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Short Paper

Observation of colony development and mortality in front of honeybee colonies and chemical residue analysis of dead bees

Beobachtungen zu Volksentwicklung und Totenfall bei Honigbienenvölkern sowie chemische Analyse von toten Bienen

Osservazione dello sviluppo di famiglie di api e mortalità all'apiario e analisi chimica di api morte

Benjamin Mair[1], Manfred Wolf[1]

[1]Laimburg Research Centre, 39040 Auer/Ora, Italy

ABSTRACT

This study presents the results of the monitoring of apiaries in the vicinity of fruit production areas in South Tyrol from spring to autumn (March-October) between 2017 and 2020. According to the results in previous studies regarding honey bee colony development, dead bees and contamination of pollen pellets with plant protection products harmful to bees we were interested in a more detailed analysis of dead bees accumulating in front of bee hives and the colony development. In this context, our investigations focused especially on colony development and mortality. At those points in time when mortality increased rapidly and abnormally, dead bees were analysed in a laboratory to search for plant protection product residues. In each apiary, increased mortality was observed whenever residues of at least one plant protection product harmful to bees was detected on the dead bees. Most of the peaks in mortality were observed in spring and the highest number of products harmful to bees were also found during this period. In spring, products primarily used for apple production and, to some extent, cherry production were detected. Residues originating from vineyards were identified only in autumn. At the same time, our investigations observed colony development and to our knowledge this is the first time such observations have been made from March to September in the area of concern. Comparing the results on colony development with those on mortality, we noted that a higher number of dead bees in front of the hives (expressed as a % of the colony strength), corresponded to fewer bees present in colonies in June, when the population generally peaked. However, no effect on colony development was observed due to the contamination of pollen pellets with plant protection products harmful to bees, based on the calculated daily pollen hazard quotient (PHQ).

KEYWORDS

honeybee, colony development, mortality in front of beehives, plant protection products harmful to bees

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CORRESPONDING AUTHOR

Benjamin Mair Laimburg 6, Pfatten/Vadena, 39040 Auer/Ora, BZ, Italy, benjamin.mair@provinz.bz.it, +390471969643

Tab. 1: Mean and maximum values as well as number of detections and LD50 contact for the most harmful products to bees found in the residue analyses on dead bees during the monitoring period. LD50's were taken from the Pesticide Properties DataBase and the Bio-Pesticides DataBase.

Active substance	max [mg/kg]	mean [mg/kg]	st. dev. [mg/kg]	no. of detections	LD50 contact [µg/bee]
Chlorpyrifos-ethyl	0.24	0.14	0.14	2	0.059
Chlorpyrifos-methyl	0.39	0.06	0.09	30	0.15
Dimethoate	0.48	0.17	0.27	3	0.1
Etofenprox	4.5	1.16	2.23	4	> 0.038
Imidacloprid	0.7	0.1	0.15	34	0.081
Indoxacarb	0.18			1	0.08
Phosmet	7.58	0.91	1.82	37	0.22
Spinetoram	0.07			1	0.024
Spinosad	2.88	0.8	1.23	5	0.0036
Sulfoxaflor	0.15	0.07	0.04	8	0.379

INTRODUCTION

South Tyrol is home to the largest contiguous apple producing area in Europe [1]. These apple orchards are mostly on the valley floor; aside from apple orchards, vineyards and a few other fruit producing cultivations, such as cherry plantations and pear orchards are also present [2]. Approximately 14 000-15 000 honeybee colonies placed in the surrounding areas [3] also form part of these agricultural systems. Considering that more than 90% of the leading 107 global crop types are pollinated by bees [4], it is obvious that bee colonies and most agricultural fields interact contiguously over an agronomic season, most when the crop is in flower but also when other flowers are present in the understory [5]. This particularly important connection between orchards and beekeeping, bearing in mind that apple orchards are 90% reliant on insect pollination to produce fruit [6], was made in 2014 and represents the starting point for more detailed research activity at Laimburg Research Centre in the beekeeping sector and particularly with regard to bees and the use of plant protection products harmful to bees [7] [8]. Insecticides harmful to bees and their effect on honeybee colonies placed close to crops where they were used has been the main argument of many recent studies focused on the contamination of collected matrices by foragers with active substances, such as in pollen [9] [10] [11] or nectar or honey [12] [13], while others have been concerned with the effects of using plant protection products harmful to bees on colony development [13] [14] as well as on mortality [15]. However, in the majority of studies, only the effect of the measures in one single crop cultivation (for example apples [16] or oilseed rape [17]) or a combination as a consequence of migratory activity [18] was observed, whereas studies where the effects of different crop cultivation measures were observed at the same apiary during one agronomic season are less frequent [11] [19] [20]. In the area of our study, we have the situation of different crops being cultivated relatively close to one another; therefore, during one season, honeybees are able to forage on different crops without migrating to other apiaries. The requirement to use different plant protection products harmful to bees made it necessary to observe possible effects on honeybee colonies in their vicinity for the whole growing season (approximately March-October), and not just to limit the observations to the period from March-June as was the case in the Apistox I project [7]. Moreover, there is no information on colony development or the number of dead bees per colony per day in South Tyrol available for summer and autumn which could be useful in view of possible changing environmental conditions in the future, e.g., the introduction of non-native animals such as *Vespa Velutina* [21].

MATERIALS AND METHODS

From 2017 to 2020, at least three apiaries with a minimum of five colonies in each were monitored every year. In the period from April to June in most years, the capped drone brood was extracted twice to reduce Varroa destructor infestations. No other measures with a potential influence on colony development (such as extracting brood combs for building up new colonies) were taken. The period of observation of the different apiaries varied over the years (Tab. 2 SUPMAT). In 2017, colonies were observed only from March until June, whereas from 2018 to 2020, they were observed from March until October. Every 21 days, a scientific assistant from Laimburg Research Centre estimated the adult bee population and the number of brood cells present in the colonies using the Liebefeld method. The accumulating number of dead bees in front of the hives was observed using underbaskets. Generally, dead bees were counted and collected twice a week, but often every day. If a specific point in time or a notable increase in mortality was of

interest, a sample was taken and stored in a freezer for later chemical residue analysis. The residue analyses were done by the pH laboratory (Tavernelle Val di Pesa, Italy) following the QuEChERS-method [22]. Data were analysed and all figures created using the R computer software [23].

RESULTS

Figure 1 shows the different numbers of brood cells and bees present in the colonies during ten colony evaluations from March until September. At the end of March, colonies start with a median population of bees (8841) and brood cells (8031). Then, during spring, the number of brood cells and bees increases rapidly, achieving a maximum mean number of brood cells at the beginning of May (28 409) and a maximum mean number of bees (28 700) at the beginning of June. The highest number of brood cells and bees were present in the colonies in the period from the middle of May until the end of June. The strongest decrease in the number of brood cells and bees was observed in July. In the middle of July, the number of adult bees decreased to a mean number of 21 200 and decreased to less than 20 000 by the eighth evaluation at the beginning of August. At the end of the observations in mid-September, the number of bees decreased to a mean number of about 16 000 (15 978). A very similar trend was observed for the number of brood cells, but it should be mentioned that the decline at the beginning of August was

