Manuscript on agrochemical and pathogen effects on individual bumble bee health

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

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Preface

Wild bees suffer from a range of different stresses (Goulson et al., 2015). While this is natural and bees are adapted to respond to this, the addition of agrochemical pesticides as a novel stressor has the potential to be damaging to bees. This is true not just for pesticides as stressors alone, but additionally, as a potential synergist of other stressors (Siviter et al., 2021). If stressors do synergise together then their negative effects are exacerbated, potentially contributing to the declines in bee populations observed globally. Alternatively, if agrochemicals act as antagonists of other, natural stressors, they could reduce their impact on bees. Consequently, understanding interactions between stressors and how they impact the health of bees is a key and pressing question.

Bumble bees are a charismatic and important group of pollinators that play a significant role in providing pollination services to agricultural systems (Garibaldi et al., 2013). As wild pollinators, their health cannot be managed through veterinarian interventions. Consequently, understanding the impact of agrochemical pesticides and parasites on them is important for developing sustainable landscapes that minimise these stresses. Bumble bees are commonly parasitised, with a range of parasites reaching high prevalence in their wild populations (Schmid-Hempel, 1998). Likely the most prevalent is *Crithidia bombi*, a trypanosome gut parasite (Shykoff & Schmid-Hempel, 1991). *Crithidia bombi* has low impacts on otherwise healthy bees (Brown et al., 2000), but for bees under stress, such as through nutritional deprivation, it can have strong impacts on survival and fitness (Brown et al., 2000; Brown et al., 2003).

As such, if *Crithidia bombi* were to be exacerbated by any pesticide, this would increase its prevalence in the environment and impact bee fitness. Further, if the stress caused by pesticide exposure were to interact with the stress caused by *Crithidia bombi* infection, it is possible that this would exacerbate the negative consequences of the pesticide.

Here we test three pesticides, sulfoxaflor, glyphosate and azoxystrobin (in formulation as Amistar) in a series of fully crossed experiments with *Crithidia bombi* that focus on examining impacts on (i) survival in individual workers and males, (ii) *C. bombi* loads in workers and males, (iii) learning in workers, and (iv) hibernation and colony foundation in queens.

Summary

Interactions between agrochemicals and other stressors, including parasites, have been suggested to be drivers of declines in bees. Consequently, experimental studies looking at these interactions are urgently needed. Here we report on a series of laboratory experiments that were designed to uncover whether the combination of each of three different agrochemicals and a common bumble bee parasite would have negative impacts on a range of bee health metrics.

Experiments with worker bees found no marked effects of these agrochemicals on parasite load. In addition, they found no impact of the agrochemicals, the parasite, or their combination on worker mortality or food consumption. Experiments with males similarly found no impacts of these stressors on bee health, and no impact of agrochemicals on parasite load. Using a modification of the PER response, we found no evidence for combined impacts on learning abilities in bees. Finally, while we found agrochemical effects on aspects of colony founding by queens, we found no interactive effects of the two stressors.

We conclude that, under the experimental conditions used here, there are no meaningful interactions between 3 different agrochemicals and a common bumble bee parasite. This suggests that risk assessment of these agrochemicals does not require integration of parasitism. However, we note that other conditions, and other, more virulent parasites, may still be a source of interactive impacts on bee health.

1. Methods

1.1. General bumble bee acquisition, screening, and maintenance

For queen and worker experiments, commercial *Bombus terrestris audax* colonies were maintained on *ad libitum* sucrose and honey bee collected pollen. On arrival, 10 workers per colony were removed and their faeces screened for micro-parasites (Rutrecht & Brown, 2008). No infections were detected, and all colonies were thus retained for experiments. Males were acquired from the same commercial source for all male experiments and immediately entered into experiments; post-experiment dissections confirmed initial parasite-free status for these males.

1.2. Interactions between glyphosate and *Crithidia bombi* in workers: General Methods

All acute experiments (described below) used the doses shown in Table 1.

