Manuscript on the interactive effects of agrochemicals, nutrition and pathogens on novel wild bees

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Sara Hellström[1], Alexandre Barraud[2], Denis Michez[2], Robert J. Paxton[1]

[1] *MLU*

[2] *UMONS*

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Summary

The morphology, physiology and ecology of wild bees differ greatly among species, and with that, the impact of stressors on their health. It is therefore of great importance to conduct research on a broader range of species, and determine what stressors might be of particular concern for them. In order to conduct such research, additional model species need to be developed. In this report we outline method development and results from pilot studies on the ivy bee *Colletes hederae* and the hairy-footed flower bee *Anthophora plumipes* exposed to nutritional, pathogen and pesticide stressors. As we lay the groundwork for further studies on the risks of known bee pathogens on the host *Anthophora plumipes*, we expose *A. plumipes* to two viruses (deformed wing virus genotypes A and type B), a trypanosome gut parasite (*Crithidia mellificae*) and a microsporidian (*Nosema ceranae*), as these are pathogens that bees are likely to encounter in the natural environment. We could not confirm that any of the pathogens tested could readily replicate in *A. plumipes,* after controlled inoculation in the laboratory. A subsequent experiment combining the trypanosome gut parasite and a fungicide stressor yielded no conclusive results, since the pesticide stressor caused 100% mortality, preventing assessment of infection intensity.

The ivy bee (*Colletes hederae*) was collected in the larval stage and attempts were made to modulate the larval food sources, but larval mortality was 100% in all trials.

We did not perform any successful interaction experiments of agrochemicals and other stressors since the establishment of methods for rearing and handling these novel species are still in the early stages. Of the two species tested in the laboratory, *A. plumipes* is a promising candidate for further laboratory and field based experimental work, as their rearing and handling is functioning well across the PoshBee project's duration. Despite the limitations presented here, we provide important insights on pathogen stressors on a hitherto unexplored host bee species in the Anthophorine subfamily, and lay the groundwork for further research into the health effects of stressors on ground-nesting bee species, a significantly under-studied group.

1. Introduction

There is widespread concern about declines in wild bee abundance and diversity across Europe and the globe, with a considerable fraction of species in decline, or data deficient, while other, mainly generalist species, are stable or increasing (Nieto et al., 2014; Powney et al., 2019). The causes of bee declines are multifactorial, with habitat and resource loss (associated with agricultural expansion), pesticide use, climate change and spread of disease-causing organisms generally considered to be likely culprits (Brown et al., 2016; Dicks et al., 2021). These stressors may interact to form a complex set of risks posed to bees in the modern, anthropogenic landscape (Vanbergen et al., 2013). The vast majority of bee species are understudied in terms of how they respond to stressors. In the Poshbee project, we aim to test the two risk factors, pathogens and nutritional deficiency, on novel wild bee species, which are hitherto unexplored in this context.

Disease causing agents associated with the domesticated western honey bee (*Apis mellifera*) have been considered an emerging threat to wild pollinators (Graystock et al., 2014). The risk of disease transmission from managed pollinators is thought to depend on co-foraging on floral resources (Graystock et al., 2015). Artificially dense populations of managed pollinators may result in increased transmission of pathogens and thus in higher disease prevalence (Fürst et al., 2014). The presence of pathogens associated with managed pollinators in wild conspecifics and other host species has been identified through various field studies, with up to 80% of wild bees harbouring at least one known honey bee-associated virus (Ravoet et al., 2014; Dolezal et al., 2016). The impact of these pathogens

on novel host species fitness (i.e., pathogenicity), or whether their presence in field samples indicates true infection (i.e., replication of the pathogen in the novel host as opposed to mere harbouring of the pathogen), is poorly understood.

Laboratory studies have confirmed the infectivity of various trypanosomatids of the genus *Crithidia* in several solitary bees (Strobl et al., 2019; Ngor et al., 2020; Pinilla-Gallego and Irwin, 2022), while inoculation experiments with *Nosema ceranae* have yielded varying results (Müller et al., 2019; Ngor et al., 2020). Screenings of wild-caught *A. plumipes* have yielded positive signals for the presence of deformed wing virus (DWV) and black queen cell virus (BQCV) (Radzevičiūtė et al., 2017), and for *N. ceranae* (Müller et al., 2019).

