UNIVERSIDADE D
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Nuno Xavier Jesus Capela

The impact of landscape structure and management on the development of *Apis mellifera* colonies

A MULTIFACTORIAL APPROACH

Tese no âmbito do Doutoramento em Biociências, especialização em Ecologia, orientada pelo Professor Doutor José Paulo Sousa, Professor Doutor Christopher John Topping, e Professora Doutora Maria Alice Pinto, e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

Dezembro de 2022

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Thesis in the scope of the Doctorate in Biosciences, specialization in Ecology, supervised by Doctor José Paulo Sousa (Department of Life Sciences, University of Coimbra), Doctor Maria Alice Pinto (Mountain Research Centre, Polytechnic Institute of Bragança) and Doctor Christopher Topping (Department of Ecoscience, Aarhus University), presented to the Department of Life Sciences of the Faculty of Sciences and Technology of the University of Coimbra.

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The B-team: From two lost souls in the middle of harsh Burgos weather to sixteen amazing people making this project a dream come true.

My family: Sara and Biscoito.

Declaro que esta tese foi elaborada por mim e confirmo não ter sido previamente submetida, total ou parcialmente, para obtenção de outro grau académico. Confirmo que o trabalho descrito foi realizado por mim e pelos co-autores, no caso de publicações conjuntas, como indicado nos capítulos II, III, IV, V VI. Nestes casos, a minha contribuição está explicitamente indicada abaixo:

O trabalho apresentado no capítulo II foi concebido por mim e pelos co-autores do manuscrito. Realizei todo o trabalho de campo com ajuda pontual de outros co-autores (Artur Sarmento) e colegas que estão referidos nos agradecimentos. A análise de dados foi auxiliada por José Paulo Sousa e a primeira versão do artigo foi escrita em conjunto com Yoko Luise Dupont. As sugestões dos outros autores foram posteriormente incorporadas por mim.

O trabalho apresentado no capítulo III foi concebido por mim, José Paulo Sousa, Henrique Azevedo-Pereira e Yoko Luise Dupont. Realizei todo o trabalho de campo para a recolha de dados das colónias juntamente com Artur Sarmento e Sandra Simões (e outros colegas que estão nos agradecimentos do capítulo II). A recolha de dados de paisagem (GIS) e elaboração dos mapas foi feita por António Silva e Ruben Mina, sendo a análise da composição florística feita por Lucie Mota (paisagem de Burgos) e Sara Lopes (paisagem da Lousã e Idanha). O cálculo da oferta da paisagem foi elaborado por Mari Gigauri com auxílio de Sílvia Castro (paisagem de Burgos) e por mim (paisagem de Lousã e Idanha). A análise de dados foi auxiliada por José Paulo Sousa.

O trabalho apresentado no capítulo IV foi concebido por mim e pelos autores do manuscrito. Realizei todo o trabalho de campo para a recolha de dados das colónias e da paisagem juntamente com Sandra Simões (e outros colegas que estão nos agradecimentos do capítulo IV). As análises de laboratório, e material suplementar sobre a metodologia, foram elaboradas por Mang Xu e Jeroen Peters. A análise de dados foi auxiliada por José Paulo Sousa e Mathieu Renault. A primeira versão do artigo foi escrita por mim, sendo as sugestões dos outros autores posteriormente incorporadas.

O trabalho apresentado no capítulo V foi concebido por mim e pelo Artur Sarmento. Realizei o trabalho de campo com o auxílio de Sandra Simões (e outros colegas que estão nos agradecimentos do capítulo V). A análise de dados foi auxiliada por José Paulo Sousa. A primeira versão do artigo foi escrita por mim, sendo as sugestões dos outros autores posteriormente incorporadas.

O trabalho apresentado no capítulo VI foi concebido por mim, Xiaodong Duan e por Christopher Topping. A implementação das simulações foi elaborada por Xiaodong Duan, enquanto a paisagem (e recursos) foram criados por Elżbieta Ziółkowska. Realizei todo o trabalho de análise de dados e a primeira versão do artigo foi escrita por mim, sendo as sugestões dos outros autores posteriormente incorporadas.

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Resumo

Os serviços de polinização são essenciais para o funcionamento e estabilidade dos ecossistemas, mas podem ser colocados em causa com as recentes perdas de polinizadores (ex. *Apis mellifera*). Nos últimos 60 anos, o número de colónias de abelhas melíferas sofreu um declínio na Europa e nos USA devido a múltiplos stresses ambientais, químicos e biológicos. Assim, perceber de que forma é que esses stresses atuam e afetam o desempenho das colónias de abelhas tornou-se essencial. No entanto, a maioria dos estudos foca-se na avaliação do impacto de uma única fonte de stress sem considerar o contexto onde as colónias estão inseridas. Além disso, os protocolos de campo para medir as variáveis do desenvolvimento das colónias baseiam-se em estimativas visuais que possuem pouca precisão.

No capítulo II foi desenvolvido um protocolo para medir o desenvolvimento das colónias considerando as variáveis mais importantes, usando metodologias que conferem resultados precisos sem recorrer a estimativas que variam com o observador. O possível impacto negativo da recorrente aplicação deste protocolo, tal como a sua precisão, foram avaliados provando a sua possível aplicabilidade no futuro.

No capítulo III, o protocolo foi aplicado para medir o desenvolvimento de colónias instaladas em diferentes paisagens. O desenvolvimento das colónias apresentou variabilidade dentro da mesma paisagem e entre paisagens e os recursos utilizados foram específicos para cada paisagem. Dados relativos à disponibilidade de recursos, clima e população foram utilizados para explicar a variação de peso diária das colónias. Numa paisagem com recursos abundantes, essa variação foi significativamente explicada pelo clima e população. Na outra paisagem, onde os recursos eram escassos, apenas a disponibilidade de recursos foi significativa.

Sendo que os pesticidas podem interferir com a forma como as abelhas interagem com a paisagem, dois testes de campo foram desenvolvidos para avaliar a exposição e os efeitos de neonicotinóides (capítulos IV e V). No capítulo IV, a exposição e efeitos da acetamiprida na colónia foram avaliados num cenário real. A quantidade de resíduos de acetamiprida na paisagem e a sua acumulação nas colónias foram medidos para se calcularem níveis de risco. Não foram detetados efeitos ao nível individual (a partir do risco calculado) ou da colónia; os níveis de exposição correlacionaram-se fortemente com a área de aplicação, apesar da distribuição heterogénea de acetamiprida. Este resultado demonstra a dificuldade em avaliar a exposição a pesticidas a partir de *guidelines* para testes de campo em zonas florestadas. Ao nível individual (capítulo V), o efeito do sulfoxaflor e acetamiprida na *homing ability* das abelhas (capacidade de voltarem para a colónia após

serem alimentadas com as substâncias testadas) foi avaliado. Uma dose de sulfoxaflor 2.3 vezes inferior à concentração mais alta testada de acetamiprida levou a que apenas uma pequena fração das abelhas conseguisse voltar à colónia. Isto demonstra a necessidade de se realizarem mais testes com doses subletais para uma avaliação de risco ambiental mais forte. Ainda assim, ferramentas mais robustas e fidedignas são necessárias para que efeitos negativos ao nível individual possam ser transpostos para a colónia.

Para responder a essa necessidade, a EFSA propôs a criação de um modelo de múltiplos stresses para abelhas melíferas: o ApisRAM, composto por vários submodelos. No capítulo VI, a primeira versão do modelo de forrageamento foi criada, para se testarem possíveis estratégias para o comportamento de *scouting* e *foraging* em paisagens complexas. As abelhas melíferas foram moduladas ao nível individual, dentro de uma paisagem dinâmica onde a disponibilidade de recursos variava no espaço e no tempo. As decisões das abelhas foram feitas através de informação privada e social. Quando estas priorizam polígonos de acordo com a estratégia do ganho (*i.e.,* eficiência energética), a colónia obtém uma maior quantidade de açúcar. Por outro lado, ao priorizar os polígonos menos distantes, a colónia adquire a maior massa de pólen.

O modelo de forrageamento do capítulo VI foi o primeiro passo do seu próprio desenvolvimento; testes adicionais são necessários para obter um modelo totalmente funcional. A criação do ApisRAM, no qual o resultado obtido resulta da emergência de comportamentos individuais, é chave para uma avaliação do risco ambiental mais robusta e fidedigna. Além disso, mais informação é necessária para entender a exposição de abelhas melíferas a stresses ambientais, como é que estes se traduzem em efeitos ao nível da colónia, e as consequências para o ecossistema. Felizmente, novas metodologias estão a ser desenvolvidas se reduzir o nível de incerteza associado à informação recolhida; esforços estão também a ser feitos, ao nível europeu, para uma avaliação do risco ambiental mais robusto, de forma a melhor proteger as abelhas melíferas e outros insetos polinizadores.

Palavras-chave: *Apis mellifera; características de desenvolvimento da colónia; recursos da paisagem; avaliação de risco de pesticidas.*

Abstract

Pollination is vital for the function and stability of terrestrial ecosystems; however, recent declines in wild and domesticated pollinators (*e.g.*, *Apis mellifera*) could hamper pollination services. In the last 60 years, the number of honey bee colonies in Europe and the US have declined due to multiple environmental, chemical, and biological stressors. Nowadays, understanding the impact of this network of stressors on honey bee colonies development is crucial. Most of the existent studies focus on the impact of a single stressor, without considering *where* and *when* (context) stressors are applied. Additionally, no standardized field-test protocols that encompass all features of colony development exist, and some methodologies (based on visual estimations) do not provide accurate data.

In Chapter II, a protocol was created to measure colony development through the season, considering the most relevant variables using observer-independent methods that provide reliable and accurate data. The protocols' impact on colony development and its accuracy were analysed proving its applicability in future studies.

In Chapter III, that protocol was applied to assess how colonies responded to different landscapes. Resources availability, climate, and colony strength were used to explain daily colony weight variation. In a landscape where resources were abundant, climate and colony strength were the variables that significantly affected weight variation. When resources where scarcer, only these became significant to explain the weight variation. Additionally, colonies development showed variability within and between landscapes while the use of resources was specific to the different landscapes.

Pesticides can interfere with colony-landscape interactions. Two field studies were developed to evaluate the exposure and effects of neonicotinoids at the colony level (Chapter IV) and effects at individual level (Chapter V). In chapter IV, acetamiprid's colony exposure and effects were evaluated under a real exposure scenario. Acetamiprid residues in the landscape and their transferability and accumulation in the colonies were measured to calculate risk levels. No effects on colony or individual bees (from the calculated risk) were detected; exposure levels were strongly correlated with the sprayed area, despite the heterogeneous distribution of acetamiprid. This demonstrates the difficulty of calculating exposure using guidelines for field studies in forested areas. At the individual level (Chapter V), the effect of sulfoxaflor and acetamiprid on the bees' homing ability (*i.e.*, ability to return to the colony after being fed with the tested substances) was tested. Radio Frequency Identification (RFID) chips were used to individually mark each bee. With a sulfoxaflor dose 2.3 times lower than the highest tested acetamiprid dose, only a small fraction of bees managed to return to the colony. This

shows the need for more tests at sub-lethal doses for a stronger future environmental risk assessment (ERA). Nonetheless, robust and reliable tools are needed to translate the negative effects detected at the individual to the colony level.

To tackle this challenging task, EFSA has proposed the creation of a multi-stressor honey bee model, ApisRAM, composed of several sub-models. In chapter VI, the first version of the foraging model was created to test possible strategies used by scout and forager bees in complex landscapes. Honey bees were modelled at the individual level on a dynamic landscape with a spatiotemporal variation of resources availability. Model bees were guided by their private and social information to select were to forage. When bees prioritized polygons according to the distance, amount, and quality of the resource (gain strategy), the colony collected more sugar. However, prioritizing the closest polygons conferred the colonies with the highest mass of collected pollen.

The foraging model from chapter VI was the first step of its development and further tests are needed to obtain a fully functional mechanistic model. The creation of the ApisRAM, in which the outcome of the whole colony emerges from individual behaviour, is the key to create a robust and reliable ERA for honey bees. Furthermore, several other building blocks are needed to understand bees' exposure to environmental stressors, how these translate into effects at the colony level, and their consequences for the whole system. Thankfully, new methodologies are being developed to decrease the level of uncertainty of collected data; efforts are being made at the European level to create a more robust ERA to better protect bees and other insect pollinators.

Keywords: *Apis mellifera*; colony development traits; landscape resources; pesticide risk assessment.

Chapter I

General Introduction

David Sarmento

1. Pollination services

Pollination is vital for the functioning and sustainability of terrestrial environments being considered one of the most important ecosystem services (IPBES, 2016). About 87.5% of all flowering plant species depend on animal pollination (Ollerton *et al.*, 2011). Without pollination a reduction of 5 to 8% on crop production and diversity would be expected (Aizen *et al.*, 2009). On top of it, there has been a constant increase on pollinator-dependent crops (Aizen *et al.*, 2019). Fast-growing nations, as well as the search for healthier and more diversified nutrition, has led to a higher food demand of diverse pollinator-dependent agricultural products (Aizen *et al.*, 2009). In the last five decades, the volume of these crops has increased by 300% (IPBES, 2016). Worldwide, pollination service is provided by a range of insects, birds, bats, and other vertebrates. Insects, such as wasps, some flies, butterflies, moths, beetles, or thrips, are the most important categories of pollinators, with a particular emphasis for bees, visiting more than 90% of the leading 107 global crop types (Klein *et al.*, 2007).

Approximately 85% of the known ~20.000 bee species are solitary bees (Batra, 1984). Nonetheless, the social species are easily recognized by humans (*e.g.*, honey bees and bumble bees) due to their special status as domesticated bees. Honey bees are the most managed bees worldwide, with the use of their products dating back to 9000 years ago (Roffet-Salque *et al.*, 2015). In recent years, the need for pollination services has increased mainly due to agricultural intensification (Potts *et al.*, 2016), and the human dependency on pollinated crops (Aizen *et al.*, 2019). This has led to the domestication of other bees (as bumblebees - *e.g.*, *Bombus terrestris* - and a few solitary bees - *e.g.*, *Osmia lignaria* (Dafni *et al.*, 2010; Bosch & Kemp, 2000)). In total, there is the potential to manage at least 66 insect species for pollination purposes (Osterman *et al.*, 2021).

The use of managed species might present as a good solution to overcome the lack of pollination services in agricultural systems by rewarding beekeepers (and other bee breeders) with a payment for their service. Beekeepers, who usually (and sometimes obliviously) provide free pollination services to crops by installing their colonies near the fields to increase honey production, are particularly aware of the value of the service they offer. The use of colonies for the single purpose of pollination started in 1909 in New Jersey, USA, when a few colonies were deployed in apple orchards (Morse & Calderone, 2000) and has escalated to roughly 1.4 million colonies in 2004 just for almond production (Sumner & Boriss, 2006). In 2020, worldwide pollination services were evaluated to be worth between US$195 billion to ~US$387 billion US dollars (Porto *et al.,* 2020). However, the importance of this service varies with crops and therefore it influences economies at a regional scale.

Crop pollination cannot be sustained by managed bees alone, since crop yield and/or quality depends not only on pollinators' abundance but also on their diversity (Garibaldi *et al.*, 2014). On top of it, it is worth mentioning the negative impacts of using non-native bees for pollination services considering competition with native species (*e.g.*, Dafni *et al.,* 2010; Valido *et al.*, 2019). Managed honey bees have been blamed for disrupting wild pollinator networks due to their intense competitive pressure, a result of the high number of foragers in each colony (Mallinger *et al.*, 2017). Consequently, there is a growing debate about the spread of managed bees into natural and protected areas, and even more, their possible trivial environmental value (Geldmann & González-Varo, 2018). This debate, though valid and valuable, requires a holistic view of the pollinator network systems and the awareness of the benefits of honey bees also in natural ecosystems, as honey bees are the most frequent visitor in 13% of natural plant species and the only one in 5% of them (Hung *et al.*, 2018). Moreover, the efforts to restore the health of honey bee colonies, by implementing changes in agricultural systems, will also benefit wild pollinators, since their losses are closely related with the same factors (Alaux *et al.,* 2019). The most concerning aspect, for both wild and managed bees, is the expansion of intensive agricultural practices that undermine pollinators while increasing the need for pollination services (Aizen *et al.*, 2019). If the increasing pollination-dependent trend continues, there's a risk of pollination shortfall (Aizen *et al.*, 2019) continuing the pressure on the use of managed pollinators like honey bees.

2. Honey bees

Honey bees' ubiquitous distribution, their ability to adapt to different climates and to forage at low temperatures (of great value for crops that flower in winter or early-spring), linked to the fact they visit flowers of many shapes and sizes, focusing on the same species in each foraging flight (increased pollination efficiency), makes them the preferred pollinator in crop systems. Honey bees are not the most efficient pollinator in some crops, but this lack of efficiency is compensated by the high number of foragers that visit thousands of flowers a day.

2.1 The honey bee colony

In a colony, there is an age-dependent division of labour mechanism (age polyethism) that regulates the tasks performed by the honey bees according to their age (Johnson, 2008). Younger bees start their life cycle by cleaning the alveoli where they were born. As they get older, they participate in the construction of the colony by producing wax, they work as "nurses" by taking care of young larvae, and they act also as "guard" bees (the first ones to attack any intruder). At the same time, such division of labour is also regulated by the colony needs; in some cases, the honey bee behaviour may be adjusted (polyethism plasticity) to perform the former tasks. As an example, if the colony needs more "nurse" bees to feed their brood, the forager bees can "reverse" their function and adapt to perform as "nurse" bees again (Tautz, 2008). Likewise, if the colony needs to increase resources uptake, workers can start foraging at a younger age (early-foraging). All the major colony tasks are performed by the female sterile worker bees, which represent the majority of the colony. Besides these workers, the colony is composed by a single fertilized bee, the queen, and a few males, the drones.

From the egg stage until adulthood, the queen has a faster development cycle (15 days) than the worker bees (21 days) or drones (24 days). After hatching, all the new-born larvae are fed with royal jelly, a mixture of hypopharyngeal and mandibular-gland secretions produced by nurse bees. After three days, only the few larvae selected to become a queen will continue to be fed with royal jelly, while the others will be fed with a mixture of beebread and nectar. Interestingly, it's not the royal jelly *per se* that confers the bees their queen status, but rather the pollen that is used to feed the other bees that inhibits the development of their sexual glands, developing into sterile bees (Zhu *et al.*, 2017). After being born, the virgin queen performs a nuptial flight where she mates with approximately 16 drones, with some studies showing a maximum of 50 drones (Withrow & Tarpy, 2018). Sperm is stored in the spermatheca and used later by the queen, selecting between male and female eggs. This selection is based not only on the size of the comb cell, in which the bigger cells receive an unfertilized egg (drones) and the smaller cells receive a fertilized one (workers), but on the colony needs regarding the drones and workers colony population (Wharton *et al.*, 2007). Most of the female brood cells are positioned in the middle of the frame, while the resources, honey and pollen, are stored mostly on the upper borders of the frame. This organizational system provides nurse bees with proximity to food to feed their brood, while also protecting the nest from temperature fluctuations from the corners of the frames. On the other hand, drone brood is usually found on the side of the frames, as the colonies only produce it when they have enough food and workers to maintain the colony needs. Drones only exist during the mating season (mainly in Spring but also in

late Summer) with a lifespan of 2-4 weeks and die soon after mating or at the end of the active season (before winter), when the female nestmates expel the unmated ones from the colony to save resources (Seeley & Mikheyev, 2003). Usually, the presence of drone brood cells indicates a healthy and strong development, as drone rearing requires more energy, with no service being provided directly to the colony (Seeley & Mikheyev, 2003).

2.2 The superorganism organization

The colony development and organizational system properties are not identifiable at the honey bee individual scale; instead, they emerge from single bees' behaviour as a response to the colony status. The individual decisions (*e.g.*, feed the larva, receive nectar) are adopted at a local level, which stimulate other bees to adjust to the new reality and adapt their behaviour, leading to changes at the macro-level (Tautz, 2008). This cyclic regulation allows the colony to auto-regulate itself, without needing a centralized coordinating entity – it works almost like a decentralized democracy in which each bee decides what it needs to do, based on the overall needs. Contrary to what is commonly believed, the honey bee queen does not perform the role of leading the colony and making decisions at a colony level. Queen-produced pheromones are, instead, responsible for limiting worker ovarian development (Hoover *et al.*, 2003). As mentioned above (section 2.1), even the egg-laying location within the colony is decided both by the queen and the worker bees (Wharton *et al.*, 2007).

2.3 Landscape interaction

2.3.1 Collected resources

Honey bees forage for nectar, pollen, propolis and water (Dreller & Page, 1999). The nectar is where the bees get their energy. While visiting a flower, nectar foragers accumulate nectar in their honey stomach. When returning to the colony, foragers will pass along the nectar harvest to "nectar receivers" (Anderson & Ratnieks, 1999), which will distribute it amongst nestmates or store it inside the comb cells (DeGrandi-Hoffman & Hagler, 2000). In some regions, instead of flower nectar, bees will also bring other sugary substances (*e.g.*, honeydew). The nectar/sugar substance is slowly transformed into honey by adding enzymes produced by the bees and by reducing the water content.

After filling the whole cell with honey (moisture reduced to around 18%), the bees seal it with a wax cap to prevent water exchanges with the exterior. This preserves the honey, allowing bees to use it during winter to produce energy for warming up the colony.

While visiting the flowers, pollen foragers will accumulate pollen on their hind legs (pollen basket) and transport it into the colony. In this case, there are no "pollen receivers", therefore, the pollen foragers will be the ones that add the pollen directly into the comb cells, pressing it against the cell bottom with their head. Pollen is processed into beebread to feed protein to the larvae, guaranteeing the necessary amino acids for a healthy development (Keller *et al.*, 2005a). The processing encompasses addition of small amounts of nectar as well as enzymes, which will transform pollen into beebread, by fermentation, to preserve it and increase its nutritional value.

Propolis, collected from tree resins, is used by bees to seal the hive and, at the same time, sanitizes the colony, due to the anti-microbial properties (Hegazi *et al.*, 2000). On top of all these resources, honey bees also collect water, mainly for thermoregulation processes (Kovac *et al.*, 2010).

2.3.2 Foraging activity

The forager bees are, usually, the oldest and the most experienced individuals in the colony, as the probability of being recruited for foraging increases with age. The foraging recruitment will increase the number of foragers, which will guarantee a higher resource uptake from the landscape. Remarkably, during foraging flights, foragers will acquire experience, increasing their rate and efficiency at collecting resources (Schippers *et al.,* 2006; Klein *et al.*, 2019). Nonetheless, this comes with an associated cost, as foragers have a higher mortality rate (Rueppell *et al.*, 2007). Interestingly, this increase in mortality rate is attributed to the behavioural state (age of transition to forager bees), and not to the chronological age or the external mortality hazards (Rueppell *et al.*, 2007).

At the colony level, the number of brood cells (Dreller *et al.,* 1999; Le Conte *et al.*, 2001; Pankiw, 2004), the amount of stored pollen, and the available storage space (Dreller *et al.,* 1999) will positively influence pollen foraging. Nectar foraging is also triggered by the amount of storage space, in addition to the rate of nectar intake (Seeley & Tovey 1994). Forager bees obtain this information indirectly, by the time spent to find nectar-receiving bees. If foragers take too long to unload their nectar harvest, it means that the colony is already receiving too much nectar and therefore foraging is ceased (Seeley & Tovey 1994).

Outside the colony, climatic conditions as well the weather and availability of resources also play a role on the foraging behaviour. Foragers do not go out on rainy days or with strong wind (Hennessy *et al.*, 2020), and require minimum temperature (12º C) and solar radiation thresholds to forage (Vicens & Bosch, 2000; Clarke & Robert, 2018). While foraging, bees will scout the landscape to find rewarding flower patches, to fulfil their pollen or nectar requirements. Afterwards, they come to the colony and unload their nectar or pollen harvest. However, before travelling back to get more food, they will recruit more bees for foraging purposes, through the waggle dance. Remarkably, this recruiting process will increase the foraging intensity in the most rewarding patches, as the bees that visit these patches are more excited while performing the waggle dance, convincing more nestmates to forage there (Seeley *et al.*, 1991).

The waggle dance was thoroughly described by Karl Von Frisch, earning him the medicine Nobel prize in 1973. During his studies, he noticed that the bees performed a round dance to provide information on feeding sites close to the colony (50 to 70 m), while for more distant resources the recruited bees needed a larger and more reliable amount of information. He observed that forager bees would perform a dance pointing to different directions throughout the day, even though the feeding site was the same. He then concluded that the sun was used to aid orientation, whilst the duration of the dance would inform on the distance between the colony and the feeding site (Von Frisch, 1967). In his studies, one second performing the waggle phase would mean that the feeding site was one kilometre apart from the colony.

More recently, the use of new technologies (*i.e.*, video recording) has aided the dance analysis with a more reliable decoding for a better understanding of these movements (*e.g.*, Couvillon *et al.*, 2012) and new models for waggle dance decoding have already been proposed (*e.g.*, Schürch *et al.*, 2019; Kohl & Rutschmann, 2021). Nonetheless, the human decoding of waggle dances has been controversial, as no universal model exist for all landscapes and honey bee species or subspecies, due to a lot of intra (Couvillon *et al.*, 2012) and inter-dance variability (Schürch *et al.*, 2016). This variability could derive from the presence of other cues that cannot be decoded by humans while (visually) analysing solely the waggle dance. While performing the dance, foragers will also transmit vibrations through the wax comb (Tautz, 1996) and share scents with their nestmates (Thom *et al.*, 2007). Additionally, foragers use landscape landmarks to orient themselves, which also modify the way they perceive the travelled distance (Kheradmand & Nieh, 2019).

3. Multiple stressors

The number of honey bee colonies in the United States and Europe suffered a decrease in the 80s and 90s, respectively, despite a worldwide overall increase, due to increases in Africa, Asia and South America (FAOSTAT, 2022). Even though the number of colonies have recovered in the last 10 years, there are still reports of winter losses above the 15% usual levels since the arrival and establishment of the ectoparasitic mite *Varroa destructor* (Potts *et al.*, 2010b). These losses vary strongly by country, province, and year. Moreover, there were several reports of unexplained rapid losses of adult population that were classified as Colony Collapse Disorder (CCD; Underwood & Vanengelsdorp, 2007). So far, there is no single cause for the CCD reports and the increased mortality. Instead, there are several stressors appointed as the main drivers of these losses, including environmental, biological, and chemical stressors.

3.1 Environmental stressors

Environmental stressors responsible for the increased mortality include climate change, habitat fragmentation, shortage of flower resources mainly through the intensive use of monocultures, and beekeeping practices/management (Steinhauer *et al.*, 2018). Climate change can shift flowering patterns, desynchronising the normal colony development and the amount of resources available. Also, in areas with an expected reduction in precipitation and increase in temperature (*i.e.*, Mediterranean areas), reduced plant nectar secretion can occur (Takkis *et al.*, 2018). Honey bee distribution ranges can also be affected, as well as their interaction with parasites and pathogens (Le Conte & Navajas, 2008). Climate change can lead to movements of different species and races of honey bees and their associated pathogens, bringing them to contact with pathogens that they did not co-evolve with (Le Conte & Navajas, 2008).

Intensive farming systems create a fragmented landscape with larger fields and monocultures, reducing flower diversity. Flower patches rich in resources to pollinators, such as field margins, hedgerows and grasslands, are becoming increasingly limited or seized to exist (Nicholls & Altieri, 2013). This has led to less fallow land for flowers to grow and less food resources for the bees outside the crops' flowering season. The maintenance of green infrastructures naturally present in the landscape (*i.e.*, field margins, hedgerows, fallow areas), as well as the implementation of enhanced

green infrastructures (*i.e.*, floral stripes or forest islets) in agricultural landscapes is proposed as a solution to increase flowering resources, fields connectivity, and to offer nesting sites for wild bees (Benayas & Bullock, 2015). This would provide managed and wild bees with a continuous offer of flower resources while the nectar-rich crops (*e.g.*, oilseed rape and sunflower) are not yet flowering. So far, the implementation of such green infrastructures has been a success: sown wildflower strips have higher insect abundance and diversity than grassland habitats, sown grass strips, or margins with natural regeneration (Feltham *et al.*, 2015; M'Gonigle *et al.*, 2015; Martin *et al.*, 2019). Hence, these local scale restorations, using strategic revegetation approaches, are a great conservation tool to halt biodiversity loss in intensively managed landscapes (M'Gonigle *et al.*, 2015; Benayas & Bullock, 2015).

The recovery and implementation of green infrastructures would therefore shift the wild pollinator decline (Ricketts *et al.*, 2008), and at the same time help maintaining healthier honey bee colonies, which are highly dependent on flower diversity mainly while foraging for pollen sources. Honey bees need a diversified pollen diet to acquire different amino acids and proteins for normal larvae development (Brodschneider & Crailsheim, 2010), as well as to ensure their immunocompetence against pathogens (Alaux *et al.*, 2010), due to the offer of essential nutrients. Pollen shortage at the beginning of the season can lead to a reduction in brood population and to lower amounts of food reserves and adult population by the end of the season (Requier *et al.*, 2017). Hence, complex flowering landscapes will offer a better place for healthy colony development (*e.g.*, Ricigliano *et al.*, 2019).

Beekeeping practices, although usually discarded has playing an important role on colony development and health, can have critical impacts on colonies survival (*e.g.*, kulhanek *et al.*, 2021). Depending on their goal, beekeepers can choose from a high array of practices from lower to higher intervention levels (Underwood *et al.*, 2019). Curiously, the professional beekeepers that are usually perceived as more aggressive towards colony management (*i.e.*, use of chemical treatments, supplementary feeding, migration), report less winter mortality when compared to small scale beekeepers (Gray *et al.*, 2020).

3.2 Chemical stressors

Modern farming practices rely on the use of pesticides, monocultures, and large continuous crop areas. As stated above (section 3.1), intensively managed areas create a shift on the flower resources offer, creating poorer and simplified landscapes for pollinators to nest and feed. On top of it, the use of pesticides represents a further pressure on pollinator communities (Vanbergen *et al.*, 2013; Goulson *et al.*, 2015; Potts *et al.*, 2016). Understanding the true impact of pesticides is one of the biggest challenges for the scientific community.

One of the most used classes of pesticides is the neonicotinoids (Bass *et al.* 2015). Neonicotinoids are of great concern for honey bee health because they are specific for insects, affecting their nicotinic acetylcholine receptors (nAChR), deterring insects' movement and ultimately leading to their death (Tomizawa & Casida, 2005; Simon-Delso *et al.*, 2015; Ihara & Matsuda, 2018). Neonicotinoids can be applied as foliar insecticides, in seed coating or with root drench application (Pisa *et al.*, 2015), being translocated to all the parts of the plant due to their systemic nature (Simon-Delso *et al.*, 2015). This includes the pollen and nectar that will be collected by honey bees and transported to the colony, potentially affecting its development and survival. Nonetheless, if the effects are delayed or sub-lethal, it is challenging to connect the colony failure/weakness to the pesticide exposure. Fortunately, several studies have shed light on the sub-lethal effects of neonicotinoids, showing that these substances can affect several stages of the bee development, from the immune system to the effects on learning and orientation (Desneux *et al.*, 2007). Henry *et al.* (2012) used radio frequency tags to measure homing failure on honey bees, showing that the exposure to commonly encountered doses of thiamethoxan (that did not lead to lethal effects), reduced bees' ability to return to the colony. In another independent study, Coulon *et al.* (2018) found that exposure to the same substance also reduced bees' tolerance to viral infections. Another widely used neonicotinoid substance is thiacloprid. Honey bee workers exposed to this substance under field conditions had impaired foraging behaviour, as well as reduced homing success and social communication (Tison *et al.*, 2016). Negative effects have also been documented in queens; when fed with food contaminated with thiacloprid and clothianidin during larvae development, queens showed a reduction of immunocompetence that can affect their resistance to diseases (Brandt *et al.*, 2017). Moreover, queens exposed to thiamethoxan and clothianidin experienced fewer mattings than non-exposed queens, leading to lower genetic diversity inside the colony, known to negatively affect its development and strength (Forfert *et al.*, 2017). Above all, honey bees cannot actively distinguish between contaminated or uncontaminated sources. In a study where honey bees had to choose

between uncontaminated sucrose solutions or contaminated with the three most common neonicotinoids (imidacloprid, thiamethoxam and clothianidin) they preferred to feed from the imidacloprid and thiamethoxam solutions than from the sucrose alone (Kessler *et al.*, 2015). All this research had an impact on the world perception of neonicotinoids, shading some evidence on the causes of the uncommon colony losses, leading to the application of severe use restrictions of almost all neonicotinoids' substances, at a European level (see section 5).

Pesticides like neonicotinoids are not the only chemical stressors for honey bees. Beekeepers regularly apply treatments to control varroa mites' infestation. These in-colony chemicals (acaricides) are helpful to keep varroa constrained at low levels. Nonetheless, a negative effect on honey bee health after exposure to some of these acaricides has been proved (Boncristiani *et al.*, 2012, Frost *et al.*, 2013, Johnson *et al.*, 2013). Some of the most used products (thymol, coumaphos and formic acid) change the detoxification gene expression pathways, as well as some components of the immune system (Boncristiani *et al.*, 2012). Tau-fluvalinate (another popular acaricide) increases its toxicity when in combination with other acaricides (Johnson *et al.*, 2013) and affects honey bee learning behaviour, memory, and survival at high oral doses (Frost *et al.*, 2013). In most European countries, as well as in the United States, this substance is no longer used, after findings of flauvalinate-resistant mites (Sammataro *et al.*, 2005). The main problem about these substances is their degradation lifetime and subsequent metabolites. From the most used pesticides in the 00' decade (amitraz, bromopropylate, coumaphos, chlordimeform, cymiazole, flumethrin, and tau-fluvalinate), only amitraz showed high degradation rates while the others were stable for at least 9 months (Korta *et al.*, 2001). These substances will accumulate in the colony, mainly in the wax (composed by lipophilic substances) and later might contaminate the honey. Moreover, since wax is recycled by beekeepers, these substances can persist and be already present in new colonies. Considering these problems, natural acaricides like organic acids and essential oils have been tested. These products have a low risk of accumulating on colony products (most of them are water soluble or volatile) but can have dangerous effects when not applied correctly (*e.g.*, when applied at high temperatures; Rosenkranz *et al.*, 2010).

3.3 Biological stressors

Honey bee colonies are affected by several biological stressors – the parasite *V. destructor*, pathogens (including nosemosis, foulbrood and viruses), and/or predators (including *Vespa velutina* and *Aethina tumida*). *V. destructor* is one of the most relevant biological stressors, with a large impact on colony losses. *V. destructor* was originally reported in Asia, in *Apis cerana* colonies. It arrived in Europe and in South America in the 1970's and by the 1990's had already spread across all Europe and entered the United States. Therefore, its distribution, impact and behaviour are extensively known. Even so, adequate treatments, fulfilling all the criteria of being safe, effective, and easy to apply, are yet to be found (Rosenkranz *et al.*, 2010).

V. destructor life cycle is closely related to the honey bee cycle. In the first phase (phoretic phase), the mites travel attached to the bees (between the abdominal segments), where they feed on the fat body tissue (Ramsey *et al.*, 2019), releasing themselves into drone and worker cells containing the oldest larvae right before capping. After capping, the mites feed on the honey bee larvae and will start laying eggs (reproductive stage). When the honey bee pupae hatches, at least two new mites and their mother will go into the phoretic stage (Rosenkranz *et al.*, 2010).

Besides feeding on the bee´s fat tissue (Ramsey *et al.*, 2019), varroa mites also act as a reservoir and incubator of viruses, transferring them to the bees while feeding. The most commonly varroa-transmitted virus, the Deformed Wing Virus (DWV), is also the widest spread (Martin *et al.*, 2012). Moreover, there is a newly emerged genotype of DWV that is more virulent and causes a faster collapse of the colony (McMahon *et al.*, 2016). Other common viruses, like the Acute Bee Paralysis Virus (ABPV), Kashmir Bee Virus (KBV), Sacbrood Virus (SBV) and the Israeli Acute Paralysis Virus (IAPV), are also transmitted by varroa mites (Brutscher *et al.*, 2016). In total, there are over 24 different viruses identified in honey bees (De Miranda *et al.*, 2013; Brutscher *et al.*, 2016), not all vectored by varroa. For example, the Black Queen Cell Virus (BQCV), which affects the queen cells instead of the worker cells, is associated with infection by *Nosema apis* (Bailey, 1982), a Microsporidia fungus that is responsible for the nosemosis disease. Nosema ceranae, which also causes the nosemosis disease, was recently identified in Europe (Higes *et al.*, 2006) and can also lead to the colony collapse (Higes *et al.*, 2008).

Colonies are also exposed to bacterial diseases like the American (AFB) and European (EFB) Foulbrood. AFB is caused by a spore-forming bacterium *Paenibacillus larvae*, affecting larvae during the early stages (12–36 h after egg hatching). The bacteria multiply rapidly after the larvae have been capped, eventually killing the infected larvae at the pre-pupal or pupal stage. This will create an

irregular and patchy brood pattern, and a sunken and perforated cell capping (Genersch, 2010). EFB is caused by the bacterium *Melissocccus plutonius* and affects mainly unsealed brood, killing honey bee larvae usually when they are 4–5 days old, changing its colour from pearly white to brown. If a high proportion of the larvae die, the brood pattern also appears patchy (Forsgren, 2010). Both diseases are detected by a foul or sour smell and are highly resistant and infectious (*e.g.*, the spores from AFB remain infectious for more than 35 years), so beekeepers need to burn their colonies and contaminated materials to prevent Foulbrood spreading into other colonies (Genersch, 2010).

Additionally, there are emergent pests affecting colony development. *Aethina tumida*, also known as the Small Hive Beetle (SHB), feeds on honey bee brood, pollen, and honey, leading to honey fermentation and brood death. This invasive species originates from Africa and has proven to be a serious pest in the USA and Australia. In Europe, it was first detected in Portugal in 2004 but the colonies were successfully eradicated until September of 2014 when it was found in Italy (EFSA, 2015a). The natural spread of the beetle is slow but could be facilitated by transporting infested colonies to other countries (EFSA, 2015a). So far, other European countries have not reported its presence.

Another important pest, the hornet *Vespa velutina nigrithorax*, originally from Asia, arrived in France in 2004 and has already spread to Italy, Spain, Portugal, Germany, Belgium, Switzerland, Netherlands, and Great Britain. Having such a swift expansion and by preying on honey bees (using the protein to feed their brood), *V. velutina nigrithorax* became one of the toughest problems for beekeepers to solve. Apiaries, which concentrate large food sources in relatively small areas, have become the preferred place for the hornets to hunt. On top of it, the limited knowledge about this species biology represents a disadvantage in the establishment of management plans (Monceau *et al.*, 2014). Recently, new methodologies have been explored for a faster detection of the hornets' nests (*e.g.*, Maggiora *et al.*, 2019), and national authorities have created special local task forces to eliminate the nests, mainly by the use of chemical control. Other environmentally friendly methodologies have also been explored (*e.g.*, Ruiz-Cristi *et al.*, 2020), but chemical control remains as the only efficient solution for nests' elimination.

3.4 Interaction between stressors

The list of stressors affecting honey bee health and development is long and the general assumption is that there is not a single factor responsible for the unusual colony losses. Researchers have agreed on a multiple stressor theory to explain this phenomenon (Underwood & Vanengelsdorp, 2007; Vanengelsdorp *et al.*, 2009; Steinhauer *et al.*, 2018), and in the last years a growing number of studies have focused on the effect of multiple stressors and their interaction. Tosi *et al.* (2017) reported, for the first time, the existence of a synergistic relation between poor nutrition and exposure to neonicotinoids, leading to reduced (i) survival, (ii) food consumption, and (iii) hemolymph levels of glucose. Similarly, thiacloprid exposure caused synergistic interactions with *Nosema ceranae* and BQCV, leading to increased mortality, more so than with the combination of just *N. ceranae* plus thiacloprid (Doublet *et al.*, 2015). Even antibiotics (verapamil) can increase mortality in the presence of common acaricides (coumaphos and t-fluvalinate) and neonicotinoids (imidacloprid, acetamiprid and thiacloprid) by inhibiting the "drug resistance" transporters that are responsible by the transport and excretion of toxins (Hawthorne & Dively, 2011). Recently, a synergistic effect between neonicotinoids and varroa was also found for the first time. The presence of neonicotinoids in colonies infested by varroa had significant negative synergistic effects on honey bee body mass and longevity (Straub *et al.*, 2019). However, most of these studies were conducted under laboratory conditions and on individual bees. There is still a lack of semi-field and field studies in which honey bee colonies are exposed to environmentally realistic concentrations and exposure pathways of neonicotinoid insecticides (Heimbach *et al.*, 2017).

4. Risk assessment in honey bees

Outlines of risk assessment are made by tiered approaches that start with laboratory studies (low tier) and later use semi-field and field studies (high tier). For honey bees, pesticide risk assessment tests take into consideration the colony survival and development (measuring effects on larvae and behaviour) and substances are approved if the exposure is negligible or if it has acceptable acute or chronic effects (Rortais *et al.*, 2017). For each bee life stage, specific trigger values are established in accordance with risk managers, to extrapolate to colony level effects (Rortais *et al.*, 2017), creating a strong first tier assessment. Nevertheless, by not considering multiple exposures or combined stressors, and ignoring additive or synergistic effects, such ERA process is based on the fundamentally

wrong and outdated assumption of "single substance, single use", *i.e.*, pesticides being assessed individually and for use on a single crop (Topping *et al.*, 2020). In a landscape, where bees forage on several hectares, several pesticide applications with several mixtures are applied through the year, and bees transport these substances into their colonies (Zioga *et al.*, 2020).

4.1 Risk assessment in Europe

The current guideline for risk assessment on honey bees was created in 2013 by the European Food and Safety Authority (EFSA, 2013). A tiered approach scheme is suggested, in which the appropriate level of protection needs to be achieved at each tier. In this document, EFSA opted for a high level of protection establishing the specific protection goals (SPGs) and the associated trigger values on the lowest values of background mortality (EFSA, 2013). It was defined that an effect of a pesticide was negligible if its magnitude was < 7% on the colony/population size reduction, as the existing technology and beekeepers' expertise did not allow effects to be detected at a lower level. This was an improvement in the protection levels compared to the previous guidelines, allowing a 20-25% population decline (EU, 2002). However, EFSA (2013a) guidelines were never fully implemented by many member states, so current risk assessment schemes are based on 20-years old science. EFSA is currently revising the risk assessment scheme, which was open for public consultation between July and October 2022 and will soon be presented to the European Commission. Considering the newly available technologies (*e.g.*, bee counters - Odemer, 2022), SPGs conferring a higher level of protection were to be expected. Nonetheless, a 10% population decrease as an acceptable effect was proposed by the European commission (27-04-2021 in a letter by a Health and Food Safety member of the European Commission). If implemented, the 10% SPG will increase the current protection level but falls short regarding the agreed levels in 2013 (Simon-Delso *et al.*, 2021a).

To set the new SPGs, the European Commission requested EFSA to review the Guidance Document, based on the assessment of a single substance/single use assumption (More *et al.*, 2021). EFSA proposed the use of four different approaches to calculate the SPGs:

- Approach 1 – to establish acceptable effects based on long-term colony survival.
- Approach 2 – to derive a threshold of acceptable effects on colony size based on the normal operating range.
- Approach 3 – to establish acceptable effects, based on pre-defined levels, on colony/population size.