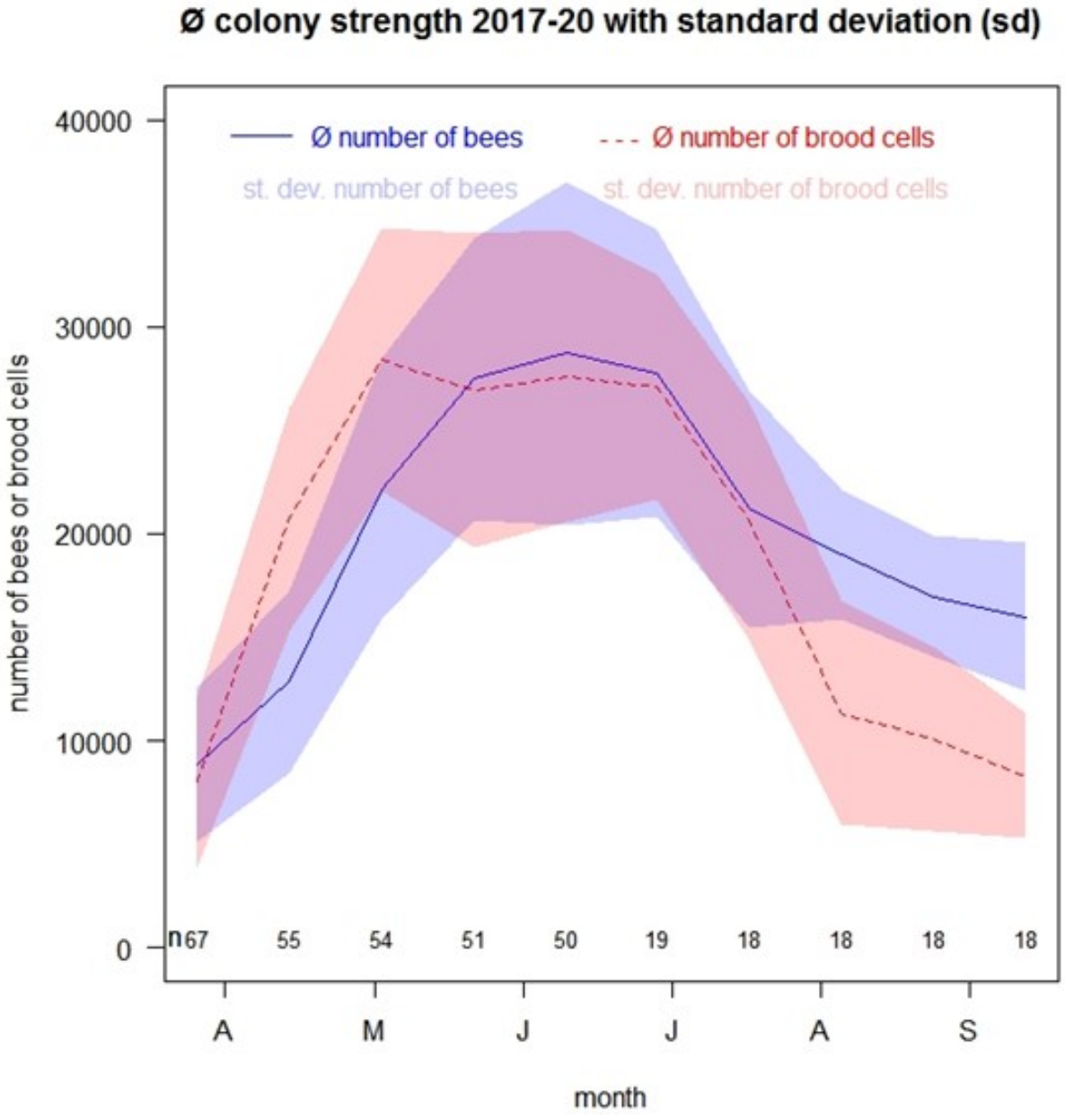

Fig. 1: Mean adult bee population (solid blue line) and mean of total brood cells (sum of open and capped brood cells; represented with a red dashed line) present in the colonies at 10 evaluations of colony size from March-September 2017-2020. Blueish and reddish areas around the lines indicate the standard deviations. The number of colonies which were taken into consideration at each colony evaluation for this analysis are displayed above the x-axis (in the row of “n”).

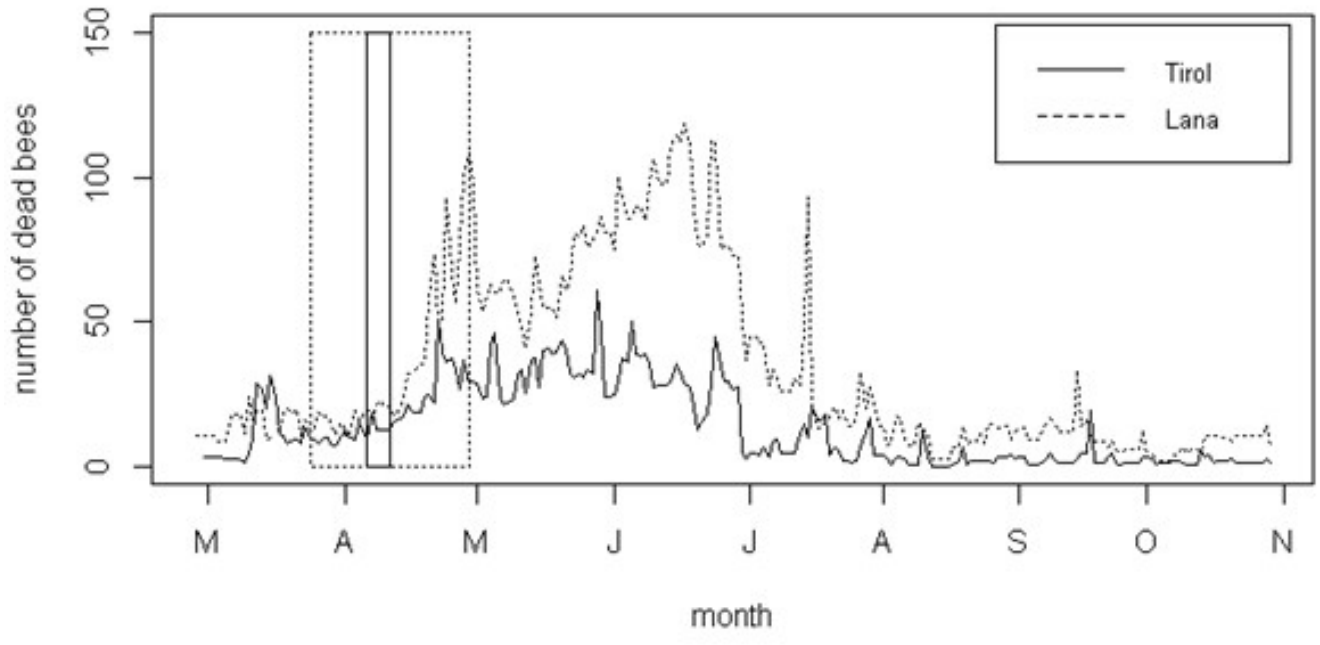

Fig. 2: Lines show the mean number of dead bees per colony for the period March-October from 2017-2020 in the apiaries in Lana and Dorf Tirol. The spotted rectangles indicate the earliest begin resp. the latest end of the apple blossoming, whereas the rectangle with the solid line corresponds to the period where from 2017-2020 was always apple bloom.

stronger than that for the number of bees. At the beginning of August, colonies had a mean number of brood cells of 11 360 and in mid-September, the mean number of brood cells (8285) was clearly below 10 000.

Figure 2 represents the mean number of dead bees per colony per day for two selected apiaries: Lana, where the highest number of dead bees was registered and Dorf Tirol, where the number of dead bees observed was generally lower in comparison with other apiaries. The earliest and latest start and the earliest and latest end of the apple blossoming period between 2017 and 2020 are indicated by dotted rectangles. The period between the latest start and the earliest end of the apple blossoming period is marked with a solid black line. This corresponds to the time span during which, in all four years of observation, there was always blossom on the trees. Before the apple blossom, and after mid-July, the mortalities of these two apiaries were comparable. The means were always clearly below 50 and generally after mid-July, values tended to be slightly higher in Lana. It is interesting that in the period before the trees came into blossom, the mean number of dead bees per colony was in part higher than in the subsequent period during blossoming. This is particularly evident in the Dorf Tirol apiary: between March 14 and March 18, the median was between 19 and 31 whereas between March 28 and April 1, it was between 7 and 10. The situation in the period after the apple blossom period until July is different. The earliest end of apple blossoming was in 2017 on April 14, and this marks the moment when the number of dead bees started to rise sharply, with peaks at both apiaries at the end of April. In Lana, the median for dead bees in the period from the end of April until the end of June was mostly over 50, whereas in Dorf Tirol, a median of 50 was exceeded only three times (April 24 with 51, May 30 with 61, and June 7 with 51). At the end of April and the beginning to middle of June, mortality in Lana was also above 100 and therefore twice as high as in Dorf Tirol.

From some observed mortality peaks, samples were examined in a laboratory for chemical residues. Table 1 summarises some information on the active ingredients with an LD50 < 10 µg/bee found on the dead bees. Ten different products were found in diverse concentrations and frequencies. The three most frequent products were Phosmet (37), Imidacloprid (34), and Chlorpyrifos-methyl (30). Products like Indoxacarb and Spinetoram were found only once. The highest concentrations measured were of Phosmet (7.58 mg/kg), Etofenprox (4.5 mg/kg), and Spinosad (2.8 mg/kg).