Additionally, there are likely still a large number of undiscovered microorganisms, including viruses, present in wild bees, some of them disease-causing agents, as suggested by high-throughput RNA sequencing studies (Schoonvaere et al., 2018). Overall, there is a dearth of controlled laboratory studies on wild bee species investigating the infectivity and potential negative impact (i.e., virulence) of disease-causing agents, and in turn, their impact on wild bee populations cannot be accurately assessed.

Agricultural expansion, resulting in landscape simplification, has been identified as an important driver of pollinator decline (Winfree, 2010; Persson et al., 2015; Vray et al., 2019). This can directly or indirectly affect the quality, the quantity and the diversity of floral resources and thus the food sources of bees (e.g. Roger et al., 2017). This makes the abundance, distribution/availability, quality and diversity of these resources potentially a main proximal pressure explaining bee population trends (Roulston and Cane, 2000; Vaudo et al., 2020). Bees obtain their carbohydrate nutrient intake mainly from nectar, and their protein and lipids from pollen (Roulston and Cane, 2000; Nicolson, 2011). The chemical composition of pollen is highly variable across floral species, with between 2-60% and 1-20% for protein and lipid content, respectively (Roulston and Cane, 2000; Vaudo et al., 2020). Field and semi-field studies showed that this chemical composition can be related to bee health (e.g. in the honey bee *A. mellifera*: Alaux et al., 2010; Brodschneider and Crailsheim, 2010; Di Pasquale et al., 2013; in the mason bee *Osmia bicornis*, Bukovinszky et al., 2017). Generalist bees seem able to assess pollen's chemical quality and balance multiple macronutrient resources when making foraging decisions (Vaudo et al., 2016, 2018; Kraus et al., 2019; Ruedenauer et al., 2020). Based on a large quantity and diversity of samples, Vaudo *et al.* (2020) showed that honey bees collected pollens with a 1:1 and 2:1 protein to lipid (P:L) ratio. The honey bee therefore appears to occupy a different nutritional space compared to *Bombus impatiens* and *Osmia cornifrons*, which collect at P:L ratios of 4:1 and 2:9, respectively. Furthermore, to satisfy the food intake of colonies with numerous individuals, honey bees must collect large amounts of pollen. Therefore, honey bees collect pollen from 'generalist flowers', those with open floral morphologies such as those typically found in mass blooming trees (e.g. the anthers of *Quercus* spp., *Salix* spp., *Prunus* spp.) and wild herbs with a high production of pollen (e.g. Asteraceae), which may have a nutritional make up that falls in the lower P:L values (i.e. 1-3:1 P:L) (Vaudo et al., 2020). Bumble bees are much more picky in their choices, since many species mainly forage on Fabaceae pollen showing a high P:L ratio value (Leonhardt and Blüthgen, 2012; Wood et al., 2021). In contrast to honey bees and bumble bees, *Osmia cornifrons,* a solitary foraging bee with a short flight period, has mixed preferences for Rosaceae and Fabaceae pollen (Haider et al., 2014; Nagamitsu et al., 2018), with average P:L ratios of 1.6 ± 0.3 and 3.8 ± 0.5, respectively. Specialist species, such as *Colletes hederae*, feed almost exclusively on a single plant family, resulting in less variability in their diet. Few studies have been carried out on these oligolectic bees, and it would be interesting to know more about the effects of a change in pollen diet, especially in the context of a decrease in plant diversity that could lead to a decrease of host plants.

Regarding chemical profiles, particular lipids and proteins seem more important for bee nutritional requirement. For example, sterols (e.g. β-sitosterol) are essential to synthesize ecdysteroid, involved in the moulting of larvae and maturation of the ovaries of female imagos. In the case of sterol deficiency, a delay in moulting can be observed (Regali, 1996). Additionally, a good amino acid balance is also crucial for bee development (Moerman et al., 2016). They are involved in growth, survival, flight ability and in immunity (Regali, 1996; Carter et al., 2006; Moerman et al., 2016). Some amino-acids (methionine, lysine, threonine, histidine, leucine, isoleucine, valine, phenylalanine, tryptophan) and sterols (24-methylenecholestrol and β-sitosterol) cannot be synthethised by the bee and are therefore considered essential, meaning that it is necessary to obtain them through pollen consumption (De Groot, 1953; Svoboda et al., 1978; Behmer and Nes, 2003). It is largely unknown how oligolectic species, which are used to feeding on pollen with a specific chemical profile, would develop on other pollen diets.