- Approach 4 – to establish acceptable effects on colony/population size based on levels of acceptable impact on the provision of ecosystem services.

While the "approach 3" was used in previous studies to set the SPGs, EFSA is relying on the "approach 2" for the current review, in which the Beehave model (Becher *et al.*, 2014) was used to model the possible background variability on colony strength. Nonetheless, this model had been previously discarded by the same authorities due to its simplicity and shortcomings regarding the stressors, landscape, and climate variables (EFSA, 2015b).

While revising the Guidance Document, EFSA was also requested to issue an opinion on the systems-based approach to make an assessment focused not only on the single crop/use, but on the context of multiple stressors. This document is based on monitoring and modelling strategies, in which it is proposed the use of sentinel colonies to retrieve data from colony development under different scenarios and the use and constant update (using the environmental data collected in the sentinel colonies) of the ApisRAM model (EFSA Scientific Committee, 2021) were proposed.

Colony assessments under field conditions are therefore valuable for the envisaged systems-based approach (sentinel hives) and for the risk assessment guidelines, in which, the higher tier (field) is the reference tier for decision making. Nevertheless, the colony assessment methodologies are still prone to bias, being observer-dependent.

5. Colony assessment

Field-testing has several constrains regarding the control of the experimental variables, which are not found under laboratory conditions. Under field testing, the climatic conditions and the landscape influence colony development and behaviour, leading to higher data variability thereby compromising the statistical power of the obtained data (Rortais *et al.*, 2017). On top of it, despite the vast number of studies with honey bees, there is a lack of studies evaluating the normal colony development concerning the environment where the colonies are installed (*e.g.*, Hatjina *et al.*, 2014). Contrary to the standard laboratory studies, there are no standard universal protocols to assess colony strength and health under field conditions. Moreover, most of the available methods are based on visual assessments, which are therefore observer-dependent and prone to bias (*e.g.*, the Liebefeld method and/or adaptations; Delaplane *et al.*, 2013). To standardize methodologies and create efficient

protocols to harmonize data collection, EFSA published a document that gathers the most common and effective methodologies to measure each feature of colony development (EFSA AHAW Panel, 2016). However, no standard protocols have been proposed in this document.

During the last decade, the use of novel technologies aided the analysis of colony strength (*e.g.*, Alves *et al.*, 2021), production (Lecocq *et al.*, 2015, Meikle & Weiss, 2017), behaviour (*e.g.*, Henry et a., 2012), activity (see the review by Odemer *et al.*, 2022) and health (*e.g.*, Mayack *et al.*, 2022). These could pave the way for a more efficient and observer-independent data collection, allowing the implementation of standard protocols. Additionally, increasing the methods' accuracy used for colony monitoring will help to detect effects of lower magnitude, increasing field testing reliability.

Currently, colony assessment methods are focused on the analysis of brood, provision, and adult bee population inside the colony (Delaplane *et al.*, 2013). To get a fine-scale time-series data on these parameters, hive scales enabling nectar/honey provision measurements are already being used. On the other hand, the number of brood cells or bees is only known by opening the colony, creating additional stress. Therefore, efforts should be made to understand the impacts of the applied protocols and/or search for ways to reduce that impact, exploring novel technologies (*e.g.*, Ramsey *et al.*, 2020) that could indicate the colony status without opening the hive.

6. Colony modelling

To understand how different stressors act and interact to affect honey bee colonies development, one needs to start by exploring the normal colony development. Honey bee colonies are composed by several individuals engaged in different tasks, which regulate the colony temperature, pollen and nectar reserves, brood rearing, and the queen well-being. All these tasks lead to colony growth and provision accumulation, with spatiotemporal changes on provision and nest distribution and size. Such complexity prevents researchers from using simple models to predict colony development, by creating a parsimonious model that could be simple to create, easy to interpret, and with great explanatory predictive power. Instead, the colony behaviour and plasticity require the development of complex models. The main challenge, while developing such models, lies in the number of factors used to create the system. The goal is to create a system by deploying only the most relevant factors and avoiding spending time and resources to implement factors that are not important for the system. While that is true, this simplification can lead to exclusion of important factors and those might

become useful in predicting ecological systems (Topping *et al.*, 2015), as they usually fluctuate around organisms' plasticity/acclimation to new environmental challenges.

So far, honey bees have been extensively studied and several models have been created regarding (i) foraging strategies (Schmickl & Crailsheim, 2004; Dornhaus *et al.*, 2006; Baveco *et al.*, 2016), (ii) colony population dynamics (Khoury *et al.*, 2011), (iii) effect of infections on colony failure (Betti *et al.*, 2014) and (iv) effects of varroa and other stressors on population dynamics (Becher *et al.*, 2014). Despite the extensive knowledge on honey bees and the developed models to date, these models do not mechanistically link the several variables that affect the colony development rates - colony population dynamics, in-hive stressors like varroa and other viruses, spatiotemporal distribution of resources in the landscape, climatic variables, the colony interaction with the landscape - to predict the effects of multiple stressors. Consequently, EFSA (2016a) proposed the development of a conceptual model for the risk assessment of pesticides and other stressors in honey bee colonies. EFSA suggested which modules would need to be created to develop a fully functional model for risk assessment. After a year, EFSA (2017) specified which data would need to be collected to contribute for the development and corroboration of the model. Furthermore, in 2021 it was proposed the implementation of a monitoring system (see section 4.1 for more details) to collect even more data to feed the modelling system (EFSA Scientific Committee, 2021). Within this report it is also proposed a timeline for the modelling system: the ApisRAM.

The ApisRAM aims at linking all the variables that affect the colony development rates to predict the effects of multiple stressors. The ApisRAM formal model (Duan *et al.*, 2022) contains a detailed description of all these modules that compose the ApisRAM and how they interact with each other. The model follows the rules of the individual agent-based models (ABM), in which each individual (honey bee) is modelled as an agent that affects and it's affected by other agents, the intrinsic internal colony features and the external environmental variables. According to EFSA (EFSA Scientific Committee, 2021), the ApisRAM model will be fully developed by 2026. However, an interim version of the model that only addresses single stressor/single use risk assessment will be available in summer of 2024.

7. Objectives and thesis outline

The main goal of this dissertation is to assess how spatiotemporal changes in the landscape affect honey bee colonies development rate and how (mainly) chemical stressors can affect the honey bee behaviour under field conditions.

Knowing that colony development and health depend on both temporal and spatial scales, according to the colony needs and the resources offered by the landscape, there is a need for a close follow up of colony development throughout the season. For that purpose, this study is focused on the honey bees' response under real scenarios, for which five main scientific experiments were conducted, each one representing an attempt to address specific goals (Chapters II-VI), as follows:

Chapter II - Create a comprehensive and result-efficient protocol to collect reliable and detailed data on several colony attributes (development and health status) under field conditions without compromising the normal colony development by monitoring or sampling.

To compare colony development under different conditions and through time, reliable and objective methods for obtaining colony status metrics are needed. In this first experiment, several methodologies were evaluated based on their importance to colony development and health, reliability, feasibility, and accuracy. Priority was given to methods that are not observer-dependent, and which can be applied across different regions independently of the observer. A protocol was developed using in-colony real-time sensors, digital photography, and frame weighing every 20 days for colony development monitoring through the season. The protocol was then evaluated for its accuracy, its possible impact on the normal colony development, and the pros and cons of using it compared to methods used by other studies.

Chapter III - Evaluate the relationship between colony development and the external landscape variables, as climate and spatiotemporal offer of resources, to identify which are the most relevant variables affecting colony development and floral resources use throughout the season.

To evaluate the development of colonies located in different areas with different landscape compositions, the protocol developed in chapter II was employed. Three landscapes were used varying from mostly composed by agricultural areas to being dominated mostly by natural vegetation,

differing in their spatial and temporal availability of food resources. The landscape composition, as well as the climatic variables, were used to explain the differences in the colony development of each apiary. Furthermore, these variables and colony strength were explored to evaluate the daily nectar income and pollen diversity collected by the colonies.

Chapter IV: Assess the colony exposure and possible effects to acetamiprid in a real exposure scenario

Pesticides are often indicated as a key driver for changes in colony developmental rates in space and time, hence, in this chapter the exposure to a neonicotinoid insecticide was evaluated. The neonicotinoids are a group of pesticides, that are specific for insects and have been criticised for their negative effects on honey bees and other pollinators. Nonetheless, the exposure routes and concentration of these substances after field exposure can have large fluctuations because they are context dependent. Therefore, a field experiment was performed in areas where acetamiprid, the only neonicotinoid still allowed for outdoor uses in Europe, was applied. To measure the exposure levels both in the landscape and in the colony, flowers and colony products were sampled, and residue levels were used to derive risk values.

Chapter V: Assess the effects on individual homing ability after exposure to sulfoxaflor and acetamiprid

Recently, severe restrictions were applied to most neonicotinoids in the European Union, leaving acetamiprid as the only neonicotinoid that can be applied outdoors. However, new products that act in a similar way, including sulfoxaflor, are replacing the old neonicotinoids. Since the colony development rates can also be affected by sub-lethal effects usually caused by exposure to lower doses, we tested the sub-lethal effects of these substances. Individual honey bees were orally exposed to acetamiprid and sulfoxaflor spiked solutions to evaluate their ability to return to the colony after being exposed to sub-lethal doses. The Radio Frequency IDentification (RFID) was used to evaluate the honey bee's ability to return to their original colony after being released 1 km away.

Chapter VI: Contribute to the calibration and improvement of the honey bee model ApisRAM, by evaluating the foraging strategies of honey bees to create a functional foraging module to integrate in ApisRAM.

The main challenge in environmental risk assessment is linking the individual effects (lethal and sub-lethal) to effects at the colony level. The colony intrinsic mechanisms and the external environmental variables play a substantial role in how the pesticides affect the colony development rates. To link these variables and predict effects at the colony level from pesticide exposure, ApisRAM is being developed. ApisRAM is composed of several sub-models/modules that interact with each other, to create a fully functional model on colony development, considering the landscape and stressors. Honey bee colonies interact with the landscape through the forager bees. These are the ones that decide where and when to forage. In this study, the first stage of the foraging module was developed to evaluate how the possible forager bees' strategies affect the overall colony collection of pollen and nectar in a dynamic landscape. The landscape characteristics as the field size, the amount, quality and distribution of flowering resources, as well as the distance from the colony to the food source were explored to evaluate the different foraging strategies. Furthermore, it was explored how the amount of social colony information could affect these strategies.

In the future, the detailed data collected in *Chapter III* from the different landscapes (from the colonies, landscape structure and land management) will contribute to model calibration and improvement, allowing to enrich model application to southern European zones.

Chapter VII integrates the results of all five chapters in a General Discussion, from which future lines of enquiry are proposed.

Chapter II

High accuracy monitoring of honey bee colony development by a quantitative method

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Abstract

Honey bees are key insect pollinators, providing important economic and ecological value for human beings and ecosystems. This has triggered the development of several monitoring methods for assessing the temporal development of colony size, food storage, brood and pathogens. Nonetheless, most of these methods are based on visual assessments that are observer-dependent and prone to bias. Furthermore, the impact on colony development (invasiveness), as well as accuracy, were rarely considered when implementing new methods. In this study, we present and test a novel accurate and observer-independent method for honey bee colony assessment, capable of being fully standardized. Honey bee colony size is quantified by assessing weight of adult bees, while brood and provision are assessed by taking photos and conducting image analysis of the combs with the image analysis software Deepbee®. The invasiveness and accuracy of the method were investigated using field data from two experimental apiaries in Portugal, comparing results from test and control colonies. At the end of each field experiment, most of the tested colonies had the same colony size, brood levels and honey production as control colonies. Nonetheless, continuous weight data indicated some disturbance in tested colonies in the first year of monitoring. The overall accuracy of the image analysis software was improved by training, indicating that it is possible to adapt the software to local conditions. We conclude that the use of this fully quantitative method offers a more accurate alternative to classic visual colony assessments, with negligible impact on colony development.

Introduction

Pollination is vital for the functioning and sustainability of terrestrial ecosystems and is considered one of the most important regulating ecosystem services (IPBES, 2016). Pollinators are responsible for the maintenance of many terrestrial ecosystems, since the service they provide allows, directly or indirectly, for other species to co-exist and develop. It is estimated that 87.5% of all flowering plant species are to some extent dependent on animal pollination (Ollerton *et al.*, 2011). Amongst pollinators, the Western honey bee (*Apis mellifera*) has been introduced worldwide and is a key species in crop pollination (Klein *et al.*, 2007; Garibaldi *et al.*, 2013). Furthermore, honey bees are complementary pollinators in natural habitats, as they are the most frequent visitor in 13% of plant species and the only flower-visitor observed in 5% of the plants (Hung *et al.*, 2018).

The increasing use of honey bees for crop pollination, derived from the rapid expansion of pollinator-dependent crops (Aizen *et al.*, 2009), has led to an increase in the number of colonies worldwide (FAO, 2022). Even the decreasing trend in the number of colonies due to sudden colony

losses and winter losses in Europe and North America from the 1990's onwards, has been inverted since 2008 (FAO, 2022; Osterman *et al.*, 2021). Nonetheless, these figures only cover the total number of colonies, without considering their health status or strength, and high winter losses (>15%) have been reported since the occurrence of the ectoparasitic mite *Varroa destructor* outside of its native range (Potts *et al.*, 2010b; Potts *et al.*, 2016). In addition to varroa, multiple drivers of honey bee colony losses have been identified, including historical land use changes leading to scarcity of flower resources, use of pesticides, detrimental beekeeping practices, and increased pressure from other pests and parasites (VanEngelsdorp *et al.*, 2009; Steinhauer *et al.*, 2018). Due to the diversity and interaction of stressors, the main challenge is to understand their impacts, in isolation and in combination, in addition to confounding factors such as landscape context and climate (EFSA Scientific Committee, 2021).

The economic and ecological value of honey bees and the urgent need to understand colony losses have motivated the implementation of research methods to assess a temporal development of colony strength and health (Delaplane *et al.*, 2013; Human *et al.*, 2013; EFSA AHAW Panel, 2016). These methods provided researchers with numerous useful tools for colony assessment. However, universal methods and protocols for the field assessment of honey bee colonies have not been established yet, due to local variation and constraints imposed by climate, honey bee genetic diversity (subspecies and ecotypes), beekeeping practices and landscape. Therefore, the results obtained using different methods are not necessarily directly comparable. As one example, honey production may be estimated by calculating the number of honey cells in a comb or by weighting the honey frames, however, no simple conversion exists between these two measures.

Most protocols for colony strength assessment are based on the Liebefeld method or adaptations from it (Delaplane *et al.*, 2013). The Liebefeld method consists of a visual estimate of the number of adult bees on each side of the frame, in addition to a visual estimate of the comb surface area (dm^2) containing open brood, capped brood and provision (Dainat *et al.*, 2020). The method has been enhanced by training observers using images of combs with known cell content (*e.g.*, Hernandez *et al.*, 2020; Dainat *et al.*, 2020), or by the use of a grid or a measuring tape to estimate the brood ellipse (*e.g.*, Odoux *et al.*, 2014). These adaptations improve the accuracy of the visual estimates, although estimates are still observer-dependent.

The need for quantitative data, which are both accurate and observer-independent, has been increasing in recent years. As a quantitative measure of honey bee adult population, the weight of the frames with and without bees has been used as an alternative to visual assessments (*e.g.*, Odoux *et al.*, 2014; Meikle & Weiss, 2017). Semi-automatic or automatic analysis of comb images has also been

evaluated as an alternative to visual assessment of comb cell content. However, a range of challenges have hindered the use of image analysis methods. Some software/algorithms only reliably identified capped brood cells (Yoshiyama *et al.*, 2011; Rodrigues *et al.*, 2016), while others could detect different cell content but with a low accuracy level (Liew *et al.*, 2010), or extensive time was needed for the analysis (Meikle & Weiss, 2017). A recent development, DeepBee© (Alves *et al.*, 2020), is an open-source software capable of distinguishing different comb cell contents (eggs, larvae, capped brood, beebread, nectar, honey and others) with high accuracy (94.3% overall accuracy according to Alves *et al.*, 2020).

Despite these technological advancements, which enable quantitative assessments of honey bee colony strength and provision, no standard protocols are available which provide reliable and accurate colony analysis. Such an analysis should include a detailed assessment of the most important indicators of colony development and health, based on accurate, observer-independent data. Furthermore, most of the existing protocols have, in general, not been assessed for their invasiveness, *i.e.*, their potential impact on colony development. If the monitoring method adversely affects colony development, the resulting data will not reflect normal colony growth. Hence, a standard protocol should be based on high quality quantitative data, using monitoring methods, which do not impact colony development.

The Animal Health and Welfare (AHAW) Panel of the European Food Safety Authority (EFSA) published a scientific opinion that mapped existing colony indicators and assessment methods, known as the "HEALTHY-B" toolbox (EFSA AHAW Panel, 2016). Based on the most important colony health status indicators from this document, relevant methods were selected and used in a large-scale field study, in order to develop a field protocol, which involved collecting accurate quantitative empirical data on colony size (adult bees), brood development, provision, and health (diseases and parasites loads) (Dupont *et al.*, 2021; supplementary material, section II.1).

In the current study, we tested a new quantitative method, as an alternative to the widely used Liebefeld method. In two apiaries located in Portugal, we assessed colony strength and development using weight and image analysis of cell combs to quantify colony size, brood and provision and their dynamics across two field seasons. To evaluate the image analysis usefulness and adaptation to local conditions, we tested the accuracy of the cell detection made by the software DeepBee©. Furthermore, we tested whether disturbance induced by frequent and invasive monitoring had a measurable impact on colony development by comparing monitored (test) colonies and non-monitored (control) colonies.

Materials and methods

Experimental setup

Experimental apiaries were installed in two distinct landscapes: Serra da Lousã (40°02'53.6"N 8°14'38.9"W) and Idanha-a-Nova (39°51'33.0"N 7°09'49.7"W), Portugal. The landscape in Lousã was dominated by forested areas and scrubs, with a high diversity of nectar and pollen resources available from March to October, with a peak in May. In Idanha, the landscape was dominated by pastures, cork-oak forest and cereal crops for fodder, containing floral resources from March to July, with a peak in April/May (Dupont *et al.*, 2021).

Each apiary included seven colonies of local honey bee populations, *i.e.*, *Apis mellifera iberiensis* in Langstroth hives. The colonies were established in autumn 2018 using varroa-treated package bees (provided by a local professional Beekeeper). To minimize variability among colonies due to genetics, colonies originating from sister queens produced in 2018, were used in both apiaries. All colonies were managed using local standard beekeeping practices. Colonies were treated against varroa mites with Apivar (amitraz) in January 2019, with Apiguard (thymol) in August 2019 and again with Apivar in February and August 2020 and visually screened for disease symptoms at every visit.

In each apiary, five colonies (hereafter denoted test colonies) were subjected to regular colony assessments (see supplementary material section II.1 for details), throughout two field seasons, between March and September of 2019 and 2020 guaranteeing low levels of varroa mites. Test colonies were assessed approximately every ±19 days, to guarantee a snapshot of all the brood cycles within the worker bee brood development cycle of 21 days. Two colonies (hereafter denoted control colonies) were only subjected to colony assessments at the beginning and the end of the field season, in both years and to regular beekeeping practices during the season. In 2020, monitoring started later due to COVID-19 restrictions and cold weather in Lousã.

Continuous monitoring of hive weight

All hives were equipped with an Beeyard stand-alone hive scale for continuous logging of the hive weight. Hive weight was monitored continuously throughout two field seasons, from 05 April 2019 to 31 December 2020 in Lousã and from 30 March 2019 to 31 December 2020 in Idanha. Data on hive weight was logged continuously and automatically once per hour. However, to reduce diurnal fluctuations in weight due to foraging, the hive weight at midnight was used as the daily measure of total hive weight. For both years, cumulative weight (increase/decrease) from the beginning of each field season until honey harvesting was used to compare the development of test *vs*. control colonies,

by calculating the weight difference between the cumulative mean weight of control colonies and the cumulative mean weight of test colonies.

Colony assessment

During colony assessment, smoke was applied to keep the bees on the frame. Afterward, each comb frame was hanged on a fixed hanging scale, to ensure the scale stability, and weighed with adult bees, and set aside in a separate box. In a second step, bees were gently removed from the comb by brushing them off into the original hive, and the empty comb was weighed again. Brood and food resources were assessed through image analysis of photos taken from both sides of each comb frame. To provide homogenous light conditions, images were obtained using a digital camera (DSRL Nikon D3300, 24.2 MP) installed inside a photography tunnel (for further details see Alves *et al.*, 2020). This procedure was repeated for all the frames in the colony, before returning them to the original hive. To avoid heat loss during monitoring conducted early in the season, colony assessments were carried out when weather conditions were favourable, *i.e.,* >14 °C, with week wind and dry weather. Monitoring was carried out as quickly as possible, to minimize disturbance of the colony. Furthermore, care was taken to cage the queen during monitoring, to avoid physical damage or exposure to cold or hot external temperatures.

The number of adult bees was calculated based on a mean weight per bee. The mean bee weight was estimated by weighing 50 individual bees, randomly collected after applying smoke to the colony (supplementary material, section II.2).

Recorded images were assessed for comb cell utilization using an upgraded version of the DeepBee© software, adapted to local conditions (see next section – DeepBee© analysis training). The software automatically detected cells in the comb images and classified them into the following categories: eggs, larvae, capped brood, pollen, nectar, honey, and others (Alves *et al.*, 2020). Although the number of honey and nectar cells was quantified by the DeepBee© software, the weight of honey or nectar varied with cell depth. Therefore, we estimated honey/nectar provision (honey production) for each comb frame by subtracting the weight of the foundation and other components (capped brood, larva and beebread) from the weight of the frame comb without bees. The mean weight of empty frames was calculated by weighing 50 nest, and 50 honey super, Langstroth comb foundation frames. Beebread mean weight was calculated by individually weighing 100 beebread cells on a precision scale, while capped brood and larva means weight was calculated based on Zółtowska *et al.*

(2011), in which the body weight of the successive developmental stages (for both larvae and pupae) was determined (Supplementary material II.2).

To detect potential disturbance of colony development due to handling during the detailed colony assessment, the number of adults, the number of cells containing brood and the amount of provision (honey/nectar) were used as proxies of colony performance. The amount of beebread cells was not used for comparison since honey bees have a preference to consume fresh pollen and ignore old beebread (Carroll *et al.*, 2017). During the experiment, care was taken to verify that all the colonies had enough beebread cells in the colony.

For each study year, the performance of test and control colonies was compared at the beginning and end of the field season. For Lousã and Idanha in 2019, due to loss by swarming of one control colony, a one-sample *t*-test was carried out to compare the colony size, brood cells, and honey production of test colonies. For Lousã 2020, an independent samples *t*-test was performed to compare test with control colonies.

Image analysis training

The original version of the DeepBee© software has a high level of accuracy (F1 score of 94%) compared to visual assessments, although error rates varied among the different classes, with the least accurate classes being "eggs" (84% correctly identified cells), followed by larvae (88%) (Alves *et al.*, 2020). Since the software is based on deep learning, it is possible to increase its performance and to adapt it to local conditions such as variations in colours, the structure of pollen and wax, or luminosity during image capture. To improve the performance of the software, in particular for the detection of eggs and larvae, we selected 25 images containing these cell classes from our colonies. In this training set of images, the automatic classification of each cell was carefully examined and manually revised and corrected whenever needed. After the training, the accuracy of the upgraded version was assessed using 40 random images from our colonies by comparing the automatic output with the manually corrected output. First, the error for each class was calculated as:

$$error = \frac{manual\ correction - software\ output}{manual\ correction}\ x\ 100$$

Secondly, the overall accuracy of each class was calculated as:

$$accuracy = 100 - abs(error)$$

Results

Colonies that swarmed or became queenless during the experiment were removed from the analysis. This resulted in data being available for analysis from three test colonies and one control colony in Lousã and Idanha in 2019, four test colonies and two control colonies in Lousã in 2020, and five test colonies and no control colonies in Idanha in 2020. Therefore, Idanha 2020 data were discarded from the analysis.

Hive scale data

Seasonal change in hive weight (Fig. II.1) was calculated using the automatic hive scale data. These data reflect diurnal changes in weight due to nectar flow and pollen collection, consumption of provision, and changes in adult and brood populations. A decrease in the weight of the test colonies was observed in Lousã 2019 and Idanha 2019 compared to the control colonies by the end of the season. The difference in weight between control and test colonies in the end of the season in Lousã 2019 was approximately 6 kg, which represents approximately 9% of the total colony weight. In Idanha 2019, a similar weight difference (approximately 5 kg) representing approximately 5.5% of the total colony weight was found. In Lousã 2020, the weight patterns of the test colonies were within the range of the control ones.

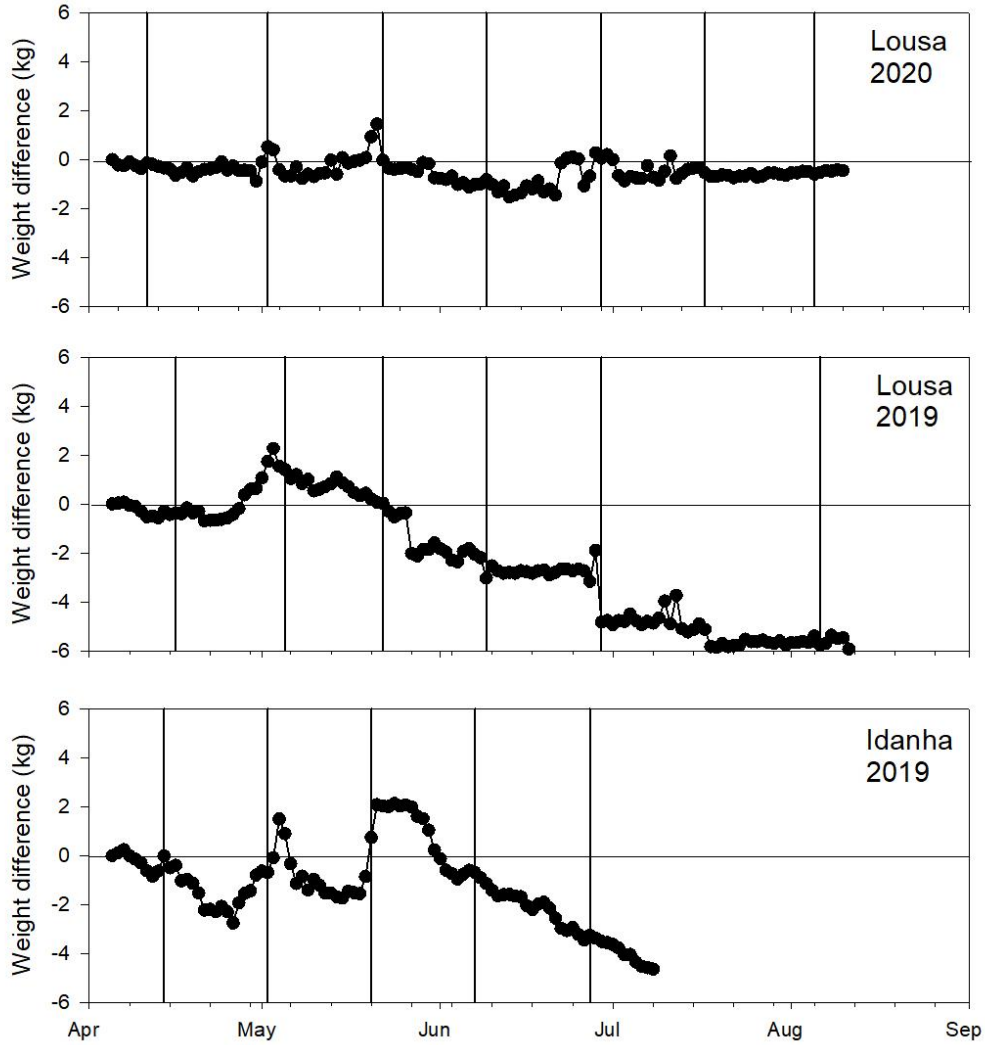


Figure II.1: Seasonal change in the mean weight difference between the mean weight of test colonies and mean weight of control colonies, from the beginning of the experiment until honey collection in the Lousã apiary in 2020, Lousã apiary in 2019, and Idanha apiary in 2019. The honey collection in Idanha is earlier than in Lousã.

Colony assessment

Colony performance parameters (colony size, number of brood cells, kg of honey/nectar at the beginning and end of the season) showed no significant differences (independent samples *t*-test) in Lousã in 2020 (Fig. II.2c). Only the number of initial brood cells in test colonies was significantly higher than control (one sample t-test, $p=0.036$) in Lousã in 2019 (Fig. II.2a). In Idanha in 2019, the initial and final numbers of adult bees were lower in test colonies compared to control colonies (one sample t-test, $p<0.01$ in both cases). Similarly, the final honey production was lower in test colonies compared to control ones (one sample t-test, $p<0.01$; Fig. II.2b).

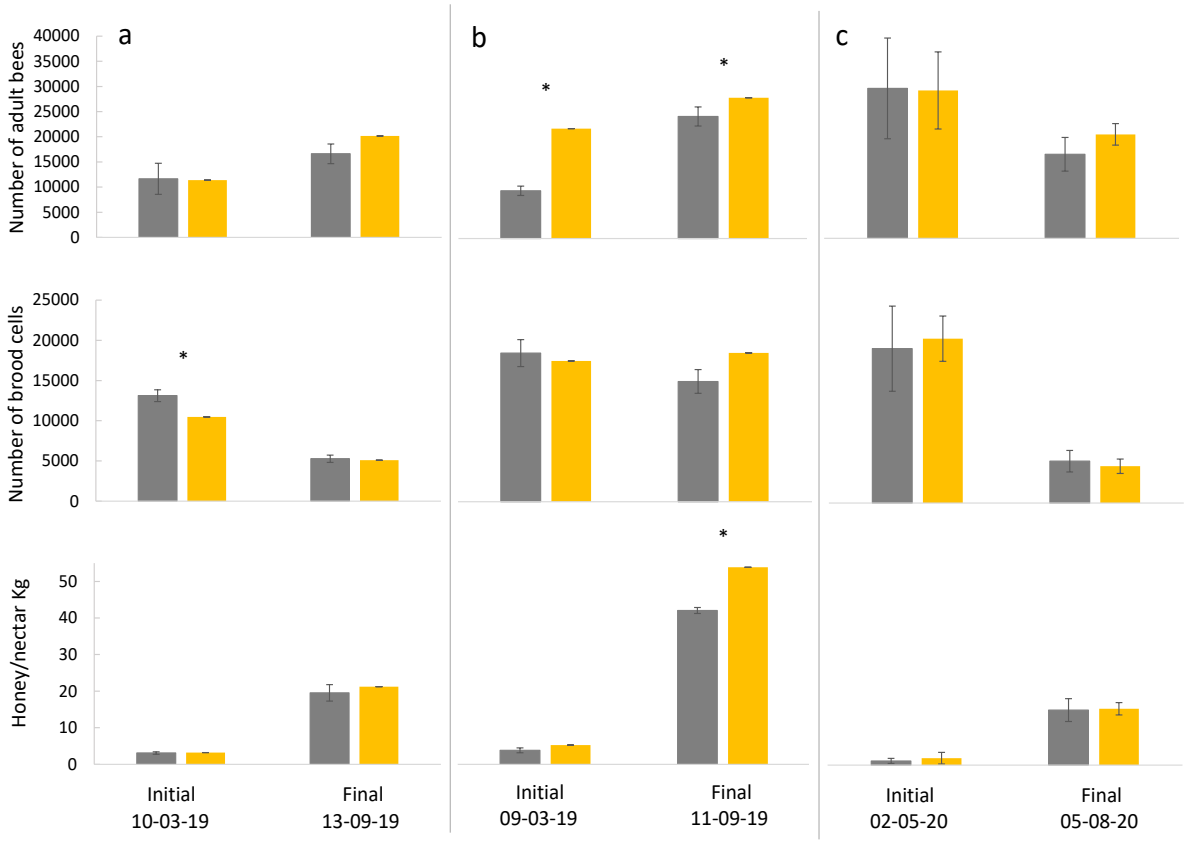


Figure II.2: Mean number and SD of adult bees, brood cells (eggs + larvae + pupae) and honey/nectar present in test (grey) and control (yellow) colonies. (a) Lousã apiary in 2019 - 3 test vs. 1 control colony; (b) Idanha apiary in 2019 – 3 test vs. 1 control colony; (c) Lousã apiary in 2020 – 4 test vs 2 control colonies. (*) represents significant statistical differences.

Image analysis software accuracy

Several training sessions of DeepBee© were carried out to improve the accuracy of the identification of egg cells and larvae. Comparing the original (Fig. II.3a with data from Alves *et al.*, 2020) and the upgraded version after the training session in the current study (Fig. II.3b, from our own set of 40 random pictures after training), the image classification resulted in the preservation of a near-perfect capped brood detection, and in a better performance in detecting eggs, larvae and honey, albeit a poorer performance in pollen, and nectar classification (Fig. II.3b). With an overall increase in the software accuracy, the "other" class accuracy, that usually is associated with empty cells, also increased. The upgraded version of DeepBee© allowed for a more accurate assessment of brood development with a minor impact on provision quantification as honey/nectar were estimated using weight.

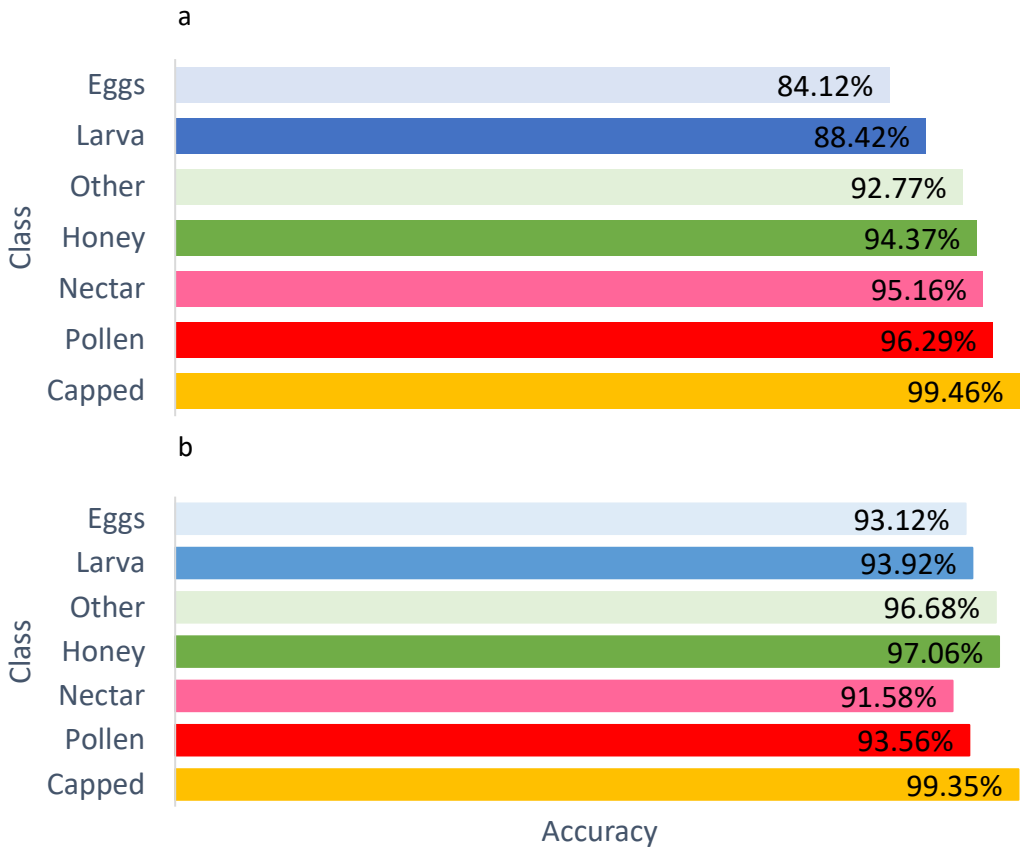


Figure II.3: DeepBee© accuracy (a) in Alves et al. (2020) and (b) after training with a set of comb images recorded in the current study. Eggs, larvae, other and honey classification was improved while pollen and nectar were reduced. Capped cell classification remained above 99% accuracy. In the original DeepBee© version, young larvae and eggs are the main source of inaccuracy. Nevertheless, the upgraded version increased the detection of eggs and larvae above 93%.

Discussion

In this study, we gathered detailed data on colony size, brood, and provision, monitored at regular intervals during the field season from test colonies and only in the beginning and end of the season in control colonies. This was combined with continuous daily colony weight measured by automatic hive scales. Both sets of data were used to assess if the disturbance induced by frequent and invasive monitoring had a measurable impact on colony development. Also, the image method accuracy and adaptation to local conditions were evaluated by measuring the image analysis software accuracy.

In general, comparing the performance of test and control colonies in the beginning and end of the season, only indicates minor impacts due to implementation of the protocol every ±19 days in some scenarios. In Idanha 2019, test and control colonies differed in the final colony size and honey

production. However, this may be related with the initial status (the control colony had a higher population in spring 2019) and not with any impaired development. Nonetheless, the small number of colonies and high variability in colony development may mask subtle effects of monitoring mainly when comparing only two data points in time. To overcome this concern, we used the scale data to assess variation during the season.

We had expected that the higher number of bees and brood cells in the beginning of the experiment would result in a larger population of foragers for collecting resources during peak flowering in spring. Nevertheless, the 2019 scale data showed a decrease in the weight of test colonies compared to control ones in both apiaries, although the decrease in weight started only after spring. This possibly means that the initial status did not play a role in the final production nor on colony size and there is a measurable impact caused by the regular colony inspections. Nonetheless, the negative impacts of monitoring on test colonies could only be detected after several assessments. This tendency was not observed in 2020, as the seasonal patterns of weight change of the test colonies were within the same range as the control ones. Furthermore, no differences were detected between the initial and end-of-season parameters when comparing the test and control colonies (Fig. II.2a). This suggest that the level of disturbance due to monitoring is not affected by the methodology *per se*, but on the handling by the observer. Unexperienced observers spend more time on each assessment, increasing the colony stress by preventing the colony from a faster return to their original state (before the stress), and by increasing the brood temperature fluctuations, which, above certain levels, cannot allow the brood to recover from the stressor (Ramirez *et al.*, 2021). Also, the gentleness used in frame handling and to brush the bees can play a role on decreasing these stressors. Possibly, our experience in colony assessment acquired during the 2019 field season resulted in a more swift and effective monitoring and induced less disturbance to the test colonies in 2020. The registered colony weight loss in 2019 that was not registered in 2020 could therefore be explained by the higher amount of energy used to restore the colony after the suffered stressor (*e.g.*, Schott *et al.*, 2021).

We hence conclude that conducting detailed colony assessments every ±19 days during the field season is likely to induce some stress, although effects are subtle when comparing colony performance due to colony feedback mechanisms and to inter-colony variability. Such feedback mechanisms may allow stressed colonies to spend more energy and resources on recovering (*e.g.*, Schott *et al.*, 2021). Reducing the number of visits/colony assessments can compromise the temporal resolution of data points, but will decrease the induced stress, increasing the data reliability on the specific days of monitoring. Likewise, the colonies only seem to be impacted after a few colony assessments. Therefore, the use of the method during a short timeframe (*i.e.*, less than 3 months)

would not compromise the quality of the data. Finally, we recommend training sessions on non-experimental colonies before an experiment is carried out, to improve the observers' skills in colony handling.

Table II.1: Comparison of available methods for honey bee colony assessment, from visual assessments (Liebefeld) to quantitative ones (the proposed method).

Parameter	**Liebefeld**	**Coleval (Hernandez *et al.*, 2020)**	**ECOBEE (Odoux *et al.*, 2014)**	**Quantitative method**
Data accuracy	Low	Intermediate	Intermediate	High
Distinguish eggs/larvae/pupae	Yes	No (open *vs*. closed brood)	No (open *vs.* closed)	Yes
Identification of pollen, nectar and honey	Yes	Yes	No	Yes
Colony size assessment	Yes	Yes	Yes	Yes
Observer dependent	Yes	Yes	No	No
Time spent on assessment	Slow	Fast	Fast	Slow
Experience setting	Simple	Simple	Simple	Laborious
Observer training	Laborious	Laborious	Simple	Simple
Adequate for large experiments	Yes	Yes	Yes	No

The proposed protocol allows the user to assess colony size, brood development and provision using quantitative assessments that are independent of the observer. Compared to other existing methods (Table II.1), the proposed protocol requires the construction of a photography tunnel for recording comb images (see construction details in Alves *et al.*, 2020), and an investment in the tunnel and a digital camera. We believe that these constraints are easily overcome by researchers, but financial and logistic challenges may limit the implementation by beekeepers while doing regular colony assessments. When compared to the Liebefeld-based methods, the quantitative method allows the acquisition of accurate cell count data on all brood stages (from eggs to pupae), different

food reserves (nectar/honey and pollen) and number of adult bees. The method can be applied anywhere in the world without any previous training for comb content extrapolations (observer-independent), and the comb images can be stored, and hence re-analysed and/or used in future training of DeepBee©. However, although much more accurate data are obtained by image analysis, the recording of images is more labour intensive than conducting a Liebefeld assessment. Disregarding the time required to set up the photography tunnel (10 minutes, if the tunnel is installed in the apiary during the season), monitoring takes two people approximately 25 (±5) minutes, 32 (±5) minutes and 49 (±2) minutes for a colony with one, two boxes or three boxes, respectively. Our team achieved similar assessment times in another project in which an enhanced Liebefeld method (using a grid) was used. However, the time spent on both assessments is not directly comparable, as we had more experience in the quantitative method.

In addition to being capable of recognizing and distinguishing the different cell contents, DeepBee© is an open-access and user-friendly software. Moreover, it can be upgraded and adapted for different image acquisition conditions, including different comb frame dimensions, colours, or photographic light conditions. For instance, the software could be adapted to different pollen colours and structure of each cell originating from different bee subspecies and landscapes, as reported by Dupont *et al.* (2021). However, for optimizing the performance of the software, we recommend following the recommendations of the DeepBee© developers for the acquisition of images, taking into consideration lux intensity, LED positioning (see Alves *et al.*, 2020 for details) and adjusting the tunnel dimensions to fit the frame dimensions (Dupont *et al.*, 2021). Previously developed methods reported that images acquired under field conditions often suffer from poor and variable light conditions. For instance, in the study by Meikle & Weiss (2017), only capped brood was identified with high certainty. Furthermore, DeepBee© detects and identifies the content of each single cell, which is more accurate than extrapolating areas, and hence avoid over-estimating *e.g.*, the number of capped brood cells, due to the empty cells in the middle of the brood area (*i.e.*, Bargen *et al.*, 2020). One drawback of the image analysis compared to visual assessment methods, is the time required for software training and processing. On the other hand, the training allowed us to improve the accuracy of DeepBee© above 93% for all cell classes, even for the low frequency cells (*i.e.*, eggs and larvae) and the improved version performed well in the remaining images acquired during this two-year study. Also, DeepBee© can analyse a comb in less than 30 seconds and the software can run a batch of images automatically overnight, whereas in Bargen *et al.* (2020), 20 minutes were required to analyse one frame comb using the HiveAnalyser© software.

The logistic constraints of the protocol (*e.g.*, using a tunnel for images acquisition) can also be a challenge in studies with many colonies and apiaries. Therefore, we recommend the use of this protocol in studies with <10 colonies per apiary, and a limited number of apiaries. To avoid robbing events we suggest monitoring colonies far from each other and to clean and/or smoke the materials to remove any pheromones that can trigger defensive behaviours.

