# Manuscript on agrochemical and pathogen effects on individual honey bee health

## Deliverable D6.1

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**PoshBee**
**Pan-european assessment, monitoring, and mitigation of stressors on the health of bees**

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## Summary

Reports of increased global losses of honey bee colonies are alarming and cannot be explained by a single driver. Most likely a wide array of stressors including ubiquitous diseases, pesticides, as well as malnutrition are taking their toll on honey bee health. Subsequently, much focus has been laid upon investigating the effects of pathogen and pesticide exposure which often revealed detrimental effects at an individual bee and colony level. While there is a plethora of studies on individual effects, far less is known about how these individual stressors interact when bees are exposed concurrently. Therefore, further research is needed to improve the understanding of how such stressors act individually and in combination on honey bees.

D6.1, a manuscript on agrochemical and pathogen effects on individual honey bee health addresses experiments conducted within Work Package 6, which studies the effects of agrochemical-pathogen interactions on bee health in the laboratory. Here, under Task 6.2, we tested the effects of agrochemical-pathogen interactions in honey bees to gain further insight into this highly relevant topic. We conducted fully-crossed laboratory experiments covering all honey bee life stages, using a selection of key pests and pathogens, as well as three agrochemicals selected within the PoshBee project framework. Tests were primarily conducted on female (worker) honey bees. However, additional studies were performed on the sexuals (i.e., male drones and queens) to respond to the current ecotoxicological knowledge gap for honey bees. In Sweden, research focussed on larvae using *Paenibacillus larvae*. In Switzerland and Italy, studies focussed on *Varroa destructor* and deformed wing virus (DWV) on worker larvae/pupae as well as adult workers. In Spain, focus was laid upon *Nosema ceranae* and adult workers. Additionally, *N. ceranae* was tested on males (drones) in Switzerland and queens in France. All experiments were performed using standard protocols and applying field-realistic concentrations of the selected pesticides (i.e., azoxystrobin, coumaphos/glyphosate, and sulfoxaflor).

For *V. destructor* we measured larval/pupal mortality, mite mortality, fertility and fecundity, as well as adult bee long-term survival. For *P. larvae* we measured larval mortality, pathogen loads and development in time, and larval immune gene expression. For *N. ceranae* we measured consumption, survival, pathogen loads and immune- and detoxification-gene expression levels in workers. Additionally, we measured survival and pathogen loads in drones and survival in queens. Evolutionary lineages and admixture ancestry were determined for each colony used in the different countries.

## 1. Introduction

Insects, such as western honey bees (*Apis mellifera*), provide key ecosystem services as they are responsible for pollinating roughly 84% of the European Union's crops (Klein et al., 2007; Williams, 1994). Therefore, bees play an immense ecological and economic role as they are responsible for the pollination of numerous native plants and crops (Gallai et al., 2009; Klein et al., 2007). However, there are increasing reports suggesting drastic temporal and regional losses in managed honey bee colonies (Gray et al., 2019; Neumann and Carreck, 2010; Potts et al., 2010a).

A variety of factors can negatively affect the health and survival of managed honey bee colonies, including the spread of pests and pathogens, the simplification of habitat structure, reduced availability or quality of food resources, beekeeping practices, as well as exposure to xenobiotics such as industrial agrochemicals (O'Neal et al., 2018). Although no single driver has been defined as the cause of the declines, the simultaneous exposure to multiple stressors is believed to be the cause of recent global losses of honey bee colonies (Potts et al., 2010b; Ratnieks and Carreck, 2010). Arguably, concurrent exposure to pesticides and parasites is believed to be a major contributor to the recent losses of honey bee colonies (Bird et al., 2021). The most important parasites for managed honey bees

are the bacterium *Paenibacillus larvae*, the microsporidian *Nosema* spp., and the parasitic mite *Varroa destructor*.

Prior to the arrival of the *V. destructor* mite, the most important disease of honey bees worldwide was the bacterial brood disease, American foulbrood (AFB). The gram-positive spore-forming *Paenibacillus larvae* bacteria is the causative agent of AFB (Genersch et al., 2006). AFB is still among the most harmful bee diseases, and is particularly deceptive as the spores can remain infectious for more than 35 years even if exposed to harsh environmental conditions (e.g., extreme temperatures or humidity) (Haseman, 1961). Larvae infected with AFB are likely to die and the highly contagious spores can swiftly spread throughout a colony causing the entire colony to collapse. By exchanging hive and bee material between colonies, and the global trading of queens, colonies and honey, the spread of the disease is facilitated (Genersch, 2010). AFB is a notifiable disease that must be reported by beekeepers to their local authorities and upon reporting, immediate adequate measures must be implemented.

Since its introduction, the ectoparasitic *Varroa destructor* mite is considered the most significant pathological threat to the western honey bee, *A. mellifera* (Dietemann et al., 2012; Traynor et al., 2020). *V. destructor* is a nearly ubiquitous ectoparasite of Asian origin that is the main biological cause of honey bee colony mortality worldwide. This is primarily because of viral infections vectored by this parasite (e.g., deformed wing virus (DWV)) as well as due to an array of negative sublethal effects (e.g., impaired immune response or reduced body mass) (Amdam et al., 2004; Dainat et al., 2011; Noël et al., 2020). Without treatments colonies are believed to die within a few years (Boecking and Genersch, 2008). Therefore, beekeepers globally have treated against this parasite by using synthetic acaricides for more than 40 years (Koeniger and Fuchs, 1989; Rosenkranz et al., 2010). However, mite resistance is continuously increasing to such common commercially applied chemicals (Hernández-Rodríguez et al., 2021). Furthermore, there are increasing reports of honey bee colonies surviving *V. destructor* infestation without it being treated, yet these reports remain restricted to certain locations. The underlying mechanism responsible for the bees' tolerance towards the mite remains to be fully understood (Le Conte et al., 2020, 2007; Locke, 2016; Mondet et al., 2020).

The microsporidian *Nosema ceranae* is an intracellular midgut parasite of *A. mellifera* that was first described in the eastern honey bee, *Apis cerana* (Fries et al., 1996). It spread from the original host to *A. mellifera* at least two decades ago and has since developed a nearly ubiquitous distribution worldwide (Higes et al., 2009; Paxton et al., 2007). This parasite infects adult honey bees and is transmitted *per os* (spore ingestion) (Fries, 1997). Ingested *N. ceranae* spores penetrate the midgut where proliferation and spore accumulation occurs (Ptaszyńska et al., 2014). The impact of infections is highly debated. While various studies show strong effects on mortality in the laboratory and the field (Higes et al., 2008, 2007; Klee et al., 2007; Martín-Hernández et al., 2007) others report no impact of *N. ceranae* on honey bee colony losses in the field (Dainat et al., 2012).

Aside from bees being frequently confronted with parasites and pathogens, there is also an increasing exposure to pesticides (Wintermantel et al., 2020). Managed honey bee colonies are often chronically exposed to sublethal doses of xenobiotics (e.g., industrial pesticides), and residues are frequently detected in bee products (Traynor et al., 2021). Chronic pesticide exposure can, for instance, impair individual development (Friedli et al., 2020), learning, and flight behaviour (Aliouane et al., 2009), as well as increase overall mortality rates (Rondeau et al., 2014). Given the ubiquitous exposure of pesticides to bees and other pollinating insects, xenobiotic exposure plays a critical role in the evaluation of bee health.

Indeed, a variety of interactions amongst pathogens and pesticides have been reported for bees (Bird et al., 2021). However, results are often too inconsistent to draw general conclusions. For details on interactions between chemicals and *V. destructor* see: (Annoscia et al., 2020; Blanken et al., 2015;

Morfin et al., 2020; Sprygin et al., 2016; Straub et al., 2019); for *N. ceranae* see: (Alaux et al., 2010; Aufauvre et al., 2012; Dussaubat et al., 2016; Kairo et al., 2017; Retschnig et al., 2015, 2014a; Tesovnik et al., 2020; Vidau et al., 2011); for *P. larvae* see: (Hernández López et al., 2017). The nature of such interactions depends on the pesticide's mode of action, the tested concentration, the chosen model pathogen, as well as the selected endpoint measurements and the susceptibility of the host (Bird et al., 2021; Laurino et al., 2013; Siviter et al., 2021). Therefore, there is an urgent need to further strengthen our understanding of how pesticides and pathogens interact and affect bee health. To shed light on this highly complex topic, future research efforts should be focused on understanding how these interactions vary across different life stages of the bees, as well as to compare effects between and within different bee species and subspecies.

Here, we investigated the effects of the three main pathological honey bee threats in combination with the selected chemicals axozystrobin, glyphosate/coumaphos, and sulfoxaflor. Experiments were conducted on different life stages (i.e., larvae, pupae, and adults), varying *A. mellifera* subspecies (*A. m. carnica*, *A. m. iberiensis*, *A. m. ligustica*, and *A. m. mellifera*) depending on the country the assays were performed in, as well as on different sexes and castes (queens, drones, and workers).

# 2. Elucidating agrochemical-pathogen interactions in honey bees

## 2.1. *Varroa destructor*-pesticide interactions (UNIUD & BERN*)*

### 2.1.1. Material and Methods

Experiments were conducted at UNIUD and BERN following the same protocol, to gain insights into the interaction of *Varroa destructor* and pesticides on the survival of immature and adult bees infested (or not) by the parasite, and the reproduction of the infesting mites. Therefore, pesticides were administered orally, through larval food, at doses that were regarded as sub lethal for larvae, based on existing scientific literature and on the experiments performed in Bern in spring 2019 (**Table 1**). Three different pesticides were tested: azoxystrobin (As), sulfoxaflor (Sf) and coumaphos (Cp). Note that, as agreedat PoshBee's first AGM, glyphosate (a pesticide chosen by all PoshBee members), was replaced with coumaphos: a substance commonly found in hives, as a result of its use for mite control. In addition, we also tested the combined effect of two pesticides (Sf+Cp), to assess any additive or synergistic effects between them.

**Table 1: Overview of pesticide treatment groups.** For each chemical the applied concentration is given in ppm as well as in µg/larva.

| Treatments | Concentration | |
|---|---|---|
| | *ppm* | *µg/larva* |
| Coumaphos | 20 | 1 |
| Sulfoxaflor | 0.5 | 0.025 |
| Azoxystrobin | 100 | 5 |
| Coumaphos + Sulfoxaflor (Co) | 20+0.5 | 1+0.025 |

Larvae L4 were manually collected and transferred into sterile petri dishes (Ø = 9 cm) containing 10 grams of clean or pesticide-treated diet (**Fig. 1a**). Larvae were kept in these petri dishes for 24 hours. L5 larvae were then cleaned from the larval food and transferred into gelatine capsules (Agar Scientific Ltd., Ø = 6.5 mm) with 1 mite (+V) or no mites (**Fig. 1b**), and maintained at 35 °C, 75% R.H. until eclosion (12 days). In total, 2868 larvae (255 to 329 L5 per experimental group (C, C+V, As, As+V, Sf, Sf+V, Cp, Cp+V, Sf+Cp, Sf+Cp+V)) were prepared by the two laboratories (**Table 2**); however, technical problems precluded the possibility of carrying out fully balanced experiments in BERN (**Table 2**); this should be

considered when interpreting possible differences between labs. Mites were collected from brood cells capped in the preceding 15 h.

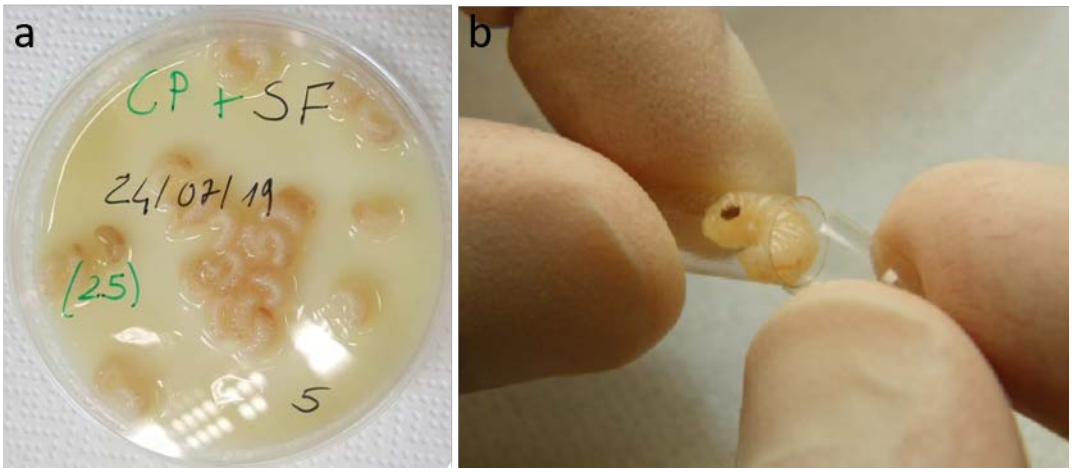


**Figure 1: a:** Western honey bee, *Apis mellifera*, larvae L4 (N~25) reared in a petri dish with 10 g of artificial diet containing 20 ppm coumaphos (Cp) and 0.5 ppm sulfoxaflor (Sf). **b:** Artificial infestation: female *Varroa destructor* and larva L5 reared in gelatine capsule.