Figure 3 provides an overview of when most of the plant protection products harmful to bees were detected over the four years. Most detections were made in May. More than one detection of a substance in one day (day of year) was observed twice in March (for Etofenprox and Chlorpyrifos-ethyl) and once in April (for Chlorpyrifos-ethyl), whereas in May on several days more than one substance was detected. In July, August and September, only a few analyses were made, as the number of dead bees rarely reached levels that were comparable with those

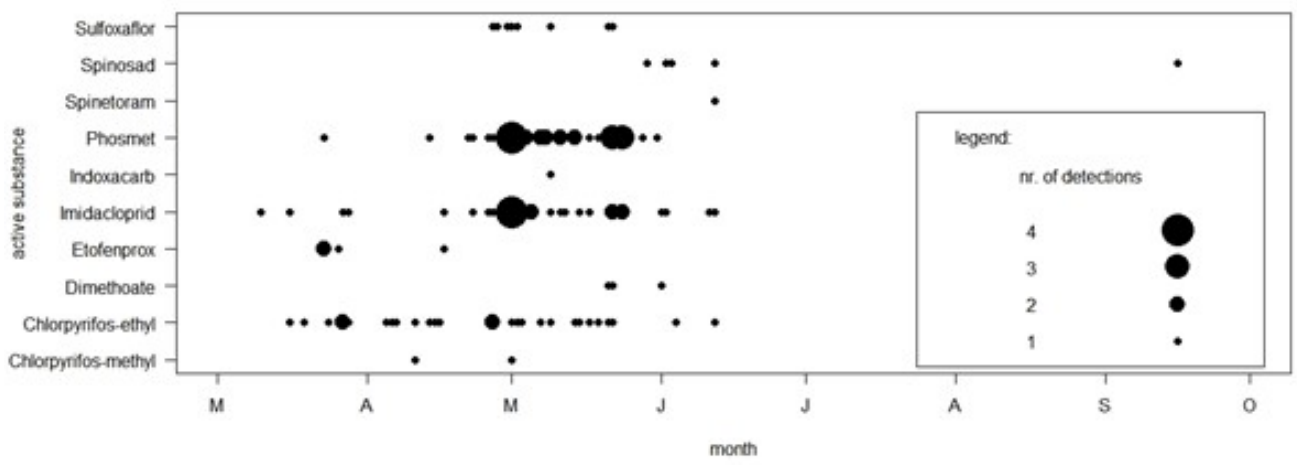

Fig. 3: For each substance, it is possible to see when the most detections of an active substance along the months from March-October were observed.

in spring (Fig. 2). Nevertheless, from July until October seven analyses were conducted on days on which the number of dead bees exceeded expectations according to the observations on other days for the corresponding apiary and period. No plant protection products harmful to bees were found in the samples from July and August, and the active substance Spinosad was found only once in September at Dorf Tirol apiary.

In Figure 4, we checked if the mortality registered in front of the colonies had an impact on their development. The regression between the number of adult bees at the time of the 5th evaluation (which corresponds to the time when the peak adult population is normally achieved) correlates significantly with the % sum of dead bees measured relative to colony strength from the 1st-5th evaluation. The higher the % of dead bees measured relative to colony strength, the lower the colony strength at the 5th evaluation.

Another negative impact on development could have been expected from a diet of pollen contaminated with plant protection products harmful to bees (degree of contamination already shown in a previous publication [8]). The negative correlation between the maximum bee population at the 5th evaluation and the PHQ, calculated in the pollen pellets collected daily (originating partly from wild species and cultivated crops such as apple trees) by foragers is not significant (Fig. 5). Therefore, we cannot confirm whether a higher contamination of pollen loads with products harmful to bees, similar to those reported in Table 1 (exact contaminations are shown in Table 1 of the publication from Mair & Wolf 2023 [8]), increase the likelihood of colonies achieving only reduced colony strengths.

DISCUSSION

This monitoring in the field had no experimental approach, but provided important information for beekeepers and farmers in the area, helping gain a better overview of the effects of using bee-harmful plant protection products in fruit production in South Tyrol on honeybee colonies placed in their vicinity. To our knowledge, these are the first documented observations of colony development in South Tyrol for the period from July to October.

Regarding colony development, similar results were observed to those already reported in the 2022 article by Mair & Wolf [7] for the spring and early summer period. The highest mean production and losses were around 1500 bees/-day [14] [7] and the strongest increase in population was observed in interval 2 (period around mid-April). Many factors, such as a favourable site and weather conditions, the number of adult bees within colonies and an abundant store of food influence colony development [24]. The presence of *Varroa* destructor and the treatments against this parasite also have an impact [25]. We observed that even prior to the *Varroa* treatments (which began every year immediately after the 7th evaluation in mid-July), the number of adult bees within the colonies decreased strongly. The same trend is observable for the number of brood cells. However, it should be mentioned that the strong decline in brood cells at the beginning of August (Fig. 1) was partly caused by the effects of the formic acid treatment which damages eggs and open brood and by the total removal of brood before dribbling oxalic acid into the hive.

The number of dead bees registered in front of the hives varied between apiaries and by time (Fig. 2). A variation in line with changing colony strength during the season was expected, as larger populations would naturally lead to more dead bees. However, contrary to this assumption, mortality was higher prior to the blossoming period than during the subsequent blossoming period. This could often be attributed

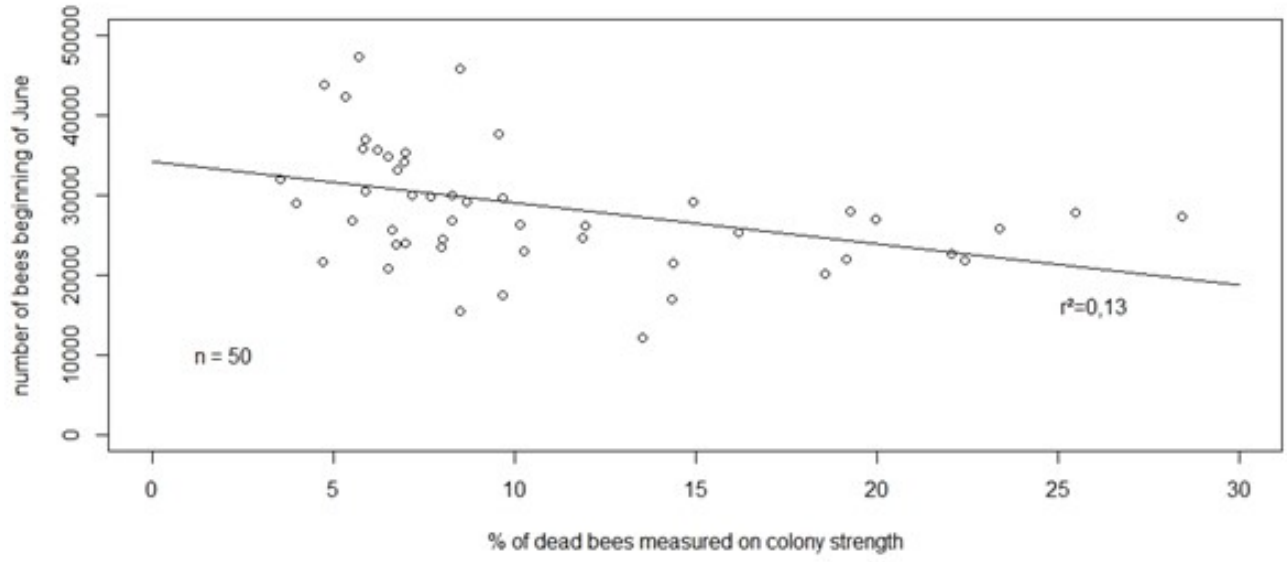

Fig. 4: Regression shows the effect of the sum of dead bees (expressed as % of their colony strength) from the 1st until the 5th colony strength evaluation, on the colony strength at the 5th evaluation (beginning/mid of June). Correlation (r= - 0,38) between the two variables is significant (p-value: 0.006).

to the use of bee-harmful plant protection products during the apple pre-blossoming period, which is a common practice in other apple-producing areas, such as in New York [6]. The highest numbers of dead bees were observed in all apiaries between the end of April and July. The mean number of dead bees per colony per day was high when exceeding 100, even if individual colonies were also often reaching mean numbers exceeding 300 (maximum registered: 576 bees). These numbers are comparable with intoxifications observed in maize fields with Clothianidin-baited seeds [15]. The accumulation of dead bees in front of hives is a natural phenomenon [14] and depends on factors like site, the method of registering dead bees (size of underbasket or plane), and the colony itself, particularly its size. Continuous information about the number of dead bees in front of hives for a specific apiary and period is therefore crucial for correctly interpreting increased mortalities. The generally lower number of dead bees detected in the second half of the year (from July until November) is likely due to wasps collecting dead bees from the underbaskets. Parts of the dead bee bodies were often found in the underbaskets, and some may have been transported away entirely, making it difficult to determine the exact number of dead bees. Nevertheless, due to the declining colony size at this time of the year, a decrease in the number of dead bees is to be expected.

Over the course of this four-year study, 129 samples of dead bees were analysed and in 78 (60.5%) of them residues of plant protection products harmful to bees were found. The most frequently detected product was Phosmet (37), which was also the substance with the highest measured concentration (7.58 mg/kg). It is not totally clear why no residues of products harmful to bees were found on approx. 40% of the samples. It could be that they were present only in low concentrations or degraded too quickly for detection. However, there may also be other reasons why mortality increased, as we observed that beekeepers' measures (such as estimation of colony strength) as well as colony behaviour (such as robbing) could also be responsible for an immediate rise in dead bees.

