distributed, and therefore a generalized linear model with gamma distribution was fitted. Because of a difference among batches in the weight of the pollen load, batch was included as a covariate. The model was fitted on the level of individuals but nested within hives. Significance values were obtained from conducting wald-chisquare test with the help of the Anova function of the car package in R studio. Since marginal significance was found dependent on the treatment, pairwise comparisons between the treatments were conducted using the pairs and emmeans functions in R studio.

To obtain data over the protein content of the individual samples, data from two datasets were combined: most data was extracted from Weiner et al. (2010), by adding both protein and free amino acid content per species. However, since not every plant family found in our data was present, the protein content from Vaudo et al. (2020) was used for the following families: Adoxaceae, Fagaceae, Hypericaceae, Orobanchaceae, and Poaceae. Since the protein content in both datasets was on the basis of individual species, mean values per plant family were calculated using all available species. Even after combining both datasets, not all plant families in the pollen analysis had known pollen protein contents. But since a mean of 92% of pollen per sample was accounted for, all other families that were only present in the collected pollen in very small ratios were excluded. This protein data was then multiplied with the ratios of pollen abundance obtained from the pollen analysis in 7.2 and summed per sample. Since none of the datasets covered all plant families that the bees had visited, this protein content per sample was then divided by the ratio of all the pollen for which protein contents were available. For example, if in a particular sample, the protein content was known for 90% of the species, the sample was divided by 0.9. This way for every collected individual sample, it was possible to estimate the amount of protein in µg per mg of pollen.

This protein content data was then used to estimate effects of treatment on the pollen protein content. First, modelling the protein content per individual pollen sample dependent on treatment with batch and site as covariates was attempted. However, this model had heterogenous and non-normally distributed residuals, that could also not be improved by transforming the data. Therefore, the individual model was discarded in favor of aggregating the data on a colony level. I then fitted a linear model using the mean protein content per mg pollen per colony, which resulted in a well-fitting model. In this model, batch did no longer have an impact on the protein content and therefore, site was the only covariate.

The plotting of the plant families of origin of the collected species was done through adapted code by Natalia Davila. Since the raw data after the pollen scanning both categorized some pollen to the species level, while others only to the family level, the data was aggregated to the family level.

2.6.3 Hive Dissection

To assess possible effects on larvae survival, a binomial model was fitted comparing the ratio of live to dead larvae found in the colonies. Due to overdispersion, a generalized linear model with quasibinomial distribution was selected. Significance estimates for all dissection data were obtained using the Anova function of the car package in R studio, which performed a likelihood-ratio chisquare test. Covariates here were batch and initial weight of the hives.

A similar model was fitted to assess worker survival, by comparing the ratios of alive to dead larvae. Batch was the only statistically significant covariate.

Moreover, effects of treatment on overall larval and worker count were evaluated by fitting generalized linear models with quasipoisson distribution. There was no statistically significant covariate for larval count, and batch was the only covariate for worker count.

3 RESULTS

3.1 EXPLORATORY ANALYSIS

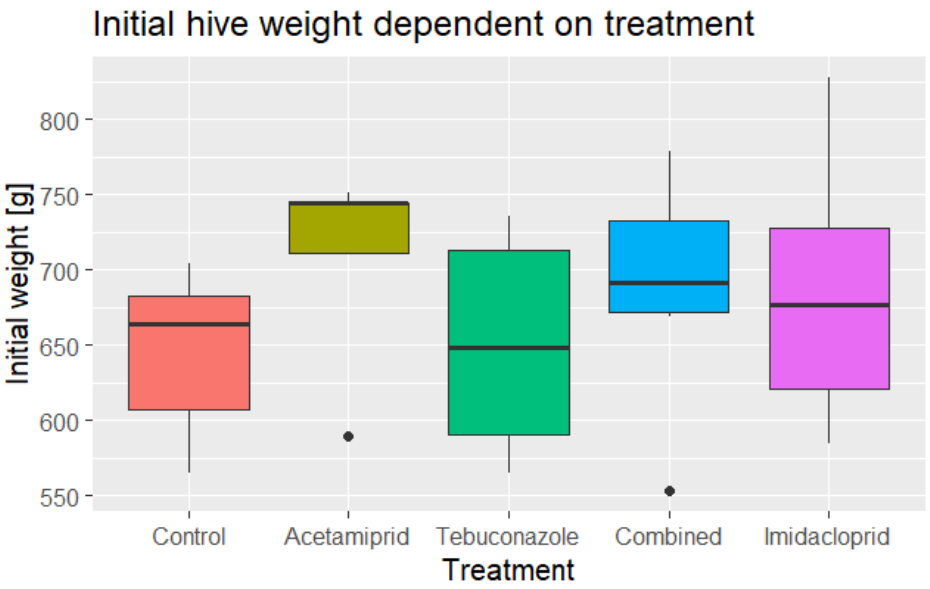

Figure 5 Initial hive weight dependent on treatment. Initial weight is the weight including the plastic nest boxes on the first day of exposure. The combined treatment is the mixture of Acetamiprid and Tebuconazole.

Figure 5 shows that there was no difference between in the initial weight at the day of exposure between the treatments. This is also shown by the One-way ANOVA: $F(4,26)=0.80$, $p=0.54$.

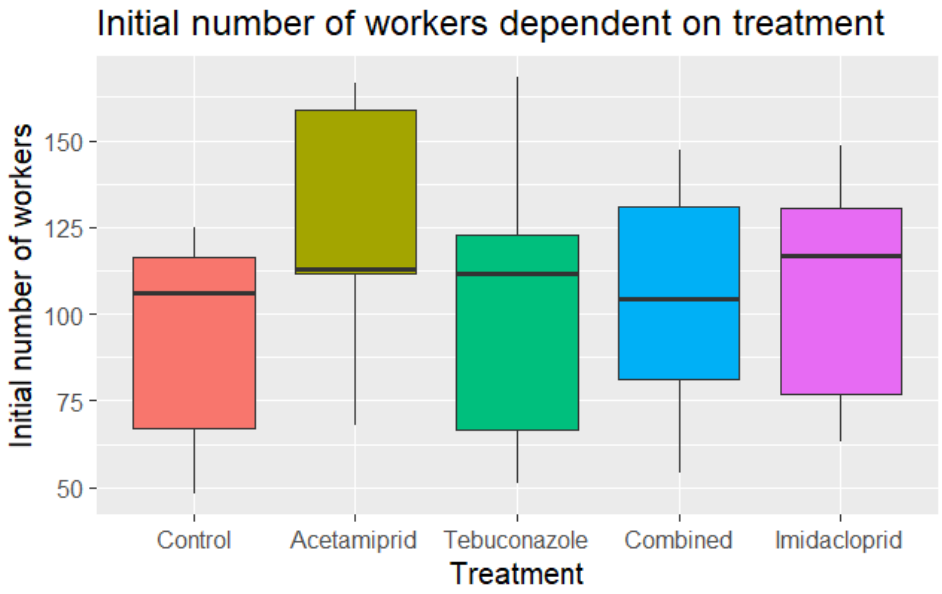

Figure 6 Initial number of workers dependent on treatment. Initial number of workers represents the estimated number of workers at the start of exposure. The combined treatment is the mixture of Acetamiprid and Tebuconazole.

Figure 6 shows as with the initial weight that there was no difference between the treatments concerning the initial number of individuals on the day of exposure. This is confirmed by the One-way ANOVA: $F(4,26)=0.52$, $p=0.72$.

Figure 7 Initial number of workers dependent on initial weight, shows both parameters per hive at the day of exposure. The initial number of workers was estimated from the % of tagged individuals on the day of exposure and the known number of tagged individuals. The regression line was fitted using a linear regression model.

Figure 7 shows that there is a strong correlation between the initial weight and the initial number of workers, wherein initial weight can predict the initial number of workers, $R^2=0.56$, $F(1,29)=39.7$, $p<0.001$.

3.2 HIVE WEIGHTS

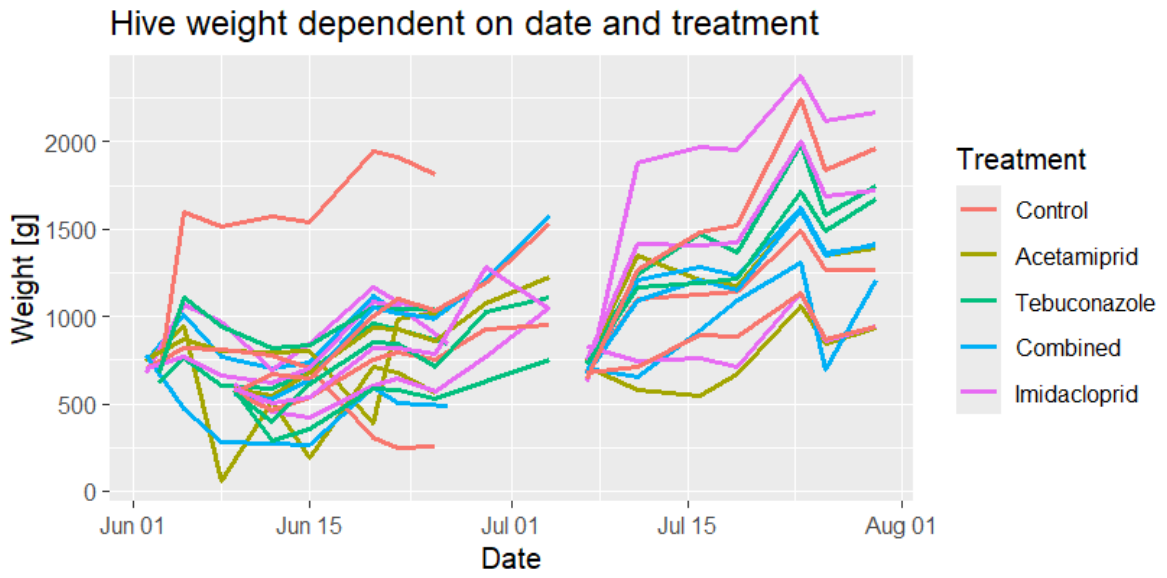

Figure 8 Showing the Hive weights [g] dependent on date and coloured by treatment. Visible are the starting dates/dates of first exposure for all three batches: 2.6. (batch 1), 9.6. (batch 2), 7.7. (batch 3). The first measurement was done in the lab before the first exposure and the last just the day before the hives were frozen. The hive weights after the first measurement were obtained by subtracting the weight of the wooden nest boxes from the total weight measured in the field.