**Table 2: Treatment groups that were assessed in both locations, UNIUD and BERN.** Treatments, absence or presence of *Varroa destructor* (+/-), the number of tested western honey bee larvae per replicate (*N (larvae)* per replicate) are shown for each laboratory (UNIUD, BERN).

| Treatments | *Varroa destructor* | UNIUD | | | | BERN | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | *N (larvae) per replicate* | | | | *N (larvae) per replicate* | | | | |
| | | *R1* | *R2* | *R3* | *TOT* | *R1* | *R2* | *R3* | *R4* | *TOT* |
| Control (C) (C+V) | - | 46 | 50 | 37 | 133 | 66 | 65 | 34 | 30 | 195 |
| | + | 48 | 49 | 40 | 137 | 66 | 62 | 34 | 30 | 192 |
| Coumaphos (Cp) (Cp+V) | - | 46 | 52 | 35 | 133 | 64 | 0 | 60 | 0 | 124 |
| | + | 47 | 53 | 44 | 144 | 65 | 0 | 0 | 60 | 125 |
| Sulfoxaflor (Sf) (Sf+V) | - | 43 | 54 | 39 | 136 | 65 | 0 | 60 | 0 | 125 |
| | + | 45 | 56 | 44 | 145 | 64 | 0 | 0 | 60 | 124 |
| Azoxystrobin (As) (As+V) | - | 49 | 53 | 39 | 141 | 0 | 0 | 60 | 60 | 120 |
| | + | 51 | 56 | 41 | 148 | 0 | 0 | 60 | 60 | 120 |
| Coumaphos + Sulfoxaflor (Cp+Sf) (Cp+Sf+V) | - | 46 | 52 | 35 | 133 | 66 | 51 | 60 | 0 | 177 |
| | + | 47 | 53 | 36 | 136 | 64 | 53 | 0 | 60 | 177 |

Daily, dead larvae were removed and counted. Upon eclosion, mite mortality and reproduction (i.e., fertility and fecundity) were measured by inspecting each capsule. Once separated from the infesting mite (if present), newly emerged adult bees were transferred into plastic cages (UNIUD: 18.5 × 10.5 × 8.5 cm, BERN: 80 $cm^3$) with water and sugar candy (Apifonda®) *ad libitum*, and maintained there until death to assess survival. Additionally, at eclosion 6-7 adult bees per experimental group were sampled and stored at -80 °C for future analysis aiming to assess the DWV load and host gene expression levels (not shown here, as analyses are incomplete at the time of publishing).

Data regarding the effects of treatments on larval survival, mite mortality, fertility and fecundity were analyzed by means of generalized mixed models (GLMMs) with replicate number as a random factor (note that any statistical differences reported below are referred to the control column). Results are reported as average ± standard deviation (SD). Adult survival was studied with the Cox model test. Data from BERN and UDINE laboratories were studied separately.

### 2.1.2. Results

2.1.2.1. L4 mortality

Mortality between L4 and L5 was <10% in both laboratories; no apparent difference among treatments was observed (**Fig. 2ab**).

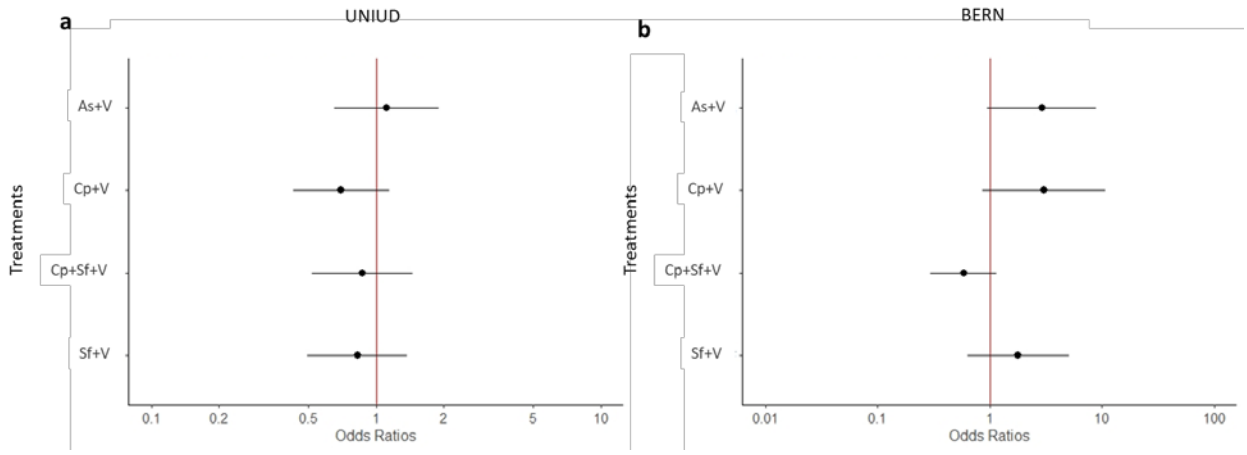


**Figure 2: Effects of chemical treatments on western honey bee L4 larval mortality**. GLMMs did not highlight any significant difference among treatments. **a**: UNIUD mortality results (C mortality = 0.08 ± 0.08 SD; Cp mortality = 0.10 ± 0.11 SD; As mortality = 0.08 ± 0.09 SD; Sf mortality = 0.09 ± 0.10 SD; Cp+Sf mortality = 0.09 ± 0.08 SD); **b**: BERN results (C mortality = 0.03 ± 0.03 SD; Cp mortality = 0.01 ± 0.01 SD; As mortality = 0.01 ± 0.01 SD; Sf mortality = 0.02 ± 0.03 SD; Cp+Sf mortality = 0.05 ± 0.05 SD). Differences are referred to the control column.

2.1.2.2. Mortality during development

Mortality during development (from encapsulation to emergence) was higher in UNIUD individuals, similar to the results from L4-L5 larvae (**Fig. 2**). Results from UNIUD indicate a general negative effect of *V. destructor* on bee survival during pupation in all treatment groups, although the difference is not statistically significant. In both labs, the strongest effect was caused by the combination of sulfoxaflor and coumaphos (i.e., Cp+Sf and Cp+Sf+V; **Fig. 3ab**). This result concurs with the negative effect of sulfoxaflor in infested honey bees observed in UNIUD (Sf+V; **Fig. 3a**) and the negative impact of coumaphos on both uninfested and infested honey bees observed in BERN (Cp and Cp+V; **Fig. 3b**).

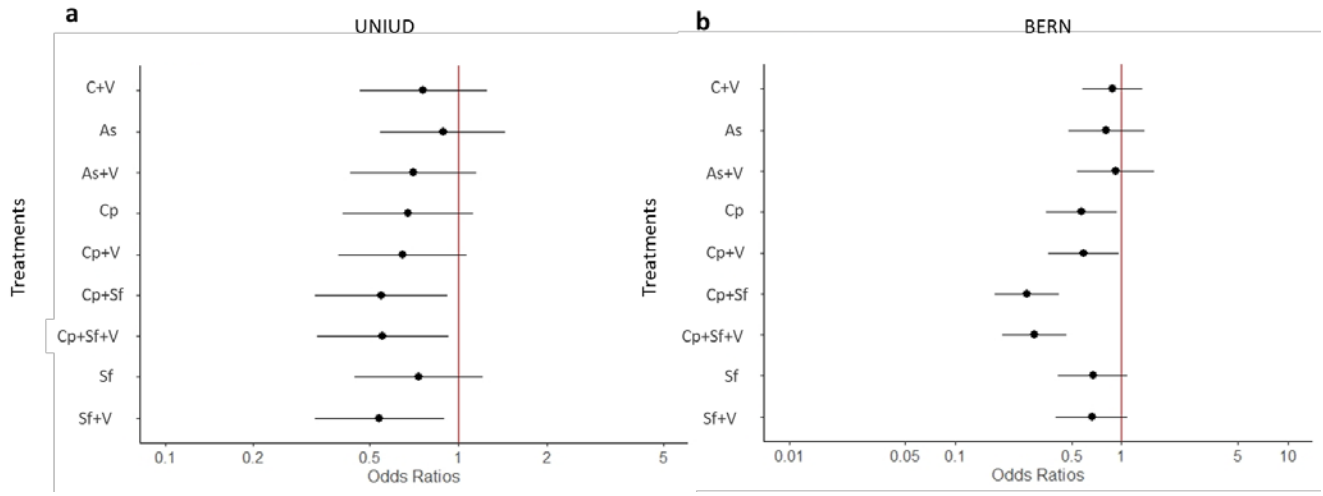


**Figure 3: Effects of chemical treatments during worker development** (between L5 to emergence). **a**: Results of UNIUD show a negative effect of *V. destructor*, regardless of pesticides. Sulfoxaflor has a negative effect when in combination with *V. destructor* (Sf+V), while the combination of coumaphos and sulfoxaflor has a negative impact in both uninfested and infested bees (Cp+Sf and Cp+Sf+ V). Mortality results: C mortality = 0.58 ± 0.14 SD; C+V mortality = 0.64 ± 0.08 SD; Cp mortality = 0.66 ± 0.08 SD; Cp+V mortality = 0.68 ± 0.07 SD; As mortality = 0.61 ± 0.16 SD; As+V mortality = 0.66 ± 0.11 SD; Sf mortality = 0.65 ± 0.11 SD; Sf+V mortality = 0.71 ± 0.16 SD; Cp+Sf mortality = 0.70 ± 0.20 SD; Cp+Sf+V mortality = 0.71 ± 0.12 SD) **b**: Results obtained in BERN confirmed the negative effects of the combination of coumaphos and sulfoxaflor (Cp+Sf and Cp+Sf+V). In contrast to UNIUD, BERN results indicate a negative effect of coumaphos in both infested and uninfested bees (Cp and Cp+V). Mortality results: C mortality = 0.34 ± 0.20 SD; C+V mortality = 0.37 ± 0.21 SD; Cp mortality = 0.50 ± 0.02 SD; Cp+V mortality = 0.47 ± 0.21 SD; As mortality = 0.28 ± 0.01 SD; As+V mortality = 0.27 ± 0.02 SD; Sf mortality = 0.45 ± 0.10 SD; Sf+V mortality = 0.44 ± 0.15 SD; Cp+Sf mortality = 0.69 ± 0.12 SD; Cp+Sf+V mortality = 0.66 ± 0.16 SD). Differences are referred to the control column.

2.1.2.3. Adult survival

In general, the shapes of the survival curves revealed that the lifespan of *A. mellifera* honey bee workers was shorter in UNIUD than in BERN. This could be related to the generally stronger effects of treatments on larval survival at UNIUD (**Fig. 3a**). Despite these data, a clear negative effect of *V. destructor* regardless of pesticides was found in both laboratories (**Fig. 4ab**). Also, the combination of coumaphos and sulfoxaflor had a negative impact on adult survival in both laboratories (Cp+Sf; **Fig. 4ab**). Surprisingly, both UNIUD and BERN labs confirmed a somewhat beneficial effect of coumaphos compound on adult lifespan (Cp; **Fig. 4ab**). This could be due to a negative effect of coumaphos on *V. destructor* (highlighted by mite mortality results by UNIUD) which, by reducing *V. destructor* damage on pupae, could indirectly increase adult bee survival. Please note that the lack of a similar effect by the combination of coumaphos + sulfoxaflor may depend on the fact that the negative effect of sulfoxaflor on larval survival (highlighted by larval mortality by UNIUD) could hinder, in this case, the indirect positive effect of coumaphos.

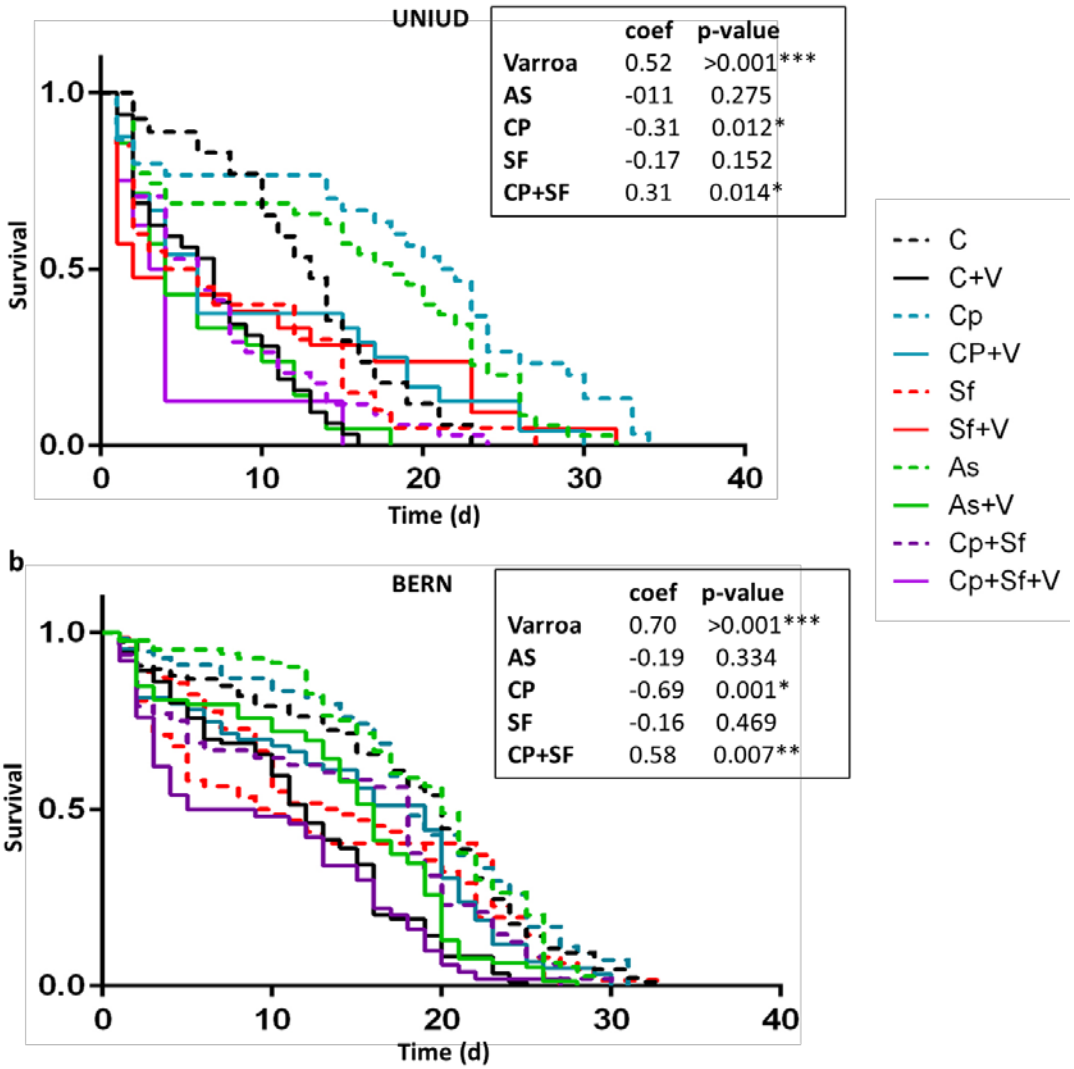


**Figure 4: Survival of adult *A. mellifera* workers** infested or not by *V. destructor* and treated with different pesticides during the larval stage. **a**: UNIUD results show a clear negative effect of *V. destructor* (Varroa) and the combination of coumaphos and sulfoxaflor (CP+SF). Coumaphos alone (Cp) had a positive impact on adult workers. **b**: BERN data confirm UNIUD results.