In order to elucidate the effects of pathogens and nutritional stress in a wider variety of species, the PoshBee project has developed protocols to rear and handle two solitary bee species: *Anthophora plumipes* (Apidae:Apinae) and *Colletes hederae* (Colletidae) under controlled conditions. *Anthophora plumipes* is a widespread, univoltine ground-nesting species active from March to June across Europe. It is polylectic with a preference for flowers with a long corolla such as *Lamium* spp. We performed experimental inoculation of pathogens and later co-exposure to agrochemicals in *A. plumipes,* using two viruses (deformed wing virus genotype A and type B), a trypanosome gut parasite (*Crithidia mellificae*) and a microsporidian (*Nosema ceranae*), as these are pathogens that bees are likely to encounter in the natural environment. *Colletes hederae* is a species that has recently expanded its geographic range and is oligolectic on ivy pollen (*Hedera helix*), active late in the season between August and October. In order to elucidate the relationship between diet composition and health in an oligolectic bee, we aimed to manipulate its larval provisions in order to measure the effects of diet on emergence and body size, with the aim to later include agrochemical stressors in the larval provisions.

This is, to our knowledge, the first controlled laboratory study with *A. plumipes,* and the first attempt to inoculate an anthophorid bee with pathogens. It is also the first attempt to rear and maintain adults and larvae of *C. hederae* in the laboratory.

2. Methods: Pathogen exposure of *Anthophora plumipes*

2.1. *Anthophora plumipes* acquisition and maintenance.

This study was conducted on the hairy-footed flower bee *A. plumipes.* All individuals were collected from brood cells taken from soil-packed nesting units placed in the field close to an existing nesting aggregation of the species in the Botanical Gardens of the University of Halle, Germany (MLU). The brood cells were extracted from the soil matrix by hand and subsequently overwintered at 4°C. At the start of experiments, brood cells were transferred to a 21°C incubator and emergence from brood cells was checked daily (Figure 1). Freshly emerged adults were transferred back to 4°C until enough individuals had emerged to populate the tests. Bees were housed in groups of 4-10 individuals, separated by sexes, in hoarding cages of clear plastic (24*16*14 cm) fitted with a 5 mL Eppendorf tube filled with 50% w/v sucrose solution. Paper towels and sometimes clay cells from which bees had emerged were added as enrichment. Cages were kept in room temperature (i.e. 23±3°C) and daylight conditions.

Figure 1. Station for individual feeding of *A. plumipes* (left) and recently emerged male with brood cell (right).

2.2. Exposure to deformed wing virus genotype A & B

2.2.1. Preparation of inoculum and viral injection

Purified virus inocula propagated from a local honey bee stock (MLU, Germany, see Tehel et al. 2019) was diluted to 10^7 viral particles/µL with phosphate buffered saline (PBS). Bees were cold-anesthetized for 20 minutes at 4˚C before 1 µL of solution was injected between the second and third tergite of the abdomen using a Hamilton syringe. The control treatment was an injection of 1 µL PBS. An additional trial with BQCV injection was planned within the scope of Deliverable D4.2 but later excluded due to lack of individuals to populate the test. *A. plumipes* males and females were housed separately (see above). As a control to ensure that viral inocula were infective, *A. mellifera* were similarly inoculated with DWV and then housed in metal cages with ad libitum 50% w/v sugar, with n=20 bees /cage and at 25˚C. Bees were sampled at day 6 post-inoculation and directly frozen at -80˚C.

2.2.2. Molecular quantification of virus particles

Entire bees were crushed and total RNA was extracted using standard protocols (Tehel et al. 2019). Viral titre per individual was assessed via quantitative real-time PCR (QuantStudio 3) run in duplicates and using a standard curve of serially diluted purified viral inoculum of known quantity (see Tehel et al. 2019 for more details). Control bees were screened for both viral genotypes and found to be clean.

