Manuscript on agrochemical and pathogen effects on the health of the solitary bee *Osmia bicornis*

Deliverable D6.4

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Sara Hellström[1], Matthias Albrecht[2], Robert J. Paxton[1]
[1] MLU,
[2]Agroscope

PoshBee

Pan-European assessment, monitoring, and mitigation of stressors on the health of bees

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Summary

The impacts of pathogens in wild bees remain poorly investigated outside of a few bumble bee model species. The vast majority of wild bees are solitary, with a very different life history compared to eusocial or semi-social species. The prevalence and pathogenicity of known insect pathogens, and much less their potential interaction with pesticide stressors, remains largely unexplored in solitary bees. The initial challenge of Deliverable 6.4 was to establish a working model system in which to study pathogen stressors in the model solitary bee *Osmia bicornis*. We attempted inoculation via injection of the two honeybee-associated RNA viruses, deformed wing virus A and deformed wing virus B, but could not establish viral replication in *O. bicornis* over a 10-day period. Thereafter, we instead pursued the trypanosome gut parasite *Crithidia mellificae*, which has previously been shown to infect a variety of solitary bees. Laboratory-based inoculation experiments yielded positive results as the parasite infected the vast majority of exposed individuals. An interaction experiment with the novel insecticide flupyradifurone and the herbicide glyphosate was conducted, showing that neither of the substances caused increased replication of the pathogen, or significant host mortality in the short term.

In order to explore the lifetime fitness effects of *C. mellificae* parasitism on *O. bicornis* females, we moved to a semi-field cage setup to enable the lifetime reproductive output of the bees as a measure of harm induced by the parasite. This is, to our knowledge, the first controlled cage experiment testing the effects of a pathogen on a solitary bee. At Agroscope Reckenholz, Switzerland, a fully factorial experiment was set up in order to explore the fitness impacts of *C. mellificae* infection with and without spray exposure of the insecticide flupyradifurone (Sivanto™). A trap-nest design with individually marked bees and video filming at the nest entrances analysed with the BeeTracker software, developed within PoshBee, allowed for daily observations of flight behaviour and individual brood provisioning per day. We found weak evidence of increased mortality and decreased establishment success directly following *Crithidia* infection, as well as an overall effect of *Crithidia* on lifespan of infected individuals. There was no evidence of effect of insecticide spray treatment on mortality. In individual bees that successfully established nests, there was no evidence of increased mortality of the pesticide exposure alone or in combination with infection treatment. Foraging trip duration and number of attempts by a female to find the correct nest increased with time, but did not differ across treatments. When considering the number of brood cells produced per female, there was a decrease in the pathogen-only treatment, but not in the insecticide treatment or the mixed treatment. When brood cells produced were divided by number of days a mother bee was active, this effect disappeared, indicating these adverse impacts of *Crithidida* infection were primarily driven by reduced nesting female longevity rather than daily offspring production rate. Offspring survival did not differ between those produced after insecticide treatment and those that were not sprayed, nor did the sex ratio or body size of offspring differ between treatments.

In these experiments, we show that *C. mellificae* readily colonises the gut of *O. bicornis* upon oral inoculation. We find only weak negative effects of trypanosome infection on the lifetime fitness of *O. bicornis*. More importantly, we find no evidence that the two tested agrochemicals affect the parasite's intensity in the gut upon oral pesticide exposure in the laboratory, and no evidence of negative impact of flupyradifurone spray treatment on *O. bicornis* in the semi-field alone or in combination with a pathogen.

1. Infectivity of honey bee-associated viral pathogens in *O. bicornis*

1.1. Introduction

Wild bees carry a large variety of micro-organisms, including eukaryotic parasites, bacteria, fungi and viruses, many of which have not yet been characterized, and many whose role as pathogens or commensals has not yet been explored (Schoonvaere et al., 2018). The study of bee (Aculeata: Anthophila) pathogens is, by necessity, centred around the managed honey bee (*Apis mellifera*), where destructive pathogens are widely considered an ongoing threat to worldwide honeybee stocks. The link between bumble bee (*Bombus* spp.) associated pathogens and their population decline has received partial attention. Pathogens in solitary bees are rarely experimentally investigated, although recent studies have demonstrated *O. bicornis* tolerance to *Nosema ceranae* infection, and the presence of, and increased mortality caused by, *Apicyctis bombi* (Tian et al., 2018; Müller et al., 2019).

There are several viruses associated with decreased survival and compromised colony performance in honey bees. These viruses have been show to spread to wild bee populations, sometimes also replicating in other hosts (Fürst et al., 2014; Manley et al., 2019). There is evidence that certain pesticides can alter immune responses and thereby exacerbate the impact of viral infection in honey bee hosts (Doublet et al., 2015; O'Neal et al., 2019). Currently, little is known about how these viruses behave in solitary bees. Honey bee-associated viruses have recently spread across a large proportion of the world's honey bees, driven by the parasitic mite *Varroa destructor*. Deformed wing virus is a single-stranded RNA virus which, in honey bees, causes morphological impairments in adults infected at the pupal stage. Using methods adapted for honey bee and bumble bee viral research (Tehel et al., 2020), we aimed to establish whether *O. bicornis* was a permissive host for the well-studied viral pathogens deformed wing virus genotypes A (DWV-A) and B (DWV-B). The mode of infection was injection of viral particles directly into the haemolymph of the adult solitary bee in order to maximise the likelihood of infection. Using this admittedly unnatural scenario, we aimed to determine whether the pathogen has the capacity to replicate in the novel host's cells by comparing the viral titre present in a bee's body days after infection to the administered amount. Honey bees were infected simultaneously in order to i) assure that the inoculum was viable, ii) assess the viral titre in honey bee versus *O. bicornis.*

1.2. Methods

1.2.1.1. Test organisms

Osmia bicornis in diapause were supplied by PoshBee partner Wildbiene & Partner (WILD). From arrival to test initiation, cocoons were kept in the dark at 4°C. To break diapause, cocoons were incubated at 21°C and emerged bees were collected twice per day. Based on the size difference of cocoons, males and females were sorted and incubated separately to prevent mating activity. *Apis mellifera* capped brood in a frame was obtained from a local colony. The frame was incubated at 35˚C and freshly emerged workers were collected after 12-24 hours.

1.2.1.2. Infection treatment

Viral inocula propagated from those of Tehel et al. (2019) were diluted to 10^7 genome equivalents/µL with potassium phosphate buffer (PPB). 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 (hypodermic needle outer diameter: 0.235 mm). The control treatment involved injection of PPB only. Injection treatments were identical between species, and injections were administered by the same person on the same day.