Table 1. Each cell gives a treatment used in all acute exposure experiments

Control	***C. bombi* only** 10,000 cells per worker	**Positive control** 4µg dimethoate per worker
Glyphosate only 200µg per worker	**Glyphosate and *C. bombi*** 10,000 cells per worker 200µg per worker	

1.3. Interactions between glyphosate and *Crithidia bombi* in workers: modified ecotoxicological protocol OECD 247

OECD 247 (OECD, 2017) is an internationally agreed upon protocol for testing the toxicity effects of acute exposure to an oral solution in bumble bees (*Bombus spp*.). The protocol only allows for a single exposure phase, so modifications were used to include an additional parasite exposure phase.

Worker bees were housed in Nicot cages a day in advance of parasite exposure, and then ranked and allocated sequentially to treatments based on weight, with an even distribution of source colonies across treatment groups. Bees outside the range of 0.1g-0.4g were not used. Syringes with 50% (w/w) sucrose were added to the Nicot cages for sustenance. The tip of the syringe was clipped off to allow access to the sucrose.

The subsequent day, following the OECD 247 protocol (OECD, 2017), we exposed bees in the parasite treatments to an inoculum containing 10,000 cells of *Crithidia bombi*. The parasite inoculum was prepared by removing 40 worker bees from a *C. bombi* infected colony and inducing them to defecate. The faeces were then purified following Cole (1970). Purified *C. bombi* solution was then diluted in distilled water and mixed 1:1 with 50% (w/w) sucrose to produce the test solution with 10,000 cells in 40µL of inoculum. A control solution of 1:1 distilled water and 50% (w/w) sucrose was also produced. At dissection, any bees with a parasite intensity of 0 cells per µL were deemed to have a failed infection, and were excluded from the experiment. A further single worker with an intensity of 100 cells per µl, which is more likely to have resulted from contamination of the slide than an infection, was also excluded.

Sucrose syringes were removed for 2-4 hours prior to exposure to the inoculum, effectively starving the bees. Then 40µL of solution was pipetted into a fresh syringe and this was added to each cage. The bees were left to feed on the inoculum for a further four hours, at which point the syringe was removed and consumption visually verified. Bees that did not consume >80% of the solution were excluded from the experiment. Bees were returned to *ad libitum* sucrose with a syringe of 50% (w/w) sucrose and had a small ball of pollen added (~1g).

Bees were left for 7 days for the parasite infection to develop, at which point they entered the pesticide exposure phase. Here the above steps for parasite exposure were repeated, but with pesticide-laced treatment solutions replacing the parasite treatment solutions.

After exposure to the pesticide, mortality was recorded at four hours, 24 hours and 48 hours. Mortality was defined as a lack of response to physical agitation. Dead bees were discarded as their corpses degrade too quickly to be dissected for parasites.

Any bees who survived the full 48 hours were weighed (Scout SKX, Ohaus, Switzerland, accuracy limit of 0.001g), then transferred to a 2mL Eppendorf tube and frozen at -80C° for later dissection. Bees in the *C. bombi* or *C. bombi* + Glyphosate treatment groups were later dissected to isolate the ileum. The ileum was moved to a 1.5ml Eppendorf with 100µL of Ringers solution (0.8% NaCl (w/v)) and ground using a pestle for five seconds in a set pattern of movements. The ground gut was then vortexed for a single second, 10µL of the homogenate pipetted onto a Neubauer haemocytometer slide and the *C. bombi* concentration counted. All endpoints are presented as mean ± one standard deviation.

1.4. Experiment one: modified ecotoxicological protocol OECD 247: small scale

In this initial exploratory experiment only the *C. bombi* only and Glyphosate + *C. bombi* treatments were included. While bees were evenly allocated to treatments by colony of origin, but colony origin was not tracked through the experiment and as such this is not accounted for in the statistics. Due to non-feeder events and deaths prior to the glyphosate exposure stage, the final treatment groups may have had an uneven allocation of colony of origin, although this is unlikely due to the initial even distribution and low occurrence of such events. Sucrose consumption was not measured.