2.3. Oral exposure to *Crithidia mellificae* and Amistar

2.3.1. Cell cultivation, oral inoculation and Amistar exposure

A starting culture of *C. mellificae* (ATCC ® 30862™) in 0.5 mL aliquots was stored at -80˚C. To initiate culturing, an aliquot was defrosted in a 35˚C water bath and immediately added to 5 mL of 25˚C autoclaved ATCC cell culture medium in 10 mL cell culture tubes. The culture tubes were tightly sealed and incubated at 25˚C. Cultures were assessed daily by counting motile cells/µL in two replicates using a Fuchs-Rosenthal haemocytometer placed under a phase-contrast microscope. Peak density was normally reached within 3-5 days from initiation, after which the number of cells decreased.

Since previous attempts at individual feeding of *A. plumipes* had resulted in many non-feeders, and the number of individuals were limited in this experiment, group feeding was deployed. In hoarding cages (24*16*14 cm), 5-7 individuals were allowed to acclimatize for 12 hours with a fully stocked feeder. The feeder was then removed and bees were starved overnight. Oral inoculum was prepared by mixing medium containing a determined amount of cells/µL with 25% w/v sugar solution, creating a 12.5% w/v sugar solution and cell culture medium mix. Motile cells/µL were counted prior to each dose preparation. Each infective dose contained 20,000 *C. mellificae* cells in 10 µL of medium mixed with 10 µL 25% w/v sugar solution, creating a 20 µL 12.5% w/v sugar solution and cell culture medium mix. After that, an identical feeder with oral inoculum containing 40 uL of solution with 20,000 cells per individual in the box was presented (e.g. 200 µL inoculum presented to five individuals). After 6 hours, the inoculum was fully consumed in all cages, and 50% sugar solution was again provided. In half of the cages, the sugar solution was spiked with azoxystrobin (2 g/L a.i) in its commercial formulation Amistar (250 g a.i./l, SC, Syngenta, UK). After six days, remaining bees were freeze-killed and stored at -20°C until further processing. Infection intensity data from a previous experiment using *Osmia bicornis* were included as positive controls, as they had been infected with *C. mellificae* from the same cell culture line.

2.3.2. Molecular quantification of *C. mellificae*

Freeze-killed bees were thawed and rinsed in 30% sodium chloride to exclude any surface contamination. The stinger, hindgut and midgut were removed and put in 100 uL PBS buffer. DNA extraction of the removed gut was performed with a DNeasy minikit (Qiagen, Germany) using the manufacturer's protocols. The total number of *C. mellificae* cells was quantified by qPCR. Using a 2-fold increasing standard curve made of DNA extracted from cell culture samples with a known concentration of cells (10,000-80,000), the total number of cells per gut sample could be determined by qPCR. Each 10 µL qPCR reaction consisted of 1 µL DNA, 0.2 µL of each primer CriRTF2 and CriRT2 (Ulrich et al., 2011), 3.6 µL DEPC water, and 5 µL Sensi-MixPlus SYBR & Fluorescein Kit (SYBR-Green; Bioline, Luckenwalde, Germany). Reactions were run in a Bio-Rad C1000 thermal cycler (Bio-Rad, Munich, Germany).

2.4. Oral exposure to *Nosema ceranae*

2.4.1. Inoculum preparation

In this experiment, male and female *A. plumipes* were inoculated orally with a known quantity of *N. ceranae* spores in order to determine whether they are a permissive host for the pathogen (i.e. if the pathogen can replicate within the host's body). Honey bee (*Apis mellifera*) workers were infected in parallel as a positive control for the treatment. Inoculum of *N. ceranae* was prepared from local honey bees (MLU, Germany) using standard methods (Fries et al., 2013). In brief, honey bee workers sampled from a colony heavily infected with *N. ceranae* were kept in an incubator for ca. 14 days then their ventriculi removed and crushed in potassium sulphate buffer. Spores were counted in a Fuchs-Rosenthal haemocytometer placed under a phase-contrast microscope (x400) and found to be present at levels of 1.25×10^6 spores/bee. Spores were purified using previously described methods (Eiri et al., 2015).

2.4.2. Oral inoculation, quantification

As a positive control to check on spore viability, honey bee workers were inoculated by feeding with 10^5 spores per bee using standard methods for manual, individual feeding (Fries et al., 2013). *Anthophora plumipes* males and females were inoculated individually using a modified version of the

"petal method" (Ladurner et al., 2005). In brief, individuals were allowed to starve overnight before being transferred to individual feeding cups (Figure 1) and presented with the inoculum or a sham treatment pipetted into a small plastic ampoule next to a glued-down flower of *Lamium purpurea.* Individuals were freeze-killed for screening at days 5 and 10 post-inoculation.