1.2.1.3. Housing and sampling

Osmia bicornis males and females were housed separately in metal cages (10 x 10 x 6 cm; n=6 bees/cage) with *ad libitum* 50% w/v sugar solution at 21°C. *Apis mellifera* were housed in identical cages, with n=20 bees/cage and at 25˚C. Bees were sampled at days 6 and 10 post-inoculation.

1.2.1.4. Molecular detection of viral targets

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.

1.2.1.5. Statistical analysis

Viral titres were $\log_{10}$-transformed and compared between sexes of *O. bicornis* and days using Wilcoxon rank-sum exact tests. Mean titres were found not to differ significantly between day 6 and 10 ($p = 0.23$), not between sexes (DWV-A: $p=0.32$; DWV-B: $p=0.51$) and were subsequently pooled for comparison between species. Mean viral titres between species for the two genotypes were compared using a two-sample Wilcoxon rank-sum exact test, as the assumption of normality was not met in all groups.

1.3. Results

There was little direct evidence of replication of DWV-A or DWV-B in *O. bicornis*. All individuals (except one male) displayed lower viral titres than the titre present in the inoculum after both 6 and 10 days. Conversely, the viral titre in honeybees increased rapidly across the first three days post-infection, highlighting the different responses of the two hosts (Table 1. Figure 1.), and the final viral titres differed significantly between species (DWV-A: p=<0.001; DWV-B: p=0.0015).

Table 1. Mean (SD) viral genome equivalent titres per species and sex. *Osmia bicornis* values were pooled across sampling days

Species	Virus	n screened	Amount injected	Mean viral genome eq.	SD
Apis mellifera	DWV-A	6	1.0e+07	5.89e+10	4.28e+10
	DWV-B	4	1.0e+07	8.91e+09	5.31e+09
O. bicornis m	DWV-A	6	1.0e+07	9.46e+05	1.29e+06
	DWV-B	7	1.0e+07	5.87e+06	8.01e+06
O. bicornis f	DWV-A	11	1.0e+07	4.05e+05	4.05e+05
	DWV-B	6	1.0e+07	2.38e+06	2.80e+06

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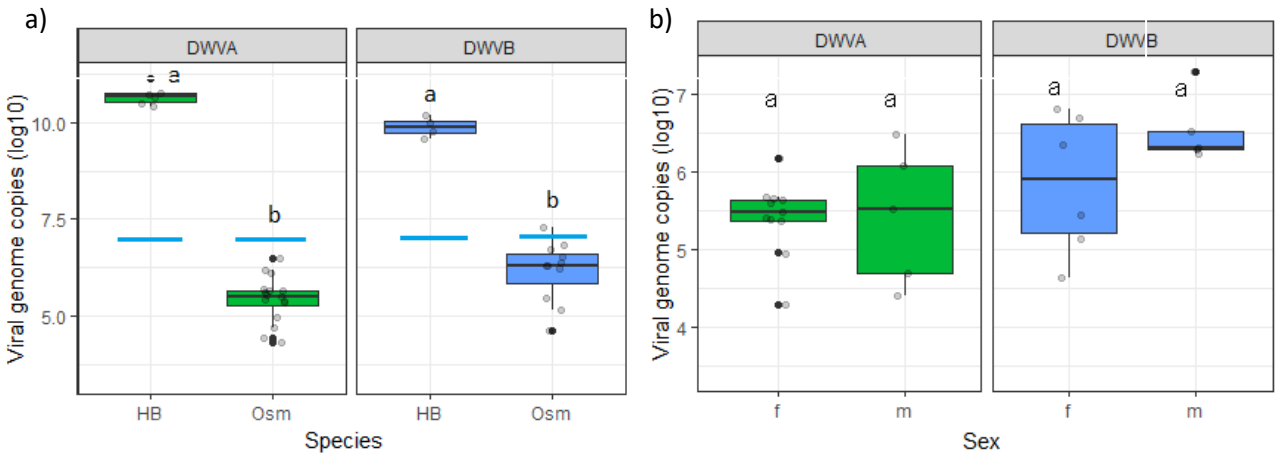

Figure 1. a) Boxplot showing viral genome copies per bee for honey bees (HB) and *O. bicornis* (Osm). b) Boxplot showing viral genome copies per bee for *O. bicornis* sexes: females (f); males (m). In species comparisons (left pair of plots), the blue bars represent the inoculum injected per bee (10^7). Different letters indicate statistical differences between means (Wilcoxon rank-sum exact test).

1.4. Discussion

We found no evidence of increase in viral titre across either sex of *O. bicornis* for the two viral pathogens DWV-A and DWV-B. The fate of the viral particles in the body of the novel host after injection into the haemolymph is not certain. The viral particles were still present at 10 days post-inoculation, but did not appear to enter an exponential growth phase, as was the case in the reservoir host, *A. mellifera*. In light of these findings, it is interesting to note that the PoshBee Deliverable 2.3: Report on exposure of bees to pathogens screened *O. bicornis* from the field for the two virus genotypes and found relatively high prevalence across countries. Whether these screened individuals have an ongoing infection of these viruses, or whether they simple carry the pathogen in their gut or on their body, cannot be determined. For the purpose of investigating the interactive effects of a pathogen and agrochemicals, we opted to not work with this viral pathogen since the outcome of this trial could not confirm establishment of infection of *O. bicornis* in the laboratory.

2. Trypanosome *Crithidia mellificae* infection in *O. bicornis*

2.1. Introduction

Having concluded that the tested viral pathogens (see above) were not causing noticeable infection after inoculation by injection, we decided to pursue the trypanosome parasite *Crithidia mellificae* as a potential model pathogen system. *Crithidia* spp. are single-cell, flagellated trypanosomatid parasites that infect the rectum and ileum in a number of insect orders, and are transmitted faecal-orally. *Crithidia mellificae*, which was first isolated from honeybee guts (Langridge and McGeeh, 1967), appears to utilize a wide variety of hymenopteran hosts, and has been detected in *Vespula squamosa* and *O. bicornis* (Schwarz et al., 2015). In honey bees, the pathogen is widespread, but its direct effect on colonies is unclear, though its presence appears to be a predictor of winter mortality (Ravoet et al., 2013). Experimental infection has shown its ability to infect multiple bee species (Ngor et al., 2020), and *C. mellificae* grew significantly in number and had a deleterious effect on male but not female survival in *O. cornuta* (Strobl et al., 2019). Flupyradifurone, marketed by Bayer Crop Science in its formulation Sivanto, is a butelonide insecticide which is not acutely toxic to honey bees at field-realistic levels, but may produce sublethal effects such as immune response modulation and increased pathogen intensity (Al Naggar and Baer, 2019). Therefore, flupyradifurone, along with the widely used herbicide glyphosate, which has also been suggested to affect honey bee immunity and the gut microbiome (Dai et al., 2018), were deployed as pesticide stressors in the following experiments. Glyphosate has specifically been shown to cause increased pathogen intensity of the trypanosome *Crithidia bombi* in its host *Bombus terrestris*, although this effect was not consistent across trials and no fitness effects could be discerned (Straw and Brown, 2021).

