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Research project on field data collection for honey bee colony model evaluation

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Abstract

As part of the MUST-B project, a research project on field data collection for honey bee colony model evaluation was carried out in 2018-2020. In a preparatory phase (2018), methods for monitoring of honey bee colonies were tested, field operators trained, and experimental colonies established. The main field experiment was conducted in 2019-2020, during which bee colonies in six experimental apiaries were closely monitored in both Denmark and Portugal. An experimental spraying (spraying of Pirimor G in 6 ha of flowering oilseed rape) was carried out at one of the sites in Denmark in 2019. During the two-year experiment, climate variables were recorded continuously, and availability of floral resources was mapped regularly in the landscapes surrounding each apiary (within an area of 1.5 km radius). Adult bee population, brood and provision were assessed approximately every three weeks in experimental colonies. Furthermore, the weight of colonies was logged continuously during the field seasons by automatic hive scales. At four sites, foraging activity was monitored continuously in 1-2 colonies in 2019 and 2020. Spatial foraging was decoded from honey bee waggle dances observed once per month in four apiaries, at the same time as floral mapping. Finally, samples for analysis of diseases (varroa, *Nosema* and viruses), pesticide residues and botanical composition of pollen were collected. All data were organized in a relational database. Whereas previous studies have monitored similar aspects of honey bee colony development and health, the current dataset is unique in encompassing a large number of variables measured simultaneously. In particular, the current study emphasized a detailed data collection on population dynamics and development for the testing and calibration of the ApisRAM model developed in the MUST-B project. Methods used encompassed manual and automatic monitoring. Recommendations for future data collection include an assessment of variables currently collected with confidence and variables in need of further development.

Key words: landscape mapping, floral resources, population assessment, observation hive, pesticides exposure, flight activity, image analysis

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Summary

This report describes the work done during the project "Research project on field data collection for honey bee colony model evaluation", which was designed for development and calibration of a honey bee colony simulation model, ApisRAM. Both field data collection and the development of the model were outsourced by ESFA for the MUST-B project (EU efforts towards the development of a holistic approach for the risk assessment on MUltiple STressors in Bees) (section 1). In the preparatory phase of the project in 2018, experimental landscapes were selected (sections 2.2, 3.1.3), methods for monitoring of honey bee colonies were tested, field operators trained, a database for compiling field data developed (sections 2.1, 3.1), and experimental colonies established (section 2.5.1). In the main experiment, intensive monitoring of honey bee colonies was carried out using six experimental apiaries across two field seasons in 2019-2020. Monitoring in and around the experimental apiaries encompassed floral mapping in the landscape (<1.5 km) surrounding the apiaries (sections 2.3, 3.2), local weather and in-hive conditions (sections 2.4, 3.3), in-hive measurements of adult population, brood and provision (sections 2.5, 3.4), identification and prevalence of infectious agents (section 2.6, 3.5), background pesticide load (sections 2.7, 3.6) and experimental pesticide spraying (sections 2.8, 3.7). Recommendations based on lessons learned during the field study are discussed in section 4.

In autumn 2018, experimental landscapes were selected and experimental apiaries were installed: four in Denmark (Northern EU regulatory zone) and two in Portugal (Southern EU regulatory zone). In each apiary, five experimental colonies were monitored, while 1-5 hives were kept as back-ups, replacing experimental colonies, which were lost during the experiment. In order to minimize variation due to genetic composition, sister queens of *Apis mellifera* Buckfast were used in Denmark, and sister queens of *A. m. iberiensis* were used in Portugal. An experimental spraying with Pirimor G (in 6 ha of flowering oilseed rape) was carried out at one of the sites in Denmark (high exposure site) during the crop flowering, in late April 2019.

For the data collection, a relational database was developed in .NET Entity Framework Core, ran on a PostgreSQL, and hosted by Amazon Web Service. The database was available for users through a web page. It consisted of six data tables: Pesticide application, Resource providing unit and landscape fitness (floral resources), Colony management, Colony inspection, SSD2 (results of laboratory analyses, i.e. diseases, pesticide residues and botanical composition of pollen), Colony observation (honey bee waggle dances). Data could be imported or entered manually in the database through a web form. In addition to the six data tables, four tables were created by an administrator prior to data collection: Users, Sites, Hives, and Polygons.

Study sites were selected within six landscapes. Two of the landscapes in Denmark (designated as "high exposure sites") were planned to be subjected to an experimental pesticide spraying event. However, only one spraying experiment was conducted in 2019 due to adverse weather conditions and small experimental colonies, and in 2020 spraying experiments were cancelled due to COVID-19 restrictions. Two other experimental landscapes matching the high exposure sites in terms of landscape, crop use and honey potential, but separated from the high exposure sites by at least 10 km, were selected as low exposure sites, where no experimental spraying was conducted. In Portugal, both experimental apiaries were located in areas with a low input of plant protection products. Each apiary was initiated in autumn 2018 with 10 colonies (five experimental and five back-up colonies). However, due to winter mortality and colony losses during the experiment, experimental apiaries were re-organized and, in some apiaries, supplemented with new colonies unrelated to the sister queens in spring 2020 (see Table 12 for details).

Landscape composition was analysed in 10x10 km quadrates and a 1.5 km circular area surrounding each apiary in 2019 and 2020. Location of crop fields changed between years, although the area composition was similar between the two study years at all sites. Only small differences were found in land use of the landscape within 1.5 km of the experimental apiaries and the landscape at larger scale (10x10 km quadrates) at all study sites. Availability of floral resources was mapped by ground truthing

within 1.5 km (in Portugal in some cases up to 3 km) of each experimental apiary, from March in Portugal and May in Denmark until September in Denmark and October in Portugal. Floral mapping was carried out approximately once per month during the field season (5 times per year in Denmark, 8-11 times per year in Portugal) in 2019-2020. Flower availability of each flower-containing polygon was recorded and scored from 1 to 3, depending on their level of richness ("1" for low, "3", "2" for medium and "3" for high). In Denmark, the flower-containing polygons identified in the field project (denoted field polygons) were digitalized by the modelling team to ALMaSS landscape model parcels with respect to geographic and thematic coincidence. In Portugal, the final landscape maps were updated with results from the field survey. Additionally, the polygons were updated using new orthophotos from 2018. The end product had a 1:1 relationship between flower-containing polygons mapped in the field and one polygon in the ALMaSS system.

Climate data (air temperature, relative humidity, precipitation, wind speed, wind direction and solar radiation) were logged automatically each hour by weather stations. Climate data were logged continuously during the experiment, from spring 2019 to autumn 2020, except during short periods of missing data due to technical problems (e.g. incidences of lightning damage).

All experimental hives were placed on ApisTech bee hive scales, which were used to continuously monitor in-hive temperature and humidity, in addition to hive weight every hour during the field seasons 2019 and 2020. Furthermore, population assessments of experimental colonies were carried out at regular intervals during each field season, approximately every 19 days, and preferentially no more than 21 days (the developmental time of workers) between two consecutive assessments. The adult population was assessed from the weight of live bees, while brood development and provision was assessed by analysing images of the combs using an image analysis software (Deepbee®). Colony assessments revealed a large variation in colony development among apiaries in different landscapes and countries, and among colonies within each apiary. At the four low exposure sites, one bee colony was installed in an observation hive in 2019. Observation hives were observed for waggle dances once per month, at the same time as the botanical mapping. However, on many observation days and study sites (approximately 50%), waggle dances could not be observed. At four sites (two in Western Denmark and two in Portugal), activity of foragers was continuously monitored by videorecording in 1-2 hives in 2019 and 2020. The activity was measured as the number of bees leaving and returning to the hive during 10 minutes. A total of 4075 hours of foraging activity (bees leaving or returning to the hive) were recorded and calibrated.

Samples were collected from all experimental colonies for disease analysis in both study years [varroa spring (February in Portugal, April in Denmark), August, October; viruses (DWV, SBV): August and October; Nosema: spring (February in Portugal, April in Denmark) and October]. Due to unusually weak development of bee colonies at the study sites in Denmark in 2019, extra samples were collected for disease analysis in spring 2019. Results indicated that 20 of the 40 colonies were infected with Nosema, although all colonies except for one had a very weak (< 500 000 spores/bee) to medium (between 1 000 000 and 2 000 000 spores/bee) infection of Nosema. However, a high prevalence of sac brood virus (SBV) was found. Level of varroa was low (much less than a critical level of 10 mites per 100 bees) in the colonies in Denmark in both study years, but high (>10 mites per 100 bees) in some colonies in Portugal in 2019. Samples collected in autumn 2019 revealed variable levels of infections by Nosema for all six apiaries. In 2019, infections by virus were generally low, except for SBV. In 2020, infections by virus were generally low, except for SBV in Denmark in all colonies. Deformed wing virus type B occurred frequently after treatment against varroa in Western Denmark and in Portugal. It may be linked to the transmission via varroa mites, and inhibition of immunity due to treatment of varroa.

Samples for multi-residue pesticide analysis for determination of background pesticide exposure were collected from experimental colonies in spring and mid-summer (after main spraying season) in both study years and in all apiaries. In 2019, few pesticides were detected in Denmark, while in 2020 samples contained traces of several pesticides, especially in Eastern Denmark. In Portugal, traces of coumaphos were detected in 2019 and DMPF in 2020.

An experimental spraying was carried out at Foulum (high exposure site in Western Denmark) on the 29th of April 2019 using a dosage of 0.9 kg Pirimor G per ha (equalling 0.45 kg a.i.). Pre-spraying samples, in addition to samples 3-4 hours, 1 day, 2 days, 3 days and 2 weeks after spraying were collected for mono-residue pesticide analysis for pirimicarb. Samples included bee bread and honey collected from experimental colonies (pre-spray and 2 weeks after spraying), in addition to pollen and nectar from foraging bees and flowers of oilseed rape (pre-spray and 3-4 hours to 3 days after spraying). Sugar content (sucrose equivalents) was measured in nectar extracted from bees and from flowers. Pirimicarb was found in both pollen and nectar from sprayed oilseed rape flowers, foragers and beebread/nectar in combs. Concentrations were higher in pollen than nectar and decreased with time after spraying.

Pollen for palynological analysis of botanical composition was collected by pollen traps (in 2019 in Denmark and Portugal, in 2020 in Portugal) or from bee bread from combs (in 2020 in Denmark). Pollen samples included pollen collected during the field seasons, in addition to pollen collected from the experimental colonies during the spraying experiment. Pollen samples were dominated by mass flowering wild flowers and crops in all landscapes.

The final data set includes data concerning pesticide application during the spraying experiment in 2019, location and management of experimental colonies in 2019-20, adult bee population assessments, and assessments of brood and provision 2019-20, hive scale data 2019-20, forager activity 2019-20, pesticide residue analysis 2019-20, pollen analysis 2019-20, prevalence of diseases 2019-20 and colony observation (waggle dances) 2019 have been uploaded to the database.

The current project aimed to support the ApisRAM model, a landscape-level agent-based model of a honey bee colony. Data collected during the field study will be used for development and calibration of the model, but more generally contribute to the understanding of development and functioning of honey bee colonies in different landscape contexts. In the field study, a large range of parameters were measured, some variables were monitored intensively, others less intensively. Whereas some aspects of honey bee colony development have been studied in previous studies, this study is unique in the large spatial and temporal scale of the experiment, and the simultaneous monitoring of a large number of variables. In particular, the study emphasized a detailed assessment of the temporal development of adult population, brood and provision in different landscapes. Two complementary approaches were used to assess colony development: automatic hourly monitoring of hive weight (frequent monitoring, but low accuracy assessment) versus detailed in-hive observations every 19-20 days (non-frequent monitoring, but high accuracy assessment).

The value of the different variables should be considered in the choice of parameters included in future monitoring schemes in field studies. Automatically collected data, such as hive scale monitoring, hold potential for standardized data collection in future monitoring schemes. However, the automatic methods used in the current study required further development and adaptation to local conditions. Moreover, automatically collected data should be carefully cleaned and calibrated.

Difficulties and challenges were met in the field data collection of the current study, and different challenges applied to the different methodologies and variable. Lessons learnt and recommendations on methods and parameters measured are listed in section 4.3.

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1. Introduction

1.1. Background and Terms of Reference as provided by EFSA

1.1.1. Background

The European Food Safety Authority (EFSA) has the mandate to improve food safety in Europe and to ensure a high level of protection of human and animal health, including bee health, and the environment, including ecosystem services such as pollination of a wide range of crops and wild plants, which is largely provided by bees.

The way that stressors (biological, chemical and environmental) affect bees and contribute to the current observed trends of population declines is not well understood, neither are the underlying mechanisms, which remain complex given the potential number of combinations and interactions among stressors (IPBES, 2016).

In 2008, EFSA conducted a survey on existing bee surveillance systems in the European Union (EU) (EFSA, 2008). Following its recommendations, the European Commission (EC) established an EU Reference Laboratory (EURL) for honey bee health (Commission Regulation (EU) No 87/2011[1]) and funded an EU-wide monitoring programme on honey bee mortalities and diseases in Europe (EPILOBEE). The results of this programme showed a geographic north-south trend in mortality (Chauzat et al., 2014), but given the large dataset, a high number of variables not yet fully analysed, and the absence of data on the monitoring of other bee stressors (i.e. chemical and environmental factors), these results remain preliminary.

At EFSA, the multifactorial aspect of bee losses and colony weakening puts this issue under the competence of the Scientific Committee, which addresses multi-sectorial issues (Article 28 of EFSA's Founding Regulation (EC) No 178/2002[2]). It is the role of the Scientific Committee and Emerging Risks (SCER) unit to develop integrated risk assessment approaches (EFSA, 2015).

In 2012, in line with its mandate, the SCER unit initiated horizontal work in bee health through the establishment of an internal and multi-disciplinary task force (i.e. the Bee TF) and through the organisation of a scientific colloquium (EFSA, 2013). The Bee TF produced an inventory of EFSA's work in the area of bee health (EFSA, 2012) and consulted a wide range of stakeholders (i.e. the European Commission and Member States) to identify knowledge gaps in research and to make recommendations to move towards an integrated and holistic approach for the risk assessment on multiple stressors in bees (EFSA, 2014). Some specific recommendations were made for future work at EU level, which is further described below.

1.1.2. Terms of Reference

The MUST-B project (EU efforts towards the development of a holistic approach for the risk assessment on MUltiple STressors in Bees) comprises several interlinked activities (see overview Figure 1) to be either continued (e.g. use of in-house expertise through the Bee TF and development of outsourcing projects and networking activities) or developed (e.g. scientific support from the Scientific Committee and external experts, collaborations with EURL on honey bee health and outsourcing the collection of scientific evidence for risk assessment and monitoring on multiple stressors in bees).

[1] Regulation (EU) No. 87/2011 415/2013 as from 6 May 2013. OJ L 29, 3.2.2011, p. 1–4.
[2] Regulation (EU) No. 178/2002 as from 28 January 2002. OJ L31/17, 1.2.2002, p. 1–24.

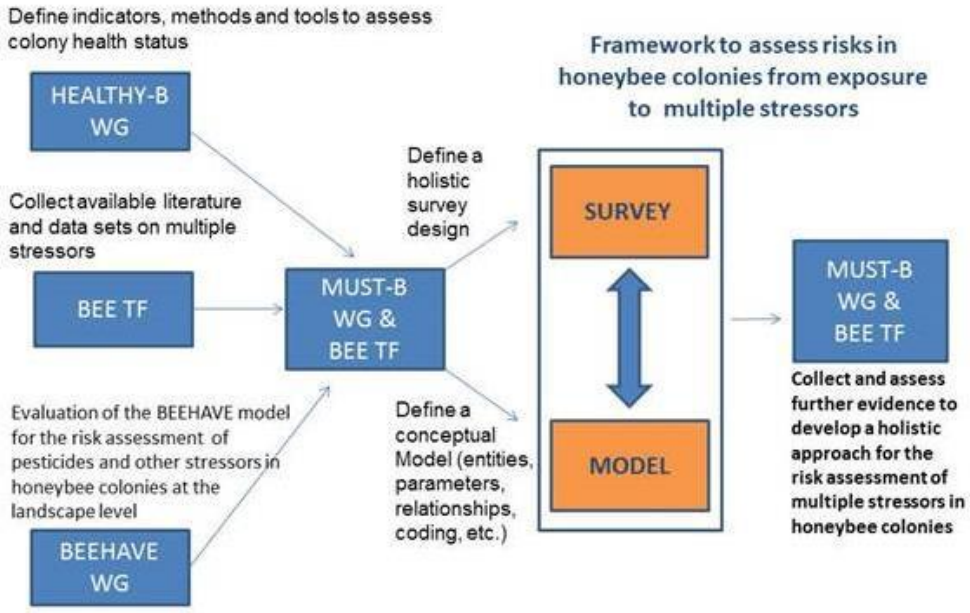


Figure 1: MUST-B project

The final goal of this project is to bring together all available expertise and knowledge in the area of bee health and risk assessment by further developing the multi-disciplinary approach initiated at EFSA by the bee task force. This will be moved forward by bringing together evidence and stakeholders for a more cohesive and collaborative approach towards the development of a holistic approach to the risk assessment on multiple stressors in bees.

The Working Group (MUST-B WG) of the Scientific Committee (SC) and the EFSA Bee Task Force (TF) have been given the following tasks:

- To develop a holistic approach for the risk assessment on multiple stressors in honey bee colonies; this will be formalised through a Scientific Opinion of the Scientific Committee. Technical reports will be produced through the analysis of specific data sets as they become available by outsourcing and other activities.
- To produce regular updates on EFSA's activities in the area of bee health.

The Bee TF is a multidisciplinary group of EFSA staff that supports all MUST-B activities and regularly reports on MUST-B progress and other relevant on-going activities, dealing with bee-health issues *via* a dedicated microsite on the new EFSA portal.

1.1.3. Interpretation of Terms of Reference

The MUST-B working group is developing a framework incorporating modelling, experimental and field-monitoring approaches. These complementary approaches are being combined to extrapolate risks from individual to colony levels, to assess the complexity of co-exposures from multiple stressors coming from both the hive environment and the landscape, and to determine their relative contribution to colony losses and weakening. The work is being conducted with input from the disciplines of ecotoxicology, population biology and landscape ecology.

A first technical report outlined specifications for a honey bee model development, drawing extensively on expert knowledge and a detailed understanding of current published information (EFSA, 2016). It is

envisaged that the model will be used as an exploratory tool for regulatory risk assessment purposes, and also to better understand the (relative) risks and impacts of multiple stressors on honey bee colonies, including the overall complexity of interactions. The model is intended to be used to aid setting protection goals and toxicity thresholds for pesticides, clarify the relative importance of different stressors to the system and answer systems-level exploratory questions (e.g. how does the impact of a pesticide on colony health change with changing climate).

Development, calibration and evaluation of a model are parts of an iterative cyclical process (Figure 2). This starts with an initial focus on individual modules (code development, unit testing with respect to module behaviour against defined criteria, further code development and further unit testing) utilising published data and bee expert knowledge. Once individual module testing proves satisfactory, modules are combined for further evaluation, including model calibration, sensitivity and uncertainty analyses and evaluation based on independent data.

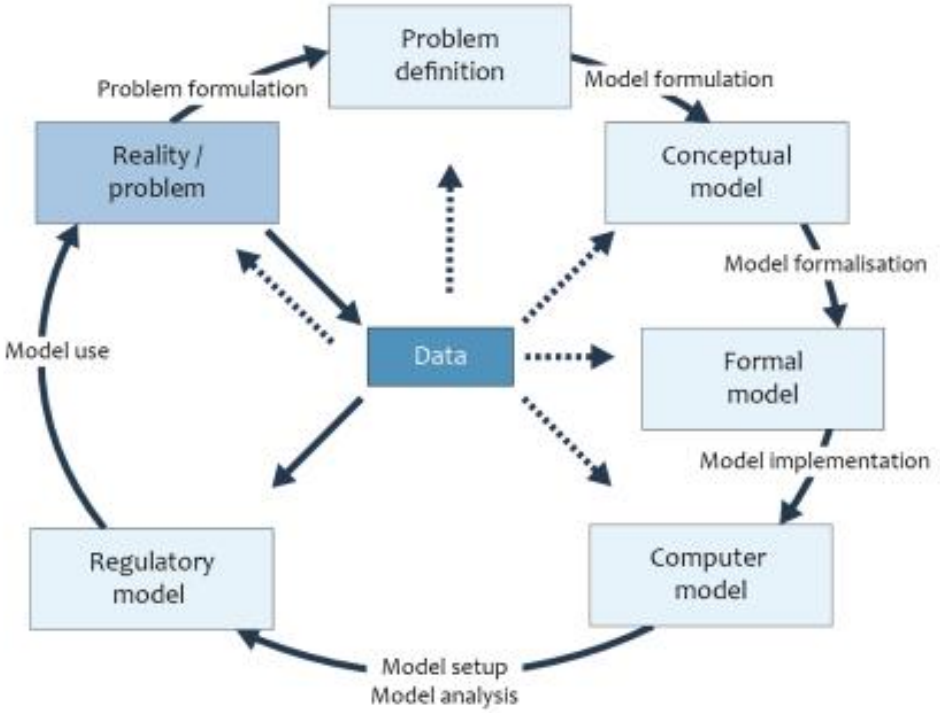


Figure 2: Modelling cycle from EFSA PPR Panel (2014)

Before a model can be used for regulatory purposes, it will need to be evaluated with respect to the intended use. This evaluation is reliant on high quality field data, representative of situations where the model is expected to be applied. It will take several years to collect the sufficient amount of high-quality field data for a complete evaluation of the model for a regulatory purpose. As a consequence, evaluation will be a lengthy process, conducted in stages.

In the early phases of modelling development, model evaluation will be conducted during model development, utilising published data, *ad hoc* data and bee expert knowledge. During this process, model structure and parameters are revised, as a result of new published information, new available data and results from the model evaluation. When the resulting computer model (see Appendix B in EFSA PPR Panel, 2014) proves satisfactory based on available literature data, the evaluation will be expanded to include the performance of the model under realistic field conditions. For this purpose, field data collected under controlled conditions, which can be replicated within the model are required.

The current external scientific report outlines specifications for field data collection, contributing to model development and evaluation. Honey bee colonies and their interactions with the environment form complex systems. The model needs to be corroborated and verified using a number of instances of these complex systems. The model should fit not just a single metric of these systems but multiple metrics in time, in parallel, thus demonstrating both system behaviour and the mechanisms behind generation of that behaviour.

To assist with this process, data will be conducted from honey bee colonies across a range of landscapes, including:

- At least two of the three regulatory zones of Europe to assess functionality over a number of geographically different environments representing the regulatory zones;
- areas with different levels and seasonal patterns of floral resource availability, to evaluate performance in resource-stressed and non-stressed colonies, and
- locations of high and low pesticide exposure, to evaluate model performance in response to exposure of bees to varying general level of pesticide use.

2. Material and methods: sites selection, protocols for field data collection and database

The project was carried out in two phases, the preparatory phase, and the main experiment. In the preparatory phase, monitoring methods were tested and developed, study sites were selected, field protocols and database for data upload were developed, field operators trained, and experimental bee colonies reared and installed in experimental apiaries. The preparatory phase was carried out during the field season 2018, and encompassed the following tasks:

- Database and field/lab forms
- Site selection and confirmation of landscape fitness
- Sister queens rearing and start of colonies
- Field protocols finalization
- Field operators training
- Colonies setting

The main experiment was carried out during the field seasons 2019-2020, and encompassed the following tasks:

- Agricultural practices and land use, cover and structure
- Local weather and in-hive conditions
- Colony management and colony observations
- Identification and prevalence of infectious agents
- Experimental insecticide spraying and quantification of chemical residues in crops, bees and hives
- Sugar concentration in nectar and floral origin of pollen
- Data collation and reporting

2.1. Database

2.1.1. Database requirements

All data collected during the project was stored in a relational database. The database was developed in .NET Entity Framework Core, ran on a PostgreSQL, and was hosted by Amazon Web Service during the whole duration of the project development. Data could be imported or entered manually in the database through a web form. Administrators could create new users and administrators, new sites, and new colonies, i.e., administrators were allowed to enter or change data of all tables. Users were allowed to enter data, and could view, retrieve, and modify their own data of all tables, except for Table III (description of experimental colonies). Administrators could view and retrieve all data. Data was retrieved in CSV and XML formats, and were structured to secure a smooth transmission of data to the Data Collection Framework of EFSA. Furthermore, data flow from the field data collection to the development of ApisRAM was secured by direct communication between the field and modelling teams.

2.1.2. Database structure

The structure of the data largely followed the tables described in the Technical Report specifications Appendices A.1 through A.7 (EFSA, 2017). Furthermore, the web form was equipped with special procedures for automatic entry of automatically registered data such as data from the logging of hive scale data, colony observation (waggle dance), forager activity and comb image analysis. Where appropriate, speed buttons were added in some tables facilitating the entry of the same basic information for many consecutive entries from for instance the same polygon or the same colony by one click on a button. The database was constructed with case specific drop-down menus (depending on business rules), to make data entry as swift as possible and to reduce the risk of errors. In order to reduce the risk of errors, the database was constructed to notify if required data fields had not been filled in. Finally, automatic range checks were implemented wherever it was found appropriate. Unique ID numbers for each entry were provided automatically by the database. In some fields of the database, it was found appropriate to have default values, and in other fields, default values were avoided to force the user to make active choices in mandatory fields.

During the construction of the database some simple business rules were implemented. Business rules that, for instance, secured relevant size of magnitudes, avoided erroneous negative values, and made the available choices in drop down menus in some fields depending on selections in previous fields. In some cases, the selections in one field made some of the following fields irrelevant and consequently they were made inactive.

The database was available for users and administrators through a web page. Login required a username and a password. The database consisted of the following six tables accessible for users:

Table I: Pesticide application, reporting data on experimental spraying events;

Table II: Resource providing unit and landscape fitness, reporting data on abundance of flowering plants in polygons mostly within 1.5 km, but in some cases up to 3 km of the experimental colony;

Table IV: Colony management, reporting the log of the beekeeper regarding input (if material was added to the hive: e.g. empty frames, chemicals for varroa treatment, sugar), output (if material was removed from the hive, e.g. honey combs, supers), queen loss, swarming, or clinical signs observed in the experimental hives;

Table V: Hive inspection, reporting data on in-hive measurements in the experimental colonies. This table contained several types of data, including:

- Data on brood development and food provision ("cell utilization") obtained from image analysis of combs;
- Data on forager activity obtained from automatic video recordings and image analysis by a bee counter;
- Data on hive weight obtained from automatic logging by a hive scale;

- Data on adult bee strength, obtained by weight assessment of combs with and without adult bees ("bees per comb data").

Table VI: SSD2, reporting data on results of laboratory analyses of pollen, pesticide residues and parasites/pathogens. These four types of laboratory analyses involved different methods and were reported according to different standards. Therefore, a number of the fields in the technical specifications for the SSD2 table (EFSA, 2017) were not applicable for records reporting results of some analyses, in particular palynological, parasite and pathogen analyses. These fields were left empty. In order to make the table suitable for palynological, parasite and pathogen analyses a choice of laboratory analysis type was added in the first field of a record, resulting in only the fields relevant for this particular analysis being shown.

Table VII: Colony observation, reporting observations of honey bee waggle dances from observation hives. Orientation denotes the angle of the waggling phase relative to the vertical axis on the comb. Direction denotes the actual direction in the landscape, as calculated from the orientation of the waggle dance.

In addition to the six field data tables, four tables were created by an administrator prior to data collection:

- Users: all users of the database had a username and password;
- Sites: the study sites were defined by site number, site name, country and UTM coordinates. Four study sites were created in Denmark and two in Portugal. The list of sites was available as a drop-down list in other tables of the database;
- Hives (Table III), a list of experimental hives (colonies). Each experimental colony (one record per colony) was defined according to study site (from drop-down menu based on the Sites table), honey bee sub-species (*Apis mellifera mellifera x* Buckfast in Denmark and *A. mellifera iberiensis* in Portugal), frame type (Norwegian in Denmark, Langstroth in Portugal), standard hive/observation hive and hive identification number. The list of experimental colonies was linked to drop-down menus in other tables of the database;
- Polygons: A list of polygons at each study site was provided by the ApisRAM team. Each polygon was described by location (UTM coordinates of centroid) and area (in km^2). The list of polygons was linked to drop-down menus for each of the study sites.

Tables I-VII were published as a separate document in the knowledge junction community of Zenodo (https://doi.org/10.5281/zenodo.4953762).

2.2. Selection of study sites

The field experiment involved a total of six experimental apiaries located in two countries, representing two different EU regulatory zones for the authorisation of plant protection products: (1) Denmark (Northern zone) and (2) Portugal (Southern zone). The field experiment encompassed four study sites in Denmark (low and high exposure sites in Western and Eastern Denmark, respectively) and two study sites in Portugal (low exposure sites in Lousa and Idanha). At high exposure sites, an experimental spraying event was planned (see section 2.8), while no experimental sprayings were carried out at low exposure sites, although pesticide use in the surrounding landscape could not be ruled out. In Portugal, the two low exposure study areas were selected differing in landscape context, in particular in relation to floral resource availability.

Due to legislation and logistics, the experimental sprayings were planned to be conducted at the two experimental farm facilities of Aarhus University (http://agro.au.dk/en/facilities/): Foulum in Western Denmark (56.49 N, 9.58 E) and Flakkebjerg in Eastern Denmark (55.33 N, 11.39 E). The spraying experiments encompassed spraying of 6 ha of oilseed rape (application rate of 0.9 kg Pirimor pr ha i.e. 0.5 kg active substance pirimicarb pr ha). Permission for the experimental spraying was granted by the

Danish EPA (journal number MST-664-00140) in 2019 and 2020. At each "high exposure" site, the experimental apiaries were placed in close proximity to the experimentally sprayed field in 2019. The spraying experiments were cancelled in 2020 because of a national lock down due to COVID-19.

Low exposure sites in Denmark were selected to conform as closely as possible to the "high exposure" sites within each region based on a GIS analysis of the landscapes. The GIS analysis was based on crop data from 2017. In each region, a 10x10 km landscape was identified, based on the following requirements:

- 10x10 km squares centered on the study sites ("high" and "low exposure" sites) should be non-overlapping, as honey bees may regularly forage at distances of several kilometers from the hive in an agricultural landscape (e.g. Garbuzov et al., 2015). However, we aimed at minimizing distances above 10 km between pairs of "low" and "high exposure" sites in order to reduce environmental variability;
- Areas with a similar oilseed rape crop field coverage within the 10x10 km area;
- Areas with similar grass/clover ley coverage within the 10x10 km area;
- Areas with similar forest cover within the 10x10 km area;
- Areas with similar coverage of flower-rich natural areas within the 10x10 km area;
- Areas with similar honey potential within the 10x10 km area. Honey potential is weighted by area. The honey potential for each type of crop and natural vegetation is an assessment of the amount of honey which can be produced in one hectare (e.g. winter oilseed rape 200 kg/ha, lingonberries 100 kg/ha, forest 25 kg/ha etc.). Honey potential of natural vegetation was assessed through literature review and expert knowledge, and for crops only insect-pollinated crops were included (Kryger and Greve, 2018). Each area was classified according to land use based on various sources (forest map, map of protected nature, field blocks etc.), and floral resources were assessed in each nature type or crop. The honey potential of natural areas was not based on specific plant species, but the estimated amount of nectar, which is available per hectare of a particular nature type. For agricultural fields, honey potential was estimated based on information from the subsidy applications, which specifies the crop of each field. Furthermore, information on high nature value was used as input and distinguishes e.g. meadow from meadow with high nature value (Kryger and Greve, 2018). The honey potential is not equal to the amount of honey harvested, but the potentially available amount of nectar;
- Total number of different crops;

Within the 10x10 km quadrates which were identified as suitable "low exposure" landscapes matching the "high exposure" landscapes in Denmark, suitable hosts for experimental apiaries were identified.

2.3. Agricultural practices and land use, cover and structure

2.3.1. Landscape analysis

At each of the six study sites, landscape composition was analysed by GIS in the 10x10 km landscapes surrounding the experimental apiaries, in addition to a 1.5 km circular area centred on the experimental apiaries, within which floral mapping was carried out. Additionally, in Portugal, polygons outside the 1.5 km circle but within 3 km of the apiaries were included in the assessment if the land use type was not present within 1.5 km radius and to guarantee a better representation of existing land use types within this area. Furthermore, landscape composition was compared between pairs of "low" and "high exposure" sites (areas of 10x10 km surrounding the experimental apiaries) within each region in Denmark, and between the two study years 2019 and 2020. In Denmark the Integrated Administration and Control System (IACS) registration each year includes information on the specific crop grown in each field parcel (through Land Parcel Identification System - LPIS). This data is freely available. The

landscape analysis was carried out using crop data from 2019 and 2020, respectively. In Portugal, available land-use maps were used (COS – Carta de Ocupação do Solo).

In the analysis of the landscape in Denmark, areas potentially containing forage for bees were quantified (Appendices A and B). These included:

- Natural areas classified as high nature value (HNV, High Nature Values are scores between 0 and 13, high values indicate areas of high biodiversity). These areas potentially contain wild flowers important as bee forage;
- Forests, including deciduous and conifer forests. Aphids in forests may provide a source of sugar for honey bees at certain times of the year;
- Oilseed rape fields provide an important nectar resource in early spring;
- Clover fields, including seed fields and grasslands with clover, provide an important nectar resource for honey bees during the summer.

In the analysis of the Portuguese landscapes, a diverse range of land use types were included, including permanent land cover types and temporary crops.

2.3.2. Floral mapping

Mapping of floral resources was carried out in a circular area of 1.5 km radius surrounding the experimental apiaries using available maps (and, in Portugal, expanding to 3 km radius to include typologies not present within 1.5 km radius). Polygons containing flowers of importance to honey bees were identified in the field, by annotating print out of maps or by confirming the GPS coordinates in an app. In Denmark, sparse floral resources (covering a small area and/or rare and scattered flowers) were not registered, as these were not considered important for honey bees. In Portugal, the landscape was dominated by natural habitats with sparse and highly variable vegetation through the year and, thus, all flower resources were registered (even sparse floral resources). The polygons identified in the field were linked to polygons in landscapes of ALMaSS, the system used by the ApisRAM model.

Assessment and mapping of floral resources in the landscapes were done using a semi-quantitative method, which was previously developed by the Danish team following EFSA's requirements (EFSA, 2017). In the preparatory phase of the project, a training session of the field operators in charge of the botanical surveys in Portugal and Denmark took place at the University of Coimbra (9-12 July, 2018). Members of the GIS team of the University of Coimbra (involved in the development of the Portuguese ALMaSS landscapes) also participated in the session to link the structure of the data collection protocol with the information required for ApisRAM. During the training session, the method for floral mapping was improved and adapted to include habitat types and floral resources for honey bees in the Portuguese landscapes. The training session included an initial planning meeting and field visits to the two sites selected in Portugal (Lousa and Idanha), followed by meetings to discuss adjustments of the methodology and structure of the database for data collection. Communication among GIS experts, database experts and botanists continued throughout the project, in order to link data from GIS and floral resources registered by ground truthing.

On each observation day, all field polygons, i.e. areas potentially containing floral resources of importance to honey bees were visited and characterized. Flowering plant species were always determined visually (never by DNA analysis), and no pollen or nectar samples were collected for further analysis. Each flowering species was categorized according to species growth form (herbs or dwarf shrubs, shrubs and trees) and when relevant by species type (crop, weed, wild plant). The amount of floral resources available in a field polygon was characterized by classifying each flowering species according to three categories: low (category 1), medium (category 2) high (category 3). The categorization was based on flower density of the plant species within the area (abundant or low, which

can relate either to ground cover for herbs or surface area for shrubs and trees), combined with the area covered by the flowering species (herbs) or number of plants (shrubs or trees) (Table 1). A small number of plant species (e.g. *Salix* spp. and *Prunus spinosa*) occurred both as a shrub and a tree. In that case, the species was registered twice. Although the exact numbers of flowers were not counted, the flower categories may be converted to a coarse measure of flower density (number of flower units per unit area). A flower unit is a single flower, flower head, basket or inflorescence. We propose the following density classes: 3 corresponds to 1000 flowers per m^2; 2 corresponds to 100 flowers per m^2; and 1 corresponds to 10 flowers per m^2.