Future directions and developments

Automatic and semi-automatic, non-invasive, real-time and accurate data are optimal for keeping track of the health status and development of a honey bee colony. New technologies including scales and other sensors (*e.g.*, temperature, humidity, sound, vibration (Eouzan *et al.*, 2019; Ramsey *et al.*, 2020)) are attractive, as they are associated with minimal colony disturbance. These technologies can be used for colony evaluation by beekeepers or researchers, and are promising tools for obtaining standardized, large-scale, and long-term data of colony development. However, the output from these sensors needs to be validated to be transformed into colony development and health status. If these sensors are calibrated with low accuracy data, a high error rate will be associated with the output, and the user will not get accurate information on the colony development and events. In a similar vein, computer simulation models which predict colony development, are highly dependent on accurate field data for validation and calibration. EFSA proposed the development of a honey bee model as a predictive tool to assess the impact of multiple stressors on honey bee colony development (EFSA, 2016a). The model (ApisRAM) is composed of several modules that represent the honey bee colony, the landscape management, resources and pesticide fate, and other stressors (*e.g.*, infectious agents, pests and predators) that affect colony health (EFSA, 2016a). The combination of field data, which is collected at fixed points in time, with agent-based models having high predictive power, will represent a huge step forward allowing early detection and prevention of colony mortality (Requier, 2019). The proposed protocol was developed to gather accurate data for calibrating the ApisRAM model (Dupont *et al.*, 2021). However, we envisage the use of the protocol in other studies across Europe and elsewhere, as it can encompass heterogeneity with regards to bee subspecies, climate, landscape, etc. Despite the possible downsides associated with a higher workload, we believe the protocol has the potential to provide reliable data, guaranteeing sound knowledge to help future decision-making.

Chapter III

Landscape influence on honey bee colony development

Introduction

Honey bees play a major role as pollination service providers to crops and natural ecosystems (Hung *et al.*, 2018) due to their domestication and worldwide distribution. They are responsible for about 35% of all agricultural production, increasing the outputs of 75% of all crops (Klein *et al.*, 2007). Worldwide, the total number of honey bee colonies is increasing on a yearly basis, although not growing as fast as the human population, leading to a reduction of colonies per capita (Phiri *et al.*, 2022). Furthermore, this increase is slower than the overall demand for pollination services (Aizen & Harder, 2009).

Considering regional trends, however, the overall number of honey bee colonies has declined in Europe and the United States for the last 60 years, with events of sudden colony losses being constantly reported (Colony Collapse Disorder; Underwood & VanEngelsdorp, 2007). These losses, also happening in other regions, show high temporal and spatial variability, and although no clear factor has yet been appointed as the main cause, the overall agreement within the scientific community points to a combination of factors including diseases, pests, exposure to chemicals, and changes in the landscape (VanEngelsdorp *et al.*, 2009; Steinhauer *et al.*, 2018). Intensive agriculture, in particular, has resulted in high inputs of fertilizers and/or pesticides, leading to high levels of disturbance and resulting in less pollinator-friendly agricultural landscapes (Potts *et al.*, 2010a; Potts *et al.*, 2016; Powney *et al.*, 2019). Flower-rich semi-natural areas, including green infrastructures such as field margins, hedgerows, and grasslands, are also being reduced or eliminated (Nicholls & Altieri, 2013), leading to habitat fragmentation and loss (IPBES, 2016). As a result, honey bees are exposed to a reduced or altered floral resource availability and distribution in agricultural landscapes (IPBES, 2016), together with shifts in flowering phenology and nectar production related with other factors as climate change (Takkis *et al.*, 2018). Hence, in intensive farmland areas, honey bees may experience short periods of high resource availability followed by long periods of food shortage, rather than a continuous and diverse availability of floral resources during the entire season (Di Pasquale *et al.*, 2016). This may result in inadequate nutrition and, ultimately, in colony losses (Di Pasquale *et al.*, 2016; Requier *et al.*, 2017).

Colonies experiencing nutritional stress in flower-poor landscapes are more prone to failure due to other stressors (Alaux *et al.*, 2010; Di Pasquale *et al.*, 2016). Colony development is therefore context-dependent, with regional differences in external conditions interacting with the colony's behaviour and plasticity (*e.g.*, Hatjina *et al.*, 2014). Nonetheless, field studies that evaluate colony development under different landscape scenarios are scarce (Hatjina *et al.*, 2014; Odoux *et al.*, 2014) and spatial patterns, including resources availability in the surrounding landscape and climate

conditions are usually poorly considered (Rogers & Staub, 2013). Landscape composition has been used to explain colony production (Naug, 2009, Lecocq *et al.*, 2015, Sponsler & Johnson, 2015), wintering mortality (Kuchling *et al.*, 2018), and even to evaluate colonies' development success to provide pollination services (Smart *et al.*, 2019). In most studies, satellite images are used to categorize general landscape composition (*e.g.*, urban vs agricultural; Lecocq *et al.*, 2015); however, these usually have low accuracy (Gallant *et al.*, 2014) and the qualitative categories resulting from satellite image analysis lack ecological relevance compared to quantitative measures of floral resources available to honey bees in different habitat types.

To fill these gaps, we measured the seasonal honey bee colony development using the protocol developed in Chapter II under three different Southern European landscapes located in Portugal and Spain. These landscapes differed in temporal and spatial patterns of resource availability and weather patterns, potentially influencing colony development. Furthermore, these areas presented a gradient from being dominated by agricultural fields intensively managed to mostly forested/shrubland areas with almost no arable land. To overcome the landscape simplification caveats from previous studies, the landscape was firstly categorized based on land cover (*e.g.*, crop areas. grassland or field margin) and field surveys were carried out to assess the amount and diversity of flowering resources in each category. Moreover, the influence of climate variables on the colony behaviour and resources collection was assessed by calculating the available number of foraging minutes per day.

The aims of this chapter were (i) to monitor the colony development in the three landscapes, (ii) to analyse which are the most important plant species used as nectar and pollen resources within these landscapes, (iii) to document the temporal offer of resources, and (iv) to evaluate how variables, from intrinsic colony parameters to external environmental variables (weather and resources offer), affect the amount of daily collected nectar.

Materials and methods

Study areas and experimental design

The study was carried out across three different years: 2018 in Burgos (Spain) and 2019 and 2020 in Idanha-a-Nova and Lousã (Portugal). The *Burgos* study window (42.280667, -3.767417) is mainly composed of agricultural fields, highly dominated by cereal crops, alfalfa, and sunflower. The study window from *Idanha* (39.859172, -7.163818) is an agricultural area, composed mostly of cattle

pastures, with a few intensively managed crop fields. On the other hand, the *Lousã* study window (40.048209, -8.244142) is composed of broadleaf and deciduous forests, with large areas of shrubland.

In each 10 x 10 km study window an experimental apiary was installed in the central point (visual representation of the study windows can be found on Supplementary material section III.1). Each apiary consisted of five *Apis mellifera iberiensis* colonies in Langstroth hives provided by a professional beekeeper. All queens were born in the year before the settlement of the apiaries; colonies were managed according to local beekeeping practices and treated for varroa mites in the beginning and at the end of the season. Supplementary feeding by sugar paste was added at the beginning of the season in all apiaries due to the cold temperatures. All colonies were equipped with an Beeyard® scale that transmitted weight and temperature data on an hourly basis. In each apiary, rainfall, wind speed and direction, solar radiation, temperature, and relative air humidity were monitored every 15 minutes by a meteorological station (Watchdog 2900ET).

Environmental external variables – resource availability

The landscape composition and flowering patterns of each plant species were evaluated using botanical mapping methodologies. Each study window was mapped using open-source GIS databases, following the use of CORINE land cover (level 3) to classify each polygon according to its *land use* category. Land cover of each polygon was later confirmed through field observations. To evaluate the cover, abundance, and diversity of flowers in each *land use* category, several polygons from each category (within a 1.5 km radius from the apiary) were visited and visually assessed. At the *Burgos* landscape, this assessment was performed only for three months during the season, whereas at *Idanha* and *Lousã* it was performed simultaneously with colonies assessment (once every 20 days for five months; rainy days were avoided). For the *Burgos* landscape, a 1 m^2 square was used to estimate the overall polygon diversity and the abundance of each flower species. Since both sampling frequency and the method to assess polygon diversity and abundance in the *Burgos* landscape were found to be insufficient to capture the spatiotemporal resource availability, landscape data from Burgos was removed from the analysis and composition comparisons were made only between *Idanha* and *Lousã*.

For the *Idanha* and *Lousã* landscapes, the polygon diversity and abundance of each flower species was evaluated using an estimation at the polygon level by selecting a representative area. To evaluate the resource availability at each sampling point, a *resource score* per m^2 was also calculated. Each flowering species was classified according to the "Bee friendliness (BF) value" from the Deliverable 3.1 of the Horizon 2020 B-GOOD project (Alves da Silva *et al.*, 2021). To the species that

were not on this list, the average BF value of the corresponding genus or family was attributed. The "bee friendliness value" was multiplied by the flower abundance per m^2. To evaluate the total *resource score* of a certain *land use* category, the *resource scores* per m^2 of the polygons belonging to the same *land use* category were averaged and multiplied by the whole area of that category in the landscape.

Therefore, for each date, each land use category was associated with a semi-quantitative value of resource offer (*resource score*), which was used to assess the temporal change in resource availability in each study window.

Colonies interaction with the external environmental variables – resources use

Pollen traps (4.8 mm mesh) were installed for 24 h at the entrance of the colonies during each observation day. Pollen trapping periods longer than 24 h were avoided since bees can change their behaviour (*e.g.*, increase foraging effort) if pollen is continuously removed (Webster *et al.*, 1985). After harvest, pollen samples were cleaned for debris, weighted (± 0.01 g), and kept at -20 °C. Later, samples were dried at 40 °C for 48 h. For each date and apiary, pollen samples from the five hives were homogenized and a common sample was sent to a certified laboratory (LabApis®) for palynological analysis. These were also performed on honey samples, collected from the extracted honey from all colonies, to assess the most used plant species for nectar foraging. A qualitative palynological analysis was carried out following Louveaux *et al.* (1978), with a total of 1200 grains identified in each sample. Counting 1200 grains allowed to have a good representation of the collected species and the consideration of rare pollen species that can be underestimated when analysing only small portions of the slide.

To examine the amount of collected pollen for each species and avoid the overrepresentation of smaller grains (if only grain numbers were considered), pollen diameter was considered and the weight of each species was calculated as follows (Requier *et al.*, 2015):

$$Mass_{i,j} = \frac{(n_i \; x \; d_i)_j}{\sum_i (n_i \; x \; d_i)_j} x \; Mass_j$$

in which *i* is the contribution of each pollen species to total mass (Mass, g) of collected pollen in a sample *j*, by weighting the species' occurrence frequency (n_i, number of pollen grains of species *i* averaged between the two subsamples) by the pollen grain species-specific diameters (d_i).

Colony development parameters

From each apiary, the five study colonies were subjected to health, strength, brood, and provision assessments every 14 to 20 days, according to the protocol described in Chapter II. Population was assessed by weighing all the frames from the nest and honey suppers with and without bees, multiplying the weight difference by the weight of a single bee. The weight of a single bee was assessed by individually weighing 100 bees collected from the brood box. Brood and beebread were assessed by analysing high quality pictures of each comb using the DeepBee® software (Alves *et al.*, 2020) and classifying each alveoli by its content (eggs, larvae, pupae, nectar, honey, pollen, or "other"). Since nectar/honey cells are highly variable in weight, nectar/honey weight was calculated by subtracting the weight of wood frame, wax, brood, and beebread from the total frame weight. Disease prevalence was controlled by detecting clinical signs of the most common diseases. The levels of varroa mites were measured at each visit by counting the natural mite fall over a 48 h period, using the bottom board method (Flores *et al.*, 2015), ensuring low varroa levels for the duration of the study. All colony and beekeeping events (*e.g.*, unusual behaviour, supplementary feeding, adding honey supper) were registered.

The hourly scale data was used to calculate the daily colony weight variation by subtracting the colony weight registered at 1 a.m. of each day with the value from the previous day at 1 a.m.

Environmental variables – climate driving available foraging time

Climate variables (considered for all landscapes from March 1st to September 21st) were transformed into potential foraging minutes for each apiary by using the formula below (Vicents & Bosh 2000):

$$r_s = 2261.9e^{-0.164t}$$

where t is the external temperature (°C) and r_s is the solar radiation threshold in w/m^2. When the environmental solar radiation was higher than the calculated threshold r_s, it was assumed that bees were able to carry out foraging activities.

Furthermore, to distinguish between good environmental conditions for foraging that could lead to high colony activity from weak environmental conditions that allow foraging but at a lower rate, a ratio between the real solar radiation and the threshold value was calculated. Environmental conditions were considered as leading to low foraging potential whenever the ratio was below 3.

Data analysis

To assess which variables could affect overall colony weight dynamics, General Linear Mixed Models (GLMM) were used in which daily available foraging minutes, minutes of low and high foraging activity, landscape resource score, and colony strength (number of adult bees) were used as explanatory variables and the daily weight variation (dWt) was used as the response variable (Log X +10). For this analysis, only the productive season was considered (in here considered to be 120 days starting from when the colonies' weight started to increase): in Lousã 2019, from 25 April to 22 August and in Idanha 2020, from 19 March to 16 July. The daily foraging minutes and the weight variation were calculated for each day using daily data while the resources and colony strength were interpolated based on the assessments every 20 days. For interpolation, a FORECAST function (linear regression) was applied between two consecutive data points. Colonies that swarmed during the study were removed from the analysis. Therefore, for daily weight variation and colony strength, tree colonies were considered for Lousã 2019 and five colonies for Idanha 2020.

Colony ID was used as random factor. For this analysis, only the Lousã 2019 and Idanha 2020 datasets were used. Each dataset was analysed separately, and explanatory variable selection was done by performing a data exploration and eliminating variables with high variance inflation factor values (VIF > 5). Testing models with different explanatory variables was performed by comparing Akaike values. Analyses were performed using Brodgar software (version 2.7.5).

Results

The overall resource score for each of the tested landscapes (*Lousã* and *Idanha*) showed not only a clear difference in the amount of resources available, but also in their temporal dynamics (Fig. III.1a,b). *Lousã* landscape presented a much higher resource availability, with a peak in late Spring followed by a strong decline (Fig. III.1a). In contrast, at *Idanha* landscape resource availability was much lower, presenting a peak in Spring followed by an almost linear decrease until Summer (Fig. III.1b).

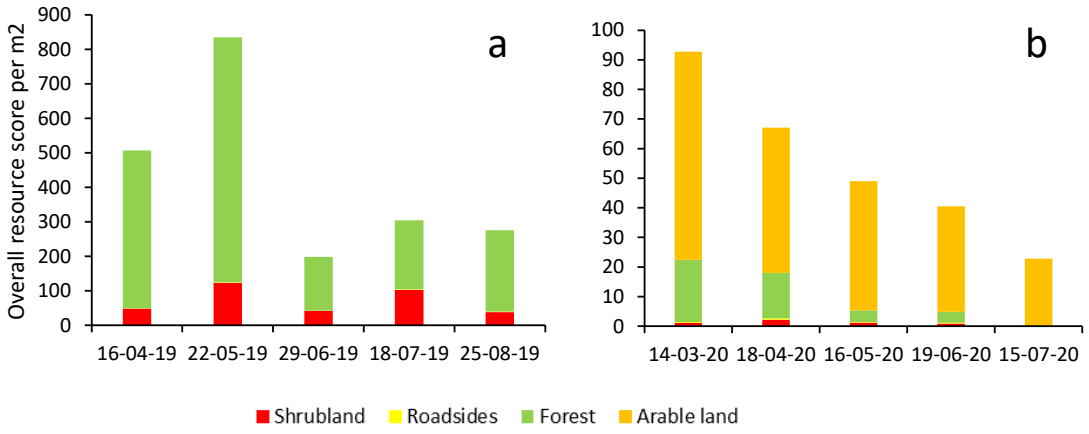


Figure III.1: Resource score for the (a) Lousã 2019 *and (b)* Idanha 2020 *landscapes. Please note the difference in scale of the resource score between landscapes.*

Considering the three landscapes, the colonies foraged for pollen in at least 37 different species throughout the season (Fig. III.2). The genera *Rubus*, *Eucalyptus*, and *Trifolium* were collected in more than one landscape, while other genera as *Erica* and *Castanea* were only found in *Lousã*.

In the *Burgos* landscape, sunflower (*Helianthus annus*) and clover (*Trifolium sp.*) were the most visited species for nectar collection. Clover was also the main important nectar resource in the *Idanha* landscape, while in *Lousã* the bees foraged on heather (*Erica sp.*) and chestnut trees (*Castanea sativa*) for nectar collection (Fig. III.2).

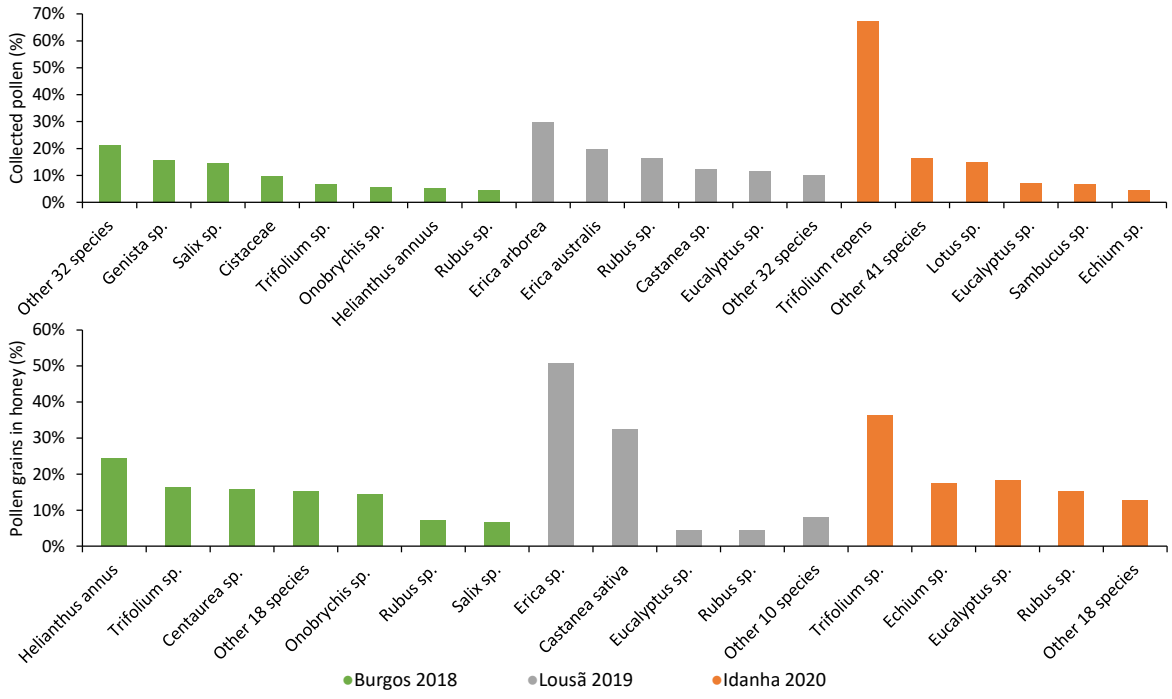


Figure III.2: Relative percentage of collected pollen and pollen grains in honey for each landscape.

The colony development rate showed different trends considering the landscape and year (Fig. III.3). Furthermore, colonies in the same environmental conditions (*i.e.*, landscape and year) showed distinct variability in their development. In all landscapes, the colony strength increased in Spring. The peak was achieved around July in the *Burgos* landscape but at the beginning of May in *Idanha* and *Lousã*. The total number of brood cells also varied within colonies and landscapes. Contrary to colony strength, the number of brood cells in all apiaries had a similar peak around 20000 cells. Nonetheless, the nest size in *Burgos* was maintained within these high levels for a longer period. Regarding food provision (*i.e.*, nectar/honey and beebread colony production) bees produced more honey in the *Burgos* landscape than in *Idanha* or *Lousã*, in which the nectar/honey collection pattern was similar for both years and with lower values in the *Lousã* landscape. The number of beebread cells was kept at low levels while the colony was growing, and the beebread peak was usually achieved one to two months after the brood peak.

Overall (from March 21 to September 21), the weather conditions allowed more daily foraging minutes in *Idanha* (M = 660, SD = 151), followed by *Lousã* (M = 585, SD = 206) and *Burgos* (M = 556, SD = 210) (Fig. III.3). On the other hand, when considering only the productive season, *Burgos* climatic conditions granted more daily foraging minutes (M = 648, SD = 122) than *Idanha* (M = 629, SD = 182) and *Lousã* (M = 629, SD = 178).

In *Lousã*, colony strength, the total amount of foraging time and the time of low foraging significantly explain the variation in colony weight (dWt), with positive relationships found on the first two explanatory variables and a negative relationship in the last one (Fig. III.4). However, resource availability over time, although positively related with dWt, did not explain a significant proportion of its variation (Table III.1, Fig. III.4). On the contrary, at *Idanha*, only resource availability was able to significantly explain the variation in colony weight (Table III.1), with foraging times and colony strength not playing a significant role on colony weight variation over time.

Table III.1: Summary of GLMM analyses on the effects of selected environmental variables on colony weight variation (dWt) at Lousã *and* Idanha *landscapes. Statistically significant results are in bold with F-values presenting the numerator (nDF) and denominator (dDF) degrees of freedom. Random effects variable: colony ID.*

Landscape/ Response variable	**Fixed effects**	**nDF, dDF**	**F**	**p value**
Lousã / dWt	Total foraging	1, 350	15.20	**0.0001**
	Low foraging	1, 350	17.35	<**0.0001**
	Population strength	1, 350	60.74	<**0.0001**
	Resource score	1, 350	0.15	0.6976
Idanha / dWt	Total foraging	1, 468	0.60	0.4383
	Low foraging	1, 468	1.80	0.1806
	Population strength	1, 468	0.10	0.7787
	Resource score	1, 468	166.30	<**0.0001**

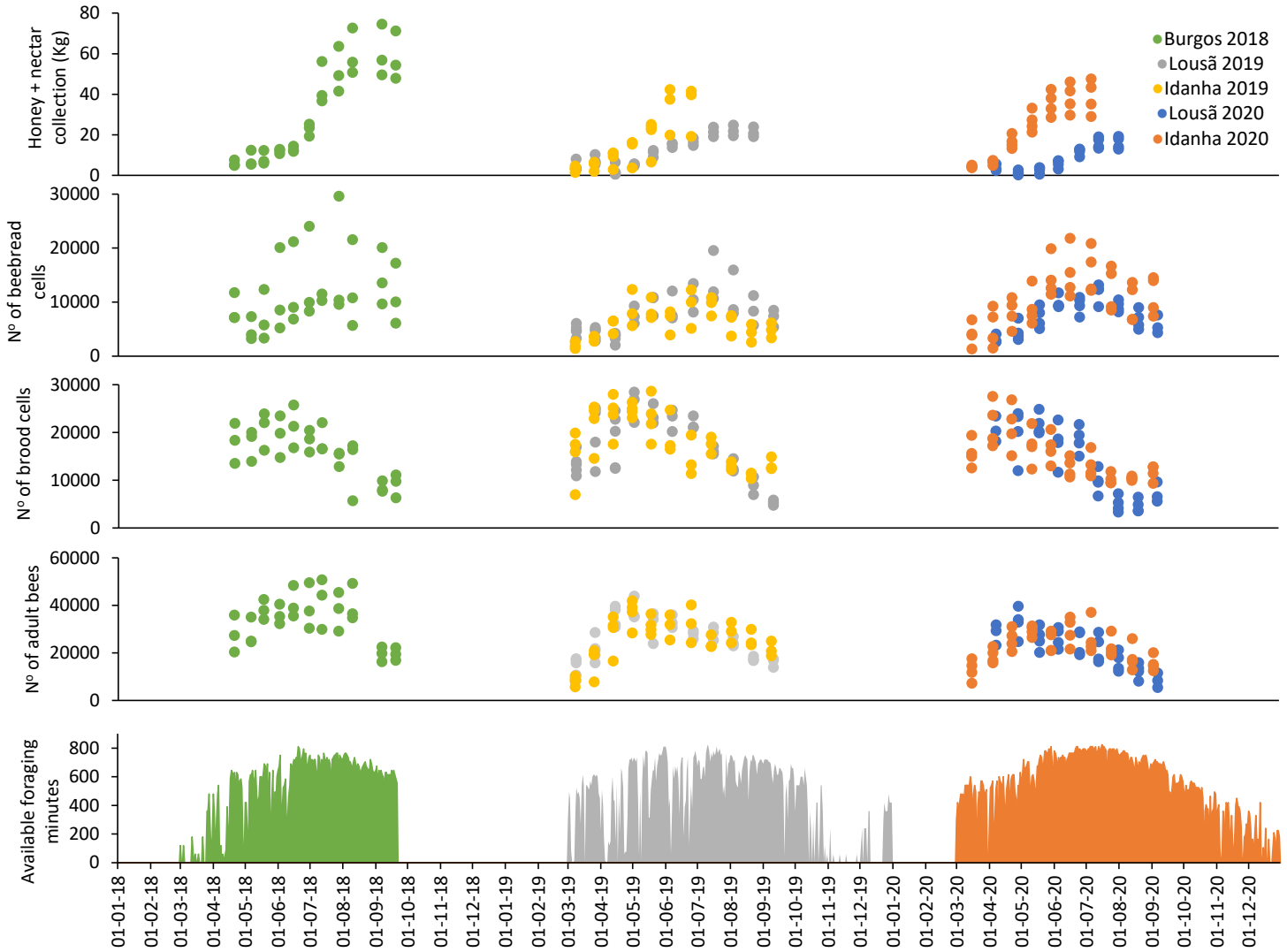


Figure III.3: Colony development parameters (honey and nectar collection, number of beebread cells, number of brood cells, and number of adult bees) for all the landscapes and available foraging minutes for Burgos 2018, Lousã 2019, *and* Idanha 2020.

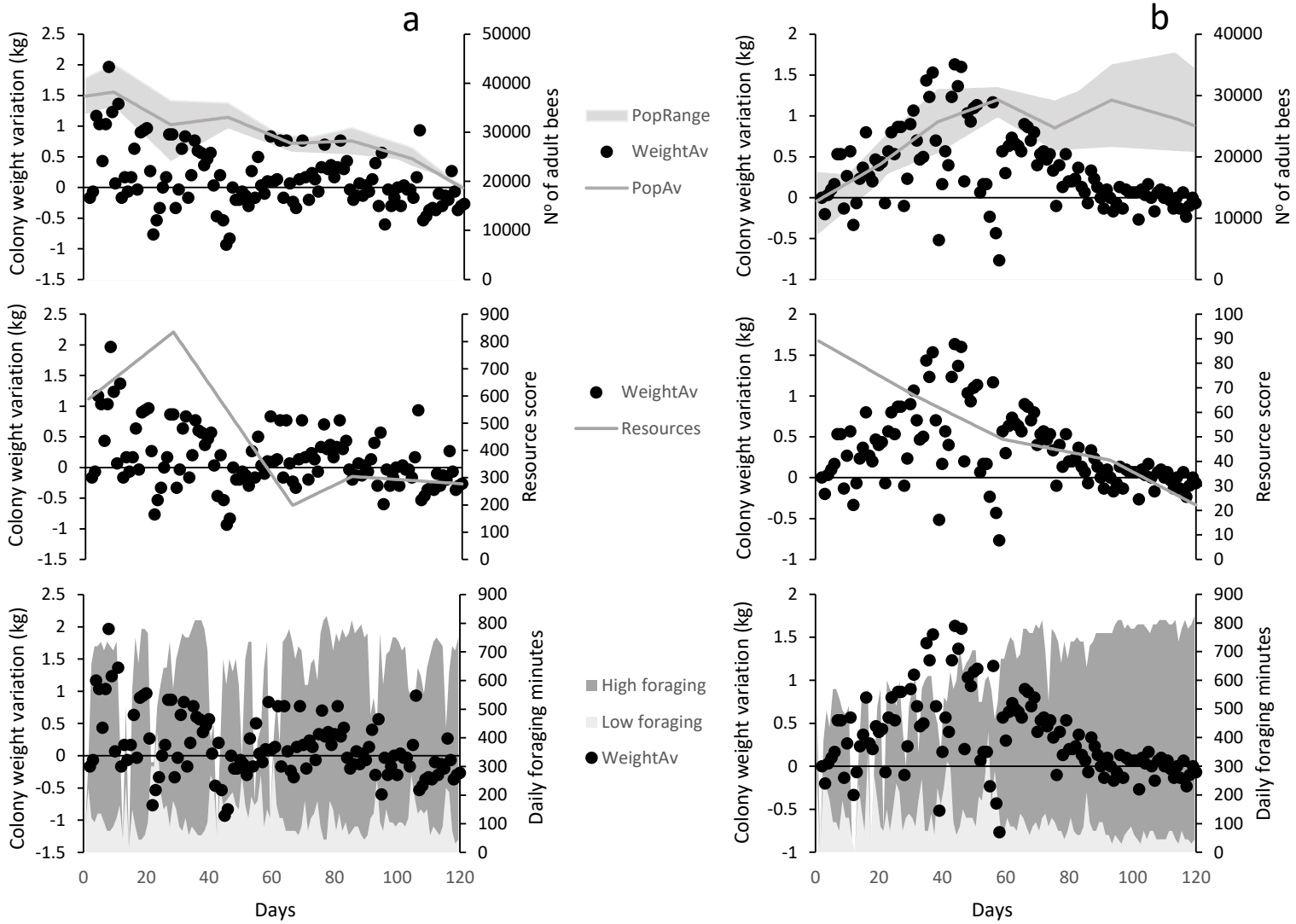


Figure III.4: Colony weight variation in relation to colony strength (number of adult bees), landscape resource score, and available foraging minutes for (a) Lousã 2019 *and (b)* Idanha 2020*. PopRange = population range from all colonies within one apiary; WeightAv = average daily weight variation of all colonies; PopAv = population average from all colonies; Resources = daily resource score for the landscape; High foraging = number of daily foraging minutes with high foraging activity; Low foraging = number of daily foraging minutes with low foraging activity.*

Discussion

Honey bee colonies showed different development rates within and between the three landscapes tested. This suggests that intrinsic internal mechanisms drive colony development, while being influenced by the external environmental variables. The resource score applied in this study allowed for a semi-quantitative measurement of the landscape suitability; low resource availability, as observed in Idanha, seems to act as a limiting factor for colony development. On the other hand, in situations with a high resource availability, as observed in Lousã, climatic variables influencing foraging time, and colony strength seem to be the major drivers of colony weight variation.

Resource availability in the landscapes

In all tested scenarios, floral resource availability was expected to be temporal and spatially dynamic; this was confirmed by field observations of flower resources in each land use category. During the first-year assessment (*i.e.*, *Burgos*), several constrains in assessing flower resources were identified: although the flowers' density in crop fields (*i.e.*, homogeneous polygons) was relatively easy to measure, the same was not true in heterogeneous polygons with diverse flowering resources unevenly distributed. To overcome this methodological problem, in the assessment performed in *Lousã* and *Idanha* landscapes in subsequent years, the evaluated area was extended to search for an area that could be representative of the polygon, instead of measuring just one square meter and extrapolating to the whole area. This approach led to a better estimate of the flowering resources in heterogeneous polygons.

In this study, the collected data was used to obtain a semi-quantitative value of production by creating a *resource score,* calculated based on the "bee friendliness value". This score was only partially connected with the nectar/pollen plant production, since it was mainly based on observations from flower-bee interactions, resulting in higher values for flower species that are visited several times. We believe that, despite not being a direct measure of flower production, this *resource score* serves as a good proxy for resource quality. Data from flower resource production is still scarce and, when available, is rather specific for a certain climatic region (Filipiak *et al.*, 2020). Therefore, to calculate the nectar and pollen offer from South European landscapes, efforts should be made to assess plant production (*e.g.*, Tew *et al.*, 2021) mainly at a regional level.

Resource collection

The used resources by the honey bees also clarifies the differences between the tested landscapes. In the *Burgos* landscape, *Genista* and *Salix* were the most visited genera for pollen collection, while the sunflower was the main resource for nectar collection. Despite the high presence of arable land (66% used for cereal crops; 9% sunflower; 3% alfalfa), the colonies relied on the use of trees and bushes to collect pollen for their colony development. Furthermore, bees also relied on other wildflowers from the genera *Trifolium*, *Centaurea* and *Onobrychis,* and from the *Cistaceae* family before the sunflower bloom. The exploration of these resources indicates that the bees' foraging mechanisms (Grüter & Farina, 2009) can lead them to find small patches in the landscape that are not used for crops production. By analysing waggle dances from a colony installed in this landscape (*i.e.*, *Burgos*), Gigauri *et al.* (unpublished) showed that bees are foraging mainly in hedgerows before the main crop (sunflower) blooms in the end of July.

In *Idanha*, the percentage of arable land was also high but mostly composed by permanent pastures. These, besides grasses, have a strong presence of leguminous plants (*e.g.*, *Trifolium* and *Echium*), which were evidently used by bees for pollen and nectar collection. A previous study conducted in the same region identified other relevant species (besides *Echium*) for honey production (*i.e.*, *Erica*, *Lavandula* and *Campanula*; Silva *et al.*, 2017), evidencing the local influence of the plant communities.

In *Lousã,* the arable land was practically inexistent, with bees relying on forest and shrubland areas to collect pollen and nectar from heather, chestnut, and brambles (*Rubus sp*.). These species are commonly found in forested areas and used by honey bees in Portugal (Morais *et al.*, 2011; Estevinho *et al.*, 2012). Moreover, in both Portuguese landscapes, bees used *Eucalyptus* flowers (Machado *et al.*, 2022). This genus is widely distributed in Portugal, with a high percentage of the forested area being covered with eucalyptus monoculture (ICNF, 2019), being used by beekeepers, mainly in the littoral area, to produce honey during winter time.

Some of the plant species in the tested landscapes (*i.e.*, *Trifolium, Salix* and *Helianthus)* are commonly used by honey bees at the European scale (Keller *et al.*, 2005a). On the other hand, this study evidences once more the need for regional scale studies. The use of sentinel honey bee colonies can be a good tool to evaluate resources availability and even potential exposure to pesticides. This approach was proposed by the EFSA scientific committee (2021) to gather data on pesticide exposure and effects for a pre- and post-approval environmental risk assessment system and is currently being applied in some European projects (*e.g.*, INSIGNIA-EU project). Furthermore, the use of sentinel

colonies could overcome the caveats associated to pollen and nectar resources availability assessments at landscape scale, as they are extremely laborious and rely on an anthropomorphic evaluation.

Colony development

The regional differences between landscape composition and weather conditions led to distinct patterns regarding the development rate of the different colonies. Different patterns can occur for several reasons other than climatic and landscape variables, namely the presence of diseases and/or pests. So, when assessing colony development in field studies, it is practically impossible to know that the measured colony traits aren't being affected by some background stressors. In this study, some of the variables, as pests and diseases, were controlled as must as possible to exclusively measure the influence of the landscape context in which the colonies were installed. Nonetheless, colonies may still be affected by several diseases or pest levels despite symptoms not being visible; in the case of varroa, even a number within "safe" levels may have an influence on colony status (Genersch & Aubert, 2010). Besides, the colony genetic variability can also play a role on their development even if the outside variables remain the same (Mattila & Seeley, 2007). Assuming a low influence of these variables, in this study we believe that colonies development patterns are strongly linked to the landscape in which they were installed, with their development traits changing according to the environmental scenarios (Hatjina *et al.*, 2014; Sponsler & Johnson, 2015; Smart *et al.*, 2018).

In *Burgos*, the colonies were able to maintain a big nest size during a long period (approximately 20000 alveoli for three months), leading to high population levels. Interestingly, the population peak was achieved right before the sunflower blooming period, ensuring that many forager bees collected resources from this nectar-rich crop, simultaneously providing the highly needed pollination services. In this scenario, both beekeepers and farmers benefit from the presence of non-arable polygons in the landscape necessary for the colonies to reach their peak strength just before sunflower blooming, thus ensuring a good productivity of both honey and sunflower seeds. In *Idanha* and *Lousã*, the maximum population levels were similar but showed a slight chronological desynchronization. This difference can be explained by the colony's response to brood rearing, as it is closely related to diet and external temperature (Seeley & Visscher, 1985; Kim *et al.*, 2017). Remarkably, in all apiaries, the levels of beebread were desynchronized with the levels of brood cells, with higher beebread levels achieved later in the season when the brood levels were lowering. As the brood pheromone (Pankiw, 2004, 2007) is one of the main drivers of colony mechanisms for pollen

foraging, a reduction in pollen collection when less brood is in the colony would be expected. Nonetheless, later in the season, when the nectar/sugar availability is reduced, the forager bees change their foraging efforts towards pollen (Arenas & Kohlmaier, 2019), leading to the accumulation of beebread in the colony, which becomes essential for winter bees rearing when pollen availability is scarce.

The colonies installed in agricultural areas (*i.e.*, *Burgos* and *Idanha*) had a higher honey yield than in the forested area (*i.e.*, *Lousã*). The mean daily available foraging minutes, as well as the achieved population levels, can partially explain these differences. Nonetheless, we hypothesised that the resources availability would also play a fundamental role on colony production levels. Unfortunately, it was not possible to assess the resource availability for *Burgos* and, due to data flaws (*e.g.*, weather station failure), it was not possible to also include all landscapes and years in the analysis. From the *Idanha 2020* analysis, it was possible to conclude that the available resources play a significant role on the daily weight variation. On the other hand, in the *Lousã 2019* analysis, the landscape resources did not explain a significant variation on colony daily weight change. We hypothesize that the resources offer was not significant as it was not a limiting factor. Bees have a constant high offer of nectar and pollen but are only allowed to forage when the external environmental conditions allow, making the weight variation (mostly from sugar accumulation/consumption) highly dependent on the climate variables and colony strength, but not on the resources offer. Furthermore, when the environmental conditions allow a low level of foraging activity (*i.e.*, *low* foraging) the colony has a poor performance and there is a negative significant influence on the weight variation. The model used to derive foraging time, although potentially being a good tool to calculate the available foraging minutes, does not have a qualitative/quantitative measurement of how it translates to foraging activity (like it was implemented by the low/high foraging). We believe this method should be further explored as it can provide ecologically relevant endpoints, since climate is one of the main drivers of bee foraging behaviour (Clarke & Robert, 2018).

Final remarks

The use of landscape composition to correlate with honey bee colony development is not new and should be further explored (Rogers & Staub, 2013), but still presents major limitations. First, most available land cover products lack sufficient local accuracy to measure the temporal and spatial shifts on honey bees' resources (Gallant *et al.*, 2014). Secondly, there's always the need to associate flowering composition to the *land use* categories. This might be easily solved for areas mainly composed of arable land by relying on national databases, which have information on farmers activities, or by using satellite images paired with other techniques (*e.g.*, NDVI) that allow the

identification of the crops present (Boori *et al.*, 2019; Park *et al.*, 2021) even when national databases are incomplete or inexistent. On the other hand, in areas where the landscape could be partially composed of several, usually small, patches used for local farming, and/or the national spatial databases are rather incomplete, this task becomes more challenging. On top of it, flower abundance from field assessments cannot be used in different areas of the same land use if the field topology and weather variables are different. Only by integrating habitat specific species composition and phenology with the weather variables (Park *et al.*, 2021) and nectar and pollen production, would it be possible to create landscapes with a continuous (*e.g.,* daily) spatial and temporal offer of resources for honey bees.

The “creation” of these landscapes is extremely important for the development of predictive models to overcome the challenges of field testing. In this study, colonies changed and adapted their behaviour in response to the external variables, leading to different development rates, showing that results from field testing is extremely context-dependent. The use of field testing for environmental risk assessment system is valuable for specific contexts but extrapolations should be made with caution.

Chapter IV

Assess the colony exposure and effects to acetamiprid in a real exposure scenario

Chapter published as an original article:

Capela, N., Xu, M., Simões, S., Azevedo-Pereira, H. M., Peters, J., & Sousa, J. P. (2022). Exposure and risk assessment of acetamiprid in honey bee colonies under a real exposure scenario in Eucalyptus sp. landscapes. *Science of the Total Environment*, 840, 156485.

David Sarmento

Abstract

Honey bee colonies have shown abnormal mortality rates over the last decades. Colonies are exposed to biotic and abiotic stressors including landscape changes caused by human pressure. Modern agriculture and even forestry, rely on pesticide inputs and these chemicals have been indicated as one of the major causes for colony losses. Neonicotinoids are a common class of pesticides used worldwide that are specific to kill insect pests, with acetamiprid being the only neonicotinoid allowed to be applied outdoors in the EU. To evaluate honey bees' exposure to acetamiprid under field conditions as well as to test the use of in-situ tools to monitor pesticide residues, two honey bee colonies were installed in five Eucalyptus sp. plantations having different area where Epik® (active substance: acetamiprid) was applied as in a common spraying event to control the eucalyptus weevil pest. Flowers, fresh nectar, honey bees and colony products samples were collected and analysed for the presence of acetamiprid residues. Our main findings were that (1) acetamiprid residues were found in samples collected outside the spraying area, (2) the amount of residues transported into the colonies increased with the size of the sprayed area, (3) according to the calculated Exposure to Toxicity Ratio (ETR) values, spraying up to 22% of honey bees foraging area does not harm the colonies, (4) colony products can be used as a valid tool to monitor colony accumulation of acetamiprid and (5) the use of Lateral Flow Devices (LFDs) can be a cheap, fast and easy tool to apply in the field, to evaluate the presence of acetamiprid residues in the landscape and colony products.

Introduction

Honey bees are exposed to a wide range of biological, environmental, and chemical stressors (Steinhauer *et al.*, 2018), that can be influenced by colony traits and the landscape where these colonies are installed (*e.g.*, Meikle *et al.*, 2020; Steinhauer *et al.*, 2021). The landscape surrounding honey bee colonies can be considered as a complex environment, with temporal and spatial shifts generated mostly by human management (in non-natural areas; Tscharntke *et al.*, 2005; Odoux *et al.*, 2014; Requier *et al.*, 2015; Meikle *et al.*, 2020). Intensive agriculture and forestry have been transforming landscapes, comprising intensively managed areas in which wild flowering resources are scarce or might not even exist (Nicholls & Altieri, 2013; Requier *et al.*, 2015). Landscape fragmentation and food scarcity or non-diverse food sources can lead to a poor diet for honey bees. Honey bees need to acquire essential amino acids and proteins for normal larvae development, which can be obtained from different plant species (Brodschneider & Crailsheim, 2010). Low diversity and/or abundance of plant species – thus potentially low diversity of nutrients – might compromise their

immunocompetence against pathogens (Alaux *et al.*, 2010). Moreover, modern agriculture (and modern forestry, to a less extent) is highly dependent on the use of plant protection products (*i.e.,* pesticides) to prevent production losses due to pests/pathogens. Pesticide product formulations have different modes of action, depending on their active substance, and can be harmful to non-target species like honey bees (Pisa *et al.*, 2015; Hopwood *et al.*, 2016). In fact, these chemicals can act synergistically (*e.g.*, Sgolastra *et al.*, 2017) and negatively contribute to the challenges caused by nutritional stress (Tosi *et al.*, 2017) or by other stressors like the ectoparasitic mite *Varroa destructor* (Straub *et al.*, 2019). To address this problem, pesticide risk mitigation measures aim to limit the exposure of the applied products to non-target organisms (*e.g.*, EU, 2009). Nevertheless, due to their broad foraging range behaviour, bees are commonly exposed to a variety of pesticides (Sanchez-Bayo & Goka, 2014, Simon-Delso *et al.*, 2017, Tosi *et al.*, 2018, Zioga *et al.*, 2020), and several studies have acknowledged a possible link between classes of pesticide exposure (*e.g.*, neonicotinoids) and the decline of bee health (Hopwood *et al.*, 2016; Wood & Goulson, 2017).