For visual quantification of spores from experimental bees, individual guts were dissected and crushed with a pestle in 100 µL potassium sulphate buffer. The presence of spores and/or quantification of spores was performed in a Fuchs-Rosenthal haemocytometer placed under a phase-contrast microscope (x400). Three samples were taken from each gut suspension and the average number of spores in the samples was used to calculate the total number of spores per bee. In cases where the spore density was too high, gut suspensions were diluted with an extra 500 µL of buffer.

3. Results: Pathogen exposure of *Anthophora plumipes*

3.1. Viral replication in *A. plumipes*

Under inoculation via injection, the DWV titres in *A. plumipes* were consistently below the titre initially injected, indicating that the virus, allthough present, did not enter an exponential growth phase. In comparison, the titres in *A. mellifera* increased from the start inoculum to an estimated mean genome equivalent titre of 5.89×10^{10} and 5.31×10^{9}, for DWV-A and DWV-B, respectively (Figure 2).

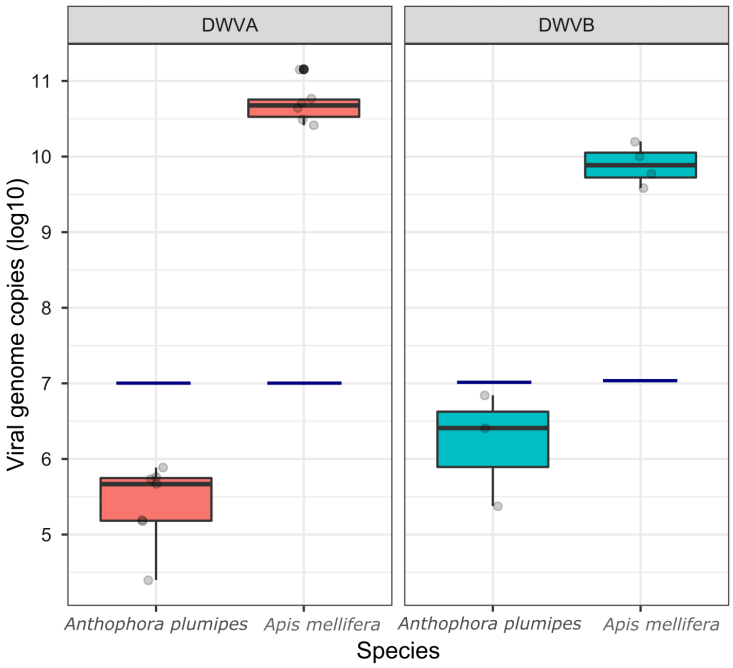

Figure 2. Comparative viral genome equivalents (log-transformed) for *A. plumipes* and *A. mellifera* infected with deformed wing virus genotype A (left) and deformed wing virus genotype B (right) respectively. The horizontal line represents the amount inoculated.

3.2. Replication of *C. mellificae* in *A. plumipes*

At day six post-inoculation, 100% of the Amistar exposed bees were dead, preventing a comparison of pathogen intensity between the two treatments. The absence of a control group also prevented the comparison of survival between infected + exposed and unexposed individuals. In the inoculated group, only 1 of 6 males and 2 of 10 females tested positive for *C. mellificae* after 6 days, but with

estimated cell counts only reaching a maximum of circa 300 cells per bee. In comparison, in a subset of *O. bicornis* screened at 6 days post-inoculation, 100% of screened individuals tested positive, with estimated titres ranging from 1.4 x 10^6 to 1.15 x 10^5 cells per bee (Figure 3).

3.3. Replication of *N. ceranae* in *A. plumipes*

Guts of *A. mellifera* screened at day 20 post-inoculation were all heavily infected, with spore titres ranging from 2.33 x 10^7 to 5.53 x 10^7 per bee. In contrast, gut samples from *A. plumipes* were devoid of spores in 8 of 10 females and 4 of 5 males screened. Where spores were present, the estimated titres reached at most 40,000 cells/bee, which corresponds to the estimated number of spores inoculated (Figure 3).