Figure 8 depicting the weight development of the bumblebee colonies over time, clearly shows the 3 batches. The first measurements in the more controlled indoor environment are very close together, and rapidly diverge between the hives as soon as the measurement takes place outdoors for the 2nd measurement after exposure. Apart from some exceptions, the weight seems to change in parallel with each other. The hives in batches 1 and 3 start at very similar weights, while the hives in batch 2 were smaller from start. Moreover, the hives from batch 3 seem to be the biggest at the end of data collection, but batch 2 seems to have grown the most over the time period (fig. 9). These findings are also in accordance with the results of an the One-way ANOVA that modelled weight differences dependent on batch: $F(2,28)= 14.82$, $p<0.001$ (See also fig. 5). Therefore, it can be concluded that the hives in both batch 2 and 3 have grown the most over the 3-week exposure period, with hives in batch 2, which started at the lowest average weight, increasing the most.

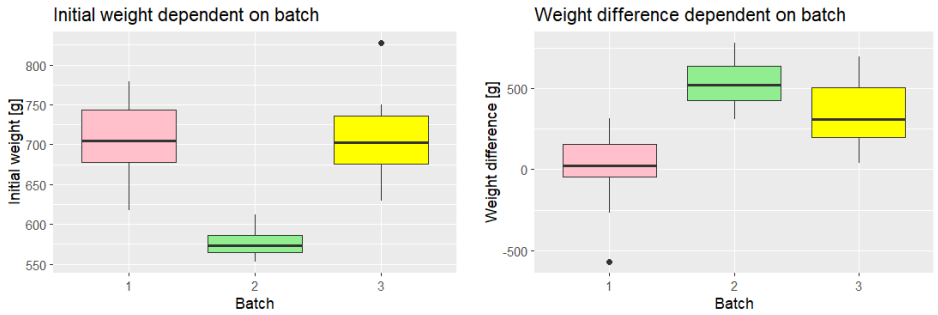

Figure 9 Initial weight (left) and weight difference (right) dependent on batch, wherein weight difference represents the difference in weight between the 2nd (the first after the initial exposure) and last measurement and batch represents the three distinct groups in which the hives were ordered, tagged, and released. Initial weight [g] represents the weight per colony on the day of exposure. Each batch contains hives from all treatment groups.

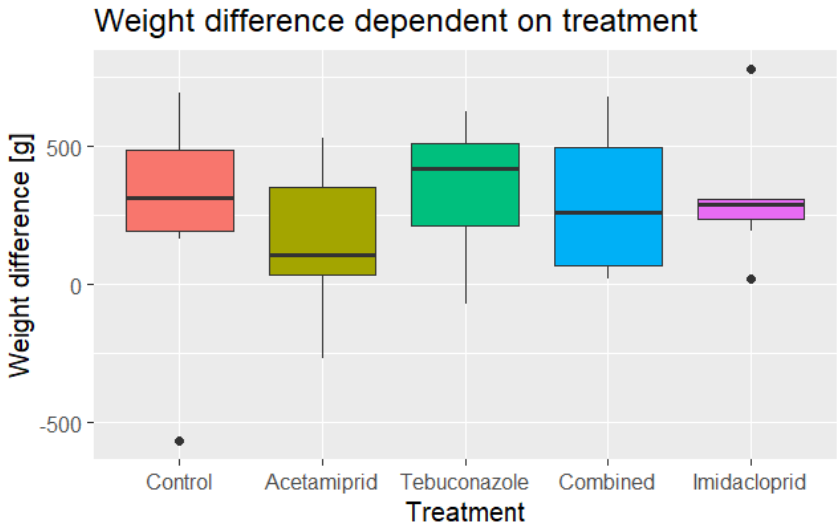

Figure 10 Weight difference dependent on treatment shows the difference in weight from the 2nd (the first after the initial exposure to the last measurement of each hive dependent on their treatment. Combined represents the mixed treatment with both acetamiprid and tebuconazole.

Both the results of the ANOVA (table 2) and the graphical analysis (fig. 10), show that no effect of treatment on the weight increase could be observed. All hives apart from few exceptions in the control, acetamiprid, and tebuconazole treatments, showed an increase in weight, with a maximum increase from a single hive of about 750 g in the imidacloprid treatment.

Table 2 results of the linear model with weight difference dependent on treatment, batch, and site, as obtained after performing a One-way ANOVA. Weight difference was calculated as the difference between the 2nd (the first after the initial exposure and last measurement per hive. The degrees of freedom (Df) are given as: Df (residual Df).

PREDICTORS	DEGREES OF FREEDOM	F-VALUE	P-VALUE
TREATMENT	4 (21)	0.59	0.67
BATCH	2 (21)	12.24	>0.001
SITE	3 (21)	0.40	0.75

3.3 POLLEN ANALYSIS

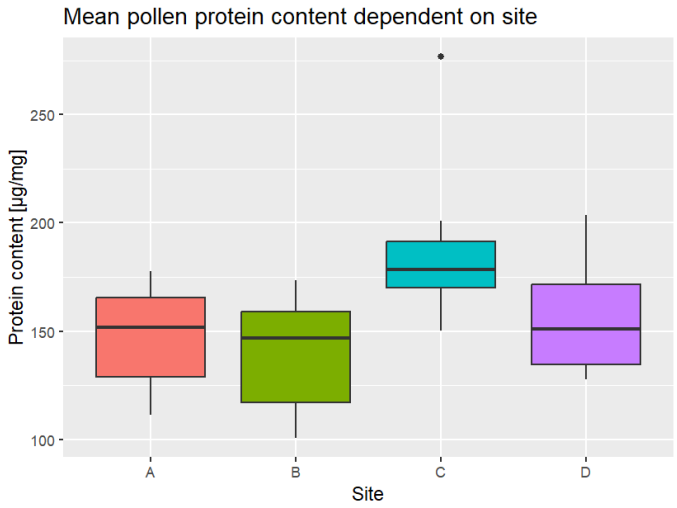

Figure 11 Mean pollen protein content dependent on site. The pollen content is given as protein content in µg per mg pollen. The data was aggregated per hive. The site represents the position around the ecology building accord to the hives map given in the methods section (fig. 1).

The results of the preliminary analysis of the protein content dependent on the site (fig. 11) show that there is an effect of site ($F(3,29)= 5.00$, $p= 0.006$). Here, the protein content per mg of pollen is significantly increased in site C.

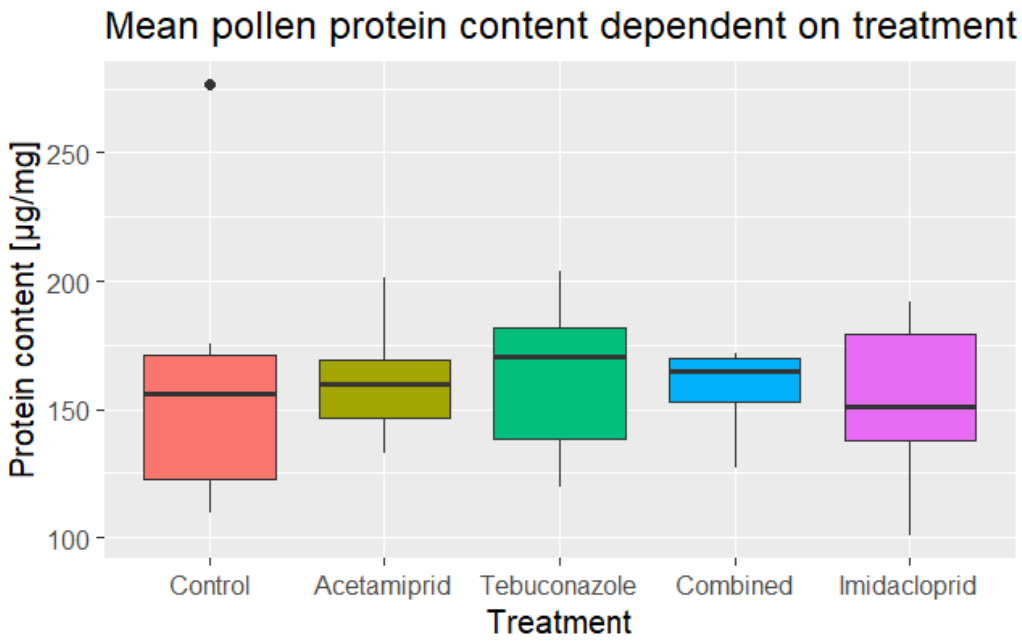

Figure 12 Mean pollen protein content dependent on treatment. The pollen content is given as protein content in µg per mg pollen. The data was aggregated per hive. Treatment represents the pesticides to which the hives were exposed, wherein control means no pesticides and combined is the mixed exposure to both acetamiprid and tebuconazole.

Treatment has no effect on pollen protein content. As both shown in the statistical analysis, where $F(4,25) = 0.08$ and $p = 0.99$ and visually confirmed in figure 12, where there are no

differences between treatments. The covariate site has a much larger effect in the model (fig. 11) with F(3,25) = 4.39 and p = 0.01.