Neonicotinoids (*e.g.*, acetamiprid, thiamethoxam, imidacloprid, thiacloprid and clothianidin) have become the most used insecticides worldwide, with a market share of 25% (Bass *et al.*, 2015). These systemic insecticides remain in the vascular system of the plants for several days, protecting the crop from a variety of pests. Such protection occurs by blocking impulse signals on insects' central nervous system, thus limiting their activity (*i.e.*, paralysis), and therefore causing death (Tomizawa & Casida, 2005). Due to their systemic nature, nectar and pollen of plants treated with these pesticides might also contain the active substance, which can be ingested by insects while feeding (including non-target organisms like honey bees and other pollinators; Simon-Delso *et al.*, 2015; Hopwood *et al.*, 2016). Even so, and despite the long history of usage of neonicotinoid-containing products, most of the studies linking effects of this class of pesticides on colony health were published only after 2011 (Lu *et al.*, 2020). These pesticides show low lethal effects when sprayed and honey bee colony resilience can mask the loss of forager bees (Schmickl & Karsai, 2017). By inducing mainly sub-lethal effects at different stages of honey bee development, the effects at colony level are not immediately detected, being visible only after prolonged exposure (Lu *et al.*, 2012; Rondeau *et al.*, 2014). Over the past years, the use of several pesticides containing neonicotinoids have been severely restricted in the European Union (imidacloprid [EU, 2018b], clothianidin [EU, 2018c], thiamethoxam [EU, 2018d], thiacloprid [EU, 2020]), leading eventually to the withdrawal of these pesticides. Besides the emergency authorizations for some neonicotinoids, currently, only acetamiprid is authorized for outdoor crop protection, having been renewed until 2033 (EU, 2018a). In fact, acetamiprid is considered safer for honey bees as it has a LD_{50} (by contact) 100 times higher than the one reported

for thiamethoxam (Lewis *et al.*, 2016) and when compared to this later substance and imidacloprid, is the least transferred neonicotinoid into the hives via pollen and nectar (Niell *et al.*, 2017).

The European Food Safety Authority (EFSA) stated that acetamiprid has "a low risk to bees", considering that a ban or further restrictions of this substance was not scientifically or legally appropriate (EFSA, 2016b). Nonetheless, this decision was based on data from tests developed under laboratory conditions (tier I), since semi-field (tier II) and field (tier III) studies, had major drawbacks: short duration, lack of exposure assessment (unavailability of data on relevant metabolites in pollen and nectar) and low number of colonies used (EFSA, 2016b). This implies that there is still a lack of relevant semi-field and field studies regarding exposure pathways and effects of environmentally realistic concentrations of acetamiprid-based insecticides (Heimbach *et al.*, 2017). To tackle the need for field studies (tier III), risk assessment for honey bees at a landscape level rely on exposure predictions based on landscape composition (field sizes and crop attractiveness), sprayed area and honey bee foraging ranges (Appendix E in EFSA, 2013). However, such schemes can be difficult to apply in some landscapes, yet field studies are necessary to infer the real exposure pathways and calculate risks/toxicity.

In Portugal, acetamiprid has been used to control the spread of *Gonipterus platensis* in eucalyptus areas, by spraying the formulated products directly to the tree canopies (Valente *et al.*, 2004). *G. platensis* is a known eucalyptus weevil whose larvae feed on eucalyptus leaves and young shoots. Since eucalyptus trees do not flower during May, they do not provide resources for pollinators, thus one cannot use the landscape level approach used in risk assessment. Possible exposure can derive from flowering plants from the understory vegetation, that can come in contact with the insecticide via aerial drift or wash-off, which makes pollinator exposure hard to predict.

In the present study, we used several study windows dominated by eucalyptus plantations, to capture a range of acetamiprid exposure situations to which colonies might be subjected to. Exposure can be conditioned by the colonies' location ((un)protected by vegetation), wind speed and direction, density of understory vegetation (mainly shrubs) and trees, and also by colonies' foraging area in relation to the sprayed area. We hypothesized that (1) there is a gradient of acetamiprid accumulation in the understory vegetation from the most exposed vegetation (near the roads used for spraying) to the most protected vegetation (inside eucalyptus stands) and (2) apiaries installed in landscapes with larger sprayed area transport more acetamiprid residues into the colony. Our aim was to understand the range of honey bees' exposure to acetamiprid residues under realistic field conditions in this type of forest habitat, and the implications when deriving risk values.

Materials and Methods

Test areas and setting the experimental hives

Five eucalyptus-dominated stands presenting high incidence and prevalence of *G. platensis* were selected in articulation with Altri Florestal®, among the several forest areas managed by this company. In these parcels, the commercial formulation Epik® SG (SIPCAM; 20% p/p acetamiprid) was applied (during May 2020) to control the eucalyptus weevil (200 g/ha; 40g a.s./ha). Pesticide application was done by aerial spraying from the existing forest roads, using an ultra-low volume canon (debit of 50 L/h) attached to a pick-up truck, aiming at the top of the trees (where *G. platensis* larvae feed) and always considering the topography of the selected parcel and current weather conditions (*e.g.*, absence of rain in the 24h after spraying, temperature < 30°C, and wind speed < 11 km h^{-1}). From the spraying events done in the selected parcels, five locations (supplementary material, section IV.1) were chosen based on the size of the sprayed area (ranging from 0.25 to 4.39 km^2) to seek a gradient of area of exposure upon which the colonies were foraging on. Each study window comprised an area of 2500 m radius from the central point where the colonies were installed (total foraging area of 19.63 km^2) and encompassed the parcel that was sprayed; a common feature of all the five study windows is that the colonies were always installed within the sprayed parcel. The total area of the study window was calculated based on foraging information from previous field work in a similar forest habitat (Domingues *et al.*, 2022). Four of these study apiaries (A1 to A4) were installed within an area of 40 km^2 while the fifth apiary (A5) was 25 km Northwest.

All the selected study windows (supplementary material, section IV.1) are managed forested areas used for intense *Eucalyptus sp.* plantation, intended to provide raw material for paper/pulp industry. Spraying areas were previously defined to guarantee total spraying coverage in the marked area. Non-eucalyptus riparian vegetation neighbouring small intermittent streams (dry during the course of the experiment) as well as large forest clearings, were not marked for spraying.

To evaluate the most common flowering resources and their hotspots, a survey of existing flowering species was performed the day before the experiment, by scouting through all the roads inside the spraying area (a band of 30m to each side was inspected). To assess exposure to acetamiprid due to foraging, potential honey bee resources were collected by random sampling the identified hotspots, considering flowering patches that presented plant species with known honey bee attractiveness (species described in Caravela *et al.*, 2019).

Two days before being transported to the field, colonies were selected among two levels of biological status (weak vs strong colonies), focusing on the major colony traits: population was

assessed by weighing all the frames with and without bees, while brood and resources were calculated by photographing the frames and using the DeepBee® software (Alves *et al.*, 2020). The health status was also evaluated, using a list of all the known disease symptoms (Appendix D – EFSA AHAW Panel, 2016), with a special focus given to *Varroa* mites to ensure low levels of infestation (sticky bottom method; Flores *et al.*, 2015). Since colony population size determines colony behaviour and interaction with the landscape (*e.g.*, Beekman *et al.*, 2004), one strong (approx. 30,000 bees) and one weak (approx. 20,000 bees) colony were installed inside each of the selected study windows, one week before the spraying event, to allow the colonies to acclimate to the new landscape. After being installed, each colony was equipped with a pollen trap, which was only activated during specific periods for pollen collection. Data about the health/strength of the colonies, before (approximately 1 week) and after (approximately three weeks) the spraying event, can be found on supplementary material (section IV.2).

Sampling events

Four sampling events (Table IV.1) were determined: one day before spraying (day -1) on the day of pesticide application immediately after the spraying event (day 0), one day after pesticide application (day +1) and 15 days after the application (day +15).

One day before the spraying experiment (day -1), beebread, honey, pollen (≥ 5 g) and forager bees (≥ 20 bees) were collected from all colonies. From the surroundings of each apiary, one pooled soil sample (from 4 locations), one pooled eucalyptus leaves sample (from 3 trees), 4 flowers and 4 leaves samples were randomly collected from flowering plants with high beekeeping potential (See Table IV.1 for the total number of samples). All the samples were collected into glass vials and stored in a cold and dark container (with ice bags), until being frozen at -20^{o}C in the same day. Sampling site characteristics (distance to closest spraying point [meters], position considering spraying map [mark/unmarked for spraying]) and coordinates were annotated. Before spraying (early in the morning), the colonies were closed (covering the entrance with foam) after some foragers had already left the colony. Immediately after spraying (day 0), all foraging honey bees arriving at – or in the vicinities of – the closed colonies were captured and stored, to account for exposure by contact.

On day +1, a pooled sample of at least 20 forager bees were collected to measure oral exposure using a sweep net. Additionally, one pooled sample of fresh nectar (≥ 2ml - by gently squeezing forager bees to collect nectar from their honey stomach - Gary & Lorenzen, 1976) and a sample of fresh pollen were collected from each colony, using pollen traps (≥5g; traps activated from

7/8 a.m. to 6/7 p.m.). In the sprayed area, regions with high potential for honey bee visits (plants with high beekeeping interest) were screened and 10 flower samples were randomly collected from different shrubs. The aim for collecting different types of samples was to gather knowledge on pesticide distribution in the flower resources and its transferability (via pollen and nectar) into the colonies.

On day +15, pesticide residues in flowers from each study window were analysed by randomly collecting 10 samples of flowers following the same procedure as stated above (day +1). Therefore, flower samples were not necessarily collected from the same plants as before. Pesticide residues in the colony were assessed by collecting a pooled sample of beebread, honey (≥ 5g of each) and forager bees (≥ 20 bees) that where stored and later analysed. The flower resources-colony residues transferability was assessed by the analysis of fresh pollen (≥ 5g) collected from each colony using the pollen traps.

Sample analysis

Samples collected on day -1 (before the spraying event) were analysed by a qualitative (presence/absence) screening, using a paper-based LFD immunoassay containing specific antibodies for acetamiprid (Wang *et al.*, 2017; Liu *et al.*, 2019). All the samples collected after spraying, from colony and landscape matrices, were analysed by a matrix-matched semi-quantitative xMAP acetamiprid immunoassay (*e.g.*, Guo *et al.*, 2013; Hamza *et al.*, 2014; Peters *et al.*, 2014). Additionally, a subset of the extracts was also screened using the aforementioned LFDs. More details of the used methodologies can be found in the supplementary material (section IV.3).

Exposure and risk assessment

Colony's age polyethism can lead to different exposure levels, since honey bees adapt their feeding needs according to the task they perform in the colony (Johnson, 2008; Brodschneider & Crailsheim 2010; Rodney & Kramer, 2020). Therefore, for each bee class, daily Residue Intake (RI; mg/day) was calculated using the following formula, adapted from EFSA (2013a):

$$RI = \frac{(R_{Pollen} x\ C_{Pollen}) + R_{nectar} x\ C_{nectar}\ x\ \frac{d_s}{m_s})}{1000}$$

where the R_{pollen} and R_{nectar} are the 'residues concentrations' (mg/kg) in pollen and nectar, respectively. C_{pollen} (consumption rates of pollen), d_s (mg/day; intake rate of sugar), and m_s (kg/kg; sugar content in nectar) were based on the estimations from table J1 in EFSA (2013a). Since the sugar concentration in nectar was not measured, a worst-case scenario was assumed and a low threshold value for nectar and honey (20 and 50%, respectively) was used. Additionally, to calculate larvae exposure, the total consumption of pollen and nectar during the 5 days of larvae development was considered.

Exposure Toxicity Ratio (ETR) values (risk values) were calculated for each colony and for each class of bees (*i.e.*, forager, nurse, winter, and larvae), using the exposure values measured in the present study, and toxicity values obtained from EU (2004), and compared with the trigger values (obtained from table 3 in EFSA, 2013). ETR values below the trigger values indicate an acceptable risk.

Statistical analysis

Acetamiprid residues in flowers were compared at several levels via Linear Models (LM) and using log transformed data. LMs were chosen after checking the best distribution fit for each dataset and after analysis, data normality and model fit were checked via Q-Q plots and residue analysis. Comparisons were done in acetamiprid values between the five study windows at day +1, between day +1 and day +15 (considering all study windows), and to assess the effects of distance from the spraying location (nearest road) and if the sample was collected inside an area marked for spraying (spray Y or N) (also considering all study windows).

The relation between the sprayed area and the acetamiprid levels in pollen and nectar at day +1 were analysed by performing a simple linear regression (nectar residue data was log transformed). Also, across all study windows, differences in acetamiprid values between pollen and fresh nectar at day +1 were compared by a LM using log transformed data. The same method was used to compare pollen residues between day +1 and day +15, and the residues between beebread and honey on day +15.

Analyses were performed with *R Studio version 1.4.1106* using the nlme (Pinheiro *et al.*, 2019) and ggplot2 (Wickham, 2016) packages.

Results

Flowering resources and acetamiprid residues

In all study windows, the most abundant flower resources with known attractiveness for honey bees (by providing pollen and/or nectar) were *Cytisus striatus*, *Cistus ladanifer*, *Lavandula sp*., *Rubus sp.* and *Echium sp.*. *C. striatus* and *C. ladanifer* were only sampled on day +1, as they were already reaching the end of the flowering period (few flowering buds), translating to a lower sampling effort. *Lavandula sp.* and *Echium sp.* were the most abundant flowering resources throughout the experiment, while *Rubus sp.* was flowering only on day +15. None of the samples collected before spraying had any residues of acetamiprid (table IV.1).

Table IV.2: Number of collected samples in all the apiaries/landscapes, during the experiment, and the respective acetamiprid residues measured (mean; min. – max.).

	Type of sample	**Before spray**	**Spraying day**	**After spray**	**After spray**
		Day -1	**Day 0**	**Day 1**	**Day 15**
Colonies	Beebread	10 (0)			10 (408; 15-1980)
	Pollen	10 (0)		10 (262; 15-592)	10 (21; 1-70)
	Honey	10 (0)			10 (23; 0-114)
	Bees	10 (0)	10 (359; 41-1000)	10 (123; 4-608)	10 (7; 2-21)
	Fresh nectar			10 (41; 10-180)	
Landscape	Flowers	20 (0)		50 (350; 10-1000)	50 (81; 0-586)
	Leaves	20 (0)			
	Soil	4 (0)			
	Eucalyptus leaves	4 (0)			

On day +1 (after spraying), no statistical differences on acetamiprid concentrations in flower samples ($p = 0.82$) were found between the different study windows. Acetamiprid residue concentrations ranged from 11 to 1000 ppb (Fig. IV.1). Despite the significant reduction in acetamiprid residues from day +1 to day +15 in all areas ($p << 0.001$; Fig. IV.1), almost all flower samples still contained acetamiprid residues in concentrations >1 ppb (mean = 81 ± 360).

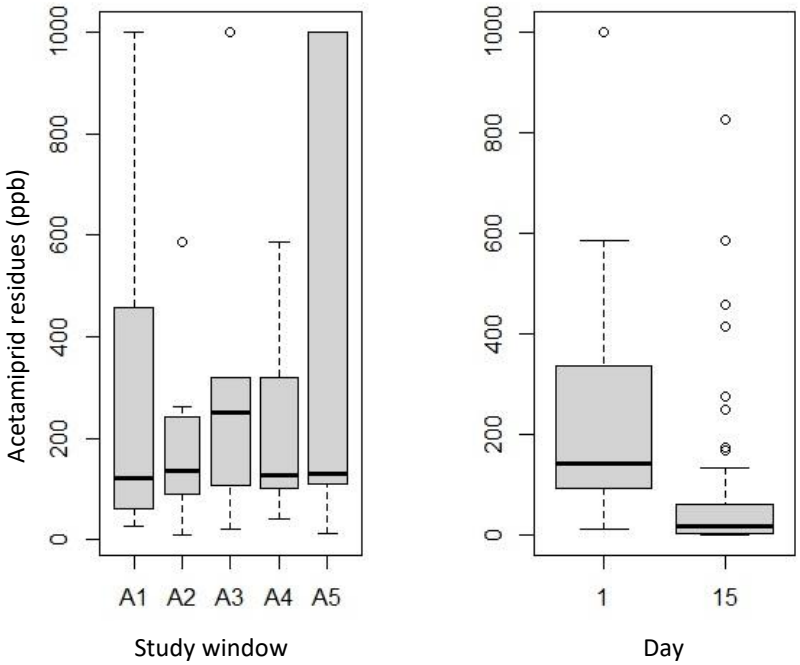


Figure IV.3: Acetamiprid residues found on flowers on the day +1 within the different apiaries/landscapes (left) and acetamiprid residues on all flower samples from day +1 and day +15 (right).

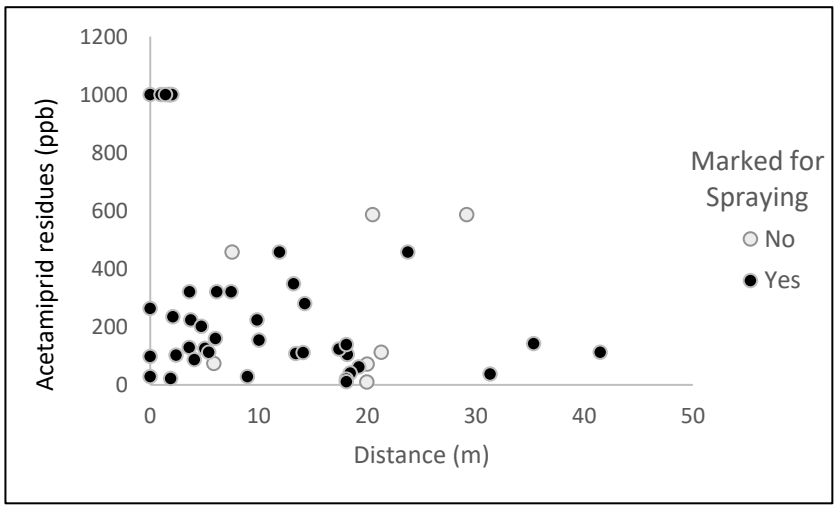


Figure IV.4: Acetamiprid residues on flowers considering their position regarding the distance from the spraying point (x axis) and if it was previously marked for spraying (black dots).

None of the sampling point characteristics ([1] distance to closest spraying point, and [2] position considering spraying map [mark/unmarked for spraying]) could explain the amount of acetamiprid residues present in the flower samples (p = 0.07, and p = 0.22, respectively). Even in areas where spraying was avoided (variable: marked/unmarked), acetamiprid residues above 10 ppb were found in all samples in day +1 (Fig. IV.2).

Acetamiprid residues in the colonies

As expected, the chances of foraging on sprayed plants increased with the size of the spraying area (Fig. IV.3): nectar residues (M=40.6 SD=55 ppb) had a strong linear relationship with the area (R^2 = 0.7, p ≤ 0.05, Fig. IV.3a), while the pollen residues (M=262.4 SD=218 ppb) had a moderate positive linear relationship with the size of sprayed area (R^2 = 0.55, p ≤ 0.05, Fig. IV.3b). At day +1. the amount of acetamiprid residues on pollen is significantly higher than on nectar (p<<0.001; Table IV.1). After 15 days, acetamiprid residues in pollen pellets were 12.5 times lower (day +1 M=262 SD=218; day +15 M=21 SD=18 ppb; Table IV.1), when compared with day +1 (p << 0.001).

Despite the reduction of acetamiprid residues in flowers between day +1 and day +15, bees kept collecting resources during this period, and the residues are likely to have accumulated on bee matrices. On Day +15, mean residue levels found in honey (M=23 SD=33, from 0.3 to 114 ppb; table IV.1) were 17 times lower than the mean residues found on beebread (M=407 SD=666, from 15 to 1980 ppb; table IV.1) (p<0.01).

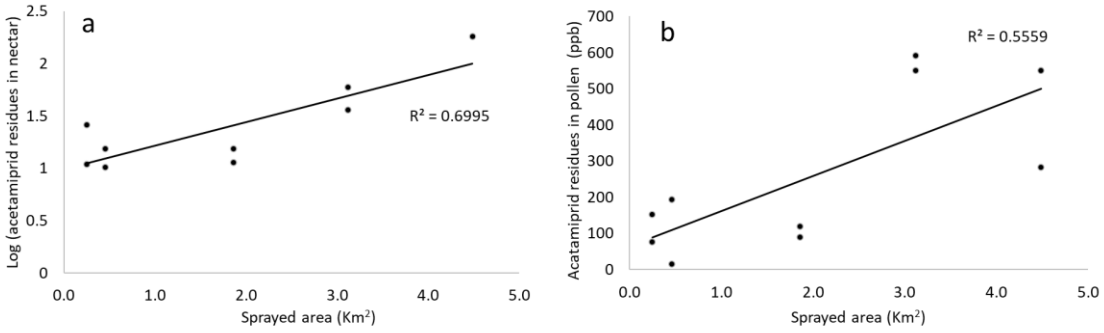


Figure IV.5: Acetamiprid residues in nectar (a) and pollen (b) samples collected on the day +1, considering the amount of area in which the insecticide was sprayed.

Exposure and risk assessment

The tested landscapes allowed us to capture several exposure scenarios (high to low density of eucalyptus foliage protecting the colonies) for forager honey bees and to calculate the amount of active substance that bees might be exposed by contact (forager bees residues after spraying range from 40 to 1000 ppb, mean = 359 ± 368 ppb). Oral exposure of forager honey bees was calculated on day +1 and day +15: forager bees were carrying 17.5 times less residues from day +1 to day +15 (from M = 123 ± 192 to M = 7 ± 5 ppb).

The calculated ETR values were below the trigger values for all the colonies, both considering residue levels found on pollen and fresh nectar on day +1, as well as on honey and beebread on the day +15 (Table IV.2).

Table IV.3: Exposure Toxicity Ratio (ETR) values for different classes of bees considering their food consumption (nectar and pollen) and the LD/LDD 50 and NOEL for the acetamiprid. Only the highest (from all the colonies) calculated ETR values are reported. If the ETR values are smaller than the trigger value then the specific protection goals are achieved (acceptable risk).

Type of assessment	Endpoint	Class of bee	ETR	Trigger values
Acute oral exposure adult bees	LD50 = 8.85 µg a.s./bee	Forager bees	0.0033	0.2
		Winter bees	0.0016	0.2
		Nurse bees	0.0028	0.2
Chronic oral exposure adult bees	LDD50 = 11.7 µg a.s./bee	Forager bees	0.0025	0.03
		Winter bees	0.0012	0.03
		Nurse bees	0.0022	0.03
Chronic oral exposure larvae	NOEL = 5 µg a.s./bee	Larvae	0.0204	0.2

Discussion

Landscape heterogeneity (*e.g.*, spatial distribution of the flower resources in the sprayed area), together with the spraying method (*e.g.*, non-uniform pesticide application and drift), could be behind the observed variability in acetamiprid in flower samples, originating no statistical differences between the tested study windows. Fifteen days after the application, the acetamiprid concentration in all the different matrices collected were reduced, in average, by 3.3-fold. Of note, when flower samples were collected for pesticide analysis, preference was given to flowering resources being visited by honey bees at that point in time, thus sampled plant species were not the same between the two sampling periods. Consequently, extrapolations about pesticide degradation in the flower samples should be done with caution.

Management practices in the study windows included the removal of vegetation between eucalyptus lines by ploughing. These practices, along with the high tree density, can lead to low flowering resources inside the eucalyptus stands (shrubs were only present in *Eucalyptus* plantation lines). Therefore, most of the flowering resources/sampling points were within the forest clearings and small water channels (Domingues *et al.*, 2022), and not inside the eucalyptus areas. Despite spraying maps (supplementary material, section IV.2) indicating these sensitive areas were to be avoided (not sprayed), acetamiprid residues were detected on flower samples from these areas (18% of all sampling points). The unintentional contamination of non-target areas is a common event almost at every pesticide application (Fishel & Ferrell, 2010). To control the level of drift, besides the use of buffer areas there are several other mitigation strategies (*e.g.*, avoid spray with strong wind, use of crop specific delivering methods, use of windbreaks) that can be taken into consideration (Ucar & Hall, 2001). In this study, the spraying event only took place when wind speed was lower than 11 km/h, while the pesticide solution was applied at the top of the eucalyptus trees using a nebulizer cannon that created small droplets with a charge that easily adhere to the eucalyptus foliage, reducing the washoff towards the soil and understory vegetation. This means that even with tight safety spraying measures, implemented to reduce cross-contamination of non-target areas, air drift did occur, resulting in exposure of honey bees due to foraging.

The amount of acetamiprid residues found in the different matrices collected in the sprayed area gives a proxy of how organisms can be exposed. Honey bees forage in these landscapes and bring nectar and pollen (among other elements, like resins and water) into the colonies, either from the sprayed area or outside of it. As the spraying area increased, higher honey bee's exposure was expected and confirmed by an increasing amount of acetamiprid residues found in pollen and nectar with an increase of the area sprayed. This confirms that the acetamiprid concentration found on pollen

pellets provides a representative image of flower contamination at a moment in time, making the honey bee colonies good indicators of environmental pollution by xenobiotics (Bargańska *et al.*, 2016; Niell at al., 2018).

Concentrations of acetamiprid found in pollen pellets were much higher than the ones found in nectar one day after spraying ($p<0.05$). This can be explained by the fact that bees metabolize at least 50% of acetamiprid in the digestive tract just 30 minutes after nectar ingestion (Brunet *et al.*, 2005). However, when this nectar is transformed into honey, the acetamiprid degradation is significantly impeded (half-life of 200 days in multiflower honey; Yeter & Aydin, 2019). As for pollen (and beebread), the presence of higher acetamiprid concentrations is possibly related with these matrices' ability to retain both lipophilic and hydrophilic chemicals (Lozano *et al.*, 2019). The samples collected after 15 days agree with these assumptions, considering the higher acetamiprid residue levels found in beebread when compared to honey. In these samples, colonies from the apiary 4 have significantly higher amounts of acetamiprid residues when compared to the other colonies. The landscape surrounding this apiary has several intermittent streams covered with shrubs of *Rubus ulmifolius*, whose flowering period only started after the spraying event. Considering the systemic characteristic of this class of pesticides, it is expected that plants might have been exposed to acetamiprid during the spraying event, with the insecticide entering the vascular system through the leaves/roots, traveling into the nectar and pollen, and thereafter being transferred into the colonies after blooming. This specific case also demonstrates the difficulties in calculating bee's exposure to neonicotinoids in such heterogenous landscapes when considering flower resources (even though all landscapes were mainly composed by eucalyptus plantations).

Taking into account that beebread and honey reserves are commonly consumed by the colonies during the most critical period for survival (*i.e.* winter; *e.g.*, Brodschneider *et al.*, 2018), that the winter bees live longer than summer bees (as their activity is highly reduced and body fat is highly increased; Amdam & Omholt, 2002), and that at the same time, the colony nest is highly reduced/non-existent leading to a few/no bees being born, stressors that affect the colony equilibrium are able to cause the colony to collapse (*e.g.* Van Dooremalen *et al.*, 2012). In previous reports (EFSA, 2013), winter bees, larvae, nurse bees, and foraging bees are the classes of honey bees that are more exposed to pesticides through oral exposure. In this study, for all of these classes, despite assuming the worst-case scenario (*e.g.*, honey with 50% sugar concentration), the ETR values were considerably lower than the trigger values, even with those having been conservatively calculated based on the lowest background mortality levels found in the literature (EFSA, 2013). This indicates that under these specific conditions, there are no honey bee classes seriously harmed by these spraying events. Other

studies have also shown that field applications of acetamiprid did not harm honey bees (no increase in mortality nor behavioural changes) when using the recommended dose (Stanley *et al.*, 2015; Dworzańska *et al.*, 2020). The absence of significant effects on adult bee mortality also occurred in laboratory studies performed by Stanley *et al.* (2015) when the recommended dose was used. Furthermore, similar doses to the ones found in the fresh nectar do not affect honey bees homing ability (Capela *et al.*, 2022a). Negative effects of acetamiprid were only detected on honey bee lifespan and foraging behaviour after exposure to 2 µg/a.s./bee (exposure by contact; Shi *et al.*, 2020a), and on larval development after exposure to 5ppm (oral exposure; Shi *et al.*, 2020b). In the current study, the maximum value detected in colony samples, was 1980 ppb in beebread samples, which is approximately 2.5 times lower than the harmful values reported by Shi *et al.* (2020b). Interestingly, acetamiprid structure is being used to search for alternative pesticides that are less harmful for honey bees (Crisan *et al.*, 2020).

The wide range of acetamiprid residues found in all sites also shows how extremely difficult it is to predict pesticide exposure in this type of landscapes. The amount of pesticide residues that bees transport into the colony (as nectar and pollen) can be seen as a measure of the real exposure conditions that honey bees might be subjected to. This assessment gains an even higher relevance since food sources vary spatially and seasonally (Berenbaum, 2016), and exposure may depend on the foraging behaviour (visited plants). Also, when assessing exposure to honey bees, it can be misleading to only evaluate residue levels immediately after pesticide application. Not only can they be found in plants several days after application, but the collected nectar (as honey) and pollen (as beebread) will also be stored in the colony, thus originating an accumulation of pesticide residues in these matrices for a long time (*e.g.*, Tong *et al.*, 2018), and being an exposure source for bees inside the colony. Therefore, sampling bee products becomes essential to determine the real colony exposure and the effects of the accumulated residues which will later be used to feed future generations (Colin *et al.*, 2019a). In this study, the maximum residue levels detected in pollen and nectar were 592 ppb and 180 ppb, respectively, which are much higher than the detected maximum residues in other studies. In a review from Zioga *et al.* (2020), the maximum residues found in pollen were 0.82 ppb while in nectar where 7.60 ppb. These differences may arrive from the lower application rates (20g a.s./ha vs. 40g a.s./ha in our study), timing and sampling methods used in the different studies (not specified in the review) since we measured the fresh nectar and pollen transported by the bees in the day after spraying. When compared to other neonicotinoids, acetamiprid seems to be the least transferred into the colonies. In Niell *et al.* (2017), acetamiprid residues found in the colony (honey bees) were 10 times lower than the other tested neonicotinoids (thiamethoxam and imidacloprid). The authors hypothesized that bees could detect the presence of acetamiprid due to the high volatility of the

insecticide. Nonetheless, acetamiprid repellent effects were detected for termites (Rust & Saran, 2008) while for honey bees there are no evidence of such effects. The available data also confirms that the maximum acetamiprid residues found in colony products (nectar and pollen) have a lower concentration than other neonicotinoids (pollen / nectar maximum residues concentration (ppb) for each neonicotinoid in Zioga *et al.*, (2020): acetamiprid – 0.82 / 7.60; imidacloprid – 159 / 6588; thiamethoxam – 95.2 / 11; thiacloprid – 78 / 65.6; clothianidin – 11 / 2992). Therefore, acetamiprid seems safer than other neonicotinoids due to its lower toxicity and lower transference into the colonies.

Despite the lower toxicity from acetamiprid we advocate that to avoid unacceptable risks derived from pesticide exposure to honey bees (in general), not only should it be considered that mitigation measures may and can be applied to decrease the exposure of non-target plants, instead of just following the "non-attractive crops" requirement (Simon-Delso *et al.*, 2017), but also that collecting residue data from flower resources and from the colony is fundamental to better predict exposure. In this study, eucalyptus trees (the sprayed "crop") were not flowering, which might lead one to infer that spraying events in these areas and during this season can be safe for pollinators when considering oral exposure. Nonetheless, under the sprayed "crop" and next to it (surrounding areas), there are important food sources and even nesting places that sustain pollinator communities. The acetamiprid residues found on the analysed matrices show that these sources also get pesticide residues input and that honey bees transport those xenobiotics into the colonies. This makes the honey bee colonies good indicators of environmental pollution by xenobiotics (Bargańska *et al.*, 2016; Niell at al., 2018) being essential the use of sentinel hives to monitor not only effects but also exposure to these compounds (EFSA Scientific Committee, 2021).

Based on the obtained data and considering the cost of residue analysis, the implemented xMAP acetamiprid immunoassay has shown to be a fast, medium-high throughput and reliable option to analyse a large set of diverse samples. Nonetheless, considering sampling efficiency (cost, time spent on sampling, and output), we defend that the analysis of colony products like honey and beebread through time can be used as a tool to evaluate landscape suitability regarding pesticides presence, while fresh pollen can provide a snapshot in time. Additionally, the LFD prototype has proven to be a reliable and useful tool for in-field and lab-based pre-screening (Posthuma-Trumpie *et al.*, 2009) for the presence of neonicotinoids in landscape and colony matrices. Beekeepers and other stakeholders can use it for a fast and cheap screening to assess the spread of residues in the landscape and evaluate if honey bees are bringing pesticides residues into the colonies. For now, this device is

validated for detecting six neonicotinoids (imidacloprid, acetamiprid, clothianidin, thiacloprid, nitenpyram and imidaclothiz) in several bee-related matrices.

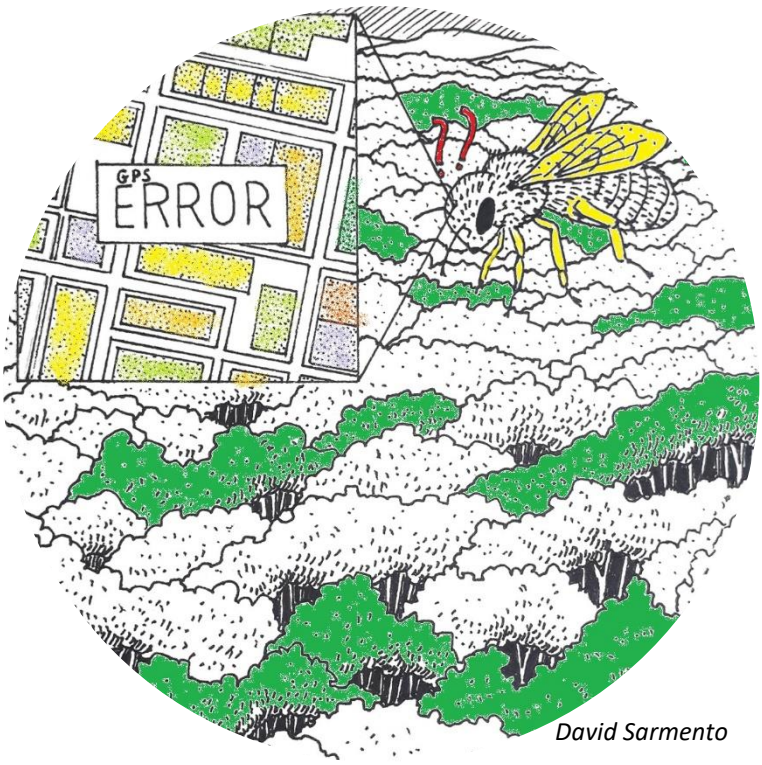
Conclusions

In this study, real landscape-based scenarios where acetamiprid is usually applied and beekeepers usually install their colonies were tested. The low ETR values found show that spraying events covering up to 22% of the honey bees foraging ranges towards the end of the flowering period of the main flower resources present a negligible risk to the honey bee' colonies. Nevertheless, these results must be interpreted with caution, since (1) in all study windows, honey bees had non-sprayed areas to forage; (2) no other crop fields with pesticides' input were inside the study areas, limiting cross contamination and possible synergistic effects caused by other chemicals (*e.g.*, Wang *et al.*, 2020); and (3) the study was performed in a period where nectar and pollen income might not be so high as during the peak of flowering.

The range of acetamiprid exposure was successfully assessed showing how acetamiprid residues behave in the landscape and follow their fate in honey bee colonies by measuring its accumulation on several colony matrices. On the other hand, no clear pattern was detected between the most exposed (near roads) flower resources and the ones inside the eucalyptus area, despite the low p-value (0.07).

Chapter V

Assess the effects on individual homing ability after exposure to sulfoxaflor (new neonicotinoid-like substance) and acetamiprid



David Sarmento

Chapter published as an original article:

Capela, N., Sarmento, A., Simões, S., Azevedo-Pereira, H. M., & Sousa, J. P. (2022). Sub-lethal doses of sulfoxaflor impair honey bee homing ability. *Science of The Total Environment*, 155710.

Abstract

Agricultural intensification has increased the number of stressors that pollinators are exposed to. Besides increasing landscape fragmentation that limit the supply of flower resources, intensive agricultural practices relying on the use of pesticides to control agricultural pests also affect non-target organisms like honey bees. The use of most pesticides containing neonicotinoids has been severely restricted in the European Union, leaving pesticides containing acetamiprid as the only ones that are still authorized. In the meantime, new substances like sulfoxaflor, that have a similar mode of action acting on the insect's nicotinic acetylcholine receptors (nAChR), have been approved for agricultural use. In Europe and USA, the use of pesticides containing this active ingredient is limited due to toxic effects already reported on bees, but no restrictions regarding this matter were applied in other countries (*e.g.*, Brazil). In this study, homing ability tests with acetamiprid and sulfoxaflor were performed, in which honey bees were fed with three sub-lethal doses from each substance. After exposure, each honey bee was equipped with an RFID chip and released 1km away from the colony to evaluate their homing ability. No significant effects were detected in honey bees fed with 32, 48 and 61ng of acetamiprid while a poor performance on their homing ability, with only 28% of them reaching the colony instead of 75%, was detected at a 26 ng/a.s./bee dose of sulfoxaflor. Although, both pesticides act on the nAChR, the higher sulfoxaflor toxicity might be related with the honey bees detoxifying mechanisms, which are more effective on cyano-based neonicotinoids (*i.e.*, acetamiprid) than sulfoximines. With this study we encourage the use of homing ability tests to be a suitable candidate to integrate the future risk assessment scheme, providing valuable data to models predicting effects on colony health that emerge from the individual actions of each bee.

Introduction

Neonicotinoids are a worldwide commonly used class of insecticides (Bass *et al.* 2015) with systemic properties, which allows them to stay active in the plant vascular system during several days, protecting the plant from pests (Simon-Delso *et al.*, 2015). Due to their biochemical characteristics neonicotinoids are specific to insects, acting on the nicotinic acetylcholine receptors (nAChR), located on the central nervous system, deterring insects' movement and ultimately leading to their death (Tomizawa & Casida, 2005; Simon-Delso *et al.*, 2015; Ihara & Matsuda, 2018). Although applied to control pest species, non-target organisms - such as honey bees - can also suffer from sub-lethal and lethal effects caused by contact (during or immediately after pesticide application) or ingestion (when foraging for resources in contaminated floral species) of these compounds. Foraging ability (Henry *et*

al., 2012; Matsumoto, 2013; Tison *et al.*, 2016; Gomes *et al.*, 2020), life span (Shi *et al.*, 2019), queen reproductive success (Williams *et al.*, 2015) are just some examples of the sublethal effects caused by neonicotinoids. Moreover, these might be enhanced via synergistic effects with other stressors (*e.g.*, Tosi *et al.*, 2017). Due to their toxicity to non-target organisms like bees, the European Commission has restricted the use of most neonicotinoids (4A IRAC MoA sub-group; *e.g.*, EU 2013; EU 2018b,c,d), leaving acetamiprid as the only neonicotinoid substance from that group that can still be applied outdoors. In North America, it was also applied restrictions on the use of products containing these substances, including the restriction to spray during blooming, or even in crops attractive to pollinators. On the other hand, in African countries, there has been recent developments to evaluate the use and effects of neonicotinoids, but no restrictive measures have been applied (NASAC, 2019). In South America, for example in Brazil, which is the fourth agricultural producer worldwide, the scenario is similar, in which most of the products containing these active substances have unconditional uses (*e.g.*, Friedrich *et al.*, 2021). Even though acetamiprid has been presented as having low toxicity to honey bees (Zhu *et al.*, 2015), recent findings have shown that this active substance has potential to decrease honey bee lifespan, to affect learning, memory, and honey bee's ability to return to their colony (Shi *et al.*, 2019), and to impact larvae development (Shi *et al.*, 2020a). Nevertheless, when compared with other commonly applied neonicotinoid (thiamethoxam), acetamiprid is 100 times less toxic to honey bees (LD50 by contact; Lewis *et al.*, 2016); and considering the available data, the European Food Safety Authority (EFSA) concluded that acetamiprid presented a low risk to bees even in field applications (EFSA, 2016b; EFSA, 2022).

The severe restrictions on most neonicotinoids created a void on the supply chain, as producers do not go forward with their renewal applications, in a stage where the demand for pesticides is increasing (Simon-Delso *et al.*, 2015). Therefore, new plant protection products are arriving to the market every year, containing synthetic or natural compounds/active substances (a.s.) with proven effectiveness against pests (Umetsu & Shirai, 2020). Sulfoxaflor is one of these new active substances that were registered for use in at least 81 countries (Brown *et al.*, 2016). It was registered by the United States Environmental Protection Agency in 2013 and by the European Union in 2015. This compound has been presented and classified as belonging to a new chemical group - IRAC MoA sub-group 4C sulfoximines – although its mode of action is similar to neonicotinoids, acting as agonists of the nicotinic acetylcholine receptor (nAChR) (Sparks *et al.*, 2013; Bacci *et al.*, 2018; Watson *et al.*, 2021). Therefore, sulfoximines are considered as a subgroup of the neonicotinoid's family by some authors (Cutler *et al.*, 2013; Bacci *et al.*, 2018). Interestingly, the honey bee health impact of the products containing this a.s., appears to be greater than acetamiprid: sulfoxaflor effects are more

related with nitro-based neonicotinoids while acetamiprid is a cyano-based neonicotinoid, to which the cytochrome P450 enzymes of honey bees are more efficient in detoxifying (Bacci *et al.*, 2018).

In 2019, EFSA emitted a peer review on the pesticides containing sulfoxaflor as active substance, establishing it as high risk for honey bees and bumblebees in field conditions (under some specific scenarios like spraying before or during flowering) and as low risk in permanent greenhouses (EFSA, 2019). Subsequently, by late 2019, the French Agency for Food, Environmental and Occupational Health & Safety (ANSES) withdraw the use of all pesticides containing sulfoxaflor due to its negative effects on pollinators (ANSES, 2019). At the same time (2019), the United States Environmental Protection Agency (USEPA) announced a long-term approval for the pesticides containing sulfoxaflor, removing some of the restrictions of use applied in 2016, which were in place to reduce the potential risk to pollinators (USEPA, 2019).

For approval, these compounds need to comply with a series of safety requirements (*e.g.*, EFSA, 2013) to ensure that their effects on non-target organisms are negligible and/or within previously defined threshold values. Nonetheless, honey bees' risk assessment scheme still has room for improvement (*e.g.*, tests on queen performance, homing ability, and development of hypopharyngeal glands; Rortais *et al.*, 2017) to overcome the challenges of field testing (*e.g.*, different sources of uncertainty and inter-colony variability, leading to a low statistical power; Rortais *et al.*, 2017) without compromising the established specific protection goals. Hence, information to support decision-making when it comes to the authorization and renewal of products containing these substances, including the definition of restrictions on their use, is extremely relevant. Acetamiprid has been shown to negatively impact the foragers' ability to return to their colony (Shi *et al.*, 2019) - homing ability - while there is still no information on sulfoxaflor impact on this behaviour, despite the similar mode of action of both compounds. To fill this knowledge gap, in this study we assessed the effect sub-lethal doses of acetamiprid and sulfoxaflor on their homing ability by using the radio frequency identification (RFID) technology. We also aimed at discussing the importance of the experimental setup when performing such experiments.