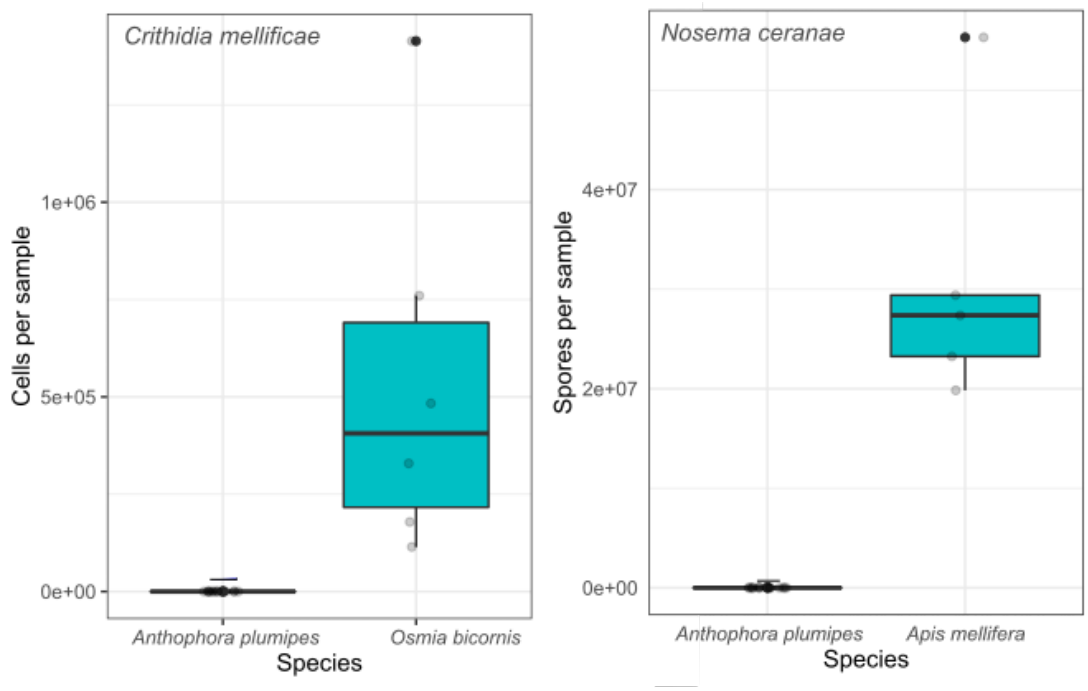

Figure 3. Comparative cell/spore counts for *A. plumipes, O. bicornis* and *A. mellifera* infected with *C. mellificae* (left) and *N. ceranae* (right) respectively.

4. Methods: Nutritional modulation in *C. hederae*

In August 2018, we first tried to induce reproduction of *Colletes hederae* under semi-field conditions. To do so, greenhouses were erected in the UMONS campus (Figure 4).

Figure 4. *C. hederae* greenhouse, with ivy flower and nidification surface in the back.

A net-greenhouse was built in the UMONS campus and positioned to receive as much sunshine as possible. A layer of 40 cm of sandy soil was deposited in the back of the greenhouse to allow bee nesting, and ivy flowers were collected and added in bouquets for foraging (Figure 4). Approximately 200 bees were captured from neighbouring field sites and put in the greenhouse.

The bees successfully started to mate and dig a nest (Figure 5), but almost no brood cells were found in the soil.

Figure 5. Female of *C. hederae* nesting in a semi-field cage.

Due to the difficulty of inducing reproduction under semi-field conditions, we proceeded to dig up newly formed brood cells in which the egg had not yet hatched. To do so, brood cells (Figure 6) were collected at a large nesting site in Belgium called “La grande Bruyère”. Approximately 100 brood cells were collected during September-October 2019/2020, and used for laboratory assays.

Figure 6. Brood cells of *C. hederae.*

We first tried to manipulate the pollen resource of the larvae. To do so, the top of each brood cell was cut with a scalpel and the egg or larva was removed from the brood cell using a small brush. Each egg/larva was then put on either a new or its own pollen resource in a 48 well plate, placed in an incubator at 21°C.

In another attempt, we tried to keep the egg in the brood cell and sucked up the pollen resource (brood provisions) with a syringe to replace it with a new one. Unfortunately, the viscosity of the pollen resource did not allow us to do so without damaging the brood cell.