2.1.2.4. Mite mortality

*V. destructor* mortality rates on control pupae were 10-15% in both laboratories. Significant differences were revealed in UNIUD for coumaphos, azoxystrobin and the combination of coumaphos with sulfoxaflor. In these cases, mite mortality was higher than the control group. In contrast, sulfoxaflor did not affect mite survival (**Fig. 5a**). Interestingly, in BERN sulfoxaflor was the only compound that increased mite mortality (**Fig. 5b**).

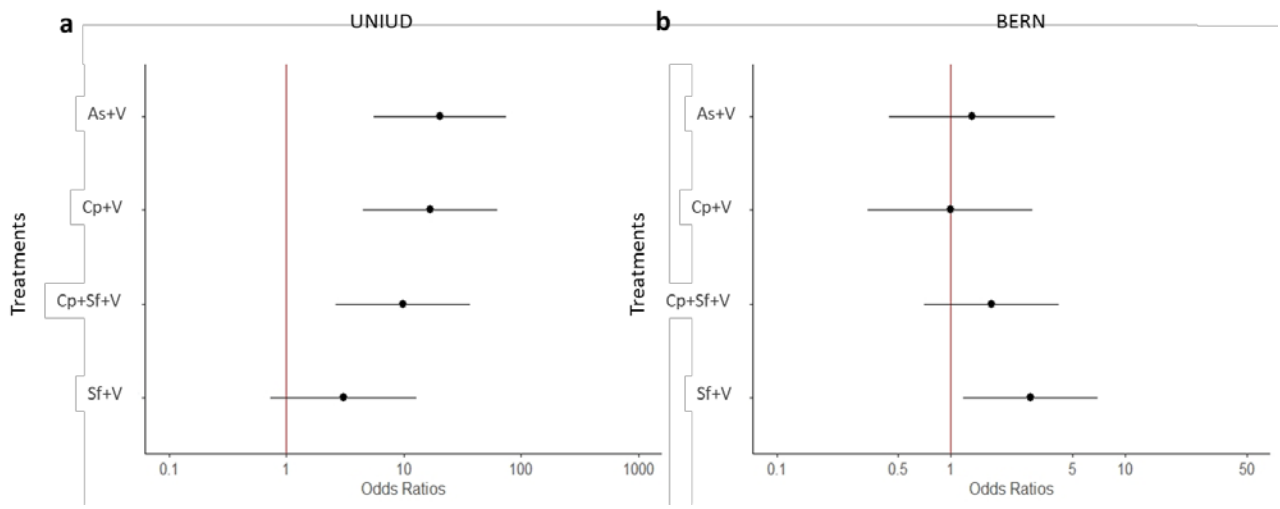


**Figure 5: Effects of chemical treatments on mite mortality** in UNIUD **(a)** and BERN **(b)**. **a**: Coumaphos (Cp+V), axozystrobin (As+V) and the combination of coumaphos and sulfoxaflor (Cp+Sf+V) increase mite mortality in UNIUD. Mite mortality results: C+V mortality = 0.11 ± 0.12 SD; Cp+V mortality = 0.25 ± 0.22 SD; As+V mortality = 0.25 ± 0.09 SD; Sf+V mortality = 0.13 ± 0.06 SD; Cp+Sf+V mortality = 0.32 ± 0.16 SD) **b**: Sulfoxaflor increases mite mortality in BERN (Sf+V). Mite mortality results: C+V mortality = 0.14 ± 0.11 SD; Cp+V mortality = 0.12 ± 0.10 SD; As+V mortality = 0.09 ± 0.00 SD; Sf+V mortality = 0.27 ± 0.13 SD; Cp+Sf+V mortality = 0.30 ± 0.23 SD). Differences are referred to the control column.

2.1.2.5. Mite fertility

In general, *V. destructor* fertility (the ratio between the number of mites producing at least one offspring and the total number of mites that survived until bee eclosion in each experimental group) was higher in UNIUD. However, the only compound that significantly increased mite fertility at UNIUD was sulfoxaflor (Sf+V, **Fig: 6a**). No differences in mite fertility were observed in BERN (**Fig. 6b**).

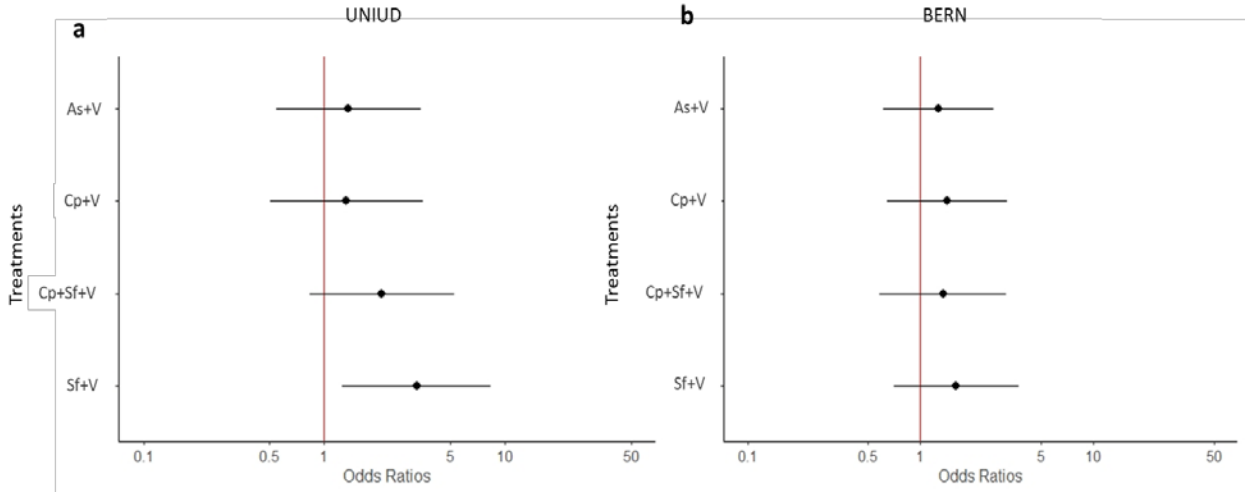


**Figure 6: Effects of chemical treatments on mite mite fertility**. **(a)** UNIUD mite fertility results: C+V fertility = 0.44 ± 0.13 SD; Cp+V mortality = 0.67 ± 0.10 SD; As+V mortality = 0.57 ± 0.09 SD; Sf+V fertility = 0.68 ± 0.06 SD; Cp+Sf+V fertility = 0.64 ± 0.19 SD). **(b)** BERN mite fertility results: C+V fertility = 0.36 ± 0.11 SD; Cp+V fertility = 0.28 ± 0.28 SD; As+V fertility = 0.28 ± 0.19 SD; Sf+V fertility = 0.24 ± 0.13 SD; Cp+Sf+V fertility = 0.29 ± 0.20 SD). Sulfoxaflor significantly increased mite fertility at UNIUD (Sf+V) but not in BERN. Differences are referred to the control column.

2.1.2.6. Mite fecundity

*V. destructor* fecundity (the number of offspring produced per fertile mite) followed the same pattern of *V. destructor* fertility. Fecundity was generally higher in BERN, but no differences between treatments were observed here. Instead, in UNIUD, sulfoxaflor (Sf+V, **Fig. 7a**) significantly increased mite fecundity (**Fig. 7ab**).

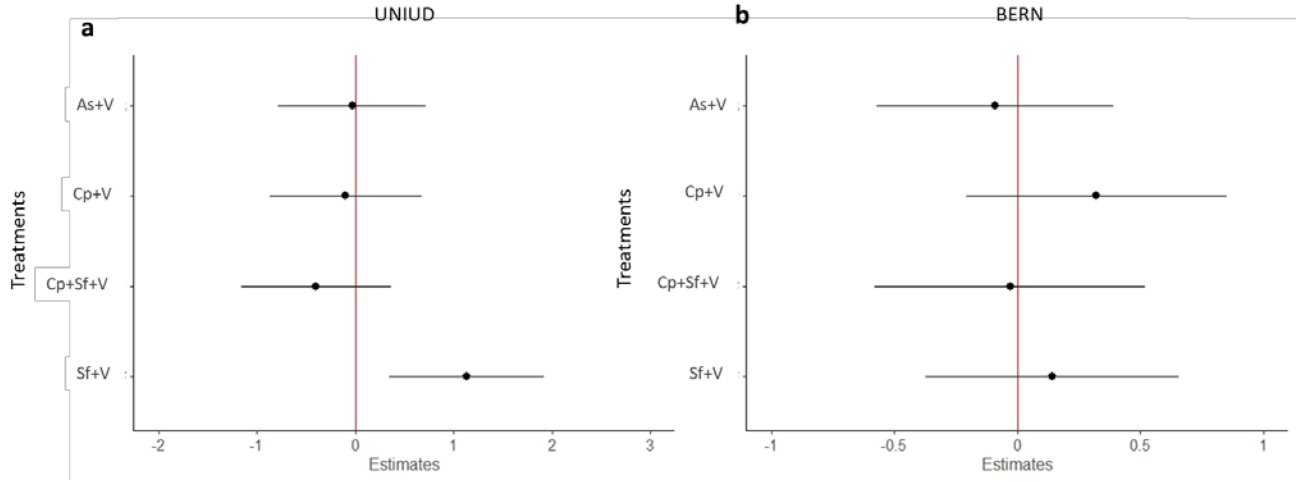


**Figure 7: Effects of chemical treatments on mite fecundity**. **(a)** UNIUD mite fecundity results: C+V fecundity = 1.71 ± 0.95 SD; Cp+V fecundity = 1.70 ± 0.86 SD; As+V fecundity = 1.76 ± 0.31 SD; Sf+V fecundity = 2.85 ± 0.35 SD; Cp+Sf+V fecundity = 1.36 ± 0.56 SD). **(b)** BERN mite fecundity results: C+V fecundity = 2.77 ± 0.09 SD; Cp+V fecundity = 2.97 ± 0.61 SD; As+V fecundity = 2.63 ± 0.56 SD; Sf+V fecundity = 2.89 ± 0.34 SD; Cp+Sf+V fecundity = 2.74 ± 0.59 SD). Only sulfoxaflor, in UNIUD laboratory, influenced the fecundity of the mites (Sf+V). Differences are referred to the control column.

### 2.1.3. Discussion

2.1.3.1. Mortality during development

The tested compounds did not cause any significant acute toxic effect on larvae, confirming the appropriateness of the concentrations chosen for these experiments. Overall, mite infestation revealed a small but consistent effect on larval survival in UNIUD. This trend may have been unnoticed in BERN due to the unbalanced experimental design. Both coumaphos (BERN) and sulfoxaflor (UNIUD) seem to cause higher mortality in bee pupae, also indicated by the significantly higher mortality rates in the combined treatment groups in UNIUD as well as BERN. Except for the case of sulfoxaflor in UNIUD, in both laboratories, *V. destructor* infestation does not seem to significantly aggravate the effect of chemicals on bee survival at the pupal stage.

2.1.3.2. Adult mortality

On the contrary, a clear negative impact of *V. destructor* on the survival of adult workers infested at the pupal stage was recorded. Also, the combination of sulfoxaflor and coumaphos applied to larvae significantly affected the survival of emerging adult bees. In contrast, the positive effect of coumaphos on adult bee survival may be a side effect of the negative impact of *V. destructor* survival in UNIUD, but BERN could not show this effect.

2.1.3.3. Mite mortality, fertility and fecundity

*V. destructor* mortality rates were significantly higher in all treatment groups except for sulfoxaflor in UNIUD. The contrary was observed in BERN, where the only negative impact on mites was revealed in

the sulfoxaflor treatment group. A significant positive effect of sulfoxaflor on mite fertility and fecundity was observed at UNIUD. This is in line with recently published data on the effect of neonicotinoids on *V. destructor* reproduction (e.g., Annoscia et al., 2020). However, BERN could not confirm these findings, likely due to differences between the mite populations.