Materials and Methods

Test sites and used colonies

Experiments were conducted within 2 different landscapes, in which the tested products – pesticides containing acetamiprid and sulfoxaflor a.s. - are usually applied. For the acetamiprid test, the landscape was located in the central region of Portugal (Alcafozes; 39.974739, -7.066327) and was mainly composed of eucalyptus stands with medium density of understory vegetation (mainly shrubs). The experiment took place in May, when acetamiprid is usually applied to control the eucalyptus weevil (*Gonipterus platensis*). As for sulfoxaflor, the experiment was also performed in May, in a non-intensive agricultural landscape inside a region known for its watermelon (Cucurbitaceae) production (sulfoxaflor is commonly applied in this crop), also in the central region of Portugal (Idanha-a-Nova; 39.859172, -7.163818).

Queenright colonies of *Apis mellifera iberiensis* (with queens born in 2019) were installed one year prior to the experiment. To avoid colony drift, the colonies were isolated from other apiaries by at least a 1.5 Km radius. At the time of the experiment, each study colony had ca. 40000 individuals, free of any disease symptoms, and with low to no varroa mites' infestation. In total, 2 colonies were used in this experiment.

Four dummy RFID readers were installed in front of the colony one week before the experiment for foragers acclimation, to avoid bee accumulation on the entrance or behavioural changes that could jeopardize the results. On the first day of each experiment, the dummy readers were replaced by four functional RFID readers which recorded bee activity every day during the full duration of each experiment, including 5 days after the last release day, to account for bees that did not return to the hive on the same day of release.

Used pesticide solutions and experimental setup

Sucrose solutions containing 50% sugar spiked with 3 different concentrations of the formulated products Epik® SG (a.s. acetamiprid; 200g/kg (20% w/w), supplied by Sipcam Portugal) and Closer® (a.s. sulfoxaflor; 120 g/L (11.3% w/w), supplied by Corteva Agriscience) were prepared before each field experiment. For acetamiprid, three sub-lethal doses were used: 3200, 4800 and 6100 ppb (µg/a.s./L), corresponding to 32, 48 and 61 ng/a.s./bee. For sulfoxaflor, also three sub-lethal doses were used (720, 1200 and 2600 ppb (µg/a.s./L), corresponding to 7.2, 12 and 26 ng/a.s./bee. The doses tested for each compound were selected to be within a potential field exposure range calculated

based on the daily maximum nectar consumption (up to 640 µL, assuming a 20% sugar content in nectar; Table J1 at EFSA, 2013) and the range of measured concentrations on forager collected nectar on the day of application or on the next day. For acetamiprid the concentration in the nectar ranged between 10 – 180 ppb (Capela *et al.*, 2022b), originating an interval of potential exposure concentrations between 6.4 – 115 ng/bee/day. For this compound, a dose near this maximum value (100 ng/bee/day) was tested but discarded due to bee mortality during the captivity period. For sulfoxaflor, the measured concentration in forager collected nectar varies between 9.11 – 46.7 ppb (USEPA, 2016), giving a potential concentration exposure range between 5.8 – 30.06 ng/bee/day.

Test solutions were stored under -18°C, protected from light exposure and sent to an independent chemical analysis laboratory to validate the contaminant concentration by Ultra Performance Liquid Chromatography tandem Mass Spectrometer (UPLC-MS/MS) (Limit of quantification: 10 µg/L). The tested doses of acetamiprid and sulfoxaflor mentioned above are the measured values by UPLC-MS/MS.

Forager bees were captured at the entrance of the hive during the morning period (from 9am to 9:30am). The colonies were closed with forager bees on the outside. These bees were captured using tweezers, by gently grabbing the bees on their hind legs, and stored in a black box container until use. After capture, all the bees were fed *ad libitum* for at least 2h with a 50% sucrose solution (control) to synchronize their diet state. Subsequently, bees were starved for 1:30h before feeding on the contaminated solution. A solution of 10µl containing only the sucrose solution (control group) or a spiked solution (50% sucrose solution with pesticide) was given individually to each bee, using a micropipette. Individuals that did not consume the whole solution were discarded from the experiment. After feeding, honey bees were kept for an additional 30min period to ensure the assimilation of the contaminant and to register any abnormal behaviour. Honey bees that did not perform a flawless flight (*e.g.*, either flying towards the ground or not even being able to fly) were discarded from the study. After exposure and selection of bees, RFID tags (mic3®-TAG 64-bit, iID2000, 13.56 MHz system, 1.0 × 1.6 × 0.5mm; Microsensys GmbH, Erfurt, Germany) were glued onto the honey bee thorax using dental cement to avoid glue toxicity (temporary cement polyvinylsiloxane, Temposil®2, Coltene®) (Henry *et al.*, 2012). The readers at the entrance of the hive registered the time stamp and the code of the RFID chip once it passed though the reader (iID® MAJA RFID system, Microsensys GmbH, Erfurt, Germany). To confirm that the used doses or the handling of organisms did not cause mortality, a group of honey bees (n = 15) per treatment were fed individually with the solutions and kept in captivity during at least 2h.

Tagged bees were released from 6 different points equally spaced on a 1km radius from the colony (15 bees per tested dose and control, *i.e.*, 60 bees per point). The distance from the feeding site was fixed at 1km which considers the common foraging window (Couvillon *et al.*, 2015), and the use of several release points were considered to account for landscape familiarity.

Each experiment last for 8 consecutive days, in which the first 3 days consisted of the pesticide exposure and releases and the last 5 days to control for bees that returned to the colony on the days after exposure.

Assessed parameters and data analysis

For each bee, the time difference between release and arrival to the colony was calculated using the first timestamp registered by the RFID readers installed at the entry of colony. The cumulative homing ability was therefore calculated based on both the number of honey bees that arrived at the colony and the time that took them to get there.

Fisher's exact tests were performed to compare (1) the effect of pesticide dose by using the overall number of bees that arrived and that were lost (observed values) at each tested pesticide dose with those arrived/lost in the controls (expected values); (2) the influence of release points by using the observed control values (arrived/lost) versus the expected (arrival/lost) considering a proportion of 14:1 (this was used instead of the expected 15:0, where every bee arrives at the colony, to account for predation and possible old age of foragers), and (3) the effect of pesticide dose in each release point by comparing the controls (expected) with the tested doses (observed) in each release point. From all these analyses, one of the release points on the acetamiprid test (coded as point D) was excluded from the analysis, as the recorded lower homing ability was apparently related with other factors besides the exposure to the pesticide during the experiment. For all the tests performed, a significance level of $p < 0.05$ was adopted. The statistical software Statistica®13.3 (TIBCO, 2017) was used for all analyses.

Results

Considering the total number of bees that arrived at the colony per treatment and release point, the tested acetamiprid doses do not seem to cause negative impacts in bees' orientation, even at the highest (61 ng/bee) tested dose (Fisher's exact test, $p=0.42$). It is possible to observe (Fig. V.1a) that bees took more time to get home but eventually arrived, as only 58% reach the colony in the first 2h compared to 78% in the control. Unexpectedly, bees fed with the lowest doses (32 ng/bee) had a better homing ability performance (93% got home) than bees fed with the control solution (82% got home), although this difference was not statistically significant (Fisher's exact test, $p=0.08$).

On the other hand, sulfoxaflor had a significant negative impact on foragers' homing ability (Fig. V.1b) when fed with 26 ng a.s. solution (Fisher's exact test, $p<0.001$). Among the 73 bees released, only 28% reached the colony. Also, it is noteworthy - but not significant (Fisher's exact test, $p=0.24$) - the slight decrease in the percentage of bees that reached the colony after being fed with 12 ng of sulfoxaflor (65% instead of 75%). The lowest tested dose (7.2 ng/bee) did not hamper their ability to return to the colony when compared to the control (Fisher's exact test, $p=0.86$). In this landscape, only 75% of the bees from control reached the colony. From these, 86% reached the colony in less than 2h. This tendency is maintained despite being fed with the two lowest sulfoxaflor doses (89% and 87% in 7.2 and 12 ng/bee, respectively, during the first 2 hours).

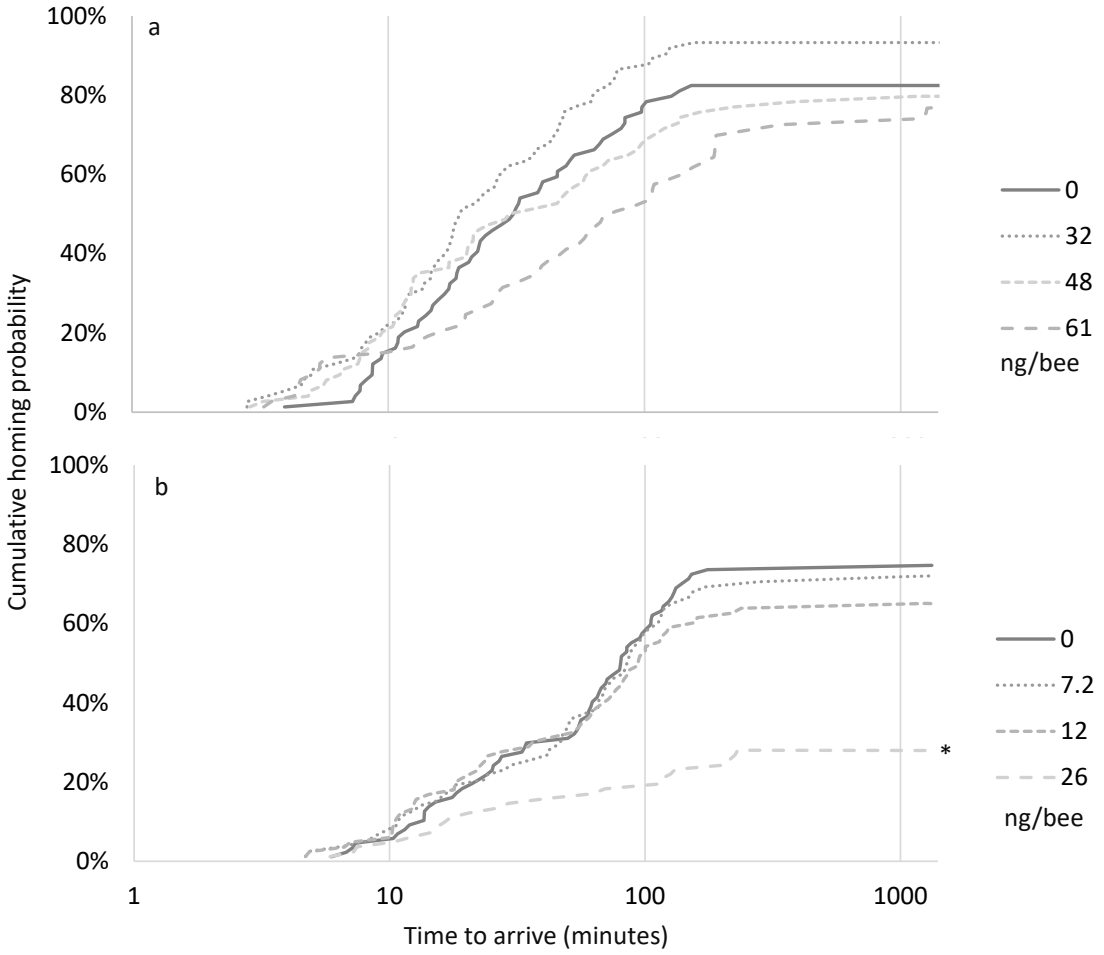


*Figure V.1: Cumulative homing probability of honey bees exposed to different doses of acetamiprid (a) and sulfoxaflor (b) through time. Honey bees were released 1km away from the colony, after being fed with a control or pesticide solution. The time to arrive back to the colony was calculated using RFID technology. * = statistical differences from control.*

In the acetamiprid landscape (Fig. V.2), no significant differences were found between the controls, and the expected proportion of arrival/lost (14/1) in the different release points. Nonetheless, on the landscape where sulfoxaflor was tested, the release points influenced the bee homing ability. In the point "E" (Fig. V.3E), only 57% of the control bees reached the colony (Fisher's exact test, p=0.03) and in point "B" (Fig. V.3B), despite not being significant (Fisher's exact test, p=0.08), only 60% of the bees from the control reached the colony.

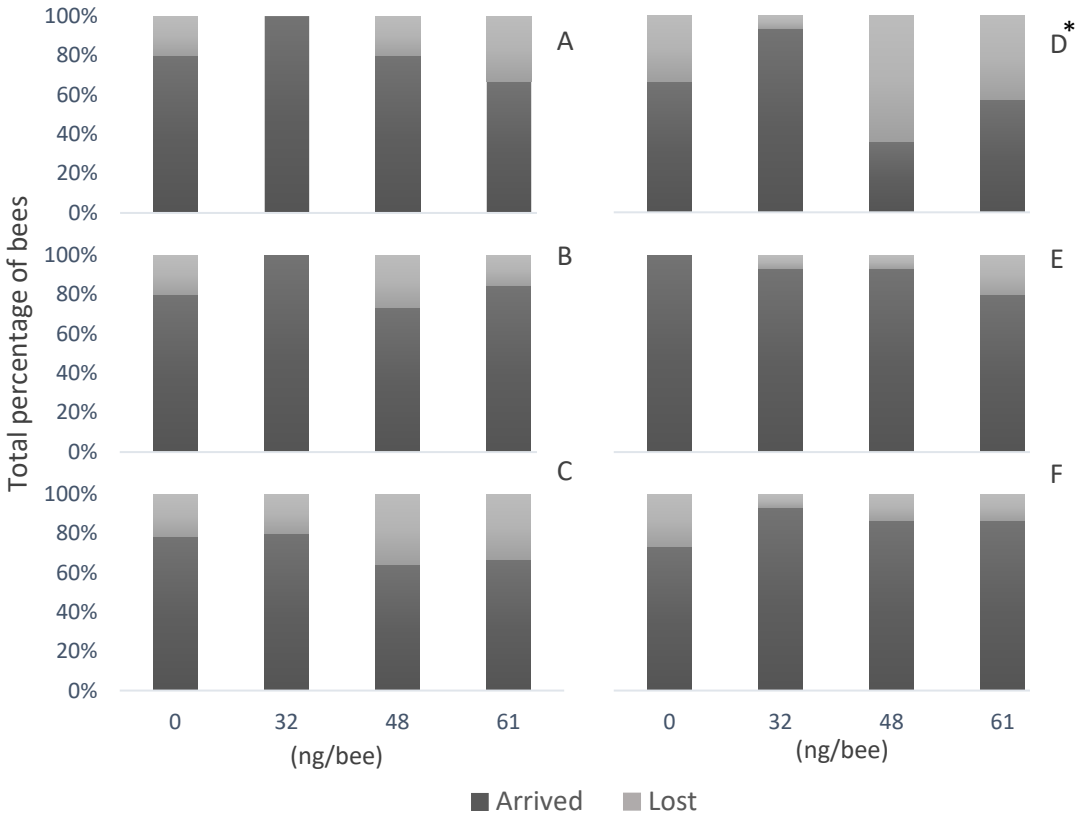


*Figure V.2: Total percentage of bees that have arrived back at the colony or got lost and never managed to come back after being fed with a sugar control or acetamiprid spiked solution. Bees were released from 6 different points (A-F) equally distributed around the colony, at 1km. *Release point D was excluded from the analysis because the higher homing failure registered in the 48 and 61 ng/bee doses, could be related with the release time (after 19h) and not with the pesticide effects.*

After analysing each release point in the acetamiprid landscape (Fig. V.2), the tested doses did not differ significantly from control in any of the release points. On the sulfoxaflor exposed bees, we observed that in release points B and E (Fig. V.3B, V.3E) there was no statistical differences between the control and none of the tested doses. On the other hand, in release point D (Fig. V.3D), individuals fed with 12 ng also showed homing ability impairment when compared with the control ones (Fisher's exact test, p= 0.03).

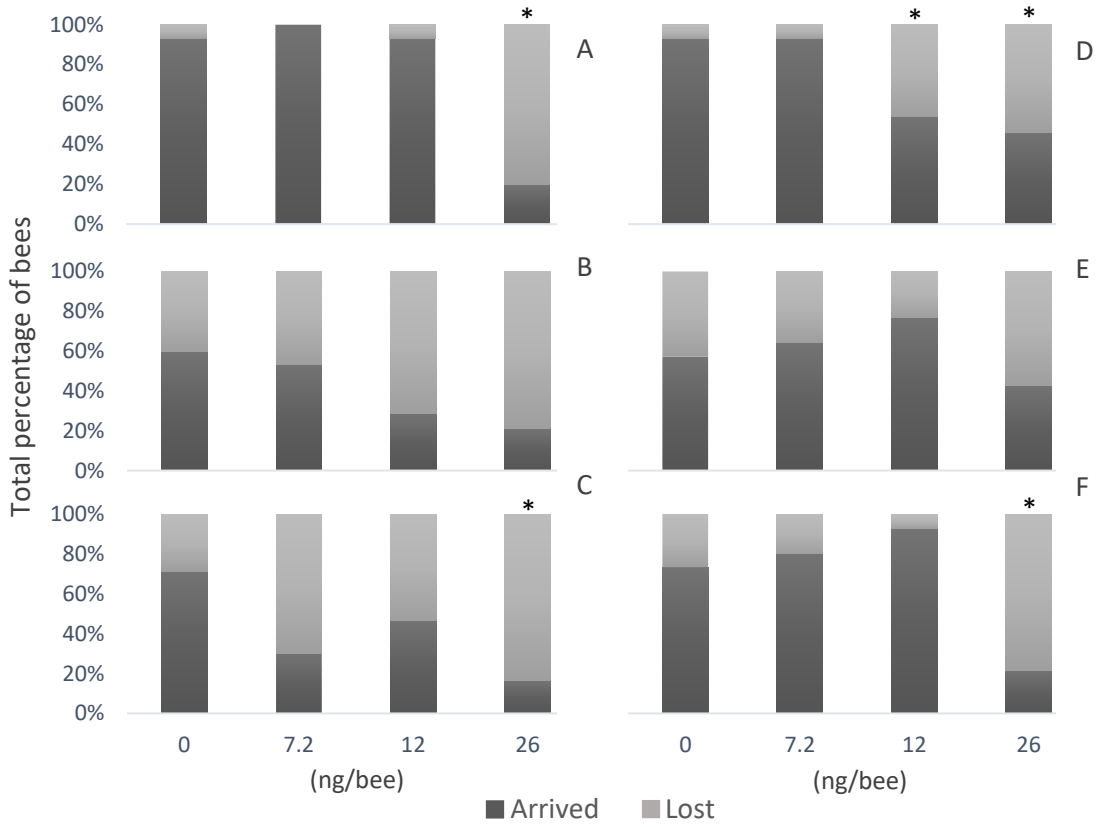


*Figure V.3: Total percentage of bees that have arrived back at the colony or got lost and never managed to come back after being feed with a sugar control or sulfoxaflor spiked solution. Bees were released from 6 different points (A-F) equally distributed around the colony, at 1km. * = statistical differences from control.*

Discussion

Effects of sulfoxaflor and acetamiprid on homing ability

Although most pesticides containing neonicotinoids have been severely restricted in the European Union market, in the past years (*e.g.* EU, 2013; 2018b,c,d), new substances that act in a similar way are replacing them and being applied in farming and forest areas (Simon-Delso *et al.*, 2015; Bacci *et al.*, 2018; Azpiazu *et al.*, 2021). Currently in the EU, acetamiprid is the only neonicotinoid (4A ICRAC MoA sub-group, nAChR agonists), that is allowed to be applied. According to EFSA (2016b; 2022), the current available studies could not be used to draw any firm conclusion on the acetamiprid risk to honey bees. As for sulfoxaflor (4C ICRAC MoA sub-group, nAChR agonists), it was already pointed out by EFSA as a high-risk substance for honey bees when it is sprayed during the flowering period (EFSA, 2019). These restrictions have proven effective to protect honey bees if sulfoxaflor is applied 5 to 6 days before flowering (Tamburini *et al.*, 2021) and application methods are correctly pursued.

Our data confirms a higher toxicity of sulfoxaflor when compared to acetamiprid as reported by other authors (*e.g.*, Azpiazu *et al.*, 2021). The highest acetamiprid dose tested (61 ng/bee) is approximately 2.3 times higher than the highest sulfoxaflor dose (26 ng/bee) and, despite this difference, bees fed with 26ng of sulfoxaflor exhibited high levels of disorientation – presenting homing ability failure – while bees fed with acetamiprid apparently did not exhibit this abnormal behaviour. Nonetheless, despite the higher sulfoxaflor toxicity when compared to acetamiprid, the tested dosages are 18% (sulfoxaflor) and only 1% (acetamiprid) of the respective LD50 (oral acute LD50 of 0.146 µg/bee and 8.85 µg/bee, respectively). Similar sublethal doses of sulfoxaflor administered to young honey bees (16 and 60 ng) have also proven to reduce bees daily flight activity and the number of flights when they turn into foragers (Barascou *et al.*, 2022), and to negative effects on the reproductive success and worker production in bumblebees after chronic exposure to 5ppb of sulfoxaflor (Siviter *et al.*, 2018). The difference in bee behaviour observed in this study between both pesticides could be related with the honey bees' detoxification mechanisms. These can better cope with cyano-based neonicotinoids like acetamiprid in comparison with nitro-based neonicotinoids and sulfoximines (Bacci *et al.*, 2018). Homing performance tests with bees orally exposed to 1.26ng of a nitro-based neonicotinoid (thiamethoxam) also showed a poorer performance (between 10.2 and 31.6% less bees returning to the colonies) in returning to their colony (Henry *et al.*, 2012). Comparing the result reported by Henry et al (2012) and the one obtained in this study, we could say that sulfoximines are more toxic than cyano-based neonicotinoids but less toxic than the nitro-based neonicotinoids. Sulfoximines are known to be more effective against insects than cyano-based neonicotinoids because these molecules are poor substrates for the metabolic enzymes involved on the degradation processes of chemicals (Sparks *et al.*, 2013).

Despite the higher toxicity in comparison to acetamiprid, it would be unlikely a scenario where bees would be exposed to 26ng of sulfoxaflor under field conditions considering a daily feeding on contaminated nectar since, as shown in the calculations presented in the Materials and Methods section, this value corresponds to a potential exposure when considering that forager bees would ingest their maximum daily requirements feeding only on the contaminated nectar. Although unlikely to occur, this scenario could be possible in countries without restrictions to pesticides containing sulfoxaflor (*e.g.*, Brazil), while at the European level, the applied restrictions (can only be applied when crops are not blooming) would prevent such exposure scenario.

On the other hand, the tested acetamiprid doses did not have a negative effect on their orientation. Interestingly, but not statistically significant, honey bees fed with the lowest tested dose (32 ng/a.s./bee) outperformed the control group (Fig. V.1a). We hypothesize that the low acetamiprid

dose could have led to an increase in movement, potentializing their success to arrive home, which might be due to an hormetic response from these individuals (Mattson, 2008) to balance their homeostasis. In fact, low doses of pesticides such as neonicotinoids can promote stimulations of biological functions of pests (Qu *et al.*, 2015) and even on non-target arthropods (Rix & Cutler, 2020). In honey bees it was already documented an increase on bee locomotor activity after being exposed to small acetamiprid doses when bees where topically exposed to doses of 0.1 and 0.5 µg/bee (El Hassani *et al.*, 2008). Besides, a higher dose (1 µg/bee) did not affect their locomotion. This kind of favourable responses at low doses have also been reported when bees where fed with flupyradifurone (Tosi & Nieh, 2019), imidacloprid (Lambin *et al.*, 2001) and even with a mixture of imidacloprid with coumaphos (Williamson *et al.*, 2013). At higher acetamiprid doses (48 and 61 ng/a.s./bee), we observed a slight, but non-significant, decrease in homing ability, which might not be relevant for the colony health, considering the percentage of lost bees or the amount of acetamiprid that can be found on honey bee's collected nectar (maximum of 180 ppb). These findings go in accordance with previous studies that compare the effects of acetamiprid and other neonicotinoid pesticides, in which acetamiprid poses a lower risk to honey bees (Iwasa *et al.*, 2004; Christen *et al.*, 2017).

Extrapolations to colony level

Homing ability tests are a great tool to assess behavioural changes at an individual level (Jeker & Grosser, 2020). The disorientation levels suffered by the forager bees and their eventual dead in the field is hardly perceived while evaluating the colony strength and the bee mortality measured in front of the colony. These two endpoints are usually applied in risk assessment to assess pesticide effects and are valid to evaluate the effects at the colony level after exposure to a toxic substance, since the colony is the entity to protect. Nonetheless, the mechanisms behind the detected effects are not perceived by evaluating these endpoints. The use of homing ability tests can help to understand the causes responsible for the measured effects at the colony level. Nonetheless, despite the high ecological relevance of these tests to understand the deteriorating effects on individual bees (disorientation), the rate of foragers loss that could lead to a critical reduction in colony size is not straightforward due to compensation mechanisms (*e.g.*, Schott *et al.*, 2021) on the colonies development.

An extrapolation of the impact of the reduced homing ability has already been done by Henry *et al.* (2012) in which a considerable negative impact at a colony level (by using the model by Khoury *et al.*, 2011) was predicted after an exposure to only 1.36ng of thiamethoxam. Nonetheless, the same authors, after conducting afield study showed that the colonies did not suffer any considerable impact, a result that could be explained mainly by the colony compensating mechanisms (Henry *et al.*, 2015).

Based on our study, in the unlikely event of an exposure to 26ng of sulfoxaflor, it could be hypothesized that the considerable loss of foragers could lead to new bees being recruited for foraging (early foraging; Colin *et al.*, 2019b; Shi *et al.*, 2020a), which can lead to reduced longevity (lifespan), decline in brood rearing, decreasing colony's population and normal development, and consequently reduction of the pollination services it provides, as well as the nectar and pollen colony's income (Khoury *et al.*, 2011; Russell *et al.*, 2013). On the other hand, in a feeding study where colonies were exposed to 0.5 mg/kg of sulfoxaflor (EFSA, 2019), there were no effects detected at the colony strength and hive mortality (measured in front of the colony) which means that individual effects (detected in this study at 26ng) can probably be diluted at a colony level. Nonetheless, these extrapolations could only be confirmed with the use of validated models that consider the whole colony development, including feedback/compensating mechanisms, and the environmental factors surrounding the colony. This need has already been identified by EFSA, who is developing a model that can entail all the population's dynamics and feedback mechanisms, that will allow to predict responses at a colony level based on individual responses to stressors (Rortais *et al.*, 2017; EFSA Scientific Committee, 2021).

Landscape familiarity

Approximately, 12% of all the bees from both control groups (*i.e.*, acetamiprid and sulfoxaflor field tests) never managed to reach the colony after being released, which was expected considering the landscape familiarity and exposure to predation. This percentage is in line with the median daily forage mortality rates calculated by EFSA (ap. 12.6%; EFSA, 2020). Our analysis of the control groups in the several release points shows that the landscape familiarity plays a role in our findings, with significant impact on bee's ability to return to their colony in some release points (*i.e.*, Fig. V.3E, V.3B). Forager honey bees learn specific routes from previous foraging trips by using landmarks (Dyer, 1991; Kheradmand & Nieh, 2019), which means they acquire experience at each foraging flight (Klein *et al.*, 2019) and increase their homing ability when visiting familiar places (*e.g.*, Henry *et al.*, 2012). On top of it, results from the analysis of each release point also sustain the landscape familiarity theory (Degen *et al.*, 2016; Kheradmand & Nieh, 2019), in which some of the release points have lower success rates (*i.e.*, Fig. V.3B, V.3D, V.3E). Therefore, extrapolations made from a single release point could originate a bias in the data. Bering this is mind, we suggest the use of several release points to account for landscape familiarity, increasing data reliability. Recently, it was also pointed out the use of familiar release points in honey bee homing flight tests (OECD, 2021). Despite the use of a different experimental design because our experiment was conducted prior the release of the OECD guideline, our study managed to successfully fulfil the key OECD requirements and test conditions for the homing

flight tests. The main divergences arrived from (1) the use of 10ul to feed the bees instead of 20 to 30ul and (2) the lack of a reference substance. The fact that bees were fed with less food can be compensated by the increase in the sucrose solution concentration, from 30% to 50%. This could be advantageous in energetic terms, providing enough energy to return to the colony, as it was proved by the control bees that managed to reach their colony within the expected mortality range. We agree that the use of a reference substance would indeed increase the test reliability but like in other homing ability studies (Henry *et al.*, 2012; Shi *et al.*, 2019) it does not invalidate the conclusions.

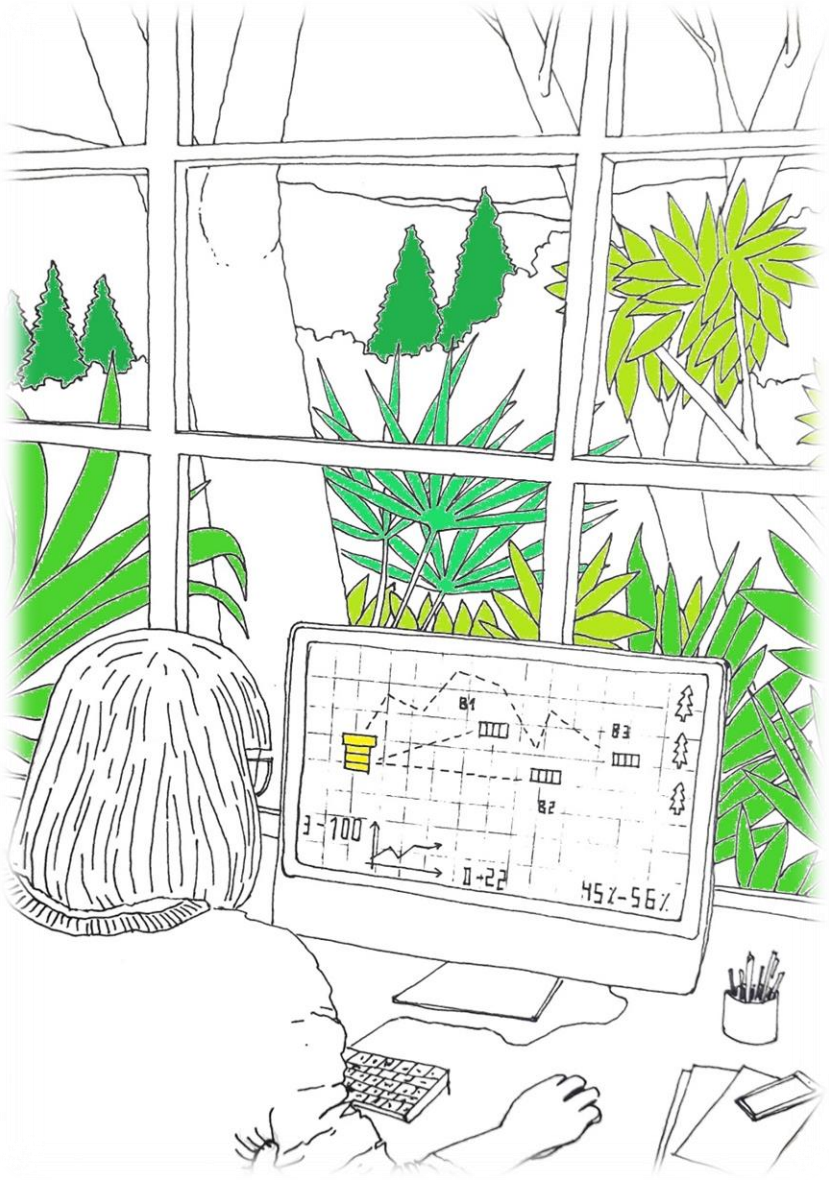
Conclusion

For approval, active substances need to pass through a series of steps by performing different tests following a tiered approach. These tests should provide sound data for plant protection products to be safely applied in the respective crop. Nonetheless, the link between pesticides and effects on pollinators (including honey bees) has been extensively reported (Vanbergen *et al.*, 2013; Goulson *et al.*, 2015; Potts *et al.*, 2016; Kopec & Burd, 2017; Powney *et al.*, 2019), evidencing the need to improve pollinators protection even when exposed to low pesticide doses. In this study, at the sub-lethal dose of sulfoxaflor of 26 ng/bee, forager bees exhibit such high degree of disorientation that cannot reach the colony, leading eventually to their death. Nonetheless this scenario would only be achieved in countries in which the spraying of these products is not properly regulated, leading to high levels of exposure. If protection measures are implemented to reduce bees' exposure (*e.g.*, in Europe and US), the application of products containing sulfoxaflor will not lead to negative effects on bees' homing ability. As for acetamiprid, it has proven to be less harmful for honey bees than sulfoxaflor. Honey bees can reach their colony even when being exposed at high acetamiprid doses (61 ng/bee).

Homing failure/homing ability has been considered by EFSA to be used for the evaluation of sub-lethal effects in free-ranging (forager) honey bees (Rortais *et al.*, 2017). With this study, we provide another example of how homing ability tests can be used as such by recommending the use of several release points to account for landscape familiarity. Regarding the role of homing ability tests within a risk assessment scheme we believe that the results from these tests may not be used on their own, to trigger further tests, but rather to feed model approaches that rely on the actions of individual bees, in which the effects at the colony level are the emergence of those individual actions considering the environment in which they are installed (*i.e.*, the honey bee colony model – *Apis*RAM - being currently developed by EFSA; Duan *et al.*, 2022).

Chapter VI

Foraging model



David Sarmento

Chapter submitted as an original article:

Capela, N., Duan, X., Ziółkowska, E., Topping C. J. (2022). Modelling foraging strategies of honey bees as agents in a dynamic landscape representation. *Food and Ecological Systems Modelling Journal.*

Abstract

Both intrinsic colony mechanisms and external environmental variables affect the honey bee colony development rates and response, and a key aspect of this is the use of resources within the landscape by honey bees. Although several models have been developed to explore the foraging behaviour of bees, none of these fully considered the spatial and temporal dynamics of landscape resources, and the role of the colony in the process. Here we introduce a new honey bee foraging model being developed as a part of the ApisRAM honey bee colony model. Using agent-based modelling we used a dynamic ALMaSS landscape model enhanced with floral resource modelling to assess the impacts of weather conditions and resource availability on the possible foraging behaviour of honey bees. Several possible mechanisms (defined based on honey bee traits) for scouting and foraging were investigated, separately for nectar and pollen collection, including prioritizing foraging polygons according to their *distance* to the colony, *area*, total *amount* of resources, and the *quality* or the *gain* (only in case of foraging for nectar). Furthermore, the influence of social information was tested for several of these scout-foraging strategies. When model scout bees implemented trait-based strategies, the overall colony resource collection increased. However, if model foraging bees prioritised the polygons based on their distance from the colony, the number of unsuccessful flights increased compared to other tested strategies. This resulted in reduced foraged nectar mass, meaning that model bees were not able to find the most profitable foraging sites. On the other hand, the collected pollen mass was not so strongly impacted and was similar for all tested strategies. Like in other models in which the energetic costs were considered, when forager bees implement the *gain* strategy, the overall nectar production was increased. The amount of social information impacted the colony's resource collection. Information-sensitive strategies showed a greater dependence on the resources provided by the surrounding landscape. Variability in the mass of collected nectar and pollen was found within the same scout-forager strategy and between different combinations. This showed that the foraging mechanisms and the colony position (surrounding landscape) influence the overall resource collection. These outputs were only possible thanks to the implementation of a dynamic landscape model and dynamic spatiotemporal resource model, as well as the implementation of a social communication mechanism for bees to share information about the resources. Information of plant nectar production and quality is essential to predict honey bee foraging distribution. In future model development, the implementation on pollen quality should also be explored to evaluate if it influences the overall pollen collection.

Introduction

Honey bee colonies are under stress due to land use/land cover changes causing loss, fragmentation, and degradation of habitats, and altering the spatial and temporal distribution, diversity, and abundance of flower resources (Tscharntke *et al.*, 2005; Alaux *et al.*, 2017). Some level of infectious or parasitic agents almost always occur in honey bee colonies (*e.g.*, *Varroa destructor* and *Nosema ceranae*). Colonies may also suffer from poor beekeeping management practices (Rortais *et al.*, 2017; Stanimirović *et al.*, 2019), and are exposed to pesticides and other chemicals (Zioga *et al.*, 2020; Xiao *et al.*, 2022). Understanding the mechanisms behind each stressor and how they interact with each other has been a challenge for the scientific community: although the impact of a certain stressor (*e.g.*, the active ingredient of a plant protection product) can be measured on an individual bee, the complex behavioural system within the colony hinders the assessment of how these individual impacts transfer to the colony level (EFSA Scientific Committee, 2021).

Seeley (1995) in his book *Wisdom of the Hive*, identified the need to model this complex honey bee colony system and predict its response to stimuli using computer simulations, more than 25 years ago. Recently, the European Food Security Agency (EFSA) endorsed the development of a mechanistic model of the honey bee colony as a basis for the environmental risk assessment of multiple stressors to bees at the European level (EFSA, 2016; EFSA Scientific Committee, 2021). The ApisRAM model, currently under development (Duan *et al.*, 2022), uses an agent-based modelling (ABM) approach to deal with complex systems with dynamic feedback mechanisms and interactions among many agents (Grimm *et al.*, 2005; Stillman *et al.*, 2015). ABMs are based on a bottom-up approach, in which relevant information regarding the individual level (bottom) is gathered to formulate theories about its behaviour. This information is then used to create a digital twin of the individual (agent). The model outcome will then be the result of the emergence (up) of those agents' behaviour based on their intrinsic characteristics and external environmental conditions (Grimm *et al.*, 2005). In ApisRAM, the agents (bees) interact with and react to both other bees and the resources in the colony, the hive's physical and chemical properties, and the external environment. This model is composed of several sub-models that entail specific mechanics of the colony, such as the foraging behaviour (Duan *et al.*, 2022).

The foraging sub-model simulates the interactions between the foraging individual agents and the environment, based on the coded behavioural rules for the acquisition and transportation of food (*i.e.*, nectar and pollen) into the colony in each specific scenario. Over and above bee behaviour, such an approach also requires detailed modelling of patterns of food resources and stressors (*e.g.*,

pesticide loads) in space and time and the interactions with other environmental variables (*e.g.*, weather).

The modelling of honey bee foraging behaviour is not a novel idea. Several other models have been developed, exploring the metabolic costs of foraging (Schmid-Hempel *et al.*, 1985), the exploitation of the most rewarding resources (Camazine & Sneyd, 1991), the behavioural rules and states while foraging, including the impact of foraging recruitment (de Vries & Biesmeijer, 1998; Sumpter & Pratt, 2003; Dornhaus *et al.*, 2006), and the role of feedback mechanisms (*i.e.*, nectar receivers) that control foraging (Schmickl & Crailsheim, 2004). However, these models do not fully integrate the various impacts of environmental conditions on foraging. The landscape representation in which bees were modelled was rather simplistic and, in most cases, included only a few food sources (Camazine & Sneyd, 1991; de Vries & Biesmeijer, 1998; Schmickl & Crailsheim, 2004). Weather variables were not fully contemplated, either because the simulations spanned over only a few hours (de Vries and Biesmeijer, 1998; Dornhaus *et al.*, 2006; Baveco *et al.*, 2016), or because no interlinks were considered between the modelled landscape and the weather variables (Becher *et al.*, 2014). Notably, none of these previous foraging models integrated the spatial and temporal dynamics of landscape resources. They also did not fully consider the role of the colony in the foraging process - which impacts the forager bees' numbers and behaviour - either because a limited number of foragers was modelled or because the foragers were not modelled at an individual level.

The foraging sub-model developed within the ApisRAM aims to overcome these limitations by performing a much more detailed simulation of the bees and the environment in which they are foraging. The environment in which the colony and the bees are modelled is implemented as a detailed, spatiotemporal landscape representation within the Animal, Landscape, and Man Simulation System (ALMaSS). Detailed simulation of the bees requires, however, knowledge of the mechanisms driving foraging preferences and distribution and those are still poorly understood (Grüter & Farina, 2009). In this paper, we evaluated how different theoretical foraging strategies (mechanisms) affect honey bees' collection of resources. These foraging strategies were defined based on honey bee traits/behaviour that possibly determine their foraging ability and communication. Furthermore, we assessed how the outcome of each strategy is affected by the level of social information available to foragers allowing them to exchange information on known forage resources.

These goals were achieved by performing computer simulations in which the number of forager bees and their strategies were independent of in-hive mechanisms, *e.g.*, the number of brood cells or receiver bees. Bees were only affected by their behaviour (the strategy applied) and environmental characteristics. In the future, the results obtained from these simulations will be used

to create the final foraging sub-model of ApisRAM, in which the nectar and pollen production from the colony will also be influenced by in-hive colony dynamics, emerging from the individual honey bee foraging decisions (bottom-up approach).

Material and methods

Defining the modelling environment

ALMaSS landscape and floral resource model

To properly model the impacts of environmental conditions on foraging activities, a detailed, spatiotemporal landscape representation within the ALMaSS modelling environment (Topping *et al.*, 2003; Topping *et al.*, 2015) was used. This landscape representation combines detailed land use/land cover mapping with information on farming systems, farm practices, weather, and plant growth (Fig. VI.1). The ALMaSS landscape simulation typically operates on a 10 by 10 km window, with a spatial resolution of 1 m^2. Each 1 m^2 belongs to one single landscape element (being a polygon of rather homogenous properties) of a specific land use/land cover type (*e.g.*, forest, building, field in rotation, river, etc.). To account for crop diversity, field boundaries are also mapped, with each field belonging to a given farm unit (managed by the same farmer). Farm units are classified into different types (*e.g.*, cattle, pig, or arable farms) based on the structure of crops grown and animals present in the farm. Each farm type has an associated crop rotation plan, which allows to realistically model patterns of crop types changing in space and time. Crops husbandry is described by country-specific management plans consisting of time windows and probabilities of occurrence of all farming operations including soil cultivation practices, as well as fertilizer and pesticide applications. Associated vegetation growth models for all modelled vegetation types and crops supply vegetation height, green and total, and biomass daily, and are driven by weather conditions (mean daily temperature, mean daily wind speed and the daily precipitation). Such a structure reflects the dynamic character of the agricultural landscape, allowing it to map patterns of vegetation changing in space and time together with associated management actions.

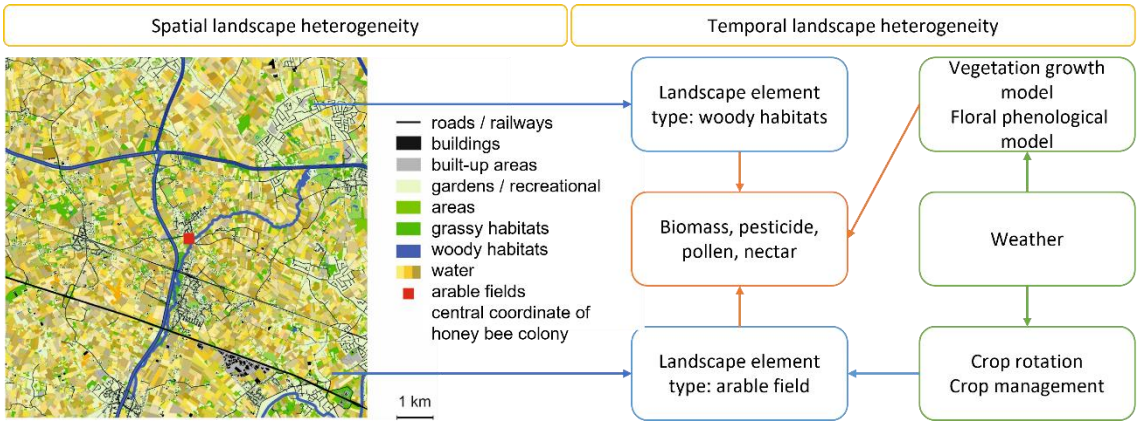


Figure VI.1: Components in ALMaSS landscape model. The blue arrow represents the access to landscape information at a 1m^2 resolution. In this example, one element has woody habitats, while the other one is an arable field. The information about each element depends on its type and the temporal factors described in the green boxes. The orange box shows some of the factors that are derived from the landscape element type, its management, and the weather.