Most of the products harmful to bees detected in March and April can be attributed to treatments applied in apple orchards (especially Chlorpyrifos-methyl, Etofenprox, Imidacloprid, Phosmet, and Sulfoxaflor). This assumption is confirmed by the following factors: the timeframes of the observed incidents; the products recommended by the most important consulting group for apple production; and the detection of other molecules (mostly fungicides) which are often used in apple production. For May and June, it was again possible to attribute most detections to treatments for apple production (for the same reasons as mentioned before), but we probably should also attribute some increased mortalities to treatments applied in cherry plantations (especially of Spinosad). Unfortunately, no pollen in the body hairs from dead bees was analysed in our monitoring; this could have provided additional information about the plants on which they were foraging when they came into contact with certain substances. However, the detection of Spinosad in September can, with a high degree of probability, be attributed to treatments applied in vineyards to protect grapes against attacks from

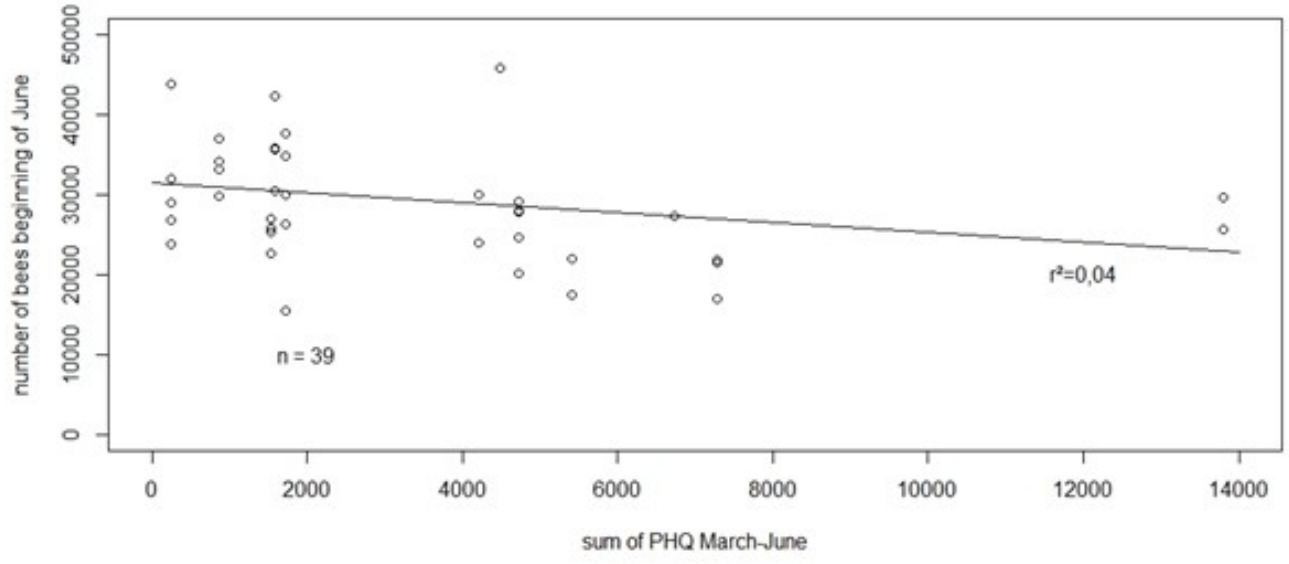

Fig. 5: The regression between number of adult bees at the 5th colony strength evaluation and the PHQ resulting from the analysed pollen pellet samples at the three different apiaries showed a correlation of -0.25 (not significant).

Drosophila suzukii. Residues of this product were also detected in the analyses of bee bread from September and October [8], which in addition confirms that bees regularly come in contact with the active substances in vineyards.

We observed that the higher the number of dead bees relative to colony size in front of the hives, the lower the number of bees at the beginning of June when population maximum was reached (Fig. 4). This would indicate that an additional increase in mortality caused by contact with bee-harmful products would have a negative effect on colony size. However, the real effect of poisoning events induced by plant protection products harmful to bees on colony size is difficult to analyse [14], not least because it is not possible to quantify the real number of dead bees – we were only able to monitor the number of dead bees accumulating in the underbaskets and have no information on dead bees elsewhere, such as on the way to the plants where they are foraging. Moreover, it has often been observed that bees transport their dead fellows a few metres away from their hive, though the prevalence of this behaviour remains unknown. To resolve this issue, electronic bee counting devices positioned at the entrance to a hive could probably generate additional data, but for the moment it is not possible to find a device of this kind guaranteeing the requisite precision; this is a problem with which different researchers are currently dealing [26] [27]. No negative effect of high PHQ measured in corbicular pollen loads collected daily on colony strength was observed (Fig. 5) even if the concentrations of toxic substances for bees reached levels of more than 1 mg/kg [8]. This corresponds with results obtained by other researchers which showed no reduction in development or performance following exposure to plant protection products harmful to bees in free flying colonies [28] [13].

For the first time, we observed colony development and mortality in different apiaries in South Tyrol for the whole agronomic season. These investigations showed that the use of plant protection products harmful to bees for different crops such as apples, cherries and grapes may have an impact on honeybee colonies placed in their vicinity at different times of the year. We concluded that it is necessary to continuously collect data on single incremental mortality events at a specific apiary to correctly interpret poisoning events. Nevertheless, it remains important whenever products that are harmful to bees are used in any plot, that attention be paid to the possible risk for honeybees.

Due to the time lapse between the period when this work was conducted (2017-2020) and its submission date (2025), we must reassess the regulatory status of the active ingredients (AIs) or products discussed in our study. With the exceptions of Spinosad and Etofenprox (detailed below), all AIs mentioned have already been withdrawn from field use in apple-growing regions or are undergoing processes that will result in a permanent ban by the end of 2025.

- Imidacloprid: this AI was banned for outdoor use in 2018 due to its high risk to pollinators, particularly honeybees [29].
- Sulfoxaflor: officially withdrawn from the market in May 2023, Sulfoxaflor had a special authorisation for essential use in 2024 on a restricted basis. The European Union limits its use to indoor applications to mitigate potential harm to pollinators, including bumblebees and solitary bees. Discussions regarding its essential use in 2025 are ongoing, with a decision expected by December 2024 [30]. Finally special authorisation until 06/08/2025.

- Spinetoram: the EU authorisation for Spinetoram expired on June 30, 2024. No renewal application has been submitted, meaning its approval will lapse. Member states may allow a grace period to phase out existing stocks, but Spinetoram use is expected to cease by the end of 2025 [31].

- Indoxacarb: authorisation in the EU was not renewed as of December 2021, effectively banning its use in 2022. Maximum residue levels (MRLs) were also reduced, limiting its presence in agricultural practices [32].

STATUS OF PHOSMET, CHLORPYRIFOS AND CHLORPYRIFOS-METHYL

- Phosmet: the authorisation for Phosmet was not renewed in the EU as of 2022. This decision was based on concerns about operator safety, dietary risks and significant environmental impacts. Although Phosmet had been used with restrictions to mitigate risks to pollinators, its ban reflects the EU's shift toward stricter safety standards for older chemical substances [33].

- Chlorpyrifos: Chlorpyrifos was banned in the EU in 2019 after the European Food Safety Authority (EFSA) concluded it did not meet the criteria for continued approval under updated safety regulations. The primary reasons for its withdrawal were its potential risks to human health, including neurodevelopmental effects, and its environmental impact [34].

- Chlorpyrifos-methyl: authorisation for Chlorpyrifos-methyl was withdrawn in January 2020. The decision was primarily driven by concerns over human health risks, particularly dietary exposure, and not directly related to pollinator safety [35].

Although these three organophosphate esters raised concerns about wild pollinator protection and honeybee toxicity, their use had been repeatedly allowed over the years with restrictions. Ultimately, the bans were driven by broader ecotoxicological and human health concerns rather than pollinator risks specifically. These decisions reflect the EU's cautious approach to substances with significant safety or environmental concerns.

CURRENT STATUS OF SPINOSAD AND ETOFENPROX

- Spinosad: Spinosad remains approved for agricultural field use in 2025; however, its use is subject to strict regulations due to its high toxicity to bees. Applications on flowering crops or where a flowering understory is present are prohibited. These restrictions are designed to mitigate the risk to pollinators, as Spinosad is classified as severely bee-toxic. The European Union has also tightened its risk assessment guidelines, requiring additional mitigation measures or modifications in application practices in order to allow continued registration in the future [36].