2.2. Infectivity and trypanosome cell growth in the laboratory with agrochemical exposure

We aimed to compare the infectivity and replication rate of *C. mellificae* in its established host *A. mellifera* with that of the hitherto unexplored host *O. bicornis.* Honey bees were used as a baseline by which to compare parasite growth between species and across treatments. Two separate sets of experiments were carried out using flupyradifurone pesticide stressor exposure scenarios in the two species.

2.2.1. Methods

2.2.1.1. Source of test organisms

As in section 1.2.1.1., only female *O. bicornis* was used across experiments.

2.2.1.2. Cell cultivation

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 counting chamber (Rosenthaler). Peak density was normally reached within 3-5 days from initiation, after which the number of cells decreased.

2.2.1.3. Pathogen treatment

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 10,000 *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. The low sugar concentration was chosen in order to minimize stress to the living *C. mellificae* cells prior to inoculation (Folly et al., 2020). Recently emerged, unfed and meconium-free *O. bicornis* females were selected for the test. The inoculum was presented to each bee individually using a modified "petal method" protocol for feeding of solitary bees (Ladurner et al., 2005). Inoculum was checked after 30 minutes and a similar number of motile cells were noted, confirming that only a negligible mortality of parasite cells had occurred prior to consumption and making 30 minutes an acceptable timeframe from dose administration to consumption. Bees that did not consume inoculum within 30 minutes were excluded from the experiment. Sham inoculation was sterile medium only mixed with sugar solution and presented in an identical manner. Honeybees were fed with identical 20 µL doses individually by hand using standard methods (Williams et al., 2013).

2.2.1.4. Flupyradifurone exposure and sampling regimen

Infected and control bees were housed in groups of 9 in metal cages (10 x 10 x 6 cm) and fed *ad libitum* with 50% v/w sugar solution from a 2 mL Eppendorf tube. Cages with *O. bicornis* were incubated at 21˚C, 16:8 light:dark cycle and 60% RH (Memmert). Honey bee workers were maintained in similar cages and incubator, but had 30 bees per cage and were kept at 30˚C and 60-80% humidity. At day 6 post-inoculation, a subset of infected bees was sampled to measure baseline infection intensity. The remaining infected individuals were divided into a pesticide-pathogen interaction group and a *C. mellificae*-only group. The mixed group was exposed to flupyradifurone (Pestanal, CAS no. 951659-40-8) in the following manner: untreated sugar solution was replaced with 40% w/w sugar solution containing 1.5 mg/kg of flupyradifurone dissolved in water, based on maximum levels of flupyradifurone found in apple nectar after spray treatment on open flowers (US EPA, 2014). Feeders were replaced once daily in order to prevent excessive breakdown of the active ingredient. The other *C. mellificae* inoculated group received 40% w/w sugar solution only. Based on daily average consumption values collected in a previous cage experiment, the average dose active ingredient consumed per day was 29.9±1.73 ng/bee/day, a chronic dose which did not cause increased mortality across ten days in a honey bee cage assay (Tosi et al., 2021). After a five-day exposure phase, individuals from the pesticide-pathogen treatment group and pathogen-only treatment group were sampled for screening of their pathogen intensity (11 days post inoculation). The remaining infected bees were housed with 50% v/w sugar solution for an additional 10 days, then sampled at a final time point (21 days post inoculation). At each of the sampling events, identically housed non-infected bees were sampled as negative controls. Bees were freeze-killed and stored at -20˚C until further processing.

2.2.1.5. Glyphosate exposure and sampling regimen

Individual *O. bicornis* females were inoculated and housed as described above (1.2.1.4). At 7 days post-inoculation, half of the infected bees were orally exposed to an acute dose of 100 µg glyphosate (TraceCERT, CAS no. 1071-83-6) dissolved in water and mixed with 20% sugar solution (see PoshBee Deliverable 3.2 for more details). For the following 8 days (days 8-16 post-inoculation), one bee was sampled per cage for each treatment, freeze-killed and stored at -20˚C until further processing.

2.2.1.6. Molecular quantification

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 Qiagen DNeasy minikit and then *C. mellificae* quantified 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). Using a standard curve made of DNA extracted from cell culture samples with a known concentration of cells, the total number of cells/gut sample could be determined by qPCR. Doses used for inoculation were also screened by qPCR to confirm the presence of ca. 10,000 cells/dose.

2.2.1.7. Statistical analysis

Cell counts were $\log_{10}$-transformed and compared between days and treatments for *O. bicornis* and *A. mellifera* separately using Wilcoxon rank-sum exact tests. Mean cell counts were found not to differ significantly between days 11 and 21 and were therefore pooled for comparison between species. Mean cell counts between species were compared using a Wilcoxon rank-sum exact tests.

2.2.2. Results

2.2.2.1. Pathogen replication endpoints with and without flupyradifurone exposure

Both bee species inoculated with *C. mellificae* became infected with the trypanosome. All screened individuals from the inoculation treatments tested positive for *C. mellificae*. Extrapolated parasite quantities exceeded the 10,000 cells inoculated in the majority of screened individuals (*O. bicornis* = 93.5%; *A. mellifera* = 88.1%), thus indicating parasite replication in both hosts.

There was no statistical difference in parasite cell number between the *C. mellificae* plus flupyradifurone treatment and the parasite-only treatment at either time point for *O. bicornis* females. Data from the two treatments were thus pooled for further analysis. Compared to the number of parasite cells inoculated, *C. mellificae* cell count per individual increased 80-fold for honey bees and 33-fold for *O. bicornis*. There was a significant increase in cell number between day 6 and day 11 (post treatment) for both species, and an increase in cell number between day 11 and day 21 for honeybees but not for *O. bicornis*. For honey bees, there was an additional 0.6-fold increase in cell count from day 11 to day 21, while *O. bicornis* sampled on day 21 showed a 0.15 fold decrease in average cell count. Thus, when comparing replication of the trypanosome between species, *O. bicornis* carried on average 2.4-fold fewer parasite cells than honey bees on day 11, and 4.6-fold fewer at day 21.

The rate of mortality of infected *O. bicornis* across treatments between inoculation and the last sampling point (day 21) was 6.6% and did not differ between *C. mellificae* plus flupyradifurone treatment and the parasite-only treatment.