Table 1: Categories 1-3 of floral abundance

Herbs and dwarf shurbs		**Area covered by the species**		
	Density of flowers	≥75%	25-75 %	≤25%
	Abundant	3	2	1
	Low	2	1	1
Shrubs		Number of shrubs relative to size of polygon		
	Density of flowers	Abundant	Intermediate	Low
	Abundant	3	2	1
	Low	2	1	1
Trees		Number of trees relative to the size of polygon		
	Density of flowers	Abundant	Intermediate	Low
	Many	3	2	1
	Few	2	1	1

In Denmark, mapping was carried out once per month within each 1.5 km of the four apiaries during the main flowering season, from late March/early April to late August/September, five times in 2019 and five times in 2020, in each of the four landscapes (Hinnerup, Foulum, Krænkerup and Flakkebjerg). On a given observation date, all flower species attractive to bees were registered. However, if buds were observed on a plant species on one sampling occasion, but wilted on the subsequent visit to the polygon, the species were registered as flowering on an intermediate date.

In Portugal, floral mapping was carried out eleven times from the beginning of March to mid-September in 2019 and eight times from mid-March to mid-October in 2020, during the main flowering season in each of the two landscapes. Priority was given to polygons within 1.5 km of the apiaries, although some polygons within 3 km were also visited. In Lousa, accessibility was the main problem, limiting the sampling points to areas close to available tracks that could be covered within the time frame of the project. In Idanha, the main problem was the presence of private properties at the sites, which limited access to some of the polygons. Although the team has worked continuously with landowners, authorizations could not be obtained in some places. Floral availability in inaccessible polygons was estimated from extrapolation. Because the Portuguese landscape is highly heterogenous and is highly dynamic (e.g. forest cuttings, new plantations, fire events) impacting the land-use types, in the survey of 2019, the two following sampling schemes were followed with different objectives: 1) validation scheme, and 2) monitoring scheme. In the validation scheme, the sampling points were defined with the aim of validating the polygon typology/class and homogeneity; these polygons were visited only once or twice during the survey period, and only in 2019. In the monitoring scheme, the sampling points were defined with the aim of evaluate temporally the variation in floral resources across the entire survey period; these polygons were visited every twenty days in 2019 and once per month in 2020 during the field seasons. In both sampling schemes, all the flower resources present at the time of the sites visits were evaluated and recorded.

2.4. Local weather and in-hive conditions

2.4.1. External climate (weather stations)

Weather variables were logged continuously at all study sites from early spring 2019 until October 2020. Climate data were obtained from weather stations of different brands, but the climate variables measured were standard climate variables, and comparable across sites. At Flakkebjerg, Foulum and Ødum (8 km from Hinnerup), the following parameters were obtained from climate stations run by the national weather service in Denmark (Danish Meteorological Institute):

- Average air temperature in two meters height (°C);
- RH, relative air humidity in two meters height (%);
- Rainfall (mm);
- Global radiation (W/m2);
- Average wind direction at ten meters height (degrees);
- Average wind speed at ten meters height (m/s).

(for detailed descriptions of the parameters with units, please refer to: https://confluence.govcloud.dk/pages/viewpage.action?pageId=26476616)

At Hinnerup and Krænkerup (Denmark), Cumulus model Cu6-DDffttRRrhLt climate stations were installed, and at Idanha and Lousa (Portugal) Spectrum Technologies: WatchDog 2900ET model with data logger climate stations were used. Cumulus and Spectrum Technologies weather stations logged the same climate parameters as listed above, although average wind direction and average wind speed was measured at three meters height. We suggest that wind speed at 10 meters height is extrapolated to three meters height for Flakkebjerg, Foulum and Ødum, as three meters height is more relevant for bees.

Weather stations were running from 1 January 2019 and throughout the experiment in Foulum and Ødum (Western Denmark) and Flakkebjerg (Eastern Denmark). In Krænkerup (Eastern Denmark), the weather station was installed on 17 January 2019, and in Hinnerup (Western Denmark) from 20 February 2019. At the Portuguese study sites, weather stations were installed on 26 February 2019.

2.4.2. In-hive climate (hive scales)

Hive scales of the brand ApisTech (https://apistech.eu/en/) were installed for automatically monitoring hive weight (see section 2.5.4) and in-hive climate in experimental bee hives (five hives per study site). The hive scales were equipped with two temperature sensors for measuring external and internal temperature of the hive, in addition to a sensor for measuring internal air humidity. The internal temperature sensor was placed inside the brood area of the frames, while the humidity sensor was placed on the top of the frames. Logging was carried out automatically once every hour and transmitted to an online platform through a SIM card. Good coverage was found at all six study sites. Each scale was powered by a battery, which was charged by a small solar panel placed on top of the hive.

Up to two non-experimental back-up hives (kept as replacements in case an experimental colony died during the experiment) at each of the study sites in Denmark were equipped with a scale of the brand Capaz (https://www.capaz.de/), which also measured the weight and internal temperature (comparable to data from ApisTech scales) every hour during daytime (6-22h). In Portugal, all non-experimental back-up hives, were equipped with ApisTech scales.

2.5. Colony management and colony observations

2.5.1. Honey bee colonies

In order to reduce variability between colonies due to genetics, sister queens were reared by professional queen breeders during the field season 2018 for both experimental and back-up colonies to be used for the data collection in 2019-2020. A total of 22 sister queen colonies of *A. m. iberiensis* were produced in Portugal, and 40 colonies of *A. m. mellifera x* Buckfast in Denmark. Due to practical reasons, during the winter 2018/2019, when colonies were not monitored, all colonies in Western Denmark were placed at Hinnerup, and all colonies in Eastern Denmark overwintered in Krænkerup.

In Denmark, the sister queens were produced in a mating station on the island of Glænø. Due to the isolation of the mating station from mainland populations of honey bees, the produced queens were highly likely to be sisters. In Portugal, the queens were produced by an authorized queen breeder, who certified that the queens are of the sub-species *A. m. iberiensis.* Due to limited budget, the genetic origin of the queens could not be confirmed using molecular markers.

Due to unexpected winter losses in 2019/20 in Idanha, replacement colonies, installed in March 2020 were not sister queens.

In Denmark, colonies were started from a mated queen and two full frames of brood covered with adult bees, and 2.5 kg of apifonda. The nucleus was not treated against varroa. In Portugal, the colonies started from "package bees" (3 kg of bees per colony), that were treated against varroa. Colony management has followed the standard procedures in each of the two countries.

In early spring 2019, eight colonies were moved from Hinnerup to Foulum (8 April), eight colonies were moved from Krænkerup to Flakkebjerg (15 April), and two colonies were moved from Krænkerup to Hinnerup (15 April). The field experiment started in spring 2019, when the six experimental apiaries were installed; the numbers of colonies per apiary is indicated in Table 12.

At the end of the field season 2019 (late October), colonies were prepared for overwintering. In Western Denmark, all colonies in Foulum were moved to the apiary in Hinnerup for the winter. In Eastern Denmark, the apiary in Krænkerup was not moved during the winter, while in Flakkebjerg, the apiary was moved to a location closer to the experimental oilseed rape field in 2020. In Portugal, the colonies overwintered in the experimental apiaries. In spring 2020 (23 March), the apiary in Foulum was re-installed, moving back the colonies, which were placed in Foulum in 2019 (and hence exposed to experimental spraying). Management of bee colonies in the experimental apiaries followed local beekeeping practices in Denmark and Portugal. All input (e.g. adding a new super), output (e.g. removing honey) and other beekeeping management tasks (e.g. detection of clinical signs) were recorded every 2-3 weeks. During both field seasons, the development of colonies was highly variable in Denmark. In particular, in 2019, some colonies were constantly small, possibly due to disease (see section 3.4.8). Winter loss was assessed in spring (February in Portugal and April in Denmark) in 2019 and 2020.

2.5.2. Varroa treatment

At all study sites, colonies were treated with ApiGuard (active ingredient thymol) in August/September 2019. In one treatment, 50 g of gel containing 12.5 g of thymol was evaporated for two weeks and the treatment was repeated again after two weeks. However, at one of the Portuguese sites, three colonies experienced a sudden large weight loss (around 3 to 4 kg in a few days) following treatment with ApiGuard. It has been reported that thymol can be released too rapidly at high temperatures and result in toxicity effects on the brood and adults, strong agitation of the colony, and in some cases, bees may abandon their hive (absconding) (Giaomelli et al., 2016; Gunes et al., 2017). In Denmark, a member of the consortium experienced a loss of 9-11 colonies due to varroa in 2019, in spite of treatment with ApiGuard. Furthermore, in Portuguese colonies, varroa levels were higher after the treatment than before, reaching critical levels. As an additional treatment of varroa, oxalic acid treatment was applied in all experimental apiaries (Denmark and Portugal) in December 2019. Dead colonies were replaced by new colonies in 2020. Thymol treatment was repeated in August/September 2020 in the apiaries in Denmark. In Portugal, colonies were treated with Apivar in 2020. In Lousa, treatments were conducted

from 23 March to 22 May and from 12 August to 21 October 2020. In Idanha, colonies were treated from 5 March to 15 May and from 21 July to 18 September 2020. Colonies with high levels of varroa following an Apivar treatment were treated with 15 ml of Varromed on 29 October 2020.

2.5.3. Experimental set-up

Experimental apiaries were installed at a total of six study sites, four in Denmark (Foulum and Hinnerup in Western Denmark, and Flakkebjerg and Krænkerup in Eastern Denmark), and two in Portugal (Lousa and Idanha). Experimental spraying was carried out in Foulum in 2019 (see section 2.8), while no experimental spraying was done at the remaining sites. In each experimental apiary, 10 colonies were installed in autumn 2018. Within each region (Western and Eastern Denmark, and Portugal), all colonies were assessed visually (a rough estimate of brood and adult population) in early spring 2019, ranked in sequence of size, and distributed evenly among study sites. At each site, colonies were designated as:

- Experimental colonies: 5 colonies were chosen from the middle range of colony strengths. Experimental colonies were subjected to intensive monitoring of adult population, brood and reserves approximately every 19-20 days throughout the field season;
- Control colonies: 2 colonies preferentially comparable in size to the experimental colonies. Control colonies were managed by standard beekeeping during the season, and only subjected to intensive adult population, brood and reserves monitoring in the beginning and the end of each season (hence less disturbed);
- Observation colony: a small colony was placed in an observation hive.

The remaining colonies were designated as back-up colonies. If experimental colonies were lost during the experiment, and no back-up colonies were available as replacements, control colonies were used as experimental colonies, in order to obtain data from five colonies per apiary during the experimental period.

Hives were placed with some distance in between (3-10 meters), to have working space, to avoid honey robbing during summer and to minimize the risk of drift of workers and spread of infectious agents between colonies. To further reduce the risk of robbing, experimental hives, which were opened more frequently and during a prolonged period, were separated by back-up hives, every second hive being experimental. When possible, measures to reduce drift were used: hive entrances were oriented in different directions, the landing boards of the experimental hives were painted in different colours, or different coloured shapes were placed near the entrance to help bees finding their own hive.

All hives were marked with permanent numbers. Each super was numbered (starting with bottom super = 1), and each frame was marked with a number on the top using a thick marker, and on both sides (A and B). Hence, each comb was named by a unique code, and could be identified by study site, colony number, super number, frame number and comb side.

2.5.4. Hive weight

Each experimental hive was placed on an ApisTech hive scale, which automatically logged the weight of the hive, in addition to in-hive climate recorded on an hourly basis (see section 2.4.2). Hive scales were installed, and weight monitoring initiated at the onset of the 2019 field season (i.e. in early April in Denmark), and continued until the end of the field season (i.e. until the end of October in Denmark). Hive scales were removed from the apiaries during the winter in Denmark (from late October 2019 until March 2020), due to sensitivity of the hive scales to adverse weather and frost (to prevent battery damage and given that solar panels do not work below 0ºC, batteries could not recharge during winter). Hive scales were re-installed in late March 2020, and monitoring continued until the end of the experiment (i.e. until the end of October 2020). In Portugal, hive scales were installed at the onset of the field season 2019 in mid-March and monitoring continued during the winter period 2019/2020 until the end of the experiment (i.e. until late October 2020).

2.5.5. Assessment of adult population, brood and provision in experimental colonies

Assessments of adult bees, brood and food provision were done regularly during the field seasons 2019 and 2020, starting in spring (early mid-March in Portugal, late April in Denmark), and ending in late summer to early autumn (September in both Portugal and Denmark). In-hive monitoring was adapted to weather conditions, as monitoring was not possible when raining or under strong wind. Furthermore, precautions were taken when temperatures were low i.e. below 14 °C).

In Portugal, monitoring was done only during days with no rain and no strong wind, and when temperatures were above 14 °C. In Denmark, it was not possible to meet these weather requirements in early and late season. However, an initial colony assessment was made in early April 2019. This monitoring event did not involve removing bees from the combs, in order not to damage the brood. However, each colony was assessed according to the following criteria:

- Presence of a queen;
- A rough assessment of food provision (Apifonda sugar doug was added in colonies with sparse food provision);
- Number of frames with bees;
- Number of frames with brood.

These criteria were used to compare the colonies within each of the two overwintering sites (Hinnerup in Western Denmark and Krænkerup in Eastern Denmark), assigning colonies randomly to two groups, i.e. colonies in the two apiaries in Western Denmark were comparable, as were the two apiaries in Eastern Denmark. Furthermore, the initial colony assessment was used to designate colonies to experimental, control, observation and back-up colonies at each study site.

Following the initial assessment and installation of experimental apiaries at the four study sites in Denmark, detailed population assessments were carried out for all experimental colonies approximately every two-three weeks throughout the field season (Table 2). Mean number of days between two consecutive assessments were 18-19 days in 2019 and 2020. Care was taken not to exceed 21 days (the developmental time of a worker bee) between two consecutive monitoring events, especially during early and mid-field season. Although population assessments were carried out mostly on the same day for all colonies in each apiary, in Denmark monitoring events took place during several days on some occasions. This was due to adverse weather conditions, or to honey robbing behavior. Assuming that each bee roughly weighted 100 mg. For illustration, we report the total number of bees (bee strength) per colony per observation date, although in the data set, the numbers of adult bees per frame are reported.

The population assessment of adult bees was done by weighting all frames, supers and hive bottoms with and without bees. The number of bees per frame was estimated from the total weight of bees,

Brood and provisions were assessed by photographing combs and using a newly developed method, automatic detection and classification of honey bee comb cells using deep learning (Alves et al., 2020). During adult population assessment, every comb of the colony was inspected. Except for pure honey combs and empty combs, both sides of all combs were photographed with a digital camera inside a wooden tunnel with a built-in LED lighting, as in Alves et al. (2020). During the preparatory phase of the project, the set-up, which was originally developed under Portuguese conditions and for Langstroth frame measures, was adapted for Danish conditions and the Norwegian frame measures used in the experimental hives. This required adjustment of the tunnel length and a precise 3D print of the plastic holders for keeping the frames in an 11-degree angle inside the tunnel. Furthermore, lighting conditions were adjusted.

Table 2: Dates of population monitoring at the six experimental apiaries in 2019 and 2020.

	Denmark				**Portugal**	
Region	**Eastern Denmark**		**Western Denmark**		**Portugal**	
Site	**Krænkerup**	**Flakkebjerg**	**Hinnerup**	**Foulum**	**Lousa**	**Idanha**
2019	3 April[1]		4-5 April[1]		10 March	9 March
	26 April	25 April	23-24 April	25 April	28 March	27 March
	15 May	13 May	14 May	15 May	16 April	14 April
	27 May	29 May	29 May	3-4 June	5 May	2 May
	11+14 June	12+14 June	18-19 June	20+24 June	22 May	20 May
	26 June	28 June	8-9 July	10-11 July	9 June	7 June
	15 July	17 July	29 July	30 July	29 June	27 June
	29+31 July +1 August	30-31 July	14+16 August	19-20 August	18 July	16 July
	30 August	29 August	6 Sept.	4-6+9 Sept.	6 August	4 August
	25 Sept.	26 Sept.	18 Sept.	23 Sept.	25 August	25 August
					13 Sept.	11 Sept.
Average days between visits[2]	19.00	19.25	18.38	18.88	18.78	18.67
SD	6.19	5.95	3.42	1.55	0.89	1.13
2020	17 April[3]	16 April[3]	23-24 April	25 April		19 March
	2 May[3]	3 May[3]	14 May	15 May	11 April	08 April
	20 May[3]	20 May[3]	29 May	3-4 June	02 May	26 April
	20 July[3]	20 July[3]	18-19 June	24 June	22 May	15 May
			8-9 July	10-11 July	09 June	02 June
			29 July	30 July	29 June	20 June
			14+16 August	19-20 August	17 July	10 July
			9 Sept.	5-6+9 Sept.	5 August	29 July
					24 August	18 August
					11 Sept.	07 Sept.
Average days between visits[2]			19.12	18.57	19.25	19.11
SD			2.03	1.61	1.04	0.93

[1] initial colony assessment at overwintering sites, not included in the average ± number of days between visits

[2] if colony assessments spanned several days, the median observation day was used to calculate average and standard deviation (SD) of days between two consecutive colony assessments

[3] Visual assessments only

During photographing, special care was taken not to damage the queen, hence the queen was caged before image capture. Bee colonies, especially brood and the egg laying queen, are sensitive to cooling

and over-heating. In particular, cold weather was a challenge in Denmark in spring, due to low temperatures and windy conditions. Furthermore, in spring colonies were small, and hence colonies risked losing heat, as they contained only few worker bees to generate heat. Due to the risk of affecting the colonies negatively by the monitoring, weather conditions were assessed carefully before every monitoring event, and in cases of an adverse change in weather, monitoring was ended and resumed when possible (later the same day or the following days). Population monitoring was not possible in early spring and late autumn because of low temperatures and/or strong wind. Preferably, the method of photographing combs was used only when air temperatures exceeded 14 °C, although assessments in Denmark were carried out on non-windy and sunny days with temperatures down to 11-12 °C. Under cold conditions, care was taken to first weigh food combs without brood. As the last task before closing the hive, bees were removed from combs containing brood and photographed, minimizing the time that brood combs were not heated by workers.

Photographed combs were placed in the original nest box and covered by a blanket, to minimize heat loss. When cleaning combs of bees, prior to photographing, bees were gently brushed directly into this nest box, and combs were quickly photographed before being returned to the super. This reduced the time that brood was exposed, and furthermore limited the number of flying bees around the hives during monitoring. In hot periods (mainly in Portugal), care was taken not to overheat the brood and queen. The caged queen was kept in the shade to avoid over-heating. Opening the hives for a prolonged period, particularly during late summer, could result in honey robbing. In order not to leave the experimental colonies vulnerable to honey robbing during the monitoring, the workers were brushed gently into the original nest box and covered by a blanket. Hive tools and gloves were disinfected or changed before moving to the next experimental colony, in order to reduce transmission of diseases from one colony to the other within the apiary. With some exceptions, two consecutive monitoring events were separated by no more than 19-20 days, in order for the sampling frequency to cover the developmental time of workers (21 days from egg to adult). During the mid and late summer periods, in-hive monitoring was started early in the morning (6 am), in order to reduce risk of robbing.

Images were analysed using the software DeepBee® (registration N.º 2765/2018 - IGAC. 2018, Alves et al., 2020). The software detected and classified the comb cells as follows: eggs, larvae and capped brood, pollen, nectar, sealed honey and other cells (including empty cells) from images. Deepbee® was trained in order to optimize cell detection to local conditions. Separate training was performed using images from Portugal, Western and Eastern Denmark.

2.5.6. Observation hives

One observation hive per site was placed at each of the low exposure sites in Denmark, Hinnerup (Western Denmark) and Krænkerup (Eastern Denmark), in addition to the two sites, Lousa and Idanha, in Portugal. Furthermore, as part of a students' project, an observation hive was installed in Foulum (Western Denmark), and waggle dances were recorded during the spraying experiment (Jeppesen & Frederiksen, 2020). Originally, the aim of recording and decoding waggle dances was to assess the landscape "fitness" for honey bees, i.e. the suitability of the landscape (EFSA, 2017). In low fitness landscapes, foragers were expected to have longer foraging distances, due to lack of sufficient floral resources in the near vicinity of the hive, compared to high fitness landscapes, where abundant resources are found (EFSA, 2017).

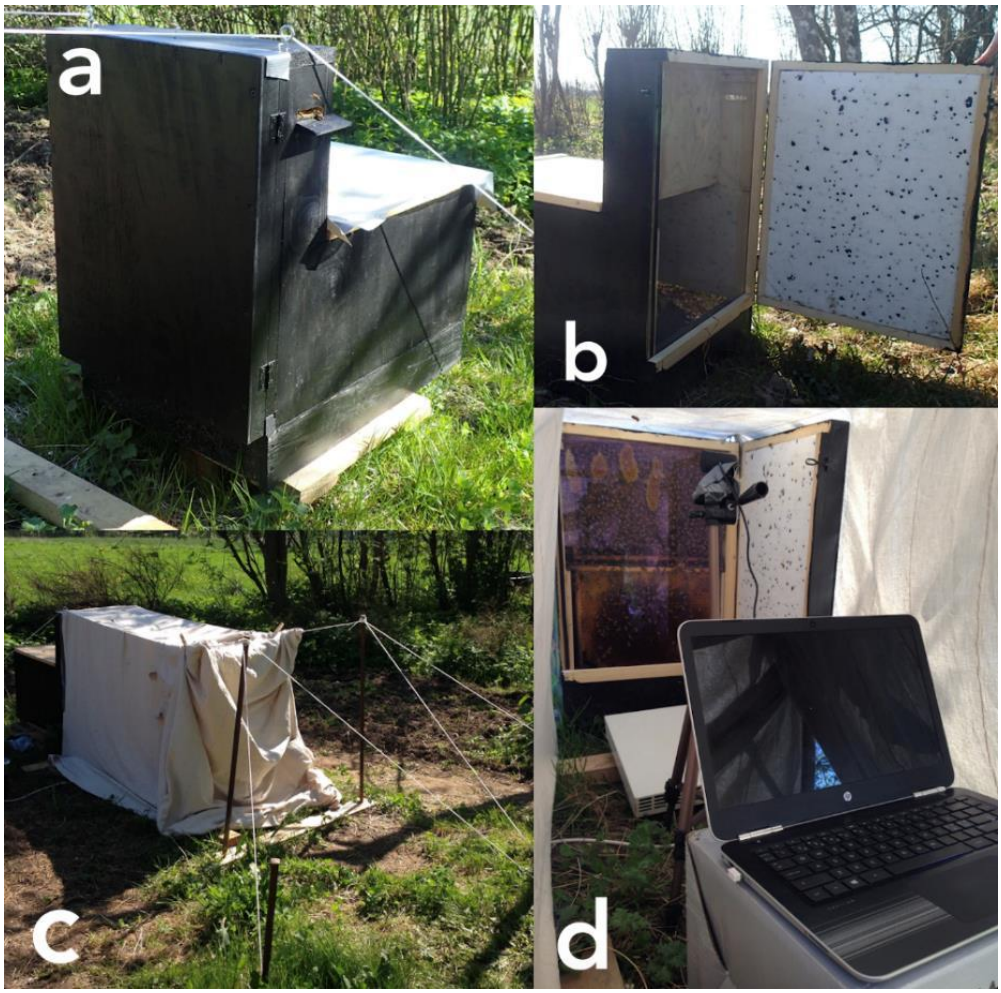
External pollen traps were installed at the hive entrance of the observation hives (in Denmark) and the back-up hives (in Portugal). Pollen traps were activated during one day, once per month (Denmark) or once every 17-20 days (Portugal), preferably at the same time as (or within a few days) of video recording of waggle dances. Because of lack of resources and time, observation hives were only used in 2019.

Observation hives were observed during the field season (April-September in Denmark and March-September in Portugal), preferentially within one week of the floral mapping. Waggle dances were

observed 3 out of 8 observation days in Hinnerup (Denmark), 4 out of 7 observation days in Foulum (Denmark), 0 out of 10 days in Lousa (Portugal), and 8 out of 10 days in Idanha (Portugal). In Krænkerup (Denmark), the observation hive was lost due to robbing.

Waggle dances were recorded and decoded using a set-up and semi-automatic method developed in 2018. A special observation hive was built. Whereas conventional observation hives only contain a few combs, the observation hive in the current study is extended to consist of two compartments: an observation compartment (where the returning foragers were visible through a glass door) and a hive compartment (Figure 3a, Figure 3b). Although the two compartments were connected, the hive compartment was similar to a standard hive, and additional supers could be added as the colony expanded. Hence, the observation hive housed a normal-sized colony, without spatial restrictions.

We used a semi-automatic method for decoding honey bee waggle dances. During observations, a tent was set up in front of the observation compartment, to exclude sun light (which would interfere with the decoding) and for insulation (Figure 3c). Inside the tent, a heater was placed to avoid condensation on the glass door of the observation hive, and in order for the colony not to lose too much heat, disturbing the bees. Videos were recorded using a web-cam connected to a computer (Figure 3d). Videos were recorded in intervals of 30-60 minutes when bees were actively foraging, i.e. from 9h30 to 17h00 on days with good weather conditions (no rain and no strong wind).



(a) The observation hive seen from the outside, the observation compartment is the tall box at the front. This is connected to the box behind, which has the same dimensions as a super, and additional supers can be placed on top. (b) A look into the observation hive from the observation compartment. (c) Experimental set-up during video-recording, seen from the outside. (d)

Experimental set-up showing the set-up with the web-cam and a computer inside the tent. (Photos by Julie Ø. Frederiksen, Annika S. Jeppesen and Yoko L. Dupont).

Figure 3: The observation hive and the experimental set-up

Recorded videos were decoded using the Excel macro LetsDance version 7 developed in this project and used by Jeppesen (2018) and Jeppesen et al., (2018). Flight distances were decoded using the universal calibration suggested by Schürch et al. (2019). Flight direction was decoded by interpreting the angle of waggling relative to the vertical axis on the comb, as equivalent to the angle between flight direction and the direction of the sun. The decoded angle was calibrated by the observation time (time of the day), in order to correct for diurnal change in solar position. Only bees, which were relatively constant in dancing direction and distance were used, and dances disrupted by other nest mates bumping into the dancer were discarded. Each dance was decoded as a mean angle and mean duration of four consecutive waggle runs, but not including the first nor the last waggle run, because these are known to be significantly more variable than the middle runs (Couvillon et al., 2012, Carr-Makrell et al., 2020). Decoding dances resulted in estimates of approximate, but not exact, locations.

2.5.7. Pollen

Samples of pollen from the apiaries were collected during the field seasons 2019 and 2020, in order compare with the floral mapping data and information obtained from decoding of waggle dances.

Pollen was collected from an external pollen trap, which was installed on the outside of the entrance of the observation hive in Denmark, and in control hives in Portugal. External pollen traps were activated for 3-10 hours when collecting pollen (Figure 4). When the device was active, the bees were forced to enter the hive through small openings. When squeezing through the small holes, some of the bees lost the collected pollen pellets, which were collected in a tray.

The observation compartment is the tall box to the right. The external pollen trap (yellow device) was fitted to the entrance hole. When the pollen trap was active (as shown on the image), bees squeeze through the grid, and some lost their pollen pellets, which were collected in the brown tray underneath the yellow grid (Photo by Yoko L. Dupont).

Figure 4: Observation hive at Hinnerup (Western Denmark)

In 2019, pollen was collected from the external pollen traps (one pollen sample per apiary) approximately once per month (Denmark) or once every three weeks (Portugal) during the field season, at the same time as population monitoring of the experimental colonies. In 2020, pollen was collected using external pollen traps from control hives (one pollen sample per apiary) once every three weeks during the field season in the two Portuguese apiaries. In the four Danish apiaries, newly collected bee bread was collected directly from the combs of the experimental colonies (bee bread from all five experimental colonies were pooled in one sample), approximately once every three weeks, at the same time as population monitoring. Newly collected bee bread was identified as fresh pollen deposited by the bees close to the brood area. Pollen collected in a pollen trap reflects the pollen collection by bees during the relatively short period that the pollen trap was active (few hours to one day). Care was taken to collect bee bread newly collected by the bees. However, bee bread may reflect pollen collection during a longer collection period. Hence, the two methods are slightly different.

Pollen samples were kept in the freezer (-18 °C) until drying. Samples were dried shipped to the palynological laboratory, and analysed based on the German DIN-Norm-10760 (DIN-Norm-10760 2002) (as detailed in section 2.8.3). The pollen samples were completely homogenized in water. All pollen types were identified in a sub-sample of about 2.5 µg, mostly identified to plant family, genus or species. Botanical composition is reported as % of 500 pollen grains counted in the sample. Pollen types represented by <3% of the sample are considered as minor, and are not reported.

2.5.8. Monitoring of foraging activity (bee counter)

During the field seasons (early spring to autumn) 2019 and 2020, continuous monitoring of foraging activity of bees was carried out by video-recording activity at the hive entrance, and later analyzing videos using image analysis. In 2019, raw data of forager activity was recorded during the field season from two colonies in Foulum (Western Denmark), Idanha (Portugal) and Lousa (Portugal), and one in Hinnerup (Western Denmark), although one colony in Foulum was very small. In 2020, raw data of forager activity was recorded during the field season from one colony in Foulum, Idanha and Lousa, and two colonies in Hinnerup, respectively. Unfortunately, technical difficulties were encountered in Portugal in 2020 (defect LED lights in the bee counter set-up, rain and unexpected battery discharge). Furthermore, one of the colonies swarmed. From Portugal, recorded video data only from 2019 and for Idanha was calibrated and uploaded to the data base.

During 2019 and 2020, 4075 hours of video were recorded (for all study sites), and most of these were analysed using an image analysis program designed to trace the bee movements using frame subtraction. The analysis generated output files for the movements at one-minute intervals, which was used for manual calibration, in order to calculate the movement of bees. Following calibration, data on foraging activity based on 10-minute interval observations were uploaded to the database. Some of the videos were recorded at times of no or low foraging activity (evening/night/early morning and during inactive periods). In particular, in late season, crowding of bees at the entrance limited the capability of detecting movement by image analysis. However, 49.000 data records reflecting 10 minutes values of foraging activities were uploaded to the database.

For long time series such as these, there is a tradeoff between the precision of calibration and calibration effort. If the precision of each counting event is maximized, the effort used in calibration increases proportionately. Calibration macros were developed in order to optimize the efficiency of the manual counting effort.

Photo: Peter Borgen Sørensen.

Figure 5: Video image used for analysis

Figure 5 shows a typical video image in which the entrance to the hive is at the bottom. In a manual calibration, ingoing and outgoing bees were counted during the first one minute of the video selected

for calibration. Incoming bees were counted as number of bees crossing the bottom line and outgoing bees as the number of bees crossing the top line.

The automatic counting was pixel-based and used a principle of subtracting single frames in the videos (30 frames per second). All images that were the same between the two frames would disappear after frame subtraction. Movements that took place during 1/30 of a second were detected when a pixel changed from light to dark. When a bee was moving, dark pixels were generated in the direction of movements. The image analysis assumed a linear relationship between the generation of dark pixels by frame subtraction and the number of passing bees.

Calibration was automated to minimize the calibration effort, although a close manual inspection of each day was still needed. A final quality assurance in form of a filter and graphical interface to control of each day for outliners and quality check system was applied before uploading the forager activity data to the database. Especially in the late period of the season, the bees tended to crowd on the scene, making image analysis impossible.

2.6. Identification and prevalence of infectious agents

2.6.1. Infestation by *Varroa destructor*

Bees were sampled from all colonies (experimental, control, observation and back-up colonies) at least three times during the field season 2019 and 2020 for varroa quantification (early spring, August and October).

In spring 2019 and 2020 (March in Portugal, April in Denmark, during the flowering of *Salix* spp.), the number of varroa mites was assessed by counting the number of varroa mites falling on the bottom of the hive. Dead bees on the boards were checked carefully because varroa mites tend to stick to the dead bees. This method is less invasive when the colonies are small in early spring. In Denmark, a white plastic tray was placed at the bottom of the hive (below the frames containing brood). The mite downfall was checked once per week during three consecutive weeks. In Portugal, white paper sheets were glued to the bottom board with vaseline or cooking oil (spread with a brush), and the number of varroa mites on the boards was counted once, after 48 hours on every visit during the season. For logistical reasons, mite downfall could not be checked every week for three weeks, and leaving the tray under the hive for three weeks would result in too much debris accumulating on the tray, covering the mites. In the two Portuguese apiaries and the two apiaries in Western Denmark, mite downfall was assessed during every population monitoring event in 2020. Varroa counts using the mite downfall method at different time intervals cannot be compared between different sites. However, the main purpose of these measurements was to have an early detection system to ensure that the colonies were well treated, low counts of varroa indicating that the colonies were healthy.

In August (shortly before varroa mite treatment) and in October or November 2019 (two months after varroa mite treatment), colonies were assessed for varroa using the soapy water method: 300 internal bees (as recommended by Lee et al., 2010) were taken from the first comb adjacent to the brood nest and placed in a labelled plastic container. Very small colonies were not sampled, and in a few colonies <300 bees were sampled due to small colony sizes. Samples were stored at minimum -18°C and shipped on ice to the laboratory in Denmark for further analysis. The number of varroa mites were assessed by the soapy water method, in which varroa mites are counted after washing the bee sample in soapy water. In October/November 2019, the soapy method was used for varroa assessment in the four apiaries in Denmark. In the two Portuguese apiaries, some of the colonies had been weakened by absconding behaviour following treatment with thymol (see section 2.5.2). Hence, in these two apiaries, varroa was assessed in November as mite downfall in the bottom of the hive, not to negatively affect the small colonies by sampling. In July/August 2020 (before varroa treatment), varroa was assessed using the mite downfall methods in the apiaries in Portugal and Western Denmark, and using the soapy

water method in the apiaries in Eastern Denmark. The soapy water method was used to assess varroa in October/November 2020 (after varroa treatment) in all apiaries.

Bees used for varroa assessment using the soapy water method were re-used for the analysis of prevalence of *Nosema* spp.

2.6.2. Viruses and *Nosema* spp.

The planned sampling scheme for viruses and *Nosema* infestation was twice a year for *Nosema* spp. (early spring and late autumn) and twice a year for viruses (August and October). However, due to poor health status, all colonies were tested for prevalence of viruses and *Nosema* spp. in April 2019. A sample of 60 adult bees (collected in a 20 mL vial) was collected from all colonies (experimental, control, observation and back-up colonies) for this analysis. To minimize disturbance of the colonies, bees were collected from frames not adjacent to the brood. *Nosema* infection was not quantified in five colonies in Western Denmark, which were dead in early spring. One colony in Eastern Denmark, which was queenless but alive in early spring 2019, was included in the analysis.

Before (July/August) and after (October/November) varroa treatment, 60 bees (a sub-sample of bees collected for varroa quantification) was weighed, freeze dried, macerated and stored, and later analysed for *Nosema* and viruses, as described in the Epilobee project (https://www.anses.fr/en/content/european-epilobee-programme). The samples were controlled and quantified for Acute Bee Paralysis Virus, Deformed Wing Virus type A and type B, and Sacbrood virus. Sixty bees were freeze dried, grinded down. Each sample was analysed with real-time PCR of RNA extracted from the samples, in order to calculate the mean viral load per bee. Infection by *Nosema* was quantified using double determination, i.e. spores counted in two different droplets from the same suspension. The identity of the *Nosema* species was determined by conventional PCR. The virus loads of the samples were quantified using real-time-qPCR of RNA extracted from the samples.