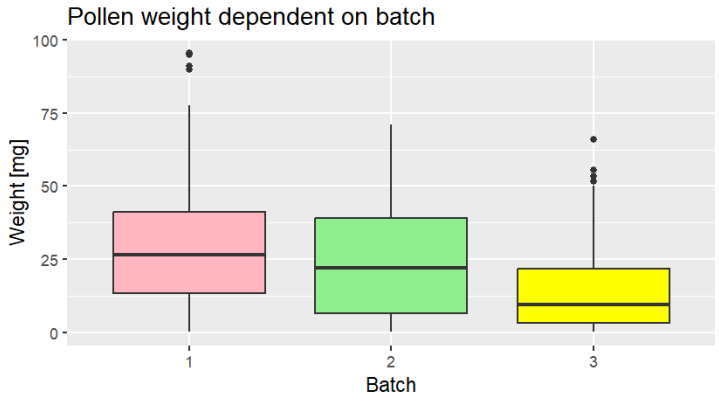

Figure 13 Weight of the pollen dependent on batch. The weight indicates the mass of the pollen that was collected from the returning bumblebee in front of the hive in mg. Batch represents the 3 distinct rounds that the hives arrived and were exposed in. Batch one and two overlapped by about two out of three weeks, whereas batch 3 was after both one and two.

The results of the wald chisquare test show that batch has the strongest effect (chisquare(2)= 23.51, p<0.001), where the weight of pollen in batch 3 is lower than in batches 1 and 2 (fig. 13). Treatment however does seem to have a marginally significant effect (chisquare(4)= 8.81, p=0.07). The subsequent pairwise comparisons between all treatment groups show that the pollen weight in the imidacloprid treatment was marginally lower than in all the other treatment groups (fig. 14).

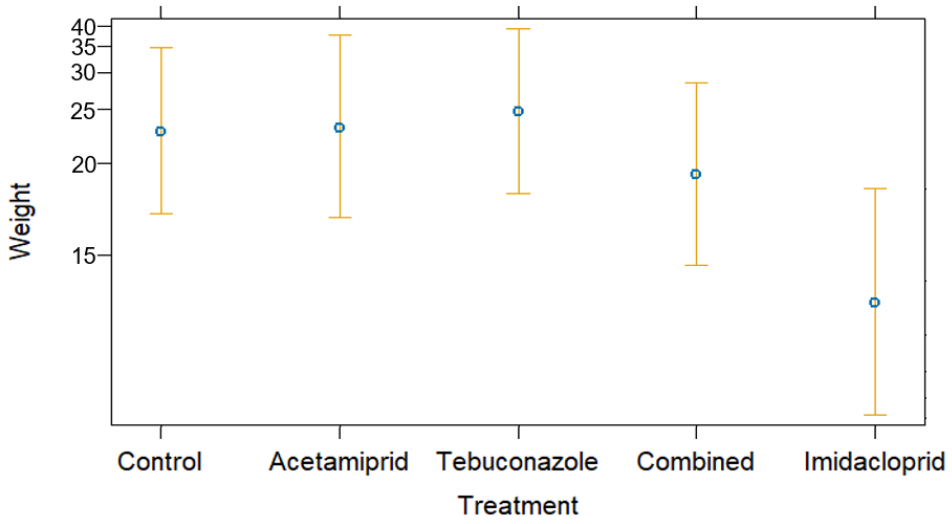

Figure 14 Estimated mean and 95% confidence interval of pollen weight dependent on treatment. The effect estimates were obtained from the effects package in R studio. The weight indicates the total mass of the pollen that was collected from the returning bumblebee in front of the hive in mg. Treatment represents the pesticides to which the hives were exposed, wherein control means no pesticides and combined is the mixed exposure to both acetamiprid and tebuconazole.

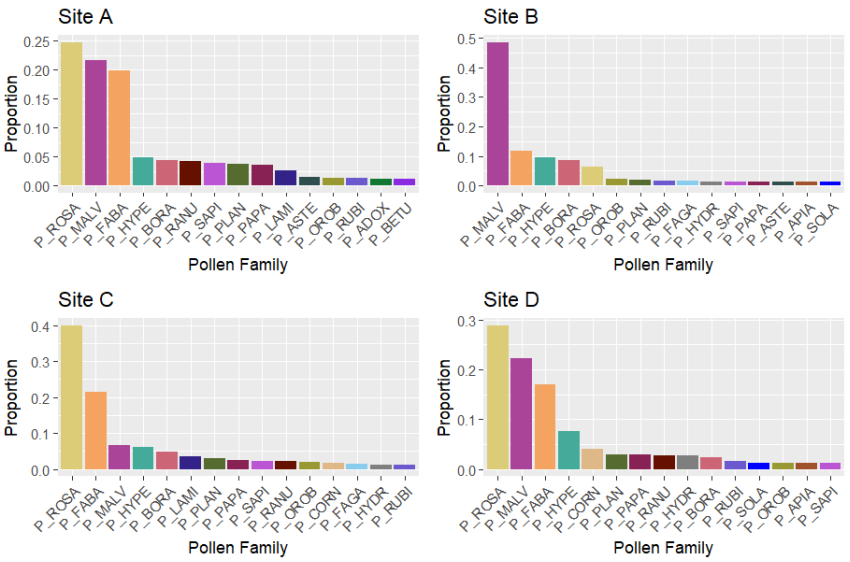

Figure 15 Overview of the top 15 pollen species dependent on site. Site represents the four areas around the ecology building on which the clusters of colonies containing all pesticide treatments were placed. Proportion represents the average proportion of the pollen samples that consisted of that specific pollen. Pollen family represents the family of origin of the pollen, whereas the first 4 letters are always given after "P_". Families represented are: Rosaceae, Fabaceae, Boraginaceae, Malvaceae, Lamiaceae, Hypericaceae, Papaveraceae, Ranunculaceae, Plantaginaceae, Orobanchaceae, Sapindaceae, Fagaceae, Cornaceae, Betulaceae, Euphorbiaceae, Solanaceae, Hydrangaceae, Asteraceae, Juglandaceae, Poligonaceae, Crasulaceae, Rubiaceae, Adoxaceae, Apiaceae, Scrophularicaceae

Figure 15 shows the proportion of the family of origin across all treatments and batches but dependent on site. Site A and D, which are sites in close proximity to each other have very similar pollen profiles with Rosacea, Malvaceae, and Fabaceae pollen being dominant. Site C shares Rosaceae and Fabaceae as dominant families, however compared to the other 3 sites, Malvaceae are much less important with only ~6% of pollen coming from Malvaceae. Site B looks to have the most difference compared to the other 3 sites, even though it is physically close to sites A and D. Malvaceae pollen is clearly dominant, whereas the next most common family is Rosaceae but with only 11%.

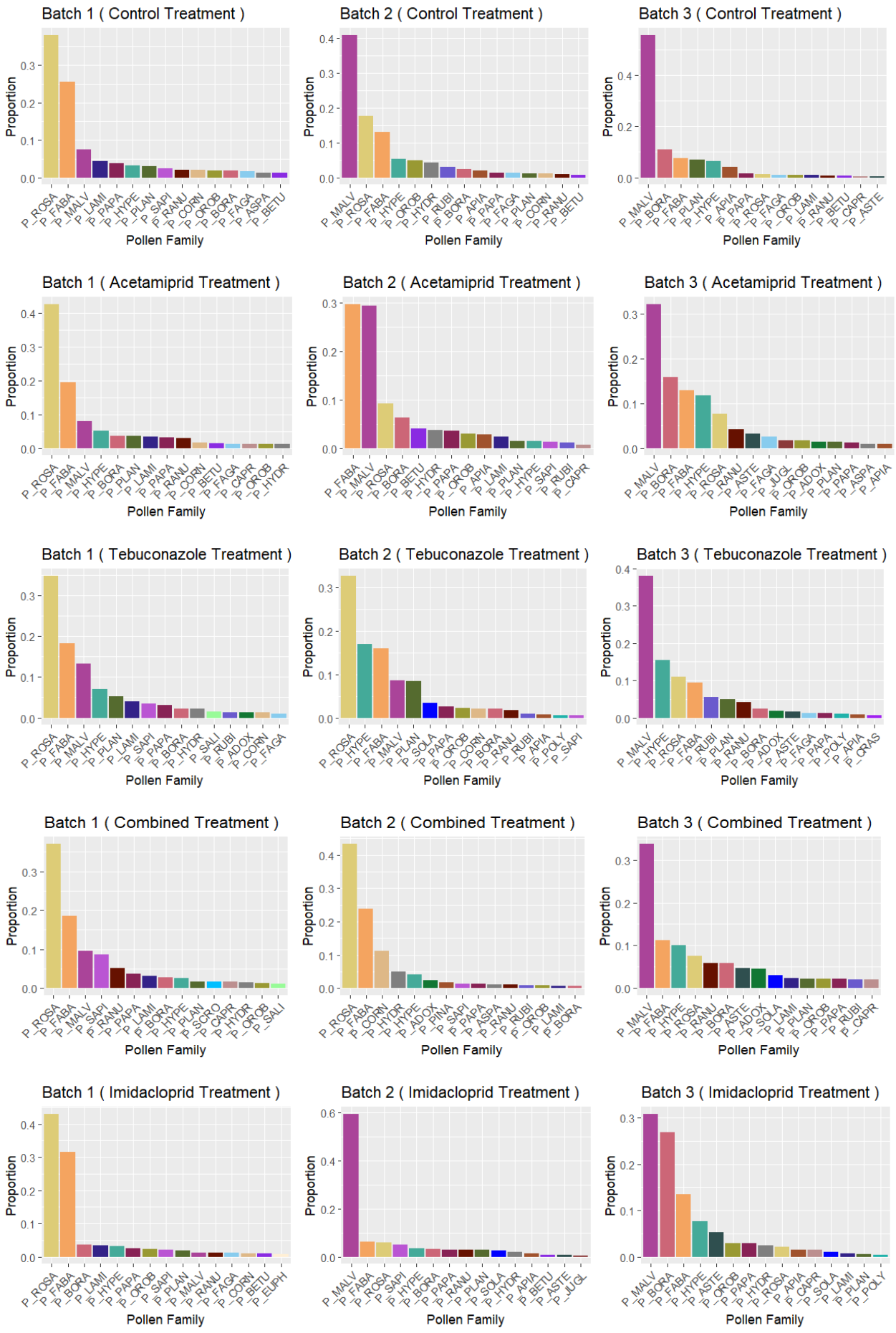
Batch 1 (Control Treatment)
Batch 2 (Control Treatment)
Batch 3 (Control Treatment)
Batch 1 (Acetamiprid Treatment)
Batch 2 (Acetamiprid Treatment)
Batch 3 (Acetamiprid Treatment)
Batch 1 (Tebuconazole Treatment)
Batch 2 (Tebuconazole Treatment)
Batch 3 (Tebuconazole Treatment)
Batch 1 (Combined Treatment)
Batch 2 (Combined Treatment)
Batch 3 (Combined Treatment)
Batch 1 (Imidacloprid Treatment)
Batch 2 (Imidacloprid Treatment)
Batch 3 (Imidacloprid Treatment)
Proportion
Pollen Family