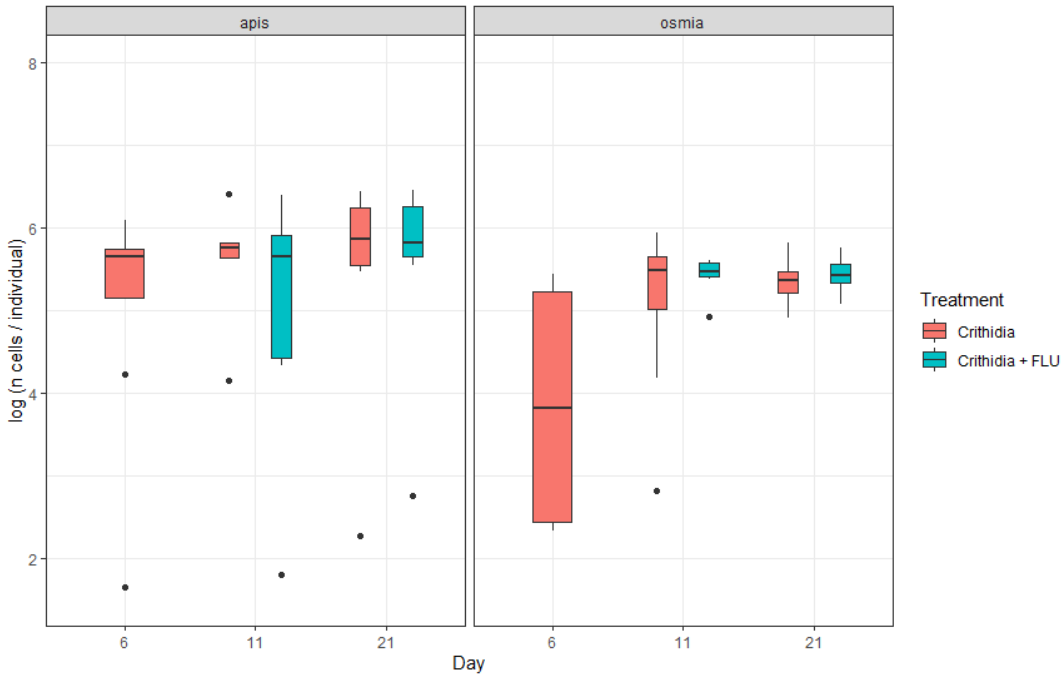

Figure 2. Boxplot showing log-transformed *Crithidia mellificae* cell count in *A. mellifera* (left) and *O. bicornis* (right) females collected at 6, 11 and 21 days post-inoculation. Red boxes represent *Crithidia* treatment only, blue boxes *Crithidia* + flupyradifurone (FLU in legend) treatment. Day 6 represents samples taken before start of flupyradifurone treatment.

2.2.2.2. Pathogen replication endpoints with and without glyphosate exposure

All screened *O. bicornis* showed an increase in cell count from the dose inoculated. There was no interaction between sampling day and cell count in either control infected or glyphosate-exposed infected bees (Figure 3). When pooled, there was no evidence of increased cell count in glyphosate-exposed bees compared to infected only bees. Mortality rate among inoculated bees between inoculation and acute glyphosate exposure (7 days) was 10.4%. No control group was present. Mortality after glyphosate exposure was not recorded since daily sampling was ongoing.

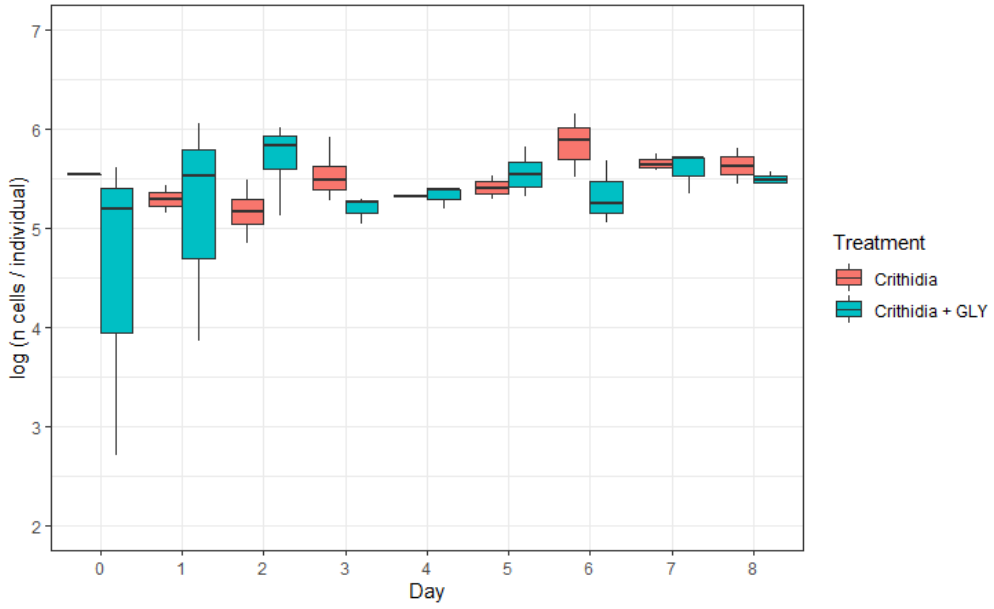

Figure 3. Boxplot showing log-transformed *C. mellificae* cell counts in *O. bicornis* females collected at 0-8 days post exposure to an acute dose of glyphosate (GLY in figure legend). Red boxes represent *Crithidia* treatment only, blue boxes *Crithidia* + glyphosate treatment. The x-axis represents day after glyphosate exposure.