2.7. Background pesticide load at all study sites

For quantifying background pesticide exposure, samples were collected at all six study sites for multi-residue pesticide analysis. Separate bee bread and honey samples were collected from all experimental colonies. A minimum of 1 g bee bread (0.5 g is the limit for lab analysis) and 2 g of newly collected honey were collected. Whenever possible, each sample consisted of several different sampling points. Care was taken to sample newly collected pollen and honey. In particular, for honey samples, old sealed cells with honey were avoided, in order not to collect sugar fed to the bees during early spring. Samples were placed in glass vials (not plastic), and immediately placed in a cool box, and kept in the lab at -18 °C.

Samples of bee bread and honey were collected for multi-residue analysis (early season samples) in spring 2019 (12-14 March in Portugal, 23-26 April in Denmark) from all experimental colonies and all sites. Samples for multi-residue analysis were collected from all experimental colonies after the main spraying season in Foulum and Hinnerup (Western Denmark) (late June/early July 2019) and Lousa and Idanha (6-8 June 2019) (Portugal). No end of spraying season samples for multi-residue analysis were collected in Eastern Denmark in 2019 (Table 3). Finally, duplicate samples of wax from each wax source used in the experimental apiaries (one source in Denmark and one source in Portugal) were collected in spring 2019.

Table 3: Samples collected for multi-residue analysis during the field season 2019

Region	Study site	Spring		End of spraying season		
		Bee bread	Honey	Bee bread	Honey	Total

Western Denmark	Hinnerup	5	5	5	5	20
	Foulum	5	5	5	5	20
Eastern Denmark	Krænkerup	5	5			10
	Flakkebjerg	61	61			12
Portugal	Lousa	5	5	5	5	20
	Idanha	5	5	5	5	20

[1] In Flakkebjerg, one of the experimental colonies died during the field season and was replaced by a back-up colony. Samples from both colonies were included in the multi-residue analysis.

In 2020, samples for multi-residue analysis were collected from all experimental colonies in spring (15 April in Hinnerup and Foulum and 7 May in Krænkerup and Flakkebjerg (Denmark), 2 April in Lousa and 29 May in Idanha (Portugal)) and after the main spraying season in June/early July (22 June in Hinnerup, 18 June in Foulum, 8 June in Krænkerup and Flakkebjerg (Denmark), 3 July in Lousa and 20 June in Idanha (Portugal) (Table 4). In addition to bee bread and honey from combs, two samples of wax were collected in spring 2020 at the two study sites in Portugal and summer 2020 at the two sites in Western Denmark. For multi-residue analysis, records of the database only contain detected substances, while non-detected substances were not reported.

Table 4: Samples collected for multi-residue analysis during the field season 2020

Region	Study site	Spring		End of spraying season		
		Bee bread from combs	Honey from combs	Bee bread from combs	Honey from combs	Total
Eastern Denmark	Krænkerup	6[1]	6[1]	6[1]	6[1]	24
	Flakkebjerg	5	5	5	5	20
Western Denmark	Hinnerup	6[1]	6[1]	6[1]	6[1]	24
	Foulum	5	5	5	5	20
Portugal	Lousa	5	5	5	5	20
	Idanha	5	5	5	5	20

[1] In Hinnerup, one of the experimental colonies died during the field season and was replaced by a back-up colony. Samples from both colonies were included in the multi-residue analysis.

Multi-residue analysis was conducted by mass chromatography GC-MS/MS (LAB 1-01-80) and liquid chromatography UPLC-MS/MS (LAB 1-01-128). Procedures were performed by extraction with modified QuEChERS and detection by GC-MS/MS and UPLC-MS/MS respectively. Reference documents: regulation (EC) no 396/2005 of the European Parliament and of the council of 23 February 2005 on maximum residue levels of pesticides in or on food and feed of plant and animal origin and amending Council Directive 91/414/EEC; and its successive amendments. All pesticide residue analyses were performed by LAB (Laboratorio Analítico Bioclinico, Spain). A list of compounds included in the screening in the multi-residue analyses by MR GC-MS/MS and UPLC-MS/MS is provided in Appendix C.

2.8. Experimental insecticide spraying

2.8.1. Experimental set-up

Experimental spraying was planned at two study sites in Denmark, Foulum (Western Denmark) and Flakkebjerg (Eastern Denmark) during the peak flowering period of oilseed rape in 2019 and 2020.

However, the spraying experiment in Flakkebjerg was cancelled due to exceptionally small honey bee colonies in combination with unfavourable weather conditions, and the spraying experiments in 2020 were cancelled due to the COVID-19 situation. The weather requirements for the spraying experiment were no rain on the day before spraying, no strong wind (<4 m/s or 14.4 km/h) during spraying, and optimal foraging conditions for honey bees (no rain, no strong wind, and temperatures above 12 °C) during the five-day experiment. Flowering of oilseed rape is usually highly affected by spring temperatures, and due to unusually high temperatures in March 2019, an early flowering of oilseed rape was expected, with a flowering peak at the end of April. As weather is highly unpredictable in early spring in Denmark, timing of the spraying experiment was difficult.

The spraying experiment was conducted in Foulum (Western Denmark) on the 29 April 2019 during the flowering of 6 ha of oilseed rape. For the experiment, the product Pirimor G (500 g/kg pirimicarb) was used at a dosage of 0.9 kg Pirimor G per ha (equalling 0.45 kg a.i.), i.e. three times the normal field application rate on oilseed rape. This compound was selected from a choice of three compounds shortlisted by EFSA, and met the requirements for calibrating the model, which included (1) measurable effects are expected on experimental colonies, and (2) the compound to be used should have a higher acute oral toxicity than contact toxicity (see section 3.4.1 of EFSA Report[3] for further details). Of the three compounds suggested by EFSA, only pirimicarb was authorized for use in Denmark. The application rate was decided considering the requirements in the permission granted from Danish EPA for conducting the experimental spraying. Permissions for conducting experimental spraying events were granted for both 2019 and 2020 by the Danish EPA (Journal number MST-664-00140). Technical details of the spraying experiment are reported in the dataset (Table I of the database).

In order to inform neighbors of the experimental facilities and the public in general prior to the experiment, two popular papers were produced. One of the papers (Dupont and Kryger, 2018) was published in "Tidsskrift for Biavl", the journal of the Danish Beekeepers' Association, the largest national beekeepers' society with >90% of beekeepers being members (Vejsnæs, 2011). The purpose of publishing this short paper was firstly to announce the spraying experiment in order to detect apiaries/beekeepers in the landscapes surrounding the experimental facilities, secondly to inform beekeepers about the project. The paper also announced three public information meetings in January-February 2019 in the two experimental areas. The second popular paper described the project in further detail and was focused on the scientific methods and general approach of the project (Dupont et al., 2019). Furthermore, local beekeepers identified within 3 km of the sprayed site were warned on the 27 April 2019 about the exact timing of the spraying experiment at Foulum.

General guidelines on good practice for application of pesticides by boom-sprayers, a standard procedure used by farmers in Denmark, was followed for the spray application (Syngenta, 2019). This encompassed certain weather requirements: no rain on the day before spraying, and no rain and maximum wind speeds <4 m/s (14.4 km/hour) during the spraying event. Hence, spraying was conducted in early morning when wind speed is generally low. Experimental spraying started at 8h45 and ended at 10h30 on 29 April 2019. Spraying of the field was started from the field border closest to the experimental apiary. Flowering of oilseed rape was assessed at approximately 30%, corresponding to the early peak flowering period of the crop.

2.8.2. Sample collection for pesticide residue analysis during spraying experiment

Prior to (25-26 April 2019) and during (29 April – 2 May 2019) the experimental spraying, samples of pollen and nectar from foragers returning to the experimental hives as well as pollen and nectar from flowers from the treated crop were collected for mono-residue analysis for pirimicarb and the metabolite

[3] http://onlinelibrary.wiley.com/doi/10.2903/sp.efsa.2017.EN-1234/epdf

pirimicarb-desmethyl (Table 5). Due to a general scarcity of foragers during the spraying experiment, and a required minimum sample size of 0.5 g, single samples (not duplicates) were collected. The timing of sampling was 3-4 hours, 1 day, 2 days and 3 days after spraying as required by EFSA (2017).

Internal pollen traps were installed in all experimental hives in Foulum during the spraying experiment. The traps consisted of a metal sheet with small holes, placed between the hive bottom and the bottom super, in addition to a tray placed in the bottom of the hive. When bees entered the hive through the hive entrance and moved to the bottom super through the small holes, some of the bees lost the pollen pellets, which were collected in the tray. The internal pollen traps were active throughout the spraying experiment, from 23 April to 2 May 2019. Pollen traps were emptied every morning (at around 9h00).

From each experimental colony, foragers were collected by blocking the hive entrance and collecting returning bees, which had accumulated at the entrance after at least 5-10 minutes. Bees were then anesthetized with CO_2 in the collection vial. Nectar was extracted from honey sacs by squeezing the bees gently, which made them regurgitate the honey from the honey sac, and this was collected using micro capillary tubes (0.5 g honey from at least 50 bees per sample). Foragers usually survived this treatment and were able to fly away after a few minutes. However, it is unknown how it affected their subsequent behavior. A total of 0.5 mg of nectar from honey sacs was collected per experimental colony, equivalent to around 50 nectar-containing bees. However, as many bees did not contain nectar (pollen collectors or empty bees), and the amount of nectar extracted per bee was highly variable (up to four drops per bee), approximately 150-200 bees per colony had to be captured and handled. On day 2 and 3 after the spraying, foraging activity was very low. As almost no foragers were returning to the smallest colony (colony number 4), foragers were not collected from this colony on day 2 and 3, in order not to negatively affect the colony by sampling.

Table 5: Samples collected for mono-residue analysis (pirimicarb) prior to, during and after the spraying experiment in Foulum (Western Denmark)

		Season start	**Spray**	**Post-application sampling**						**Total**
Object	**Matrix**	**Spring**	**0 h**	**3-4 h**	**1 d**	**2 d**	**3 d**	**2 w**	**End season**	
Colonies	Honey	5						10		15
	Bee bread	5						10		15
Foraging bees	Nectar	5		5	5	1	4			20
	Pollen	5		10	6	0	0			21
Flowers	Nectar	2		2	2	2	2			10
	Pollen	2		2	2	2	2			10
Other	Tank mixture		2							2
	Honey (beekeepers)								3	3
	Empty vials, all types		3 of each type							6

A total of five samples of nectar and two samples of pollen were collected from different locations within the experimental oilseed rape field. Oilseed rape flower stalks (approximately 500 flowers per sample) were covered with fine mesh cloth around noon, in order to exclude flower visiting insects. Covered flower stalks were collected at the end of the day (18h00), brought back to the laboratory in a cool box, refrigerated, and centrifuged no later than noon the following day to obtain nectar samples. Furthermore, pollen samples from the sprayed oilseed rape field were collected by shaking 20-30 flower stalks inside a plastic bag. In the laboratory, pollen adhering to the inside of the bag was scraped off

using the edge of a ruler. Due to the highly time-consuming task of collecting pollen from flowers, only two pollen samples could be collected per day.

Finally, the following samples were collected for pesticide residue analysis at the sprayed site during the spraying experiment:

- A duplicate sample from the tank mixture, after the pesticide had been mixed into the water;
- Single samples from honey produced by other beekeepers within 3 km from the sprayed field;
- Duplicate samples of wax from each wax source used in the experimental apiaries (one source in Denmark and one source in Portugal).

Table 5 is a summary of samples collected for mono-residue analysis (pirimicarb) during the spraying experiment. In the database, all results have been reported, including positive and negative results of the mono-residue analysis.

Pirimicarb and the metabolite pirimicarb-desmethyl were identified and quantified by UPLC MS/MS, as described for multi-residue analysis (section 2.7).

2.8.3. Other samples collected during spraying experiment

Nectar extracted from returning worker bees, and nectar collected from flowers of oilseed rape collected in the experimental field (Table 5, 6) were tested for sugar content. Sugar concentration was determined using a handheld Bellingham & Stanley® refractometer for sugar analysis (handheld refractometers for sugar determination operate in the ranges 0-30% and 30-100% sucrose equivalents). Sugar content was measured in nectar extracted from 10 bees per colony, starting in the morning when the first foragers with pollen loads were observed, as this indicated that foraging bees were returning to the hive from the field.

Pollen samples (one sample per experimental colony, i.e. five samples per study site per day) were collected from the experimental colonies 24, 26, 29 and 30 of April and 15 May 2019 (3 and 5 days before spraying, 0 and 1 day, and two weeks after spraying, Table 6). Pollen traps were emptied in the morning (9h00) on the day of the experimental spraying and each of the following three days (Table 5, 6). Pollen was collected in late afternoon each day, and the sample thoroughly mixed. Collected pollen samples were kept in the freezer (-18 °C) before drying. Samples were dried at 35 °C, and weighed after 24 hours, weighted after an additional two hours of drying, finally the samples were weighted a third time after an additional two hours of drying. Samples were considered dry when they did not lose additional weight by drying.

Palynological analysis was conducted based on the German DIN-Norm-10760 (DIN-Norm-10760 2002). All pollen types of a sample were identified following melisso-palynological literature and databases (Beug 2004, https://www.paldat.org, see section 2.5.7). A quantitative assessment of pollen types mostly at the level of plant genus, more rarely at plant species or family level, was carried out by determination of 500 pollen grains per sample. Pollen types represented by <3% of the sample were considered as minor and not reported.

In-hive mortality was measured as the number of dead bees in the black trays placed in front of the hive entrances. Dead bees were counted in late afternoon each day during the spraying experiment.

Table 6: Sampling scheme and numbers of pollen samples for palynological analysis (five experimental colonies and one observation hive) and nectar samples for determination of sugar content during the experimental spraying at Foulum (Western Denmark) in 2019

		Season start	Pesticide application	Post-application sampling			
Analysis	**Matrix**		**0 h**	**3-4 h**	**1 d**	**2 d**	**3 d**
Pollen identification	Foraging bees	6		6	6	6	6
Sugar concentration in nectar	Foraging bees	25		25	25	25	25
	Oilseed rape flowers	10		10	10	10	10

3. Results

3.1. Database

3.1.1. Development of database

The tables were developed and adapted to the project from the specifications outlined in the EFSA technical report, appendices A.1-A.7 (EFSA, 2017). In particular, specifications for Table II (RPU) and Table VI (SSD2 lab analyses) needed substantial revision and development to fit the purpose of the data collection. Table II required input from the ApisRAM team in order to link field-identified polygons to polygons in the ALMaSS modelling landscapes (see section 3.2.2). Table VI (SSD2) was adapted specifically for palynological data, varroa, *Nosema* and viruses.

The database was presented to all field operators from the three study regions (Western and Eastern Denmark and Portugal) during a field training course 6-7 June 2018 organized in Flakkebjerg (Denmark). Following an initial testing phase, the database was improved based on input from field operators and the three laboratories involved in data generation (LAB for chemical analysis, LIB for palynological analysis and AU-Agro for pathogen/parasite analysis). An updated version of the database was tested by consortium members in addition to members of the MUST-B steering group. Furthermore, a test data set and users' manual were completed in November 2018, prior to the first field season of the main experiment.

The user interface of the database was improved regularly throughout the project to correct minor errors, add selection possibilities to drop down menus, improve or implement relevant business rules, and make it as user-friendly as possible.

Data could be retrieved from the database in CSV and XML formats.

3.1.2. Data collation and reporting

Available data from the field seasons 2019 and 2020 were uploaded to the database and delivered to EFSA in several lots in time in 2019, 2020 and 2021. The final data set encompassed:

Table I: Pesticide application, which contained data on the experimental spraying event. The table included all data from the field season 2019. In 2020, no spraying experiments were carried out, due to COVID-19.

Table II: Resource providing unit and landscape fitness (RPU), contained data on abundance of floral resources in polygons within 1.5 km of the experimental hives. Floral mapping data from all landscapes

from 2019 and 2020 were uploaded. In Denmark, many of the field identified polygons were split into several sub-polygons (up to 46 sub-polygons per field polygon) referring to polygons in the ALMaSS system. In the table, floral data were only reported for one sub-polygon per field polygon, as the floral assessment applied to the entire field polygon. Lists of polygons and their centroids and area are found in the polygon table, which is linked to the RPU table.

Table III: Hives, contained the geographical location of the apiary of each experimental hive two sites in Portugal and four sites in Denmark, including the site of a new apiary in 2020, "Flakkebjerg 2020". Site numbers were linked to the sites table, which contained the UTM coordinates of the locations.

Table IV: Colony management, contained the log of the beekeeper regarding input, output or clinical signs observed in the experimental hives. The table contained all data from the field seasons 2019 and 2020.

Table V: Hive inspection, contained data on assessments of the adult bee populations, brood, food provision, hive weight and forager activity. A java program was developed to detect and remove duplicates from this table and label all observations with a unique identifier.

Hive scale data: These data contained hive scale logging from all six experimental apiaries from 2019 and 2020. The delivered data were raw data logged by the hive scales from the Danish apiaries, and cleaned data from the Portuguese apiaries (removing data from days of hive management and monitoring, and other external disturbances, extrapolating missing data, etc.). Files were named by the country, apiary and year. Each file contained the hive scale data from one apiary per year. However, due to installation of the new apiary in Flakkebjerg (called "Flakkebjerg2020" in the data set) in early field season 2020, in addition to the re-organization of the apiaries in Eastern Denmark (moving all strong sister queen colonies to Krænkerup), the following details need mention:

The file DK Flakkebjerg2020 contains data from the original sister queen colonies in the "Flakkebjerg 2019" apiary (called "Flakkebjerg" in the database) from onset of the field season (16 March 2020) until 23 April 2020, and from the new unrelated colonies in the new "Flakkebjerg 2020" apiary from 24 April 2020 until season end 10 October 2020. It was discovered that the hive scale of colony 11 was unstable. Hence, it was replaced by a hive scale of the brand Capaz on the 3 May 2020. Using this hive scale, an hourly monitoring similar to the logging by ApisTech scales was conducted, although not at night (22h-6h).

The file "Krankerup 2020" contains data from the original sister queen colonies in Krænkerup in 2020. Colony 11 is identical to Flakkebjerg hive 2 (which was logged in the Flakkebjerg 2019 apiary until 23 April 2020), and colony 12 is identical to Flakkebjerg colony 6 (which was logged in the Flakkebjerg 2019 apiary until 23 April 2020). Hives 21 and 22 (logged from 17 March to 23 April in Krænkerup) are back-up hives in Krænkerup, which were eventually not used in the experiment.

Number of adult bees: The dataset contained population assessments of adult bees from the field seasons 2019 and 2020 for the two apiaries in Western Denmark (Hinnerup and Foulum) and Portugal. Due to COVID-19, population assessments in Eastern Denmark (Flakkebjerg and Krænkerup) were carried out as visual assessments. As the format of these data do not fit to the database format, these were delivered as a separate file (XLS).

Forager activity data: These data included 42,000 records of bee counts, each record covering bees departing from or entering the hive during a 10 min interval, equivalent to 3500 hours data from a total of seven colonies from study sites in Western Denmark and Portugal. Due to some technical issues, gaps (equivalent to missing data) were found in both seasons and at all study sites. Videos were recorded in the field in 2019 and 2020, and analysed using an image analysis program designed to trace the bee movements using frame subtraction. Data were filtered and checked using a graphical interface to control each observation day for outliners. Data in 10 min intervals should be regarded replicates of the traffic on the mentioned hour, which means the specific minute is not important. This is a

scientifically sounder way of utilizing the data instead of having just one figure for each 10 min interval. Figures in 10 min intervals fluctuate erratically, and may not relate in itself to internal or external factors.

Comb data: Data included results of the Deepbee® analysis of comb images obtained in the field seasons 2019 (all apiaries) and 2020 (Portugal and Western Denmark). The software was trained during several training sessions and separately for each region (Western Denmark, Eastern Denmark and Portugal). Training of the software involved input of manually corrected comb images to improve the cell recognition by deep learning, resulting in a higher performance of the software for images from all three study regions. Images in which <3000 cells were detected were corrected manually.

Table VI: SSD2, contained data on results of laboratory analyses of pollen, pesticide residues and parasites/pathogens.

Pollen: Results of palynological analysis of samples collected in 2019 and 2020 from all study sites.

Varroa, Nosema, virus: disease analysis from all apiaries in 2019 and 2020. Varroa infestation was assessed as counts of varroa mites in spring, summer, and autumn. Analysis for *Nosema* included microscopic spore counts, in addition to determination of *Nosema* species by PCR for samples collected in spring and autumn 2019 and 2020. Infection by viruses were assessed by qPCR for RNA and DNA quantification for samples collected in summer and autumn 2019 and 2020, in addition to spring 2019. Furthermore, records included samples taken when clinical signs were observed.

Pesticide: Results of pesticide residue analysis of samples collected from all apiaries and both study years. All data were quality checked for errors. For multi-residue analyses, only compounds detected were reported. For mono-residue analysis of pirimicarb, both positive (detection) and negative (absence of detection) were reported.

Measurements of sucrose (from spraying experiment in 2019) could not be reported in the SSD2 table, and have been submitted separately (XLS).

Table VII: Colony observation, contained observations of honey bee waggle dances from observation hives in 2019. On several observation dates and study sites, waggle dances were not observed, in spite of optimal weather conditions. Waggle dance data were not collected in 2020.

Furthermore, climate data (CSV) were collected in 2019 and 2020 from weather stations at the four study sites in Denmark and the two study sites in Portugal. The climate data were obtained from weather stations of different brands, but the climate variables measured are standard climate variables, and comparable across sites (although wind speed at 10 meters height will need to be extrapolated to three meters height for Flakkebjerg, Foulum, and Ødum, as three meters height is probably more relevant for bees). All data were raw data, were not corrected, except for the wind direction in Hinnerup (for this parameter, 180 degrees were added during the period 20 February to 23 July 2019, because the wind sensor was placed wrong during this period). As the weather station in Hinnerup (Western Denmark) had several break downs, in particular due to lightning incidences in 2019, climate data from a nearby weather station (Ødum) were included in the data set.

3.1.3. Selection of study sites

3.1.3.1. Study sites in Denmark

All study sites in Denmark were typical agricultural landscapes dominated by crop fields, which are typical for Western and Eastern Danish farmland areas, respectively. Below are detailed descriptions of the four 10x10 km squares within which study sites were selected for the main experiment:

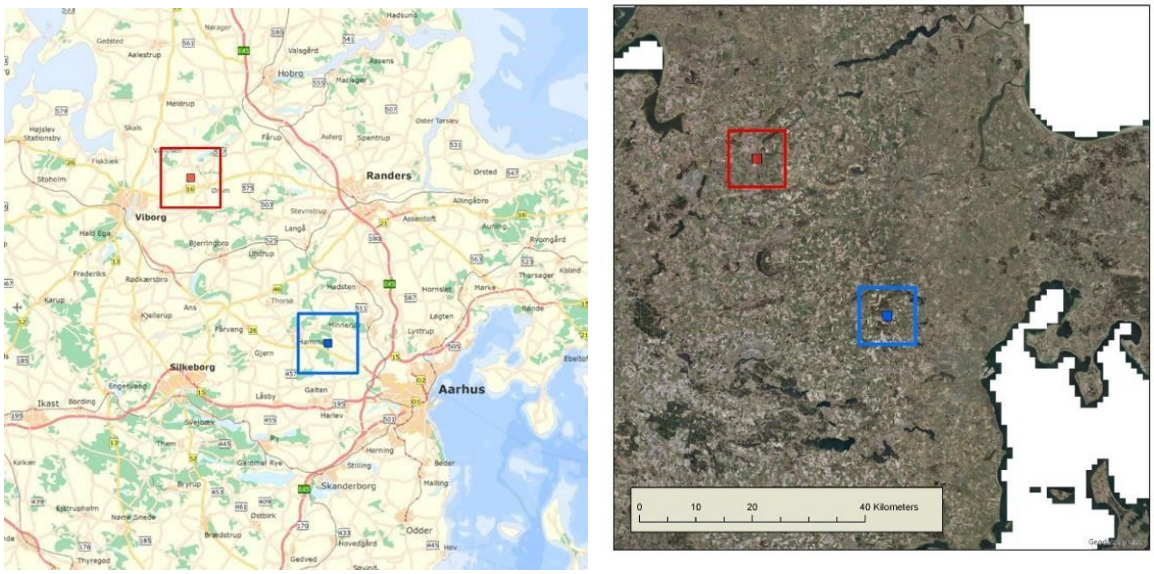
- High exposure area at Foulum (Western Denmark)

The experimental area in Foulum was predominantly sandy clay soil (JB4) and located in an agricultural area dominated by large fields, including clover ley fields for livestock, cereal and corn fields in crop rotation systems (Figure 6, Appendix A). Within a 10x10 km square area centered in the experimental

field, a total of 90 different crop types were found. The total honey potential of the 10x10 km area was 159 663 kg per year.

- Low exposure area at Hinnerup (Western Denmark)

The low exposure area at Hinnerup was located 36.2 km from the high exposure area at Foulum (centre to centre of the 10x10 km squares) (Figure 6). It had a similar landscape structure and land use (Appendix A) being an agricultural area dominated by large cereal fields and clover leys. Within the 10x10 km square area, a total of 74 different types of crops were found. The total honey potential of the 10x10 km area was 142 576 kg per year, and hence very similar to the high exposure site at Foulum.



The red square is centred in the high exposure study site at Foulum. The blue square shows the suggested low exposure area (10x10 km) matching the high exposure landscape. The low exposure study site Hinnerup was located within the blue square.

Figure 6: Study areas (10x10 km) in Western Denmark, topographic map (left) and orthophoto (right)

- High exposure area at Flakkebjerg (Eastern Denmark)

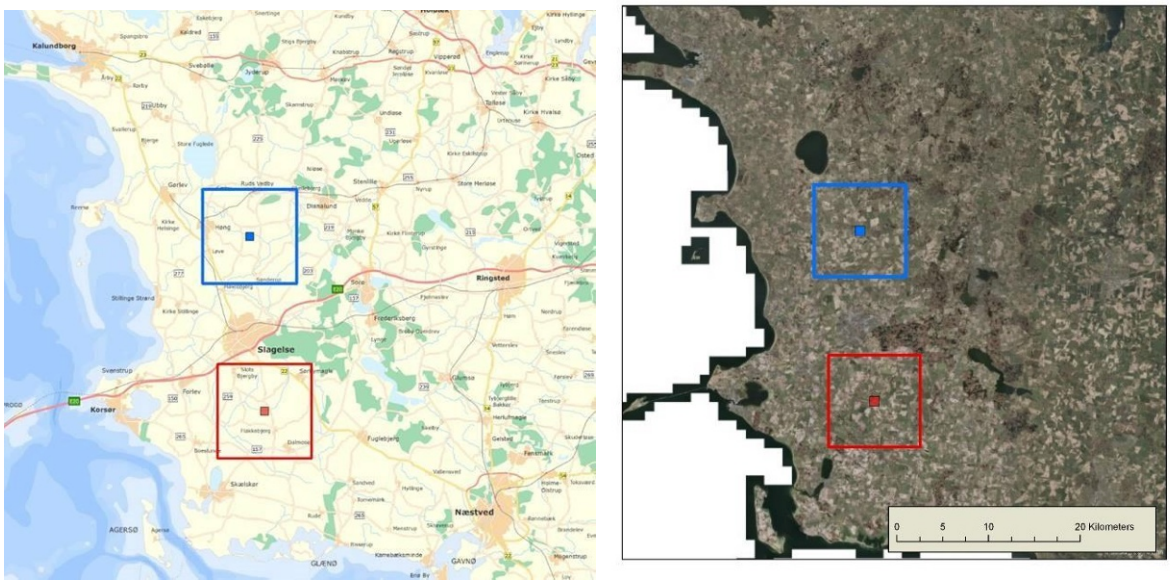
The soil type of the experimental area in Flakkebjerg was predominantly heavy clay soil (JB6-JB7), and the surrounding landscape Flakkebjerg was dominated by smaller crop fields, in particular fields for clover seed, fruit and vegetable production, and less cereal/corn fields, fields for livestock and forest (Figure 7, Appendix B). Within the 10x10 km area, a total of 82 different types of crops were found. The total honey potential of the 10x10 km area was 215 338 kg per year.

- Low exposure area at Krænkerup (Eastern Denmark)

The low exposure area at Krænkerup was located 18.6 km from the high exposure site at Flakkebjerg (centre to centre of the 10x10 km squares) (Figure 7). The landscape structure and land use was similar to the high exposure site (Appendix B). Within the 10x10 km square, a total of 80 different types of crops were found. The total honey potential of the 10x10 km area was 221 239 kg per year, a level comparable to the high exposure site at Flakkebjerg.

The total honey potential of the study areas showed that the areas in Eastern Denmark had a higher potential availability of nectar compared to the areas in Western Denmark, in early summer. However, honey bee colonies did not have the strength to harvest all the available nectar. No information was found on pollen availability or quality in the landscapes. Due to the short flowering season of clover

seed fields compared to clover ley fields, the study areas in Eastern Denmark were generally considered a lower fitness landscape for honeybees than the study areas in Western Denmark.



The red square is centred in the high exposure study site at Flakkebjerg. The blue square shows the suggested low exposure area (10x10 km) matching the high exposure area. The low exposure study site Krænkerup was located within the blue square.

Figure 7: Study areas (10x10 km) in Eastern Denmark, topographic map (left) and orthophoto (right)

At all four study sites in Denmark, a minimum of 6 ha flowering oilseed rape was found within 300 m of the experimental apiaries in both 2019 and 2020. Care was taken to match the cultivars of oilseed rape used, as different cultivars may differ in timing of flowering. However, due to an extended drought during the summer 2018, followed by a short rainy period in mid-August, and a requirement for oilseed rape to be sown before 20 August in Denmark, farmers were forced to take fast decisions. This made a coordinated effort difficult, as seeds had to be ordered and delivered during a very narrow time window. Hence, cultivars were matched between high and low exposure sites within each region, but not at all four sites in Denmark. At the two sites in Western Denmark, the diploid cultivar "Butterfly" was sown, while at the two sites in Eastern Denmark, the hybrid cultivar "DK Exlibris" was sown.

At all four study sites in Denmark, oilseed rape is grown conventionally, i.e. using herbicides, fungicides, slug repellants and insecticides against *Psylliodes chrysocephala*, which is a pest species of oilseed rape, mostly in early seedling stages. Apart from this, no systemic pesticides, including neonicotinoids, were used.

3.1.3.2. Study sites in Portugal

Study sites in Portugal encompassed two areas differing in landscape, in particular relating to crop type, pesticide use and other management practices, landscape structure and other environmental variables. Both sites were low exposure sites, i.e. with no experimental spraying, and areas are characterized by low pesticide input. The study site Idanha a Nova (hereafter Idanha) was located in a predominantly agricultural and pasture landscape in the district of Castelo Branco (inner central Portugal). Availability of floral resources were generally considered low ("low fitness landscape"). The study site Serra da Lousã (hereafter Lousã) in the district of Coimbra, central Portugal was located in a mountain area with predominantly natural area. Availability of floral resources were generally considered high ("high fitness landscape"). In both locations, the local landowners and beekeepers were contacted and the position

of the experimental apiaries was decided and registered at the Portuguese National Authority (DGAV). During the negotiations with beekeepers from both study areas it was agreed that the position of neighboring apiaries complied with the distance established by Portuguese authorities (800 m) in order to avoid competition between apiaries. Competition effects are generally not detected beyond 800-1200 meters from an apiary, possibly due to dilution effects as the density of bees generally declines away from the apiary (Mallinger et al., 2017; Henry et al., 2018). In Lousa, it was also agreed that, for the duration of the study, the apiary densities would be kept low.

3.2. Agricultural practices and land use, cover and structure

3.2.1. Landscape analysis

3.2.1.1. Landscape analysis in Denmark

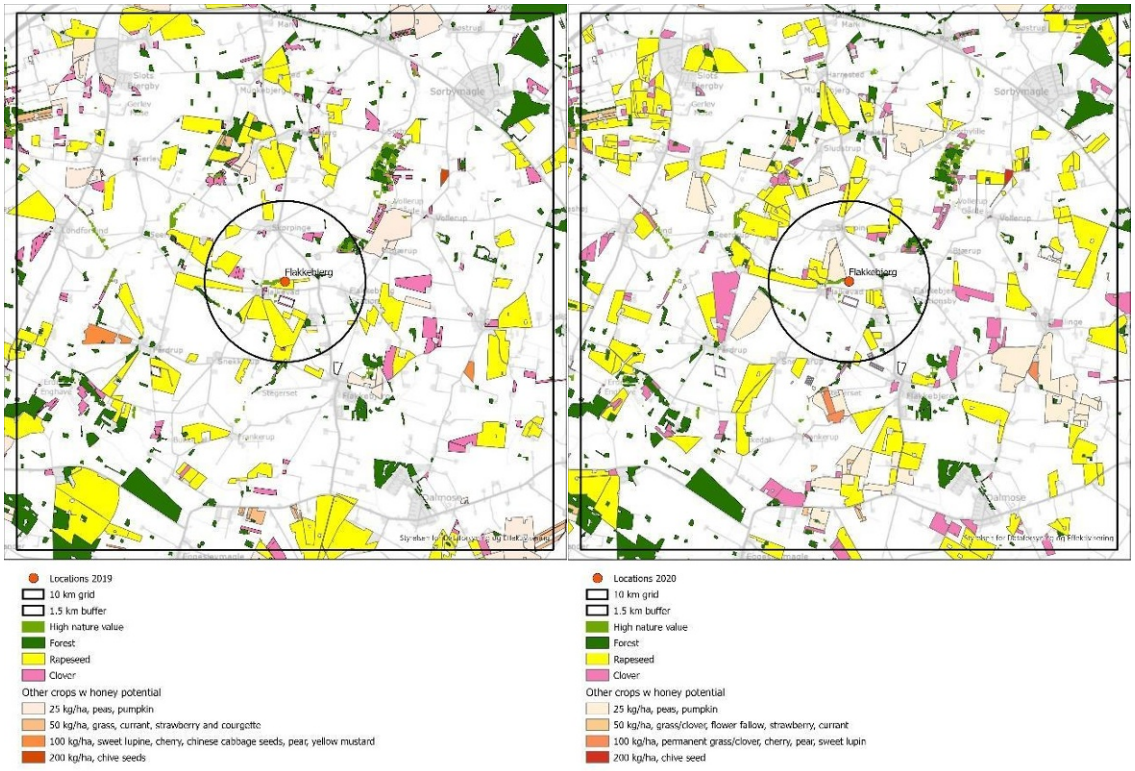
All four study landscapes were dominated by farmland, having low proportions of natural areas. However, the agricultural landscapes in the near surroundings (1.5 km) of the experimental apiaries in Foulum and Flakkebjerg constituted experimental areas having small experimental patches and fields, hedgerows, thickets and lawns, compared to standard farmland. Natural areas were generally limited (0.4-1.7% of area covered), although slightly higher for Foulum (6.3% of the area) in the 10x10 km landscape (Table 7). In general, forest cover was low (1.7-5.6% of the area), except for Hinnerup, which was dominated by forest within the 1.5 km circle (43.2% of the area). However, forest cover was similar in the two 10x10 km squares of Hinnerup (23.7%) and Foulum (17.1%), the high and low exposure sites in Western Denmark (Table 7). Area coverage of nature and forest did not change from 2019 to 2020. Oilseed rape fields had a high and comparable coverage at the two sites in Eastern Denmark, coverage was similar between the 1.5 km circle and the 10x10 km landscape, and among years (2019-2020). In Western Denmark, oilseed rape had a moderate coverage (4.5-4.7%) within the 1.5 km circle at both study sites, although the area covered by oilseed rape was slightly lower in 2020 in Foulum (2.2%). The 10x10 km landscape in Foulum had a similar (2020) or lower (2019) coverage by oilseed rape, while the 10x10 km landscape in Hinnerup had a higher coverage of oilseed rape than found in the 1.5 km circle surrounding the experimental apiaries (Table 7). Coverages of clover fields, including clovers for seed production and permanent grasslands sown with grass-clover mixtures, were similar for the two sites in Eastern Denmark (Krænkerup and Flakkebjerg) and Hinnerup, and at both spatial scales (0.7-2.9% of the area), but higher in Foulum both within the 1.5 km circle and 10x10 km quadrate in both study years (Table 7). Areas of clovers may, however, not be comparable in terms of floral resources, as abundance of flowers is highly variable, floral abundance and temporal availability of flowers varies between seed production fields and grasslands, and the proportion of clovers in grasslands are variable. Although location of crop fields changed from 2019 to 2020, the composition (areas) of land use types was similar in the two study years in Eastern Denmark (Figure 8) and Western Denmark (Figure 9).