Overall, the experiments revealed some interesting results with slight differences between laboratories. Some of the above hypotheses may be further supported by future studies on immune gene expression and DWV titre analyses.

## 2.2. *Paenibacillus larvae*-pesticide interactions (SLU)

### 2.2.1. Material and Methods

2.2.1.1. Larval mortality

Freshly hatched larvae were obtained from three different hives of the same honey bee sub-species (*A. mellifera carnica*) following a slightly modified version of the OECD-237 standard protocol. The larvae were reared in polystyrene grafting cells containing 20 µl of pure larval diet A (50% weight of fresh royal jelly + 50% weight of an aqueous solution containing 2% weight of yeast extract, 12% weight of glucose and 12% weight of fructose). The test group of larvae exposed to the pathogen bacteria were grafted in diet A spiked with a solution of *Paenibacillus larvae* (ERIC I genotype) spores to deliver a final dose of circa 10 spores/larva. The bacteria were previously plated on MYPGP agar plates (Dingman and Stahly, 1983) to check viability and to count the amount of spores per ml in solution. After two days of larval rearing, each larva was fed with 20 µl of diet B (50% weight of fresh royal jelly + 50% weight of an aqueous solution containing 3% weight of yeast extract, 15% weight of glucose and 15% weight of fructose) spiked with the three different agrochemicals to deliver a final dose of 3.8 µg/larva of sulfoxaflor (pure compound dissolved in acetone), 72 µg/larva of azoxystrobin (pure compound dissolved in acetone) and glyphosate 30 µg/larva (pure compound dissolved in water), plus a solution of diet B containing 3% of acetone was used as solvent control and a solution of diet B + dimethoate (9 µg/larva) served as positive control. After the exposure to the chemicals, larvae were fed with diet C (50% weight of fresh royal jelly + 50% weight of an aqueous solution containing 4% weight of yeast extract, 18% weight of glucose and 18% weight of fructose) for an additional 3 days and mortality was assessed every 24 hours for a total of 5 consecutive days. Larvae were kept in a desiccator containing potassium sulphate ($K_2SO_4$), to ensure a minimum of 95% humidity, and enclosed in an incubator maintained at 34.5° C for the duration of the test.

Treatment groups were: *P. larvae* (P), sulfoxaflor (S), azoxystrobin (A), glyphosate (G), *P. larvae* + sulfoxaflor (P+S), *P. larvae* + azoxystrobin (P+A), *P. larvae* + glyphosate (P+G), *P. larvae* + acetone (P+Ace), solvent control (Ace), negative control (Neg) and positive control (D). A total of 16 larvae were used per treatment.

Larval mortality was assessed in terms of percentage of dead larvae in the treated group adjusted by the mortality of the control group, following the formula:

$$M = \frac{(\%T - \%C)}{100 - \%C} \times 100$$

Where %T is the percentage of dead larvae in the treated group; %C is the percentage of dead larvae in the control group.

To check for presence and amount of *P. larvae* in the samples after the end of the test, DNA was extracted from single larval individuals and qPCR analyses were conducted using PL2 primers (Martinéz 2010). Larvae were washed before homogenization and DNA extraction with a 1% aqueous

solution of sodium hypochlorite (bleach) to remove viable cells and spores on the external surface of the cuticle.

2.2.1.2. Immune gene expression

Larval samples were grafted from the same frames used for the mortality test into petri dishes filled with 400 µl of either pure diet A, diet A spiked with *P. larvae* spore solution, diet A spiked with the agrochemicals, or a combination of the two. Each petri dish represented one specific treatment and each treatment was set up in triplicate. Treatment groups were identical to those used in the mortality test, except for dimethoate, which was absent. 20 larvae per petri dish were collected (**Fig. 8A**). At the time of grafting, 10 samples of 5 larvae each were pooled in 2 ml screwcap micro-centrifuge tubes, immediately frozen in liquid nitrogen and stored in a freezer at -80° C; these samples were not exposed to any of the treatments and represented the time zero point of the experiment. Treated larvae were sampled at three different time points: 6, 24 and 48 hours post exposure. For each time point, 5 larvae were pooled together in 2 ml screwcap tubes (**Fig. 8B**), immediately frozen in liquid nitrogen and stored in a freezer at -80° C. Only living larvae were selected at the different time points.

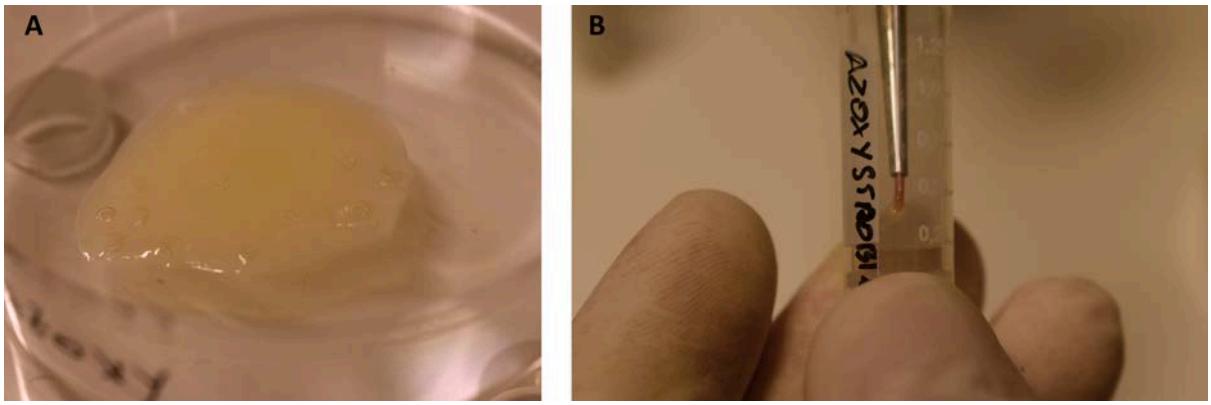


**Figure 8: Experimental setup for immune gene expression.** Petri dish containing 20 larvae over 400 µl of diet A as single treatment (**A**). Collection of larvae in 2 ml screwcap micro-centrifuge tube to be frozen (**B**).

After collecting samples for all time points, pooled larvae were washed with 1% bleach solution, homogenized in each tube and RNA was immediately extracted and converted to cDNA for qPCR immune gene expression analysis. Additionally, DNA was extracted afterwards to check for the presence and development of *P. larvae* in the samples.

Variation in immune response was assessed using *Apis mellifera abaecin* and *apiadaecin* as target genes (Evans, 2004; Li et al., 2016), and *GAPDH* as the reference gene.

### 2.2.2. Results

2.2.2.1. Mortality test

At the end of the test (5 days after exposure) a general trend of increased larval mortality was observed for the groups treated with both bacterial pathogen and agrochemicals (**Fig. 9**).

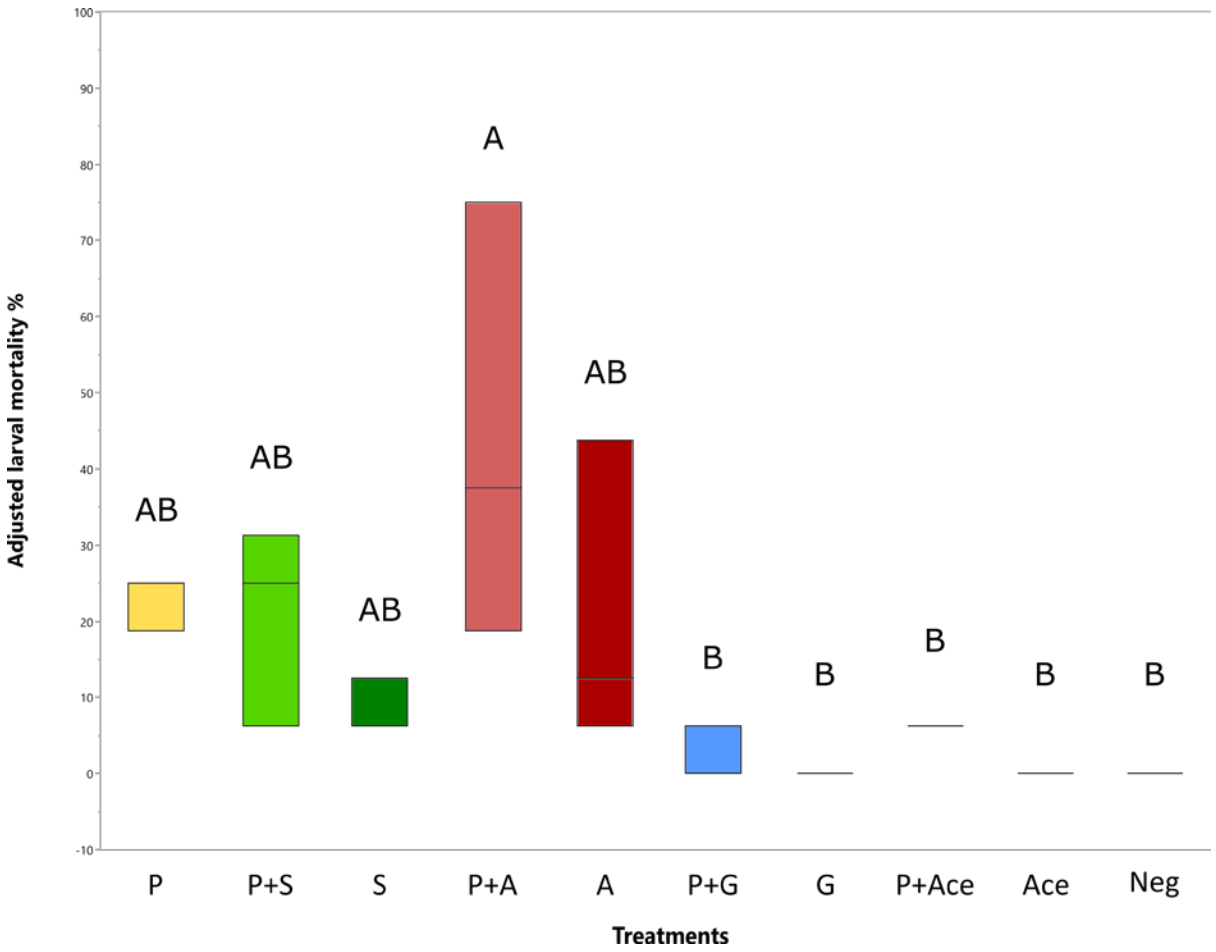


**Figure 9: Adjusted larval mortality percentage for all treatments.** Box plot showing the adjusted mortality values obtained at the end of the test for all three biological replicates for each treatment. Letters show significant statistical differences in mortality level between the treatment groups, based on the all-pairs Tukey-Kramer HSD mean comparison test. *P. larvae* (P), sulfoxaflor (S), azoxystrobin (A), glyphosate (G), *P. larvae* + sulfoxaflor (P+S), *P. larvae* + azoxystrobin (P+A), P. *larvae* + glyphosate (P+G), *P. larvae* + acetone (P+Ace), solvent control (Ace), negative control (Neg).

The treatment groups exposed exclusively to the agrochemicals showed the level of mortality expected from the given dose. Mortality levels for G exposed larvae (both chemical and pathogen + chemical) remained equal to the controls and for S no significant differences were observed between the group exposed to P+S or S. The treatment groups exposed to P+A showed an increase in mortality when compared to all the other groups, but it resulted significantly higher only compared to the control and glyphosate exposed groups (Dunnett Least Square Means differences test with the non-exposed (Neg) group; P-value 0.0017). These results were obtained from the direct comparison of all the treatment group analyzed simultaneously.

Mortality levels from the different treatment groups were also compared separately with the different control groups to assess possible interactions between the agrochemical and the bacterial pathogen. The comparison between S+P, S and the controls (P, P+Ace, Ace and Neg), did not show any significant difference in larval mortality. A significant increase in mortality was observed for P+A when compared to A using the Analysis of means (ANOM) (**Fig. 10**) and the Analysis of variance (ANOVA) with a P-value of 0.02.