- Etofenprox: Etofenprox is still authorised for use on apples under stringent conditions. In Germany, it is approved for limited applications, including on certain flowering crops, but only under specific constraints. These include prohibiting treatments during times when honeybees are active and limiting applications to two treatments per season. Etofenprox is classified as having minor bee toxicity compared to other insecticides, yet its use is carefully regulated to minimise environmental risks, particularly to pollinators [36].

The withdrawal of these AIs and the introduction of tighter application restrictions has probably already significantly mitigated the risks to pollinators, especially the honeybee (*Apis mellifera mellifera*), when beehives are placed in the vicinity of crops. We expect this based on the negative effects observed on colony development in the study area during our fieldwork between 2017 and 2020, the extent of direct bee mortality and the AI found on dead bees. The primary benefits from this include reduced exposure, e.g. fewer applications of highly toxic substances which imply a less direct exposure for the honeybee. The withdrawal of widely used insecticides necessitates the availability of effective, eco-friendly alternatives to maintain pest control efficacy.

ZUSAMMENFASSUNG

Diese Studie zeigt die Ergebnisse von Beobachtungen zu Honigbienenvölkern, welche im Zeitraum von März bis Oktober von 2017-2020 in Südtirol in der Nähe von Obst- und Rebanlagen an Versuchsbienenständen gemacht wurden. Untersucht wurde dabei vor allem die Volksentwicklung und der Totenfall. Zu Zeitpunkten, an welchen sich der Totenfall auffallend veränderte, wurden tote Bienen in einem Rückstandslabor auf chemische Pflanzenschutzmittel hin untersucht. Zumindest einmal wurde an jedem Bienenstand nach einer deutlichen Totenfallerhöhung wenigstens ein bienengefährliches Pflanzenschutzmittel nachgewiesen. Die meisten Totenfallerhöhungen wurden im Frühjahr gefunden, genauso wie die meisten bienengefährlichen Pflanzenschutzmittel. Die meisten der nachgwiesenen Mittel stammten aus dem Obstbau, wenige auch aus dem Kirschanbau. Nur im Herbst wurden Rückstände gefunden, welche ihren Ursprung im Weinbau vermuten lassen. Gleichzeitig wurde auch die Volksentwicklung der Bienenvölker beobachtet. Es sind unserer Recherche nach die ersten Beoachtungen zur Volksentwicklung in Südtirol für den Zeitraum von März bis September. Werden die Entwicklungsdaten mit jenen des Totenfalls verglichen, so beobachteten wir eine schwächere Volksstärke im Juni, wenn sie für gewöhnlich ihr Populationsmaximum erreichen, wenn mehr toten Bienen (tote Bienen prozentual gemessen an der Volksstärke) vorhanden waren. Kein Effekt auf die Entwicklung konnte hingegen durch die Kontamination der Pollenhöschen mit bienengefährlichen Pflanzenschutzmitteln auf Basis der täglich berechneten PHQ (Pollen Hazard Quotient) beobachtet werden.

RIASSUNTO

Questo studio presenta i risultati delle osservazioni sulle colonie di api mellifere condotte in Alto Adige nel periodo marzo-ottobre 2017-2020. La ricerca si è focalizzata su apiari sperimentali situati nei pressi di frutteti e vigneti, con particolare attenzione allo sviluppo e alla mortalità delle colonie. Le api morte sono state analizzate in un laboratorio specializzato nell'analisi dei residui per individuare la presenza di prodotti fitosanitari. Queste analisi sono state eseguite nei periodi in cui si osservavano significativi aumenti del numero di api morte. In ogni apiario, almeno una volta, è stato rilevato almeno un prodotto fitosanitario pericoloso per le api, confermato dalle analisi successive a un incremento rilevante delle morti. Il tasso di mortalità è risultato più elevato in primavera, periodo in cui è stata rilevata anche la maggior parte dei prodotti fitosanitari pericolosi per le api. La maggioranza dei residui identificati proveniva da coltivazioni frutticole, mentre una parte minore proveniva da campi destinati alla coltivazione delle ciliegie. Solo in autunno sono stati rinvenuti residui associabili all'uso di prodotti fitosanitari in viticoltura. Parallelamente, è stato monitorato lo sviluppo delle colonie di api. Secondo le informazioni disponibili, questo rappresenta il primo studio che documenta lo sviluppo delle colonie in Alto Adige nel periodo compreso tra marzo e settembre. Confrontando i dati sullo sviluppo con quelli relativi alla mortalità, si è osservato un indebolimento della forza delle colonie a giugno, il mese in cui solitamente si raggiunge il picco della popolazione, accompagnato da un aumento significativo delle api morte (in percentuale rispetto alla forza della colonia). Al contrario, non sono stati rilevati effetti significativi sullo sviluppo delle colonie legati alla contaminazione dei pellet di polline con pesticidi pericolosi per le api, secondo il PHQ (Pollen Hazard Quotient) calcolato giornalmente.

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APPENDIX: SUPPLEMENTAL MATERIALS

MATERIALS AND METHODS

APIARIES

The exact number of colonies and detailed information on the position of apiaries is summarised in Table 1 SUPMAT. The colonies were provided and managed by the Lehrbienenstand Dietenheim (contact Stefan Tasser) of the Landesdomäne agency in the province of South Tyrol.

BEE COLONIES

The bee colonies were kept in Zander beehives. No detailed information on the age of queens and their genetics was available. Some queens originated from mating locations in South Tyrol and others mated at the apiary where they were hatched, as is often the case for beekeepers in South Tyrol.

OBSERVED PERIOD

Table 2 SUPMAT, provides an overview of the observed periods per apiary.

OBSERVATION OF MORTALITY

Underbaskets made it possible to catch dead bees from each colony, protecting them and making it difficult for them to be transported away by wind or rain.

EVALUATION OF COLONY SIZE

The 21-day interval may have deviated by one or two days in the event of bad weather conditions or organisational issues, but in most cases, there were intervals of 21 days between two inspections. They were made early in the morning before the normal flight activity of the colonies commenced [37] [38]. To estimate the real numbers of adult bees, sealed and open brood cells by using the covered frame areas, the following parameters were utilised: 1.25 bees/cm² and 4 brood cells/cm² [39].

CHEMICAL RESIDUE ANALYSES ON DEAD BEES

The laboratory performed a multi-residue QuEChERS method analysis searching for 505 substances (Table 3 SUPMAT). In 2017 and 2018, only 503 substances were searched for because Sulfoxaflor and Flupyradifurone were only introduced in 2019. For the ten substances considered in the scope of this article, in Table 1 of the article, an LOQ of 0.01 mg/kg was applied. The laboratory utilised the following method for analysing the dead bees: mechanical homogenisation of dead bees with liquid nitrate to powder. 2.5 g of that powder was then mixed with 10 ml water and 10 ml acetonitrile in a 50 ml flacon for five minutes. Magnesium sulphate (4 g), sodium chloride (1 g), sodium citrate (1 g), and trisodium citrate (0.5 g) salts were then added, and this mixture combined again for five minutes before being centrifuged. Next, the supernatant was transferred into a vial and was ready for GC or LC analysis depending on the molecule of interest.

STATISTICAL ANALYSIS

The effect of the number of adult bees in the colonies at the middle/end of March (1st evaluation) on the number of adult bees in the colonies at the beginning/middle of June (5th evaluation) (Fig. 1 SUPMAT) was analysed using a linear model and a subsequent one-way ANOVA. The same procedure was also followed for the effect of the sum of dead bees expressed as a % of their colony strength on the colony strength at the 5th evaluation (Fig. 4 in the article) and the effect of the PHQ in pollen pellets on the number of adult bees at the 5th evaluation (Fig. 5 in the article).

RESULTS

It was not possible to consider all colonies for each analysis. For example, if a colony was swarming or if a queen had to be replaced, they were considered only when the colony development was expected to be normal (Tab. 4 SUPMAT).

Figure 2 SUPMAT shows the number of bees per day produced (green boxes) and lost (red boxes) from March to September. On the basis of these two parameters, the daily difference between production and losses (represented in the blue boxes) was then calculated. For production and losses, the same trend is true: both parameters show a strong increase in spring and achieve their maximum values during interval five (production) and interval six (losses), with a median of approximately 1500 bees/day. They then begin decreasing again until interval nine. Looking at the difference, we see mostly positive differences until interval five, after which they were negative for two intervals until reaching a median of almost zero for interval nine. The highest median daily difference was observed in the second interval, with 429 bees/day, whereas the lowest value was observed in interval six at -257.