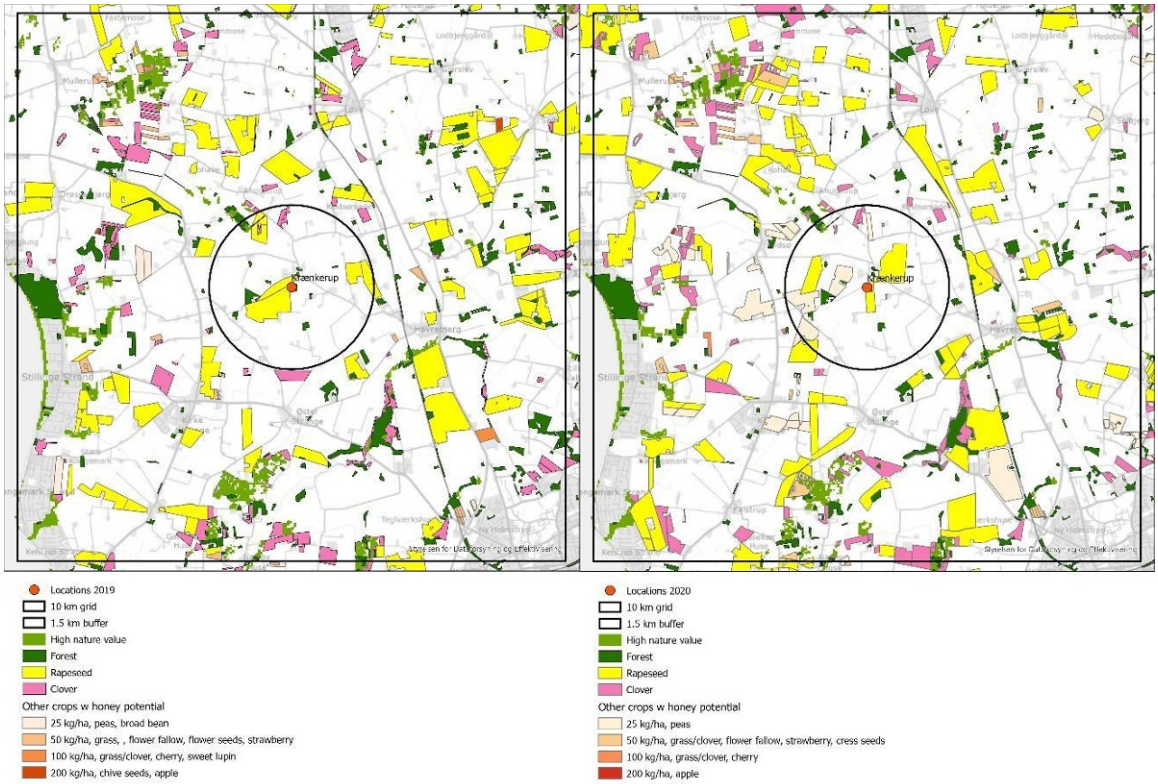
Based on the landscape analysis, it was considered if experimental apiaries should be moved in 2020, in order to secure comparability of pairs of landscapes within Western Denmark and Eastern Denmark, respectively, in terms of floral resource availability and pesticide exposure (EFSA, 2017). Mapping of floral resources in the field detected fewer floral resources within 1.5 km distance of the apiary in Krænkerup (low exposure site, Eastern Denmark), compared to the landscape surrounding the apiary in Flakkebjerg (high exposure site, Eastern Denmark). However, honey bees are known to regularly fly longer distances than 1.5 km during foraging (e.g. Couvillion et al., 2014; Garbuzon et al., 2015), and the 10x10 km landscapes were more similar in terms of coverage of oilseed rape, clovers, natural areas and forest compared to the 1.5 km circular areas (Table 7). Honey harvests of the colonies, which reflect the availability of flowers were nearly equal in the two apiaries. Floral resources were more abundant and continuous at Foulum compared to Hinnerup, mainly due to availability of clovers in grasslands. However, honey harvests were comparable in 2019. Hence, it was decided to keep the study sites from 2019 in 2020. However, due to a high mortality in Eastern Denmark, a new apiary in Flakkebjerg was

used in 2020. This apiary was located few hundred meters from the experimental apiary in 2019, and therefore considered as being in the same landscape.

Table 7: Areas (in ha) of land use types within a circular area of 1.5 km surrounding each experimental apiary and a 10x10 km area centred on each experimental apiary, for study sites in Denmark. Clover include areas for seed production, in addition to grasslands with clover

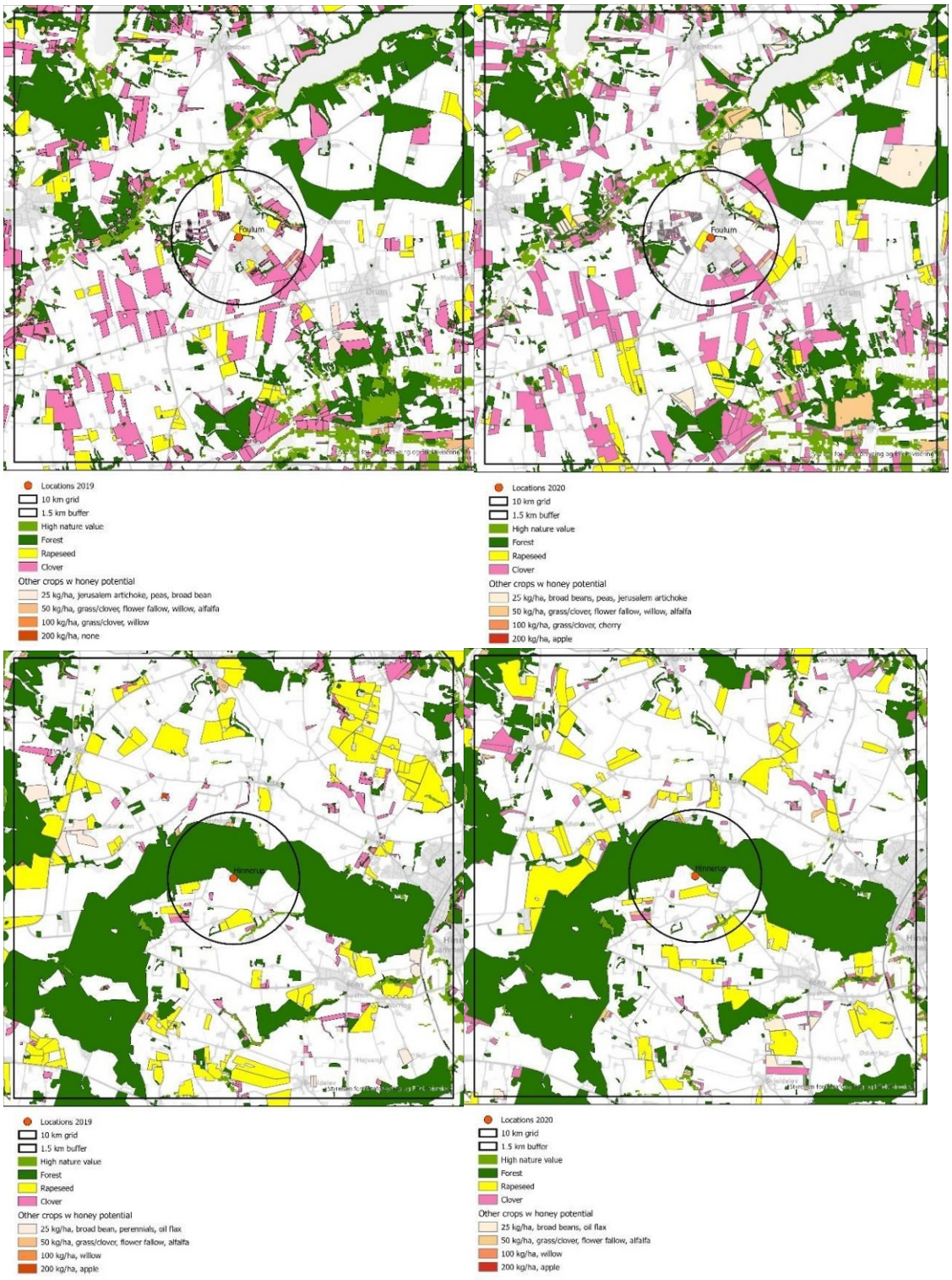
ha (%) in 2019	Eastern Denmark		Western Denmark	
Site	**Flakkebjerg**	**Krænkerup**	**Foulum**	**Hinnerup**
High Nature Value > 5, 1.5 km circle	11.8 (1.7%)	3.0 (0.4%)	12,1 (1.7%)	10,2 (1.4%)
High Nature Value > 5, 10x10 km	85.7 (0.9%)	253.8 (2.5%)	631.0 (6.3%)	193.5 (1.9%)
Forest 1.5 km circle	15.6 (2.2%)	11.8 (1.7%)	39.3 (5.6%)	304.9 (43.2%)
Forest 10x10 km	407.0 (4.1%)	336.1 (3.4%)	1713.4 (17.1%)	2368.3 (23.7%)
Oilseed rape in 2019, 1.5 km circle	99.8 (14.1%)	70.7 (10.0%)	31.7 (4.5%)	32.4 (4.6%)
Oilseed rape in 2019, 10x10 km	947.3 (9.5%)	869.2 (8.7%)	304.4 (3.0%)	913.5 (9.1%)
Clover in 2019, 1.5 km circle	14.3 (2.0%)	5.0 (0.7%)	92.0 (13.0%)	6.5 (0.9%)
Clover in 2019, 10x10 km	212.9 (2.1%)	265.7 (2.7%)	1027.3 (10.3%)	185.2 (1.9%)
Oilseed rape in 2020, 1.5 km circle	57.4 (8.1%)	56.0 (7.9%)	15.4 (2.2%)	33.4 (4.7%)
Oilseed rape in 2020, 10x10 km	1009.6 (10.1%)	904.3 (9.0%)	245.0 (2.5%)	771.6 (7.7%)
Clover in 2020, 1.5 km circle	11.7 (1.7%)	5.2 (0.7%)	90.1 (12.7%)	8.1 (1.1%)
Clover in 2020, 10x10 km	264.4 (2.6%)	286.3 (2.9%)	998.8 (10.0%)	204.0 (2.0%)





Areas providing nectar and/or pollen for bees is shown in colours, areas in white are areas without floral resources, mainly wind pollinated crops. The circular areas (radius 1.5 km from apiary), within which floral resources were mapped, are indicated.

Figure 8: 10x10 km landscapes surrounding the experimental apiaries in Eastern Denmark, Flakkebjerg (upper panels) and Krænkerup (lower panels) in 2019 (left) and 2020 (right)

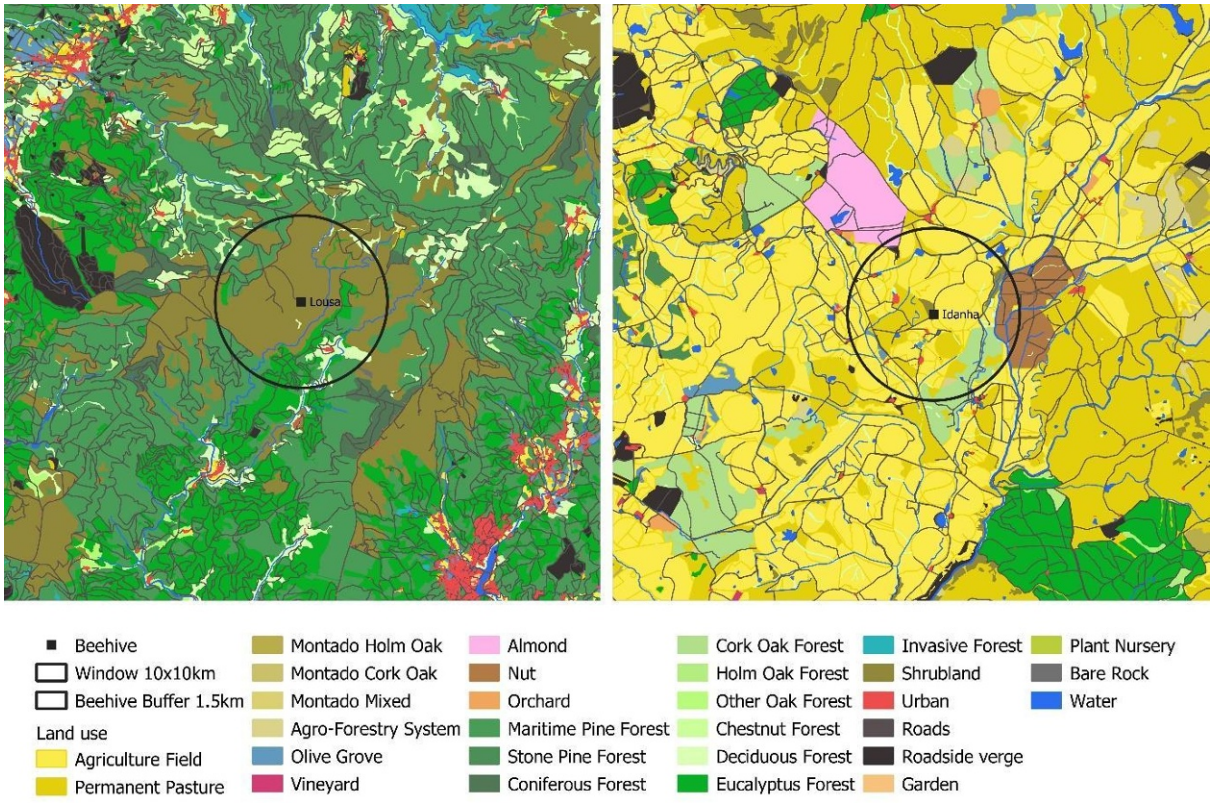


Areas providing nectar and/or pollen for bees is shown in colours. The circular areas (radius 1.5 km from apiary), within which floral resources were mapped, are indicated.

Figure 9: 10x10 km landscapes surrounding the experimental apiaries in Western Denmark, Foulum (upper panels) and Hinnerup (lower panels) in 2019 (left) and 2020 (right)

3.2.1.2. Landscape analysis in Portugal

The two Portuguese study sites differed in landscape context (Figure 10). The landscape surrounding the apiary in Lousa was dominated by a diverse plant community of nectar and pollen rich species, flowering from March/April to October, with a peak in May. The vegetation was dominated by shrub and forested areas. The landscape surrounding the experimental apiary in Idanha was dominated by farmland, in particular permanent and temporary pastures, Montados (Agro-forestry system and Cork-oak forest), and cereal crops for fodder.



The circular areas represent the radius of 1.5 km from apiary.

Figure 10: 10x10 km landscapes surrounding the experimental apiaries in Portugal, Lousa (left panel) and Idanha (right panel)

An analysis of the landscape structure and composition showed that the Lousã 10x10km landscape was dominated by maritime Pine (42.7%) and eucalyptus forest (20.8%) and shrubland (17.1%) with a very low percentage of farmland (1.8%). The 1.5km radius area from the apiary keep the same land use structure although shrubland occupies a larger percentage (62.2%) (Table 8, left panel). In Idanha, the 10x10 km landscape was dominated by farmland composed of temporary crops (46.7%), permanent pasture (27.7%) and cork oak forest/agro-forestry system (montado, 8.2%). The 1.5 km radius area from the apiary had a similar landscape composition (Table 8, right panel).

Table 8: Landscape structure and composition (land use) in Lousa and Idanha in the 10x10 km landscape and in the 1.5 km radius circular area surrounding the experimental apiary. Values represent area in ha and percentage (in parenthesis).

	Lousã			Idanha-a-Nova	
	10x10 km	**1.5 km**		**10x10 km**	**1.5 km**
Maritime Pine Forest	4270.5 (42.7%)	125.4 (17.7%)	Temporary crops	4666.9 (46.7%)	446.9 (63.2%)
Eucalyptus Forest	2083.8 (20.8)	98.0 (13.9%)	Permanent Pasture	2767.0 (27.7%)	103.1 (14.6%)
Shrubland	1711.5 (17.1%)	439.9 (62.2%)	Eucalyptus Forest	673.3 (6.7%)	2.0 (0.3%)
Chestnut Forest	566.8 (5.7%)	0.0 (0.0%)	Cork Oak Forest	625.4 (6.3%)	64.1 (9.1%)
Deciduous Forest	398.6 (4.0%)	41.1 (5.8%)	Almond	213.0 (2.1%)	3.7 (0.5%)
Coniferous Forest	318.1 (3.2%)	0.8 (0.1%)	Deciduous Forest	196.0 (2.0%)	18.4 (2.6%)
Temporary crops	179.5 (1.8%)	0.0 (0.0%)	Agro-Forestry System	189.0 (1.9%)	3.6 (0.5%)
Urban Vegetation	177.4 (1.8%)	1.1 (0.2%)	Nut	159.4 (1.6%)	35.7 (5.0%)
Other Oak Forest	97.0 (1.0%)	0.0 (0.0%)	Shrubland	143.2 (1.4%)	14.9 (2.1%)
Other	202.3 (2.0%)	0.5 (0.1%)	Other	361.6 (3.6%)	13.9 (2.0%)

In both Portuguese 10x10 km landscapes, the most common temporary crop was the temporary meadows (Table 9). In Lousa, no temporary crops were present within the 1.5 km radius of the apiary (Table 8 and 9, left panels). In Idanha, the dominant temporary crops within the 1.5 km radius of the apiary were temporary meadows (56.4%) and fodder (fodder mix, turnip, and vetch for grassing).

Table 9: Different temporary crops present in Lousa and Idanha in the 10x10 km landscape and in the 1.5 km radius area surrounding the experimental apiaries. Values represent area in ha and percentage (in parenthesis). The information is based on the crops present in 2019 as information on crop data for 2020 was lacking due to the COVID-19 pandemic

	Lousã			Idanha-a-Nova	
	10x10 km	**1.5 km**		**10x10 km**	**1.5 km**
Temporary Meadows	5.5 (35.0%)	0.0	**Temporary Meadows**	3170.8 (69.2%)	246.6 (56.4%)
Other Vegetables	3.3 (20.7%)	0.0	**Fodder Mix**	306.4 (6.7%)	7.9 (1.8%)
Fallow	2.2 (13.9%)	0.0	**Clover**	182.9 (4.0%)	6.9 (1.6%)
Oat	2.0 (12.4%)	0.0	**Fallow**	19.9 (3.9%)	0 (0%)
Fodder Mix	1.2 (7.8%)	0.0	**Triticale**	150.2 (3.3%)	0 (0%)
Corn	0.8 (5.4%)	0.0	**Turnip**	131.8 (2.9%)	102.2 (23.4%)
Potato	0.4 (2.5%)	0.0	**Ryegrass**	87.2 (1.9%)	15.7 (3.6%)
Ryegrass	0.2 (1.4%)	0.0	**Bean**	79.4 (1.7%)	3.5 (0.8%)
Other	0.1 (0.9%)	0.0	**Barley**	65.4 (1.4%)	0 (0%)
			Vetch	51.7 (1.1%)	46.6 (%)10.7
			Sorghum	48.6 (1.1%)	8.0 (1.8%)
			Yellow lupin	46.3 (1.0%)	0 (0%)
			Other	84.2 (1.8%)	0 (0%)

The landscape composition in Lousa and Idanha was not expected to change in terms of land use between study years, *i.e.* 2019 and 2020. Land use was generally stable across the study years and

with a slow turnover rate in the two regions. This is due to the presence of a very high percentage of natural areas (forest and shrubland) in Lousa and a very high percentage of permanent pasture (kept for more than 5 years in the same place) and temporary meadows (normally kept for a 5 years in the same place) in Idanha.

3.2.2. Mapping of floral resources

3.2.2.1. Landscapes in Denmark

In the four 1.5 km circular landscapes surrounding the experimental apiaries in Denmark, floral resources were found in 20 field polygons in Krænkerup, 42 field polygons in Flakkebjerg, 28 field polygons in Hinnerup and 29 field polygons in Foulum in 2019. In 2020, an additional three field polygons were found in Krænkerup, 12 field polygons in Flakkebjerg, two field polygons in Hinnerup and one field polygon in Foulum were detected. These were mainly flowering oilseed rape fields. In ALMaSS landscapes, the field polygons corresponded to 59 polygons in Krænkerup, 159 polygons in Flakkebjerg, 63 polygons in Hinnerup and 46 polygons in Foulum. Except for flowering fields of oilseed rape and white clover for seed production, field polygons were more or less permanent habitats such as grasslands, hedgerows, field borders and roadsides containing floral resources in both 2019 to 2020.

All landscapes in Denmark were dominated by farmland, although the landscape at Hinnerup contained large areas of forest (Table 7 and Figures 6, 7). Furthermore, the two landscapes Foulum and Flakkebjerg are not traditional farmland, since the landscapes were dominated by experimental fields and cultivated areas surrounding the research facilities of Aarhus University. For all landscapes, periods with little floral resources were observed, and except for periods with mass flowering crops, flower rich habitats were confined mainly to small, uncultivated areas. Descriptions below apply to both study years (2019 and 2020), as land use types did not change much between years (Table 7, Figures 6, 7), and the apiaries were not moved from one year to the other at all sites except for Flakkebjerg (Eastern Denmark). However, in Flakkebjerg, the experimental apiary used in 2020 was located only a few hundred meters from the experimental apiary used in 2019.

The landscape surrounding the apiary in Krænkerup (within 1.5 km) was a traditional Eastern Danish plant production area. The landscape was dominated by intensively cultivated agricultural fields. Hedgerows were rarely found between fields, and roadsides and field borders were, in general, very narrow and frequently cut. Apart from hedgerows, roadsides and field borders, uncultivated semi-natural habitats were rare in this landscape. In April and May, white-flowering shrubs and trees such as *Prunus cerasifera* and *P. avium* found in hedgerows, amounted to a rich resource when present. Besides from this, floral resources were mainly found in the fields. In May, mass-flowering oil seed rape and in July (2019) one field with white clover for seed production, constituted rich resources. Outside these periods, floral resources were limited both in early spring (April), mid-summer (June and July) and late summer (August). Willow (*Salix* spp.), which is known to be an important floral resource in early spring, was lacking in this landscape.

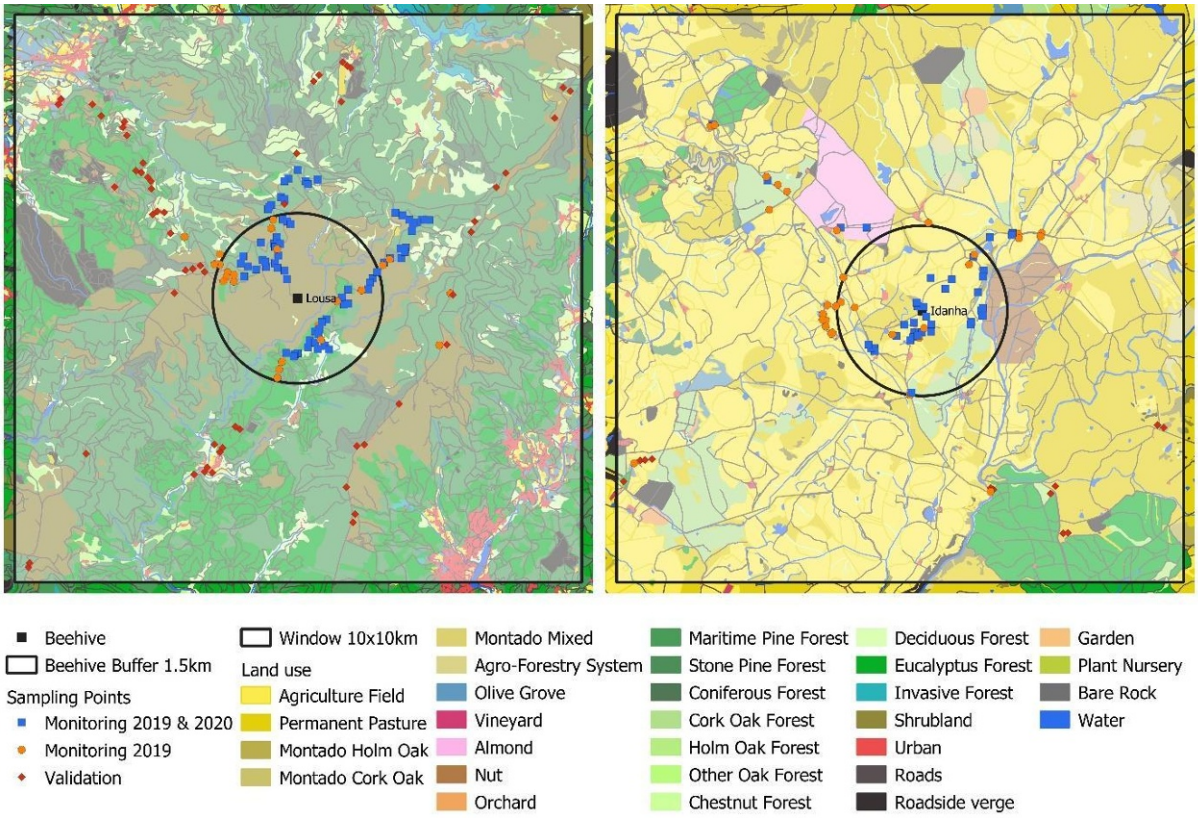
The landscape surrounding the apiary in Flakkebjerg (within 1.5 km) was, in general, relatively rich in floral resources. In addition to the cultivated areas surrounding the research center and the experimental fields, the landscape encompassed many small, uncultivated habitats and horse grazed fields that contained floral resources during part of the season. Both willow (*Salix* spp.) and white-flowering trees/bushes (*Prunus* species e.g. *P. cerasifera, P. spinosa, P. avium, and P. padus, Amelanchier lamarckii* and *Crataegus laevigata*) were found in spring and early summer and made up rich resources. Additionally, flowering *Taraxacum* spp. were common in grazed fields in May. In May, oil seed rape was mass-flowering both on agricultural fields and on experimental fields. However, less floral resources were found in mid- and late-summer (June, July and August) compared to spring and early summer. Species such as *Rubus idaeus*, *R. fructicosus*, *Cirsium arvense*, *Cichorium intybus, Rosa rugosa* and *Trifolium repens* were present. Except for *T. repens* that made up a rich resource in July all other plants only contributed minor resources.

The landscape surrounding the apiary in Hinnerup (within 1.5 km) encompassed large forest areas, where little floral resources were found throughout the season. In April, willow (*Salix* spp.) made up a rich but area-wise limited resource. Additional, white-flowering bushes and trees such as *Prunus cerasifera* and *P. avium* found in hedgerows, amounted to a rich resource in April and May. Furthermore, the landscape was dominated by large areas of oil seed rape that made up a rich resource in May. From June and onwards, floral resources were limited. Fields grazed by cattle and horses and roadsides contained some resources for part of this period, mainly contributed by species such as *Taraxacum* spp., *Ranunculus repens*, *R. acris*, *Cirsium arvense* and *Vicia cracca*. Across the forest, a wet meadow with *Cirsium palustre* made up some resources throughout the period June-August.

The landscape surrounding the apiary in Foulum (within 1.5 km) was, in general, relatively rich in resources. In addition to the cultivated areas surrounding the research center and the experimental fields, the landscape in 2019 contained a large construction site with soil covered by resource-rich wild plants contributed mainly by *Tripleurospermum inodorum* and *Cirsium arvense*. In 2020, the construction work was finalised and part of the area was covered by lawns as were the area in between the Campus buildings. Here *Trifolium repens* made up a rich resource throughout the summer when not recently cut. In April and May, hedgerows and uncultivated areas with thickets contained rich floral resources (willow, several *Prunus* species e.g. *P. cerasifera, P. spinosa* and *P. avium* and *Cratageus laevigata*). In May, mass-flowering oil seed rape made up a rich resource. Floral resources were found throughout the season in this landscape, although to a lesser extent and in more restricted and smaller areas in July and August.

3.2.2.2. Landscapes in Portugal

In 2019, a total of 111 polygons were surveyed at the two Portuguese sites (Figure 11). In Lousa, a total of 56 polygons were surveyed from which 20 polygons corresponded to the monitoring scheme (visited regularly once per month during sampling period) and 36 polygons to the validation scheme (visited only once or twice during the sampling period). In Idanha, a total of 55 polygons where surveyed, 47 polygons of the monitoring scheme and 8 polygons of the validation scheme. In 2020, sampling was affected by the COVID-19 pandemics since mid-March. Consequently, in 2020 a total of 43 polygons of the monitoring scheme were surveyed, 17 polygons in Lousa and 26 polygons in Idanha.



Sampling points surveyed in 2020 were a sub-set of those in 2019, due to COVID-19 restrictions. The circular areas represent the radius of 1.5 km from apiary.

Figure 11: 10x10 km landscapes surrounding the experimental apiaries in Portugal, Lousa (left panel) and Idanha (right panel) with the sampling points surveyed in 2019 (monitoring scheme and validation scheme) and 2020 (monitoring scheme)

The landscape surrounding the apiary in Lousa (within 1.5 km), consisted mainly of a large shrub area, although areas of pine, eucalyptus and hardwood forest were also well represented. There was also a windfarm with specific management practices, including the cutting of the shrubs around the wind turbines and in the boarders of the accesses. Within this area, there was also a small village where it was possible to observe ornamental plants and crops (mostly *Prunus* species). In the shrub areas the dominant species were shrubs such as *Erica* spp., *Ulex* spp., *Genista* spp. and *Rubus* sp. The same species occurred in the forest areas but in lower densities. In denser forests the resources were scarce. In hardwood forests, pollen and nectar providing species such as *Salix* sp., *Betula* sp., *Quercus* sp., *Castanea sativa*, *Frangula alnus* and *Rubus* sp. were found.

The landscape within 3 km of the experimental apiary was very similar to the one observed in the 1.5 km area. However, it is relevant to highlight the existence of more forest areas, more specifically birch and chestnut forests which also include, in some areas, individuals of *Pyrus cordata* and *Crataegus monogyna*. In the 3 km area, there is also another village with ornamental and crop species and some small agricultural areas.

In both 2019 and 2020, May was the month with the highest number of species flowering, and included species like *Erica* spp., *Eucalyptus globulus*, *Frangula alnus*, *Genista* spp. and *Rubus ulmifolius*. The availability of resources started to decrease from May onwards but pronounced declines in resources were observed in June and until the end of the season. The main resources available in the beginning

of the season were *Erica australis*, *Erica umbellata*, *Erica arborea*, *Acacia dealbata*, *Eucalyptus globulus*, *Salix* sp., *Quercus robur* and *Ulex micranthus*. In mid-season, the main resources were *Erica cinerea*, *Erica umbellata*, *Rubus ulmifolius*, *Castanea sativa* and *Ulex minor*. At the end of the season, the resources available included some individuals of *Erica ciliaris* and E. *cinerea*, *Calluna vulgaris* and *Ulex minor*.

Some differences were found between the two years of sampling. In 2019, individuals of *Erica australis* were observed flowering from March to mid-May, while in 2020 flowering occurred mainly in March. Furthermore, flowering of *Eucalyptus globulus* was recorded from the beginning of March to the beginning of August (except in July) in 2019, whereas in 2020 *Eucalyptus globulus* flowering was only recorded in March, April and May.

The landscape surrounding the apiary in Idanha (within 1.5 km) was mostly composed of permanent and temporary pastures and cereal crops for fodder where different herbaceous species grow. These included some Asteraceae (*Andryalla* spp., *Anthemis* sp.), *Echium plantagineum*, *Brassica barrelieri*, *Diplotaxis catholica*, *Echium* sp., *Trifolium* spp. and *Vicia* spp. There were also smaller areas composed of shrubs such as *Cytisus striatus*, *Retama sphaerocarpa*, *Lavandula* sp., *Rubus ulmifolius*, *Rosa micrantha*, *Crataegus monogyn*a and *Quercus rotundifolia* accompanied by other herbaceous species. Other smaller areas were characterized by having *Salix* sp., *Fraxinus angustifolia*, *Rubus ulmifolius* and *Rosa micrantha.* In addition, there was a large cork oak plantation area with some holm oaks, where some of the shrub species mentioned above were also found although in lower densities.

When analysing the landscape in the 3 km area, the landscape was very similar to the one observed in the 1.5 km area. However, it is important to highlight the existence of two large orchards: one of walnut and one of almonds. In these areas, several of the herbaceous species listed above were growing in-between the tree lines.

In both study landscapes, some polygons were assessed in points along the roadsides of national roads, secondary roads and on dirt roads. The species observed were mostly herbaceous species already mentioned above, some shrubs such as *Adenocarous lainzii*, *Cytisus* sp., *Rubus ulmifolius* and *Lavandula pedunculata*. These roadsides are cut periodically.

In 2019, the highest number of species flowering was detected in the beginning of May, while in 2020 it was in April. The decrease in the availability of resources occurred from July onwards in both years. The main resources available in the beginning of the season were *Echium plantagineum*, *Salix* sp., *Jasione montana*, *Trifolium* spp., *Quercus* sp., *Anthemis* sp., *Chamaemelum* sp., and *Brassica barrelieri* and *Diplotaxis catholica*. In mid-season the main resources were *Echium plantagineu*, *Jasione montana*, *Trifolium* spp., *Retama sphaerocarpa*, *Rubus ulmifolius* and Asteraceae. At the end of the season, the resources available were some Asteraceae species (e.g., *Carlina* spp., *Dittrichia* spp., among other) and other small herbaceous species (few individuals with few flowers).

In both the Danish and Portuguese landscapes, ground truthing revealed the need to update the polygons in digitalized landscapes used by the ApisRAM model with polygons identified in the field. In Denmark, the flower-containing polygons identified in the field project (denoted field polygons) were paired by the modelling team to ALMaSS landscape model parcels with respect to geographic and thematic coincidence. Field polygon to ALMaSS parcel relationships included "1:1", "1:<1" and "1:many" cases. ALMaSS parcels were edited (splitting, merging) to achieve, as far as possibly, a 1:1 relationship for each field polygon item. However, some "1:many" cases remained, e.g. large field polygons where merging of the parcels would seriously reduce the integrity of the ALMaSS landscape model, and long narrow field polygons such as roadside verges since these often comprised many small parcels that cannot be merged without reducing the integrity of the ALMaSS landscape model. The ALMaSS parcels associated with the field polygons were each labelled with a unique polygon ID (denoted UniquePolyID), which is a unique ID based on country, site, year, month, field polygon number and ALMaSS parcel. For each study site, a list of polygons (containing information on UniquePolyID, area of the polygon and UTM centroid) was produced and imported in the database.

In Portugal, the problems of matching field identified polygons with model parcels in the ALMaSS landscapes were normally due to spatial inaccuracy or temporal inaccuracy. Spatial accuracy problems were caused by spatial information used in the field survey was produced at a different spatial scale and that consequently the land cover/use classes had different levels of generalization. Temporal accuracy problems aroused from the fact that the spatial information used was produced at a different point in time than the field surveys and ultimately reflected the changes of land cover/use across time. The final landscape maps were adjusted by linking the geographical landscapes information with the results of the 2019 field survey. The specific GIS tasks that were implemented to fix these problems were the splitting/division of polygons, merging of polygons and typology adjustment of polygons. Additionally, whenever needed the landscape was validated using the Orthophotos of 2018. The final landscapes produced due to the field validation and necessary GIS corrections performed were able to capture the reality, generating high quality landscapes for ApisRAM. A one-to-one relationship was obtained for all polygons identified in the field, i.e. one polygon in the ALMASS system had one correspondence in the flower mapping field surveys.