Figure 16 Overview of the top 15 pollen species dependent on both batch (in columns) and treatment (in rows). Proportion represents the average proportion of the pollen samples that consisted of that specific pollen. Pollen family represents the family of origin of the pollen, whereas the first 4 letters are always given after "P_". Families represented are: Rosaceae, Fabaceae, Boraginaceae, Malvaceae, Lamiaceae, Hypericaceae, Papaveraceae, Ranunculaceae, Plantaginaceae, Orobanchaceae, Sapindaceae, Fagaceae, Cornaceae, Betulaceae, Euphorbiaceae, Solanaceae, Hydrangaceae, Asteraceae, Juglandaceae, Poligonaceae, Crasulaceae, Rubiaceae, Adoxaceae, Apiaceae, Scrophularicaceae

Figure 16 shows that there is a lot of difference in the pollen origins dependent on batch and treatment, with the most visible differences between the three batches. Across all hives there are 2-4 dominant plant families that comprise the majority of pollen in that group with many other families making up 10% and less per family. By far the most common species in batch one are Rosaceae, Fabaceae and Malvaceae. In batch 2 the ratio of Malvaceae pollen has increased so that it is the dominant family in 2 out of 5 treatments, followed by Fabaceae and Rosaceae pollen. The tebuconazole treatment also shows a high (~17%) ratio of Hypericaceae pollen. In the 3rd batch, Malvaceae are the dominant plant family, with Rosaceae and Fabaceae pollen still present, but far less frequently. Boraginaceae pollen was the 2nd most common pollen in 3 out of 4 treatments, while Hypericaceae pollen increased slightly compared to the 2nd batch, but largely remained stable. Overall, the treatments show very similar pollen profiles within the same batch, excepts for the tebuconazole and combined treatment in batch 2 where they have much lower Malvaceae and higher Rosaceae and Hypericaceae content compared to the other three treatments.

3.4 HIVE DISSECTION

Table 3 Overview over the models fitted for the hive dissection data. Response variable contains the dissection data. Predictors are the treatment groups, and other covariates that were found in previous analyses to be significantly affected the response variable. The degrees of freedom (residual degrees of freedom in parentheses), value of the test statistic, and p-value were obtained from the ANOVA table. The test statistic contains either F-values (in the case of total workers), or likelihood-ratio chisquare values (in all others).

RESPONSE VARIABLE	PREDICTORS	DEGREES OF FREEDOM	TEST STATISTIC	P-VALUE
LARVAE SURVIVAL	Treatment	4	4.46	0.35
	Batch	2	12.50	0.002
	Initial weight	1	1.30	0.25
TOTAL LARVAE	Treatment	4	3.62	0.45
WORKER SURVIVAL	Treatment	4	10.51	0.03
	Batch	2	11.44	0.003
TOTAL ALIVE WORKERS	Treatment	4 (24)	1.02	0.41
	Batch	2 (24)	5.73	0.009

To evaluate colony performance, 4 variables were considered: larvae survival as the ratio of alive larvae in overall larvae, the total number of larvae, worker survival as the ratio of alive workers in overall workers, and the total number of alive workers. Preliminary analyses found 3 covariates to be relevant to improve fit in some of the models (table 3), those include batch, and initial weight. No effects of treatment were observed, however some of the covariates had statistically significant effects with $p<0.05$ (table 3).

3.4.1 Larvae

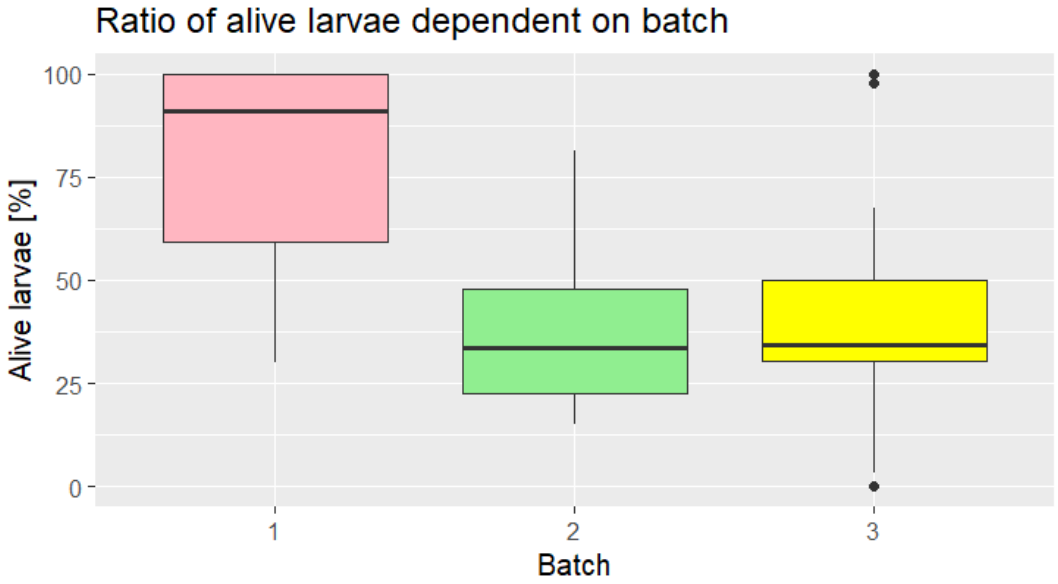

Figure 17 Ratio of alive larvae dependent on batch. The ratio was calculated by dividing the alive larvae by the total number of larvae and then multiplying by 100. Batch represents the 3 distinct rounds that the hives arrived and were exposed in. Batch one and two overlapped by about two out of three weeks, whereas batch 3 was after both one and two.

Figure 17 shows ratio of alive larvae dependent on batch as a measure of larval survival. It can be seen that batch 1 seems to have the highest larval survival rate with about 90%, whereas the survival rate in batch 2 and 3 is very similar to each other but significantly lower than 1 (LR chisqr(2)= 23.52 , p<0.001).

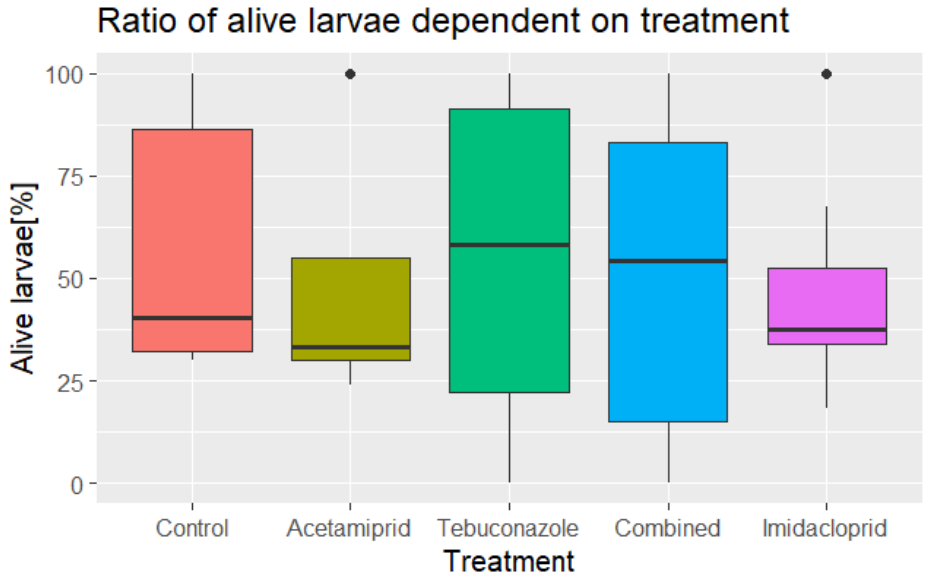

Figure 18 Ratio of alive larvae dependent on treatment. The ratio was calculated by dividing the alive larvae by the total number of larvae and then multiplying by 100. Treatment represents the pesticides to which the hives were exposed, wherein control means no pesticides and combined is the mixed exposure to both acetamiprid and tebuconazole.

Looking at figure 18 and the statistical results of the model (tab. 3), it can be seen that there is no effect of treatment on the ratio of alive larvae. The same is true for the total amount of larvae (fig. 19 and tab. 3).