The size of the adult population at the 5th evaluation of colony size (beginning to middle of June) correlates significantly with the size of the adult population at the first determination (+ 0.57). For 2018, for these analyses only five colonies could be taken into account. In 2017, no colony immediately after the winter was above 10 000 bees, whereas in the other years, at least one colony always had more than 10 000 adult bees at the middle/end of March. The year when most colonies had more than 10 000 bees at the 1st evaluation in March was 2020 (red crosses in Fig. 1 SUPMAT), whereas the year with the most colonies with an adult bee population below 5000 bees was 2019. Over the course of these 4 years of observation, only 3 out of 50 colonies were composed of more than 15 000 bees, and only 7 of less than 5000. The lowest number of bees in a colony in March was 1800, and the highest number was 16 300 (both in 2019). The highest number of bees at the 5th evaluation was registered in 2018, with 54 100, and the lowest in 2020, with 12 000.

DISCUSSION

As already shown in the paper by Mair et. al. 2022 [7] and in other studies [40] [39], colony size in early spring, i.e. immediately after the winter, is an important factor for the colony's development in the following season, that is, the more bees that are present in the colony at the end of March, the more are present at the beginning of June when population maximum is achieved (Fig. 1 SUPMAT).

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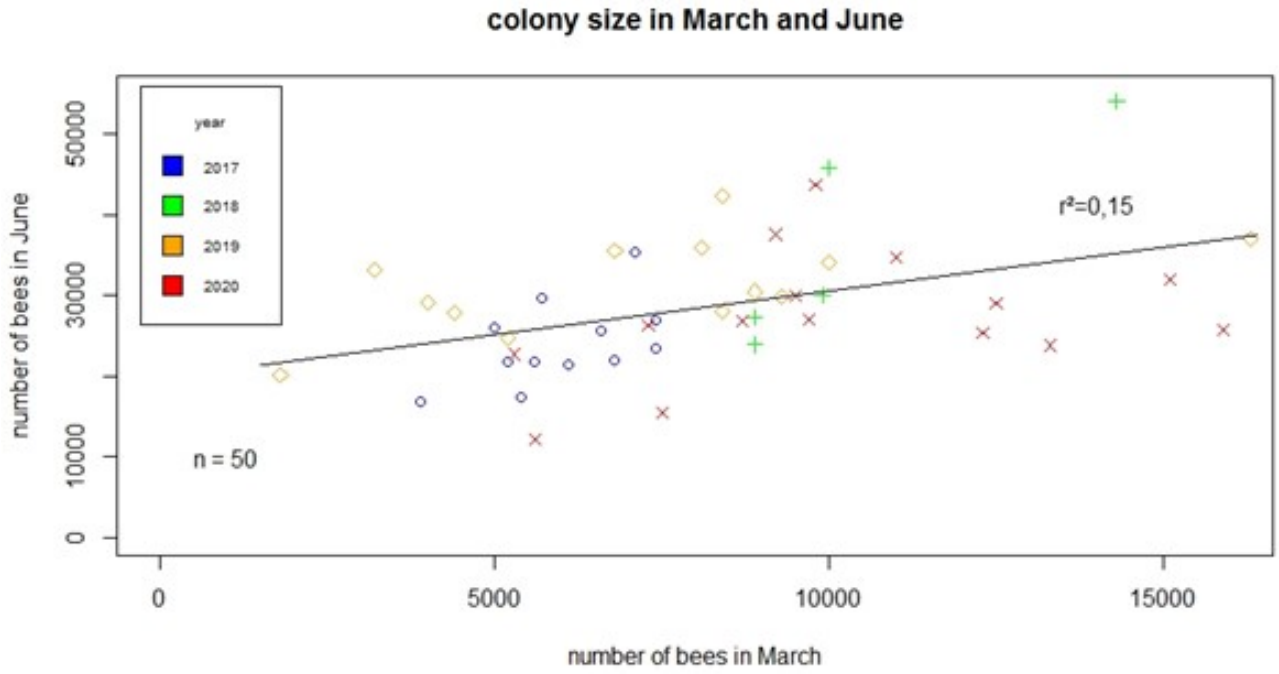

Fig. 1SUPMAT: Regression between the adult bee populations in March (corresponds to the start of the observation every year) and the populations at the beginning of June (corresponds to the moment when maximum bee population is going to be achieved). Blue spots represent data from 2017, green crosses data from 2018, orange squares data from 2019, and red x data from 2020.

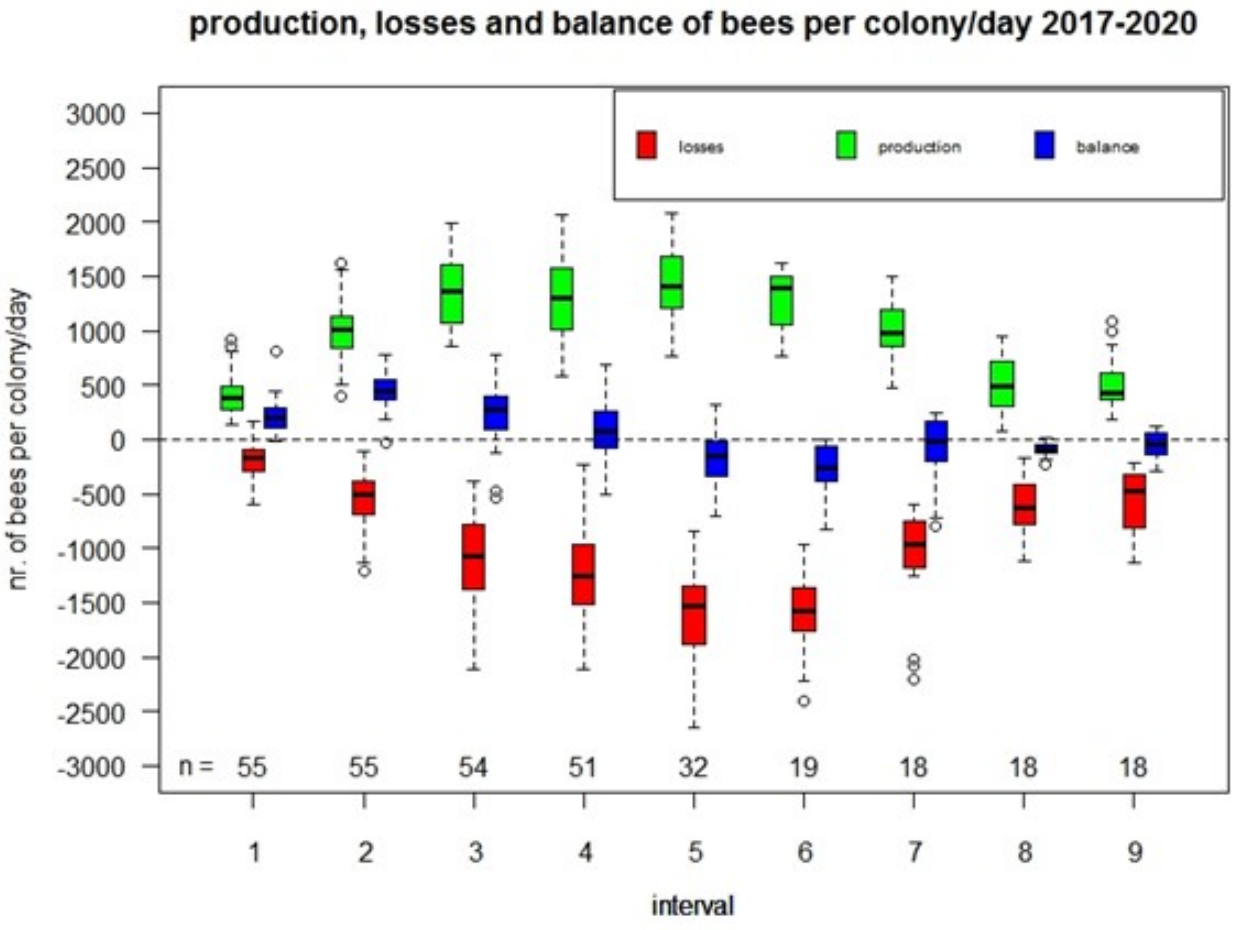

Fig. 2SUPMAT: Production, losses, and balance of bees/day in the colonies for the nine intervals between the ten evaluations from March-September 2017-2020.

Tab. 1SUPMAT: Overview of the number of observed colonies each year within the monitoring.