3.3. Local weather and in-hive conditions

3.3.1. External climate (weather stations)

The two weather stations in Portugal and three of the weather stations in Denmark (Krænkerup, Flakkebjerg and Foulum) were running continuously and largely without problems throughout the experiment, until 30 October 2020. However, the weather station at Hinnerup had multiple breakdowns in 2019, while no major problems were encountered in 2020.

Two of the weather stations were damaged by lightning, once in Krænkerup and twice in Hinnerup, resulting in loss of data for wind speed during a period, until the weather stations were repaired (error discoved 8 July 2019, corrected mid July 2019). During repair, it was discovered that the wind sensor had been installed opposite in Hinnerup, i.e. wind measurements from 20 February until 23 July 2019 were corrected by 180 degrees from the time of installation until 23 July 2019 (this was corrected in the raw data set). In mid-July 2019, the wind speed sensor in Hinnerup was disconnected due to a break in an electric cord.

A second incidence of lightning damage in Hinnerup resulted in a total breakdown of the weather station in late August 2019, due to damage of the solar panels. To monitor local weather data until the end of the field season in September 2019, the weather station was run on batteries (by-passing the solar panels) for one month. Hence, for the 2019 field season, there are periods of missing values for wind data, and in several periods completely missing weather data for Hinnerup. To secure a reliable data logging of climate data during the winter and the 2020 field season, the weather station, including the defect solar panels, was returned to the supplier in mid-September until re-installation 25 October 2019, for a thorough repair and service check. Following this repair, the weather station was fully functional. From re-installation in October 2019, it ran continuously until October 2020, except for short periods during December and January, during which batteries could not be adequately re-charged due to short days and adverse weather. The resulting data set included raw data from all study sites, in addition to Ødum.

3.3.2. In hive climate (hive scales)

In-hive temperature and humidity were logged by the ApisTech hive scales in experimental hives at all study sites and in both study years. Due to technical challenges, at the onset of the experiment, periods of missing data occurred for at least some scales in all apiaries (for details, see section 3.3.3 local weather and in-hive conditions, missing data). The number of records, i.e. hourly measurements, per year was approximately 20,000 to 24,000 in Denmark and 27,000 to 35,000 in Portugal (Table 10). The higher number of data points in Portugal was due the prolonged field season

in Southern Europe.

Table 10: Total number of data points logged by hive scales at each study site and year.

		Number of data lines	
Region	**Site**	**2019**	**2020**
Eastern Denmark	Flakkebjerg	23844	21891
	Krænkerup	23264	23571
Western Denmark	Foulum	22244	20715
	Hinnerup	20044	19707
Portugal	Idanha	31213	34334
	Lousa	26963	35479

The in-hive climate data set includes raw data from all study sites.

3.3.3. Local weather and in-hive conditions, missing data and mitigation

3.3.3.1. External climate (weather stations)

Weather stations at the two Portuguese (Lousa and Idanha) and Danish (Krænkerup, Flakkebjerg and Foulum) sites operated continuously and without any major problems during the experiment, until 30 October 2020. In Krænkerup, the weather station was struck by a lightning once in June 2019, but quickly repaired, resulting in only a small data gap. The weather station in Hinnerup had multiple breakdowns in 2019, while no major problems were encountered in 2020.

Since long periods of missing data are problematic for ApisRAM, additional climate data were obtained from the nearest weather station run by the National Meteorological Institute (DMI). This weather station was in Ødum, 8 km from Hinnerup. Whereas cloud cover and precipitation are generally local, and may differ between Hinnerup and Ødum, wind speed, wind direction and temperature are expected to be similar.

3.3.3.2. In-hive climate (hive scales)

Several problems were encountered with the ApisTech scales during the first months of monitoring in 2019. In particular, sudden changes in weight and missing data were observed at all sites. These instabilities of automatic monitoring were due to a software bug, which required an update of the devices by ApisTech. Furthermore, in Denmark, the battery of several scales was water damaged due to defect battery boxes. In Portugal, software problems were solved on the 30 of March 2019 in Idanha and on the 5 April 2019 in Lousa. In Denmark, the hive scales were repaired on the 26-27 June 2019, during the repair, the hive scale software was upgraded and the battery boxes changed. This thorough repair improved the stability of the automatic logging considerably.

In 2020, three hive scales (in Hinnerup, Foulum and Flakkebjerg, respectively) were temporally unstable, possibly due to weak antennae. The scale in Flakkebjerg was replaced by a scale of another brand (CAPAZ scale) in early May. The other two unstable scales were moved within the apiary in order to obtain a better coverage, however, periods of missing data occurred (Table 10).

The external and in-hive climate data delivered are raw data, automatically logged by the weather stations and hive scales. For external temperature measurements, we recommend using the climate data from the weather stations rather than from the hive scale external sensors. Firstly, data monitored by weather stations were standardized (e.g. by above ground height), secondly, external temperature

data from hive scales generally were slightly higher than weather station data, possibly due to heat generation by the colonies in the hives.

3.3.4. Hive weight

Hive scale data were obtained from early April to late October 2019 and from late March to late October 2020 in Denmark. Due to the problems of monitoring in-hive climate in early and late season (see section 3.3.3.2), combined with experiences that the batteries of the hive scales were sensitive to frost, it was decided to remove the hive scales during the winter (starting late October in Denmark). Although it was considered to continue the hive scale logging throughout the winter, previous experience showed that variations in hive weight is mostly caused by rain and snow accumulating on top of the hive (Per Kryger, pers. com.). Furthermore, a large population of pheasants was found at the overwintering site at Hinnerup, and the birds were often observed sitting on the hives, hence resulting in sudden weight changes. The weight of the bee colony is expected to be relatively constant during the winter, even if the colony dies. In Eastern Denmark, hive scales had to be re-arranged following the re-arrangement of colonies in the apiaries (see section 3.4.1), and hence in the new apiary in Flakkebjerg, hive scales were installed in late April 2020. In Portugal, hive scales were running continuously from mid-March 2019 until the end of the experiment in late October 2020. Further description on the number of data points, missing data and mitigation measures in relation to hive scale logging is provided in section 3.3.3.

The hive weight data delivered in the current project are raw data monitored automatically by the hive scales. Disregarding the missing data, monitoring of colony weight is generally confounded with sudden weight changes due to external factors, e.g. heavy rain, snow and birds (pheasants) resting on the hives. In addition to these external disturbances, weight data should be cleaned for changes due to management practices, e.g. when frames, supers and sugar feed are added or removed, and during days of colony monitoring.

3.4. Colony management and colony observations

3.4.1. Honey bee colonies

The table below (Table 11) reports the numbers of colonies at each study site before overwintering (September/October 2018), at the onset of the field season (February in Portugal, April in Denmark), and at the end of the field season (September in Portugal and Denmark) in 2019 and 2020.

Winter loss 2018/2019 was null in Portugal (0%) and low in Eastern Denmark (5%), but high in Western Denmark (25%). In the EU project EPILOBEE, winter colony mortality rates varied among member states and years. Both Denmark and Portugal had intermediate winter mortality rates, with colony mortality rates between 10 and 20% (Laurent et al., 2015, De Graff et al., 2016). Only a few colonies were lost during the field period (summer) 2019 at all sites, although in Portugal, several experimental colonies swarmed. During swarming, the original sister queen is replaced by a new queen, which is usually a daughter, and hence genetically related to the original queens. After varroa treatment in late summer 2019, two experimental colonies (colonies #1 and #3) in Idanha (Portugal) exhibited absconding behavior which lead to high population losses, and one colony died (observation hive in Lousa, Portugal). In addition, two colonies in Idanha (one control colony, one back-up colony) were lost due to failure of queen replacement after swarming (Table 11).

Approximately two colonies were lost per study site during the winter 2019/20, except for Idanha (Portugal), where seven colonies were lost (Table 11). In Eastern Denmark, several of the surviving colonies were weak. It was decided to move the remaining two strong colonies from Flakkebjerg (colonies #2 and #6) to Krænkerup (re-named Krænkerup colonies #11 and #12), hence obtaining five strong sister queen colonies in Krænkerup. These two strong colonies were moved from Flakkebjerg to Krænkerup on 24 April 2020. Due to a generally high national winter mortality of honey bees during the

winter 2019/20, combined with the COVID-19 situation, new colonies could not easily be obtained. However, an existing apiary was included in the experiment in Flakkebjerg, consisting of seven colonies, and located only 300-400 meters away from the apiary used for the experiment in 2019 (the new apiary was named "Flakkebjerg 2020" in the data set). Queens of the new colonies were unrelated to the original sister queens, but the colonies had overwintered in the same landscape, and five strong colonies could be designated as experimental colonies.

The total numbers of colonies in the experimental apiaries during the course of the experiment are summarized in Table 12.

Table 11: Colony loss during the experimental period, from September 2018 to September 2020

Study site	Region	Winter loss 2018/19	Summer loss 2019	Winter loss 2019/20	Summer loss 2020
Krænkerup	Eastern Denmark	0	0	2	2
Flakkebjerg	Eastern Denmark		1	3	0
Hinnerup	Western Denmark	5	0	4	1
Foulum	Western Denmark		0		0
Lousa	Portugal	0	0 (41)	2	0
Idanha	Portugal	0	0 (11)	7	0

[1] Colonies swarmed, i.e. sister queen was replaced by a new queen, although the new queen is likely to be a daughter of the original sister queen

Table 12: Total number of colonies (including experimental and back-up colonies) at all study sites. The number of colonies in brackets are the number of strong colonies in Krænkerup and Flakkebjerg in April 2020

		Colonies with original sister queens				Colonies with new queens	Total colonies 2020	
Study site	Time	Sep-2018	Apr-2019	Sep-2019	Apr-2020	Apr-2020	Apr-2020	Sep-2020
Krænkerup	Eastern Denmark	20	8[1]	7[2]	5	0	7[3]	5
Flakkebjerg	Eastern Denmark		9	8[2]	5[3]	7	7	7
Hinnerup	Western Denmark	20	9[1]	9	7	0	7	6
Foulum	Western Denmark		8	8	6	0	6	6
Lousa	Portugal	11	11	10	8	2	10	10
Idanha	Portugal	11	11	9	2	8	10	10

[1] Two back-up colonies were moved from Krænkerup to Hinnerup at onset of field season. Hence, the 9 colonies in Hinnerup included 7 colonies that over-wintered in Hinnerup, in addition to two colonies that over-wintered in Krænkerup, and only 8 colonies were left in Krænkerup.

[2] One back-up colony (observation hive) was moved from Krænkerup to Flakkebjerg during the field season due to severe honey robbing.

[3] Only two of the five colonies that overwintered at Flakkebjerg were strong. These were moved to Krænkerup in April 2020.

3.4.2. Assessment of adult population, brood and provision in experimental colonies

Population development of honey bee colonies was highly variable. Differences were found between countries (Denmark *versus* Portugal), between the two regions in Denmark (Western and Eastern Denmark), in addition to among-colony variation within each apiary. Among-country differences in colony development, e.g. patterns of seasonal rhythm of brood development and peak colony size, may relate to the different subspecies of *Apis mellifera* (*A. mellifera* x Buckfast in Denmark and *A. m. iberiensis* in Portugal) (Ruttner, 1988), in addition to differences in flowering phenology and length of the growing seasons between Northern and Southern Europe. In general, colonies in Western Denmark obtained higher colony sizes and collected more provision than colonies in Eastern Denmark. This difference was expected, based on calculations of honey potential of the surrounding landscape (section 3.1.3.1). Due to a concentration of nectar availability in early spring, when the strength of the colonies floral resource availability during the summer are generally low, , Eastern Denmark was expected to have a low "landscape fitness". This was mirrored in generally by smaller colonies, and a higher level of winter mortality in the second year of the experiment (winter 2019/2020). High among-colony variations were found in all experimental apiaries. Since all experimental colonies in each country were genetically related (i.e. by having sister queens, and initially similar in size), differences in colony development for colonies having access to the same landscape, are likely to relate to differences in health status, pesticide exposure, or stochasticity (Figure 12 and 13).

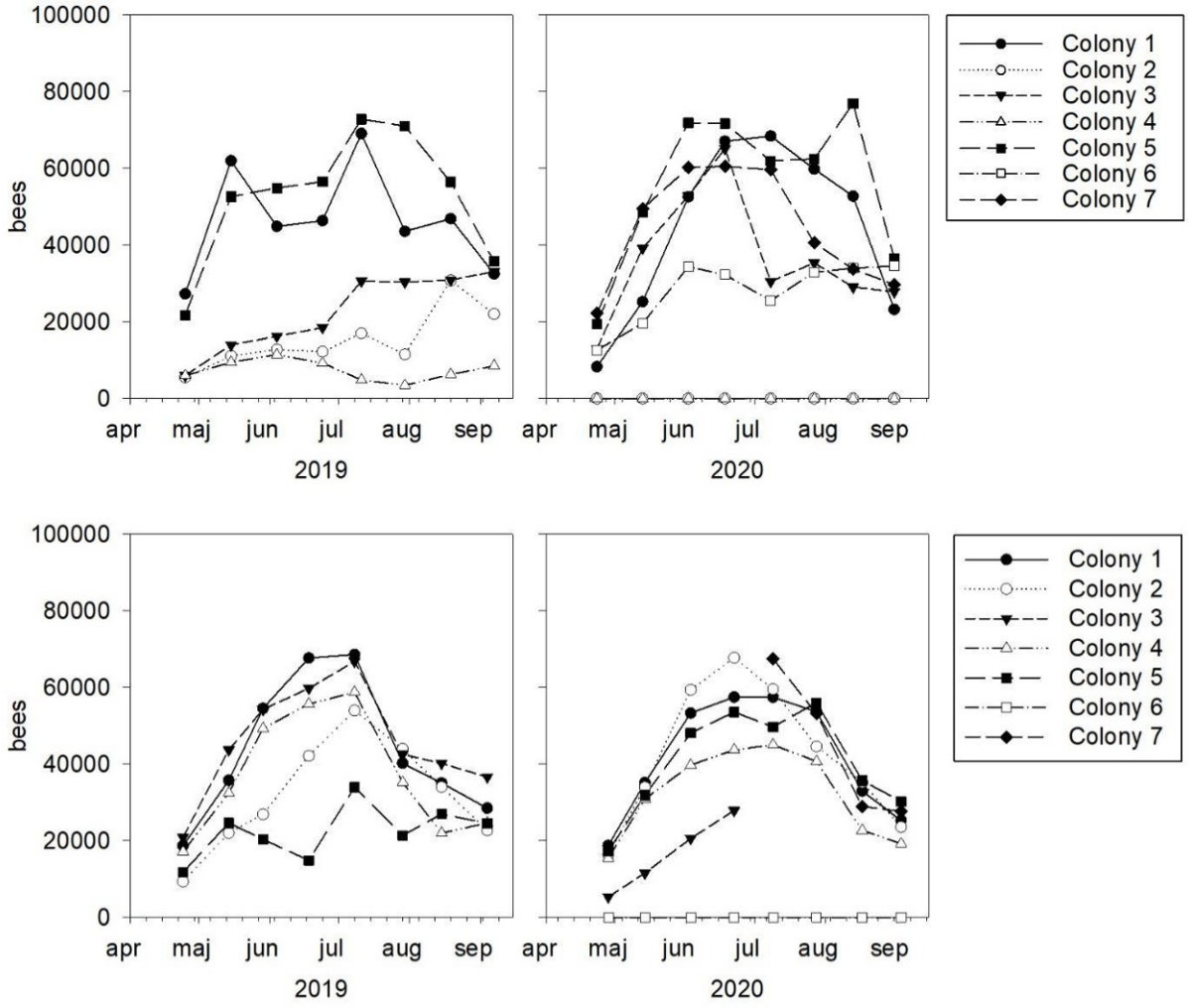


Figure 12: Population development of adult bees during the field seasons 2019 (left) and 2020 (right) in Foulum (upper panels) and Hinnerup (lower panels), respectively

Adult population size of colonies was assessed as weight of bees, and bee strength estimated using 100 mg per bee resulted in high population estimates compared to expectations from rough visual assessments. Weight of individual bees are highly variable (e.g. Jay, 1963), depending on the amount of provision carried by each individual (pollen loads and nectar in honey crops). Furthermore, drones have a higher weight than workers. In the current study, the weight per bee was estimated from the total weight of 50 bees (in Portugal) or 60 bees (in Denmark) 1-2 times during each field season. These bees were collected from the hive, hence the sample consisted mostly of in-hive bees (not foragers), which represent a large proportion of the colony. In Portugal, bees weighted an average of 124 mg per bee and in Denmark 128 mg per bee. The relatively high weight of individual bees in the current study may be due to the bees taking up nectar from the combs when smoke was applied during monitoring and management. As individual bee weights are likely to vary within and among colonies and among different localities/countries and through the season, we suggest using an estimate of 125 mg per individual bee when converting total weight of adult bees when estimating the number of bees in a colony.

The Deepbee® software classified the comb cell contents into the seven classes. The original version of Deepbee®, which was developed using images from Portugal, managed to classify each class with an accuracy of at least 84% (when compared to manually annotated images), with the lowest performance on eggs and larvae (Alves et al., 2020). In the current project, the software was initially trained using images from the Danish apiaries in 2018 and 2019. However, the software had a low performance for distinguishing pollen and empty cells, in addition to capped brood and honey, possibly due to differences in colour and texture of wax and pollen between Portugal and Denmark. In order to optimize the software for local conditions, additional training of the software was carried out separately for each region in 2020 (Portugal, Western Denmark, Eastern Denmark). Under Portuguese conditions, additional training of the software was performed in order to improve the performance of distinguishing young larvae from eggs. The training of the software involved manual annotation of images from each of the three regions (Western and Eastern Denmark, and Portugal), resulting in three different versions of the software adapted for local conditions.

The output of the analysis with deepbee® was screened for images in which <3000 cells were detected. As combs generally had approximately 3200 cells per side, <3000 detected cells indicated that major areas of the combs were missing in the detection. Furthermore, combs in which considerable areas were not correctly classified (mostly honey classified as capped brood or eggs classified as 'other') were corrected manually. In these images, the cell classification was corrected manually. Images having only minor errors were not corrected manually. Manually annotated images were used for additional training of the software for each region, further improving the performance of the software. Error rates were assessed by comparing results of the automatic detection and cell classification by image analysis with a manual assessment of images. At least eight random images were selected from each apiary (representing different dates and different cells types).

Overall performance of the software was improved, using a set of 25 random pictures from Portugal (14 from brood frames) with a total of at least 4700 cells per each class. For Danish conditions, two versions of the software were developed. In Eastern Denmark, a set of 15 pictures with a total of at least 3000 cells per class were used to upgrade the software. For Western Denmark, a set of nine pictures with a total of at least 2000 cells per class was enough to upgrade the software. Accuracy after training was also visually evaluated by using a set of 20 pictures from a whole colony for all versions of Deepbee®.

Similar to the adult population, seasonal brood development and provision of the experimental colonies differed among colonies within the same apiary. In Portugal, several experimental colonies swarmed in 2019. This is reflected in the brood development curves, showing a decrease in brood development following a swarming event (colonies #2 and #3 in Figure 11).

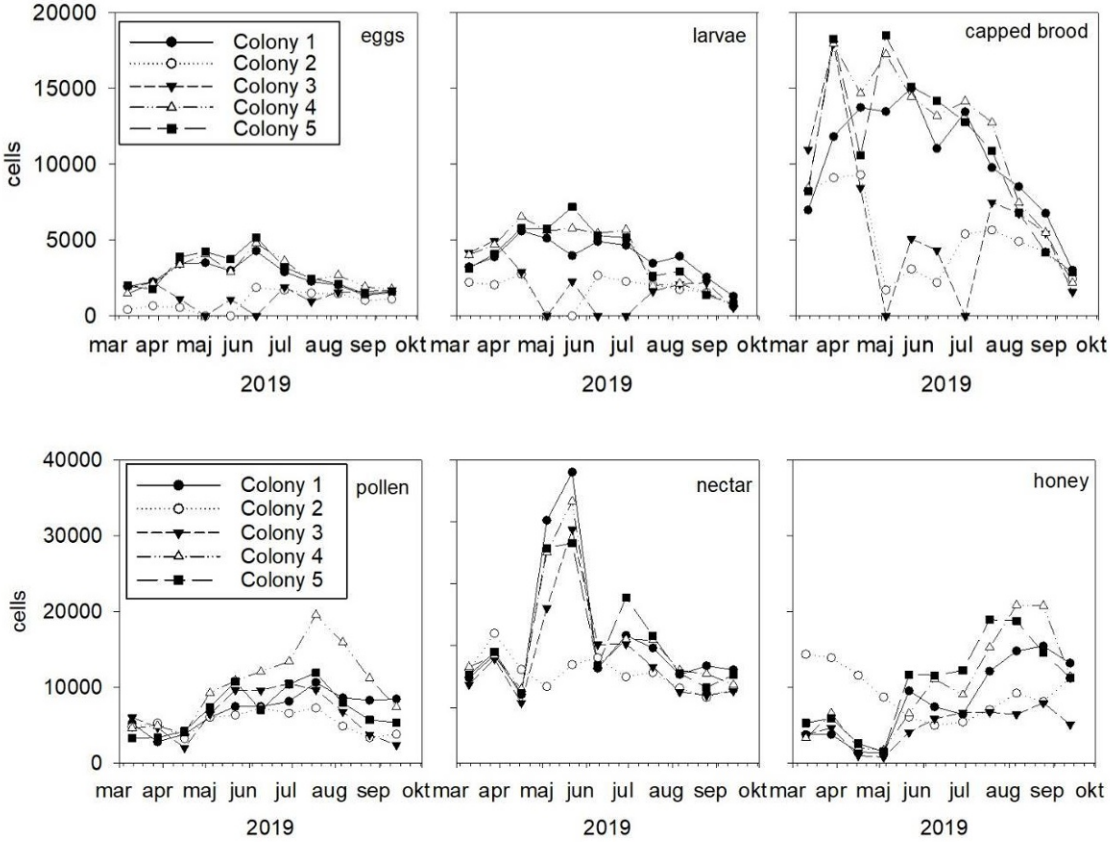
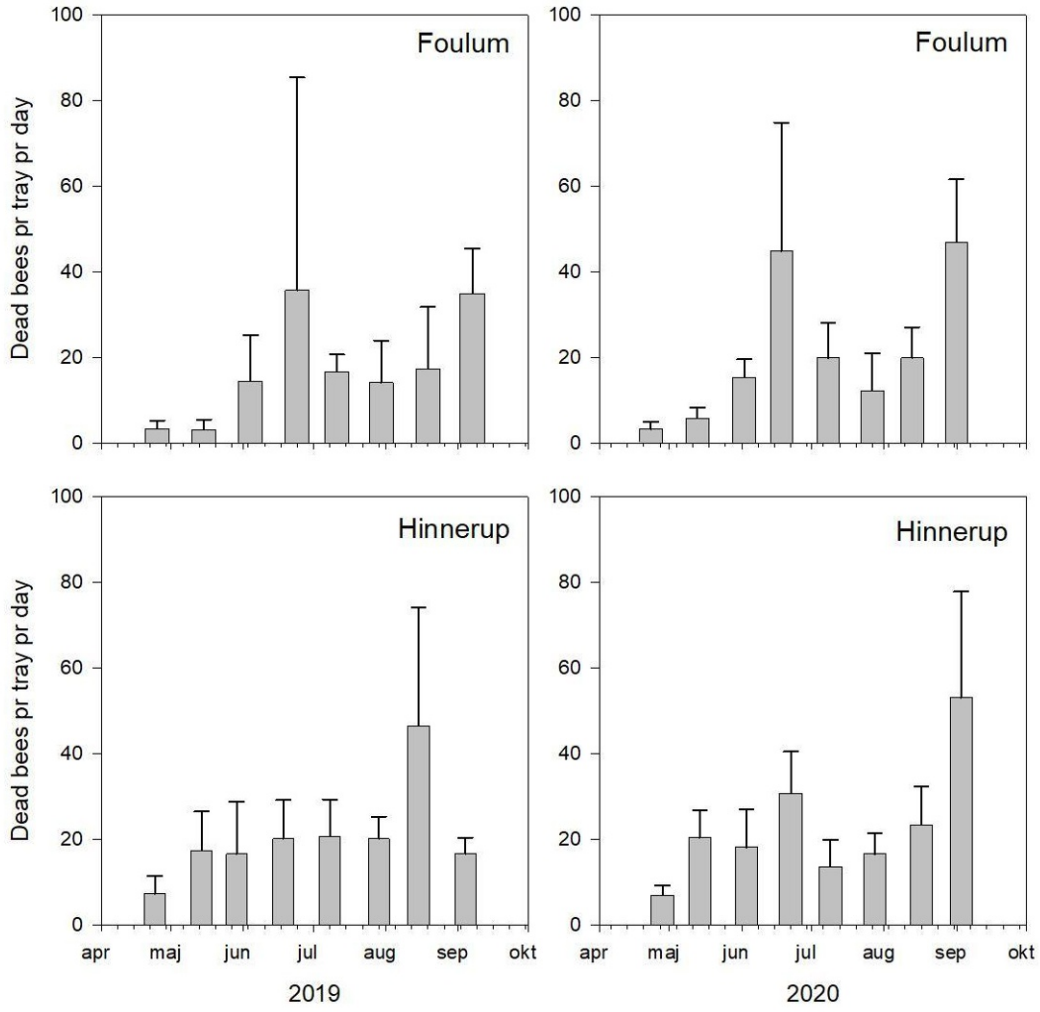


Figure 13: Development of brood (eggs, larvae and capped brood, upper panels) and provision (pollen, nectar and sealed honey) in combs of experimental colonies in Lousa in 2019

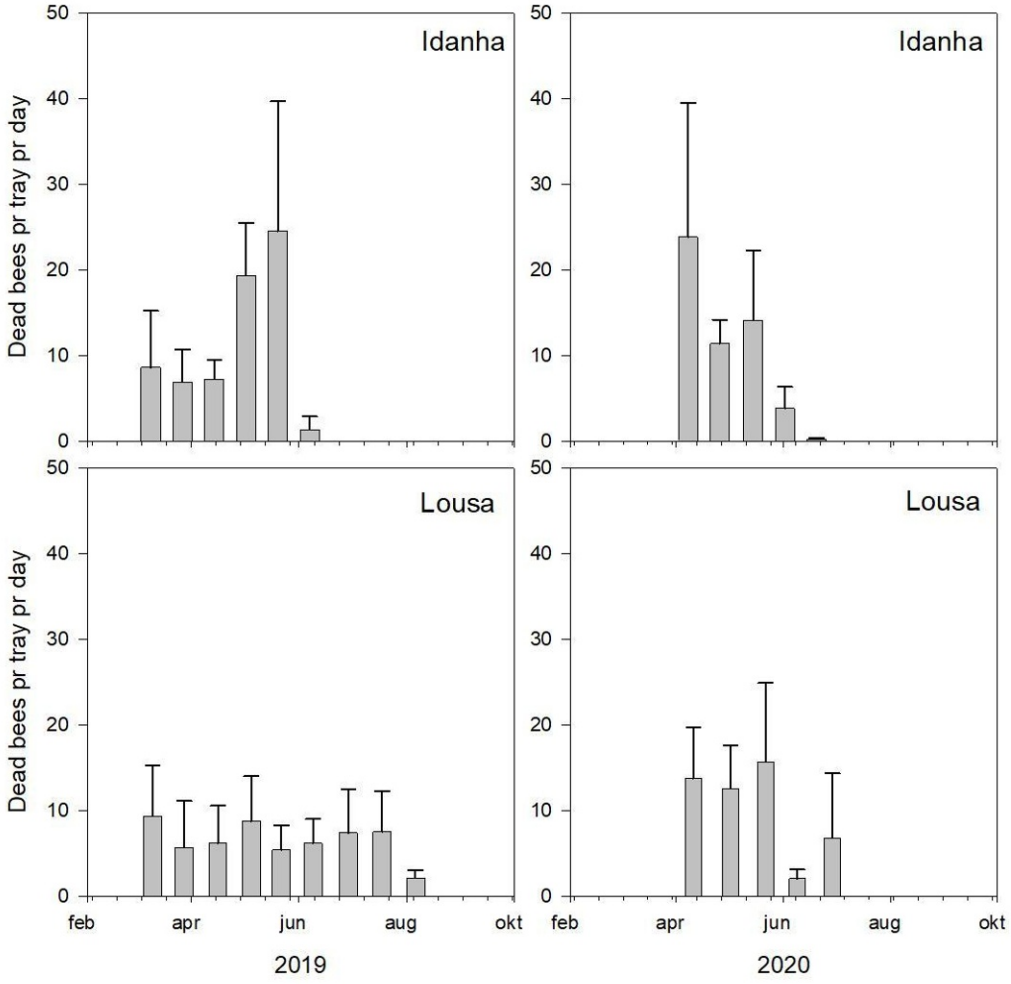
3.4.3. In-hive mortality

In the two apiaries in Denmark, two peaks in dead bee disposal by the experimental colonies were observed in both study years and for both apiaries: early mid-summer (late June/early July) and late season (September) (Figure 14). In the Portuguese apiaries, only one peak was detected in early mid-summer (late May-early June) (Figure 15). Possibly, a late season peak was not detected, as monitoring of in-hive mortality ended in mid-September.



In-hive mortality was not monitored in the period from 1st October 2019 to 1st April 2020.

Figure 14: Number of dead bees accumulated in trays per day in front of experimental bee hives in 2019-2020 in Hinnerup (upper panel) and Foulum (lower panel) in Western Denmark



In-hive mortality was not monitored from 1st October 2019 to 28th February 2020.

Figure 15: In-hive mortality (number of dead bees per tray per day) during the two field seasons (2019 and 2020) in the two Portuguese apiaries (upper panels, Idanha, and lower panels, Lousa)

Dead bees appeared to be well preserved in the trays at the study sites in Denmark, and hence not degraded due to prolonged submergence in rainwater. However, in late season (August and September), dead bees were partly degraded, and difficult to count. On these two occasions, 25-50 bees were counted and weighted, and the total number of dead bees estimated from the total weight of the sample. During dry periods of the field season, dead bees, which became dry and light, may have been blown away by the wind, although we could not assess to what extent this occurred.

In Portugal, the influence of rain and wind on mortality also could not be assessed. Furthermore, particularly in Idanha, ants entered the tray to collect the dead bees. To overcome this issue, bee heads were counted, as ants usually left the head of the bees behind. Nonetheless, as ants sometimes collected entire bee carcasses, counting bee heads may to some degree under-estimate the number of dead bees. In 2020, oil supports (traps) were used to avoid ants from entering the tray.

Data for in-hive mortality were included in Table IV (colony management) of the database, and this type of data was classified as 'other' output.

3.4.4. Observation hives

Waggle dances were recorded and observed at the two study sites in Western Denmark (Foulum and Hinnerup), and at Idanha in Portugal. Even under optimal weather conditions, waggle dances were not always observed (Table 13). This could be due to weak colonies (due to disease or recent swarming), lack of (rich) floral resources in the surrounding landscape, or the bees performing waggle dances on combs not visible in the observation compartment.

In Denmark, recordings of waggle dances were challenged by a cold and rainy April 2019, observation hives could not be installed before late April (23-26 April 2019), as the spatial arrangement of combs in the observation compartment would split and expose the brood area to cold temperatures, possibly damaging the brood. In Portugal, cold weather also negatively affected the observation hive at Lousa, and the colony did not fully develop during the season. Consequently, on most observation dates, the colony was poorly active, and no waggle dances could be observed in the observation compartment. Following varroa treatment, the colony was lost due to absconding.

The observation hive installed in Krænkerup (Eastern Denmark) was exposed to a vigorous robbing event on the 5 June 2019, during a period of low floral resource availability in the landscape. Due to multiple robbing events following the first major robbing, the colony was moved to another location (Flakkebjerg). Deprived of all honey stores, the colony had serious problems recovering, and no waggle dances could be observed.

Table 13: Observation dates for waggle dance observations

Idanha	Waggle dances observed	Hinnerup	Waggles dances observed	Foulum[1]	Waggle dances observed
09 March	Yes	30 April	No	26 April	Yes
02 April	Yes	11 May	No	29 April	Yes
14 April	Yes	14-15 May	Yes	30 April	Yes
02 May	Yes	29 May	No (few)	1 May	No
20 May	Yes	18 June	Yes[2]	2 May	No
7 Jun	Yes	27 June	No	5 June	No
27 Jun	Yes	10 July	Yes	11 July	Yes
16 Jul	Yes	6 August	No		
04 Aug	No				
23 Aug	No				

[1] Data obtained from Jeppesen & Frederiksen, 2020.

[2] Waggle dances present but could not be decoded due to too short video sequences.

A total of 508 dances were decoded from the apiaries in Denmark and 262 from the apiaries in Portugal. The minimum decoded distance was 250 meters in Denmark and 209 meters in Portugal, while the maximum distances were 2870 meters in Denmark and 6138 meters in Portugal. At all sites, bees regularly foraged 1000-2000 meters from the hive. At Idanha, waggle dances were recorded and decoded throughout the season 2019. Foraging distances were variable, but most foraging trips were less than 2000 meters (Figure 16). Foraging distances appeared to decrease during the season, although fewer dances were observed and decoded in late season (Figure 16). Other studies have documented seasonal patterns of foraging distances in honey bees, although foraging distances peaked in late summer (Couvillon et al., 2014).

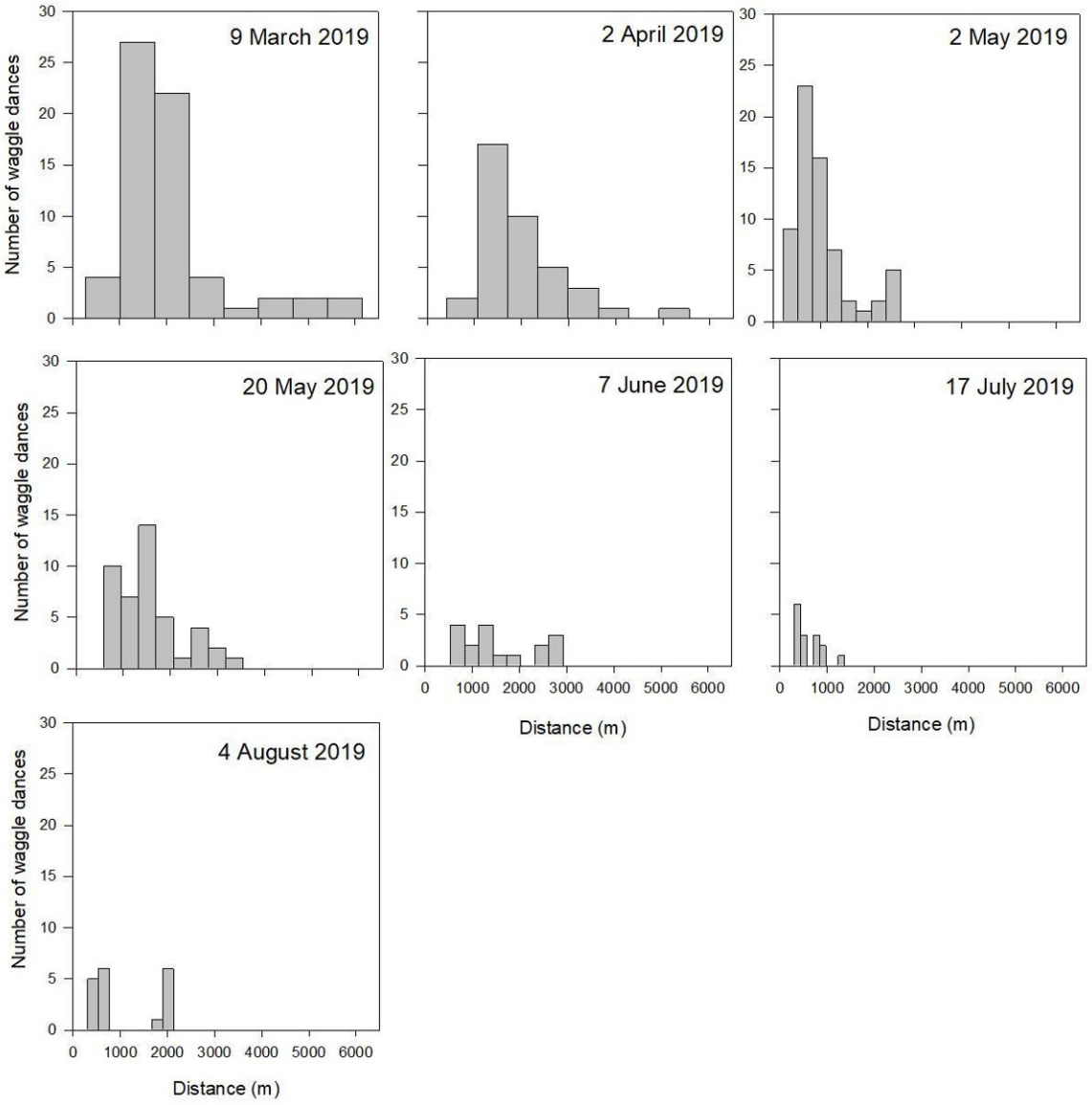


Figure 16: Foraging distances, as decoded from the waggle dances decoded at Idanha (Portugal) during the field season 2019

During the spraying experiment in Foulum, decoded foraging distances changed slightly after spraying. Median foraging distance increased from 825 meters prior to the spraying of the experimental oilseed rape field in close proximity to the apiary, to 1380 meters on the day of the spraying and 1485 meters the following day (Figure 17).