The spatiotemporal pattern of floral resources available for bees was simulated with floral resource models incorporated into the ALMaSS landscape representation (Ziółkowska *et al.*, 2021). These models describe pollen, nectar, and sugar production levels and their changes throughout the year for all vegetated polygons in a landscape (Fig. VI.2). The floral resource models relate flowering time, and therefore, the production of resources, to accumulated growing degree-days (GDD) based on daily average temperature. In models for annual crops, GDD are accumulated from the sowing date, and crop-specific thermal requirements for growing (*i.e.*, base and maximum temperatures) are used. For other vegetated landscape elements, floral resource models are generated by superimposing models for individual plant species composing that element, *i.e.*, they summarize the production of all plant species from a given habitat per GDD. Here, GDD are accumulated starting from the beginning of the year, and the same thermal requirements for growing are applied to all plant species composing a given landscape element type. Floral resource models provide information on nectar and pollen quantity (mg/m^2) and quality for each day of the simulation, at the polygon level. The quality of nectar is defined as the quantity of sugar (mg/m^2), while the quality of pollen was not considered in this study. This, together with real hourly weather information (*e.g.*, wind speed, rainfall, temperature, and solar radiation), is provided to the modelled foraging bees.

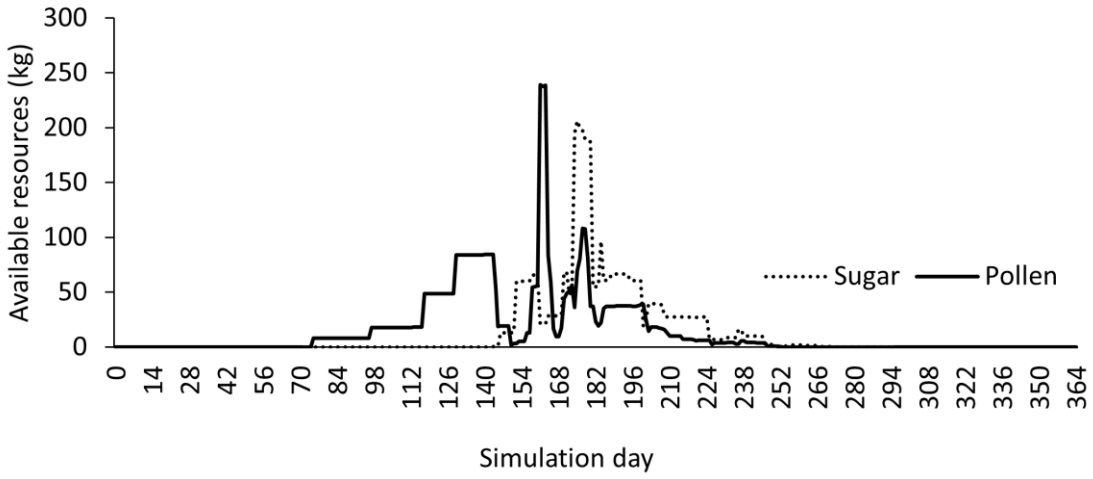


Figure VI.2: Total mass of floral resources (i.e., sugar and pollen) in the studied landscape available to bees in all the simulations. The mass of floral resources was calculated based on the production and phenology of the individual species composing habitats present in the studied landscape, and the landscape composition (see supplementary material section VI.1). Pollen availability commenced on simulation day 74 and nectar was available from day 88.

Landscape development

The foraging strategies (see below) were tested using a dynamic ALMaSS landscape representation of a 10 by 10 km area located near Merendree, Belgium. The study area was generated as a part of the H2020 B-GOOD project (no. 817622). Details of the landscape generation process can be found in an open GitLab repository (https://gitlab.com/ALMaSS/b-good-wp5). For the land cover/land use component of the simulated landscape, vector map layers from the Open Street Map project (downloaded from www.openstreetmap.be) were combined with information on forest types from the Biological Valuation Map (Biologische waarderingskaart, Instituut voor Natuur-en Bosonderzoek) and agricultural parcel boundaries from the Land Parcel Identification System (LPIS; freely available via online tools at https://www.geopunt.be/ for Flanders, and https://geoportail.wallonie.be/ for Wallonia). The landscape map units were further reclassified to the general landscape element types used in ALMaSS (Fig. VI.1, Supplementary material section VI.1). Farming structure in the study area (*i.e.*, spatial distribution and type of farms) was defined using the information on area and type of crops cultivated together with information of affiliation of parcels to agricultural holdings (provided by the Flemish and Walloon Department of Agriculture). Crop management plans for the most important crops were generated based on the opinion of three agricultural advisory agencies (Proefcentrum Groententeelt, Koninklijk Belgisch Instituut tot Verbetering van de Biet and Inagro). For

simplicity, in this study we excluded information on crop rotations from the simulations and assumed that all the fields in the landscape are managed as grasslands with clover grass cut for silage. This ensures the same distribution of floral resources for bees within the arable land in all the simulations. The landscape generation process was coded using Python and R scripts (available in the GitLab repository https://gitlab.com/ALMaSS/b-good-wp5).

For each general landscape element type identified in the study area, we defined the type of floral resource habitat (Supplementary material section VI.1) associated. The detailed description of plant composition, density of flowers, and floral resources (nectar, sugar, and pollen) produced by each plant composing each of the habitats is available in the GitLab repository (https://gitlab.com/ALMaSS/b-good-wp5). In addition, all documentation and input files related to the generation of floral resource models can be found in the GitLab repository (https://gitlab.com/ALMaSS/floral_resource_models).

Defining the foraging model rules

Environmental conditions for foraging and scouting

For the foraging model, it was defined that low temperatures (< 10 °*C*), darkness, rain, and strong winds (> 25 m/s) prevent foraging or scouting behaviour (Wenner, 1963; Hennessy *et al.*, 2020). Under favourable environmental conditions, possible foraging or scouting activities were defined by the environmental solar radiation and temperature (Vicens & Bosch, 2000; Clarke & Robert, 2018). If the average solar radiation in an hour is higher than the threshold r_s, defined by the equation from Vicens and Bosch (2000),

$$r_s = 2261.9e^{-0.164t},$$

in which *t* is the hourly environmental average temperature (°*C*), the model bees would fly out for foraging or scouting for that hour. In our model, hourly weather data was used to calculate the available foraging hours per each day (Fig. VI.3).

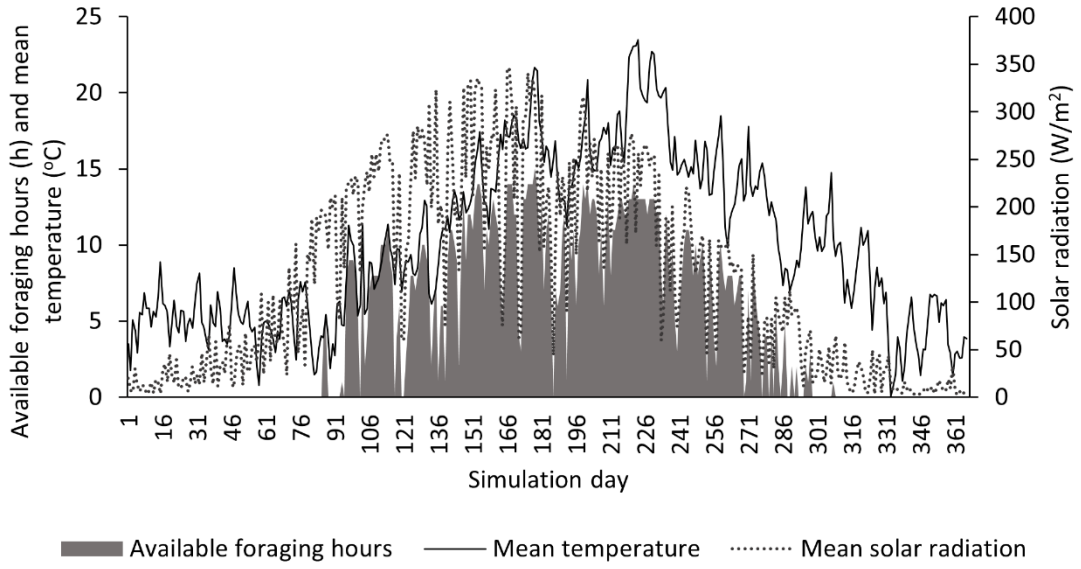


Figure VI.3: Available foraging hours and weather variables (temperature and solar radiation) for each simulation day throughout the year. Rain and wind variables are not shown but were used to calculate the number of available foraging hours.

Individual model bee behaviour

In the foraging simulation, specific rules were defined for the individual model bee behaviour. Each model bee has a maximum flying distance of 50 km per day for foraging/scouting activities. When this threshold is reached, foraging or scouting activity stops. Additionally, in each foraging flight, a scouter/forager can carry 50 mg of nectar or 1.5 mg of pollen.

When the environmental conditions allow, the model bees start to explore the landscape around the hive (scouting activity). They choose a random direction (from 0 to 359 degrees, potentially exploring the whole segment) and scout in that direction until they find a suitable polygon to forage (according to the implemented strategy; see below) or until they reach the edge of the landscape window. When successful at finding a suitable resource polygon, they will collect resources (nectar or pollen) and memorize information about their relative position (to the colony), area, resource quantity and quality, as well as the total amount of the resource in the whole polygon. Afterwards, they fly back to the colony to store the collected food. Then, the model bees will use social information to assess other possible polygons and perform foraging activities.

Social information

To mimic the communication of social information within a honey bee colony, instead of "spatially mapping" the waggle dances and other cues, we store information on polygons visited by scout/foraging model bees in the form of lists (as each polygon in a landscape has its unique identifier). Two lists are generated, separately for nectar and pollen resources (as some plants may be a valuable source of pollen but not nectar or vice versa) and they are constantly updated after each foraging flight. To control the amount of social information in the colony, the number of resource polygons stored in each of the lists is limited by a threshold. Bellow this threshold, scout model bees are actively looking for new places to forage; when the threshold is reached, the scouting activity is stopped, and foraging activity is resumed. The information on resources stored in the lists is available to all modelled bees, which mimics the flow of social information in the colony.

Each time a scout/forager model bee returns from a polygon, its resource properties are updated, both in terms of nectar and pollen (Fig. VI.4). If there is no resource left, this polygon is removed from the list. The structure of both lists is similar, *i.e.*, each element represents the properties of visited foraging polygon, including:

1. A unique identifier;
2. The angle between the hive and the polygon centroid (in degrees);
3. The distance between the hive and the polygon centroid (in *m*);
4. The area of the polygon (m^2);
5. The quantity of the resource (nectar mg/m^2, pollen mg/m^2);
6. The quality of the resource (sugar mg/m^2 for nectar, and not available for pollen);
7. The total amount of the resource in the polygon (the product of quantity and area in *mg*);
8. gain for foraging nectar (*g)* calculated as follows:

$$g = \frac{as}{qd},$$

where *s* is nectar quality, *q* is nectar quantity, *a* is the area and *d* is distance.

When foraging activity is resumed, a model bee will verify the list and then randomly choose a polygon. If this one is better than the one previously visited by the model bee (private information), they have a 50% probability of switching to the new polygon. In all simulations, this 50% chance was fixed as we still do not have enough information on how honey bees balance their private information relative to the social information (for data on private/social information see Grüter *et al.* (2008) adn Grüter & Ratnieks (2011).

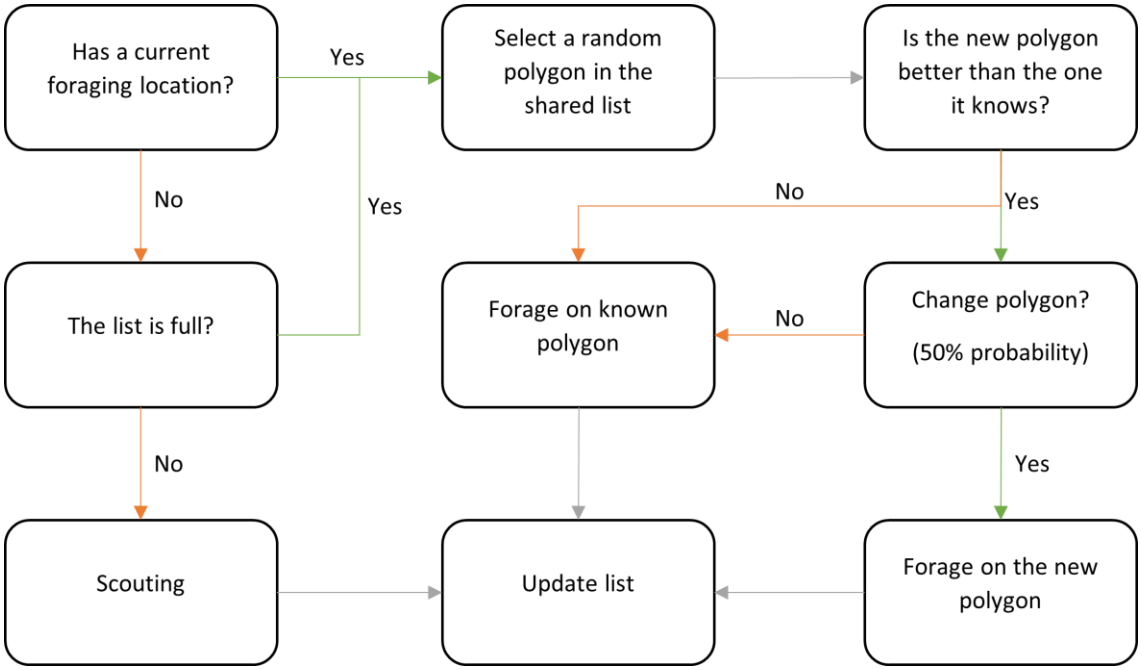


Figure VI.4: Honey bee foragers' decision-making tree. A forager bee can decide to scout if it doesn't already have a location to forage from and there are not enough foraging polygons on the list. Otherwise, the bee will forage on a known polygon or select a random polygon from the list. If the selected polygon is better than the one it already knows, there is a 50% probability of foraging from the new polygon. After a successful foraging flight, the ID number of the visited polygon will be added to the list if the list is not full, or the visited polygon is better than at least one polygon in the list. After each visit, the list is updated to account for the usage and current availability of the resources.

Selected scouting/foraging strategies

Scouting strategies

We tested the following scouting strategies:

1. Area strategy: Prioritize the polygon with the largest area (m^2)

Scout bees rely on their sense of smell to find where to forage (Farina *et al.*, 2012). Therefore, in this strategy, model bees select bigger areas as they potentially offer a stronger scent (and eventually a stronger visual stimulus once in its proximity; Srinivasan, 2010).

2. Distance strategy: Prioritize the closest polygon

Despite their ability to detect colours and patterns (Avarguès-Weber *et al.*, 2012), honey bees have poor stereo vision, hindering their perception of objects that are far away (Srinivasan, 2021). Therefore, in this strategy model bees select the closest polygons to forage.

3. Random strategy: Randomly chose a polygon from the whole landscape

As we do not yet fully understand the mechanisms driving foraging choices by scout bees (Grüter & Farina, 2009), a random strategy was also tested. In this strategy, model bees randomly select a polygon in the selected direction in the landscape.

Foraging strategies

For the foraging model bees, five strategies were defined:

1. Area strategy: Prioritize the polygon with the largest area (m^2).

As the scouting model bees used their sense of smell to find the most attractive polygons, recruited foragers also use this trait to evaluate the landscape and chose the biggest polygons.

2. Distance strategy: Prioritize the closest polygon with a resource.

As the scouting model bees selected polygons closer to the colony, recruited foragers also use this trait to select the closest polygons.

3. Amount strategy: Prioritize the polygon with the highest amount of nectar/pollen (*mg*).

As the scouting model bees can measure the total amount of food and transmit this information to the shared lists, forager model bees prioritize the polygons with more resources.

4. Quality strategy: Prioritize the polygon with better quality (sugar mg/m^2).

Scouting bees can measure the quality of the resources and share this information with the colony by sharing vibrations (waggle dance; Von Frisch, 1967; Couvillon, 2012) and gustatory cues (Thom *et al.*, 2007; Reinhard & Srinivasan, 2009). In the model, all scouting model bees have access to the lists

storing information on visited foraging polygons (see Section 'Social information'), and in this strategy foraging model bees prioritize polygons with higher *quality*.

5. Gain strategy: Prioritize the polygon with better gain

Other than the direction and distance of useful resources, honey bees can transmit information on the profitability (balance between energy gained from the resource and the energy spent to collect it) of resources by performing more intra-dance circuits during the waggle dance (Seeley *et al.*, 2000). In the model, all scouting model bees have access to the lists storing information on visited foraging polygons (see Section 'Social information'), and in this strategy foraging model bees prioritize polygons that provide a higher gain.

For nectar, all five foraging strategies were applied and compared. In the case of foraging for pollen, only the first three strategies were considered since pollen quality was not implemented.

Simulations to explore the influence of scouting and foraging strategies

Simulation setup and runs

In all performed simulations the total daily number of modelled bees was fixed and varied with time to represent a typical honey bee colony (*i.e.*, from ap. 1000 to 9600 forager bees). This daily number was obtained from field-collected data on colony strength from the EFSA OC/EFSA/SCER/2017/02 project (Dupont *et al.* 2021): 34% of all adult bees were assumed to be foragers, from which 30% were pollen foragers. For wintering months, as no data was available, a fixed (1000) number of foragers was assumed.

A total of 28750 simulations were performed to explore two main aspects of the model:

1. Test scout-forager strategies in a dynamic landscape with a different spatiotemporal resource distribution around the colony.

To explore different flower pattern scenarios around the colony, the colony was placed in 25 different landscape locations (regular grid of 25 cells of 2 x 2 km with the colony placed in the centre of each cell). For each location, each strategy combination (one scouting strategy and one foraging strategy) was tested by performing 10 multiple runs (one-year simulation for each run).

2. Test the impact of the amount of available social information (*i.e.*, the size of shared lists) on colony resources collection for each combination of scouting and foraging strategies.

Scouting (only area and distance) and all five foraging strategies were tested in all 25 different colony locations. The scouting random strategy was not tested due to the lack of ecological relevance. For each location, 10 list sizes (from 10 to 100 with a step size of 10) were tested by performing 10 multiple runs (one-year simulation for each run) for both nectar and pollen.

Simulation outputs

For each simulation, daily data on nectar, sugar, and pollen landscape offer and income were obtained, as well as the number of foraging flights and those that were successful (in which model bees collected nectar or pollen). Such daily data were used to calculate yearly amount of colony collected resources, the mean number of foraging flights, and the percentage of successful flights. For each of those outputs, data were pooled together to calculate variation of the model outputs, represented using boxplots for the respective strategy.

Results

Scout-forager strategies

The amount of sugar collected by model bees throughout the year depended on both scouting and foraging strategies (Fig. VI.5a). Overall, regardless of the implemented strategy by foragers, when scout model bees employed trait-based (*i.e.*, *distance* and *area*) vs *random* strategies, the colony collected more sugar throughout the season, while the scouting *distance* and *area* strategies gave similar results. Furthermore, not all scout-forager strategies showed the same variability. Regardless of scouting behaviour, when foragers employed the *distance* strategy, they were not as successful (Fig. VI.5c) as in other strategies, despite performing approximately twice the daily flights (Fig. VI.5b). On the other hand, the foraging success rate in the *random* scout strategy was not much lower than in the *area* strategy (and it was higher than in the *distance* strategy).

On the other hand, the overall collection of pollen was higher when scouts applied the *distance* strategy compared to the *area* and *random* strategies (regardless of the forager strategies; Fig. VI.6a). The daily mean of foraging trips was similar for *random* and *area* scouting strategies, regardless the foraging strategy, while the mass of collected pollen was higher in the foraging *distance* strategy. Once again, some scout-forager strategy combinations presented more

variability (*i.e.*, *distance-amount* and *distance-area*) than others. Also, when foragers applied the *distance* strategy (regardless of scout strategies), more flights were performed (Fig. VI.6b), with a lower success (Fig. VI.6c). Despite the lower success, the *distance-distance* strategy combination gave the highest pollen yield.

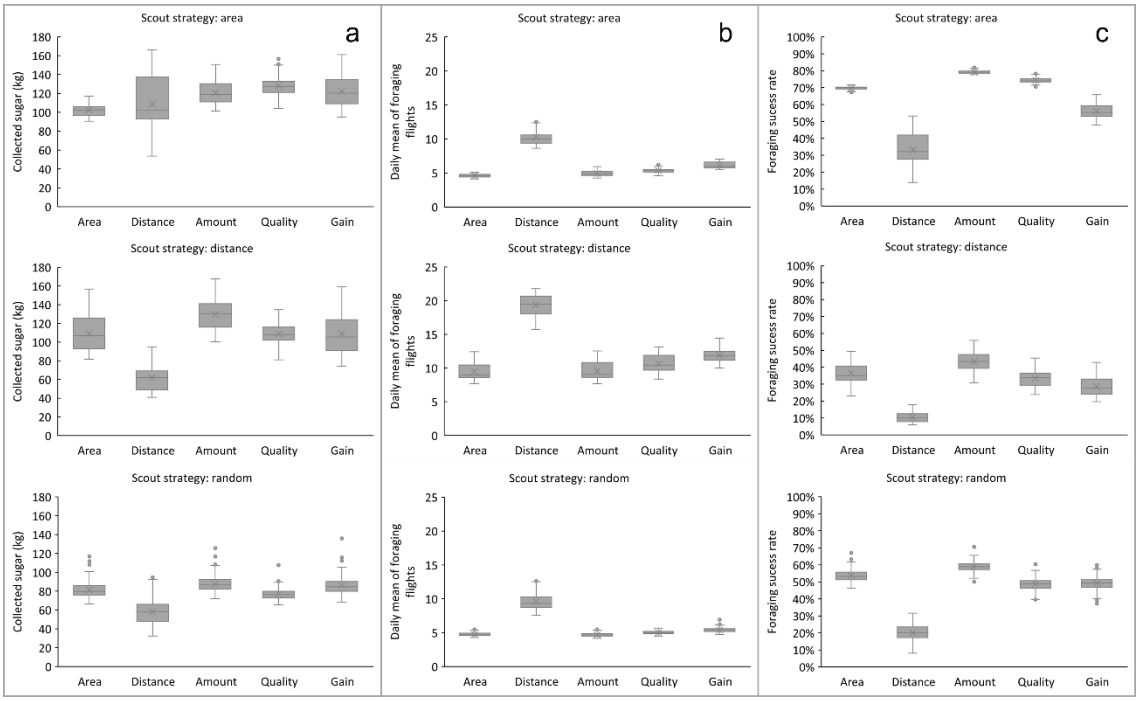


Figure VI.5: Output for each scouting (area, distance and random) and foraging strategy (x-axis: area, distance, amount, quality, gain) for nectar collection: (a) yearly amount of sugar production; (b) daily mean of performed forager flights; and (c) success rate of finding a polygon to forage. Each boxplot corresponds to data from the 10 simulations within the 25 locations (250 data points).

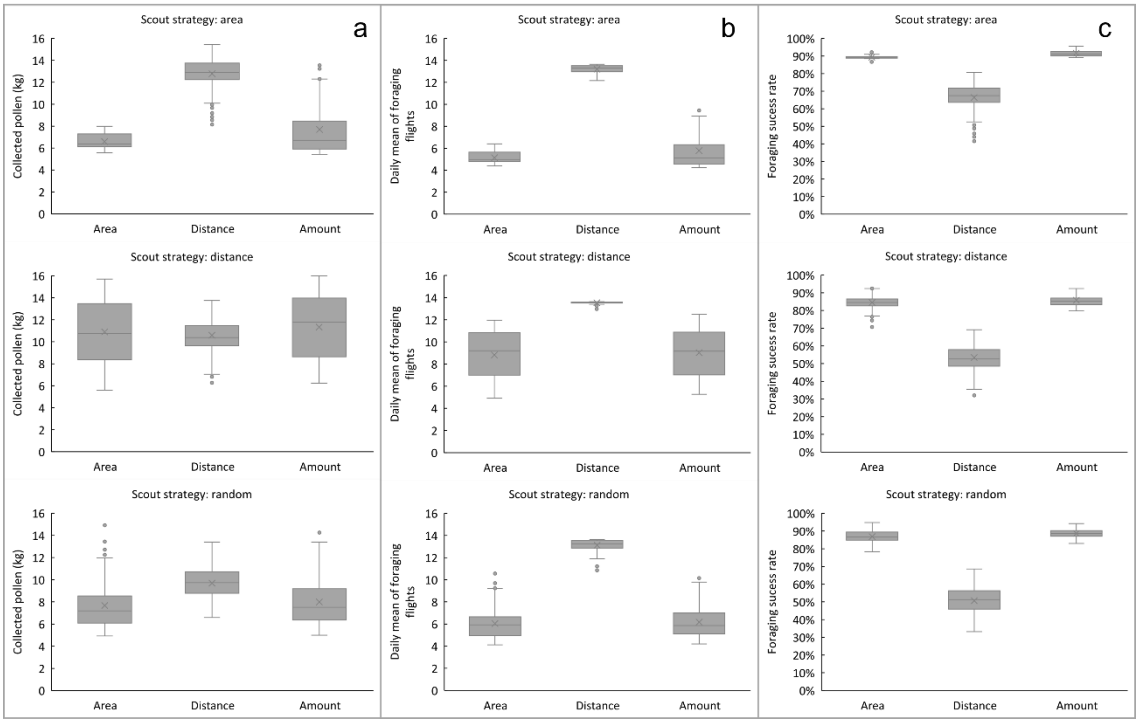


Figure VI.6: Output for each scouting (area, distance and random) and foraging strategy (x-axis: area, distance, amount) for pollen collection: (a) yearly amount of pollen production; (b) daily mean of performed forager flights; and (c) success rate of finding a polygon to forage. Each boxplot corresponds to data from the 10 simulations within the 25 locations (250 data points). The last two foraging strategies (quality and gain) were not implemented for pollen foraging due to the lack of a reliable pollen quality score.

Social information – the impact of the shared list size

Overall, there was a positive trend between the increase in social information in the colony (*i.e.*, the number of polygons stored in the lists) and the yearly sugar and pollen collection (Figs VI.7, VI.8). This trend was more accentuated regarding nectar-gathering strategies: in some (*e.g.*, *area-distance*, *distance-distance*, *distance-quality*) there was a steady linear upwards trend, while in other strategies (*e.g.*, *distance-amount, area-quality*) this positive trend reached a plateau before reaching the maximum tested list size. The variability within each scout-forager strategy showed little variation with the increase in social information (Fig. VI.7).

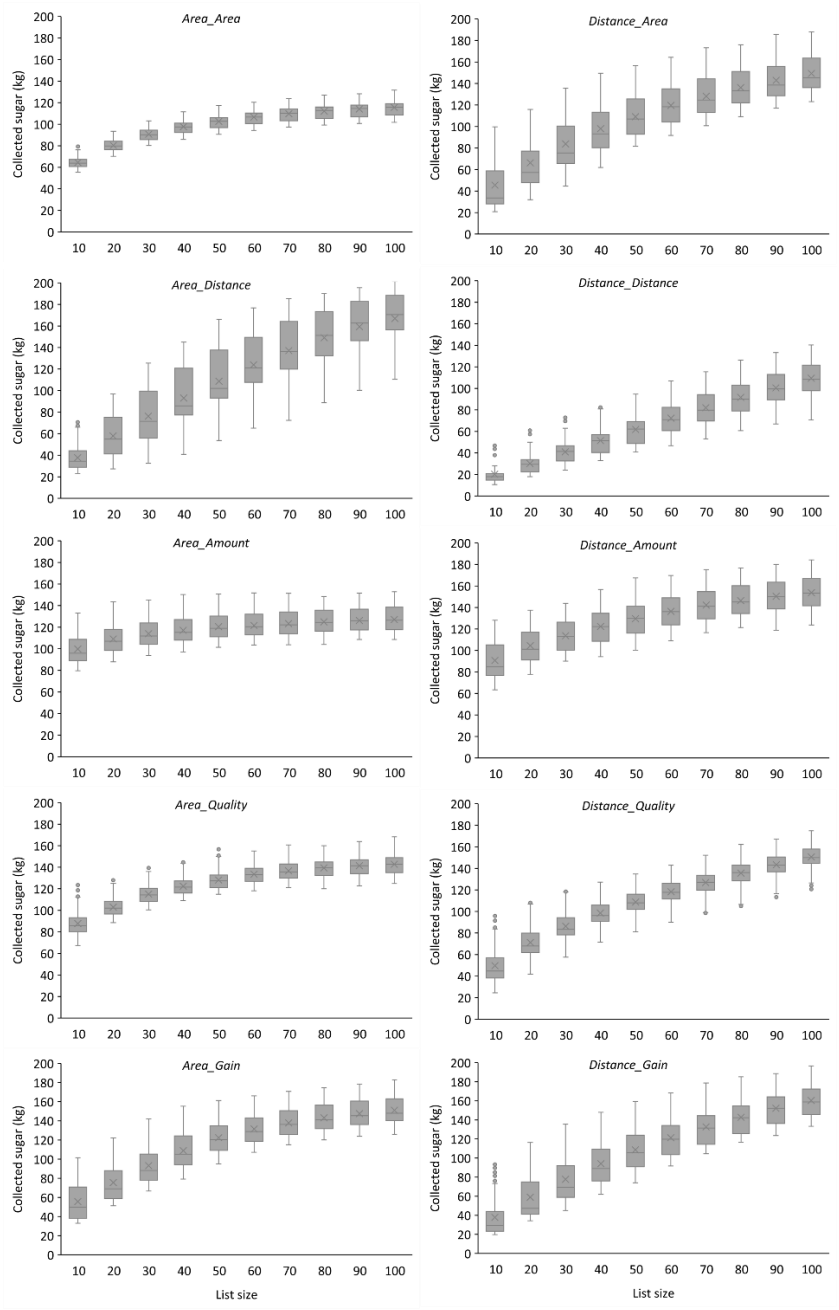


Figure VI.7: Relationship between the amount of social information in the colony (i.e., number of polygons in the shared lists) and the yearly sugar production for each scouting (area, distance) and foraging (area, distance, amount, quality, gain) strategy combination. The random scouting strategy was not simulated due to the lack of ecological relevance.

The amount of collected pollen throughout the year (Fig. VI.8) followed a strong positive trend with the increase in social information in some strategy combinations (*i.e.*, *area-distance*, *distance-distance*). In these, the increase in social information led to a reduction in variability. In other combinations of scouting-foraging strategies, the amount of pollen collected only weakly increases with the increase in social information or was even stable.

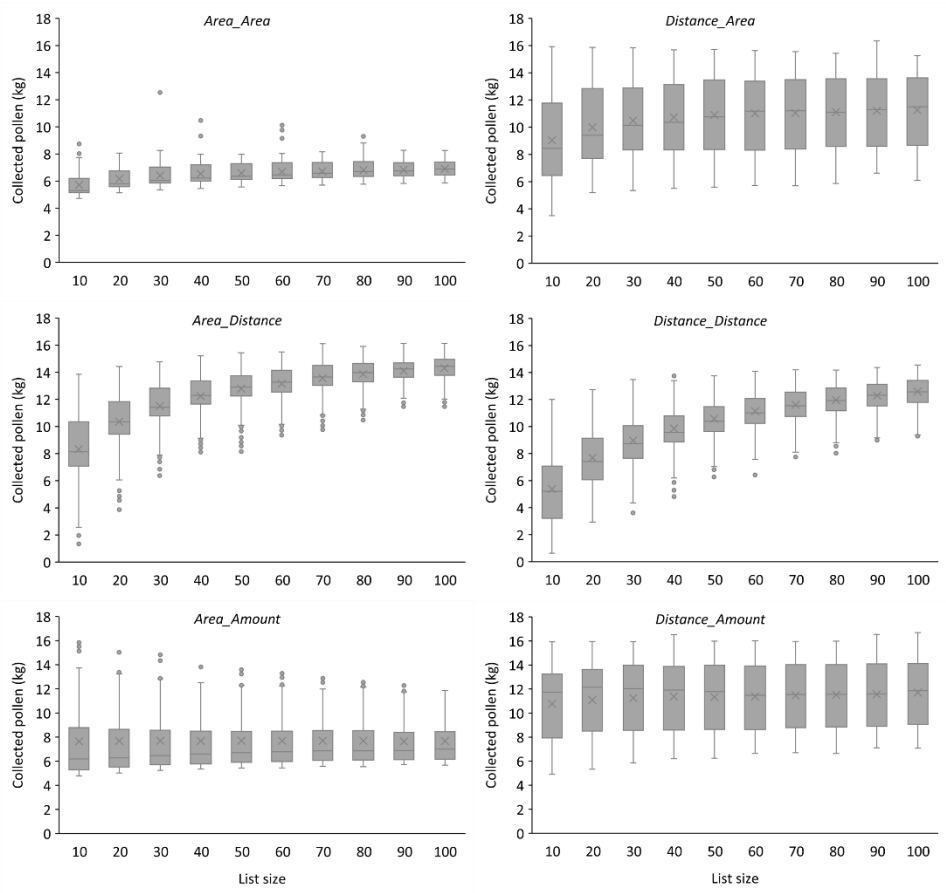


Figure VI.8: Relationship between the amount of social information in the colony (i.e., number of polygons in the shared lists) and the yearly pollen production for each scouting (area, distance) and foraging (area, distance, amount) strategy combination. The random scouting strategy was not simulated due to the lack of ecological relevance; the quality and gain forager strategies were not implemented due to the lack of a reliable pollen quality score.

Discussion

In this study, for the first time, a dynamic ALMaSS landscape model enhanced with floral resource modelling was used to assess the impacts of weather conditions and resources availability on the possible foraging behaviour of honey bees. Model bees were able to gather information on available floral resources because each mapped landscape element had associated information on habitat type and composition. The quantity and quality of floral resources available to model bees depended on habitat composition but was also influenced by weather conditions which defined the available foraging hours based on hourly weather data. This combination of factors was used to drive the outcome of the foraging strategies in terms of nectar and pollen collected.

Environmental conditions were rarely considered when evaluating the strength, production and health status of the honey bee colony (Hatjina *et al.*, 2015; Odoux *et al.*, 2014; Dupont *et al.*,

2021). Landscape structure was usually defined using very broad land use categories (*e.g.*, urban, grassland, forest; Lecocq *et al.* 2015; Sponsler & Johnson, 2015), without a quantitative assessment of its real offer (*i.e.*, resources) for bees. However, bees can actively adapt and respond to changes in the environmental conditions around the hive. Changes in environmental conditions may lead to shifts in their foraging patterns, impact bees' diet (diversity of collected resources), accumulation of resources, colony size, and energy spent for thermoregulation (Simone-Finstrom *et al.*, 2014; Sponsler & Johnson, 2015, Danner *et al.*, 2016). Therefore, including environmental conditions is essential if we aim to reflect feedback loops in the complex bee colony system and predict exposure to (and impacts of) single or multiple stressors on its strength, production, and health status (as is the goal of the ApisRAM model).

Foraging rules and individual bee behaviour

In our model, scouting model bees were only able to scout on a single direction (with 1 degree resolution). Thus, the model bees cannot change their direction during a single scouting flight. We believe that this simplification can be compensated by the swift implementation of the forager strategies once the scouting activity is finished (*i.e.*, when information lists with visited foraging polygons are full). This way, even if the model scouts cannot find the most suitable foraging polygons, the exchange of social information between foraging model bees allowed the colony to reach and focus on good foraging sites. A more realistic approach regarding flight rules was implemented in the BEESCOUT model (Becher *et al.*, 2016), although this model had the limitation of only using field size and relative position (no information on nectar production or quality) to drive forager strategies and communication of social information.

Implementation of strategies for nectar and pollen collection

In general, the implementation of trait-based scouting strategies (*i.e.*, when bees prioritized foraging polygons according to their area or distance from the colony) led to a higher sugar collection than the *random* strategy. Despite the lower role of the scout model bees (as scouting occurred only when information lists with visited foraging polygons are not full), their choices influenced the behaviour of the whole colony. Therefore, foraging models must include a reliable implementation of the scouting strategies. In our model, there was a higher overall sugar collection when the forager model bees prioritized the foraging polygons according to the amount, the *gain*, or the quality of the resource. Despite the high sugar collection achieved when foragers prioritized polygons according to the amount

of resources, there are few indications that honey bees can indeed assess the total amount of resources in a field (flowering area). They can detect the colour, shape, and fragrance of flowers (Srinivasan, 2010) and their vision has *regional* specializations to account for the specificities of the foraging tasks (*e.g.*, the dorsal rim of the eye is specialized in the perception of polarized light to aid their navigation, while the fronto-ventral region is specialized in colour vision to aid in flower recognition; Srinivasan, 2010); however, they have a poor stereo vision that hampers them from seeing at long distances.

Bees can also evaluate nectar quality (Seeley *et al.*, 1991) and transmit this information by sharing food with other bees (Farina & Wainselboim, 2005) and by performing more intra-dance circuits during the waggle dance (Seeley *et al.*, 2000). They can also transmit information on the profitability of a resource (Seeley *et al.*, 2000). Such evaluation is made by assessing the energetic gain of their visit (the ratio between the travelled distance and the received reward; Camazine & Sneyd, 1991; Seeley & Tovey, 1994). Other in silico foraging models that explore the colony-landscape interaction included this profitability principle (Schmickl & Crailsheim, 2004; Becher *et al.*, 2014; Baveco *et al.*, 2016); likewise, our study included the evaluation of nectar quality (sugar/m^2), amount and distance in the '*gain'* strategy. In this strategy, the model bees can have a higher *gain* if they forage on nearby resource polygons, even if those have lower nectar availability (nectar/m^2), due to distance costs. The highest *gain* would, however, be obtained from nearby polygons with higher sugar concentration. In this case, forager model bees would spend less time in the polygon and travelling, thus allowing them to make several high-return foraging flights on the same day. If in real life, forager bees do indeed use this strategy, it is a good way to avoid leaving the fate of the colony in the hands of the scout bees. Therefore, the use of the *gain' s*trategy to drive social information choices could be the most efficient and safe strategy for the entire colony regardless of the strategies used by the scout bees.

Intriguingly, when forager model bees were prioritizing the foraging polygons according to their distance from the colony, the overall simulation outcomes showed a lower sugar collection and an increase in the number of total and unsuccessful flights. We hypothesize that these model bees, by prioritizing polygons near the colony, did not manage to find the most profitable ones, since neither quality nor amount of food available was considered in this strategy. The foragers were able to explore habitats away from the colony only after the depletion of the closest resources. Furthermore, the increase in the number of flights performed was not profitable, as these model bees possibly foraged in closer but smaller polygons compared to other strategies. When the same strategy (*i.e.*, prioritizing the closest polygons) was applied when model bees were foraging for pollen, it also resulted in more

but less successful flights. However, pollen collection was on a similar or higher level compared to the other foraging strategies. The number of model bees collecting pollen is lower than those collecting nectar, and they can only carry 1.5 mg of pollen each flight. Therefore, the depletion of resources was slower in polygons with a large area or pollen amount, and consequently, the rate of adding new polygons to the information list with visited resource polygons was reduced. Consequently, the foraging model bees spent less time travelling, allowing a constant income of pollen. These observations also justify why model bees prioritizing foraging sites according to their area or the amount of available resources performed more successful flights. Nevertheless, this did not directly translate into higher pollen collection because model bees had to spend more time travelling farther away.

There is an important relationship between pollen availability and healthy colony growth (Keller *et al.*, 2015a, Keller *et al.*, 2015b, Mattila & Otis, 2006, Requier *et al.*, 2017) as bees need essential amino acids mainly for healthy larvae and hypopharyngeal gland development (Brodschneider & Crailsheim, 2010). In our model, the pollen quality was not implemented, and in consequence, we could not account for the need to collect pollen from diverse resources that might arise from the colony's internal needs. Despite these limitations, we believe that the tested strategies allowed the model bees to explore most of the available pollen diversity. They were able to fly in several random directions and forage in at least 50 resource polygons, changing regularly to new ones after the depletion of resources or the end of flowering. Furthermore, even if pollen quality is implemented, the available information on honey bee foraging mechanisms driving pollen selection is even more limited than concerning nectar (see Nicholls & Hempel de Ibarra, 2016). In particular, honey bees have been shown to adapt their foraging ranges to compensate for the lack of pollen diversity near the colony (Danner *et al.*, 2017), meaning that a minimum pollen diversity threshold should be explored in future model development.

Simulation of social foraging information - implementation

Honey bees, when presented with a low- or high-quality nectar source, will shift their foraging patterns at the colony level, increasing the foraging effort to visit the most rewarding sources (Seeley *et al.*, 1991; Seeley, 1995). This behaviour will eventually lead the colony to choose the most rewarding patches when food is abundant and lower its foraging thresholds when resources are scarce (Seeley *et al.*, 1991). This theory can therefore explain the emergence, at the colony level, in the use of the most rewarding patches, leading to a heterogeneous and energy-efficient-driven honey bee

distribution in the landscape. In our model, the foraging strategies defined the rewarding polygons and the bees progressively foraged more often from these.

The method we used to store and share information at the colony level (*i.e.*, via shared lists storing information on visited foraging resources) mimics the social information flow in a colony and provides a basis for model bees to adapt to landscape resource dynamics. Non-productive polygons are removed from the lists and the scouting is triggered when the lists are too small. This was effective at reducing the amount of collective memory shared by the colony, whilst allowing the colony to focus on the most rewarding polygons. Lists also allowed the colony to have dynamic thresholds, which changed according to the landscape resources availability and distribution, based on the social information shared by the model bees.

Social foraging information interaction with foraging strategy

The relationship between social information and sugar collection varied according to the strategy combinations. When scout model bees prioritized the foraging polygons based on distance from the colony, there was a positive and almost linear relationship between the length of the list and outcome, regardless of foraging strategies. This may be explained by scout model bees prioritizing the closest polygons at the beginning of the simulation, making this strategy highly dependent on the surrounding polygons for sugar collection. Consequently, if only a few options were given to the colony through the list, the model bees kept exploring them even if these polygons were poor on resources. On the other hand, with an increase in social information, model bees had more foraging choices, driving them to explore the most profitable polygons according to each strategy. For the distance scouting strategy, when the foragers also implemented the *distance* strategy, the foraged resource increase lagged behind the other strategies due to the lack of a feature considering the amount or quality of the resources.