Finally, to find out whether it was possible to reach the cocoon stage in the laboratory while keeping the brood cell intact, we placed a total of 45 brood cells in plastic boxes filled with moist soil from the nesting site (Figure 7). Boxes were placed in an incubator at 21°C. Then, each brood cell was carefully monitored every 3 days for 8 weeks to check on larval development.

Figure 7. Box filled with 1 kg of moistened soil with *C. hederae* brood cells inside.

5. Results: Nutritional modulation in *C. hederae*

After two weeks in the 48 well-plates, every individual was dead. Those being fed with their own diet from their brood cell did not survive longer than those being fed with a new diet, which suggests a problem in the rearing conditions. Larvae from entire brood cells that were put in nesting soil did not survive either, with all individuals dead after one month.

6. Discussion

In this series of pilot assays, we did not find evidence that the common honey bee-associated pathogens *C. mellificae*, *N. ceranae* and the viruses DWV-A and DWV-B, can replicate in *Anthophora plumipes*. In contrast, *B. terrestris* also belonging to the Apidae family, has been successfully infected using similar techniques and inoculum as presented here (Tehel et al., 2020). This does not, however, answer the question of whether other viruses, trypanosomes or microsporidia are present in *A. plumipes* throughout its natural range, or whether they negatively affect fitness in a potentially infected individual. Infectivity and pathogenicity can vary greatly between closely related strains of the same pathogen and between host populations, as has been shown in the *B. terrestris-Crithidia bombi* system (Barribeau et al., 2014). Thus, the assays performed here do not exclude the existence of other strain-population combinations in which the tested pathogens are infective to *A. plumipes.*

In order to design relevant pathogen and pesticide studies, ideally one should base the combination of stressors on solid screening data from multiple field populations of the host species in question. This was outside of the scope of the present work package, and little such data was previously available for *A. plumipes* (but see Radzevičiūtė et al., 2017; Müller et al., 2019). Moving forward, the interaction between fungal pathogens and agrochemical stressors requires additional attention. When looking at the natural mortality of *A. plumipes* during development, multiple entomophathogenic fungi (e.g., *Ascosphaera* sp. which cause chalkbrood disease) are the likely culprit (see Poshbee D4.1). *Ascospaera spp.* have been found in multiple solitary bee species during field sampling (Evison et al., 2012; Ravoet et al., 2014). Using the proposed nest block design developed within PoshBee, one can easily extract *A. plumipes* brood provisions, larvae and pupae at various developmental stages for subsequent experiments in the laboratory. A relevant line of research would be to develop experimental protocols by which the resistance to these fungal pathogens can be explored with or without agrochemical stressors, a combination of stressors which have been found to be additive in another solitary bee (Krichilsky et al., 2021).

So far, we did not find a way to rear *C. hederae* under semi-field conditions. It is hard to fully understand why our trials did not work, but we suppose, firstly, that the greenhouse may be stressful for the bees and, secondly, that the nesting substrate was not optimal. In fact, *C. hederae* likes deep soil and vertical sandy surfaces, and maybe our sandy nesting substrate was simply not vertical/deep enough.

Additionally, the larval rearing experiment in the laboratory was unsuccessful. We did not manage to get any pupae to develop once the brood cell was opened. This problem is probably due to the fact that the brood cell maintains the larvae under very specific environmental conditions that we failed to re-create in the incubator. As we did not see any parasite or fungus on the dead larvae, this suggests that the failure in development is probably due to the temperature or humidity to which the larvae were exposed. The next step would be to try to create artificial brood cells in which to place the egg and brood cell provisions. However, what we have tried by monitoring intact brood cells in the soil

suggests that we first need to find appropriate environmental conditions that permit larval development. It may be that colletid bees, with their cellophane-like brood cells, may not be the optimal candidate for solitary bee larval rearing and nutritional modulation experiments in the laboratory. Using the methods developed for extraction of brood cells from field populations, it may instead be possible to perform nutritional modulation experiments on *A. plumipes* larvae in the future as their provisions and eggs can be readily moved from brood cells to well culture plates, although this methodology needs further refinement.

The method development presented in this study provides solid information for further research into anthophorid bees, which have so far never been used to test the impact or infectivity of any pathogen.

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