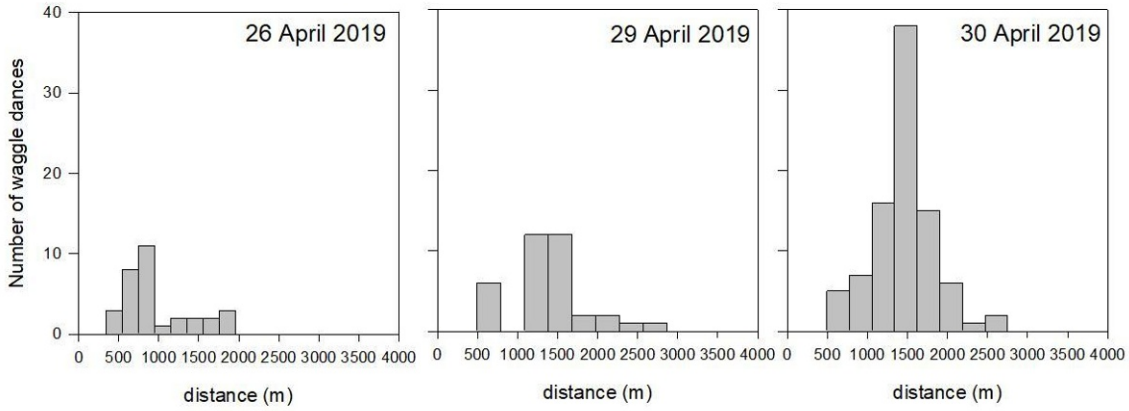


Figure 17: Foraging distances as decoded from the waggle dances decoded at Foulum (Denmark) during the spraying experiment

The decoding of waggle dances showed that spatial foraging patterns change across the season (Figure 18), and, during the spraying experiment, patterns changed even day-to-day (Figure 18). Coordinates of decoded waggle dances did not always overlap with known floral resources in the landscape. This could be due to inaccuracies in the decoding of the distance and direction of the waggle dances. It could also indicate that waggle dances do not indicate exact location of resources or demonstrate an incomplete knowledge of the floral resources in the landscapes.

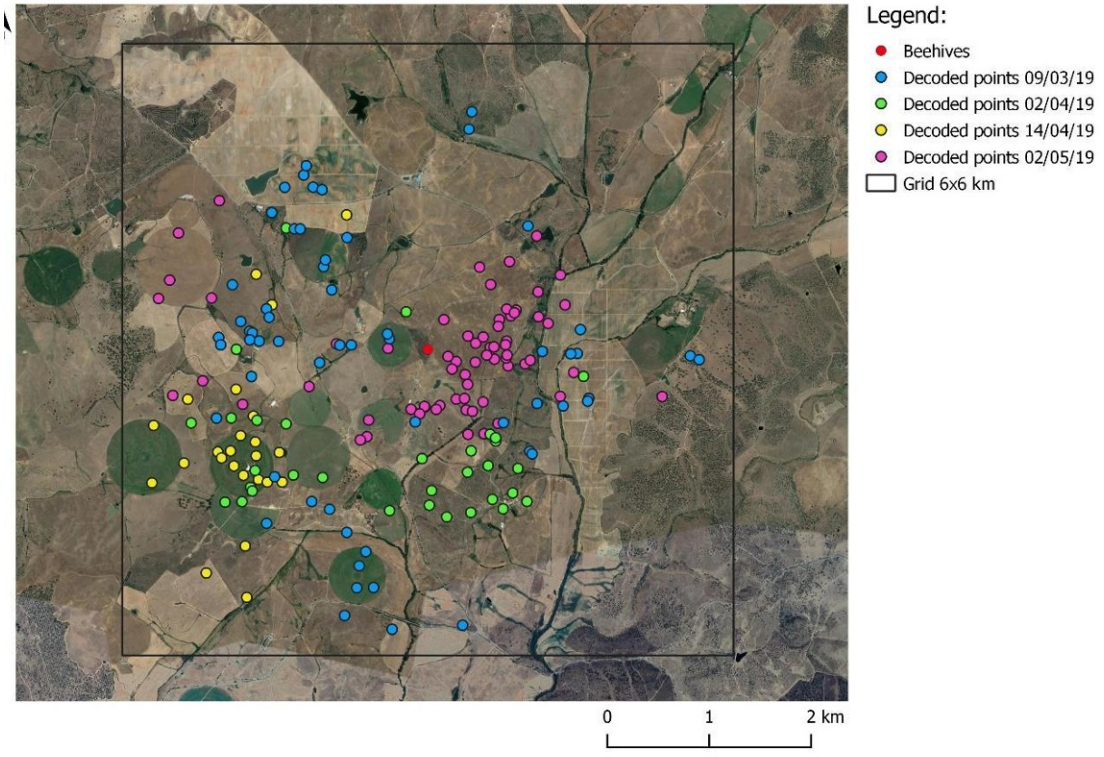
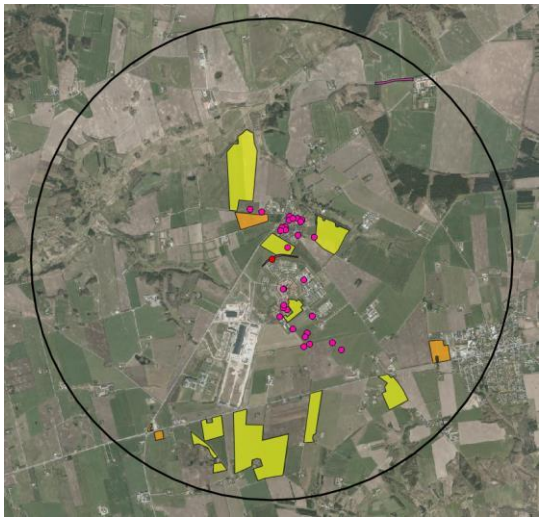
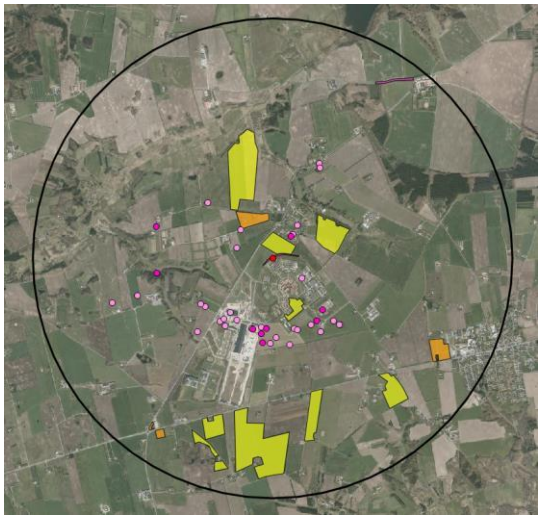


Figure 18: Waggle dances decoded at Idanha (Portugal) in March – May 2019



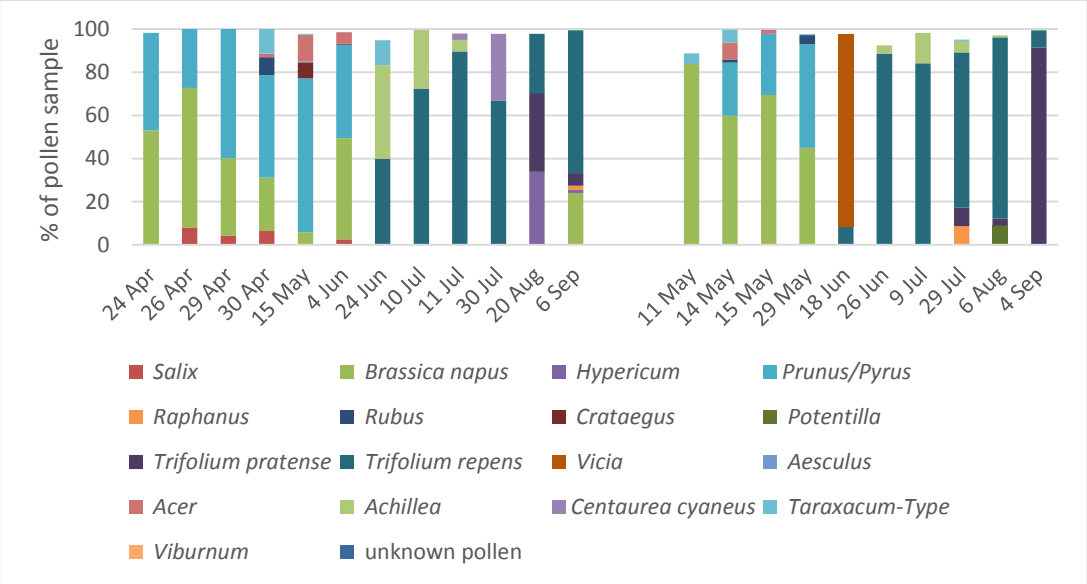


Circles define an area of 3 km in radius. Yellow and orange blocks correspond to oilseed rape crops and grasslands with dandelion, respectively.

Figure 19: Waggle dances decoded before the experimental spraying (26 April 2019, time interval 14:27-15:08), on the day of the spraying (29 April, time intervals: light pink from 14:38-15:37 and dark pink from 15:38-16:06), and one day after (time intervals from light pink to dark pink: 10:58-11:57, 11:58-12:57, 12:58-13:07 and 14:45-15:31) at the high exposure site Foulum, Denmark

3.4.5. Pollen

A seasonal change in pollen composition was observed in both Denmark and Portugal (Figures 20-23). In Denmark (Foulum and Hinnerup, Western Denmark), pollen samples collected in late April to mid/late May were dominated by oilseed rape (*Brassica napus*) and fruit trees (*Prunus*/Pyrinae). This was also supported by two pollen samples collected in the apiaries in Eastern Denmark, Krænkerup on 16 May 2019 (one sample from the observation hive: 75.2% *B. napus* and 14.8% *Prunus*/Pyrinae) and Flakkebjerg on 30 April 2019 (five samples, one from each experimental colony, 27.2% ± 7.0% *B. napus* and 63.2% ± 6.1% *Prunus*/Pyrinae). In mid-summer, the dominant pollen resources changed to white clover (*Trifolium repens*), which has a prolonged flowering, and is an abundant species in grasslands and lawns in Denmark. Furthermore, pollen of other mass-flowering crop species, *T. pratense, Vicia* and *Raphanus* spp. are temporarily common (Figures 20, 21). These findings support that the bees forage mostly on mass flowering wild plants and crop species commonly associated with Danish farmlands.



Pollen was collected from an external pollen trap installed on the observation hive (one sample per observation day), although samples from 26-30 April at Foulum were collected using internal pollen traps in five experimental colonies (in the current figure, stacked bars show the average pollen collection by the five experimental colonies). Results are reported as % of pollen grains belonging to each pollen type in a sample of 500 pollen grains.

Figure 20: Botanical composition of pollen collected at Foulum (left) and Hinnerup (right) during the field season 2019

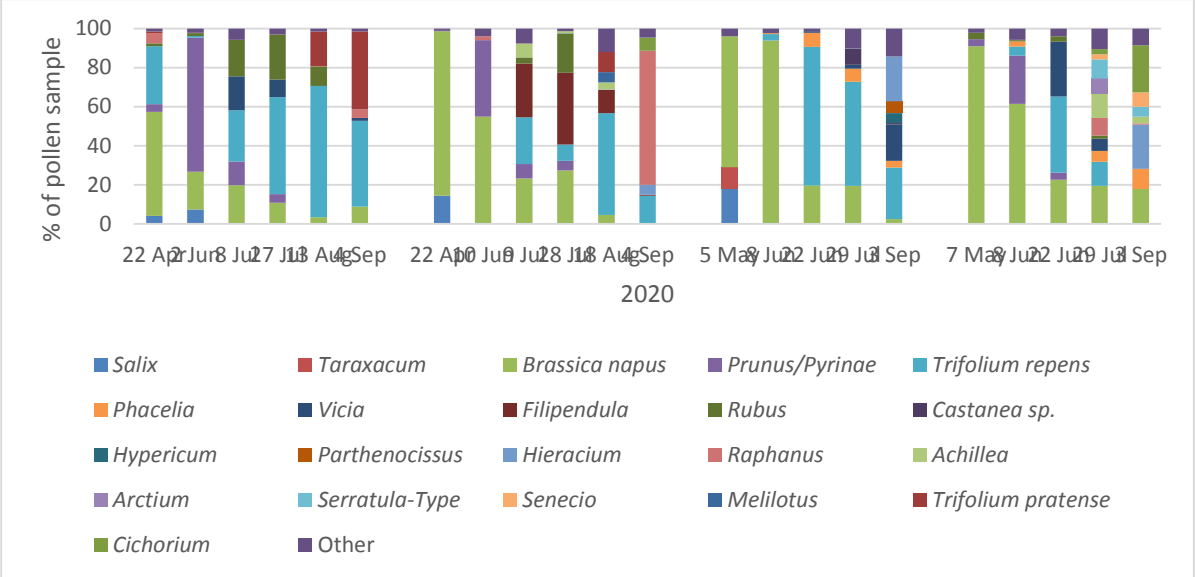
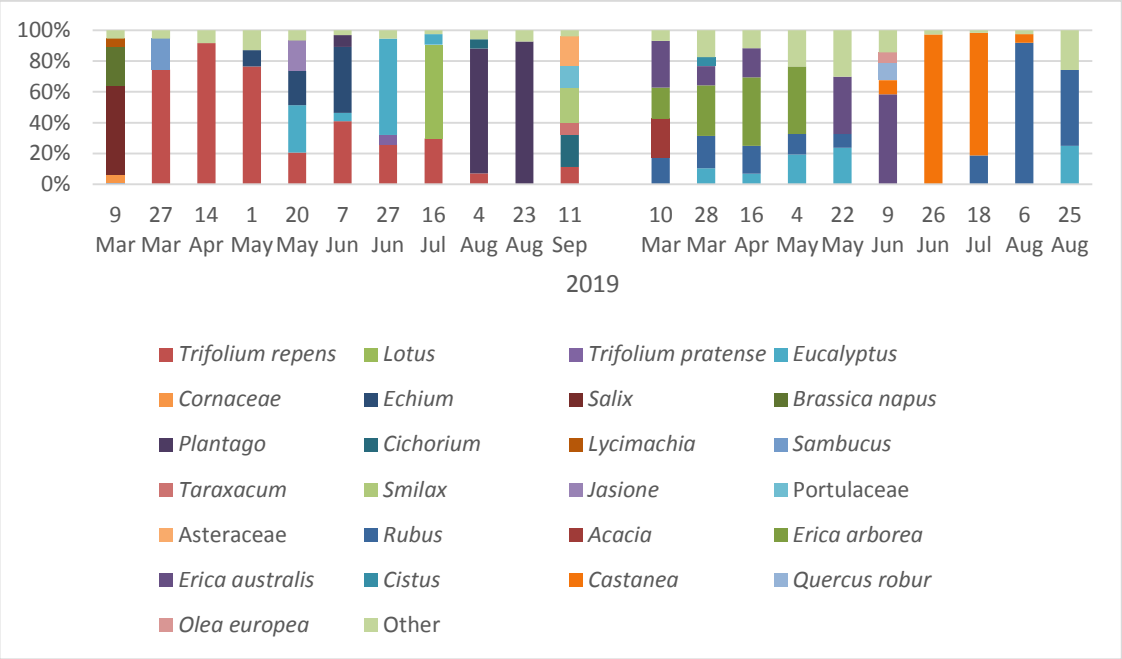


Figure 21: Percentage of pollen grains in a sample of 500 pollen grains, identified in bee bread collected from experimental colonies (pooled samples of five colonies per study site) during the field season 2020 in the four apiaries in Denmark (from left to right: Foulum, Hinnerup, Flakkebjerg and Krænkerup

In Portugal, honey bees mostly foraged on wild flowers of herbs and shrubs. At Idanha, most of the collected pollen was from herbaceous species (*Trifolium* spp., *Plantago* spp., *Echium* spp.). Nonetheless, the pollen is not considered to be from wild flowers only because some of these species are usually cultivated to produce cattle fodder. In the beginning of the season, bees collected pollen from *Salix* sp. trees. These trees are well known amongst beekeepers as an essential resource for the colonies to develop in the beginning of the season because of its early flowering period (Figure 22, upper panel; Figure 23 right). At Lousa, shrub species are the ones that offer more pollen throughout the season (*Erica* spp.). This genus has a long flowering period (the entire spring) and offers pollen and nectar resources for the colonies (data obtained by palynological analysis on honey). In summer, *Castanea* sp. trees are also known as an important source of food for bees by offering pollen and nectar, after the major nectar flow during spring (data also obtained by palynological analysis of honey samples) (Figure 22, lower panel; Figure 23 left).



Pollen was collected from an external pollen trap. Results are reported as % of pollen grains belonging to each pollen type in a sample of 500 pollen grains.

Figure 22: Botanical composition of pollen collected at Idanha (upper panel) and Lousa (lower panel) during the field season 2019

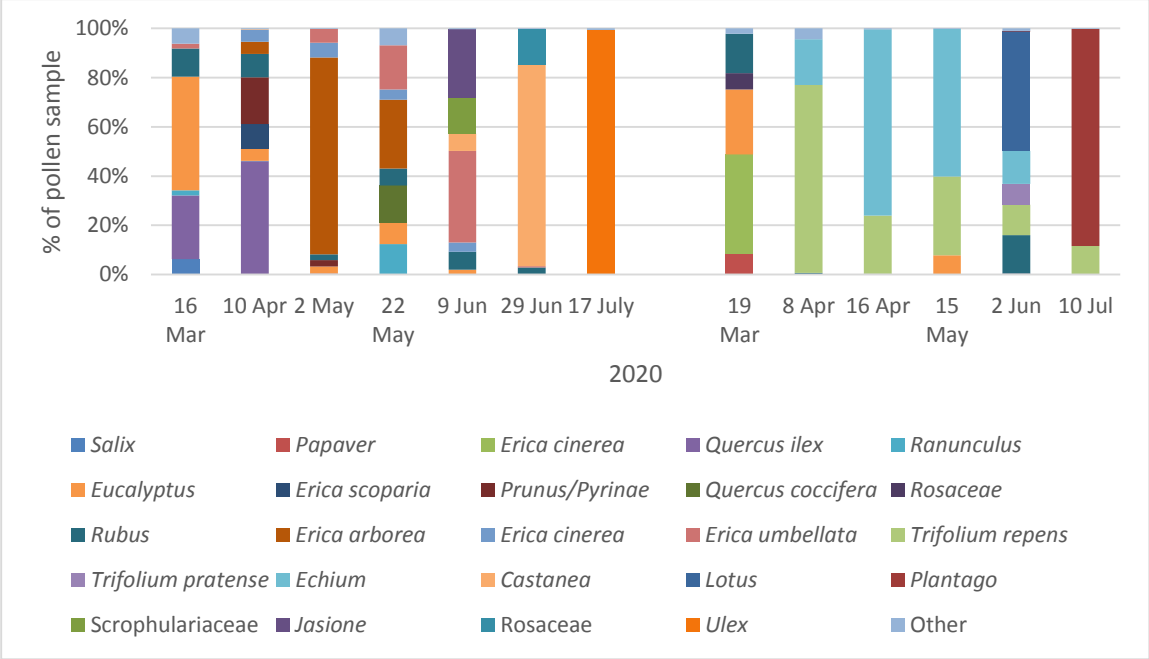


Figure 23: Percentage of pollen grains identified in pollen collected from pollen traps from control colonies (pooled samples of five colonies per study site) during the field season 2020 in the two apiaries in Portugal (left: Lousa, right: Idanha)

3.4.6. Monitoring of foraging activity (bee counter)

In video-recordings from the bee-counter, it was possible to count the dark pixels and relate the counting to directions (up, down, left, right) for one video of 1-hour length within 20 minutes using a standard laptop computer. In order to illustrate the final output by an example, output for a single day is shown in Figure 24 as number of bees counted for 10 minutes. The number of outgoing bees exceeded that of the incoming bees in the beginning of the day. At the end of the day, where the number of bees in field was decreasing, the number of incoming bees exceeded the number of outgoing bees. These results confirmed that the counts were reflecting real conditions.

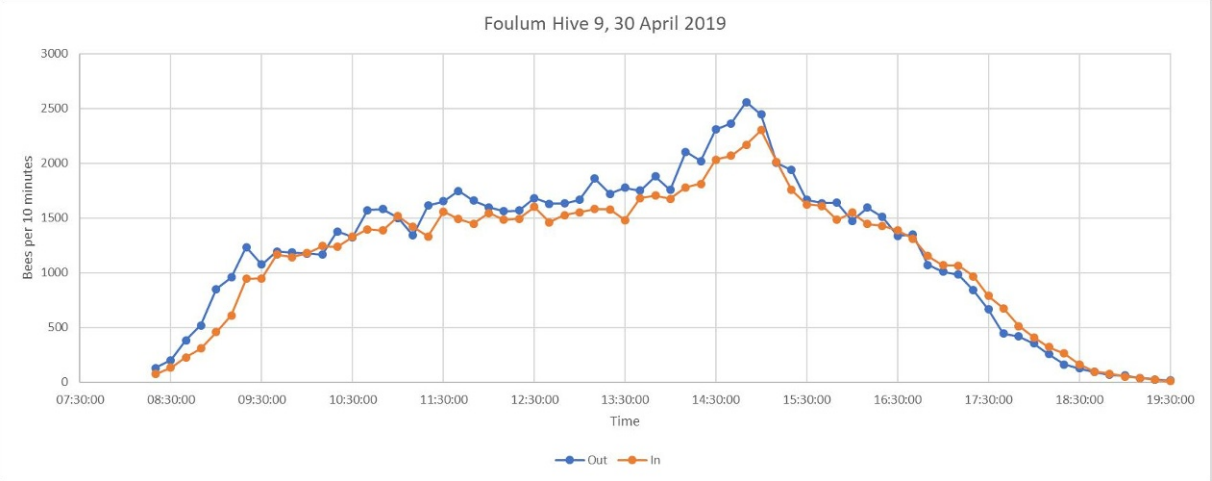


Figure 24: Simulated traffic in and out during the day per 10-minute interval

Figure 25 shows how the field data (observed bees per minute) used for calibration fits the simulation. The estimation error for the number of bees for one minute was higher than the estimation error for the number of bees during a 10-minute interval as shown in Figure 25, simply because the longer averaging time resulted in the cancelling out of the random residuals around the line. As the data was also used for simulation, it is not surprising that the total numbers tended to fit. However, the interesting conclusion from this figure is that the response from the model is linearly related to the counted number of bees. The slope of 0.9764 indicates that the simulation is a fraction of 1-0.9764=0.0236 different from the manual counting (2.6 % accuracy). However, the standard error of the slope is 0.0169, and a statistical test of the H_0 hypothesis that the slope is one yields a p-value of p=0.17, hence it is not significant ($p<0.05$) to conclude that the slope is different from one. This indicates that the model, in which pixel counts are used as a proxy of movement, is sufficiently fitted to detect the traffic.

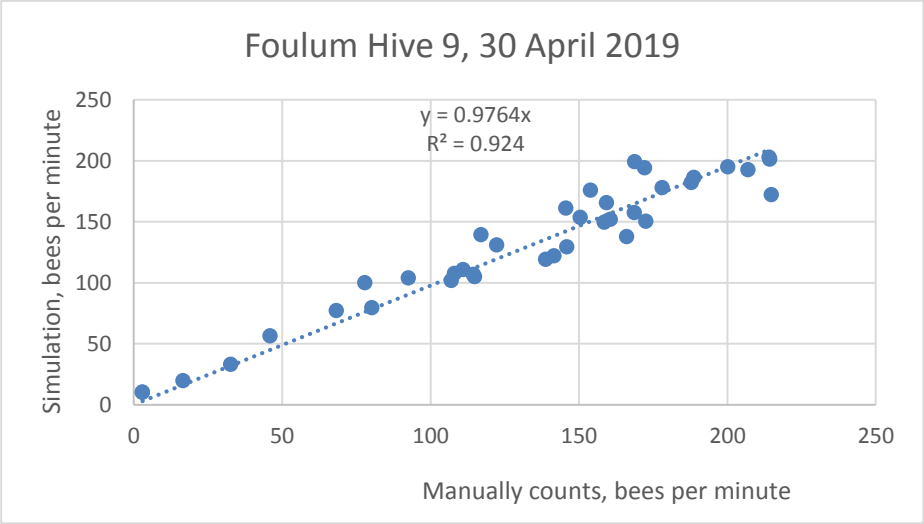


Figure 25: Manual counting during the day compared to simulated values

A typical bee count series is shown in Figure 26 for illustration. The single days in the time series is seen as maxima, having maximum values of 4-8 bees/sec as an average for 10 minutes period of counting. The calibration of outgoing and incoming bees was done separately and thus independently from each other. Hence, it is possible to compare the incoming and outgoing daily estimates of traffic with each other, having in mind that the outgoing number of bees typically will be a few percent larger value than the incoming number of bees. However, the calibration is not sufficient to ensure that the number of outgoing bees for all days is higher than the number of incoming bees event though this tendency is observed as illustrated in Figure 24.

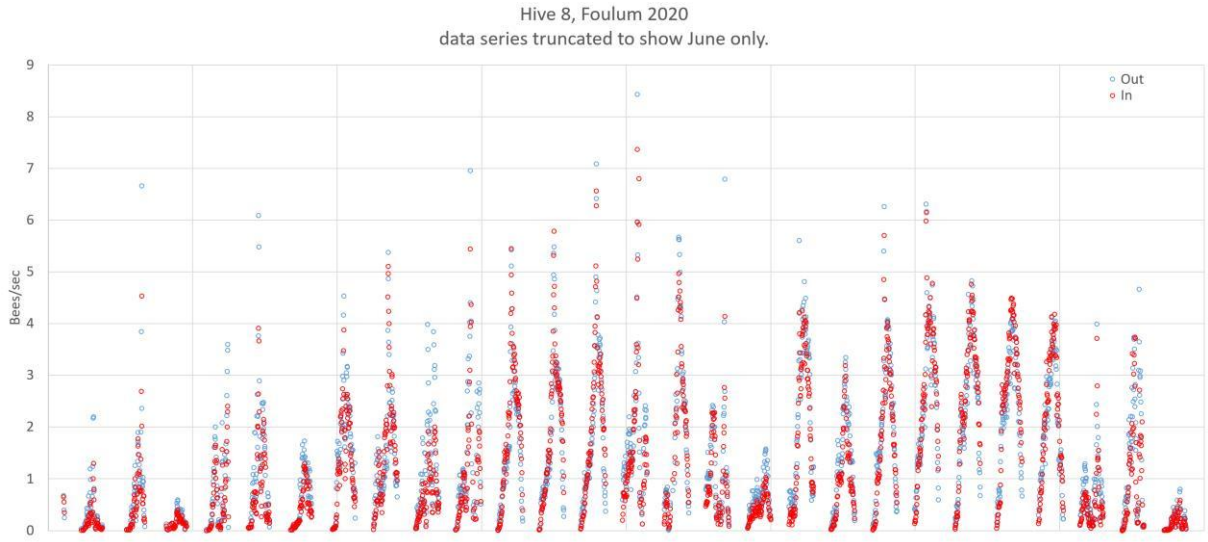


Figure 26: Bee traffic (incoming in red and ougoing in blue) at Foulum (colony #8) during June 2019 within a 10-minutes period (mean values are shown)

3.5. Identification and prevalence of infectious agents

3.5.1. Infestation by *Varroa destructor*

In spring 2019, no varroa mites were found in downfall under the brood using the mite downfall method at all six study sites. In August, before treatment with Apiguard (thymol), low levels of varroa were found in all apiaries (Table 14). In October, after varroa treatment, low levels of varroa were found in all apiaries, except for Idanha (Portugal) (Table 14). Levels of varroa were considered critical when >10 mites per 100 bees were found (Dietemann et al., 2013). Hence, varroa treatment with Apiguard appeared insufficient at Idanha.

Table 14: Table 14 Prevalence of varroa before (August) and after (October/November) treatment with Apiguard (thymol). Number of varroa mites per 100 bees (average ± SD) was determined using either the mite downfall method or the soapy water method. Level of varroa infection was assessed in all colonies, including experimental and back-up colonies (number of colonies indicated in parenthesis)

		Mite downfall method	**Soapy water method**	
			Varroa per 100 bees (average ± SD)	
Region	**2019**	**spring**	**summer**	**autumn**
Eastern Denmark	Flakkebjerg	0 (5)	0.24 ± 0.23 (5)	4.49 ± 3.24 (8)
Eastern Denmark	Krankerup	0 (5)	0.38 ± 0.26 (5)	4.0 ± 2.46 (7)
Western Denmark	Foulum	0 (5)	0 (3)	0.24 ± 0.64 (7)
Western Denmark	Hinnerup	0 (5)	1.23 ± 2.14 (3)	0.34 ± 0.76 (5)
Portugal	Idanha	0 (5)	1.84 ± 1.16 (5)	22.6 ± 23.37 (5)
Portugal	Lousa	0 (5)	3.2 ± 1.98 (5)	2.38 ± 2.24 (5)
Region	**2020**	**spring**	**summer**	**autumn**
Eastern Denmark	Flakkebjerg 2020	0.2 ± 0.45 (5)	0.16 ± 0.15 (5)	0.48 ± 0.33 (5)
Eastern Denmark	Krankerup	0 (5)	0.36 ± 0.39 (5)	0.125 ± 0.25 (4)
Western Denmark	Foulum	0 (5)	0.07 ± 0.17 (7)1	0.24 ± 0.39 (6)
Western Denmark	Hinnerup	0 (5)	0.11 ± 0.30 (7)1	0.72 ± 0.78 (6)
Portugal	Idanha	0 (5)	0 (6)	0.12 ± 0.23 (4)
Portugal	Lousa	0.35 ± 0.63 (6)	0.54 ± 0.95 (6)	0.16 ± 0.36 (6)

[1] 23 Sept 2020.

In 2020, no or low levels of varroa were found in spring, summer and autumn in all the experimental apiaries (Table 14). In general, varroa mite incidence was very low in Denmark in 2020 (P. Kryger, unpubl. data).

3.5.2. Viruses and *Nosema* spp.

Nosema infection of colonies is reported as negative or with five infection levels based on the number of spores detected per bee in the sample (Meixner et al., 2014). In spring 2019, less than half of the colonies in Denmark were negative when tested for *Nosema*. Many colonies had very weak to medium levels of infection by *Nosema* in all three study regions, and only a few colonies showed clinical signs of *Nosema* infection (traces of diarrhea). Only one colony in Western Denmark had a strong infection in spring 2019 (Table 15). In late summer and autumn 2019, the number of colonies having strong and

very strong infections by *Nosema* had increased in the two Danish regions, while in Portugal the number of strongly and very strongly infected colonies were low in both spring and autumn 2019 (1-2 colonies). All *Nosema* infections were identified as *Nosema ceranae,* while *N. apis* was not detected.

Table 15: *Nosema* infection in samples (60 bees per sample) collected in spring, late summer and autumn 2019 in the experimental apiaries

		***Nosema* infection (# colonies)1**							
Region	**Time**	**not determined**	**negative**	**very weak**	**weak**	**medium**	**strong**	**very strong**	**total**
Eastern Denmark	Apr-19	0	10	2	6	2	0	0	20
	Aug-19	0							
	Sep-19	0	5	1	2	0	3	4	15
Western Denmark	Apr-19	5	5	2	4	3	1	0	15
	Aug-19	0	6	0	0	2	3	1	12
	Oct-19	0	4	1	3	2	2	0	12
Portugal	Aug/Eep-19	0	3	4	2	0	1	0	10
	Nov-19	0	3	2	3	1	0	1	10
	spring 19	0	3	4	0	1	2	0	10

[1] Infection levels (categories from Meixner et al., 2014): Negative: No Nosema spores detected ; Very weak: < 500,000 spores per bee; Weak: between 500,000 and 1,000,000 spores per bee; Medium: between 1,000,000 and 2,000,000 spores per bee. Strong: between 2 000 000 and 5 000 000 spores per bee; Very strong: > 5,000,000 spores per bee

Table 16: *Nosema* infection in samples (60 bees per sample) collected in spring, late summer and autumn 2020 in the experimental apiaries

		***Nosema* infection (# colonies)[1]**							
Region	**Time**	**not determined**	**negative**	**very weak**	**weak**	**medium**	**strong**	**very strong**	**total**
Eastern Denmark	**Apr-20**	0	2	2	0	6	3	4	17
	Sep-20	0	8	0	0	0	0	0	8
Western Denmark	**Apr-20**	0	1	1	2	1	4	5	14
	Sep-20	0	6	2	4	0	1	0	13
Portugal	**Spring-20**	0	5	5	0	0	0	0	10
	Oct-20	0	10	0	0	0	0	0	10

[1] Infection levels (categories from Meixner et al., 2014): Negative: No Nosema spores detected; Very weak: < 500,000 spores per bee; Weak: between 500,000 and 1,000,000 spores per bee; Medium: between 1,000,000 and 2,000,000 spores per bee. Strong: between 2 000 000 and 5 000 000 spores per bee; Very strong: > 5,000,000 spores per bee

In 2020, the Nosema infections in Denmark spread further in the spring, only three out of the 29 colonies were free of infection, three colonies had a weak level of infection (< 500,000 spores/bee), eight showed a medium level (between 500,000 and 2,000,000 spores/bee), and 15 colonies exhibited a high level (above 2,000,000 spores/bee, up to a maximum of 12,000,000 spores/bee) (Table 16). In the autumn, the situation had improved, 15 out of the 22 remaining colonies were tested negative, while the level of infection was low in two colonies, and medium in five colonies. In Portugal, in spring five out of the 10 colonies tested negative, five were positive, with a weak level of infection (<500,000 spores/bee). In autumn, all colonies in Portugal tested negative. For both countries, this pattern is as expected for

Nosema, with high prevalence in spring due to the presence of many old bees. Only Nosema ceranae was found.

In 2019, virus prevalence was generally low in spring. Deformed Wing Virus (DWV) type A was not detected in the experimental apiaries in Denmark. Deformed Wing Virus (DWV) type B was detected in many colonies, but only a strong infection in one colony, which was subsequently lost. The remaining colonies had weak infections by DWV type B. No black queen cell virus or chronic bee paralysis virus was detected.