Interestingly, when the scouting strategy prioritising the largest foraging polygons (*i.e. area*) was combined with foraging strategies to prioritize polygons according to the *area*, *amount* of resources, their *quality* or *gain*, a maximum possible colony sugar collection was reached at a low threshold of social information. Under these strategies, the larger polygons offering resources were also the ones from which the model bees could collect the most sugar. Since these polygons had a higher probability of being found by scout model bees due to their size, most of these big areas were added to the list quickly. Providing more options on the social 'shared' list allowed the model bees to add more polygons, but these were smaller and thus there would be a law of

diminishing returns as each new polygon was added. This was most pronounced when both scouting and foraging bees were prioritizing foraging polygons according to the area but was modified when including a quality or gain factor (since smaller high-gain polygons could be preferred). Thus, for these strategies, despite the exploration of bigger areas, model bees were also driven by the amount and quality of the resources. This creates a more reliable prediction of bees' distribution since they use these polygon traits (*i.e.*, flowers) to choose where to forage (Seeley *et al.*, 1991; Seeley *et al.*, 2000).

Pollen strategies differed somewhat from sugar strategies. In some combination strategies (i.e., when the scouting area strategy was combined with the distance and amount foraging strategy, or when both scouting and foraging model bees were prioritizing the closest resources) there was an overall reduction of variability with the increase in social information. This indicates that these strategies are more dependent on the surrounding polygons (context-dependent) when social information is scarce. When the level of social information increases, more options are given to the model bees, decreasing the landscape context dependency. Interestingly, like in the nectar collection strategies, there was a positive relationship between the social information and the collected throughout the year when the distance strategy was applied by the foraging model bees. However, prioritizing the foraging polygons according to the distance from the colony by both scouting and foraging model bees seems to guarantee a higher collection of pollen than nectar, which can be explained, once again, by the number of pollen foragers, the lack of a qualitative assessment of pollen, and the total amount of pollen in the polygons. These results agree with previous findings on honey bee colonies, in which bees adapt their foraging distances to pollen availability in their surroundings (Danner *et al.*, 2016), suggesting that they prefer to forage near the colony if enough pollen is available. Analysing waggle dances, Couvillon *et al.* (2014) showed that bees prefer to forage closer to the colony at the beginning of the season and increase their foraging ranges later when resources become scarcer, evidencing that the colony is adapting to the dynamics of resource availability in the surroundings.

Future testing and model development

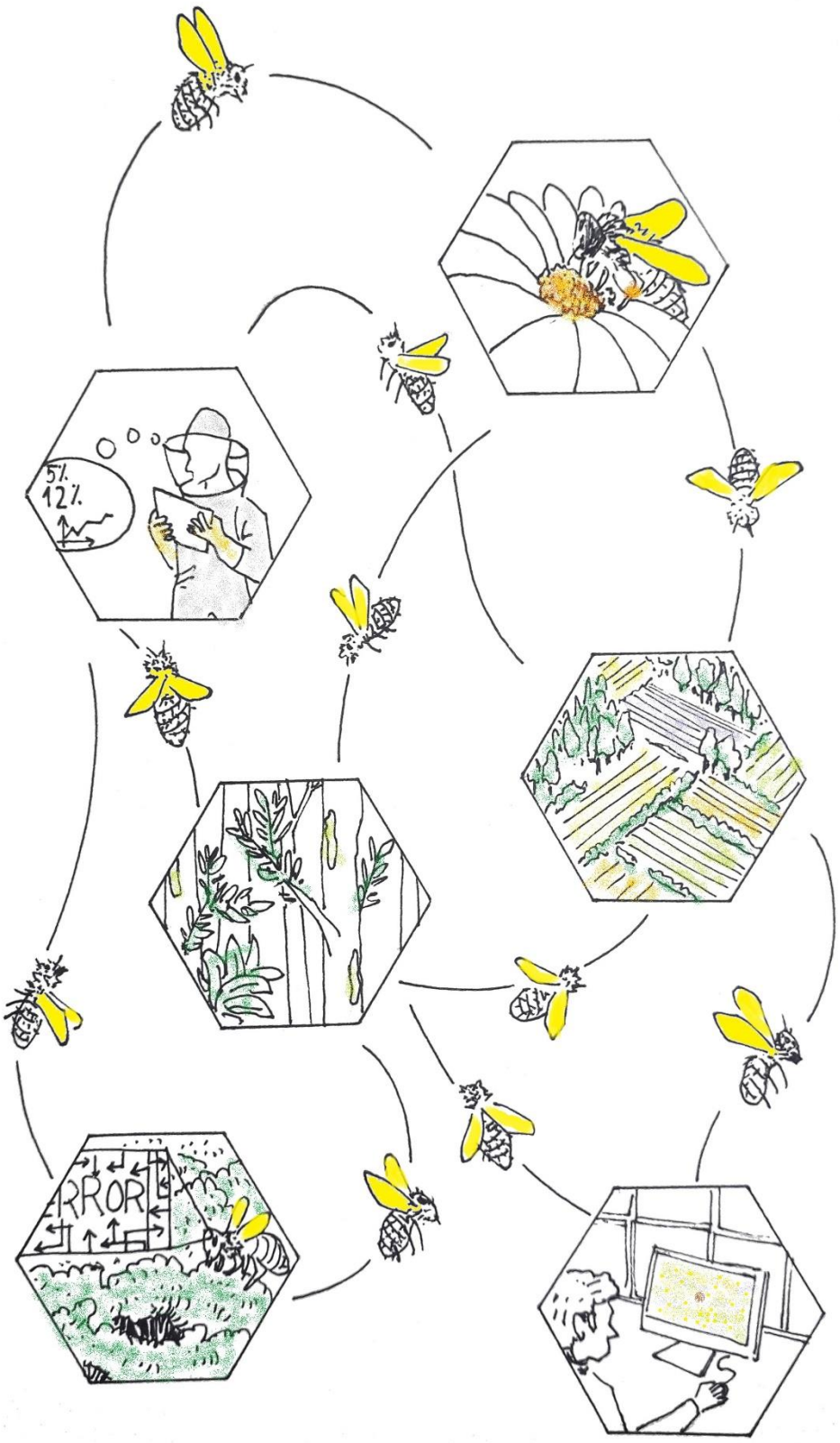
In the future development of the honey bee foraging model, we believe that the foraging for nectar should not be driven by the distance of the polygon from the colony, as it does not consider information on a polygon's profitability. Likewise, the simple strategies to prioritize the foraging polygons based on their area or the amount of available resources were explored as a consequence of an anthropomorphic vision of the system, rather than being based on the available knowledge of

honey bees' ability to perform such assessment (*i.e.*, it is not likely honey bees can measure area, nor total polygon resource).

Duan *et al.* (2022) completed the first step in the ApisRAM model development, in which the possible approaches to implementing the several components of the honey bee colony are described (*i.e.*, the formal model). In this study, we explored one of these components (foraging behaviour strategies) separately from the others. In the future development of the ApisRAM model, forager model bees will influence and be influenced by not only other foragers (as tested in this study by using lists to store information on visited resource polygons) but by other modelled bees performing other tasks in the colony, *i.e.*, processing the food (*e.g.*, Schmickl & Crailsheim, 2004), or brood rearing (Seeley, 1995). In the foraging sub-model, the next step is to use field-collected data from the EFSA OC/EFSA/SCER/2017/02 project (such as the daily number of foragers, colony population, weather, resources offer, and honey gain) to verify the tested strategies and social-list length. We aim to develop a reliable foraging model that can accurately predict the distribution of bees and their use of resources within a landscape, allowing us to properly predict possible exposure routes to pesticides and/or other stressors.

Chapter VII

General discussion



David Sarmento

Honey bees (*Apis mellifera*) are a key model species for studying social behaviour in superorganisms and are essential to global ecology through pollination (Weinstock *et al.*, 2006; Hung *et al.*, 2018). The colony's organisational system is still not completely understood. It has a structured division of labour between workers (Münch & Amdam, 2010), and it is remarkable that such dramatic distinctions in morphology, physiology, and behaviour arise within the same gene pool. Each bee performs its tasks according to its genetic predisposition and surrounding environment. This plasticity allows the colony to adapt quickly to environmental changes (Giray & Robinson, 1994; Münch & Amdam, 2010). Behavioural plasticity coupled with a phenomenal sense of orientation aided by smell, taste, vision, and touch (to sense vibrations) (von Frisch, 1967; Srinivasan, 2010; Farina *et al.*, 2012), makes the honey bee colonies efficient superorganisms for exploring and collecting the flowering resources at their disposal. Their efficiency led humans to explore such traits for honey production since historical times (Kritsky, 2017) and, more recently, to provide pollination services (Morse & Calderone, 2000). The latter is a need that arose from the constant increase in monocultures, in which the absence of areas for native pollinators to feed and shelter led to scarce pollination services (Aizen *et al.*, 2019; Osterman *et al.*, 2021). As these agricultural systems rely on pesticides, they systematically turn into even poorer landscapes for wild pollinators as well as for honey bees (Uhl & Brühl, 2019). The sudden losses in the number of honey bee colonies in Europe and the United States in the early 2000's, created an urgency for the scientific community to identify the reasons behind such losses (Underwood *et al.*, 2007). Today, most scientists agree that multiple stressors can lead to colony demise (Potts *et al.*, 2016; Stanimirović *et al.*, 2019). Nonetheless, a deep analysis of the system (colony) and all its moving parts (individual bees) is required to study multiple stressors within a colony composed of multiple individuals with multiple behaviours. Only by analysing how stressors affect individuals' behaviour will it be possible to understand the impact of stressors at the system level.

Almost 30 years ago, Seeley (1995) identified the need for in-silico methods to study and understand the complexity of a honey bee colony. He suggested a stepwise approach in which researchers should (1) identify the components of the system, (2) map their location and connection, (3) understand how each component affects the overall system, and (4) confirm if the output of the entire system matches real life. To validate such models, there is the need to capture the colony development traits, considering all the surrounding variables (*e.g.*, resources) that will affect the behaviour.

One of the major challenges to measuring colony development traits, and the rationale for the work developed in Chapter II, is the need to use observer-independent methodologies to measure such traits at the colony level. To address this challenge, a new protocol was developed in chapter II,

consisting of existing methods recommended by EFSA (EFSA AHAW Panel, 2016) and prioritising endpoints related with colony development, data accuracy, and observer-independency. Furthermore, the impact of the new protocol on the colony's development was tested, something rarely done when suggesting new approaches. It was concluded that this protocol could be applied without impacting colony development if the assessment is performed quickly. Furthermore, by performing these assessments less regularly, the potential negative effects of the protocol can be minimised. The protocol's data accuracy is relatively high and can be further improved with better model development (*i.e.*, DeepBee®) or manual corrections: researchers can keep the colony assessment pictures and manually correct them or later run them on more accurate versions of the DeepBee® software. These advantages have not gone unnoticed by the scientific community: the protocol was first developed within the Interreg Sudoe POLL_OLE_GI project and later, after further development, was successfully implemented during the EFSA OC/EFSA/SCER/2017/02 and H2020 B-GOOD project.

By implementing the protocol in Chapter III, it was possible to measure colonies' development to understand how they responded to external variables. As expected, these variables (*e.g.*, climate, flower resource availability, and diversity) drove colony development rates, with colonies showing variability within and between landscapes. The amount and diversity of the collected resources varied with the landscape. Most flowering species visited by bees for collecting nectar and/or pollen were specific to each landscape with only a few species overlap in more than one apiary. Honey production was higher in the agricultural landscapes (*Burgos* and *Idanha*) and lower in the forested area (*Lousã*) that had a higher resource availability. This outcome showed that the climate variables (*i.e.*, temperature, rain, and solar radiation) also play a decisive role on colony development. Several methodological improvements are suggested in Chapter III to increase our understanding of how the external variables can drive colony development. In future studies, flower nectar/pollen production and phenology models are needed to properly model the dynamic availability of resources and the climate variables should be further explored to predict foraging activity in a quantitative way. Above and beyond, the key message is that the evaluation of honey bee colonies should always consider context. If the *where* and *when* components are taken out of the equation, then data on colony development traits do not have ecological relevance.

The environmental context where colonies are installed is even more important if direct comparisons between healthy and stress-exposed colonies are to be made in the framework of Environmental Risk Assessment (ERA). In Europe, for an active substance to be approved (at EU level) and for the authorisation of a plant protection product (at Member State level), a series of robust tests

need to be passed to guarantee that its use does not cause unacceptable effects, fulfilling the safety requirements. The last published guidance on risk assessment for honey bees (EFSA, 2013) entails a stepwise approach in which (mostly conservative) assumptions are made to provide the desired level of protection. Unfortunately, this guidance never came to be accepted by the EU Member States and pesticides can still be authorised following the old guidelines (that use outdated assumptions). Nevertheless, even the most recent guideline (EFSA, 2013) has major limitations, making assumptions that are no longer in line with the reality as an increase in homogeneity in land use and repeated use of multiple pesticides have occurred (Topping *et al.*, 2020). The simple fact that honey bees are still being harmed by pesticides means that the current risk assessment is failing to protect them, and the assumptions are not providing the desired protection levels. Therefore, there is a need to better understand how pesticides behave in the landscape (pesticide fate) and how much is transported to the colonies (exposure), to make a better assessment/prediction of the possible effects. These predictions are even more challenging when products stay active for a long time (systemic pesticides), creating a prolonged exposure.

Pesticides from the neonicotinoid's family are systemic and have been appointed as significant stressors for honey bees (Pisa *et al.*, 2015; Hopwood *et al.*, 2016; Wood & Goulson, 2017). They cause several damages on the individual level even at sub-lethal doses (Lu *et al.*, 2020), eventually leading to colonies demise (*e.g.*, Rondeau *et al.*, 2014). In Portugal, one of these systemic insecticides (*i.e.*, acetamiprid) is used in forested areas to control the *Eucalyptus* weevil. Since the Portuguese forested area is home to the largest *Eucalyptus* monoculture in Europe (over 25% of forested area is composed of *Eucalyptus* for pulp production - ICNF, 2019), where beekeepers commonly install their colonies for honey production, exposure to this substance could lead to negative effects on the colonies. Furthermore, due to the complex distribution of flowering resources in these landscapes, it is challenging to predict pesticide exposure. In chapter IV, the exposure to acetamiprid was assessed empirically, providing knowledge on the possible negative effects considering individual bee traits (*i.e.*, their food needs). In the tested scenarios, the exposure was within safe levels and so no effects were detected from the risk calculation at the individual level and no negative effects were measured at the colony level. This outcome was in line with the EFSA opinion on acetamiprid (EFSA, 2022), in which no evidence of acetamiprid toxicity was found to justify the application of use restrictions. Once again, despite the results of this study, it is important to keep its context in mind: the results obtained from this study should only be applied for this type of landscapes, these forest management practices, and for the same time of the year.

Another substance from the neonicotinoids family that was recently approved, sulfoxaflor, has proved to be more toxic than acetamiprid to honey bees and other bees (Siviter *et al.*, 2018; Azpiazu *et al.*, 2021; Barascou *et al.*, 2022). Restrictions to the use of pesticides containing neonicotinoids were recently recommended by EFSA (EFSA, 2019). The results of chapter V are consistent with the literature as they showed sulfoxaflor to be more toxic than acetamiprid, leading to the homing failure of forager bees. This study provided one more example of how the sub-lethal doses of pesticides affect individual behaviour. The main challenge for this and other studies regarding pesticide exposure and sub-lethal effects at the individual level is how to transpose this data to effects at the colony level. As stated by Seeley in 1995, I believe that the answer lies on the use of computer models to evaluate how the effects at the individual level will emerge into effects at the system level. This need has also been identified by the European authorities, to lead the future of risk assessment into a systems-based approach (EFSA Scientific Committee, 2021).

Data on colony development traits and on the external variables surrounding the apiaries, collected in Chapter III, can be used for model validation (step 4 from Seeley, 1995). In Chapter VI, it was explored, for the first time, honey bees foraging strategies considering a landscape with a dynamic flower resource model, in which the resources changed in space and time according to the weather conditions. Hence, the implementation of a model with information on the flower phenology and resource production allowed to overcome the challenges identified in Chapter III regarding the landscape analysis. Besides the landscape novelty, the foraging model considers scouting and foraging behaviour separately and at the individual level. The need to accurately model the decisions of the scout bees was identified, as these play an important role on the overall colony success. As in other in silico models that explored the foraging mechanisms of the honey bees (Schmickl & Crailsheim, 2004; Baveco *et al.*, 2016), the gain strategy, in which they take into consideration the resource distance, amount, and quality, was one of the most successful strategies for nectar collection. On the other hand, when bees prioritise the closest polygons to the colony for pollen collection, the overall mass of collected pollen is higher than when implementing other strategies. These conclusions will be used for further model development. Remarkably, this first development step of the model (Chapter VI) is already more complex and realistic than previously developed models (*e.g.*, Becher *et al.*, 2016).

Final remarks

Chapter VI was the first of many *steps* on model development. Important shortcomings and further steps were identified in order to create a robust and reliable foraging model. We are aware that foragers will influence and be influenced not only by other foragers (as it was tested by using the shared resources list) but also by other bees performing other tasks in the colony. Therefore, these other colony components, arising from the internal colony dynamics that affect foraging behaviour, will be integrated in the model in a stepwise approach, always evaluating its impact on the system (step 3 from Seeley 1995).

The development of such a system is time-consuming and will need to be validated with more data besides the field data collected in this thesis. For the system to be reliable, with enough plasticity to respond to the imposed stressors and considering the *when* and *where* the colonies are installed, several environmental scenarios will be required. The use of sentinel colonies has been appointed as a solution to feed and update the system with real world colony data (EFSA Scientific Committee, 2021). We believe that their use is indeed useful to feed the modelling approach on colony development traits (as in Chapter III) and as bioindicators of landscape suitability (*i.e.*, availability of food) and presence of pesticides (Cunningham et al., 2022). Furthermore, the protocol created on Chapter II can be used on this monitoring scheme since it allows to evaluate the associated error from the method and, since it is not observer dependent, can be applied all over Europe. We are also aware that the protocol can be upgraded to be more efficient (less time and resources spent on the assessment) while maintaining data accuracy. The use of automatic and non-invasive methods would provide an even better alternative to explore colony development and health (including the pesticides they are exposed to). Recently, it has been shown that it could be possible to use a set of chemical traces to identify the presence of diseases or pesticides (Mayack *et al.*, 2022) and the sampling of these colonies could be done by using silicone wristbands to accumulate the chemical signatures, instead of killing several bees (Bullock *et al.*, 2020). Furthermore, sensors that are installed inside the colonies, to capture sound or vibrations (Ramsey *et al.*, 2020), also have a high potential for the future assessment of colonies development without constant colony disturbance. Accurate bee counters are also being developed, allowing for a better assessment of colonies activity and to better understand its interaction with the landscape and even measure foragers mortality (Odemer, 2022; Borlinghaus *et al.*, 2022). All these improvements will allow us to improve the measuring of colony development and possible negative effects, to raise the protection levels on honey bees using an ERA that is based on organisms' traits, behaviour, and context, reducing the use of assumptions that are in place to account for uncertainty.

The basis for the current pollinators ERA scheme is the honey bee. Nonetheless, honey bees exhibit resilience mechanisms (Schott *et al.*, 2021) that arise from their behavioural plasticity. These mechanisms are not present in other bees as most of them are solitary (Batra, 1984). Also, at the individual level, some bee species are more sensitive after exposure to the same concentration of a certain substance (Franklin & Raine, 2019). I believe that the implementation of monitoring schemes for honey bees (sentinel colonies) will undoubtedly increase protection for other insect pollinator species as well (Alaux *et al.*, 2019), but ERA needs further development to include other insect pollinators, as we do not know the full extent of their vulnerability compared to honey bees. It is therefore of utmost importance to evaluate the exposure risk and pesticide effects for more insect species. Additionally, environmental surveys to assess pollinator communities can help to evaluate pesticide effects after being approved, considering the context (*when* and *where*) of the use.

The collection of data from monitoring honey bee colonies or pollinator communities, is even more valuable if shared amongst the several stakeholders engaged on the protection of insect pollinators. The creation of a central hub that receives and stores these data is already being developed (EFSA, 2018). Furthermore, the implementation of algorithms for data analysis, which allow the automatic detection of important events or even trends, is under development (Simon-Delso *et al.*, 2021b). With such tools, the platform allows for stakeholders and decision makers to find and identify valuable data more easily. This data can provide stakeholders with sound evidence to drive a common vision for the future of European ERA. It is important to collectively agree on what we want to protect and to which extent can we do it. Nonetheless, this step is not only technical as there are constant conflict of interests within the stakeholder's community (*e.g.*, Arnold, 2021). To achieve a common vision, an ideological shift is needed for the engagement of stakeholders to work in the same direction, with the same goal: protection of biodiversity, to protect its perks (*e.g.*, ecosystem services as a whole). Furthermore, to better manage our environment, we need to analyse all the system components together instead of single species in isolation, since species co-exist and interact with each other. If we are not able to understand these interactions, our management actions could lead to defective organisms' networks, increasing the probability of collapse of the system and the services it provides (*e.g.*, Partap & Ya, 2012; Lindenmayer *et al.*, 2016). Only with a close cooperation and communication from all stakeholders (*i.e.*, academia, farmers, industry, conservationists, risk assessors and risk managers) it will be possible to steer the future of a systems-based ERA, to overcome and reverse the mistakes from the current ERA (Sousa *et al.*, 2022).

Chapter VIII

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Chapter IX

Supplementary material

Section II.1

Protocol for colony assessment

To develop the quantitative method, several colony endpoints and methods were reviewed. Based on the HEALTHY-B document (EFSA AHAW Panel, 2016), important colony health status indicators were selected for evaluation during the field season. We considered all variables classified with respect to (1) high relevance for colony health, i.e. scientific studies documented a strong association of the parameter with bee health, (2) high technical feasibility, i.e. possibility of being routinely applied by a beekeeper and (3) high priority (best benefit to effort) on the HEALTHY-B document (H-HH). On a second step, we have selected the most relevant methodologies, to acquire accurate empirical (quantitative) data. These methodologies include new, automatic technologies, e.g., video, image analysis and hive scales, combined with usual techniques on honey bee research (e.g., palynological analysis), to reduce the human error, observer subjectivity and dependency, to capture the hourly, daily, and seasonal colony changes. In this document, all the colony indicators and respective methodologies are described. Nonetheless, to assess the method impact on colony development, only data from hive scales, colony size, brood development and provision was used.

Selected variables and methodologies

1. Queen performance

1.1. Queen presence and age was detected visually, and colour marked according to birth year (Human *et al.*, 2013). The mark will allow an easier queen detection, to cage the queen early in the inspection and avoid damage of the queen.

1.2. Presence of fresh eggs was detected visually by searching for one-day old eggs. These eggs are in upwards position, placed in the bottom of the cells and indicate that the queen is present.

1.3. Natural queen replacement was detected visually if a new (unmarked) queen is observed without any signs of swarming.

2. Colony demography and provision development

2.1. Colony size (total number of adult bees) was assessed by weighting the frames with and without bees, and the number of adult bees calculated based on an average weight per bee (Delaplane *et al.*, 2013; supplementary material section II.2). Each frame was weighted with bees and moved to an empty box. To remove adult bees, each frame was then brushed (removing the bees back into the original hive), weighted without bees and returned to the hive. As in other methods, the presence of drones can influence the colony size estimates. Fortunately, a normal honeybee colony can have at its peak, a maximum of 11% of its population as drones (Rowland *et al.*, 1987). Also, the method may underestimate the number of workers, as foragers may be away from the hive, and hence not included in the total colony weight. It is recommended to close the colonies early in the morning to have all the bees inside the hive and assess their colony size properly (Delaplane *et al.*, 2013). Nonetheless, this could cause more disturbance to the colonies and affect the normal colony development during the experiment. Also, the use of automatic scales and bee counters can help to roughly quantify the number of forager bees (see more on section 2.4).

2.2. Brood development

2.2.1. Eggs, larvae, and pupae was assessed using digital photography and image analysis (Alves *et al.* 2020). While assessing the adult bee population as above (section 2.1), after removing the bees from the comb, the frame is photographed inside a tunnel with proper lighting to ensure image quality. Images are analysed using the DeepBee© software, which classifies each cell of the comb into one of seven classes: Egg, larva, capped brood, pollen, nectar, honey and other (empty cells) (see more details in Alves *et al.*, 2020). The software is available for free download and was published in an overall accuracy of 94%. Nonetheless, the software is based on deep learning, allowing users to increase its accuracy by performing training sessions with manually corrected pictures.

2.2.2. Queen cells were counted by visual assessment and removed when the old queen was present, healthy, and laying eggs.

2.2.3. Brood consistency was assessed by visual assessment of a solid/patchy brood pattern.

2.3. Provision

2.3.1. The number of beebread cells was obtained by image analysis using the DeepBee® software by the “pollen” category (see above).

2.3.2. Nectar and honey reserves were calculated by subtracting the weight of the frame materials (i.e., wood and wax), beebread, larvae and pupae cells (Annex B) from the weight of the frame without bees.

2.4. The total colony weight and thermoregulation was continuously assessed during the entire experiment using automatic scales with humidity and temperature sensors. Obtaining reliable brood temperature data requires that the temperature sensor is placed correctly inside the brood area, which may be particularly challenging in late season, when the brood area is diminishing, therefore, sensors should be installed perpendicular to the brood frames. Total colony weight reflects changes in the population of adults and brood, in addition to provision. Monitoring of daily weight changes may remotely detect swarming events, peaks of honey production, atypical events, beekeeping management events, provide a rough estimate on the amount of forager bees, and to check for normal behaviour intrinsic to the colony e.g., if the colony is consuming its reserves.

2.5. Adult bee mortality was assessed by placing a plastic tray in front of the colony, covered by a net to avoid birds' predation of dead bees. This methodology allows for the collection and counting of most of the discarded dead workers at the hive entrance and to detect an unexpected high number of dead bees. Forager's mortality cannot be assessed by this method as most of the bees die in the field.

2.6. Swarming events were detected using automatic scales when sudden weight drops occur (indicator 2.4), and visual confirmation was made on the next visit. The colony will have less bees (up to 50-60% of the bees leave the colony with the old queen) than the last monitoring event and no marked queen (section 1.1).

3. Behaviour

3.1. Colony foraging activity was assessed using video recording of forager activity at the hive entrance with later analysis performed by an automatic counter software. From the several methods that can be used to assess foragers activity (e.g., channel infra-red counters), the video-based ones allow bees to move freely in and out of the colony, hence the equipment does not disturb their normal behaviour (Odemer, 2021). Currently, there is still no available software with negligible error margins to allow the calculation of foragers' mortality (section 2.5).

3.2. Colony foraging choices were evaluated by analysing the amount and diversity of pollen and honey. At every assessment, pollen traps were installed for 24h, and fresh pollen from returning foragers was collected. Pollen samples were frozen at -20C and dried for 48 hours at 40°C. Palynological and melissopalynological analysis were carried by determination of 500 pollen grains per sample. Though, pollen content of honey does not reflect the real use of nectar sources, it provides some information of the most important species. On the other hand, the identification of the pollen botanical composition, provides a good overview of the flower preferences, temporal distribution of resources and ultimately, the pollination services provided by the colony.

3.3. Atypical behaviour was visually assessed by checking first the hive vicinity and then the combs inside the colony. A list of normal behaviours is provided by Scheiner *et al.* (2013). Trembling bees, running periods, walking in the vicinity of the hive for long time, are some of the most common atypical behaviours. Queen atypical behaviour, like laying more than one egg in one cell, is also be considered in this indicator.

4. Disease, infection and infestation

4.1. Disease and infections are assessed visually for the most common symptoms. A list of the most common symptoms is provided in the Appendix D (Clinical signs of disease) in the HEALTHY-B document (EFSA AHAW Panel, 2016). When clinical signs were detected, samples of brood/bees were collected and analysed in the lab. For monitoring purposes, honey bee samples were collected in Spring, Summer and Autumn for *Vairimorpha* (Nosema) *apis/ceranae* and virus analysis (DWV, ABPV and SBV). A sample of 300 bees is collected and bees are maintained (with sugar paste) in a plastic container until being stored at -80C to avoid virus degradation. To minimize colony disturbance, samples for disease analysis were taken at the same time as the samples for pesticides analysis (section 5.1).

4.2. Infestation levels of varroa mites were tracked during the season using sticky bottom boards, a non-invasive method. At every colony assessment, bottom boards were cleaned, and Vaseline was added to create a sticky trap for varroa mites. After one week, the number of mites that fell into the board (natural mite fall) were counted and the total number of mites in a colony estimated (Flores *et al.*, 2015). The collected samples for disease and infections analysis were also used to determine varroa infestation levels by using the soapy water method.

5. Pesticide residues in hive matrices (beebread, nectar/honey, wax)

5.1. Pesticide residues presence and amount were quantified in beebread and unsealed nectar, from samples collected in the beginning, middle (after spraying season) and end of the season and stored at -20°C. To avoid destroying wax comb and cross contamination between colonies, beebread was collected using a disposable bee bread collector similar to the one developed by Loglio *et al.* (2019) while nectar was collected with disposable plastic pipettes. A piece of wax com was also taken in the beginning of the season to check for pesticide accumulation in the past. Samples were analysed by a multi-residue array using Ultra Performance Liquid Chromatography tandem Mass Spectrometer (UPLC-MS/MS) and Gas Chromatography with tandem Mass Spectrometry (GC-MS/MS).

6. Beekeeping practices

6.1. The timing of all activities relating to colony management were registered. All input (e.g., adding supers or frames, supplemental feeding) and output (e.g., removing supers or frames, honey, wax or comb samples) were weighted, and the timing registered, in order to match beekeeping management events and data from the automatic scales (section 2.4). Beekeeping practices were performed according to local conditions.

7. Climate

7.1. Climate variables were continuously measured (every 15min) during the season. In each apiary, a weather station was installed to measure wind speed and direction, air temperature, relative humidity, precipitation and solar radiation.

Section II.2

Calculate colony size brood development and provision

During this study, honeybee colonies were subjected to regular assessments with focus on measuring their brood development, colony size and provision. To do so, all colony frames were weighted with bees and moved to an empty box. Each frame was then brushed (removing the bees back into the original hive), weighted without bees and photographed on both sides inside a photography tunnel (Figure SII.1), before returning it to the original hive position. From this assessment, we gathered data on individual frame weight with bees and without bees, and the frame cell composition by analysing the pictures with the DeepBee© software (Figure SII.2).

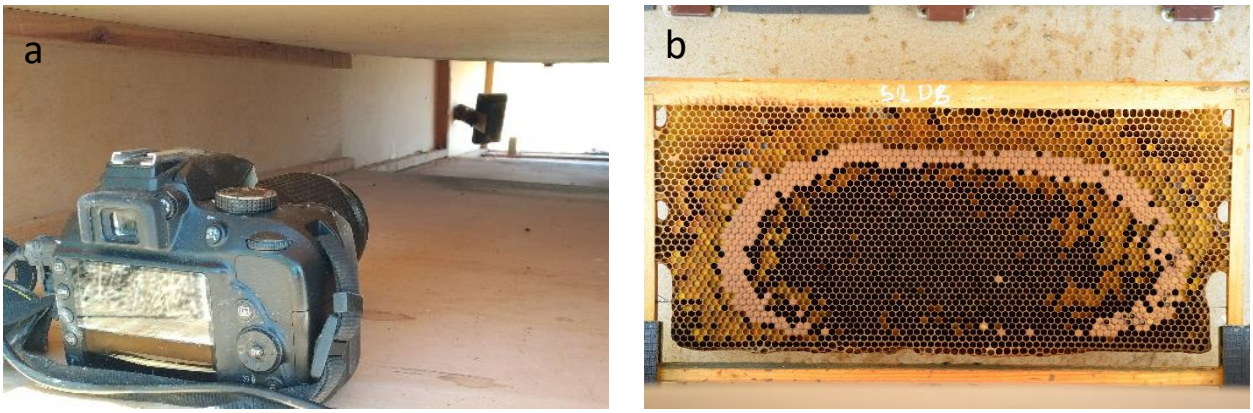

Figure SII.1: Image capture in the photography tunnel: The frame was placed in one end of the tunnel and rested on 3D printed holders that guarantee a 13° angle forward to improve the eyesight of cell content. The camera was installed in the opposite end of the tunnel (a). The tunnel was closed in both ends before taking a picture of the frame (b). Hence the only light source is the diffused light from the LEDs installed on the wall.

By knowing the number of cells with eggs, larvae and pupae in the colony (given by the DeepBee© software) we assessed the brood development. To calculate colony size, we subtracted the weight of the frame with bees from the weight of the frame without bees and then multiplied that value for the weight of each individual bee:

Number of bees = (frame with bees – frame without bees) x individual adult bee weight

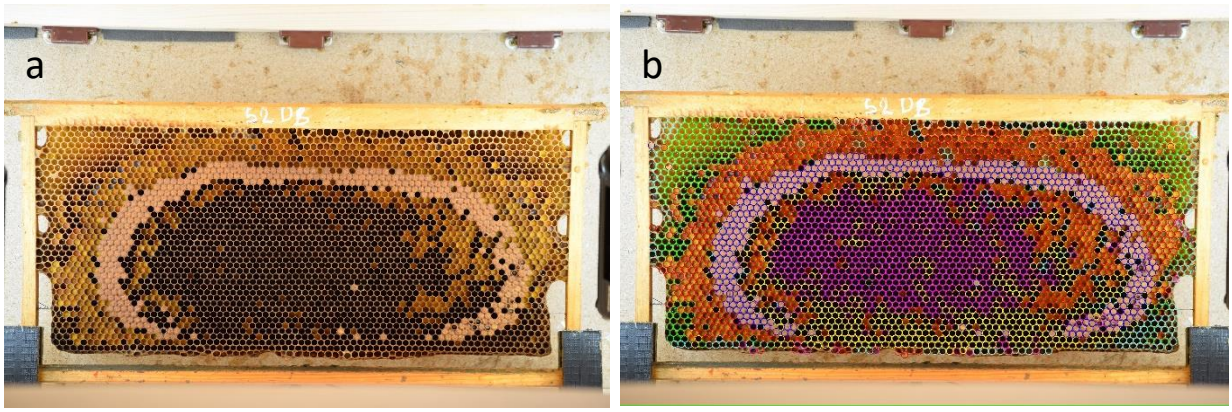

Figure SII.2: Picture taken in the photography tunnel (a) and the output of the DeepBee© software from the same picture (b). In this frame, DeepBee© identified 364 capped cells (blue), 668 larvae (purple), 573 eggs (yellow), 327 nectar cells (green), 6 honey cells (brown), 898 pollen cells (red) and 236 other (light blue).

Several studies have showed a significative variation of bee weight: 116.37mg (±0.61mg) in Bowen-Walker & Gunn (2001), 120mg in Hrassnigg & Crailsheim (2005), and 128mg (±24mg) in Meikle *et al.* (2008). Therefore, we calculated the median weight of an individual adult honey bee. To do so, 100 adult bees were collected during one colony assessment and bees were individually weighted in a precision scale (table SII.1).

To calculate provision, we can also use the software output to know the number of cells with nectar/honey. To know the amount (kg) of provision, we could use the number of cells and multiply by a mean weight per cell. Nonetheless, these cells can have a large variation in weight. To override this problem, we decided to subtract the weight of the empty frame components (wax and wood), as well as the frame cell composition (larva, pupa, beebread) from the total weight of the frame without bees:

**Provision per frame = Frame without bees – empty frame – larvae weight – pupae weight – beebread weight*

*For this calculation, the weight of the eggs was considered insignificant.

To calculate the median weight of an empty frame, 50 nest and 50 honey super Langstroth comb foundation frames were individually weighted (table SII.1).

Larvae and pupae mean weight (table II.1) were calculated based on data from Zółtowska *et al.* (2011), in which, data on several development stages is presented. To use this mean, we assume that the number of larva/pupae is evenly distributed from all the different development stages.

Beebread mean weight per cell was calculated by weighing 100 individual beebread cells on a precision scale (table SII.1).

Table SII.1: Mean weight and standard deviation (SD) in grams for: adult honey bees, beebread and empty frames calculated by weighing individual cells/frames on a precision scale. Larva and pupa individual mean weight was calculated based on Zółtowska et al. (2011).

	Mean weight (g)	**±SD**
Adult honey bee	0.124	0.012
Beebread	0.172	0.059
Capped brood (pupa)	0.117	0.016
Larva	0.103	0.071
Langstroth brood box frames (with wax fully built) *	483.3	101.1
Langstroth honey supper frames (with wax fully built) *	372.8	38.95

*After honey extraction, these frames were given to the bees to clean the remaining honey. The frames were weighed afterwards.

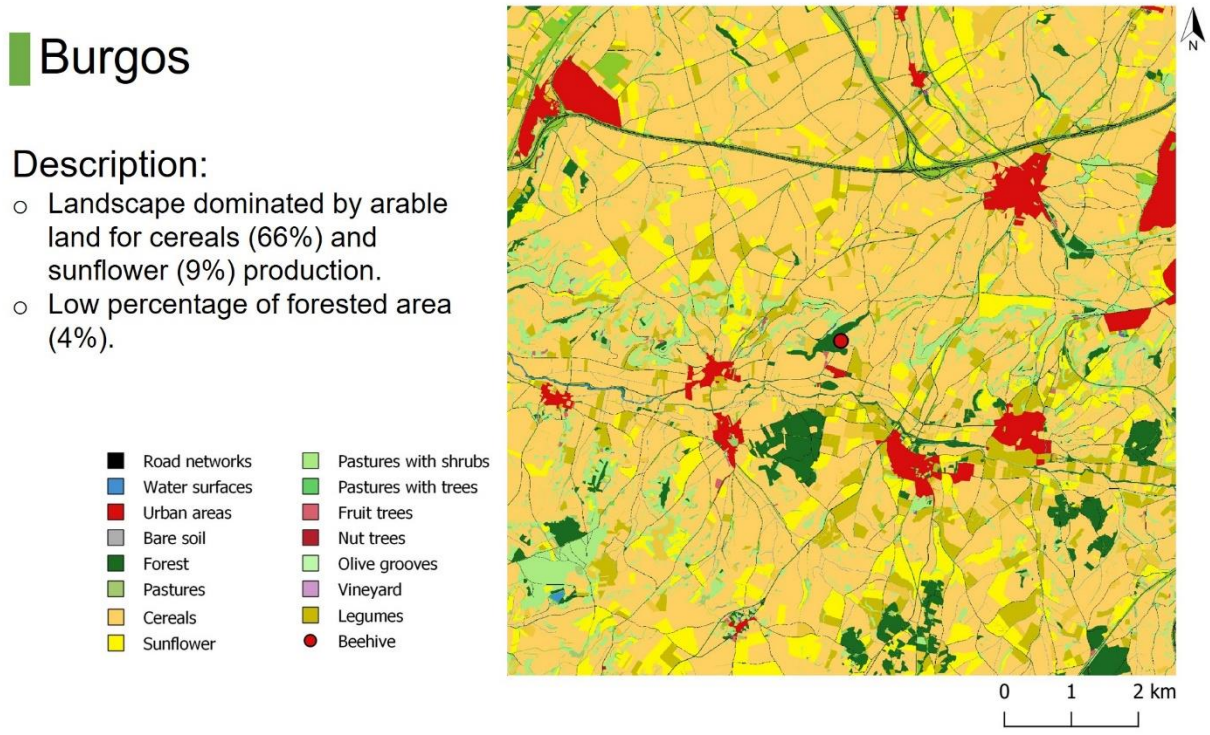

FIGURE SIII.6: LANDSCAPE REPRESENTATION OF THE BURGOS 2018 AREA.

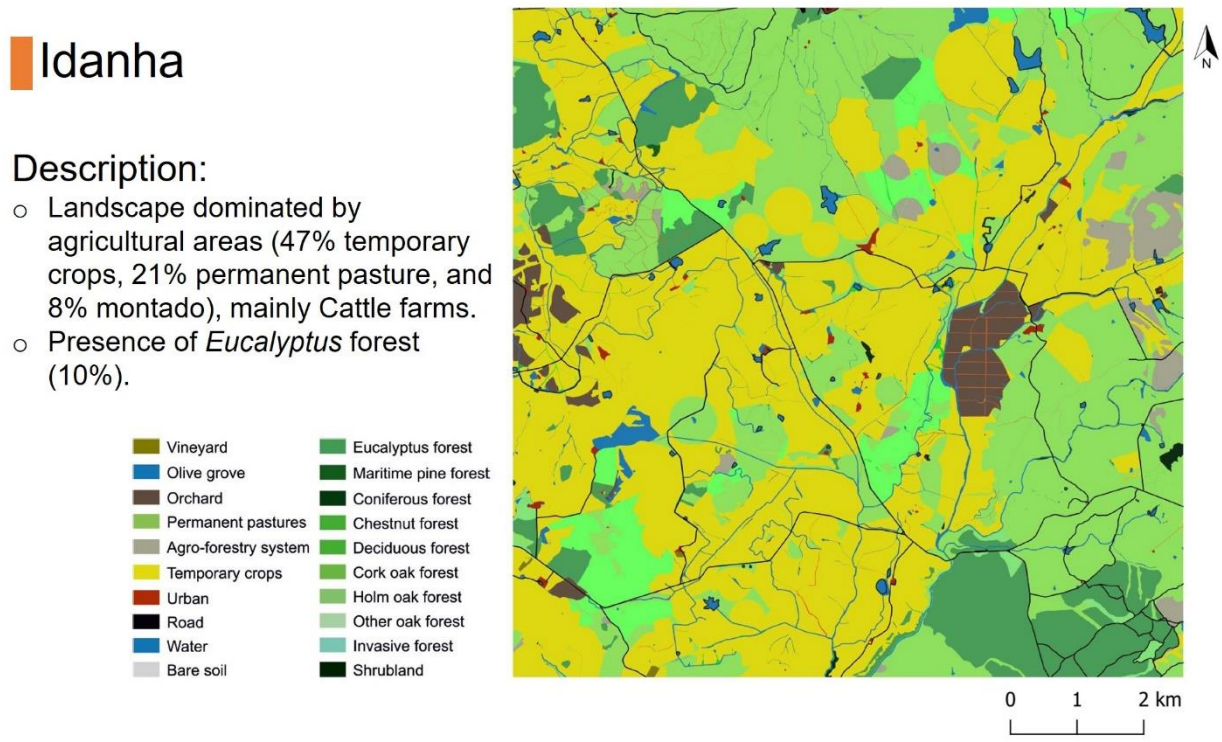

Figure SIII.7: Landscape representation of the Idanha 2020 *area.*

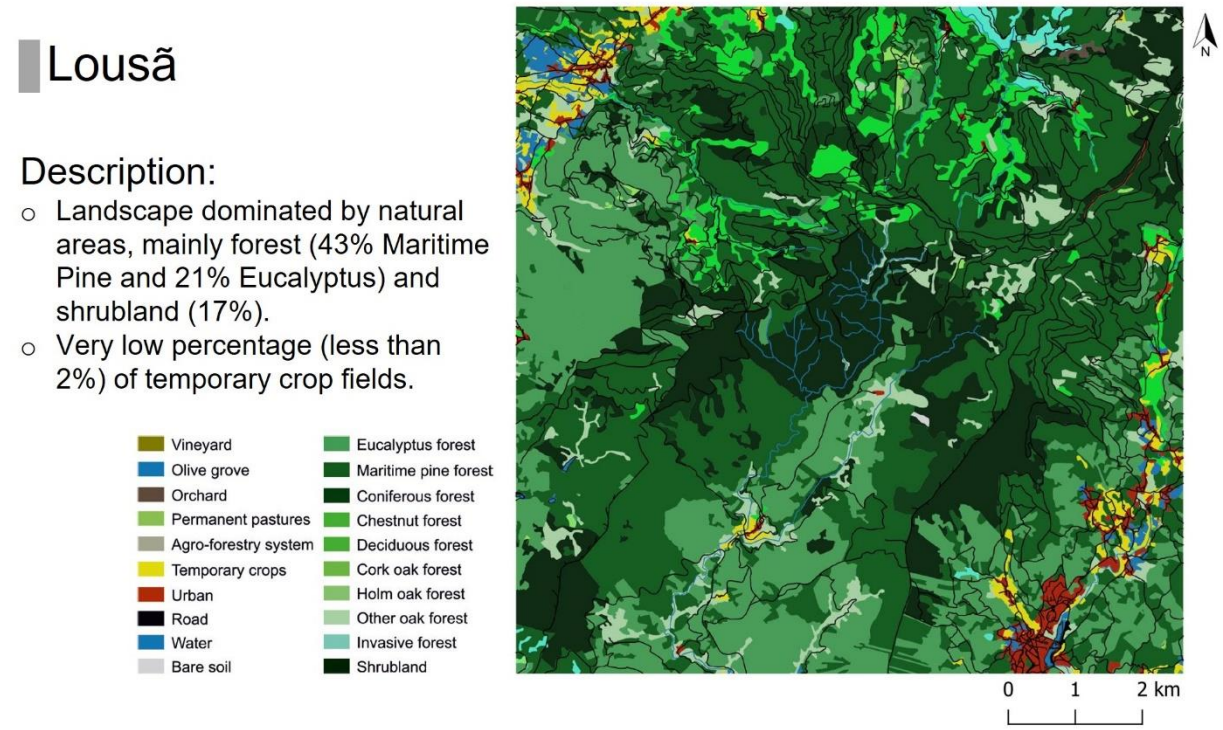

FIGURE SIII.8: LANDSCAPE REPRESENTATION OF THE LOUSÃ 2019 AREA.