Year	Location	Apiary nr.	Nr. of colonies	m a.s.l.	Coordinates
2017	Dorf Tirol	2	6	487	46°40'29.0"N 11°09'55.7"E
	Laimburg	11	5	222	46°22'46.7"N 11°17'11.7"E
	Lana	12	5	300	46°37'09.3"N 11°08'39.3"E
	Rabland	13	6	530	46°40'26.0"N 11°03'17.7"E
	Kastelbell	44	5	650	
Sum 2017		5	27		
2018	Dorf Tirol	2	5	487	46°40'29.0"N 11°09'55.7"E
	Lana	12	5	300	46°37'09.3"N 11°08'39.3"E
	Rabland	13	5	530	46°40'26.0"N 11°03'17.7"E
Sum 2018		3	15		
2019	Dorf Tirol	2	5	487	46°40'29.0"N 11°09'55.7"E
	Labers	4	5	540	46°38'18.4"N 11°11'11.8"E
	Lana	12	5	300	46°37'09.3"N 11°08'39.3"E
	Rabland	13	5	530	46°40'26.0"N 11°03'17.7"E
Sum 2019		4	20		
2020	Dorf Tirol	2	5	487	46°40'29.0"N 11°09'55.7"E
	Labers	4	5	540	46°38'18.4"N 11°11'11.8"E
	Lana	12	5	300	46°37'09.3"N 11°08'39.3"E
	Rabland	13	5	530	46°40'26.0"N 11°03'17.7"E
Sum 2020		4	20		
Total			94		

Tab. 2SUPMAT: Overview of the observed periods on the different apiaries from 2017-2020.

Apiary	Year	Month							
		March	April	May	June	July	August	September	October
2	2017	X	X	X	X				
	2018	X	X	X	X	X	X	X	X
	2019	X	X	X	X	X	X	X	X
	2020	X	X	X	X	X	X	X	
4	2019	X	X	X	X	X	X	X	X
	2020	X	X	X	X	X	X	X	
11	2017	X	X	X	X				
12	2017	X	X	X	X				
	2018	X	X	X	X	X	X	X	X
	2019	X	X	X	X	X	X	X	X
	2020	X	X	X	X	X	X	X	
13	2017	X	X	X	X				
	2018	X	X	X	X	X	X	X	X
	2019	X	X	X	X	X	X	X	X
	2020	X	X	X	X				
44	2017	X	X	X	X				

Tab. 3SUPMAT: List of active substances for which chemical residue analysis by pH was used to search in the samples of dead bees.

Rame (Cu)
2-Phenylphenol
4-Phenylphenol
Abamectin (Sum of Avermectin B1a, B1b, Avermectin B1a 8,9z)
Acephate
Acetamiprid
Acibenzolar-S-methyl (Sum)
Acibenzolar acid
Acibenzolar-S-methyl
Aclonifen
Acrinathrin
Alachlor
Aldicarb (Sum of Aldicarb and Aldicarb-sulfone, Aldicarb-sulfoxide expressed as Aldicarb)
Aldicarb-sulfone
Aldicarb-sulfoxide
Aldicarb
Allethrin
Ametryn
Amitraz (included metabolite containing 2,4-DMA expressed as Amitraz)
2,4-Dimethylaniline (2,4 DMA)
Amitraz
N-2,4-Dimethylphenyl-N'-methylformamidine [DMPF]
N-2,4-Dimethylphenyl-formamide [DMF]
Anilazine
Atrazine-desethyl
Atrazine-desisopropyl
Atrazine
Azaconazole
Azadirachtin
Azinphos-ethyl
Azinphos-methyl
Azoxystrobin
Benalaxyl (Sum of benalaxyl and benalaxyl-M)
Bendiocarb
Benfluralin
Benfuracarb
Benomyl (Sum of Benomyl and Carbendazim espressed as Carbendazim)
Benomyl
Carbendazim
Benthiavalicarb-isopropyl
Benzoylprop-ethyl
Benzoximate
Biphenyl

Bifenox
Bifenthrin (sum of isomers)
Bitertanol (sum of isomers)
Boscalid
Bromacil
Bromocyclen
Bromophos-ethyl
Bromophos-methyl
Bromopropylate
Bromoxynil-methyl
Bromoxynil-octanoate
Bromoxynil and its salts, expressed as bromoxynil
Bromuconazole
Bupirimate
Buprofezin
Butocarboxim
Butoxycarboxim
Cadusafos
Captafol
Captan (Sum of Captan and Tetrahydrophthalimide exp as Captan)
Captan
Tetrahydrophthalimide
Carbaryl
Carbofuran (Sum of Carbofuran and Carbofuran-3-hydroxy expressed as Carbofuran)
Carbofuran-3-hydroxy
Carbofuran
Carbophenothion-methyl
Carbophenothion
Carboxin
Carbosulfan
Carfentrazone-ethyl (Carfentrazone free acid expressed as Carfentrazone-ethyl)
Carfentrazone-ethyl
Carfentrazone acid
Chinomethionat
Chlorantraniliprole (DPX E-2Y45)
Chlordane (Sum of cis-Chlordane and trans-Chlordane)
cis-Chlordane
trans-Chlordane
Chlorfenapyr
Chlorfenson
Chlorfenvinphos
Chlorfluazuron
Chloridazon
Chlormephos
Chlorobenzilate

Chloropropylate
Chloroxuron
Chlorpyrifos-ethyl
Chlorpyrifos-methyl
Chlorpropham
Chlorthal-dimethyl
Chlorothalonil
Chlorthiamid
Chlorthiophos
Chlorthion
Chlorotoluron
Chlozolinate
Cyhalofop-p-butyl
Cyanazin
Cyantraniliprole
Cyazofamid
Cycloxydim
Cyflufenamid (Sum of isomer E and Z)
Cyfluthrin (Sum of isomers)
Cyfluthrin
Cyfluthrin-beta
Cymiazole
Cymoxanil
Cypermethrin (Sum of isomers)
Alphamethrin
Cypermethrin
Cyproconazole
Cyprodinil
Chlodinafop and its S-isomers and their salts, expressed as chlodinafop
Clofentezine
Clomazone
Clothianidin
Coumaphos
Coumatetralyl
DDT (Sum of o-p-DDD, p-p-DDD, o-p-DDE, p-p-DDE, o-p-DDT, p-p-DDT expressed as DDT)
o-p-DDD
o-p-DDE
o-p-DDT
p-p-DDD
p-p-DDE
p-p-DDT
DEET [Diethyl-m-toluamid,N,N]
Deltamethrin
Demeton-S-methyl (Sum of D-S-methyl, D methylsulfone and Oxydemeton-methyl exp as Demeton-S-methyl)
Demeton-S-methyl-sulfone

Demeton-S-methyl
Oxydemeton-methyl
Dialifos
Diazinon
Dichlobenil
Diclobutrazol
Dichlofenthion
Dichlofluanid
Dichlorvos
Dicloran
Dicofol
Dicrotophos
Dieldrin (Sum of Dieldrin and Aldrin expressed as Dieldrin)
Aldrin
Dieldrin
Diethofencarb
Difenoconazole
Diflubenzuron
Diflufenican
Diclofop-methyl (Sum of Diclofop-methyl and Diclofop acid expressed as Diclofop-methyl)
Diclofop-methyl
Diclofop
Diphenamid
Dimethoate
Dimethomorph (Sum of isomers)
Diniconazole
Dinitramine
Dioxacarb
Dipropetryn
Disulfoton (Sum of Disulfoton, Disulfoton-sulfone, Disulfoton-sulfoxide expressed as Disulfoton)
Disulfoton-sulfone
Disulfoton-sulfoxide
Disulfoton
Ditalimfos
Dithiocarbamates (Sum)
Diuron
Dodine
Edifenphos
Endosulfan (Sum of Alpha and Beta and Sulfate expressed as Endosulfan)
alpha-Endosulfan
beta-Endosulfan
Endosulfan-sulfate
Endrin
Endrin aldehyde
EPN [O-ethyl O-(4-nitrophenyl) phenylphosphonothioate]

Epoxiconazole
Fenvalerate (Sum of Esfenvalerate and Fenvalerate RS+SR e SS+RR)
Etaconazole
Ethalfluralin
Ethiofencarb-sulfone
Ethiofencarb-sulfoxide
Ethiofencarb
Ethion
Ethirimol
Ethofumesate
Ethoprophos
Etofenprox
Etoxazole
Etridiazole
Etrimfos
Famoxadone
Fenamidone
Fenamiphos (Sum of Fenamiphos and Fenamiphos-sulfone, Fenamiphos-sulfoxide expressed as Fenamiphos)
Fenamiphos-sulfone
Fenamiphos-sulfoxide
Fenamiphos
Fenarimol
Fenazaquin
Fenbuconazole
Fenhexamid
Fenitrothion
Fenothiocarb
Fenoxaprop-p-ethyl (Sum of Fenoxaprop-p-ethyl and Fenoxaprop- acid)
Fenoxaprop (Fenoxaprop-p included)
Fenoxaprop-p-ethyl
Fenoxycarb
Fenpyroximate
Fenpropathrin
Fenpropidin
Fenpropimorph (sum of isomers)
Fenson
Fenthion (Sum)
Fenthion-oxon-sulfone
Fenthion-oxon-sulfoxide
Fenthion-oxon
Fenthion-sulfone
Fenthion-sulfoxide
Fenthion
Fenuron
Fipronil (Sum of Fipronil and Fipronil Sulfone expressed as Fipronil)