In the spring samples, a high prevalence of sac brood virus (SBV) was found in the experimental apiaries in Denmark where some colonies were strongly infected. Because the colonies did not have a high load of varroa, and no systematic high prevalence of *Nosema* infections or other viruses were found, combined with clinical symptoms of SBV in some colonies, it was suspected that SBV infection was the main cause of poor colony development of the experimental colonies at the Danish study sites. Sac brood virus (SBV) is widespread in Denmark and elsewhere, and it is a problem for beekeeping in some years but not others. In Portugal, prevalence is generally low. Susceptibility to this virus has a heritable component. It is suggested that SBV is included in the ApisRAM model.

In 2020 the pattern of virus infection was similar to 2019. Deformed wing virus (DWV) type A was absent in Denmark East after varroa treatment, and only at very low levels before varroa treatment. In Western Denmark, the situation was reverse with DWV type A presenting very low levels after varroa treatment and absent before varroa treatment. In Eastern Denmark, DWV type B was present at low levels ($<10^4$ virus/bee) before treatment in all colonies, and absent after treatment. In Western Denmark, all but one colony tested negative before treatment; the single positive colony was found with a low level of infection ($<10^4$ virus/bee). After treatment, all 10 colonies tested positive, five at a medium level (10^4-10^7 virus/bee), five at high level ($>10^7$ virus/bee). Acute bee paralysis virus in both Western and Eastern Denmark occurred at very low levels ($<10^4$ virus/bee), in all colonies, of no biological significance. Sacbrood virus occurs widespread in all samples from Denmark, at medium and high infection levels, both in systematic samples and in symptomatic samples. The inclusion of new colonies in Eastern Denmark, did not change that situation, also the new colonies had medium to high infection levels.

In Portugal, the 10 samples for DWV type A were all positive and two at a high level, above 10^7 virus/bee. After varroa treatment the main change was that one additional colony had a virus titer of more than 10^7 per bees. Deformed wing virus (DWV) type B was present in all 10 colonies before varroa treatment, four colonies in each of the two groups showed low ($<10^4$ virus/bee) and medium (10^4-10^7 virus/bee) infection levels, and two colonies expressed high level of infection (>107 virus/bee). After varroa treatment, four colonies were found with a low level of infection ($<10^4$ virus/bee), five colonies with medium levels (10^4-10^7 virus/bee), and one colony at a high level ($>10^7$ virus/bee). In Portugal, acute bee paralysis virus, was found in nine out of the 10 colonies; seven colonies were detected with low levels ($<10^4$ virus/bee) and two with medium levels (10^4-10^7 virus/bee) of infection before varroa treatment. After treatment, one colony tested negative, nine were positive with low levels of infection ($<10^4$ virus/bee). Sac brood virus (SBV) occurred in Portugal, but not in all colonies, and only at low levels of infection ($<10^4$ virus/bee).

The virus categories are described by Amiri et al. (2015).

3.6. Background of pesticide load at all study sites

In 2019, the multi-residue analysis detected very few traces of pesticides in the samples from the three low exposure sites in Denmark. The pesticide used in the spraying experiment, pirimicarb, was not found in any of the samples (Table 17). Similar results were found for the two Portuguese sites (Table 18).

Table 17: Means and ranges of pesticide residues found in 2019 in Denmark (low exposure sites: Flakkebjerg, Krænkerup and Hinnerup and experimental site where spraying occurred: Foulum). All samples were analysed by multi-residue analyses (Appendix C). N is the number of samples analysed. A value of 0 indicates that the pesticide was not detected at the detection limit of 0.005 m/kg.

Region	Study site	Matrix	Time	Pesticides found	Mean, mg/kg	SD	Min	Max	N
Eastern Denmark	Flakkebjerg	Bee bread from combs	Spring	Boscalid	0.0022	0.0049	0	0.011	**5**
		Honey from combs	Spring	None	-	-	-	-	5
	Krænkerup	Bee bread from combs	Spring	2,4-D	0.0042	0.0027	0	0.007	5
		Honey from combs	Spring	None	-	-	-	-	5
Western Denmark	Hinnerup	Bee bread from combs	Spring	None	-	-	-	-	5
			End of spraying season	None	-	-	-	-	5
		Honey from combs	Spring	None	-	-	-	-	5
			End of spraying season	None	-	-	-	-	5
	Foulum	Bee bread from combs	Spring	None	-	-	-	-	5
			End of spraying season	None	-	-	-	-	5
		Honey from combs	Spring	None	-	-	-	-	5
			End of spraying season	Pirimicarb-desmethyl	0.011	0.0088	0	0.024	5

Table 18: Means and ranges of pesticide residues found by multi-residue analysis (Appendix C) in 2019 in Portugal. Samples were collected before (spring) and after the main spraying season. N is the number of samples analysed. A value of 0 indicates that the pesticide was not detected at the detection limit of 0.005 m/kg

Study site	Matrix	Time	Pesticides found	Mean, mg/kg	SD	Min	Max	N
Idanha-a-Nova	Bee bread from combs	Spring	Pendimethalin	0.27	0.21	0.12	0.63	5
		End of spraying season	None	-	-	-	-	5
	Honey from combs	Spring	Coumaphos	0.0036	0.0049	0	0.009	5
		End of spraying season	None	-	-	-	-	5
Serra da Lousa	Bee bread from combs	Spring	None	-	-	-	-	5
		End of spraying season	None	-	-	-	-	5
	Honey from combs	Spring	Coumaphos	0.0014	0.0031	0	0.007	5
		End of spraying season	Coumaphos	0.0006	0.0013	0	0.003	5

The pesticide content of the wax used in 2019 is presented in Table. 19. The two substances (tebuconazol and piperonyl bytoxide) found in Danish wax samples were not detected in any of the other matrices (Table 17). In the Portuguese 2019 samples, only coumaphos was found both in wax and other matrices (Table 18).

Table 19: Pesticide content of wax samples collected in spring 2019 in Denmark and Portugal. N is the number of samples analysed. A value of 0 indicates that the pesticide was not detected at the detection limit of 0.005 m/kg

Country	Pesticides found	Mean, mg/kg	SD	Min	Max	N
Denmark	Piperonyl butoxide	0.014	0.0029	0.01	0.016	4
	Tebuconazole	0.022	0.0057	0.015	0.028	4
Portugal	Chlorfenvinphos	0.13	0.014	0.12	0.14	2
	Coumaphos	0.375	0.078	0.32	0.43	2
	Dimethomorph	0.010	0.014	0	0.02	2
	Permethrin	0.014	0	0.014	0.014	2
	Propiconazole	0.0050	0.0071	0	0.010	2
	Tau-Fluvalinate	0.16	0.021	0.14	0.17	2

In contrast to the 2019 results, 2020 samples contained several pesticides, especially at the sites in Eastern Denmark (Table 20). Samples from Portugal primarily contained traces of coumaphos, as in 2019, and DMPF (a metabolite of amitraz) in 2020 (Table 21).

Table 20: Means and ranges of pesticide residues found by multi-residue analysis (Appendix C) in Denmark before and after the main spraying season of 2020. N is the number of samples analysed. A value of 0 indicates that the pesticide was not detected at the detection limit of 0.005 m/kg

Region	Study site	Matrix	Time	Pesticides found	Mean, mg/kg	SD	Min	Max	N
Eastern Denmark	Flakkebjerg	Bee bread from combs	Spring	Boscalid	0.0046	0.0047	0	0.011	5
				Fluopyram	0.24	0.151	0.096	0.48	5
				Pendimethalin	0.0076	0.017	0	0.038	5
				Piperonyl butoxide	0.0008	0.0018	0	0.004	5
				Pyraclostrobin	0.0006	0.0013	0	0.003	5
				Tebuconazole	0.0146	0.015	0	0.035	5
				Thiacloprid	0.79	0.40	0.33	1.4	5
			End of spraying season	Boscalid	0.069	0.046	0.04	0.15	5
				Fluopyram	0.0064	0.014	0	0.032	5
				Lambda-cyhalothrin	0.078	0.062	0.017	0.16	5
				Pyraclostrobin	0.0044	0.0074	0	0.017	5
				Tebuconazole	0.0016	0.0036	0	0.008	5
				Thiacloprid	0.015	0.034	0	0.076	5
		Honey from combs	Spring	Tebuconazole	0.001	0.0022	0	0.005	5
				Thiacloprid	0.013	0.015	0	0.037	5
			End of spraying season	None	-	-	-	-	5
	Krænkerup	Bee bread	Spring	Boscalid	0.81	0.60	0.29	1.7	5
				Fluopyram	0.57	0.36	0.21	1	5

		from combs		Lambda-cyhalothrin	0.013	0.012	0	0.028	5
				Pyraclostrobin	1.3	1.1	0.38	2.8	5
				Spinosad	0.12	0.26	0	0.59	5
				Thiacloprid	1.0	0.63	0.16	1.7	5
			End of spraying season	Boscalid	0.013	0.0091	0	0.025	5
				Fluopyram	0.0072	0.016	0	0.036	5
				Piperonyl butoxide	0.001	0.0022	0	0.005	5
				Pyraclostrobin	0.003	0.0028	0	0.006	5
				Spinosad	0.0016	0.0036	0	0.008	5
				Thiacloprid	0.19	0.28	0	0.68	5
		Honey from combs	Spring	Boscalid	0.001	0.0022	0	0.005	5
				Fluopyram	0.0064	0.011	0	0.024	5
				Thiacloprid	0.12	0.17	0	0.4	5
			End of spraying season	Thiacloprid	0.021	0.014	0	0.037	5
Western Denmark	Hinnerup	Bee bread from combs	Spring	None	-	-	-	-	8
			End of spraying season	Azoxystrobin	0.002	0.0057	0	0.016	8
				Boscalid	0.0034	0.0039	0	0.009	8
				Thiacloprid	0.026	0.037	0	0.088	8
		Honey from combs	Spring	None	-	-	-	-	8
			End of spraying season	Thiacloprid	0.0058	0.0066	0	0.017	8
	Foulum	Bee bread from combs	Spring	None	-	-	-	-	6
			End of spraying season	Boscalid	0.00067	0.0016	0	0.004	6
				Fluopyram	0.43	0.36	0.19	1.1	6
				Spinosad	0.0027	0.0065	0	0.016	6
				Boscalid	0.00067	0.0016	0	0.004	6
		Honey from combs	Spring	None	-	-	-	-	6
			End of spraying season	None	-	-	-	-	6

Table 21: Means and ranges of pesticide residues found by multi-residue analysis (Appendix C) in 2020 in Portugal. N is the number of samples analysed. A value of 0 indicates that the pesticide was not detected at the detection limit of 0.005 m/kg.

Study site	Matrix	Time	Pesticides found	Mean, mg/kg	SD	Min	Max	N
Idanha-a-Nova	Bee bread from combs	Spring	Coumaphos	0.0076	0.017	0	0.038	5
		End of spraying season	Coumaphos	0.0032	0.0034	0	0.008	5
			DMPF*	0.03	0.02	0.01	0.06	5
	Honey from combs	Spring	None	-	-	-	-	5
			Coumaphos	0.0034	0.0047	0	0.01	5

		End of spraying season	DMPF*	0.009	0.009	0	0.02	5
Serra da Lousa	Bee bread from combs	Spring	Acetamiprid	0.002	0.0027	0	0.005	5
		End of spraying season	DMPF*	0.02	0.01	0.008	0.04	5
	Honey from combs	Spring	Coumaphos	0.004	0.0059	0	0.013	5
		End of spraying season	None	-	-	-	-	5

* Semi-quantitative, i.e. not an exact value.

Part of the explanation for the differences between 2019 and 2020 may be the corresponding differences in pesticide residues in the comb wax (Tables 19 and 22). Furthermore, a change in crop composition in the area may have taken place.

Of the five substances found in Danish wax samples from 2020 (Table 22), all but bifenthrin were also found in other matrices. The coumaphos and DMPF found in Portugal in 2020 (Table 21) presumably stem from traces of these substances in the wax used for combs, since both substances were found in wax (Table 22). The remaining five substances found in wax were not detected in other matrices.

Table 22: Pesticide residues found by multi-residue analysis (Appendix C) of wax samples from Denmark (summer) and Portugal (spring) 2020. One sample was collected at each of the four sites, and each sample consisted of wax from several colonies.

Study site	Pesticides found	mg/kg
Foulum	Azoxystrobin	0.015
	Bifenthrin	0.1
	Boscalid	0.013
	Piperonyl butoxide	0.09
	Tebuconazole	0.026
Hinnerup	Bifenthrin	0.093
	Boscalid	0.015
	Piperonyl butoxide	0.055
	Tebuconazole	0.03
Idanha-a-Nova	Acrinathrin	0.03
	Boscalid	0.008
	Biphenyl	0.01
	Chlorfenvinphos	0.022
	Coumaphos	0.69
	DMPF*	0.43
	Tau-Fluvalinate	0.029
Serra da Lousa	Acrinathrin	0.019
	Biphenyl	0.023
	Chlorfenvinphos	0.024

	Coumaphos	0.089
	DMPF	0.14
	Tau-Fluvalinate	0.01

* Semi-quantitative, i.e. not an exact value.

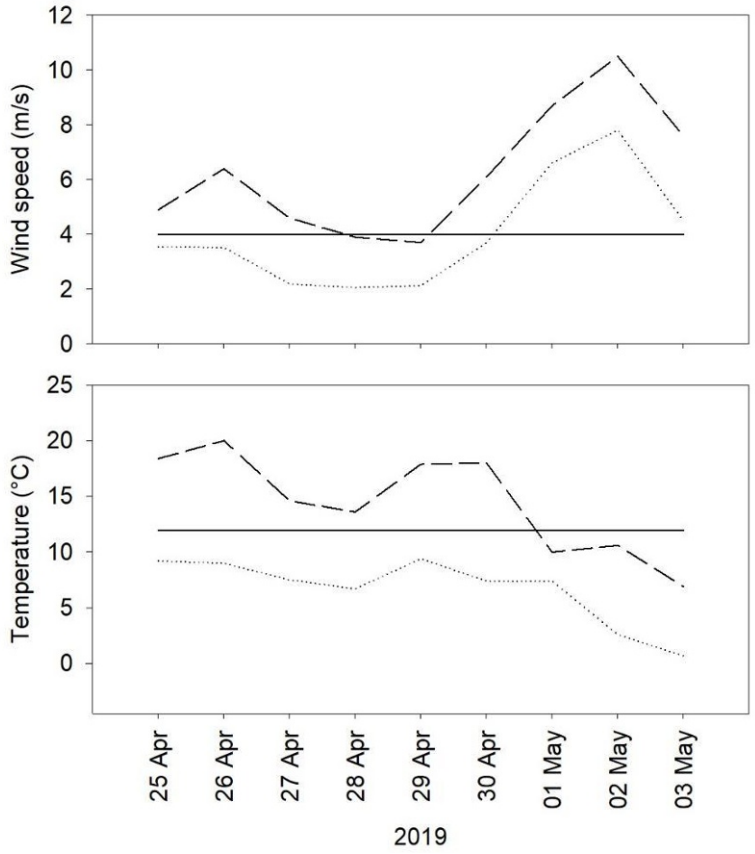
A number of pesticides not approved for use in the EU were found. As mentioned above, coumaphos and DMPF presumably stem from the Portuguese wax pool, since the substances were formerly used against varroa mites in honey bees (Premrov Bajuk et al., 2017). Some Danish 2020 wax samples contained bifenthrin, a pesticide banned in Denmark since 2014 (Danish EPA, 2020). Apparently, the substance has not been used as a varroacide, but may stem from an old pool of reused wax. This may also explain the higher number of pesticides found in the 2020 wax samples compared to the 2019 samples.

The other banned pesticides found in samples in the project were all identified in Portuguese samples. Coumaphos and Amitraz (the mother compound of DMPF) were both used as varroacides earlier but were banned several years ago. Apparently, chlorfenvinphos and biphenyl have not been used against varroa mites but are also banned for use in the EU and like the banned substances found in Danish probably also originate from the repeated reused of wax.

3.7. Experimental pesticide spraying

3.7.1. Weather conditions during spraying experiment

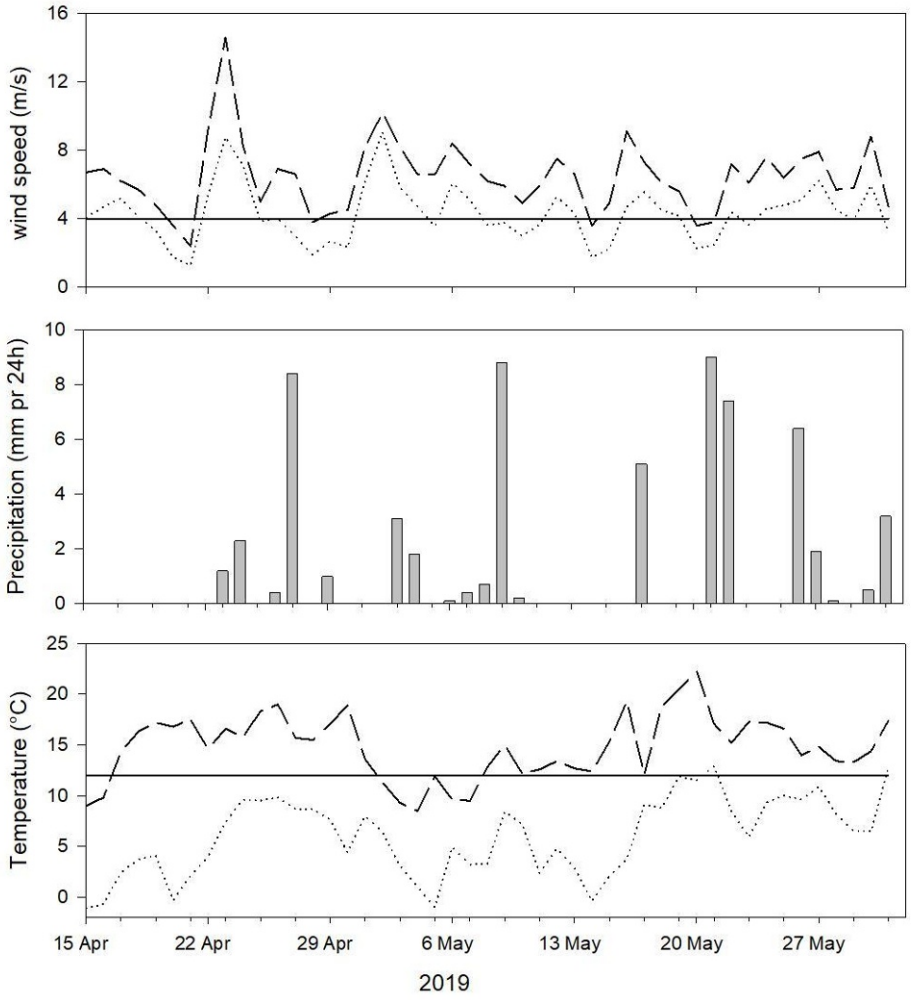
The weather was calm and sunny during the experimental spraying and the following day. However, an unexpected change of weather occurred during the last two days of the spraying experiment, the average temperature dropped down to 10 °C, and wind speed increased. Hence, day 2 and day 3 of the spraying experiment, the weather was sub-optimal for bee foraging (Figure 27). Bee foraging usually ceases when air temperature is below 12 °C, and under windy and rainy conditions. On day 2 after spraying almost no pollen was collected by foragers, and on day 3 no pollen was collected in the pollen traps. This may be a result of the pesticide exposure or the sub-optimal weather conditions. The sudden drop of temperature confounded results regarding forager activity levels.



Minimum (dotted line) and maximum (dashed line) daily temperatures, honey bees forage at temperatures above approximately 12 °C (threshold temperature for honey bee foraging is indicated by the horizontal line in the lower panel).

Figure 27: Weather conditions during the spraying experiment at Foulum (Western Denmark) in spring 2019, average (dotted line) and maximum (dashed line) wind speed, spray application of pesticides requires that maximum wind speed is < 4 m/s (threshold for pesticide application is indicated by the horizontal line in the upper panel)

A second experimental spraying event had been planned in 2019 in Flakkebjerg (Eastern Denmark). However, this experiment was cancelled due to two considerations: Firstly, weather requirements for spraying and post-spray sampling were not met, due to a long period of unpredictable, cold and especially windy period during the peak flowering of oilseed rape, from late April to mid-May (Figure 28). Secondly, the experimental colonies in Eastern Denmark were exceptionally small at the time of the planned experiment, and hence expected to be highly vulnerable to the pesticide spraying and the sampling of bees and their provision. The small colony sizes at the time of oilseed rape flowering were caused by (1) experimental colonies being small before overwintering (2) limited floral availability in early spring in the landscape surrounding the apiary, and (3) a mild winter, leading to early flowering of oilseed rape (i.e. limited time for the colonies to develop after the winter). It was assessed that there was a substantial risk of colony failure due to the pesticide treatment, combined with the removal of foragers and in-hive resources by sampling bees and provision during the experiment. Furthermore, a major concern was that the sampling of foragers for nectar extraction would affect a large proportion of the total number of foraging bees from the experimental colonies, due to their small sizes. Hence, it was expected that sampling would highly affect foraging behavior and resource collection of the experimental colonies. The consortium, therefore, recommended the experimental spraying in Eastern Denmark was postponed to spring 2020.



Upper panel: Average (dotted line) and maximum (dashed line) wind speed, spray application of pesticides requires that maximum wind speed is < 4 m/s (threshold for pesticide application is indicated by the horizontal line. Mid panel: daily total precipitation (mm). Lower panel: Minimum (dotted line) and maximum (dashed line) daily temperatures, honey bees forage at temperatures above approximately 12 °C (threshold temperature for honey bee foraging is indicated by the horizontal line).

Figure 28: Climate data in Flakkebjerg (Eastern Denmark) from mid-April to mid-May 2019

3.7.2. Quantification of pesticide residues in crops and bee matrices during spraying experiment

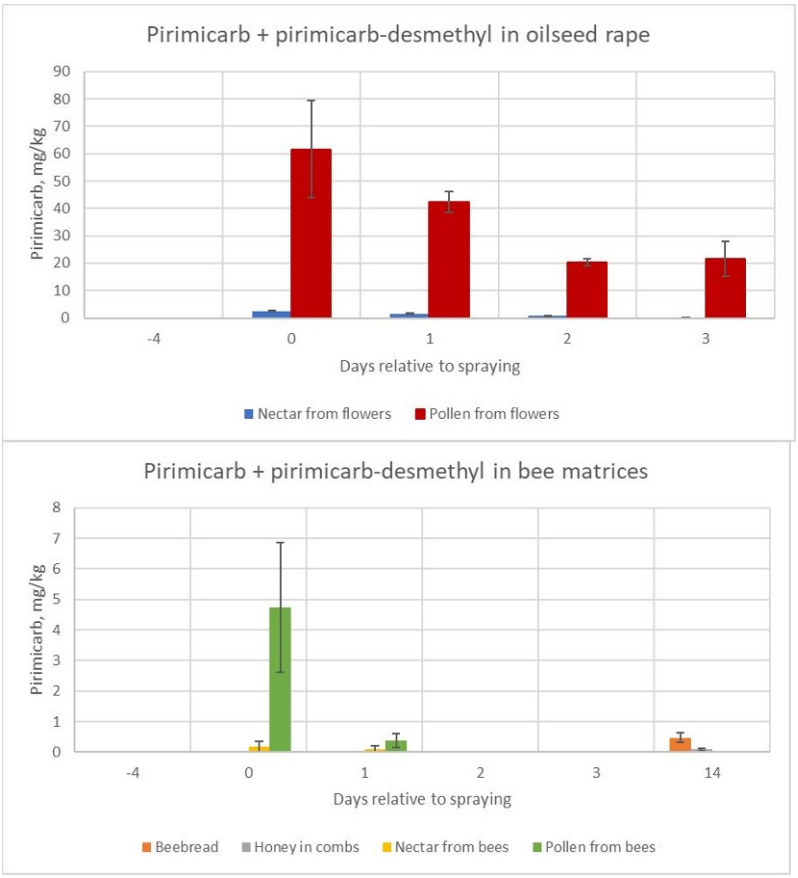
At the high exposure site in Denmark (Foulum), pirimicarb was applied on April 29, 2019 (=day 0). The duplicate samples from the tank solution contained 0.21% and 0.25% pirimicarb, respectively. Only pirimicarb and pirimicarb-desmethyl were detected in the samples of bee matrices and flowers, and only on the day of spraying or later (Tables 17 and 23). In bee bread and honey in combs, the compound did not appear before spraying, but was found on day 14. In pollen collected by pollen traps and from flowers, and nectar extracted from honey bee crops and flowers, pirimicarb was found from the day of spraying (day 0).

Table 23: Means and ranges of pirimicarb and P-desmethyl found by mono-residue analysis in bee matrices and floral resources during the spraying experiment. Means and ranges of pesticide residues found in samples from Foulum (Western Denmark) in 2019 before, during and after pirimicarb treatment as measured by mono-residue analyses (only pirimicarb and pirimicarb-desmethyl). P-desmethyl denotes pirimicarb-desmethyl

Matrix	Time	Pesticides found	Mean, mg/kg	SD	Min	Max	N
Bee bread from combs	4-5 days before spraying	Pirimicarb	0	0	0	0	5
		P-desmethyl	0	0	0	0	5
	14 days after spraying	Pirimicarb	0.22	0.078	0.078	0.35	10
		P-desmethyl	0.26	0.084	0.094	0.36	10
Honey from combs	4-5 days before spraying	Pirimicarb	0	0	0	0	5
		P-desmethyl	0	0	0	0	5
	14 days after spraying	Pirimicarb	0.013	0.0089	0	0.027	10
		P-desmethyl	0.073	0.033	0	0.11	10
Nectar from honey bees	4-5 days before spraying	Pirimicarb	0	0	0	0	5
		P-desmethyl	0	0	0	0	5
	3-4 hours after spraying	Pirimicarb	0.16	0.18	0.025	0.47	5
		P-desmethyl	0.011	0.0095	0.003	0.027	5
	1 day after spraying	Pirimicarb	0.063	0.066	0	0.16	5
		P-desmethyl	0.036	0.045	0	0.11	5
	2 days after spraying	Pirimicarb	0	0	0	0	1
		Pirimicarb-desmethyl	0	0	0	0	1
	3 days after spraying	Pirimicarb	0	0	0	0	4
		P-desmethyl	0	0	0	0	4
Nectar from flowers	4-5 days before spraying	Pirimicarb	0	0	0	0	2
		P-desmethyl	0	0	0	0	2
	3-4 hours after spraying	Pirimicarb	2.25	0.21	2.1	2.4	2
		P-desmethyl	0.30	0.035	0.27	0.32	2
	1 day after spraying	Pirimicarb	1.2	0	1.2	1.2	2
		P-desmethyl	0.40	0.035	0.37	0.42	2
		Pirimicarb	0.47	0.064	0.42	0.51	2

	2 days after spraying	P-desmethyl	0.25	0.042	0.22	0.28	2
	3 days after spraying	Pirimicarb	0.17	0.0071	0.16	0.17	2
		P-desmethyl	0.15	0	0.15	0.15	2
Pollen from pollen traps	4-5 days before spraying	Pirimicarb	0	0	0	0	5
		P-desmethyl	0	0	0	0	5
	3-4 hours after spraying	Pirimicarb	4.54	1.85	2.4	8.3	10
		P-desmethyl	0.19	0.26	0.056	0.92	10
	1 day after spraying	Pirimicarb	0.28	0.19	0.09	0.55	6
		P-desmethyl	0.10	0.048	0.044	0.18	6
Pollen from flowers	4-5 days before spraying	Pirimicarb	0	0	0	0	2
		P-desmethyl	0	0	0	0	2
	3-4 hours after spraying	Pirimicarb	57.9	16.1	46.5	69.3	2
		P-desmethyl	3.75	1.63	2.6	4.9	2
	1 day after spraying	Pirimicarb	37.7	3.312	35.4	40	2
		P-desmethyl	4.6	0.57	4.2	5	2
	2 days after spraying	Pirimicarb	14.5	0.21	14.3	14.6	2
		P-desmethyl	5.9	0.99	5.2	6.6	2
	3 days after spraying	Pirimicarb	16.8	5.94	12.6	21	2
		P-desmethyl	4.9	0.42	4.6	5.2	2

The pirimicarb applied in the experimental oilseed rape field in Foulum was found in both the pollen and the nectar of the flowers, in concentrations decreasing with time after spraying (Figure 29 upper panel). Concentrations were much higher in pollen than in nectar, probably because pollen was exposed more directly during the spraying event, and because nectar is produced continuously, and pirimicarb concentrations are thereby diluted. This difference in pirimicarb exposure is also reflected in a higher pirimicarb concentration in pollen from bees and bee bread than in nectar from bees and honey in combs, respectively (Figure 29 lower panel).



Oilseed rape pollen and nectar were sampled on days -4, 0, 1, 2 and 3 relative to the day of pirimicarb application in the field; bee bread and honey in combs were sampled on days -4 and 14; nectar from bees was sampled on days -4, 0, 1, 2 and 3; pollen from bees was sampled on days -4, 0 and 1.

Figure 29: Pirimicarb and pirimicarb-desmethyl content found in oilseed rape flowers (upper panel) and various honey bee-related matrices (lower panel) before, during and after the experimental spraying with pirimicarb in 2019

It can be concluded that the honey bees were, in fact, exposed to the applied pirimicarb, especially through the collected pollen, but also through nectar, and pirimicarb was still found in the stored resources (bee bread and honey) two weeks after spraying.

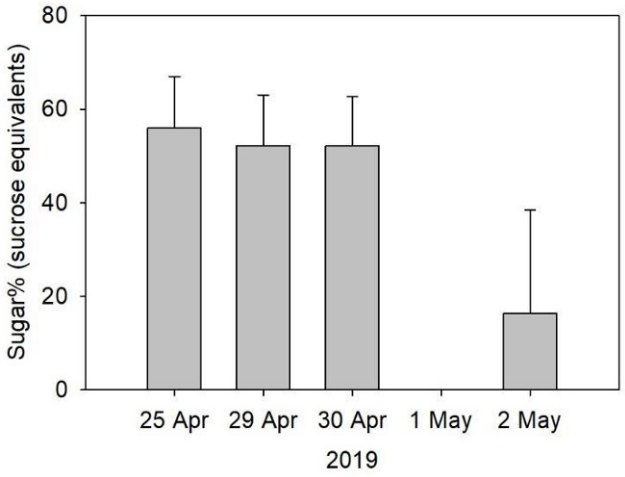
3.7.3. Nectar sugar content in flowers and honey sacs from foragers

Nectar sugar content was measured in honey sacs from foragers from each of the five experimental colonies four days before the spraying experiment (25 April 2019) and on the spraying day, day 0 (29 April 2019). Due to a scarcity of foraging bees, sugar content was measured in 10 individual bees from each of the four colonies on day 1 (30 April 2019), none on day 2 (1 May 2019), and 3 colonies on day 3 (2 May 2019).

Foraging activity is likely to be affected by the change in weather from day 1 to day 2. The weather on day 2 was cold (maximum 11 °C) and windy, and hence not optimal for bee foraging. On day 2, hive entrances were blocked, and 30 bees were caught, but only few bees carried nectar. Hence, on this day, only one sample was collected for pesticide residue analysis, pooling nectar from foragers from all experimental and control colonies (0.5 mg in total). On day 3 of the spraying experiment (2 May 2019),

the weather was still windy, but slightly warmer (12 °C), and forager activity was low, but slightly higher than in day 2. Nectar samples were collected from three colonies (colonies #1, #2 and #5), while forager activity was too low to obtain enough nectar from the remaining two colonies (colonies #3 and #4).

The weather change also appeared to influence the sugar content of the nectar collected by foraging bees. Although nectar collected during warm and dry weather conditions before spraying and in the early period of the spraying experiment generally exceeded 50% sucrose equivalents, nectar collected on day 3 was very diluted (mean 16% ± 22%) (Figure 30).



Due to very low foraging activity of bees on day 2 (1 May 2019), sugar content was not determined on this day.

Figure 30: Nectar sugar content, measured in sugar extracted by foraging bees four days before the spraying experiment (25 April 2019, N=50 bees), on the day of spraying (29 April 2019, N=50 bees), 1 day after spraying (30 April 2019, N=41 bees) and 3 days after spraying (2 May 2019, N=30 bees)

3.7.4. Botanical composition of pollen collected by bees during the spraying experiment

Following the experimental spraying on 29 April 2019, the amount of pollen collected in pollen traps dropped markedly. On day 2 (1 May 2019) almost no pollen was collected by the experimental colonies, and on day 3 (2 May 2019) no pollen was collected.

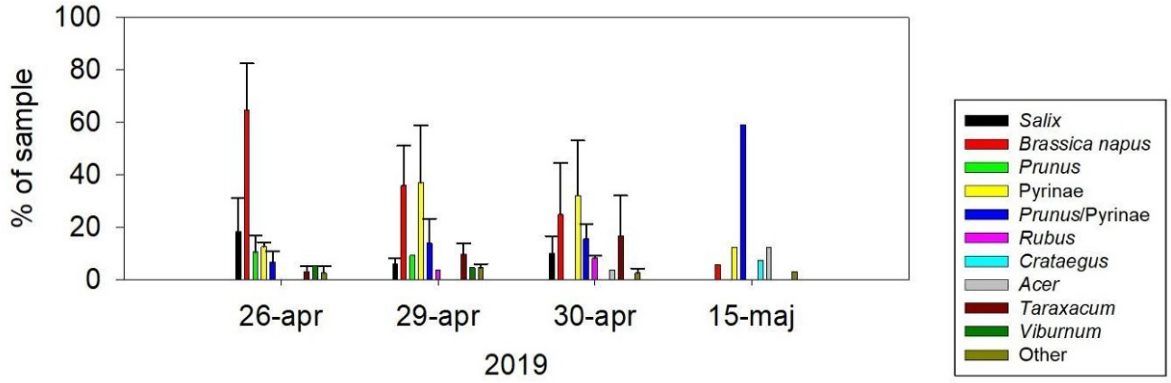


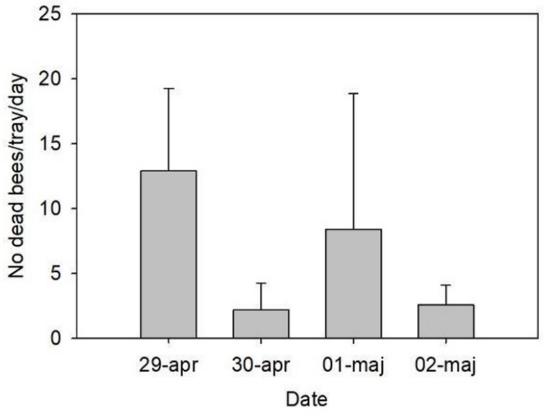
Figure 31: Botanical composition of pollen (% of a sample of 500 pollen grains) collected by experimental colonies during the spraying experiment in 2019 in Foulum, Denmark

Furthermore, the botanical composition of pollen changed following the experimental spraying on 29 April 2019. The proportion of pollen collected from oilseed rape by the experimental colonies decreased while the proportion of fruit tree pollen increased, suggesting that bees may had been repelled from the sprayed oilseed rape field (Figure 31).

These shifts in pollen collection were not observed at the nearby low-exposure site Hinnerup. Hence, the shift may indicate that pirimicarb to some extent repelled the honey bees from collecting pollen from the experimentally sprayed field. This is in contrast to the observations by Clinch & Palmer-Jones (1974), who found no repellent effect of pirimicarb on honey bees. No studies indicating repellency of pirimicarb to honey bees were found.

3.7.5. In hive mortality during spraying experiment

In-hive mortality during the spraying experiment in Foulum, Western Denmark is shown in Figure 32. The day-to-day in-hive mortality was variable, and no major short-term increase in in-hive mortality was detected during the spraying experiment.



The figure shows the in hive mortality during the spraying experiment in Foulum, Western Denmark. Experimental spraying was conducted 29 April 2019.