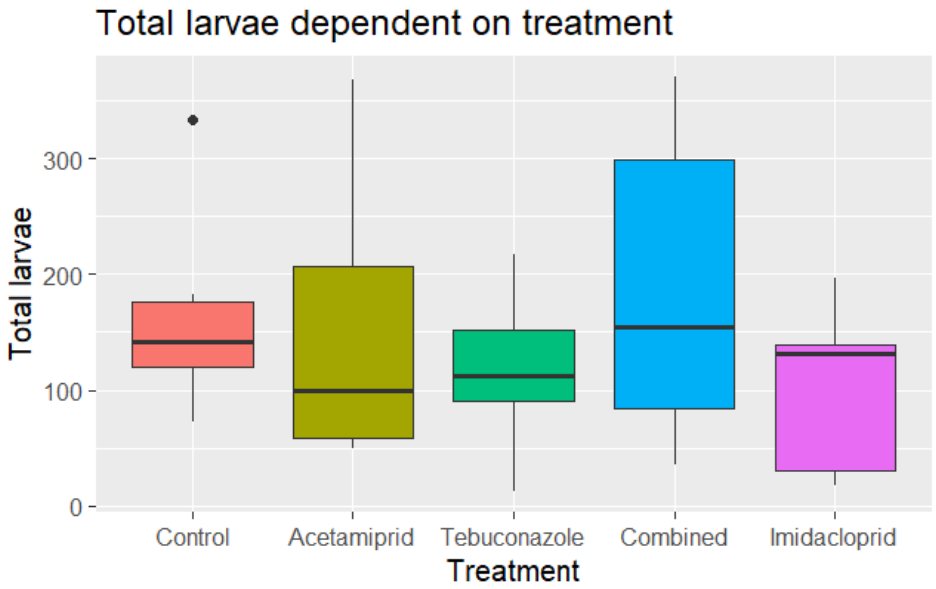

Figure 19 Number of total larvae dependent on treatment. Treatment represents the pesticides to which the hives were exposed, wherein control means no pesticides and combined is the mixed exposure to both acetamiprid and tebuconazole.

3.4.2 Workers

The results of the ANOVA show that there seems to be an effect of treatment on worker survival (fig. 20, tab. 3). However, the pairwise analysis did not detect any significant difference between the specific treatments.

Figure 20 Ratio of alive workers dependent on treatment. The ratio was calculated by dividing the alive workers by the total number of workers and then multiplying by 100. Treatment represents the pesticides to which the hives were exposed, wherein control means no pesticides and combined is the mixed exposure to both acetamiprid and tebuconazole.

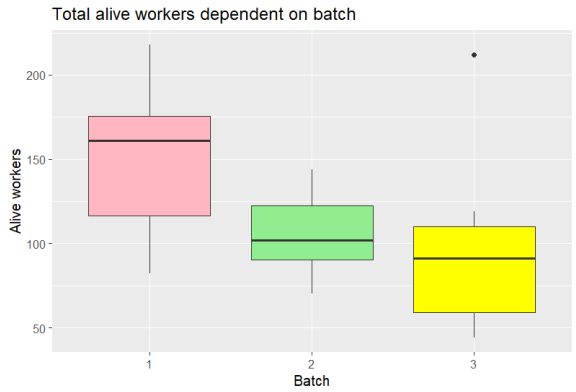

Figure 21 Total alive workers dependent batch.. Total alive workers include both tagged and untagged alive workers. Batch represents the 3 distinct rounds that the hives arrived and were exposed in. Batch one and two overlapped by about two out of three weeks, whereas batch 3 was after both one and two.

The total number of alive workers differs with batch ($F(2,28)= 5.78$, $p= 0.008$), see also figure 21. Batches two and three both have lower numbers of alive workers, with batch three having the lowest. In contrast to that, treatment does not have an effect (tab. 3, fig. 22).

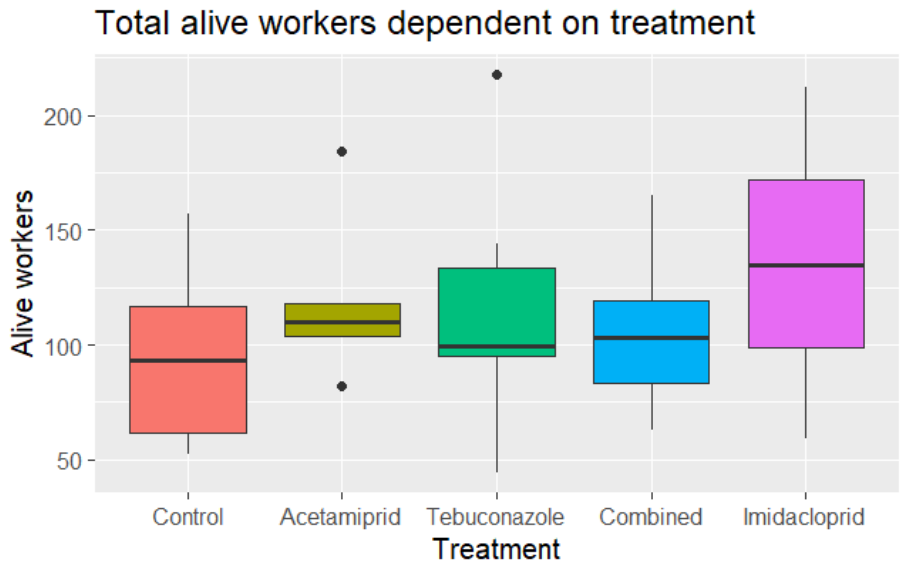

Figure 22 Total alive workers dependent on treatment. Total workers include both tagged and untagged alive workers. Treatment represents the pesticides to which the hives were exposed, wherein control means no pesticides and combined is the mixed exposure to both acetamiprid and tebuconazole.

4 DISCUSSION

Our aim was to quantify effects of acetamiprid and tebuconazole on bumblebee colony growth and foraging behaviour and investigate possible synergistic effects between acetamiprid and tebuconazole. These synergisms are expected as a result of disrupted detoxification of acetamiprid due to the effects of tebuconazole (Schuhmann et al., 2022). As discussed in more detail below, we selected imidacloprid as the positive control because of previously observed effects on colony growth, development, and larvae and worker production as well as survival, and foraging behaviour, including flower preference (Gill & Raine, 2014; Gill et al., 2012). All results considered, there were, with the exception of pollen weights and worker survival, no difference among the pesticide treatments. The lack of effect of our positive control was therefore surprising. However, while we found no effect of the pesticide treatments, other factors that we measured, like initial weight, batch, and site, had a clear impact. So why did the imidacloprid treatment not have an impact?

The most probable explanation for the lack of effect from imidacloprid is the degree of exposure. The concentrations of the pesticides and exposure method (oral) were based on the study by Gill and Raine (2014), where colonies were provided with sucrose solution by the entrance of their hive. They started with small volumes (10 ml every second day and increased this volume as the colonies grew larger to a maximum volume of 16ml). Similar to their study, we also aimed at starting the exposure with small early-stage hives. Our aim was to provide these bees with unlimited access to sucrose solution and therefore we selected a volume that was higher than their final volume, with 15-20 ml per colony every second day. However, due to factors outside our control, the hives that were delivered and exposed were standard hives with more than 50 or 100 individuals in batch 2 and batch 1 and 3, respectively. Because of miscommunication within the project group, the amount of sucrose was never adjusted to the new colony size. Therefore, the amount of sucrose (15-20 ml) given to the bumblebees was much less than expected per individual, resulting in less exposure per individual, making it entirely possible that the lack of effects is due to an insufficient degree of exposure.

Moreover, with the current oral mode of exposure – at group level rather than individual level, it was impossible to ensure that every individual consumed the pesticide sucrose solution in roughly equal amount. Only during the first 3 days of exposure, when the hives were closed, it is reasonable to assume that most individuals consumed some of the sucrose, since that was the only source of sugars available in the hives. Worsening this problem is bumblebees division of labour into foragers and nest workers - those individuals tending to the brood and fulfilling other within colony duties (Goulson, 2010). Therefore, it could be theorized that the foragers, since they regularly have access to nectar from the environment are less likely to consume the sucrose solution, having a lower exposure, whereas workers in the nest might rely much more on the sucrose to fulfil their energetic need, increasing their exposure. More information about the degree of exposure of individuals will be obtained later, as our collaborators in analytical chemistry will analyse pesticide content in individuals from whom pollen was collected. This uneven exposure shows the possibility that potential effects on an individual's level (as suggested by the effects on pollen weight, fig. 14) could be compensated by other less exposed individuals, so that effects on a colony level might be lessened. One example of a compensation was documented in Gill et al. (2012). They found that imidacloprid exposure led to an increase in forager recruitment, so that the decreased foraging

efficiency could possibly be compensated through increased forager recruitment. However, the extend to what potential compensation played a part in this study is impossible to answer with the current set of data.

Nevertheless, there were some effects treatment and other covariates documented, that will be discussed below.

4.1 HIVE WEIGHTS

This experimental setup was unable to show any effects of the pesticide exposure on the weight development and difference over the three-week experimental setup (fig. 8 & 10, tab. 2). As discussed previously, the lack of effect of other treatments should be carefully interpreted because of a non-existing effect of our positive control, imidacloprid, suggesting that the hives were insufficiently exposed to the pesticides. For acetamiprid and tebuconazole, our observation is – however –partially in line with literature: in previous studies acetamiprid could not be shown to affect bumblebee colony weight in the field (Van Oystaeyen et al., 2021) and tebuconazole, although it contributes to detrimental effects on colony growth together with other stressors like parasites and other pesticides (Botias et al., 2021), possible effects of itself have not been demonstrated. Although there have been synergistic effects observed between neonicotinoids and EBI fungicides like tebuconazole in honeybees (Iwasa et al., 2004; Schmuck et al., 2003), we find no evidence for similar synergy effects here, with the combined treatment showing no difference to the individual treatments of acetamiprid and tebuconazole (fig. 10).