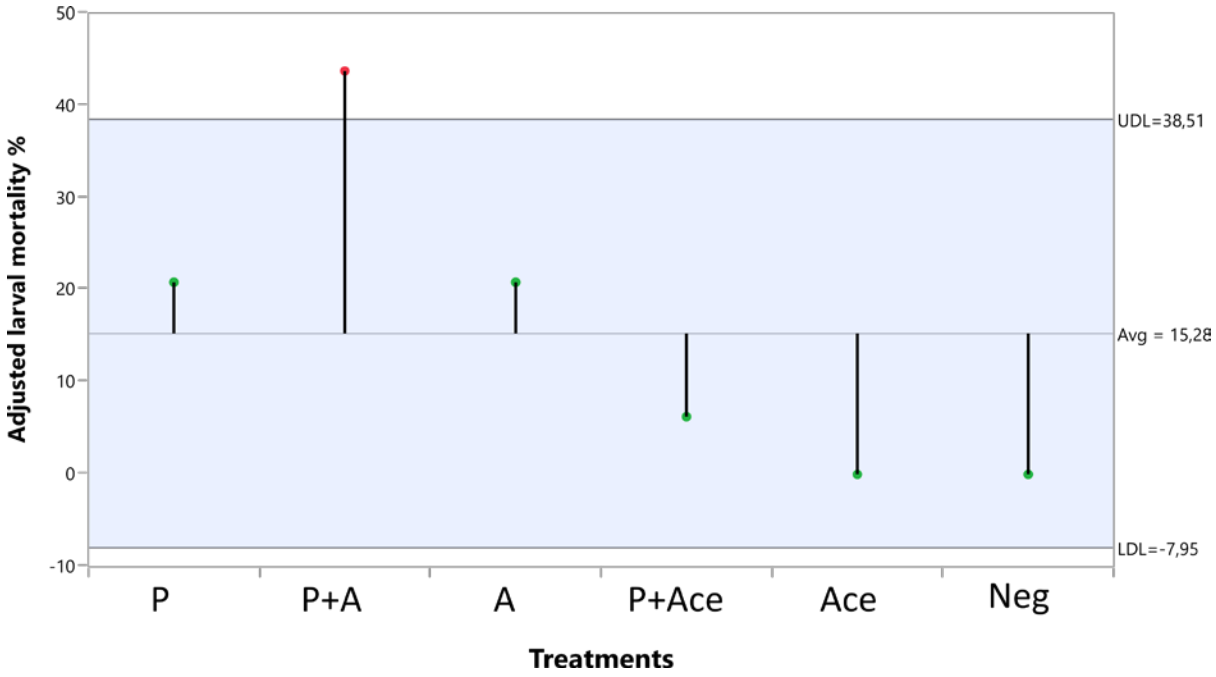


**Figure 10: Analysis of means (ANOM) for azoxystrobin - *P. larvae* interaction effect on larval mortality.** The ANOM calculates the overall mean of all the data in the dataset and then measures the variation of each sample group mean from that. On the right side of the graph, the Avg value represents the overall mean value of all the samples; UDL is the Upper Decision Line and LDL the Lower Decision Line. In the lilac shaded area are enclosed all the mean values of mortality for the treatment groups that are not significantly different from the overall mean value, therefore neither significantly different from each other (Green dots). Outside that area are located the mean values of mortality significantly different (Red dot). P+A shows a mortality level significantly higher when compared to the other treatment groups.

In the case of glyphosate, a significant difference in mortality was registered in the P+G, which was lower when compared to the samples exposed to P (ANOM analysis in **Fig. 11**) (ANOVA P-value <0.0001).

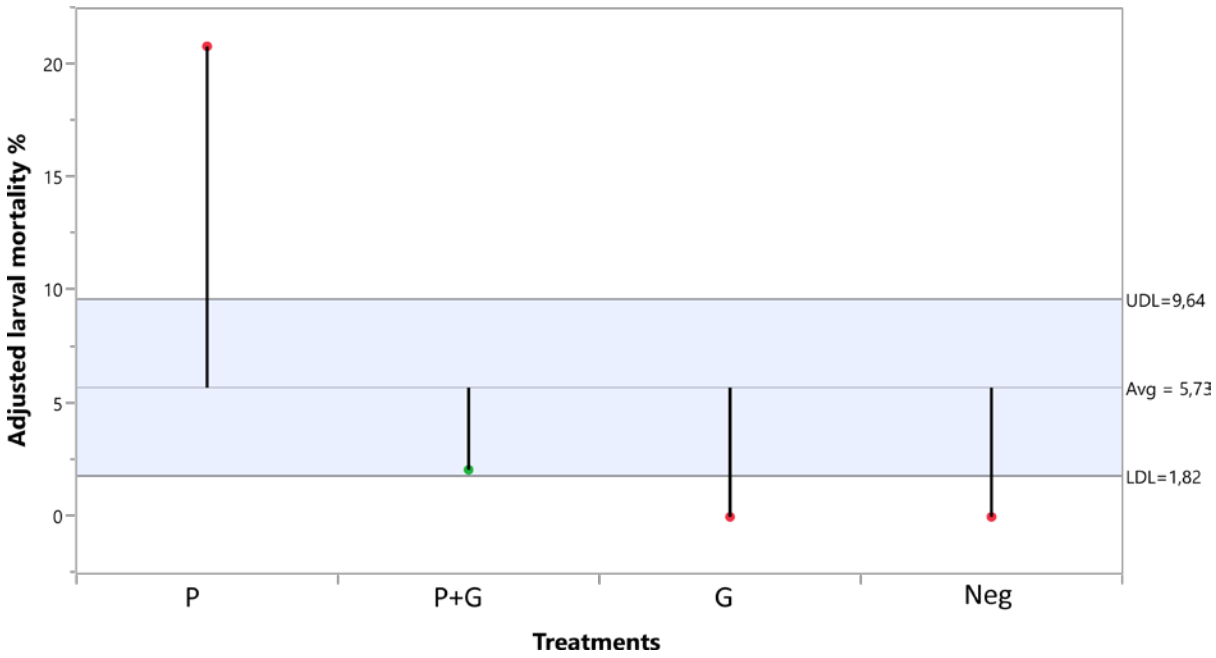


**Figure 11: Analysis of means (ANOM) for glyphosate - *P. larvae* interaction effect on larval mortality.** The mean mortality value for P is significantly higher than the overall average and all the other treatment groups. P+G is significantly different from P.

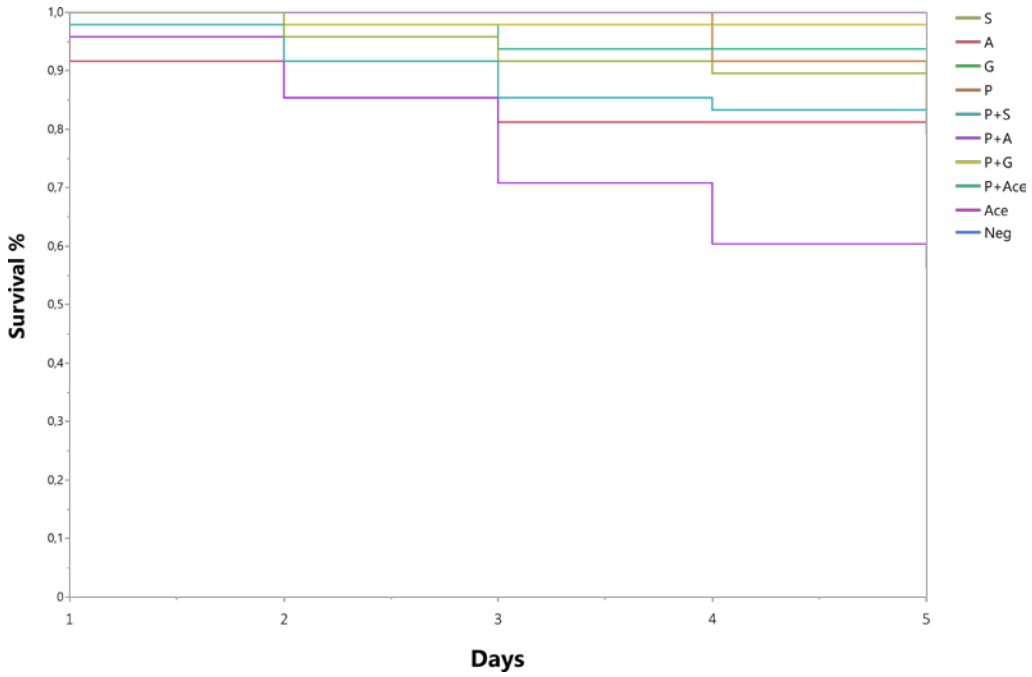


**Figure 12: Survival plot of larvae exposed to the different treatments.** Kaplan-Meier plot showing the survival of larvae exposed to the treatments. P+A treatment reduced the survival of honey bee larvae compared to the other treatments.

2.2.2.2. Immune gene expression

To assess variation in immune response between the treatment groups, *abaecin* and *apidaecin* gene expression levels were determined using the ΔΔCt method. The gene-expression fold change for the two selected targets was calculated using the formula:

$$2^{-\Delta\Delta Ct}$$

based on the difference in qPCR Ct values between the targets and *GAPDH* reference gene.

No significant variation was observed in either *abaecin* and *apidaecin* expression levels among the different treatment groups, or across the different time points.

2.2.2.3. *P. larvae* presence and load

The presence of *P. larvae* and its replication in the samples were assessed by qPCR analysis. The bacterial 16S rRNA gene was the quantification target, recorded as the number of copies. To ensure that the right PCR fragment was amplified, a melting curve was performed at the end of every qPCR run, and a 16S DNA positive standard was present in each run as a control sample. The samples exposed to the bacterial spores (P, P+S, P+A, P+G, P+Ace) were found positive by the PCR test, but none of the non-exposed samples were.

The development of the bacteria inside the larvae was assessed comparing the number of target copies found inside larvae exposed to the bacteria for 24h ($2\times10^1$ copies/larva on average) and the number of copies observed at the end of the mortality test ($8\times10^3$ copies/larva on average).

### 2.2.3. Discussion

2.2.3.1. Mortality test

The test showed the expected percentage of mortality (based on previous tests conducted within the PoshBee consortium) for larvae exposed to P, S, A and G treatments. When these results were compared with the mortality levels registered for the pathogen–agrochemical mixtures, a small increase in mortality for P+S and P+G was apparent, but it was not sufficient to reach a statistically significant level. However the result of the exposure to P+A was associated with a significant increase of mortality at the end of the test and an overall reduced survival of the larvae over time (**Fig. 12**). These results suggest an interactive effect on larval mortality when the pathogen *P. larvae* and the chemical azoxystrobin are combined.

The reduced mortality levels registered for P+G and P+Ace when compared to the P treatment were noteworthy. These data might suggest a negative interaction between the pathogen *P. larvae* and both the agrochemical glyphosate and the organic solvent acetone. This conclusion was not reinforced by data from similar experiments, especially in the case of acetone, which is extensively used as a solvent control in studies involving bee health and *P. larvae*, but further investigation of the phenomenon is surely of interest for glyphosate.

2.2.3.2. Immune gene expression

No significant difference in relative expression was registered for the selected immune response genes (*abaecin* and *apidaecin*) between the different treatment groups during this study.

2.2.3.3. *P. larvae* presence and load

Washing the external surface of the larvae with an aqueous solution of bleach, before extracting the DNA, ensured that the number of copies registered via qPCR was produced only from the bacteria developed inside the gut of the larvae (Engel et al., 2015). A comparison between the low amount registered 24 hours after infection and the amount obtained after 5 days at the end of the mortality test (almost 3 folds higher) demonstrated the replication of the bacteria inside the larval gut, indicating that the effects on mortality were driven by the pathogen.

## 2.3. *Nosema ceranae*-pesticides interactions in workers (UM)

### 2.3.1. Material and Methods

Newly emerged workers from honey bee colonies (*Apis mellifera iberiensis*) were collected and placed in microcolonies (30 °C, 70% relative humidity, sugar-water and pollen *ad libitum*). When workers reached six days of age, they were randomly assigned to one of eleven treatments combining the inoculation of *Nosema ceranae* with the exposure to three pesticides: control (Control), acetone control (Ac), azoxystrobin (A), sulfoxaflor (S), glyphosate (G), control + *N. ceranae* (Control + N), azoxystrobin + *N. ceranae* (A + N), glyphosate + *N. ceranae* (G + N), sulfoxaflor + *N. ceranae* (S + N), azoxystrobin + glyphosate + sulfoxaflor (AGS) and all stressors combined (AGS + N). At this point, bees were kept in microcolonies of 30 bees per box, with six boxes per treatment: three boxes for gene expression analyses and three boxes for sugar-water consumption, infection intensity and survivability analyses.

*Nosema* species was confirmed by PCR using primers of 218MITOC (Martín-Hernández et al., 2007) and inocula of 100,000 spores were prepared according to Fries et al., (2013) and fed to workers after 3-5 hours of starvation.

Pesticides were diluted in 50% sugar-water at low field realistic concentrations: 0.2 mg/kg of azoxystrobin (Barascou et al., 2021), 5 mg/kg of glyphosate (Thompson et al., 2014) and 0.01 mg/kg of sulfoxaflor (Jiang et al., 2020). Stock solutions of azoxystrobin and sulfoxaflor were diluted to a final concentration of 0.1 % of acetone in sugar-water. Exposure was chronic until individuals were sacrificed.

Worker individuals for sugar-water consumption, infection intensity, and survivability analyses were maintained in boxes for 14 days from the beginning of the treatments. Sugar-water consumption and mortality were recorded daily. At the end of the treatment, 10 workers were sacrificed and dissected to measure the *N. ceranae* spores in the gut.

Workers for RNA expression analyses were sacrificed seven days after *N. ceranae* infection and abdomens were homogenised in RLT lysis buffer (Qiagen) using a TissueRuptor (Qiagen). Expression of 11 immune genes comprising the main immune pathways was quantified through qPCR using *GADPH* and *Tbp-af* reference genes. The following program was used: 95°C for 10 min, 40 cycles of an initial denaturation step at 94°C for 20 s, annealing at 60°C for 30 s, elongation at 72°C for 1 min, and a step to collect fluorescence data at 78°C for 20 s.