Fipronil-sulfone
Fipronil
Fipronil-desulfinyl
Flamprop-isopropyl
Flonicamid (Sum of Flonicamid and TFNA-AM expressed as Flonicamid)
Flonicamid
TFNA-AM
Fluazifop-P (Sum)
Fluazifop-P-butyl
Fluazifop
Fluazinam
Flubenzimine
Flucycloxuron
Flucythrinate
Fludioxonil
Flufenacet
Flufenoxuron
Fluopicolide
Fluotrimazole
Fluoxastrobin (sum of fluoxastrobin and its Z-isomer)
Flupyradifurone
Fluquiconazole
Flurochloridone
Flurprimidol
Flusilazole
Fluthiacet-methyl
Flutolanil
Flutriafol
Folpet (Sum of Folpet and Phtalimide expressed as Folpet)
Folpet
Phthalimide (expressed as Folpet)
Fonofos
Forchlorfenuron
Formothion
Fosetyl-aluminium (sum of fosetyl, phosphonic acid and their salts, expressed as fosetyl)
Phosphonic acid
Fosetyl
Fosthiazate
Fuberidazole
Furalaxyl
Furathiocarb
Haloxyfop (Sum)
Haloxyfop
Haloxyfop-2-ethoxyethyl
Haloxyfop-methyl

HCH (Hexachlorocyclohexane) (Sum of isomers Alpha, Beta, Delta and Epsilon)
alpha-HCH
beta-HCH
delta-HCH
epsilon-HCH
gamma HCH [Lindane]
Heptachlor (Sum of Heptachlor and Heptachlor epoxide expressed as Heptachlor)
cis-Heptachlor epoxide
Heptachlor
trans-Heptachlor epoxide
Heptenophos
Hexachlorobenzene
Hexaconazole
Hexaflumuron
Hexazinone
Hexythiazox
Imazalil
Imidacloprid
Indoxacarb (Sum of indoxacarb and its enantiomer R)
Iodofenphos
Iprobenfos
Iprodione
Iprovalicarb
Isazophos
Isodrin
Isofenphos-methyl
Isofenphos
Isopropalin
Isoproturon
Isoxaben
Isoxaflutole
lambda-Cyhalothrin
Lenacil
Leptophos
Linuron
Lufenuron
Kresoxim-methyl
Malathion (Sum of Malathion and Malaoxon expressed as Malathion)
Malaoxon
Malathion
Mandipropamid
Mecarbam
Mepanipyrim
Metaflumizone (Sum of isomer E and Z)
Metalaxyl and Metalaxyl-M (Sum)

Methacrifos
Methamidophos
Metamitron
Metazachlor
Metconazole
Methiocarb (Sum of Methiocarb, Methiocarb-sulfone, Methiocarb-sulfoxide expressed as Methiocarb)
Methiocarb
Methiocarb-sulfone
Methiocarb-sulfoxide
Methomyl
Metidathion
Metobromuron
Metolachlor (Sum of Metolachlor and Metolachlor-S)
Metolcarb
Methoprotryne
Methoxychlor
Methoxyfenozide
Metoxuron
Metribuzin
Mevinphos (Sum of isomer E and Z)
Myclobutanil
Milbemectin (Sum of Milbemycin A4 and A3 expressed as Milbemectin)
Mirex
Monocrotophos
Monolinuron
Naled
Napropamide
Neburon
Nicosulfuron
Nitrapyrin
Nitrofen
Nitrothal-isopropyl
Novaluron
Nuarimol
Omethoate
Oxadiazon
Oxadixyl
Oxamyl
Oxyfluorfen
Paclobutrazol
Paraoxon-ethyl
Parathion-methyl (Sum of Parathion-methyl and Paraoxon-methyl expressed as Parathion-methyl)
Paraoxon-methyl
Parathion-methyl
Parathion-ethyl

Pencycuron
Penconazole
Pendimethalin
Pentachloroanisole
Pentachlorobenzene
Pentachlorophenol
Phenmedipham
Phenthoate
Permethrin
Perthane
Pethoxamid
Phorate (Sum of Phorate and Phorate-oxon, Phorate-sulfone, Phorate-sulfoxide expressed as Phorate)
Phorate
Phorate-oxon
Phorate-sulfone
Phorate-sulfoxide
Phosalone
Phosmet (Sum of Phosmet and Phosmet oxon expressed as Phosmet)
Phosmet
Phosmet oxon
Phosphamidon
Picolinafen
Piperonyl butoxide
Pyraclofos
Pirimicarb (Sum of Pirimicarb and Pirimicarb-desmethyl expressed as Pirimicarb)
Pirimicarb-desmethyl
Pirimicarb
Pirimiphos-ethyl
Pirimiphos-methyl
Procymidone
Prochloraz (Prochloraz included metabolite containing 2,4,6-Trichlorophenol exp as Prochloraz)
2,4,6-Trichlorophenol
BTS 40348
BTS 44595
BTS 44596
Prochloraz
Propham
Profenofos
Profluralin
Promecarb
Prometon
Prometryn
Propachlor
Propamocarb
Propanil

Propaquizafop
Propargite
Propazine
Propiconazole
Propyzamide
Propoxur
Proquinazid
Prosulfocarb
Pyraclostrobin
Pyraflufen-ethyl
Pyrazophos
Pyrethrins
Pyridaben
Pyridaphenthion
Pyrifenox
Pyrimethanil
Pyriproxyfen
Prothioconazole-desthio
Prothioconazole
Prothiophos
Prothoate
Pymetrozine
Quinalphos
Quinoxyfen
Quintozene (Sum of Quintozene and Penthachloroaniline expressed as Quintozene)
Pentachloroaniline
Quintozene
Quizalofop, incl. Quizalfop-P
Quizalofop-p-ethyl
Quizalofop acid
Rimsulfuron
Rotenone
S 421
Sethoxydim (Sum of Sethoxydim and Clethodim)
Clethodim
Sethoxydim
Silaneophan [Silafluofen]
Simazine
Simetryn
Spinetoram
Spinosad (Sum of Spinosyn A and Spinosyn D, expressed as Spinosad)
Spinosyn A
Spinosyn D
Spirodiclofen
Spiromesifen

Spiroxamine
Sulfotep
Sulfoxaflor
tau-Fluvalinate
Tebuconazole
Tebufenozide
Tebufenpyrad
Tebupirimfos
Tecnazene
Teflubenzuron
Tefluthrin
Terbacil
Terbufos-sulfone
Terbufos-sulfoxide
Terbufos
Terbumeton
Terbuthylazine-desethyl
Terbuthylazine
Terbutryn
Tetrachlorvinphos
Tetraconazole
Tetradifon
Tetramethrin
Thiabendazole
Thiacloprid
Thiamethoxam
Thiobencarb
Thiodicarb
Thiophanate-methyl
Thionazin
Tolclofos-methyl
Tolylfluanid (Sum of Tolylfluanid and DMST expressed as Tolylfluanid)
DMST (Dimethylaminolsulftoluidide)
Tolylfluanid
Tralkoxydim (sum of the constituent isomers of tralkoxydim)
Transfluthrin
Triadimefon
Triadimenol
Triazamate
Triazophos
Tribenuron-methyl
Tricyclazole
Trichlorfon
Trichloronat
Tridemorph

Trifloxystrobin
Triflumizole (Sum of Triflumizole and FM-6 exp as Triflumizole)
FM-6
Triflumizole
Triflumuron
Trifluralin
Triflusulfuron (6-(2,2,2-trifluoroethoxy)-1,3,5-triazine-2,4-diamine (IN-M7222))
Trinexapac (Sum of trinexapac (acid) and its salts, expressed as Trinexapac)
Triticonazole
Vamidothion
Vinclozolin
Zoxamide

Tab. 4SUPMAT: Colonies and the reasons why they were not taken into account for further analysis.

Site	Colony	Reason
	2017	
2	II	introduction of replacement queen
12	II, IV	introduction of replacement queen
13	IV	removing brood frames to build up new colonies
	2018	
2	I, III	introduction of replacement queen
	IV	queen cells present during monitoring
12	II, V	introduction of replacement queen
	III, IV	swarmed during the monitoring
13	I, IV	introduction of replacement queen
	III	queen cells present during monitoring
	2019	
2	I	introduction of replacement queen
13	II	introduction of replacement queen
	2020	
2	V	introduction of replacement queen
12	V	introduction of replacement queen
4	I, II	introduction of replacement queen
	III, V	swarmed during the monitoring