2.3. Lifetime fitness effects of trypanosome infection and flupyradifurone exposure in a semi-field cage experiment

After the initial laboratory-based assays showed high rates of infection of the trypanosome *C. mellificae* but low mortality after infection (6.6% after 21 days), the lethal and sublethal fitness impacts were investigated in a second-tier semi-field cage study. Fitness is defined as an organism's ability to pass on its genetic material, i.e. number of successful offspring raised. Thus, lifetime reproductive output is a good measure of fitness. A myriad of sublethal impairments such as less efficient foraging, hormonal or behavioural disruption may all lead to less investment in offspring production over an organism's lifespan. *O. bicornis* females produce and rear their own brood on individual nectar/pollen provisions. They continuously produce eggs and provision brood cells throughout their adult lifespan. Thus, even a temporal impairment of function, a generally lower health state, or a relatively shorter lifespan may affect the overall reproductive output of a female, with knock-on effects on population dynamics (Stuligross and Williams, 2020). Since brood cells of *O. bicornis* are provisioned individually, the body size of the offspring is determined by the size of the provision mass and, thus, a larger offspring likely represents a larger investment in time and energy. *O. bicornis* is a sexually dimorphic species in which females are larger on average than males and therefore more costly in terms of maternal investment. It is thus predicted that smaller females, females in poor condition, or females in a nutritionally suboptimal environment may produce more male offspring, since they require a smaller investment (Seidelmann et al., 2010). Offspring size, offspring number and sex ratio are thus direct measures of maternal investment. A trypanosome infection may cause females to be in a weakened state, which in turn may affect their offspring provisioning rate. The exposure to an insecticidal field spray may, though sublethal, cause diversion of energy from foraging to detoxification, thus resulting in a lower lifetime reproductive output, smaller offspring, or a skew in the sex ratio towards male offspring. Additionally, pesticide residues in pollen and nectar may lead to direct exposure of larvae, causing elevated mortality or reducing larval nutritional efficacy, also resulting in smaller adult body size. Using a full-factorial semi-field cage setup, we aimed to disentangle the effects of trypanosome infection, flupyradifurone exposure, and their combination on individual female fitness. Using individually marked bees and a camera setup, we tracked individual females throughout their lifespan to investigate per female longevity, flight behaviour and lifetime reproductive output.

2.3.1. Methods

2.3.1.1. Experimental setup

In a fully factorial cage experiment, we investigated the lifelong impacts of trypanosome infection and exposure to flupyradifurone through in-crop spraying of its commercial formulation Sivanto™ on female *O. bicornis* with the following four treatments: *C. mellificae* infection treatment, flupyradifurone (Sivanto spray application) exposure treatment, *C. mellificae* infection and flupyradifurone exposure treatment and control treatment. Experiments took place on an experiment field site near Zürich (Switzerland), managed by WBF-Agroscope. For each treatment, three 2 x 4 m cages were set up in a field sown with purple tansy *Phacelia tanacetifolia* (variety BALO, sowing rate 8 kg seeds/ha). This crop has been recommended for semi-field experiments with *O. bicornis* by the ICPPR working group (Franke et al. 2021). Each cage had a wood pulp nesting unit consisting of removable plates with 10 nesting cavities each (8mm diameter, 15 cm length), starting with a total of 50 such cavities. Additional nesting cavities were made available during the experiment if most holes were already filled with brood cells. Each plate had a transparent thin plastic layer on top of it, allowing for daily direct observations of nest construction. Using this design, daily brood cell production could be recorded after sunset each day. The nest unit was placed 1.5 m above the ground facing east with a wooden roof as protection from rain and sunshine. The cages were covered in nylon mesh and had a zipper on one end for entering. Daily observations of individually marked females took place across

31 days (6th June 2021 – 7th July 2021). Observations were terminated when cell production had ceased and only 5% of bees remained alive. Nesting units were then brought into a shed and brood (offspring) allowed to overwinter.

2.3.1.2. Pathogen treatment and handling prior to release

Individual cocoons were obtained and handled as described above (1.2.1.1.). Upon emergence, male and female bees were allowed to fly and mate in small hatching cages (0.5 x 0.5 x 0.5 m; BugDorm) under natural light for 24 hours with access *ad libitum* 50% v/w sugar solution from a 2 mL Eppendorf tube. Mating behaviour was readily observed in the cages, although successful insemination cannot be confirmed without dissection (Seidelmann, 1999). After 24 hours had passed, bees were transferred into individual plastic Nicot cages (see PoshBee deliverable 2.3 for more detail), where they were starved overnight. Inoculum preparation and inoculation took place as described above (2.1.1.2-2.1.1.3), with the notable difference being inoculation with 20,000 cells instead of 10,000. Bees belonging to treatments the two *C. mellificae* infection treatments received the inoculum, while treatments of the two non-infected treatments received a sham inoculum consisting of cell culture medium only. Bees were then sorted into cage replicates based on weight (nearest mg) and ID tags produced for the marking of honey bee queens were glued to the bee's thorax as an individual ID (Knauer et al. 2022). The following morning, bees were released into the cages and this day was considered to be day 1 of the experiment. Any bee that did not survive the night was recorded as such. An additional batch of bees was released the following day, and a final batch on day 3.

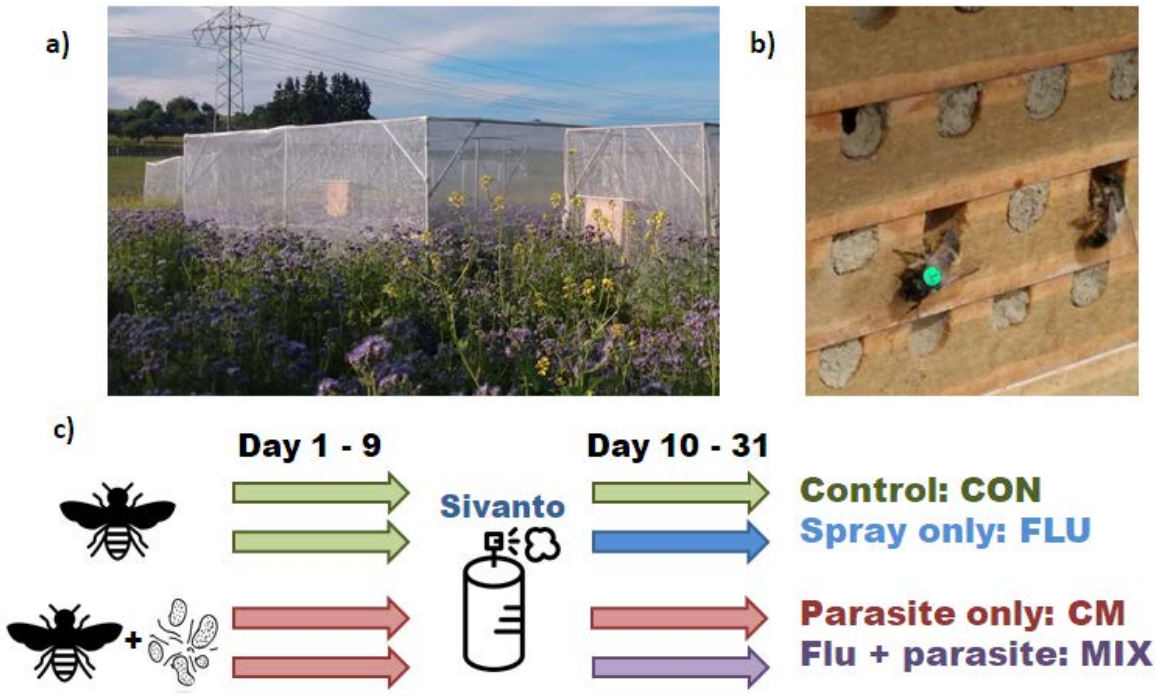

Figure 4. a) Cage setup in the semi-field, b) female at the trap nest entrance with ID tag, c) graphical representation of the full-factorial design with the four semi-field treatments and their duration.