### 2.3.2. Results

2.3.2.1. Gene expression

Multivariate analyses indicated that azoxystrobin, glyphosate and the pesticide mixture alone or in combination with *N. ceranae* did not affect gene expression. Only honey bees exposed to both

sulfoxaflor and *Nosema* infection showed a significantly altered expression of immune and detoxification genes **(Fig. 13).**

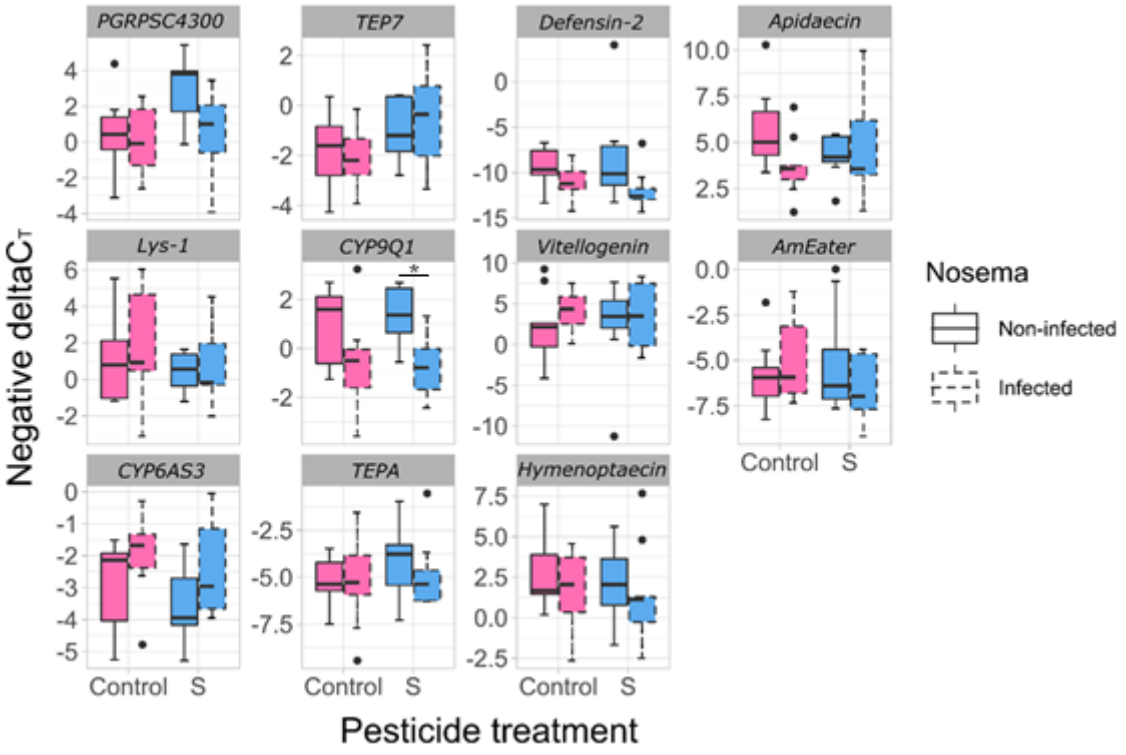


**Figure 13: Boxplot of immune and detoxification gene expression in workers exposed to sulfoxaflor and infected with *N. ceranae***. Control = control; S = sulfoxaflor. Gene expression was measured after seven days of exposure to sulfoxaflor and *N. ceranae*. The graph shows the median (bar), interquartile ranges (boxes), and maximum and minimum observed values within the Q3 + 1.5*IQR / Q1 – 1.5*IQR ranges, respectively (whiskers). *significant differences between treatments after applying Benjamini-Hochberg correction.

2.3.2.2. Sugar-water consumption

There were no significant effects of azoxystrobin, glyphosate, the pesticide mixture, or *N. ceranae* infection on sugar water consumption. Similarly, there were no significant effects of their combined treatments. However, *N. ceranae* and sulfoxaflor in combination produced an increase in daily sugar-water consumption in workers **(Fig. 14).**

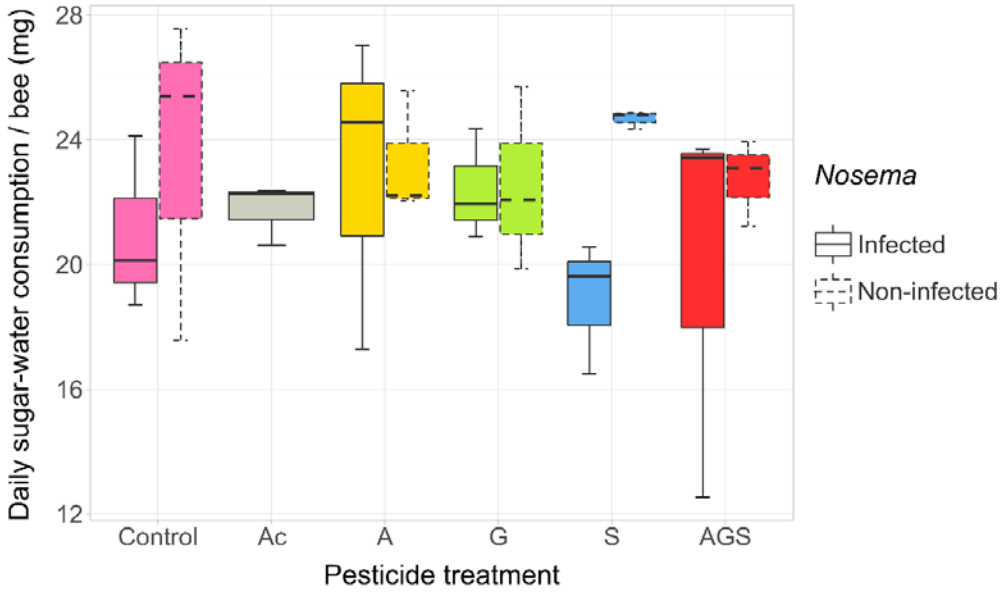


**Figure 14: Sugar-water consumption in workers exposed to different combinations of pesticides and *N. ceranae***. Control = pesticide control, Ac = acetone control, A = azoxystrobin, G = glyphosate, S = sulfoxaflor, AGS = azoxystrobin + glyphosate + sulfoxaflor N = *N. ceranae*. Sugar-water consumption was measured per microcolony for 14 days and divided by the number of living workers. Boxplot of averaged daily sugar-water consumption. The graph shows the median (bar), interquartile ranges (boxes), and maximum and minimum observed values within the Q3 + 1.5*IQR / Q1 – 1.5*IQR ranges, respectively (whiskers). *N. ceranae* infection increased sugar-water consumption on workers exposed to sulfoxaflor.

2.3.2.3. *N. ceranae* load and survivability

Exposure to azoxystrobin, glyphosate, sulfoxaflor and the pesticide mixture did not increase parasite load in the midgut. Only sulfoxaflor decreased honey bee survivability. Exposure to other pesticide treatments and infection with *Nosema* did not have a significant effect on worker survivability **(Fig. 15).**

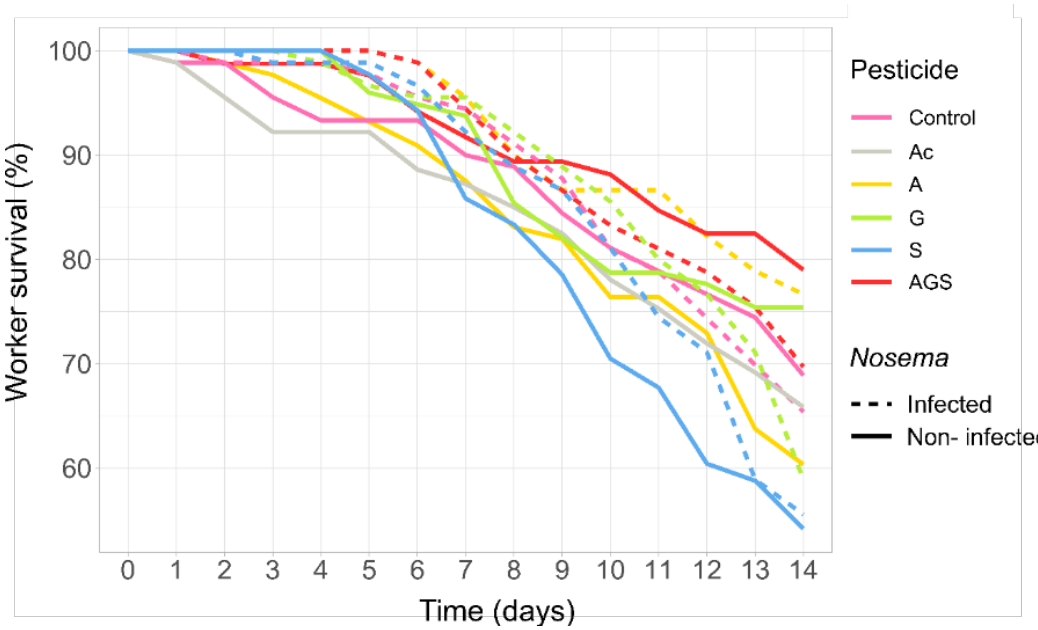


**Figure 15: Survival in workers exposed to different combinations of pesticides and *N. ceranae*.** Control = pesticide control, Ac = acetone control, A = azoxystrobin, G = glyphosate, S = sulfoxaflor, AGS = azoxystrobin + glyphosate + sulfoxaflor N = *N. ceranae*. Living workers per microcolony were measured for 14 days after the beginning of the treatments. Graph showing mean worker survival for every treatment. Exposure to sulfoxaflor decreased worker survival.

### 2.3.3. Discussion

2.3.3.1. Gene expression

Azoxystrobin and glyphosate alone or in combination with *N. ceranae* did not alter expression of immune and detoxification genes in worker honey bees. Regarding azoxystrobin, previous studies have found no significant effects on immune, detoxification and oxidative stress genes (Christen et al., 2019; Barascou et al., 2021). In reference to glyphosate, Almasri et al. (2021) found no effects on antioxidant or immunocompetence enzyme activities in honey bee adults. However, Zhao et al. (2020) found changes in the regulation of other cytochrome P450 genes and AMP genes. The difference with our results could be explained by the high response variability among colonies (Vázquez et al., 2018).

*N. ceranae* only affected gene expression in bees exposed to sulfoxaflor. Other studies have reported downregulation of immune genes upon *N. ceranae* infection (Badaoui, 2017). It is possible that our bees were only energetically stressed enough to alter gene expression when both stressors were present.

The treatments with the pesticide mixtures did not alter gene expression alone or in combination with *Nosema*. The decrease in food intake in workers exposed to the pesticide mix and infected with *Nosema*, in comparison with those exposed to sulfoxaflor and *Nosema*, could explain why no changes were observed with ingestion of the three pesticides even when bees were exposed to the same sulfoxaflor concentration.

2.3.3.2. Sugar-water consumption

We did not find any significant variation in the food intake with azoxystrobin or glyphosate alone or in combination with *Nosema,* in congruence with other studies that have also shown a lack of effect of these two agrochemicals on food intake (Tamburini et al., 2021; Almasri et al., 2021).

Sulfoxaflor exposure alone did not increase sugar-water intake, as observed by Zhu et al. (2017) and suggested by Tamburini et al. (2021), neither found an effect of sulfoxaflor commercial formulation on hive weight, used as a measure for food reserves. However, *N. ceranae* infection caused a higher increase in sugar-water consumption in workers exposed to sulfoxaflor than in control workers. It is known that *N. ceranae* infection increases energy demand in bees (Mayack & Naug 2009). On the other hand, sulfoxaflor causes oxidative stress (Chakrabarti et al., 2020), and its negative effects on honey bee survivability are reduced when bees have access to high quality pollen (Barascou et al., 2021). This suggests that sulfoxaflor exposure and infection did not pose enough energetic stress to increase sugar-water consumption separately, but that they did in combination. *N. ceranae* infection did not increase sugar-water consumption in comparison with the respective pesticide treatments except for sulfoxaflor. The tolerance to *Nosema* infection observed in this study agrees with the lack of energetic stress in most treatments.

The treatment combining the three pesticides caused a remarkable decrease in sugar-water consumption in one of the three boxes analysed. It is possible that workers from this box were able to detect and avoid consumption of the sugar-water because of the higher concentration of xenobiotics present.

2.3.3.3. *N. ceranae* load and worker survival

Pesticides used in this work did not alter *N. ceranae* spore load in the gut of honey bee workers, either alone or in combination. Another study found no effect of glyphosate on *N. ceranae* load (Almasri et al., 2021).

Azoxystrobin did not cause an increase in mortality alone or in combination with *N. ceranae*. Our results are consistent with those of Tamburini et al. (2021) and Barascou et al. (2021), which did not find an effect on bee survival. In reference to glyphosate, some studies have reported both no lethal effects and no synergistic effect with *N. ceranae* on survivability (Almasri et al., 2021). However, other studies showed significant mortality of adult honey bees (Motta & Moran, 2020). Variation in these results is likely explained by colony response variability, as shown in Vázquez et al. (2018) for larvae.

Sulfoxaflor treatments increased worker mortality. In contrast, Al Naggar & Paxton (2020) found no effect on bee survivability at higher concentrations. Barascou et al. (2021) suggest that bees exposed to high sulfoxaflor concentrations may decrease syrup consumption. It is likely that bees in this study consumed more sulfoxaflor than bees in Al Naggar & Paxton (2020) due to the low concentrations we used. Subsequently, they might have been exposed to higher concentrations which caused increased mortality. Alternatively, differences in mortality could be explained due to inter-colony genetic variability. Sulfoxaflor exposure in combination with *Nosema* infection had no worker survival, in accordance with the lack of effects of sulfoxaflor on *N. ceranae* load.

*N. ceranae* did not elevate mortality in comparison with the respective pesticide treatments. These results point to a tolerance for *N. ceranae* infection that has already been reported for some honey bee colonies (Kurze et al., 2016). Moreover, the lack of interaction with pesticides on survivability agrees with the lack of an effect on parasite load.

The treatments with the pesticide mixture did not alter worker survival alone or in combination with *Nosema*. It is possible that ingestion of the three pesticides causes a stronger activation of the detoxification system that may result in a faster metabolization of sulfoxaflor, thus decreasing its negative effect on worker survival.