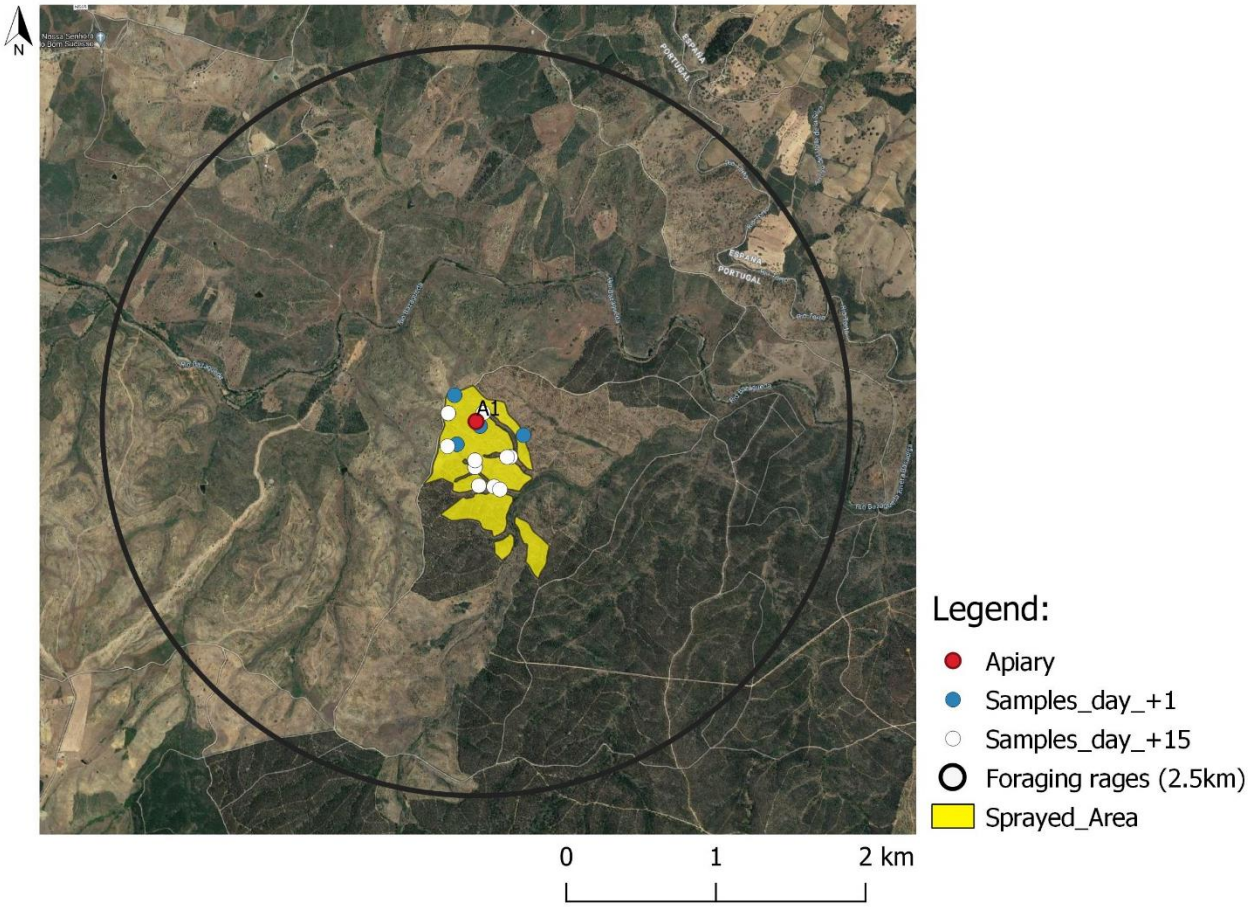

Figure SIV.1: Sprayed area (0.456 km^2) and Study Window 1 (apiary A1) location.

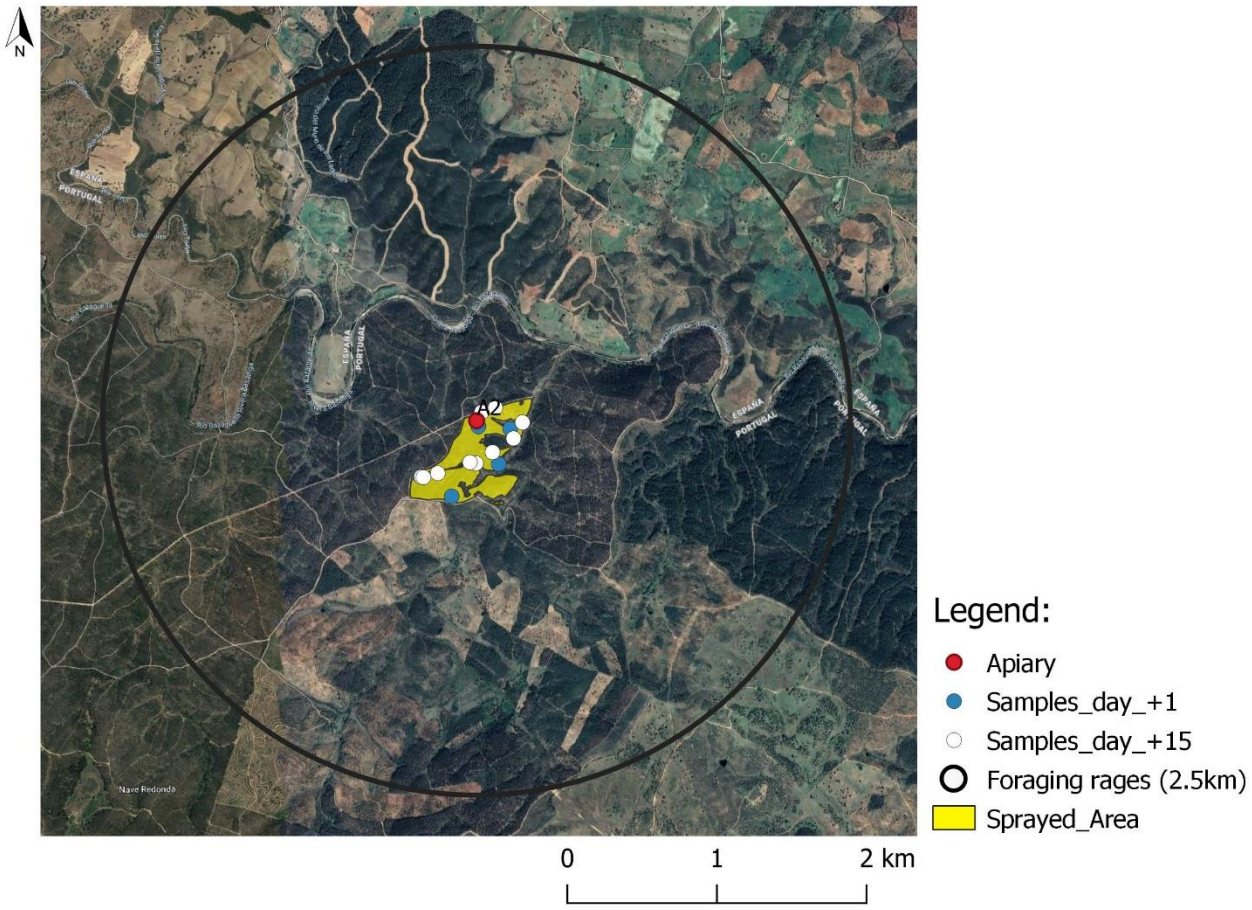

Figure SIV.2: Sprayed area (0.247 km^2) and Study Window 2 (apiary A2) location.

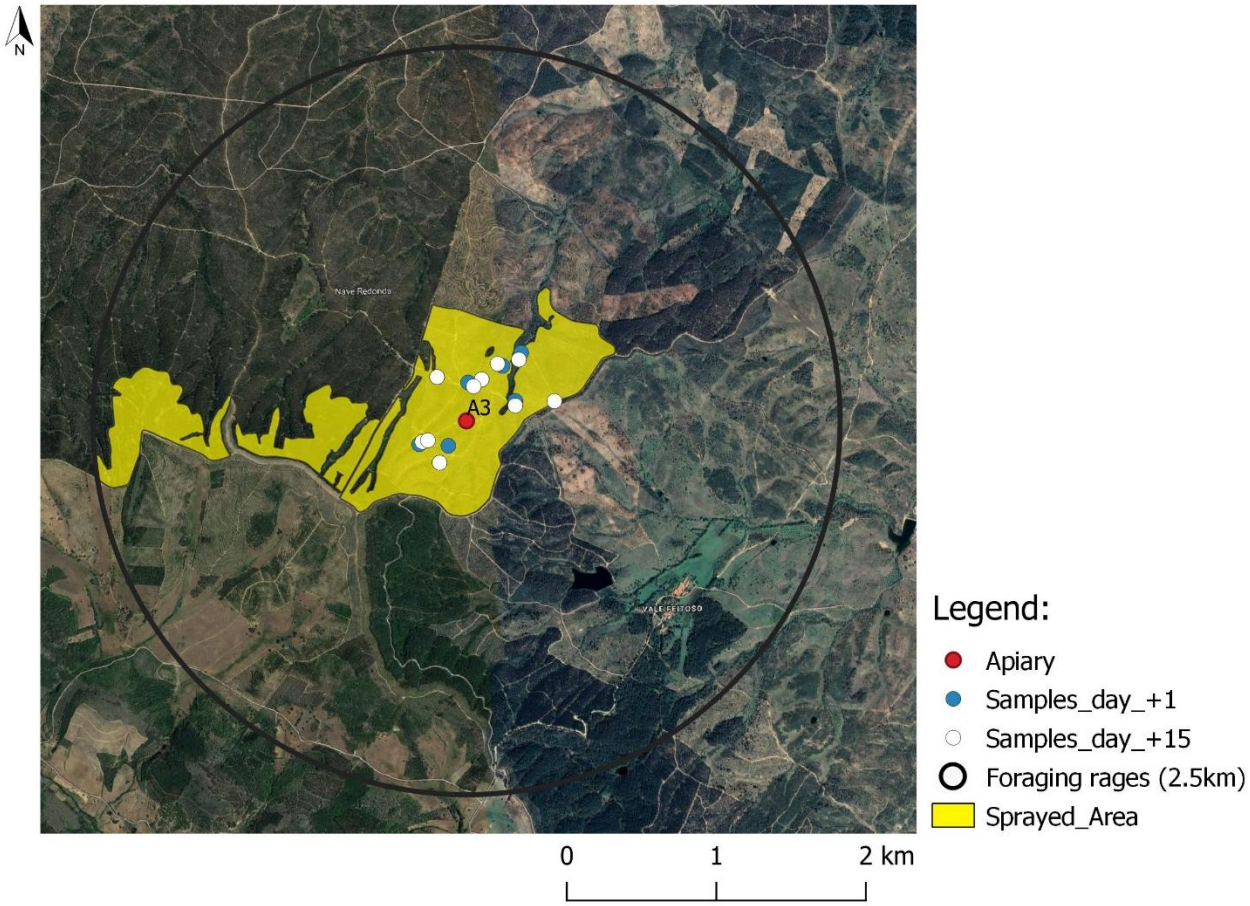

Figure SIV.3: Sprayed area (1.860 km^2) and Study Window 3 (apiary A3) location.

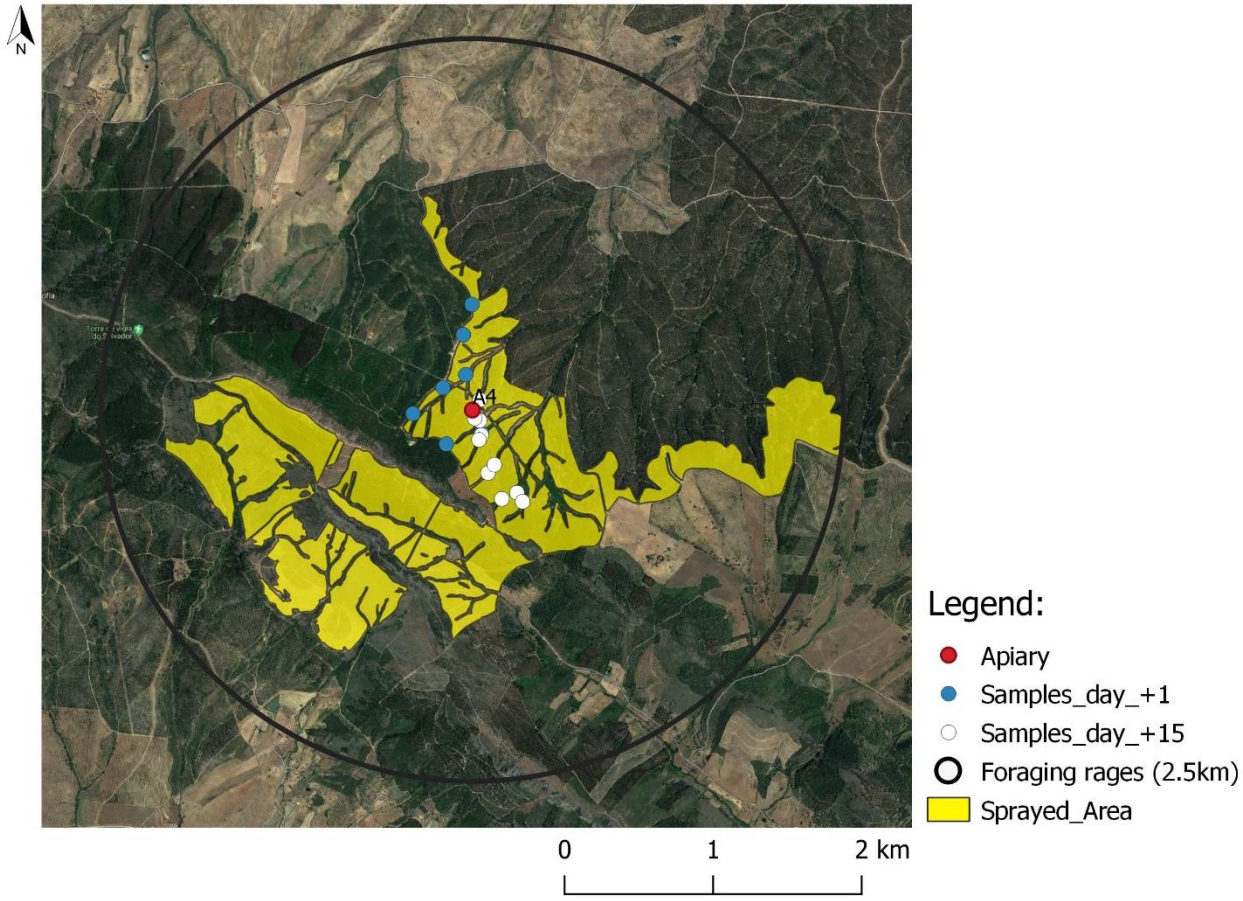

Figure SIV.4: Sprayed area (3.117 km^2) and Study Window 4 (apiary A4) location.

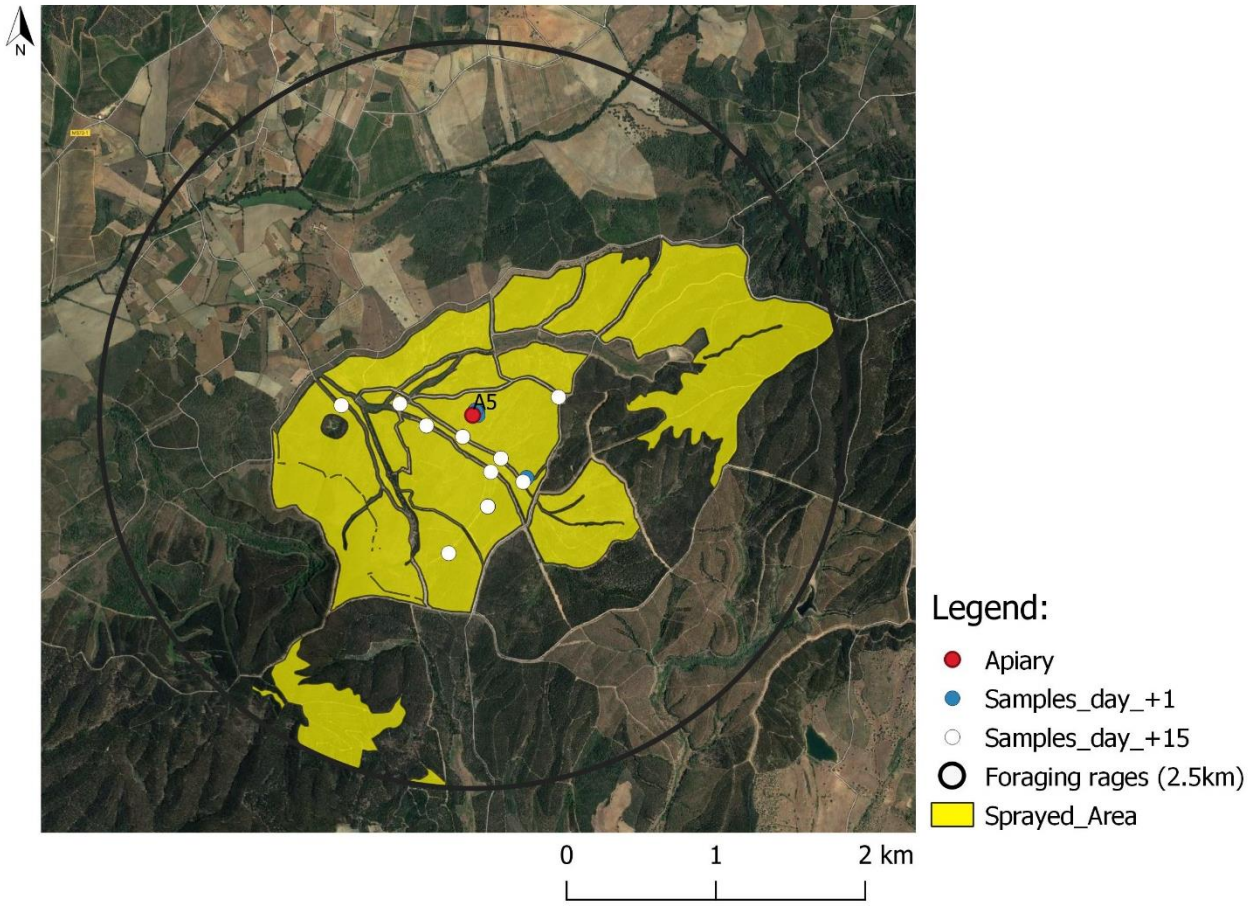

Figure SIV.5: Sprayed area (4.486 km^2) and Study Window 5 (apiary A5) location.

Section IV.2

Table SIV.4: Colony assessment was performed using the methods described in Dupont et al., 2021. Colonies were assessed one week before exposure (05-05-20) and 3 weeks after (07-06-20). For each colony, besides the visual assessment of disease symptoms, it was measured the number of adult bees (population), the amount (kg) of nectar/honey in the colony, and the number of brood (egg, larva and pupa) and beebread cells.

Date	Apiary	Colony	Population	Honey and nectar (kg)	Number of brood cells	Number of beebread cells
05-05-20	A1	3	16048	2.9	21570	4778
05-05-20	A1	10	25403	3.3	21115	7201
05-05-20	A2	5	16613	2.2	15601	3671
05-05-20	A2	6	24919	3.9	21686	4992
05-05-20	A3	1	24516	1.9	21926	3418
05-05-20	A3	7	14919	2.4	21532	4325
05-05-20	A4	4	17016	4.7	15852	4275
05-05-20	A4	8	24839	2.4	18039	6173
05-05-20	A5	2	16694	2.8	14003	6322
05-05-20	A5	9	24597	1.6	24314	4982
07-06-20	A1	3	21613	3.4	18109	7496
07-06-20	A1	10	24355	5.7	13920	10491
07-06-20	A2	5	15565	2.8	12683	4910
07-06-20	A2	6	25242	8.9	17243	4704
07-06-20	A3	1	18065	4.3	18300	7569

07-06-20	A3	7	20968	7.1	20656	6786
07-06-20	A4	4	20323	7.6	14825	4409
07-06-20	A4	8	25887	8.2	15773	5407
07-06-20	A5	2	16048	3.2	13074	6741
07-06-20	A5	9	22984	6.6	22107	5753

Section IV.3

xMAP technology for flowers and bee products residue analysis

A bead-based array platform (xMAP multi-analyte-profiling) (Hsu *et al.*, 2009) has proven to be a viable option to semi-quantify a large amount of field samples. xMAP technology has been broadly applied in various fields, including food safety, environmental pollutant monitoring and medical diagnostics (Hamza *et al.*, 2014; Peters *et al.*, 2014; Pan *et al.*, 2019). One of the first successful attempts of utilizing xMAP in pesticide detection was a competitive, single-plex assay for detecting triazophos in vegetable matrices, which was further incorporated into a multiplex assay, screening triazophos, chlorpyrifos and carbofuran simultaneously in vegetable matrices (Guo *et al.*, 2013; Liang et a., 2013). Nonetheless, these analyses require some technical and laborious procedures: samples must be collected, properly stored, transported to, and analysed in the laboratory. The use of equipment – in situ – that could reveal the presence (screening) of specific pesticides in environmental samples can give an important notice of what PPPs might be applied in a specific location. The use of lateral flow devices (LFDs) can be the answer to this quest. In LFDs, an immunoassay is powered by capillary force and uses a similar principal as a competitive enzyme-linked immunosorbent assay (ELISA): specific antibodies are used for biorecognition, and the LFD shows a colorimetric display as its readout (Posthuma-Trumpie *et al.*, 2019). Over the years, paper-based LFDs were developed for a wide range of applications for rapid screening at the point of need (Anfossi *et al.*, 2013; Huang *et al.*, 2016). In 2017, Wang *et al.* developed a quantum-dot-based LFD for the fast screening of three neonicotinoids (imidacloprid, clothianidin and imidaclothiz) in tea leaves. In 2019, the same group published an LFD for screening acetamiprid in agricultural products using the same method (Liu *et al.*, 2019). These LFDs were claimed to be portable and sensitive (limit of detection at 1 ng/ml), with a simple extraction method which only uses boiling water. This makes fully on-site sample preparation and measurement realistic (Wang *et al.*, 2017; Liu *et al.*, 2019).

To analyse the samples generated from the field experiment, we chose a paper-based rapid LFD screening immunoassay with on-site applicability and a lab-based, matrix matched semi-quantitative xMAP immunoassay, both for the detection of acetamiprid. These immunoassays were effective with only a simple hot water extraction. The lateral flow screening assay has proven to be a fast, cheap, sensitive, qualitative and reliable pre-screening method. The dual channel LFD prototype produced by Zhejiang University with a previously determined limit of detection (LOD) of 10 ng/ml has confirmed these capabilities for screening purposes in this study, and are able to detect acetamiprid below 5 ppb in the most matrix extracts (bees, pollen, bee bread and eucalyptus leaves) and below 15

ppb in the bush flowers. The Ace-mAb used for this study showed high sensitivity to acetamiprid not only in the LFD analysis, it also provided an LOD of 0.08 ng/ml in the bead-based xMAP technology (unpublished data). Despite the fact that the LFD allows easy, robust but sensitive detection of acetamiprid, its readout is only visual in this experiment and therefore is not capable of quantification. Since our field experiment produced in total 366 different environmental samples, the LFD was only implemented on a subset of samples. For the semi-high throughput acetamiprid detection in the experimental samples, xMAP technology enabled rapid semi-quantification of a large number of samples with relatively low cost and manual work when compared to instrumental analysis (Postuma-Trumpie *et al.*, 2009). Although the planar array analyser is not intentionally designed for on-site analysis, it is still portable and can be easily set up in a minimal or mobile lab environment. A wide range of samples were suitable for direct detection using the matrix-matched calibration curves. However, some samples were highly contaminated and needed dilution steps to be able to quantify. Throughout the qualitative and semi-quantitative analyses of these field samples, the matrix effect rises as the most challenging: In bees, pollen and beebread, the main matrix components are protein and fat, while in nectar, sugar is the main matrix factor. In plants, odorous essential oils as well as color pigments can be extracted together with the pesticides and introduce matrix effect.

Instrumentation and Materials

A MAGPIX planar array analyser, drive fluid and MagPlex #038 paramagnetic microspheres were purchased from Luminex (Austin, USA). Acetamiprid-monoclonal antibody (Ace-mAb) used for detection and the acetamiprid-ovalbumin (Ace-OVA) conjugate used for paramagnetic microsphere coupling were generously provided by Institute of Pesticide and Environmental Toxicology Zhejiang University. The Goat-anti-mouse IgG-R-Phycoerythrin conjugate (GAM-RPE) was obtained from Moss (Pasadena, USA). The xMAP assays were performed in Cellstar flat-bottomed 96-well microtiter plates purchased from Greiner Bio-One B.V. (Alphen aan den Rijn, the Netherlands). An electric household kettle (TOMADO) was used, for the hot water acetamiprid extraction of all samples. An Ystral polytron (Ballrechten-Dottingen, Germany) was used for homogenizing bee samples, while Whatmann 5951/2 filter paper (GE Healthcare, Germany) and 1.2 µm Durapore membrane filter plates (Millipore, Darmstadt, Germany) were used for filtering the extracts. PBS, in dissolvable pellets, was also purchased from Millipore, while MES hydrate, N-Hydroxysulfosuccinimide sodium, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, bovine serum albumin (BSA) and Tween-20 and the acetamiprid standard (purity: 99.9%) were all purchased from Sigma (Steinheim, Germany). The imidacloprid/acetamiprid dual channel Lateral Flow Devices (LFDs) were kindly produced on request

by the Institute of Pesticide and Environmental Toxicology of Zhejiang University. Graphpad Prism version 8.0.0 for Windows (GraphPad Software, San Diego, USA) was used for data analysis.

Microsphere coupling

The #038 Magplex bead stock ($1.25x10^7$ microspheres/ml) was thoroughly suspended by 10-minute vortexing. Next, $6.25x10^6$ microspheres were washed with distilled water. For activation, the microspheres were resuspended in 80 µl 100 mM monobasic sodium phosphate (pH 6.2), followed by the addition of 10 µl (500 µg) freshly dissolved N-Hydroxysulfosuccinimide sodium in deionized water and 10 µl (500 µg) freshly dissolved 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (in deionized water). This mixture was incubated for 20 minutes with gentle mixing every 5 minutes. 100 µg/ml Ace-mAb was prepared by diluting a 1 mg/ml stock (in PBS) with 50 mM MES buffer pH 5.0 and was allowed to react with the activated microspheres for two hours while gently mixing. The excess antibodies were removed, and the microspheres were blocked with PBS-TBN buffer (1x PBS, 0.1% BSA, 0.02% Tween-20, 0.05% NaN_3) for 30 minutes while gently mixing. The Ace-OVA coupled microspheres were stored in 200 µl PBS-TBN at 4°C in the dark until further use.

Sample preparation

For pollen, nectar, bee bread, plant leaves and flowers, 1 g of material was extracted with 10 ml boiling tap water for 30 minutes. Extracts were vigorously shaken every 5 minutes to ensure optimal extraction. For bee samples, 1 g of bees was weighed in a 50 ml tube, and 10 ml boiling tap water was added. Additionally, the bees were homogenized with a polytron. All crude extracts were filtered through filter paper and the flow through was collected. A subset of the extracts was used for LFD analysis, while all extracts were used for a xMAP planar array analysis. To control the samples for possible pre-existing acetamiprid contamination, Day -1 samples were measured on-site at the apiary via LFDs.

LFD analysis

For this project, prototype dual channel LFDs for the detection of imidacloprid and acetamiprid were applied on-site in the field and in the lab for the detection of acetamiprid. These prototype LFDs contain the same Ace-mAb and Ace-OVA as used in the xMAP assay. Like the xMAP method, the LFD uses an inhibition assay principle (Fig. SIV.6a)

Extracts were prepared as described previously. After the extract cooled down to room temperature, two drops of the sample extracts were added to each sample well of the LFD using a Pasteur pipette and allowed to develop for 15 minutes before visual read-out. After read-out, positive or negative results were determined (Fig. SIV.6b and SIV.6c).

Development of the acetamiprid screening assay

To determine the optimal working concentration of the Ace-mAb, 2-fold serial dilutions of the Ace-mAb were prepared in 1x PBST buffer (0.01 M, 0.1% BSA and 0.02% Tween-20, pH 7.4) in a 96 well microtiter plate in duplicate. The serial dilution of Ace-mAb was incubated with 10 µl microsphere solution (approximately 1000 microspheres, in 1x PBST buffer) for 20 minutes while gently shaking. The excess Ace-mAb was washed off, and 100 µl GAM-RPE (2 µg/ml) was added as a secondary reporter antibody and incubated for another 20 minutes. The excess GAM-RPE was removed prior to the measurement. Read out was performed by the MAGPIX planar array analyser operating on Luminex xPONENT software version 4.3. The minimum count was set to 50 microspheres. The mean fluorescent intensity (MFI) was measured and plotted against the Ace mAb concentration. The optimal concentration of Ace mAb in this assay format was determined by the corresponding concentration at 1000 MFI.

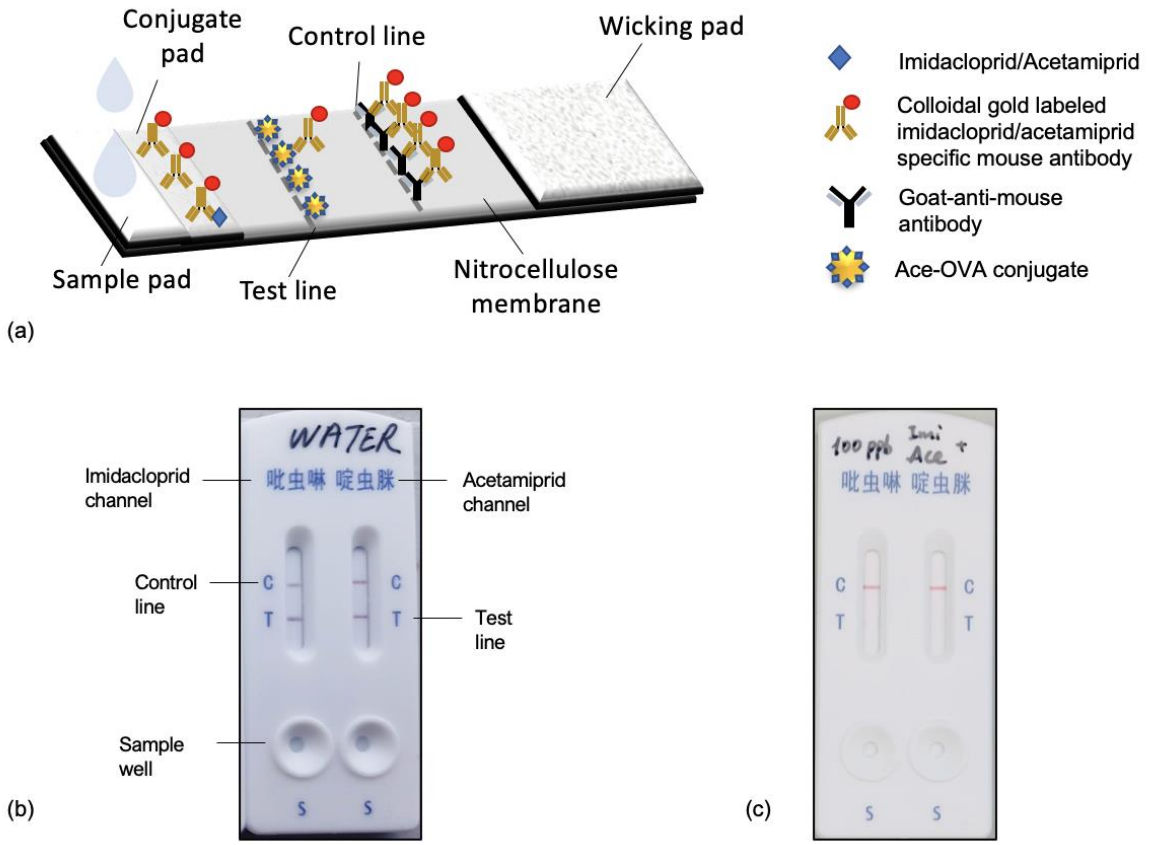

Figure SIV.6: (a) Working principle of the LFD. Control line (goat-anti-mouse antibody) and test line (Imi-OVA or Ace-OVA conjugate) are immobilized on a nitrocellulose membrane. Colloidal gold coupled imidacloprid/acetamiprid specific antibody, dried on the conjugate pad flows throughout nitrocellulose membrane via capillary flow. In a valid assay, the specific reporter antibody on the gold nanoparticles is recognized by the anti-mouse antibodies on the control line, thus the control lines should always be present as a part of a valid readout. When testing a positive sample, the analytes in the sample compete for imidacloprid/acetamiprid specific antibodies against the Imi-OVA/Ace-OVA on the test line, and therefore causing faint or absence test lines. Fig. adapted from Liu et al. (2019) and Wang et al. (2017). (b) The imidacloprid/acetamiprid dual channel LFD applied with tap water sample. Since there is no neonicotinoid present in the water, control and test lines are clearly visible. (c) Readout of the dual channel LFD after application of water samples containing 100 ng/ml imidacloprid and 100 ng/ml acetamiprid. The free imidacloprid and acetamiprid occupy the specific antibodies and prevent their binding to the control line, resulting the absence of the test lines.

Sample screening and determination of the sensitivity

Dose-response curves of acetamiprid, in the targeted matrices, ranging from 100 ng/ml to 0.01 ng/ml, were prepared by addition of acetamiprid to the blank matrix. For this purpose, the tested Day-1 samples were selected. As a negative control, and zero value, the blank matrix extracts were used. The samples and the calibration curve were filtered through 1.2 µm filter plates to remove disturbing matrix particles. Next, the dose-response curves and samples were measured in duplicate. In short, Ace-OVA coupled microspheres were diluted 1:100 with 10x PBST buffer (0.1 M, 1% BSA and 0.2% Tween-20, pH 7.4), while Ace-mAb was diluted to 10x optimal dilution in 1x PBST buffer. 10 µl of diluted microsphere solution (approximately 1000 microspheres) and 10 µl of diluted Ace mAb were incubated with 90 µl of filtered extracts or standards. This mixture was incubated for 20 minutes, and the assay was developed and measured in duplicates as previously described.

xMAP planar array data processing

Each MFI reading was normalized against the MFI of the corresponding blank matrix (B/B0). The B/B0 values of the dose-response curves were plotted against the acetamiprid concentrations, on a logarithmic scale. Four-parameter logistics nonlinear regression was implemented for curve fitting the unknown samples on the respective acetamiprid dose-response curves using Graphpad Prism version 8.0.0 for Windows. The acetamiprid concentrations in the unknown samples were calculated by the same software. The sample extracts that had concentrations above the dose-response curve's upper limit were diluted and remeasured together with the standards prepared in the correspondingly diluted matrices if necessary.

Section VI

Table SVI.1: Allocation of habitat types / floral resource curves to landscape element types in the Merendree study area. The composition of plants used by bees in different habitat types (besides gardens) was mainly on work done within the B-GOOD project. The composition for the habitat types distinguished in the EUNIS habitat classification (see https://eunis.eea.europa.eu/), i.e., broadleaved deciduous woodland, coniferous woodland, mixed woodland, temperate shrub heathland, and seasonally wet and wet grasslands, was defined based on the 'Database of Relevant Resources for Honey Bees' (later adapted to pedo-climatic zones) developed within the B-GOOD project (Deliverable 3.1 & 3.3). The composition for urban parks and riverside plants and trees was defined based on the fieldwork done by David Claeys Boúúaert within the B-GOOD project (Deliverable 3.4). Information on crop types was taken from the Land Parcel Identification System for Belgium obtained for the B-GOOD project.

Code of type of landscape element in ALMaSS	Type of landscape element in ALMaSS	Habitat type / Floral resource curve	Habitat description	Source	Share of habitat type in the 10x10 km study area (Merendree, Belgium) [%]
5	Building	none	-	-	1.7
8	UrbanNoVeg	none	-	-	1.1
11	Garden	Garden	As no detailed information on the plant composition of gardens in the study area was available to us, we based the composition on data from Baldock *et al.* (2019). We extracted data on the floral abundance in gardens sampled by Baldock *et al.* (2019) in four cities in the United Kingdom (Bristol, Reading, Leeds, and Edinburgh); and we averaged them to obtain information on the composition and floral density per m^2 of habitat. Therefore, this habitat type represents a 'typical' garden of an urban area. The main plants visited by bees modelled in this habitat were: *Myosotis arvensis*, *Bellis perennis*, *Euphorbia peplus*, *Trifolium campestre*, *Spiraea chamaedryfolia*, *Aubrieta x hybrida*, and *Hyacinthoides non-scripta*.	Baldock *et al.* (2019)[1]	18.4

17	UrbanPark	Urban Park	In this habitat type, in the Atlantic zone, the main plants (share of > 5%) visited by bees and included in the modelling were: *Fagus sylvatica*, *Hedera helix* aggr., *Populus nigra*, *Acer pseudoplatanus*, *Picea abies*, *Quercus rubra*, and *Rhododendron ponticum*.	Fieldwork in the study area done by David Claeys Boúúaert within the B-GOOD project (Deliverable 3.4)	1.0
20	Field	Managed grassland	For simplicity, we excluded crop rotations from the simulations and assumed that all the fields in the landscape are managed as grasslands with clover grass cut for silage. This ensure the same distribution of floral resources for bees within the arable land in all the simulations.	-	61.2
40	DeciduousForest	Atlantic broadleaved deciduous woodland (G1)	"Woodland, forest and plantations dominated by summer-green non-coniferous trees that lose their leaves in winter. Excludes mixed forests (G4) where the proportion of conifers exceeds 25%" (EUNIS 2019). In this habitat type, in the Atlantic zone, the main plants (share of > 5%) visited by bees and included in the modelling were: *Quercus robur*, *Hedera helix* aggr., *Rubus fruticosus*, *Fagus sylvatica*, *Quercus petraea*, *Fraxinus excelsior*, *Corylus avellana*, *Alnus glutinosa*, *Pteridium aquilinum*, and *Carpinus betulus*.	Database of Relevant Resources for Honey Bees adapted to pedo-climatic zones; B-GOOD project (Deliverable 3.1 & 3.3)	2.2
50	ConiferousForest	Atlantic coniferous woodland (G3)	"Woodland, forest and plantations dominated by coniferous trees, mainly evergreen. Excludes mixed forests where the proportion of broadleaved trees exceeds 25%" (EUNIS 2019). In this habitat type, in the Atlantic zone, the main plants (share of > 5%) visited by bees and included in the modelling were: *Pinus pinaster*, *Pinus sylvestris*, *Pteridium aquilinum*, *Rubus fruticosus*, *Pinus nigra*, *Hedera helix* aggr., *Pseudotsuga menziesii*, and *Calluna vulgaris*.	Database of Relevant Resources for Honey Bees adapted to pedo-climatic zones; B-GOOD project (Deliverable 3.1 & 3.3)	0.3

56	Orchard	Apple orchard		-	0.3
60	MixedForest	Atlantic mixed woodland (G4)	„Forest and woodland of mixed broad-leaved deciduous or evergreen and coniferous trees. Neither coniferous, nor broadleaved species account for more than 75% of the crown cover" (EUNIS 2019). The composition of this habitat type was defined as an average from Atlantic broadleaved deciduous woodland and Atlantic coniferous woodland.	Database of Relevant Resources for Honey Bees adapted to pedo-climatic zones; B-GOOD project (Deliverable 3.1 & 3.3)	2.0
90	Freshwater	none	-	-	0.3
94	Heath	Atlantic temperate shrub heathland (F4)	„Shrub communities of nemoral affinities, in which *Ericaceae* are dominant or at least prominent. Such heaths are best developed on acid soils in the Atlantic zone and also in sub-Atlantic Europe" (EUNIS 2019). In this habitat type, in the Atlantic zone, the main plants (share of > 5%) visited by bees and included in the modelling were: *Calluna vulgaris*, *Erica tetralix*, and *Vaccinium myrtillus*.	Database of Relevant Resources for Honey Bees adapted to pedo-climatic zones; B-GOOD project (Deliverable 3.1 & 3.3)	< 0.1
96	River	none	-	-	1.7
98	RiversidePlants	Riverside plants and trees	In this habitat type, in the Atlantic zone, the main plants (share of > 5%) visited by bees and included in the modelling were: *Cirsium arvense*, *Tanacetum vulgare* and *Lamium album*.	Field work in the study area done by David Claeys Boúúaert within the B-GOOD project (Deliverable 3.4)	0.7
118	Railway	none	-	-	0.2
121	LargeRoad	none	-	-	2.7
122	SmallRoad	none	-	-	0.4

123	Track	none	-	-	1.3
205	NaturalGrassWet	Atlantic seasonally wet and wet grasslands (E3)	"Unimproved or lightly improved wet meadows and tall herb communities" (EUNIS 2019). In this habitat type, in the Atlantic zone, the main plants (share of > 5%) visited by bees and included in the modelling were: *Agrostis stolonifera* and *Holcus lanatus*.	Database of Relevant Resources for Honey Bees adapted to pedo-climatic zones; B-GOOD project (Deliverable 3.1 & 3.3)	3.0
207	Stream	none	-	-	0.2
213	IndividualTree	Stand-alone trees	The type of tree was randomly assigned from the following list: *Castanea sativa*; *Tilia platyphyllos*; *Fagus silvatica*; *Quercus robur*; *Robinia pseudoacacia*.	-	<0.1
219	Pond	none		-	<0.1
222	DrainageDitch	none		-	<0.1
223	Canal	none		-	1.3

[1] Baldock, K. C., Goddard, M. A., Hicks, D. M., Kunin, W. E., Mitschunas, N., Morse, H., ... & Memmott, J. (2019). A systems approach reveals urban pollinator hotspots and conservation opportunities. *Nature ecology & evolution*, *3*(3), 363-373.