2.3.1.3. Pesticide exposure

On the 15th of June, 10 days after release of the initial batch of bees, flupyradifurone in the form of Sivanto™ (Bayer Crop Science) spray was applied to treatment cages following label instructions. Spraying took place when the dew had evaporated from the plants, while some bees were already foraging. The nest box was covered with a plastic sheet to avoid direct spraying. The product was sprayed with a rate of 205 g a.i./ha, in six of the cages (48 m^2 total sprayed surface), three cages with

uninfected bees and three cages containing infected bees. The target volume was 400 L/ha (320 mL/cage). This represents 5.125 mL of undiluted product in 2 L of water. The mixing took place directly in the field prior to application. Spraying was done using a hand-held application device (Birchmeier REC 15) at 2 bars of pressure.

2.3.1.4. Daily observations using video recording and video analysis using the *BeeTracker* software
Nests were monitored daily between days 5-15 by taking a one hour video recording each day upon signs of nesting activity. One cage (CM3) was excluded from all analyses due to netting malfunction. The BeeTracker software assigned individually marked females to their nests when an individual could be observed entering its entrance and remained inside for a minimum of 40 seconds. This duration was based on observational studies determining the amount of time a female spends in a nest during pollen deposition (Knauer et al., 2022). All brood cells constructed within that nest on that day were assigned to the specific nesting female. The software successfully assigned 29% of all produced brood cells to females across days 10-21 of the experiment. The remaining cells produced were manually assigned by observing the video recordings. This was the case for days 22-37 as well as for those individuals who lost their ID tags. Individuals of one cage (FLU 3) had to be excluded from individual reproductive output measures since bees were nesting outside the filmed arena and could thus not be correctly assigned. Excluding this treatment replicate, 98% of brood cells could be confidently assigned to individual females.

When the software successfully registered an individual exiting and entering a nesting hole to which it was assigned, the duration between the two events was registered as a foraging trip. A total of 1667 foraging trips was registered across days 11-21, when cell construction was at its most intense. The Bee Tracker software additionally registered the number of attempted entries into other nest entrances before entering the correct nest. This was used as a behavioural proxy for nest recognition.

2.3.1.5. Brood assessment
In March the following year, nest boxes were opened and their contents examined. The brood cells were classified by content (Pollen only, Pollen with dead egg, Dead larva, Dead pupa, Adult). Insect brood parasites were efficiently excluded in the cages as no evidence of their parasitism could be observed. Each cocoon was weighed to the closest 0.1 mg and opened to determine the sex of the imago.

2.3.1.6. Statistical analysis
All statistical analyses and graphical output were generated in RStudio. Loss of individuals between inoculation and release into field cages for the first batch of bees was compared using a Shapiro Wilk's test. Bees that were released were considered established if they were observed for more than one day after release. Establishment success as an effect of infection status, weight and release date was analysed with a generalized mixed-effects model with binary distribution and a log link function. Differences in the average weight of females per cage was tested with a Kruskal-wallis rank sum test and found not to differ (p=0.8).

One of the cages in the infection only-treatment had issues with netting, giving individuals the opportunity to escape. Bees in this cage were therefore excluded from longevity analysis. In order to increase statistical power of the analyses, all infected bees were pooled and compared to all uninfected bees. Likewise, all sprayed cages (*C. mellifica*-infected or not) were pooled and compared to control bees. Kaplan-Meyer survival estimates were calculated and treatments were compared with a log-rank test using the package *'survival'*. Graphical representations of the Kaplan-Meyer survival curves were made using the package *'Survminer'.* A cox-proportional hazard model was fitted, with treatment and batch as fixed factors and cage as a random factor using the package *'coxme'.* Pairwise comparisons between treatments were then carried out with Tukey post-hoc tests using the package '*multcomp'*.

For analysis of foraging flight duration, flights shorter than 4 minutes and upper outliers were trimmed from the dataset in order to exclude other types of flights such as mud collection trips and recording faults (Knauer et al., 2022). Flight duration was analyzed with a linear mixed-effects model with infection status as predictor for flight duration in seconds (log-transformed). Significance of fixed effects were derived by comparison to a null model using an ANOVA test. For the flights recorded after spraying had taken place, an identical model was used but with flupyradifurone spray as a predictor. Individual ID nested in cage was included as a random factor in all models. Number of entry attempts before entering the correct nesting hole was square root-transformed and analyzed with a general linear model assuming a negative binomial distribution. Again, significance of fixed effects was derived by comparison to a null model using an ANOVA test.

The total number of brood cells produced per female were counted based on assignment from video observation and daily nest construction measurements. From the observational data, the number of days of activity per female was determined, and the brood cells produced across the lifespan was divided by this number as a measure of cells produced per day. These two measurements were analysed with generalized mixed effects models using function *glm* from package '*lme4*' starting with a full model containing infection status, spray treatment, lifespan per female, batch, weight and their interaction terms as explanatoy variables. Using the *stepAIC* function from package *'MASS'*, the best model was selected based on the Akaike Information Criterion (AIC). The best model retained infection status, spray treatment, lifespan per female, and the interaction between infection and spray treatment. These factors were then added to a generalized linear mixed-effects model with cage as a random factor.

The weight of sons and daughters produced before spray application between the two *C. mellificae*-infection treatments and the non-infection treatments were analyzed separately with a student's t-test. Weight of male offspring produced after the spray application was compared between treatments with a Tukey's post-hoc test for multiple comparison of means. There were too few female offspring produced after the spray treatment to compare their weights.

2.3.2. Results

2.3.2.1. Mortality

There was significantly higher mortality in the inoculated group of bees upon overnight starvation prior to release into field cages compared to the non-inoculated group (Fisher's exact test; p=<0.001). Of those that were successfully released, establishment was negatively associated with infection status, with infected bees having 56% lower odds of successfully establishing upon release, although the effect was not statistically significant (p=0.06). Weight was a significant predictor of establishment success, with heavier bees being more likely to establish a nest.

When considering successfully established bees, there was an increased risk of mortality between pooled infected and non-infected individuals (Log-rank test; X^2= 3.1; df = 1; p= 0.001). However, there was no increased risk of lifetime mortality in bees treated with flupyradifurone on day 10 (Log-rank test; X^2= 0.5; df = 1; p= 0.83) (Figure 5). When treatments were considered individually with a mixed model including cage as a random factor, none of the treatments were different from controls (p=>0.05).