## 2.4. *Nosema ceranae*-pesticide interactions in queens (INRAE)

We tested the hypothesis that honey bee queens chronically exposed to an environmentally relevant sublethal dose of sulfoxaflor during their first days of life, in combination with the common parasite *N. ceranae*, experience shorter lives, are less able to mate, and lay fewer fertilized eggs. To test this hypothesis, a survivorship trial was carried out. Four groups of queens were set-up in the laboratory: *N. ceranae* infected queens (N), sulfoxaflor exposed queens (P), both *Nosema* infected and pesticide exposed queens (NP) and non-treated queens as controls (C). The experiment started in June 2021. Queens from groups N and NS only were inoculated orally at birth with 300,000 spores/queen of *N. ceranae*. Queens were then kept in cages with a group of attendant workers. The same spore dose under similar conditions was proved to be sublethal to queens in cage experiments over a comparable time lapse (Dussaubat et al., 2016). Queens and workers from groups S and NS were then exposed for 5 days to sugar syrup contaminated with 0.1 microgram/ml. sulfoxaflor. Because in nature a queen rarely feeds herself, but is fed by the attendant bees (nurses) with the royal jelly they produce in their hypopharyngeal glands, we expected an indirect exposure of queens to the pesticide. After exposure, queens were transferred into small hives used to rear queens (mating nuclei) placed in the field to naturally mate and lay eggs for 3 months.

### 2.4.1. Material and Methods

2.4.1.1. Queen rearing

40 young virgin sister queens were purchased from a local beekeeper. Each queen was placed in an individual cage and provided with 30 one-day-old attendant nurse bees obtained from combs of last stage pupae, which were incubated until emergence and then transferred into the cages.

2.4.1.2. Pesticide exposure

A sublethal dose of sulfoxaflor was given to the attendant bees and the queen, through contaminated sugar syrup for 5 days. Contaminated sugar syrup was made with a solution of 50% (w/v) sucrose and a pure standard of sulfoxaflor diluted with acetone and water to obtain a stock solution. Syrup was prepared at a concentration of 50% (w/v) sucrose and used to dilute the stock solution (sulfoxaflor 3.67 µg ml−1, 0.37% acetone) to get a final solution of 0.1 µg/l sulfoxaflor. Solutions containing sucrose and 0.37% acetone, were used as controls. Feeders with sugar syrup were replaced each day at the same time of day and weighed before and after feeding to estimate syrup consumption. Pesticide exposure began when attendant worker bees were introduced in the cages along with the queen, and continued for 5 days. Queens were then ready to start orientation and mating flights. During the 5 days of exposure, bees were provided with *ad libitum* water, but no pollen. Food and water were changed every two days.

2.4.1.3. *Nosema ceranae* experimental infection of queens.

Queens were individually inoculated with spores of *N. ceranae*. For spore inoculation, a solution was prepared containing 50% (w/v) of sucrose in water and a concentration of 150,000 spores/µl in suspension, and each queen bee was fed 2 µl of this solution using a micropipette. From a naturally infected colony, spores of *N. ceranae* were isolated by crushing the abdomens of infected bees in distilled water, filtering the suspension and centrifuging it to collect the spores. Spores were provided by Orlando Yanez, our Swiss collaborator in the PoshBee consortium (University of Bern).

2.4.1.4. Queen introduction in mating nuclei.

After *N. ceranae* spore inoculation and exposure to the pesticide in the laboratory for 5 days, each queen was colour marked on the thorax according to their experimental group (N, P, NP and C) and introduced into a mating nucleus. 40 nuclei (APIDEA®), ten for each experimental group, were established one day before the queen's introduction. To build the nuclei, workers were collected from the upper hive bodies of large colonies. Each mating nucleus contained approximately 130 g of bees, 3 small frames with foundations, and one feeding part filled with sugar candy. After introduction in the nuclei, queens were left undisturbed for three weeks, so they could mate and start laying eggs, which can take place when queens are between 10 to 19 days old depending on the time of the season. Queen acceptance by the bee population in each nucleus was verified three weeks after introduction. Every month, for 3 months, nuclei were carefully observed frame by frame to determine the presence of the queen either by direct observation or by searching for freshly-laid eggs and the presence of brood. We also recorded the unusual presence of drone cells, dead larvae, abnormal brood pattern or worker behavior and signs of diseases, queen loss, or pests. Queen survival was the only variable considered, since mating nuclei have a reduced space compared to regular hives, and are therefore less suitable to follow colony development. The mating hives were set in pairs with their entrances facing opposite directions and painted different colors to facilitate queen orientation when returning to the nucleus after mating.

### 2.4.2. Results

Queen survival across the different treatments is presented in **Figure 16**. We did not observe higher queen mortality with either the *Nosema* or the sulfoxaflor treatment or with the combined treatment compared to the control, except at 10 weeks between the control and the *Nosema* treatment. The control treatment had the lowest rate of queen survival, even when compared to the combined effect of both *Nosema* and sulfoxaflor. P and NP had the highest survival rate.

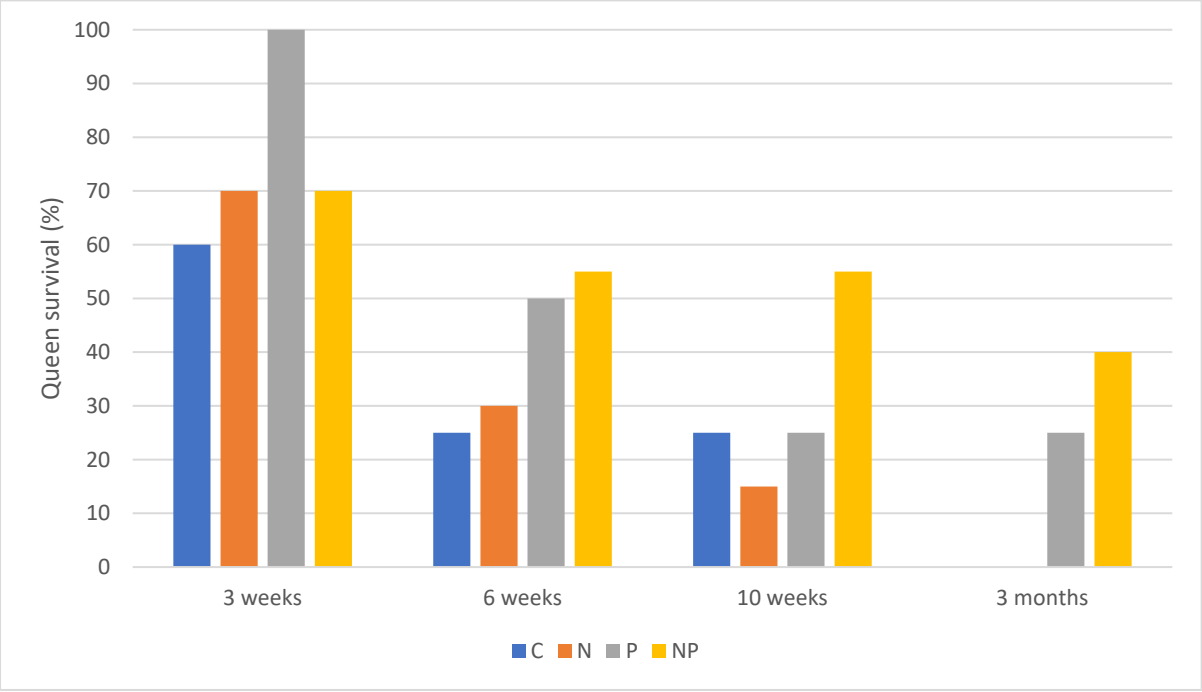


**Figure 16: Survival of the queen**. Queens were divided in four groups to be parasite-inoculated and pesticide-exposed as follows: *N. ceranae* infected queens (N), sulfoxaflor exposed queens (P), both infected and exposed to sulfoxaflor queens (NP) and control queens (C).

### 2.4.3. Discussion

In our experimental conditions, we could neither demonstrate detrimental effects of *Nosema* nor of sulfoxaflor on the survival of the tested queens, or of the combination of *Nosema* and sulfoxaflor which, in conclusion, gave the best survival rate.
The survival of the control after 6 weeks was only 25%, which suggests an experimental bias. Indeed, it could be the case that since the temperature in June was very high, it may have had an effect on queen mating.

The results suggest that the pesticide at this concentration may have limited effects on queen survival. The sulfoxaflor may have been quickly metabolized by attendant workers feeding the queen and by the queen herself, as it is suggested in other experiments (Barascou et al., 2021).

## 2.5. *Nosema ceranae*-pesticide interactions in drones (BERN)

### 2.5.1. Material and Methods

Freshly emerged drones were randomly allocated to test cages. Each cage was assigned to one of four treatment groups. 1. Control, 2. *Nosema ceranae*, 3. Sulfoxaflor, 4. Combined, and contained 10 drones. A total of 6 cages was set up for each treatment group (N = 60 drones/treatment). Treatments with *N. ceranae* (i.e., *Nosema ceranae* and Combined) received a droplet of sucrose solution (suc. sol.) containing 100'000 *N. ceranae* spores/drone, while control and sulfoxaflor cages received pure sucrose solution. Cages were placed in the incubator under standard conditions (30°C and 70%±10 relative humidity (RH)) for four hours. After full consumption of the droplet, 20 workers were added to each cage to facilitate drone attendance. Subsequently, all treatments received a feeder containing either pure suc. sol. (i.e., *Nosema ceranae* and Combined) or suc. sol. spiked with sulfoxaflor in a concentration of 0.01 mg/kg (i.e., Sulfoxaflor and Combined) as well as corbicula honey bee pollen (**Fig. 17**). Food was provided *ad libitum*. Suc. sol. feeders were changed on a daily basis and pollen feeders every three days. For a period of 14 days, bees in the sulfoxaflor and Combined treatment group received the spiked suc. sol. Chronically, which was then replaced with pure suc. sol. until the end of the experiment when the last bee had died.

**Figure 17: Experimental cage.** Each cage [100 $m^3$] containing 10 drones and 20 workers with the feeders containing sucrose solution (top) and corbicula pollen (right hand side).

Mortality was recorded every 24h and dead individuals were removed from the cages and stored at -20°C for subsequent *N. ceranae* spore counts. Spore loads were determined by crushing the whole drone abdomen in 1ml water and spore numbers were counted using a counting chamber and a light microscope following standard methods described by (Hornitzky, 2008).

All variables were tested for normality by using Shapiro–Wilk's Tests. Spore loads were non-normally distributed (Shapiro–Wilk's Tests, $p<0.05$) and values were therefore analysed using a Kruskal–Wallis One-Way ANOVA and medians and the respective 95% lower and 95% upper confidence limits are reported. Post-hoc comparisons between groups were conducted using a multiple pairwise comparisons test (Bonferroni Multiple Comparison Test). Survival analyses were performed using Kaplan– Meier cumulative survival curves and Cox-Mantel Logrank Test to compare treatment groups. Average survival in % as well as the standard error (SE) are reported for day 14 (i.e., end of exposure period). Statistical analyses and figures were performed using NCSS (NCSS version 12, Statistical Analysis Software, Kaysville, Utah, USA).

### 2.5.2. Results

2.5.2.1. Mortality

On day 14 (=end of exposure period), 70 ± 5.92 of control drones, 28.81 ± 5.9 of *N. ceranae* inoculated drones, 52.54 ± 6.5 of sulfoxaflor exposed drones, and 35.59 ± 6.23 drones in the combined treatment group were alive (cumulative survival (%) ± SE). Overall, survival of control drones did not significantly differ from the Sulfoxaflor treatment group ($p=0.663$; **Fig. 18**). Both groups showed significantly higher survival rates than the *Nosema ceranae* and Combined treatment groups ($p < 0.001$) which did not significantly differ from each other ($p=0.302$; **Fig. 18**).

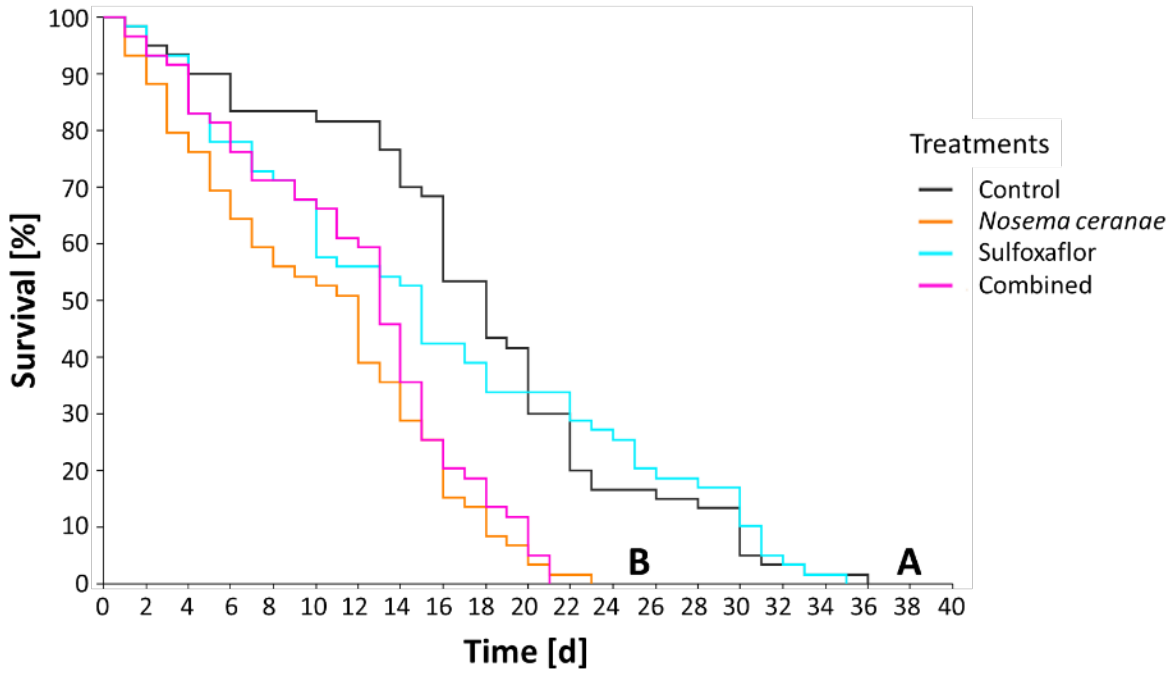


**Figure 18: Kaplan–Meier survival curves show the decline of the cumulative survival (%) over time.** Significantly reduced survival was found for *Nosema ceranae* exposed drones (N = 59, orange line) and drones from the combined treatment group (N = 59, pink line) ($p < 0.01$). Controls (N = 60, black line) and sulfoxaflor exposed drones (N = 59, blue line) showed highest survival rates and did not significantly differ from each other ($p = 0.663$). Significant differences ($p < 0.05$) are indicated by different letters (A and B).