However, unexpected is that the imidacloprid treatment has no effect (fig. 10). It has already been established in previous studies to have detrimental effects on colony growth (Gill & Raine, 2014; Gill et al., 2012) and colony weight at field realistic levels (Wu-Smart & Spivak, 2017). This lack of effect might indicate problems with the methodology of this study: Apart from problems concerning the mode and concentration of the exposure as discussed above, there were other problems specifically concerning the collection of weight data. Before the exposure under laboratory conditions, it was easy to collect weight data, with the colonies being sealed in their plastic boxes that could be weighed on a fine scale with little other influencing factors. In the field, however, collecting accurate weight data proved to be a challenge. Since the hives were placed in wooden nest boxes that themselves weighed around 12-13kg, a scale with lower accuracy had to be used. Moreover, other factors like wind influenced the weight significantly during the measurement. Especially, looking at batch 3, where the weight of the most hives fluctuates in parallel by hundreds of grams from one measurement to the other, it is reasonable to assume that those fluctuations are not a result of changes in hive weights, but other confounding factors. One possible explanation could be increased rainfall during July, that could have led the wood of the boxes to take up moisture and therefore increase in weight, even with the inside of the boxes remaining dry.

Even though pesticide exposure proved not to affect weight gain, there was a difference observed between the three batches (fig. 9), with batch 2, the smallest batch growing the most. Which is to be expected with batches 1 and 3 being larger, thus closer to their maximum weight, and therefore increasing less in mass than the smaller hives of batch 2.

However, since most of these problems could be mitigated through differences in the experimental setup, additional research could still be worthwhile. Effects of batch could be reduced by starting with smaller standardised hives according to their size. Smaller hives together with a longer period of observation would also allow for most of the life cycle of the colony to be documented. To reduce uncertainties in the weight measurement, the experiment would either have to reduce the size and weight of the nest boxes to increase the accuracy or conduct the data collection indoors under laboratory conditions.

4.2 POLLEN FAMILY AND PROTEIN

Although slightly above the commonly accepted threshold for statistical significance ($p = 0.07$), treatment seems to have an effect on the weight of the pollen clump brough back by the bumblebees after a foraging bout (fig. 14), with bees exposed to imidacloprid bringing back less pollen compared to the control. This finding is in line with previous research, where it was argued that chronic exposure to imidacloprid did negatively affect pollen foraging ability (Gill & Raine, 2014; Gill et al., 2012). This finding confirms that, in spite of problems with the route and amount of exposure that were laid out in the first chapter of the discussion, hives were indeed exposed to concentrations of imidacloprid high enough to have an effect, even if this effect is only marginally significant. Moreover, since the study by Gill et al. (2012) found that the severity of those impairments worsened over time, with statistically significant effect sizes only being present after 3 and 4 weeks, extending the length of exposure could be worthwhile in future research.

About the effects of tebuconazole and acetamiprid on pollen collection is little known. Research by Tóth and Kovács (2024) suggests there could be effects of acetamiprid, but they are largely inconclusive.

Compared to the effects of treatment, the batch still had the biggest effect on pollen weight (fig. 13). These effects however can likely be attributed to environmental factors: as batch 1 and 3 are very similar in all innate factors like initial weight and worker count, any effects of those innate differences on pollen weight would be expected in both batches. One of those environmental factors could be weather. It has already been shown that they favour pollen foraging during dry and sunny conditions, and nectar foraging during wetter conditions. Moreover, if they collect pollen during those wetter conditions, they tend to collect smaller amounts of pollen (Peat & Goulson, 2005). June 2023 with its sunny and dry weather was thus much more optimal for pollen collection compared to July 2023 with very mixed weather and frequent rain, which makes weather a likely cause for much of that difference.

The average pollen protein content per hive was not affected by the pesticide treatments, however it differed dependent on the site of the hives. This difference could be a result of different access to flowering resources depending on the site. With sites A, B, and D on the same side of the ecology building having largely overlapping access to flowers, being similar to each other, whereas site C on the opposite side having higher protein content. An additional factor could be competition between the hives, where the high density of hives on the northern side of the ecology building - that at one point in June housed 15 hives, might have led to a scarcity of pollen resources, forcing foragers to also collect pollen from less protein dense plants. On the southern side of the ecology building, only 5 hives were present, which

might have enabled the bumblebees to forage from optimal plants with high protein content, a preference which has already been shown in laboratory studies (Vaudo et al., 2016). Nevertheless, it is possible that the bumblebees from site A,B, and D were able to access the flowering resources of hive C: theoretical analyses have suggested that nectar foraging range of a bumblebee can be as far as 10 km (Cresswell et al., 2000) with a recent analysis by Kendall et al. (2022) supporting those findings. However, the same analysis also established that the realized foraging range of primitively eusocial species such as *B. terrestris* is much lower with a median of 448 m (Kendall et al., 2022). This shows that even though they likely could access flowering resources on both sides of the ecology building, it is unlikely that they did on a big scale. When also considering the plant families to which the pollen belongs, it becomes clear that these differences in protein content are likely due to differences in the type of collected pollen (fig. 14). In all four sites Fabacea, Rosacea, and Malvaceae were the dominant three plant families, however they differed in their abundance. Fabacea and Rosaceae pollen had much higher protein content (about factor 3) compared to Malvaceae pollen (see appendix), which explains why site C with lower Malvaceae content and higher Fabaceae and Rosaceae content also has higher protein content, especially compared to site B with the highest Malvaceae content and the lowest protein content.

There also does not seem to be an effect of treatment on the pollen families. Looking at figure 16, Rosaceae, Fabaceae, Malvaceae, and Boraginaceae were the dominant plant families, with shifts largely between batches from Rosaceae and Fabaceae, in batch 1 to Malvaceae and Boraginaceae in batch 3. Within the same batch, pollen profiles seemed to be very similar and shifts between batches are likely due to changes in the flowering landscape throughout June and July. That being said, the tebuconazole and combined treatment in batch 2 appears to be much closer in its profile to the same treatments in batch 1 than to other treatments in its own batch (fig. 16), however since these kinds of differences cannot be found in any other batch, it is likely not an effect of treatment, but could be an effect of different flowering resources, which is not unlikely since batch 1 and 2 had about 2 weeks overlap, so it is plausible to suggest that some of their pollen foraging profiles overlap as well. This lack of effects highlights the importance of to investigate problems with the methods used in this study, since previous research showed that imidacloprid certainly impairs foraging performance and can influence floral preferences (Gill & Raine, 2014). Other neonicotinoids (here thiametoxame) have also been documented to shift floral preferences which they speculate could be by decreasing the ability of bumblebees to forage from morphologically complex flowers (Stanley & Raine, 2016).

4.3 HIVE DISSECTION

4.3.1 Larvae

Although previous studies showed negative effects of imidacloprid on larval development, wherein larval production and survival was reduced (Gill et al., 2012), these effects could not be replicated in this study, which again highlights potential problems with the method as outlined in the beginning of the discussion. However – as also discussed above - , this lack of effects of imidacloprid on a colony level could also be a result of an effect seen in the same

paper, where Gill et al. (2012) hypothesized that the decrease in foraging efficiency, that was also seen in this study through the lower weight of the foraged pollen (fig. 14), could be compensated by the increased recruitment of foragers. This could explain why larval production and survival was unaffected here, because even though individual workers brought back less pollen per foraging bought in the imidacloprid treatment, a possibly increased number of foragers would still be able to collect sufficient pollen resources for the larvae.

In contrast to imidacloprid, the lack of effects visible in the acetamiprid and tebuconazole treatments are in line with literature. Acetamiprid was only found to negatively affect colony development by reducing reproductive output at very high concentrations, that exceeded the field realistic exposure in this experiment (Varga-Szilay & Toth, 2022) and tebuconazole has only documented effects together with other pesticides (Botias et al., 2021), its own effects are little known. Moreover, there was no detected synergistic effect of the combination of tebuconazole and acetamiprid.

Even though no treatment effects could be found, there were other covariates with significant effects on larvae production and survival. There was significantly higher ratio of alive larvae observed in the first batch, and it could be seen that not larval production, but only larval survival was affected (fig. 17, tab. 3). Since pollen is an essential resource for larval development, delivering the much needed protein (Goulson, 2010), the decrease in larvae survival in batch 2 and 3 could be due lower pollen collection in the colony, as documented in figure 14. There it is shown that the weight of the pollen loads in batch 3 were significantly lower than in batch one. However, if the reason for the decrease in larval survival is only in the pollen weights, one would only expect batch 3 to have lower survival, not batch 2, since batch 2 and 1 have very similar pollen weights. This question could be answered when also factoring in colony size: batch 1 and 3 where of similar weight at the start of exposure, batch 2 was significantly lighter. Therefore it could be hypothesized that batch 2 due to its smaller size, and batch 3 due lower pollen collection, were not able to supply enough pollen to the colony to successfully rear as many larvae as colonies from batch 1.

4.3.2 Workers

In contrast to the larvae, there seems to be an effect of treatment on either worker survival, not however on overall alive workers (tab. 3). But after conducting a pairwise analysis, it was shown that no treatment significantly differs from the control, and thus that any differences are unlikely to be a result of the pesticides themselves. These differences could be a result of randomly occurring poor colony health. Especially with the smaller sample size (6-7 colonies per treatment) few outliers that randomly perform worse than average in terms of worker survival can have large effects on the treatment group as a whole.

As with larval survival, imidacloprid would have been expected to affect worker survival (Gill et al., 2012), while there is little known about acetamiprid and tebuconazole. Interestingly, the paper by Gill et al. (2012) makes the observation that the increased worker mortality found in their imidacloprid treatment is partially due to increased forager recruitment, which they theorize is an effect of decreased foraging efficiency. Since foraging is a risky task, a higher number of foragers also increases the number of bumblebees who do

not return to the hives. The same study also found that there was no significant effect on worker mortality if the increased forager deaths were not included in the analysis. This has implications for this study, since here only dead workers in the hive were counted, not lost foragers. Which means that even if the imidacloprid treatment did in fact have effects on the worker mortality, similar to its effect on the weight of the collected pollen, those effects are unlikely to be discovered in the current study, since the experimental setup only allows to account for mortality within the hives, not mortality of foragers.