Figure 32: In hive mortality, measured as number of dead bees collected in plastic trays placed in front of the hive

4. Discussion on the lessons learnt and areas for improvement

4.1. Lessons learnt from the field data collection project

The field data collected in the current project will support the testing and calibration of the ApisRAM model, which will be used to operationalize a new system level approach in environmental risk assessments for bees (EFSA SC, 2021). In the following, lessons learned from the field study will be described. It should be kept in mind that the value and usefulness of variables may differ between a field study perspective *versus* variables most valuable for model testing and calibration. In the current study of data capture in the field, the assessment includes difficulties/challenges of obtaining data of different variables, in addition to the potential for automated data collection. In particular, separate assessments and specific recommendations given below for each of the parameters measured in the experimental apiaries of the current study, and the methods used. Experiences from the current field

study emphasize that different challenges apply to the different variables monitored in the field, and that different variables required different efforts in data collection. Hence, the value of the different variables should be considered in the choice of parameters included in future monitoring schemes in field studies.

The current study included a large number of variables and a detailed monitoring of the experimental bee colonies. For testing and calibration of ApisRAM, the data collection emphasized data on population dynamics and development of the experimental colonies in different landscapes, while other variables (e.g. floral mapping, pollen collection, etc.) were measured less intensively. Although previous studies have monitored similar aspects, the current study is unique due to the detailed and simultaneous monitoring of different aspects of colony development and health. The different variables represent different dimensions of the same entity, making the dataset extremely useful for assessing predictions made by the model.

In particular, colony assessments were made using two complementary approaches: using hive scales and 19-20 day detailed population assessments. These two types of data supplement each other in terms of temporal and spatial accuracy. Hive scale data are measured often (every hour), but weight data are very general including brood, adult bees, provision, wax etc. Population assessments, on the other hand, are only done every 19-20 days, but accuracy is very high. Combining these two types of data can be used as a strong systematic method for simulating honey bee colony development. As colony monitoring was central in this study, an assessment of the parameters measured is given below (section 4.2).

Different variables may be used as honey bee health stage indicators, including parameters related to adult population development, brood and provision, behavioural parameters (e.g. foraging activity), in addition to levels of infestation by infectious agents. Disease data may be used to calibrate ApisRAM, as data obtained in this project indicate that this factor contributes to among-colony variability observed within apiaries.

Automated tools for data collection may be an asset for standardized data collection and assessment of bee health by stakeholders. However, whereas automatic methods are likely to become widespread in future monitoring schemes of honey bee colonies, adaptation and development of currently available methods are needed. In the recommendations provided below, recommendations for future data collection includes which variables can be collected with confidence, and which variables are in need of further development.

4.2. Key data and uncertainty of variables

Assessments of the adult population by the Liebefelder method or weighting frames with and without bees can have up to 30% of error if all the foragers are out in the field collecting resources. Nevertheless, this only happens during spring when colonies are collecting large amounts of resources. In summer, the colony activity is reduced. However, using hive scales, it is possible to roughly estimate the number of foragers that left the colony prior to population assessment. Furthermore, activity data (number of bees departing from and returning to the hive) can be used to estimate the number of foragers, which have left the colony at a specific time of the day during the season.

For the assessment of food provision (i.e. honey/nectar amount in kg), negligible errors were found. For instance, in a colony with 18 frames (nest plus honey super), every comb was weighted without bees and the weight of the empty comb was subtracted. The scale had an error of 10 g. If each comb weights approximately 1000 g, having a 10 g error results in a 1% error in our assessment. Regarding the amount of bee bread cells, despite the different bee bread colours between Portugal and Denmark, the deepbee® software could be trained to recognize this class of cells due to its distinct appearance, and hence obtained a high performance.

The deepbee® software had an up to 90% of accuracy for all cell classes. The main software challenge was the recognition of small larvae (newly hatched), that even for the human eye are difficult to distinguish. In some cases, the software also could not distinguish some honey cells with capped brood. In this study, we also conducted the Deepbee® analysis using honey frames. We recommend using weight for honey assessment rather than image analysis of honey combs. This will save time during colony assessment during field work, and improve software accuracy, since honey combs will not be photographed in the field. On the other hand, the software has proven a beneficial and accurate tool for assessing numbers of eggs and larger larva (not newly hatched).

Data on hive weight measured by hive scales were associated with a low error (+/- 100 g), and gives a proxy of how the colony is developing. The main challenge lies in converting the weight data from an entire colony (including weight of bees, brood, provision and comb) into useful information. We can use this weight to calculate periods of high nectar flow, the number of forager bees leaving the colony in the morning, detect swarming events, and have an overall idea of the amount of honey/nectar in the colony. Furthermore, scale data may provide important information on population levels and brood development if paired with in-hive sensors (e.g. temperature), although weights do not directly reflect population sizes.

Colony activity data is still a challenge despite the large number of available tools (bee counters). There are numerous bee counters on the market, but data on their accuracies are still lacking. As shown during this project, a simple bee counter could be developed to measure activity with a reasonable accuracy (above 90% in some cases). Nonetheless, achieving 100% accuracy is a very laborious (and almost impossible) task. Improvements in this task would be a game-changer to evaluate the impact of stressors on colony activity and foragers mortality.

Most previous studies measuring seasonal brood development focus on open brood *vs*. capped brood which gives an indication of the temporal pattern of number of newly hatched bees and egg laying rate of the queen (Keller et al., 2005). In the current study, using image analysis, eggs, larvae and capped brood could be distinguished. This information is a step forward to understand the brood cycle by an improved prediction of the queen egg rate and the number of newly hatched bees each day.

In future studies, we should aim for automated data collection, decrease colony disturbance, increase replicates and the number of apiaries to represent more landscape categories. European landscapes can be very heterogeneous, and even small countries like Portugal have a high climate variability, which affects the phenology and the availability of floral resources even in landscapes separated by only a few kilometres. Based on patterns of spatiotemporal variability of floral resources, different scenarios can be identified, and sentinel apiaries may be created. In these apiaries, automated data collection, using hive scales and other sensors (e.g. temperature, vibrational), to collect data on colony development though the season without disturbing the colonies should be aimed for. Furthermore, detailed in-hive monitoring of colonies should be developed in order to calibrate automatically collected data. In order to obtain information on colony status without disturbing the colony, and to reduce the efforts of field assessments, further knowledge about the link between colony weight and population, brood and provision is needed. Data from the current project could be used to create the basic algorithms for these research apiaries. This way, we can create a good data flow on colony development from different landscapes for research purposes, providing beekeepers with a useful tool for decision making, in order to improve beekeeping management.

4.3. Areas for improvement

4.3.1. Database

- In the current project, the database for reporting field data was developed in the initial phase of the project, based on technical specifications for field data collection for validation of the ApisRAM model provided by the MUST-B working group. These specifications were to a large

extent based on model requirements. Due to constraints and challenges experienced in the field, the experiment was adapted continuously, including the experimental set-up, variables, methods used, dimensioning and input data formats. Hence, data often did not conform to the database structure, and considerable time was spent developing the tables of the database, in order to accommodate field data. We recommend that a database is developed in a later phase of the project, when experimental set-up, methodologies and input data formats have been decided.

- The database included features for minimizing errors due to manual entry, e.g. choice from a pre-defined drop-down menu, alerts for missing fields etc. However, it was not equipped with sorting functions, neither was it possible to delete a selection of records in one operation. Hence, finding and deleting errors was a labor intensive and sometime impossible task. The database should have been equipped with facilities for sorting, and for deleting plural selected entries at the same time.
- It would have made the work with the database much swifter if the contractor had chosen to program the database instead of hiring an IT-company to develop it. This made it rather slow to make even smaller but important changes to the database because all changes or improvements required communication with the IT-company, who had to fit the time required for the change into their workflow. This was not always as swift as the contractor would have wished.

4.3.2. Local weather and in-hive conditions, in-hive climate

- A general problem to consider, when monitoring in-hive data is that of placing the in-hive sensors. Whereas the air humidity is more or less uniform within the hive, and the sensor is placed on top of the comb frames, the temperature measured by the internal temperature sensor is highly dependent on placement of the sensor within the hive. Especially during early and late season, when the brood area is small and variable, even a small misplacement outside the brood area will result in a (steep) drop in temperature. It was not always possible to place the temperature sensor exactly within the brood, particularly in early and late season, due to changes in the small brood area, and the hives were opened as briefly as possible, not to cool down and hence damage the brood. In addition, the relative position of the sensor may change when brood area diminishes in late season, if the brood area around the sensor shrinks. Hence, we caution against using the in-hive temperature measurements in early and late season. During the main season, the temperature measurements are more reliable. More detailed in-hive climate measurements may be obtained by placing several sensors, e.g. in a grid within the hive. This has been done in the Horizon 2020 project B-GOOD.

4.3.3. Landscape analysis and floral mapping

- In future studies, a stepwise approach for mapping floral resources in intensive agricultural and other landscapes, where periods with very limited floral resources are observed, is recommended. The first year should be used to get an overview of the resource availability for honey bees across the season in the landscape within 1.5 km of the apiary. Periods with abundant floral resources as well as periods with limited resources should be identified. Different methods should be used for mapping floral resources in the two periods. During flower-rich periods, the method used in the present project seems appropriate (but see below for suggested improvements). For periods with resource scarcity in the 1.5 km circular landscape surrounding the apiary, resources further away in the mapping should be included. The mapping of such resources can easily become time consuming as the area at a distance to the apiary of e.g. between 1.5 km to 3 km increases by a factor 3. Therefore, for the mapping. other methods, including technologies as image recordings by drones should be considered.

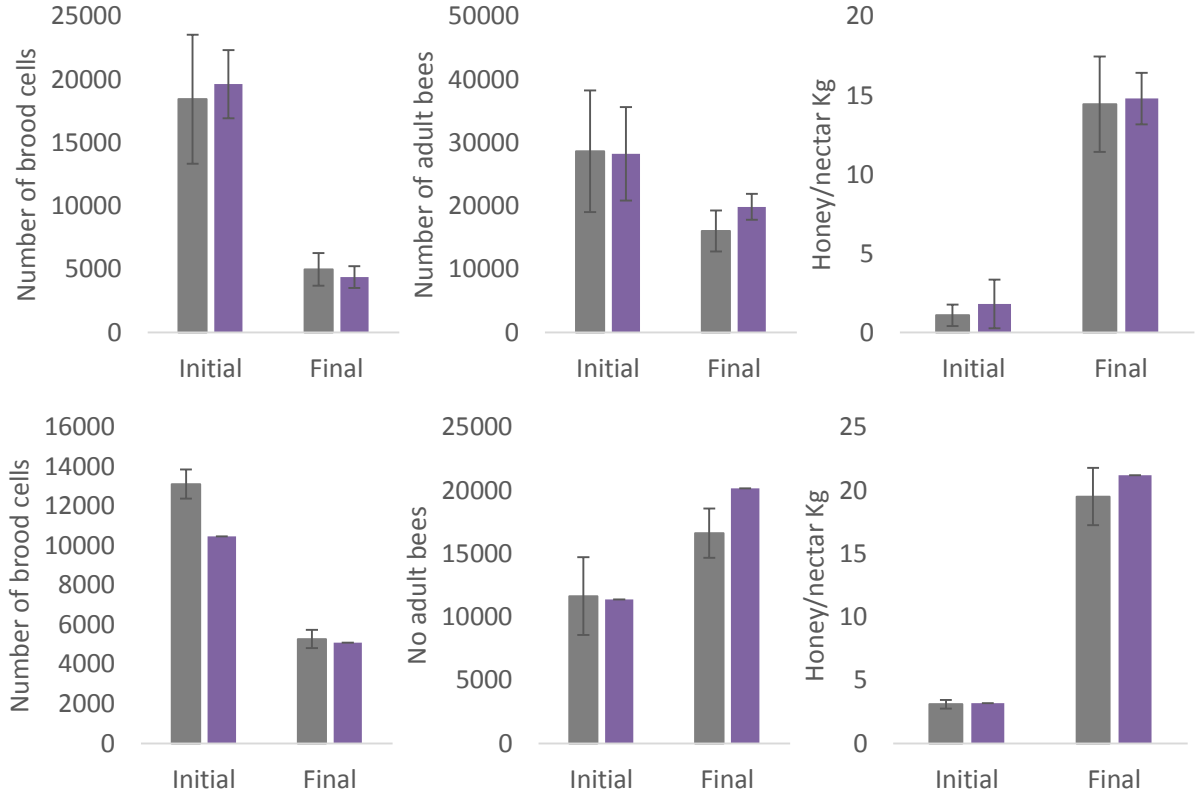
- Availability of nectar and pollen resources in different plant species is highly variable. Number of flowers per unit area, as used in the present study, forms a rough estimate of the actual resources for the honey bees. Regarding the below comment on resources, I suggest that the last sentence is changed to: "In future studies, it should be considered to map resources as kg of sugar per unit area as done by Baude et al. (2016) and kg of pollen per unit area as done by Denisow (2011), respectively."
- Length of the flowering period also varies considerably between species and the monthly sampling. As carried out in the present study, it may not encompass the flowering of important species having a short flowering period. It should be considered to include sampling of timing and duration of flowering of plant species of importance to honey bees in a first-year sampling. Thereafter, the sampling frequency should be decided in order to cover important resources more efficiently. Furthermore, in some landscapes it may be relevant to prolong the sampling season to include all relevant species.

4.3.4. Colony observations

- We caution against the use of a single sensor for measuring in hive temperature in early and late season, as the brood area is small and continuously changing in size and position within the hive. During the main season, the internal temperature measurements are more reliable (section 3.3.2). In-hive temperature measurements in the current study are not sufficient for calibrating ApisRAM.
- For image capture of combs, an initial preparatory phase is needed for adapting the method to local conditions. The length of the tunnel is adjusted for the dimensions of the comb frames. Furthermore, it is important to use a precise holder for the frames inside the tunnel, to obtain an 11-degree angle of the comb (for capturing cell contents in the images).
- In order not to affect the colonies negatively by the monitoring, care should be taken not to cool down or overheat brood and queen during image capture. Photographing should be done preferably at air temperatures above 14°C and following a protocol to reduce heat loss during cold weather or overheating under hot conditions.
- During the project, frequency of population monitoring, and brood/provision assessment was discussed. Whereas detailed monitoring data are desirable for accurate model calibration and validation, frequent population assessments also induce a high level of disturbance of the colony. In a study conducted by the Portuguese team in Burgos, Spain (INTERREG project Poll-Ole-GI SUDOE) in 2018, population monitoring was carried out every 14 days. The results from this project showed an impact of monitoring, compared to non-monitored control colonies on bee strength and nectar/honey stores. This indicates that frequent monitoring (every 14 days) induces a measurable effect on the colony development. In the current study, an interval of 19-20 days between two consecutive monitoring events was used to reduce potential disturbance. Comparing experimental colonies and control colonies (control colonies were only monitored in the beginning and at the end of the field season), no difference was found in colonies strength and honey production and control colonies in Lousa (Figure 33). Hence, we suggest using an interval of 19-20 days when monitoring population development of honey bee colonies.
- The brood and provision assessment by the Deepbee® method provides a very accurate assessment of different brood cell types (eggs, larvae, capped brood) and provision (pollen, nectar, capped honey). However, for optimum performance, the software should be adapted for local conditions. Performance can be improved by training using manually annotated images of combs.
- Monitoring of in-hive mortality is challenging, as dead bees accumulated in plastic trays in front of the hive may be lost due to predation by birds, ants and spiders. Furthermore, dry bees may

be blown away by the wind. To minimize this problem, the use of large and deep trays is recommended.

- A simple, semi-automatic method was developed, in order to assess spatial foraging patterns of a full-size colony placed in an observation hive in the experimental apiary. Whereas the videorecording of the waggle dances on certain days and sites can be challenging, the method could be a focus with further development. Specifically, the combination of waggle dance recording and decoding, sampling and analysis of botanical composition of pollen from returning foragers, and floral mapping in the surrounding landscape, is a new approach developed and tested within this project.
- The assessment of forager activity using bee counters is more accurate in the first part of the season. In late summer, the bees tend to crowd on the scene of the video recordings, challenging the count of bees with sufficient accuracy by image analysis.
- the counts of outgoing and incoming bees can be used to assess short term effects on pesticide exposure, measured as loss of foragers in the field. However, this requires a close calibration of the bee counter on every observation day. There is a trade-off between the precision of calibration and calibration effort. The effort used in calibration increases proportionately with the precision of counting events. Accurate calibration is necessary, as forager loss is calculated by subtracting two large numbers from each other (outgoing minus incoming), hence the uncertainty of the counting becomes critical.



Bars are standard deviations. Experimental colonies were subjected to intensive in-hive monitoring every 19-20 days during the field season, while control colonies were monitored only in the beginning and end of the field season.

Figure 33: Number of brood cells, adult bees and honey/nectar in experimental colonies (grey columns) and control colonies (purple columns) in Lousa in 2019 (lower panels) and 2020 (top panels)

4.3.5. Identification and prevalence of infectious agents

- The number of health indicator variables, with four different viral pathogens and parasites like varroa and Nosema, will necessitate a considerably higher number of observations to draw conclusions. In particular, the lack of independency between the two observation years on the same set of colonies is problematic in this regard, i.e. colonies that are sick in the first year, are not going to be pest free the following year.

4.3.6. Pesticide exposure

- Multi-residue pesticide residue analysis of bee matrices detected only a few pesticides from the background exposure in the experimental landscapes. Considering the costly analysis of pesticide residue analysis, the number of samples may be reduced (e.g. pooling samples from colonies in the same apiary).
- In a future study, a less invasive method for nectar collection from honey bees should be considered. One option is to use the pre-spray images of the combs to identify newly collected nectar in the combs. To validate this suggestion and make sure that nectar pesticide content is not diluted, pesticide contents of nectar from bees and newly filled comb cells may be compared. Sugar content of the extracted nectar may be checked using a hand-held refractometer, in order to avoid sampling honey or sugar feed. Newly collected pollen may be collected from the comb at the same time as nectar extraction, in order to avoid an impact of placing a pollen trap. Bees deposit the newly collected pollen closest to new, unsealed brood, and it can be collected easily using a bee bread collector (Loglio et al., 2019) or a pair of tweezers.
- We recommend that spraying experiments involving honey bee bees are conducted preferably later in the growing season. In early spring, honey bee colonies are small and hence more sensitive to sampling. Furthermore, the likelihood of adverse weather conditions decreases later in the season. The timing of experimental spraying will affect the choice of crop species and pesticide chosen for the experiment. In the present project, the consideration that data were to be used for modelling honey bee development especially early in the season was given high priority.
- A spraying experiment requires thorough planning and involves a concerted effort in field sampling. However, flexibility in the timing of the experiment can mitigate the risk of unfavourable weather during the experiment.
- To ensure exposure of the bees, a non-repellent pesticide is preferred. Results of the current study indicated a repellent effect of pirimicarb, although this has not been reported previously. Further studies are needed to conclude if high concentrations of pirimicarb are repellent to honey bees.
- For the present study, a minimum of four data points in time were required, in order to calculate the decay of the pesticide. In future studies, the number and timing of samplings should be adapted to local weather conditions, or maybe the study site should be placed in an area with more predictable weather conditions. Furthermore, frequent sampling involves a high degree of disturbance, and hence is likely to affect colony fitness.

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Abbreviations

ABPV	Acute Bee Paralysis Virus
ApisRAM	Apis Regulatory Assessment Model
ALMaSS	Animal, Landscape and Man Simulation System
AU	Aarhus University
AU-Agro	Aarhus University, Department of Agroecology
DGAV	Direção-Geral da Alimentação e Veterinária
DMI	Danish Meteorological Institute
DMPF	N-2,4-Dimethylphenyl-N'-methylformamidine, metabolite of amitraz
DWV	Deformed Wing Virus
EC	European Commission
EFSA	European Food Safety Authority
EPA	Environmental Protection Agency
EU	European Union
EURL	European Union Reference Laboratory
GIS	Geographical Information System
IACS	Integrated Administration and Control System
IATV	Instituto do Ambiente Tecnologia e Vida
LAB	Laboratorio Analítico Bioclinico
LPIS	Land Parcel Identification System
LIB	Länderinstitut für Bienenkunde, Germany
LOQ	Limit of quantification
MUST-B	EU efforts towards the development of a holistic approach for the risk assessment on MUltiple STressors in Bees
PPP	Plant Protection Products
RPLU	Resource Providing Landscape Unit
SBV	Sac Brood Virus
SC	Scientific Committee
TB	Terabyte = 10^{12} byte
TF	Task Force
WP	Work package
WG	Working Group
XML	Extensible Markup Language

Appendix A – The 10 crop types with the largest areas and honey potentials at the high and low exposure areas in Western Denmark

Western Denmark high exposure area (10x10km), Foulum			Western Denmark low exposure area (10x10km), Hammel		
Crop	**Area (ha)**	**Honey potential (kg)**	**Crop**	**Area (ha)**	**Honey potential (kg)**
Winter rape	224	44788	**Winter rape**	257	51300
Grass with clover/lucerne, under 50% legumes (in rotation)	786	39291	**Bog with high nature value**	129	12875
Forest	1258	31438	**Forest**	2646	66158
Bog with high nature value	274	27369	**Grass with clover/lucerne, below 50% legumes (in rotation)**	72	3622
Meadow with high nature value	53	5294	**Meadow with high nature value**	32	3231
MVJ not set-aside, not farmed	64	3194	**Permanent grass and grass-clover without norm, below 50 % clover**	84	2092
Permanent grass, below 50% clover/lucerne	56	2788	**Grass below 50% clover/lucerne, low yield (in rotation)**	24	1222
Permanent grass and grass-clover without norm, below 50 % clover	87	2180	**Permanent grass, below 50% clover/lucerne**	19	931
Grass and grass-clover without norm, below 50 % clover (in rotation)	34	1694	**Permanent grass, normal yield**	132	661
Bog	65	1627	**Afforestation on former agricultural land**	10	484

Appendix B – The 10 crop types with the largest areas and honey potentials at the high and low exposure areas in Eastern Denmark

Eastern Denmark high exposure area (10x10km), Flakkebjerg			Eastern Denmark low exposure area (10x10km), Krænkerup		
Crop	**Area**	**Honey potential**	**Crop**	**Area**	**Honey potential**
Winter rape	925	185038	**Winter rape**	902 ha	180300 kg
Forest	293	7322	**Forest**	607 ha	15172 kg
Bog with high nature value	47	4700	**Bog with high nature value**	69 ha	6913 kg
Grass with clover/lucerne, under 50% legumes (in rotation)	74	3684	**Grass with clover/alfalfa, under 50% legumes (rotation)**	107 ha	5356 kg
Peas, human consumption	141	3522	**Clover seeds**	23 ha	4525 kg
Clover seeds	16	3225	**Sour cherry with undergrowth of grass**	35 ha	3463 kg
Sour cherries with undergrowth of grass	22	2281	**Grass under 50% clover/alfalfa, low yield (rotation)**	34 ha	1719 kg
Apples	10	2038	**Meadow with high nature value**	16 ha	1594 kg
Grass below 50% clover/lucerne, low yield (in rotation)	41	2028	**Chive seeds**	7 ha	1363 kg
Meadow with high nature value	15	1500	**Permanent grass and clovergrass without N norm, under 50% clover**	33 ha	834 kg

Appendix C – Pesticides analysed by multi-residue analyses

Multi-residue analyses by mass chromatography GC-MS/MS (LAB 1-01-80) and liquid chromatography UPLC-MS/MS (LAB 1-01-128) procedures are performed by extraction with modified QuEChERS and detection by GC-MS/MS and UPLC-MS/MS respectively (Regulation (EC) No 396/2005 of the European Parliament[4] and Council Directive 91/414/EEC [5]). In the multi-residue analysis, the MRL is established for the sum of the parent pesticide and its metabolites, isomers, salts etc. Metabolites etc. are listed (indented) below each parent compound. All pesticide residue analyses have been performed by LAB (Laboratorio Analítico Bioclinico, S.L.U., PITA, C/Albert Einstein, nº 7. Autovía del Mediterraneo (A-7) Salida 460. 04131 Almería. Spain).

LOQ: Limit of Quantitation is 0.01 mg/kg for all compounds. The following compounds are included in the screening by the multi-residue analyses by MR GC-MS/MS and UPLC-MS/MS, respectively:

Analysis by MR GC-MS/MS (LAB 1-01-80)

Aclonifen
Acrinathrin
Alachlor
Benalaxyl
Benfluralin
Biphenyl
Bifenox
Bifenthrin
Bromacil
Bromophos
Bromophos-ethyl
Bromopropylate
Bupirimate
Butralin
Cadusafos
Captan
Tetrahydrophtalimid
Carbophenothion
Chloroneb
Cycloate
Cyflufenamid
Cyfluthrin
Cypermethrin
Cyproconazole
Clodinafop-propargyl
Chlordane
Chlorfenapyr

[4] Regulation (EC) No 396/2005 of the European Parliament and of the Council of 23 February 2005 on maximum residue levels of pesticides in or on food and feed of plant and animal origin and amending Council Directive 91/414/. Official Journal of the European Union L 70, 16 March 2005, pp. 1-16

[5] Council Directive 91/414/EEC of 15 July 1991 concerning the placing of plant protection products on the market OJ L 230, 19.8.1991, p. 1–32

Chlorfenvinphos
Chlorbenside
Chlorfenson
Chloropropylate
4-Chloro-3-Methylphenol
Chlorpyrifos
Chlorpyrifos-methyl
Chlorthal-dimethyl
Chlozolinate
Kresoxim-methyl
Crimidine
Cyanofenphos
DDT
- o,p-DDT+p,p`-TDE (DDD)[1]
- p,p`-DDE[2]
- p,p´-DDT[3]

Deltamethrin
Diazinon
Dichlofenthion
Dicloran
Perthane
Dichlorvos
Aldrin and Dieldrin
- Aldrin
- Dieldrin

Diphenamid
Difenoconazole
Difenoconazole
Diflufenican
Dimethomorph
Dimethomorph
Endosulfan
- alpha-Endosulfan
- beta-Endosulfan
- sulphate-Endosulfan

Endrin
Ethion
Ethoprophos
Etridiazole
Etrimfos
Famoxadone
Fenpropathrin
Fenamiphos

- Fenamiphos
- Fenamiphos sulphone
- Fenamiphos sulphoxide

Fenchlorphos
2-phenylphenol
Fenitrothion
Phenothrin
fenson
Fenthion
Phenthoate
Fenvalerate + Esfenvalerate
Fipronil

- Fipronil
- Fipronil sulfone

Flucythrinate
Fludioxonil
Flumioxazine
Fluotrimazole
Folpet sum
Folpet sum

- Ftalimid

Fonofos
Formothion
Phosalone

- Phosmet

Furalaxyl
Heptachlor

- Heptachlor
- Heptachlor endo-epoxide
- Heptachlor exo-epoxide

Heptenophos

- Hexachlorocyclohexane
- Hexachlorocyclohexane , alpha-isomer
- Hexachlorocyclohexane , beta-isomer
- Hexachlorocyclohexane , delta-isomer

Hexaconazole
Hexazinone
Iprodione
Isocarbophos
Isofenphos
Isofenphos-methyl
Isopropalin
Isoprothiolane

Lambda-Cyhalothrin
Leptophos
Lindan
Malathion
Metalaxyl
Methamidophos
Methidathion
Mevinphos
Myclobutanil
Mirex
Nitrofen
Nitrothal isopropil
Norflurazon
Nuarimol
o,p-DDD
o,p-DDE
Oxadiazon
Oxadixyl
Oxyfluorfen
Parathion
Parathion-methyl
  Paraoxon-Methyl
  Parathion-Methyl
Penconazole
Pendimethalin
Pentachloroanisole
Permethrin
Pyrazophos
Pyridaben
Pyridaphenthion
Pyrifenox
Pyrimethanil
Pirimiphos-Ethyl
Pirimiphos-methyl
Pyriproxyfen
Procymidone
Prochloraz
  Prochloraz
  2,4,6-Trichlorophenol
Propham
Profenofos
Profluralin
Propachlor

Propanil
Propargite
Propiconazole
Prothiofos
Pyridalyl
Quintozene
    Quintozene
    Pentachloroaniline
Silafluofen
Sulfotep
Sulprofos
Tau-Fluvalinate
Tebuconazole
Tecnazene
Tefluthrin
Terbutryn
Tetrachlorvinphos
Tetraconazole
Tetradifon
Thiobencarb
Tolclofos-methyl
Transfluthrin
Trichloronat
Trifluralin
Vinclozolin
Iodofenphos

Analysis by MR UPLC-MS/MS (LAB 1-01-128)

Abamectin
Acephate
Acetamiprid
Acibenzolar- S- methyl
Acibenzolar acid
    Acibenzolar-S-Methyl
Aldicarb
    Aldicarb
    Aldicarb-sulfone
    Aldicarb-sulfoxide
Ametoctradin
Ametryn
Aminocarb
Amisulbrom
Amitraz

Anilofos
Atrazine
Atrazine-desethyl
Atrazine-desisopropyl
Azaconazole
Azadirachtin
Azoxystrobin
Bendiocarb
Bensulfuron-methyl
Bentazone
Benthiavalicarb
Bifenazate
Bioallethrin
Bixafen
Boscalide
Bromoxynil
Bromuconazole
Butocarboxim
Butocarboxim-Sulfoxide
Butoxycarboxim
Carbaryl
Carbendazim
Carbetamide
Carbofuran
    Carbofuran
    3-hydroxym-carbofuran
Carboxin
Carfentrazone-ethyl
Carpropamide
Cyantraniliprole
Cyazofamid
Cycloxydim
Azocyclotin and Cyhexatin
Cymoxanil
Cynosulfuron
Cyprodinil
Cyromazine
Climbazole
Clofentezine
Clomazone
Chlorantraniliprole
Chloridazon
Chlorotoluron

Chloroxuron
Chlorpropham
Chlorsulfuron
Clothianidin
Coumaphos
Chromafenozide
Cyanazine
Cyflumetofen
2,4-D
DEET[4]
Desmedipham
Desmetryn
Di-allate
2,6-Dichlorobenzamide
Diclobutrazol
Dichlofluanid
Dichlorprop
Dicrotophos
Diethofencarb
Diflubenzuron
Dimefuron
Dimethachlor
Dimethenamid
Dimethoate
Dimoxystrobin
Diniconazole
Dinotefuran
Dinoterb
Disulfoton
- Disulfoton
- Disulfuton sulphone
- Disulfuton sulfoxide

Diuron
DMSA[6]
Dodemorph
Dodine
Emamectin benzoate
EPN[8]
Epoxiconazole
Spinetoram
Spirodiclofen
Spiromesifen
Spirotetramat sum

- Spirotetramat
- Spirotetramat-enol
- Espirotetramat-enol-glucoside
- Spirotetramat-ketohydroxy
- Spirotetramat-monohydroxy

Spiroxamine
Ethiofencarb
Ethiofencarb-sulfone
Ethiofencarb-Sulfoxide
Ethiprole
Ethirimol
Etofenprox
Ethofumesate
Etoxazole
Ethoxyquin
Famophos
Fenamidone
Fenbuconazole
Fenhexamid
Phenmedipham
Fenoxaprop-P
Fenoxicarb
Fenpiclonil
Fenpyrazamine
Fenpyroximate
Fenpropidin
Fenpropimorph
Fensulfothion
Fenthion
Fenthion oxon

- Fenthion oxon sulfone
- Fenthion oxon sulfoxide
- Fenthion sulfone

Fenthion sulfoxide
Flonicamid

- Flonicamid
- TFNA
- TFNG

Fluacifop sum

- Fluazifop
- Fluazifop-P-butyl

Fluazinam
Flubendiamide

Flufenacet
Flufenzin
Fluometuron
Fluopicolide
Fluopyram
Fluoxastrobin
Flupyradifurone
Fluquinconazole
Flurochloridone
Fluroxypyr
Flurprimidole
Flurtamone
Flusilazole
Flutolanil
Flutriafol
Fluxapyrosad
Phorate
- Phorate
- Phorate-sulfone
- Phorate-sulfoxide

Forchlorfenuron
Formetanate
Phosphamidon
Phosmet
- Phosmet oxon

Fosthiazate
Phoxim
Fuberidazole
Haloxyfop sum
- Haloxyfop

Haloxyfop-ethoxyehtyl
Haloxyfop-methyl
Hexythiazox
Imazalil
Imazamox
Imazapyr
Imidacloprid
Indoxacarb
Ioxynil
Iprobenphos
Iprovalicarb
Isoprocarb
Isoproturon

Isoxaben
Isoxaflutole
Lenacil
Linuron
Lufenuron
    Malaoxon
Mandipropamid
Matrine
Mecarbam
Mepanipyrim
Mepanipyrim-2-Hidroxypropyl
Mepronil
Mesotrione
Methabenzthiazuron
Methacrifos
Metaflumizone
Metamitron
Metazachlor
Metconazole
Methiocarb
    Methiocarb
    Methiocarb-Sulfone
    Methiocarb-Sulfoxide
Metobromuron
Metolachlor
Metolcarb
Methomyl
Metosulam
Methoxyfenozide
Metrafenone
Metribuzin
Metsulfuron-methyl
Milbemectin sum
Milbemectin A3
Milbemectin A4
Molinate
Monocrotophos
Monolinuron
Monuron
Napropamide
Neburon
Nicosulfuron
Nitenpyram

Ofurace
Omethoate
Oryzalin
Oxadiargyl
Oxamyl
Oxycarboxin
Oxydemeton-methyl
Demethon-S-Methyl-Sulfone
    Oxydemeton-methyl
Fenbutatin oxide
Paclobutrazol
Pencycuron
Penoxsulam
Penthiopyrad
Pethoxamid
Picloram
Picoxystrobin
Pymetrozine
Pinoxaden
Piperonyl butoxide
Pyraclostrobin
Pyraflufen-ethyl
Pyrasulfotole
Pyrethrins sum
Pyrethrin I
Pyrethrin II
Pyrimicarb
Pyrimicarb Desmethyl
Pyroxsulam
Profoxydim
Promecarb
Prometryn
Propamocarb
Propaquizafop
Propetamphos
Propyzamide
Propoxycarbazone
Propoxur
Proquinazid
Prosulfocarb
    Prothioconazole
Prosulfuron
    Prothioconazole-desthio

Quinclorac
Quinmerac
Quinoxyfen
Quizalofop, incl. quizalfop-P
Quizalofop, incl. quizalfop-P
Rotenone
Sebutylacine
Clethodim
  Clethodim
  Sethoxydim
Silthiofam
Simazine
Simetryn
Spinosad
Sulcotrione
Tebufenozide
Tebufenpyrad
Tebutam
Teflubenzuron
Temephos
Tepraloxydim
Terbufos
Terbumeton
Terbumeton desethyl
Terbuthylazine
Terbuthylazine-desethyl
Thiofanox
Thiofanox-Sulfone
Thiofanox-Sulfoxide
Thiabendazole
Thiacloprid
Thiamethoxam
Thiazopyr
Thiencarbazone-methyl
Thifensulfuron-methyl
Thiocyclam
Thiodicarb
Thiophanate-methyl
Tolylfluanid
  DMST[7]
Tolifluanide
Tralkoxydim
Triadimefon

Triadimenol
Tri-allate
Triasulfuron
Triazophos
Tricyclazole
Triclopyr
Trichlorfon
Tricresyl phosphate
Trietazine
Trifloxystrobin
Triflumizole sum
    Triflumizole
    Triflumizole FM-6-1
Triflumuron
Vamidothion
Iodosulfuron-methyl
Zoxamide

[1] DDD: Dichlorodiphenyldichloroethan
[2] DDE: Dichlorodiphenyldichloroethylene
[3] DDT: Dichlorodiphenyltrichloroethane
[4] DEET: N,N-Diethyl-meta-toluamide
[5] DMPF: N-2,4-Dimethylphenyl-N'-methylformamidine, metabolite of amitraz
[6] DMSA: Dimercaptosuccinic acid
[7] DMST: N,N-Dimetil-N`-tolilsulfonildiamida
[8] EPN: O-Ethyl O-(4-nitrophenyl) phenylphosphonothioate