2.5.2.2. *N. ceranae* load

Drones from the control group showed average *N. ceranae* spore counts of 2.25 x $10^5$ (1.07 - 6.01 x $10^5$), and sulfoxaflor exposed drones 1.25 x $10^5$ (0 – -3.6 x $10^6$) (median (95% lower - 95% upper CL)), with no significant difference between the groups (p=0.705, **Fig. 19**). Similarly, drones from the *N. ceranae* group with 1.1975 x $10^7$ (0.82 – 2.92 x$10^7$) and drones from the Combined treatment group with 1.61 x $10^7$ (1.27 – 3.05 x $10^7$) spores, respectively did not significantly differ (p=0.325**. Fig. 19**). However, both controls and sulfoxaflor drones showed significantly lower *N. ceranae* spore counts than *N. ceranae* exposed drones and drones from the Combined treatment group (p<0.001; **Fig. 19**)

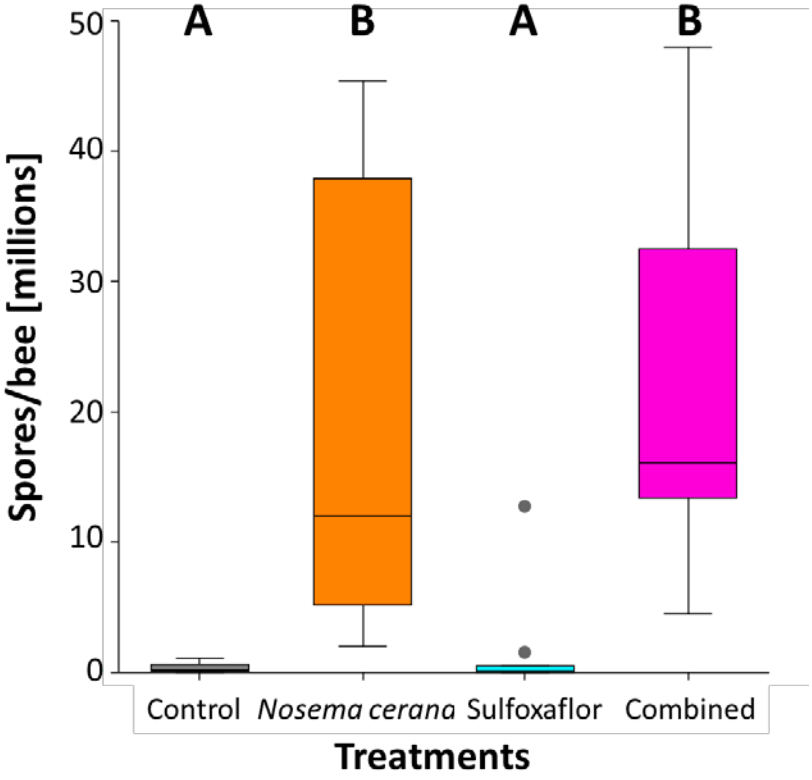


**Figure 19: *Nosema ceranae* spore counts in the different treatment groups.** Controls (gray box, N = 12) and sulfoxaflor exposed drones (blue box, N = 12) showed the lowest spore counts and did significantly differ from *N. ceranae* exposed drones (orange box, N = 12) and drones from the combined treatment group (pink box, N = 11) ($p < 0.001$). Boxplots show the inter-quartile range (box), the median (black line within box), data range (horizontal black lines above and beneath box), and outliers (gray dots). Significant differences ($p < 0.05$) are indicated by different letters (A and B).

### 2.5.3. Discussion

2.5.3.1. Mortality

Sulfoxaflor treatments at a concentration of 0.01 mg/kg did not increase drone mortality. These findings are in line with data on honey bee workers reported by Al-Naggar & Paxton (2020) and Li et al. (2021). There, field relevant concentrations did not increase worker mortality when compared to controls. In the worker experiment performed by UM (see above), sulfoxaflor exposure was the only treatment that did cause a reduced survival. This discrepancy may be due to differences in location, subspecies, or timing in the year.

*Nosema ceranae* on the contrary did significantly reduce drone survival rates. This is in line with a previous study by Retschnig et al. (2014b) where drones showed significantly lower survival rates when exposed to 100'000 *N. ceranae* cells per bee. In the same study, workers in the respective *N. ceranae* treatment group did not significantly differ from controls which is in line with the findings by UM.

Survival rates in the combined treatment group were significantly reduced when compared to controls. However, as above, sulfoxaflor did not interact with *N. ceranae* on drone survival, indicated by the non-significant difference between *N. ceranae* and combined treatment group. A study by Retschnig et al. (2014a) showed that bees exposed to thiacloprid and *N. ceranae* only showed reduced survival when thiacloprid was given in a high concentration. They conclude that the interaction between the pesticide and the pathogen seems to be dose-dependent, similarly to Alaux et al. (2009) using imidacloprid and *N. ceranae*.

2.5.3.2. *N. ceranae* load

*N. ceranae* spore counts revealed natural infections of our colonies as indicated by the low spore numbers in the controls as well as the sulfoxaflor treatment group, with the groups being statistically indistinguishable. *N. ceranae* inoculated drones showed significantly higher spore counts than drones that were naturally infected within their colonies. Again, the *N. ceranae* group did not significantly differ from the combined treatment group. Therefore, and similarly as above for UM, we conclude that sulfoxaflor does not alter *N. ceranae* spore loads in the gut of drones. Interestingly, in the study of Retschnig et al. (2014a) they reported significantly lower spore numbers in bees additionally exposed to thiacloprid, regardless of concentration. In contrast to these findings, Vidau et al. (2011) found significantly higher spore numbers in bees exposed to thiacloprid and *N. ceranae*. This disparity may be linked to differences in chemicals, location, origin and age of bees.

## 2.6. Genetic backgrounds (UM)

### 2.6.1. Introduction

The biodiversity of the honey bee is a field under continuous study and the most recent data refer to about 30 subspecies (Chen et al*.,* 2016). In Europe, 10 are naturally distributed (De la Rúa et al*.,* 2009). These subspecies are differentiated by morphological, phenotypic and molecular characters (Bouga et al*.,* 2011; Meixner et al., 2013) that allow their grouping into evolutionary lineages. Among the molecular markers, the most widely used is found in the mitochondrial DNA molecule, specifically in the intergenic region between the genes tRNA-leu and cytochrome oxidase subunit II (*cox*2). This region has a nucleotide composition and diversity that allows the subspecies to be grouped into evolutionary lineages (Garnery et al., 1993). In Europe there are honey bee populations belonging to the African (A) evolutionary lineage in the south of the Iberian Peninsula, where *Apis mellifera iberiensis* is distributed, to the western Europe (M) evolutionary lineage in central Europe where *A. m. mellifera* is naturally distributed, and to the Eastern Europe (C) evolutionary lineage in Italy (*A. m. ligustica*) and central-eastern European countries (*A. m. carnica*) and the Hellenic peninsula (*A. m. cecropia*).

Populations of these subspecies come into contact either naturally (Muñoz et al*.,* 2009; Muñoz and De la Rúa, 2021) or through intentional introduction by beekeepers (Henriques et al., 2020) leading to gene introgression events currently analysed with markers such as single nucleotide polymorphisms or SNPs (Muñoz et al., 2017; Henriques et al*.,* 2018).

### 2.6.2. Material and Methods

Samples of worker honey bees were received from the colonies included in this WP6, namely from: UNIUD-Italy, BERN-Switzerland and UM-Spain. The SLU-Sweden samples come from the same colonies as WP3. These samples were also analyzed and included in the results of WP3 genetic background.

Individuals were preserved in ethanol or in RNA-later (the latter for transport and customs reasons). On arrival at UM, they were kept at -20°C until processing.

#### 2.6.2.1. DNA extraction

Genomic DNA was extracted from individual legs of single honey bee workers according to the protocol of the Canadian Centre for DNA Barcoding Glass Fiber Plate DNA Extraction Protocol (Ivanova et al., 2006).

#### 2.6.2.2. Mitochondrial amplification

The evolutionary lineage and haplotype of the colonies were inferred by analysing the sequence variation of the mitochondrial intergenic region located between the tRNA-leu and *cox*2 genes. Given the inheritance of this molecule, the origin of the queens controlling each colony can be identified in this way (Garnery et al., 1993).

The intergenic tRNA-leu-*cox*2 region was PCR-amplified using MyTaq™ kit (Bioline, London, UK) following the manufacturer's recommendations. Briefly, 2 µL of the extracted DNA, 1X reaction buffer, forward and reverse primers (E2: 5'-GGCAGAATAAGTGCATTG-3' and H2: 5'-CAATATCATTGATGACC-3') with a final concentration of 0.2 µM each, (Garnery et al., 1993) and Taq polymerase (0.25 units) were mixed in 12.5 µL final reaction volume. The PCR cycling conditions were:

- 94°C (5 min)
- 35 cycles of a 45 s denaturation at 94°C, elongation for 45 s at 48°C, extension for 60 s at 62°C
- final extension step at 65°C for 20 min.

Amplicons of each sample were submitted for sequencing (Macrogen, Madrid, Spain).

#### 2.6.2.3. Sequence analysis

Sequences were manually checked and aligned using MEGA 6.06 (Tamura et al., 2013). Identification of the haplotypes (and hence evolutionary lineage) was performed through BLAST in the GenBank NCBI database. The identification of each haplotype was carried out by using the RFLP patterns obtained from the *Dra*I enzyme, described in Chávez-Galarza et al. (2017)

### 2.6.3. Results

#### 2.6.3.1. Haplotypes and evolutionary lineages

Three evolutionary lineages were detected: African (A-lineage), West Mediterranean and North European (M-lineage) and Central and Southeast European (C-lineage).

In Spain, only haplotypes from the A-lineage (A2) were detected. In Italy, all samples belonged to the C-lineage (C1, C2) whereas in Switzerland, only haplotypes from the M-lineage (M4, M79´) were detected (**Fig. 20**).

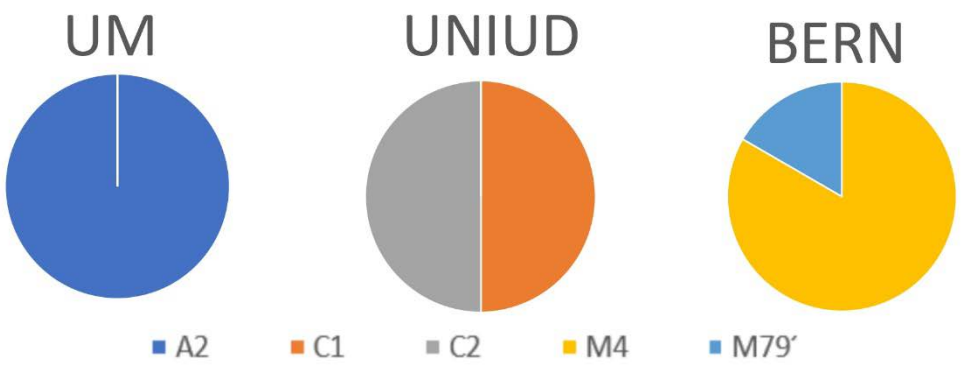


**Figure 20: Pie charts showing the frequency of haplotype** detected in the three countries included in the study.

### 2.6.4. Discussion

One of the objectives of the PoshBee project is to assess the interaction between agrochemicals used in the field and the pathogens that harm the health of honey bees, among other pollinators, with the aim of providing key information for the development of improved policies and regulations for the safe and sustainable use of agrochemicals in Europe.

The experiments for this purpose have been carried out in four laboratories with experimental apiaries whose hives should be occupied by native subspecies. In this way the results could be extrapolated to the native subspecies distributed in each country.

The results of the analysis of the mitochondrial DNA variation are presented here. Mitochondrial markers are maternally inherited and therefore indicate the origin of the queen in each colony. On the other hand, nuclear markers show the admixture derived from the genetic contribution of the drones with which the queen has mated (Meixner et al., 2013).

The expected evolutionary lineages have been detected in each of the experimental apiaries (A at UMU-Spain, C at UNIUD-Italy, M at BERN-Switzerland) (De la Rúa et al., 2009; Tihelka et al., 2020). The detected haplotypes are among the most frequently observed in naturally dispersed subspecies at these localities (Cánovas et al., 2008; Chávez-Galarza et al., 2017; Franck et al., 2000; Parejo et al., 2016; Pinto et al., 2014).

## 3. Acknowledgements

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