4.4 CONCLUSION

Even though this study was not able to characterize effects and synergisms between acetamiprid and tebuconazole on colony development and performance due to an insufficient degree of exposure, it can still function as a valuable basis for future research. Moreover, the analysis of the pollen family was able to confirm previously established floral preferences and highlight the high variability in floral preferences between hives. Past literature makes a strong case to pursue this line of study again with a few adjustments. Increased exposure adapted to the individual size of the hives and longer periods of measurement could prevent problems encountered in this study. There is already a next study being planned for this field season tackling some of those problems, especially with the mode of exposure. We are planning to expose new colonies for a week in closed hives to guarantee as sufficient exposure. Hopefully that study will yield more conclusive results and ultimately be able to contribute to understanding the complex relationship between combined pesticide exposure, foraging behavior, and colony development.

REFERENCES

Botias, C., Jones, J. C., Pamminger, T., Bartomeus, I., Hughes, W. O. H., & Goulson, D. (2021). Multiple stressors interact to impair the performance of bumblebee Bombus terrestris colonies. *Journal of Animal Ecology*, *90*(2), 415-431. https://doi.org/10.1111/1365-2656.13375

Camp, A. A., Williams, W. C., Eitzer, B. D., Koethe, R. W., & Lehmann, D. M. (2020). Effects of the neonicotinoid acetamiprid in syrup on Bombus impatiens (Hymenoptera: Apidae) microcolony development. *PLoS One*, *15*(10), e0241111. https://doi.org/10.1371/journal.pone.0241111

Crall, J. D., Switzer, C. M., Oppenheimer, R. L., Ford Versypt, A. N., Dey, B., Brown, A., Eyster, M., Guérin, C., Pierce, N. E., Combes, S. A., & de Bivort, B. L. (2018). Neonicotinoid exposure disrupts bumblebee nest behavior, social networks, and thermoregulation. *Science*, *362*(6415), 683-686. https://doi.org/doi:10.1126/science.aat1598

Cresswell, J. E., Osborne, J. L., & Goulson, D. (2000). An economic model of the limits to foraging range in central place foragers with numerical solutions for bumblebees. *Ecological Entomology*, *25*(3), 249-255. https://doi.org/https://doi.org/10.1046/j.1365-2311.2000.00264.x

David, A., Botias, C., Abdul-Sada, A., Nicholls, E., Rotheray, E. L., Hill, E. M., & Goulson, D. (2016). Widespread contamination of wildflower and bee-collected pollen with complex mixtures of neonicotinoids and fungicides commonly applied to crops. *Environment International*, *88*, 169-178. https://doi.org/10.1016/j.envint.2015.12.011

Dirilgen, T., Herbertsson, L., O'Reilly, A. D., Mahon, N., & Stanley, D. A. (2023). Moving past neonicotinoids and honeybees: A systematic review of existing research on other insecticides and bees. *Environmental Research*, *235*, 116612. https://doi.org/https://doi.org/10.1016/j.envres.2023.116612

Dong, B. (2024). A comprehensive review on toxicological mechanisms and transformation products of tebuconazole: Insights on pesticide management. *Science of the Total Environment*, *908*, 168264. https://doi.org/https://doi.org/10.1016/j.scitotenv.2023.168264

EU. (2024a). *Active Substance: Acetamiprid*. Retrieved 24.04.2024 from https://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/start/screen/active-substances/details/1050

EU. (2024b). *Neonicotinoids*. Retrieved 11.04.2024 from https://food.ec.europa.eu/plants/pesticides/approval-active-substances/renewal-approval/neonicotinoids_en#current-status

EU. (2024c). *Tebuconazole*. Retrieved 16.04.2024 from https://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/start/screen/active-substances/details/779

Gill, R. J., & Raine, N. E. (2014). Chronic impairment of bumblebee natural foraging behaviour induced by sublethal pesticide exposure. *Functional Ecology*, *28*(6), 1459-1471. https://doi.org/https://doi.org/10.1111/1365-2435.12292

Gill, R. J., Ramos-Rodriguez, O., & Raine, N. E. (2012). Combined pesticide exposure severely affects individual-and colony-level traits in bees. *Nature*, *491*(7422), 105-108. https://www.nature.com/articles/nature11585.pdf

Goulson, D. (2010). *Bumblebees: behaviour, ecology, and conservation*. Oxford University Press.

Iwasa, T., Motoyama, N., Ambrose, J. T., & Roe, R. M. (2004). Mechanism for the differential toxicity of neonicotinoid insecticides in the honey bee, Apis mellifera. *Crop Protection*, *23*(5), 371-378. https://doi.org/https://doi.org/10.1016/j.cropro.2003.08.018

Kendall, L. K., Mola, J. M., Portman, Z. M., Cariveau, D. P., Smith, H. G., & Bartomeus, I. (2022). The potential and realized foraging movements of bees are differentially determined by body size and sociality. *Ecology*, *103*(11), e3809. https://doi.org/https://doi.org/10.1002/ecy.3809

Khalifa, S. A. M., Elshafiey, E. H., Shetaia, A. A., El-Wahed, A. A. A., Algethami, A. F., Musharraf, S. G., AlAjmi, M. F., Zhao, C., Masry, S. H. D., Abdel-Daim, M. M., Halabi, M. F., Kai, G., Al Naggar, Y., Bishr, M., Diab, M. A. M., & El-Seedi, H. R. (2021). Overview of Bee Pollination and Its Economic Value for Crop Production. *Insects*, *12*(8), 688. https://www.mdpi.com/2075-4450/12/8/688

Knapp, J. L., Nicholson, C. C., Jonsson, O., de Miranda, J. R., & Rundlöf, M. (2023). Ecological traits interact with landscape context to determine bees' pesticide risk. *Nature Ecology & Evolution*, *7*(4), 547-556. https://doi.org/10.1038/s41559-023-01990-5

Kraus, S., Gómez-Moracho, T., Pasquaretta, C., Latil, G., Dussutour, A., & Lihoreau, M. (2019). Bumblebees adjust protein and lipid collection rules to the presence of brood. *Current Zoology*, *65*(4), 437-446. https://doi.org/10.1093/cz/zoz026

Migdał, P., Murawska, A., Berbeć, E., Zarębski, K., Ratajczak, N., Roman, A., & Latarowski, K. (2024). Biochemical Indicators and Mortality in Honey Bee (Apis mellifera) Workers after Oral Exposure to Plant Protection Products and Their Mixtures. *Agriculture*, *14*(1), 5. https://www.mdpi.com/2077-0472/14/1/5

Moerman, R., Vanderplanck, M., Fournier, D., Jacquemart, A. L., & Michez, D. (2017). Pollen nutrients better explain bumblebee colony development than pollen diversity. *Insect Conservation and Diversity*, *10*(2), 171-179.

Myers, S. S., Smith, M. R., Guth, S., Golden, C. D., Vaitla, B., Mueller, N. D., Dangour, A. D., & Huybers, P. (2017). Climate Change and Global Food Systems: Potential Impacts on Food Security and Undernutrition. *Annual Review of Public Health*, *38*(Volume 38, 2017), 259-277. https://doi.org/https://doi.org/10.1146/annurev-publhealth-031816-044356

Ollerton, J., Winfree, R., & Tarrant, S. (2011). How many flowering plants are pollinated by animals? *Oikos*, *120*(3), 321-326. https://doi.org/https://doi.org/10.1111/j.1600-0706.2010.18644.x

Olsson, O., Karlsson, M., Persson, A. S., Smith, H. G., Varadarajan, V., Yourstone, J., & Stjernman, M. (2021). Efficient, automated and robust pollen analysis using deep learning. *Methods in Ecology and Evolution*, *12*(5), 850-862. https://doi.org/https://doi.org/10.1111/2041-210X.13575

Peat, J., & Goulson, D. (2005). Effects of experience and weather on foraging rate and pollen versus nectar collection in the bumblebee, Bombus terrestris. *Behavioral Ecology and Sociobiology*, *58*(2), 152-156. https://doi.org/10.1007/s00265-005-0916-8

Ruedenauer, F. A., Spaethe, J., & Leonhardt, S. D. (2016). Hungry for quality—individual bumblebees forage flexibly to collect high-quality pollen. *Behavioral Ecology and Sociobiology*, *70*(8), 1209-1217. https://doi.org/10.1007/s00265-016-2129-8

Rundlöf, M., Andersson, G. K. S., Bommarco, R., Fries, I., Hederström, V., Herbertsson, L., Jonsson, O., Klatt, B. K., Pedersen, T. R., Yourstone, J., & Smith, H. G. (2015). Seed coating with a neonicotinoid insecticide negatively affects wild bees. *Nature*, *521*(7550), 77-80. https://doi.org/10.1038/nature14420

Sánchez-Bayo, F., & Wyckhuys, K. A. G. (2019). Worldwide decline of the entomofauna: A review of its drivers. *Biological Conservation*, *232*, 8-27. https://doi.org/https://doi.org/10.1016/j.biocon.2019.01.020

Schmuck, R., Stadler, T., & Schmidt, H.-W. (2003). Field relevance of a synergistic effect observed in the laboratory between an EBI fungicide and a chloronicotinyl insecticide in the honeybee (Apis mellifera L, Hymenoptera). *Pest Management Science*, *59*(3), 279-286. https://doi.org/https://doi.org/10.1002/ps.626