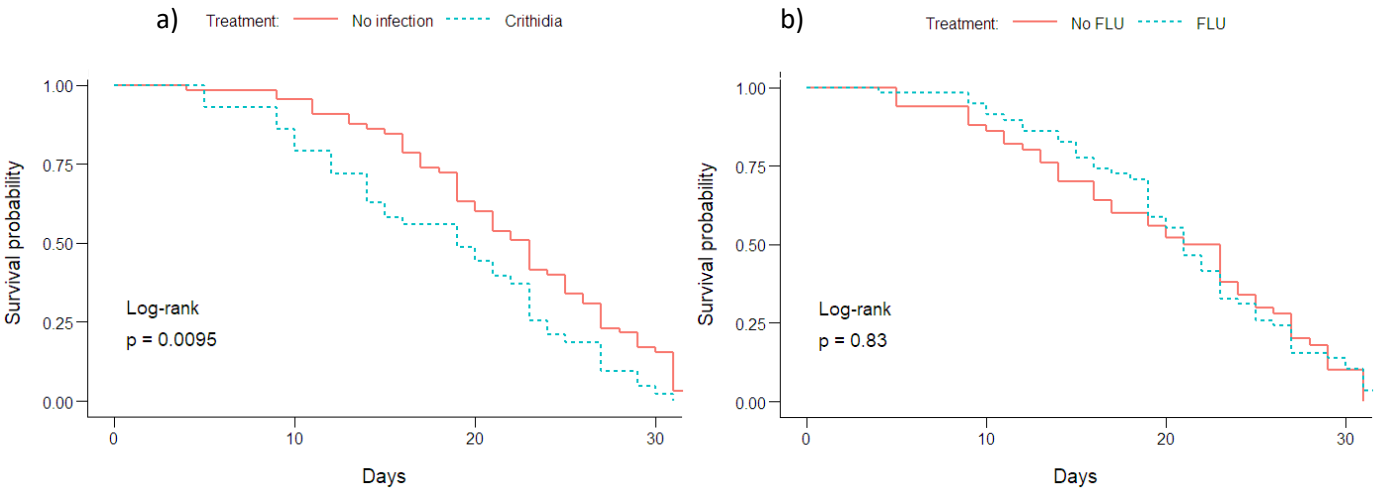

Figure 5. Kaplan-Meyer survival curves for a) uninfected vs. *Crithidia*-infected *O. bicornis* females and b) Flupyradifurone-exposed vs. control individuals.

2.3.2.2. Confirmation of infection

Five *C. mellificae* infected individuals and three uninfected individuals were censored on day 29 and 31 of the experiment and screened for pathogen intensity as described above (2.2.1.6.). All inoculated individuals were found to have a high titre of *C. mellificae* cells, while non-inoculated bees were found to be devoid of *C. mellificae*. By inference, we can thus assume that the infection treatment worked well, and the remaining individuals from the infection treatments can be assumed to be infected.

2.3.2.3. Flight behaviour

Day since the experiment started had a significant effect on foraging trip duration across the observation period, with flight increasing in length over time (Figure 6.). There was no significant main effect of pathogen treatment alone on flight duration when considered pre-spray, allthough there was a significant interaction between day and pathogen treatment on flight duration, with infected individuals making longer foraging trips with increased day (Days 5-9). The interaction between day and spray treatment did provide a better model fit, but spray treatment as a main factor did not. The number of entry attempts before correct nest entry appeared not to be significantly affected by infection status when only entry attempts after flupyradifurone had been applied were compared. However, infected bees had a tendency to make fewer probing attempts before entering a nest compared to uninfected, when compared with days pre-spray. The effect of spray treatment on nest entry attempts was not significant.

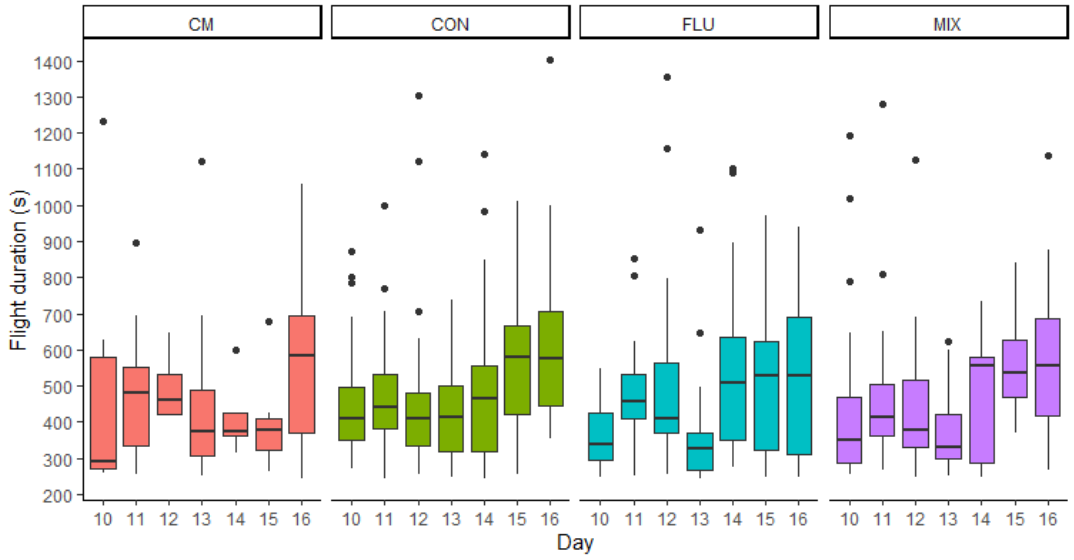

Figure 6. Boxplot showing foraging trip duration in seconds per day for a subset of *O. bicornis* females recorded on days 10-16 after spraying of flupyradifurone (Sivanto™) in the FLU and MIX treatments.

2.3.2.4. Number of brood cells provisioned per female

A total of 1721 brood cells were constructed during the experiment. In total, 42% of brood cells were already constructed on day 10, when Sivanto™ spray treatment was applied and 58% after spray treatment. In the final model, the factor infection status was not statistically significant (p=0.07), as well as the interaction between infection and spray treatment (p=0.09). The number of brood cells per female was positively correlated with lifespan in days (p=<0.001). Females of the infection only treatment produced significantly fewer brood cells compared to all other treatments. The remaining treatments did not differ between each other. However, when considering the number of cells produced per active day, neither infection nor flupyradifurone treatment had an effect (Figure 7).