Schuhmann, A., Schmid, A. P., Manzer, S., Schulte, J., & Scheiner, R. (2022). Interaction of Insecticides and Fungicides in Bees [Review]. *Frontiers in Insect Science*, *1*. https://doi.org/10.3389/finsc.2021.808335

Sharma, A., Kumar, V., Shahzad, B., Tanveer, M., Sidhu, G. P. S., Handa, N., Kohli, S. K., Yadav, P., Bali, A. S., Parihar, R. D., Dar, O. I., Singh, K., Jasrotia, S., Bakshi, P., Ramakrishnan, M., Kumar, S., Bhardwaj, R., & Thukral, A. K. (2019). Worldwide pesticide usage and its impacts on ecosystem. *SN Applied Sciences*, *1*(11), 1446. https://doi.org/10.1007/s42452-019-1485-1

Sheets, L. P. (2014). Imidacloprid. In P. Wexler (Ed.), *Encyclopedia of Toxicology (Third Edition)* (pp. 1000-1003). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-386454-3.00153-6

Stanley, D. A., & Raine, N. E. (2016). Chronic exposure to a neonicotinoid pesticide alters the interactions between bumblebees and wild plants. *Functional Ecology*, *30*(7), 1132-1139. https://doi.org/https://doi.org/10.1111/1365-2435.12644

Tosi, S., Sfeir, C., Carnesecchi, E., vanEngelsdorp, D., & Chauzat, M.-P. (2022). Lethal, sublethal, and combined effects of pesticides on bees: A meta-analysis and new risk assessment tools. *Science of the Total Environment*, *844*, 156857. https://doi.org/https://doi.org/10.1016/j.scitotenv.2022.156857

Tóth, Z., & Kovács, Z. (2024). Chronic acetamiprid exposure moderately affects the foraging behaviour of buff-tailed bumblebees (Bombus terrestris). *Ethology*, *130*(2), e13425. https://doi.org/https://doi.org/10.1111/eth.13425

van der Sluijs, J. P., Simon-Delso, N., Goulson, D., Maxim, L., Bonmatin, J. M., & Belzunces, L. P. (2013). Neonicotinoids, bee disorders and the sustainability of pollinator services. *Current Opinion in Environmental Sustainability*, *5*(3-4), 293-305. https://doi.org/10.1016/j.cosust.2013.05.007

Van Oystaeyen, A., Klatt, B. K., Petit, C., Lenaerts, N., & Wäckers, F. (2021). Short-term lab assessments and microcolonies are insufficient for the risk assessment of insecticides for bees. *Chemosphere*, *273*, 128518. https://doi.org/https://doi.org/10.1016/j.chemosphere.2020.128518

Varga-Szilay, Z., & Toth, Z. (2022). Is acetamiprid really not that harmful to bumblebees (Apidae: Bombus spp.)? *Apidologie*, *53*(1), Article 2. https://doi.org/10.1007/s13592-022-00909-6

Vaudo, A. D., Patch, H. M., Mortensen, D. A., Tooker, J. F., & Grozinger, C. M. (2016). Macronutrient ratios in pollen shape bumble bee (Bombus impatiens) foraging strategies and floral preferences. *Proceedings of the National Academy of Sciences*, *113*(28), E4035-E4042. https://doi.org/doi:10.1073/pnas.1606101113

Vaudo, A. D., Tooker, J. F., Patch, H. M., Biddinger, D. J., Coccia, M., Crone, M. K., Fiely, M., Francis, J. S., Hines, H. M., Hodges, M., Jackson, S. W., Michez, D., Mu, J., Russo, L., Safari, M., Treanore, E. D., Vanderplanck, M., Yip, E., Leonard, A. S., & Grozinger, C. M. (2020). Pollen Protein: Lipid Macronutrient Ratios May Guide Broad Patterns of Bee Species Floral Preferences. *Insects*, *11*(2), 132. https://www.mdpi.com/2075-4450/11/2/132

Wallace, D. R. (2014). Acetamiprid. In P. Wexler (Ed.), *Encyclopedia of Toxicology (Third Edition)* (pp. 30-32). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-386454-3.00091-9

Weiner, C. N., Hilpert, A., Werner, M., Linsenmair, K. E., & Blüthgen, N. (2010). Pollen amino acids and flower specialisation in solitary bees. *Apidologie*, *41*(4), 476-487. https://doi.org/10.1051/apido/2009083

Whitehorn, P. R., O'Connor, S., Wackers, F. L., & Goulson, D. (2012). Neonicotinoid Pesticide Reduces Bumble Bee Colony Growth and Queen Production. *Science*, *336*(6079), 351-352. https://doi.org/doi:10.1126/science.1215025

Woodcock, B. A., Isaac, N. J. B., Bullock, J. M., Roy, D. B., Garthwaite, D. G., Crowe, A., & Pywell, R. F. (2016). Impacts of neonicotinoid use on long-term population changes in wild bees in England. *Nature Communications*, *7*(1), 12459. https://doi.org/10.1038/ncomms12459

Wu-Smart, J., & Spivak, M. (2017). Effects of neonicotinoid imidacloprid exposure on bumble bee (Hymenoptera: Apidae) queen survival and nest initiation. *Environmental Entomology*, *47*(1), 55-62. https://doi.org/10.1093/ee/nvx175

Zattara, E. E., & Aizen, M. A. (2021). Worldwide occurrence records suggest a global decline in bee species richness. *One Earth*, *4*(1), 114-123. https://doi.org/https://doi.org/10.1016/j.oneear.2020.12.005

APPENDIX

Table 4 Results of the hive dissection.

Colonie	Treatment	Site	Position	Workers tagged	Workers untagged	Workers foreign	Males	Queens	Larvae ok	Larvae young	Larvae dead	unident.	Moth damage	Other parasites/notes
B	Acetamiprid	A	A5	46	138	0	0	1	16	39	45	11	no damage, few adults	
CD	Acetamiprid	A	A3	32	78	0	0	1	15	44	0	17	0	
E	Acetamiprid	C	C5	22	60	0	0	1	15	0	35	7	30	
L	Acetamiprid	B	B1	32	86	0	0	1	0	50	157	8	0	few earwigs
MN	Acetamiprid	D	D8	26	78	0	0	1	31	91	246	14	0	10% moldy
ÖÜ	Acetamiprid	C	C2	4	53 1g		0	1	27	15	0	70	0	
N	Combined	B	B3	20	62 1l		0	0	98	30	90	60	10	30% mold, very decomposed
ST	Combined	C	C1	31	32	0	0	0	0	0	35	60	0	few earwigs
A	Combined	A	A1	55	65 1 wpu		0	1	196	130	0	30	0	
F	Combined	C	C3	45	120	0	0	1	93	245	33	40	few adults	few other larvae
V	Combined	A	A2	32	56	0	0	1	3	0	88	60	0	few earwigs, some other larvae
X	Combined	D	D7	45	73	0	0	1	15	26	41	23	0	
G	Control	C	C1	25	90 1g, 1l		0	0	141	30	0	15	0	few other larvae
O	Control	B	B5	32	38 1g, 1p		0	0	93	55	34	0	0%	7 earwigs, spider
EF	Control	A	A5	18	35	0	0	0	0	43	99	81	0	quite moldy, many dead, many other larvae
D	Control	A	A2	32	125	0	0	1	293	10	30	17	0	5% mold
P	Control	D	D7	37	56	0	0	1	39	0	91	0	few adults	0
XY	Control	C	C4	61	58	0	0	1	19	26	66	9		0 some mold, ants
Z	Control	D	D3	14	38	0	0	1	0	25	48	70		0 very moldy
C	Imidacloprid	A	A4	39	140	0	0	1	5	4	15	21		0 few earwigs
I	Imidacloprid	C	C4	27	138	0	0	1	70	61	0	20		0 many other larvae
K	Imidacloprid	B	B4	40	66	0	0	1	0	74	123	0		0 2 earwigs, 1 spider
QR	Imidacloprid	C	C3	33	26 1w, 1wb		0	1	12	0	25	4		0
T	Imidacloprid	D	D2	42	93 2 wp		0	1	0	26	117	11		0 1 earwig
U	Imidacloprid	A	A1	12	79	0	0	1	0	6	11	2		0 no parasites, many small workers
W	Imidacloprid	D	D5	34	178 1 wb		0	1	81	10	44	30		0
ZÄ	Tebuconazole	C	C5	29	48 2 g		0	0	15	41	60	50		0 few earwigs
AB	Tebuconazole	D	D4	9	35	0	0	0	0	0	12	100		0 many fly larvae
H	Tebuconazole	C	C2	20	81	0	0	1	110	18	0	8	no damage, few some larvae	
J	Tebuconazole	A	A3	72	146	0	0	1	70	0	27	29	0	0
M	Tebuconazole	B	B2	41	103	0	0	1	39	0	49	1	0	
R	Tebuconazole	D	D8	44	54	0	0	1	24	0	136	1	0	2 earwigs
Y	Tebuconazole	A	A4	17	77	0	0	1	142	70	5	14	0	10% moldy

Table 5 protein content of the pollen dependent on the plant families.

Family	Protein content
P_ADOX	80.76558676
P_AMAR	298.39
P_APIA	163.5958688
P_ASTE	116.2784914
P_BORA	207.5827603
P_BRAS	138.1575224
P_CAMP	209.0398027
P_CAPR	161.1607486
P_FABA	190.0709915
P_FAGA	36.9940112
P_GERA	92.95109568
P_HYPE	295.1923948
P_LAMI	169.5269078
P_MALV	55.08616566
P_OROB	448.0549964
P_PAPA	218.9920142
P_PLAN	117.4797174
P_POAC	62.58826877
P_RANU	140.0444641
P_ROSA	160.7226841
P_RUBI	185.62
P_SALI	181.5765584
P_SCRO	200.3926412
P_SOLA	288.75
P_VIOL	170.82