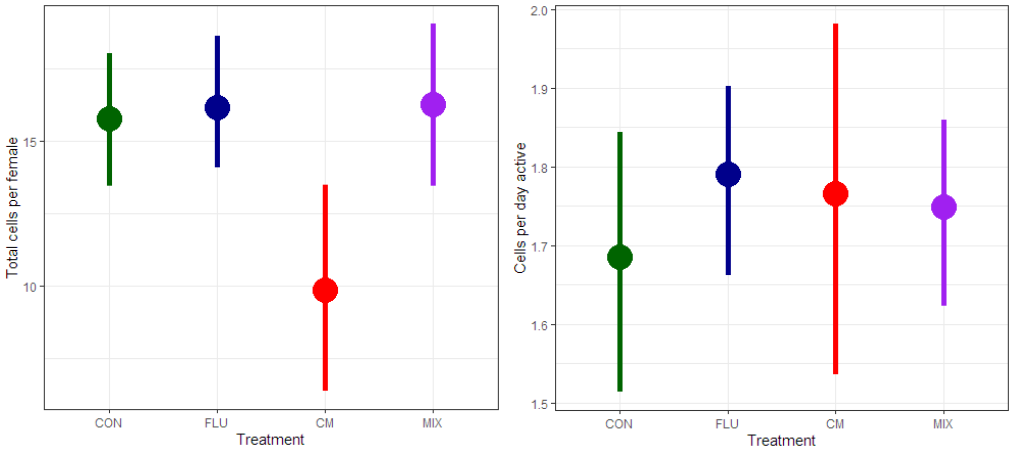

Figure 7. Total number of brood cells per *O. bicornis* female (left) and average number of cells completed per day of active nest construction per female (right) for the four treatments. Dot represents mean and lines standard error (SE).

2.3.2.5. Offspring survival, sex ratio and body size

A subset of brood cells (7.5%) was damaged in a heavy storm during the experiment and could therefore not be analyzed. The mortality during development (egg to imago) was moderate across

treatments (Control 10.3%; flupyradifurone 9.4%; *C.mellificae* 6.6%; flupyradifurone + *C. mellificae* 5.1%). The overall sex ratio of the surviving brood was 15.2% females across treatments. All tracked nesting females produced at least one daughter (range 1-9), confirming that mating had taken place. Cages with *C. mellificae*-infected bees produced overall 15.7% daughters and non-infected bees produced 14.7% daughters. When considering offspring laid pre-spray application, there were no weight difference between those offspring whose mothers belonged to the infection treatment and those who did not, for either male or female offspring. Male offspring laid after the day of the flupyradifurone application did not differ in weight between treatments.

3. Discussion

We started our exploratory work with a controlled infection treatment of two common honeybee viral variants. We could not find evidence of consistent replication for either of these pathogens when administered by injection. This is an interesting contrast when considering exposure surveys of *O. bicornis*, in which high prevalence of these and other viruses has been found (see PoshBee Deliverable 2.3). We do not rule out that other viral honeybee-associated pathogens such as black queen cell virus (BQCV), or other variants of DWV, may cause virulent infection in *O. bicornis*.

We decided instead to pursue the trypanosome gut parasite *C. mellificae* as a model pathogen, since there is previous evidence from the literature of its successful inoculation and replication in other *Osmia* species. The percent of infected individuals upon oral inoculation was, at almost 100%, higher than has been recorded in previous studies on the closely related *O. lignaria, Megachile rotundata* and *O. cornuta* (Strobl et al., 2019; Ngor et al., 2020). We found evidence of the trypanosome increasing in number up to day 11 post inoculation, thereafter stagnating and remaining at high levels until 21 days post inoculation. The additional stressor of oral exposure to flupyradifurone did not affect pathogen intensity (number of cells/bee) across the investigated time points. This lack of effect was also confirmed in *C. mellificae*'s presumed reservoir host *Apis mellifera*. An acute dose of glyphosate, likewise, did not result in higher cell counts. Survival across trials was relatively low, although a controlled survival experiment was not conducted in the laboratory.

In our semi-field cage experiment, we found a survival effect on *O. bicornis* after exposure to an inoculation of 20,000 cells of *C. mellificae* and subsequent overnight starvation. Additionally, the likelihood of successful establishment of inoculated females in the semi-field was reduced. We did detect a weak reduction in survival in the pathogen only treatment (CM) compared to all other treatments. When comparing the combined stressor treatment to control treatment, we did not, however, detect an impact. There was a reduction in total number of brood cells produced per female in the parasite treatment, but not in the combined stressor treatment. This was, however, not significant when cells produced per day active was considered. These results should be interpreted with caution since statistical power was reduced due to the loss of one cage replicate in the parasite treatment. There was no detectable difference in survival or cell production parameters between unexposed bees and bees exposed to flupyradifurone in its commercial formulation, Sivanto™.

There was no apparent decrease in brood survival between treatments, although a subset of cells had to be removed from the analysis due to damage. Likewise, there was no difference in average body weight of male offspring between treatments, and no difference in the weight of daughters between infection treatment and control treatment was observed. Likewise, sex ratio of offspring did not differ between cages with infected versus non-infected bees. This implies that eventual flupyradifurone residue in pollen was not a factor contributing to reduced survival or body weight, although such a residue analysis was not performed in this study.

A caveat of our study is that 42% of total cell production had already taken place when FLU treatment was applied, and it cannot be ruled out that early-life exposure to FLU may have caused a noticeable

effect. This is relevant to an understanding of how flupyradifurone might affect overall reproductive output in a natural setting, and especially the production of daughters, which takes place at the start of nesting. It should also be noted that the study took place in cages stocked with *P. tanacetifolia*, which is a protein-rich pollen source. Such high-quality nutrition may enhance host tolerance of a trypanosome infection. Effects of the pathogen or the pesticide treatment may thus be present if food stress is also introduced, as has been demonstrated in previous studies (Stuligross and Williams, 2020; Klaus et al., 2021). The increased mortality during overnight starvation and early establishment, shown in our study, may indicate that the virulence, i.e. the ability of the pathogen to cause harm, is dependent on nutritional status, which has previously been shown in the *Bombus-Crithidia* system (Brown et al., 2000). The cage environment also frees the bees from the energy expenditure of long foraging trips, in which suboptimal health may impact foraging efficiency.

We can, however, conclude that there are marginal negative effects of *C. mellificae* on survival, although no dramatic impact on overall fitness can be discerned from this set of experiments. We conclude that infection with *C. mellificae* can be tolerated by *O. bicornis* under certain conditions, and that no evidence of additive or synergistic interaction could be identified between the pathogen and two pesticides. This may provide evidence for the safe use of these agrochemicals in agricultural systems with regards to bee health.

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