1.5. Experiment two: modified ecotoxicological protocol OECD 247: full scale

This experiment was a full-scale repetition of experiment one, with all treatment groups included. The Modified Ecotoxicological Protocol OECD 247 protocol described above was followed with a single major deviation, in that haemolymph samples were taken from all bees at the end of the experiment. The haemolymph was sent to colleagues (PoshBee, Work Package 9) for proteomic analyses, which will be reported elsewhere. This manipulation did not affect the mortality metric as mortality was recorded prior to the manipulation. Further, it did not affect the parasite intensity measure as there was no 'by treatment' difference, and the timing of the haemolymph extraction was too near to the termination of the experiment to influence *C. bombi* levels. This experiment was conducted in two batches with just a single day stagger between them.

1.6. Experiment three: modified ecotoxicological protocol OECD 247: long term survival

To test for longer term effects, a version of the Modified Ecotoxicological Protocol OECD 247 described above was performed, with the only deviation being that bees were maintained for 20 days post exposure rather than 48 hours. Mortality checks were made daily and pollen balls renewed weekly.

1.7. Experiment four: microcolony acute exposure

To test for effects on reproduction, a microcolony experiment was performed. Bees were moved into microcolony boxes (clear acrylic boxes (6.7x12.7x4.9cm), with a plastic mesh grate bottom (6.7x7.3cm)) a day prior to parasite exposure. Initially 8 workers were added per microcolony box.

Pathogen inoculation and glyphosate exposure followed the Modified Ecotoxicological Protocol OECD 247, with bees being moved into Nicot cages for this exposure. Between treatments, bumble bees were maintained in microcolony boxes.

Due to time constraints, only bumble bees receiving a treatment were moved to Nicot cages and exposed. Bees in the control treatment were never moved to Nicot cages, bees in the *C. bombi* only

treatment and the glyphosate only treatment were moved to Nicot cages just once, and those in the Glyphosate + *C. bombi* treatment were moved to Nicot cages twice. This had the potential to cause a 'by treatment' effect because being moved to a Nicot cage is a potentially stressful experience. However, the day prior to *C. bombi* exposure, all bees were manipulated as they were moved from their source colony to a microcolony box. Similarly, on the glyphosate exposure day, all bees not moved into Nicot cages were manipulated as they were moved into a fresh microcolony box. As such it is only the marginal additional level of stress from the time in the Nicot cages that could produce a by treatment effect. Bees in the Nicot cages were also kept in their microcolony box adjacent to nest-mates to reduce stress.

Non-feeders were excluded from the experiment at each of the exposure steps, which alongside mortality led to slightly lower worker numbers in the micro-colonies (Glyphosate: 6.9 ± 1.2, *C. bombi*: 6.7 ± 1.1, Glyphosate + *C. bombi*: 6.4 ± 1.1 (SD)), versus the control (7.8 ± 0.4 (SD)). Workers who died (n = 4) or escaped (n = 5) during the experiment were recorded, but not replaced. This was accounted for in the analysis, however, with reproductive output expressed per worker present at end of experiment. Given that worker reproduction is highly dependent on the laying individual (Blacquière et al., 2012), this should robustly account for differing worker numbers.

After glyphosate exposure, bumble bees were moved to a fresh microcolony box to reset their reproductive efforts, and then provided *ad libitum* sucrose and pollen for 14 days. 14 days is shorter than the time required for a bee to develop from egg to eclosion, so all adults at the end of the experiment were those initially added to a microcolony box after glyphosate exposure.

On day 14, adult bumble bees were counted and frozen for later dissection to quantify pathogen intensity; the total number of eggs and larvae were counted, and total larval weight measured. Larval weight was chosen as the best measurement of reproductive success as it reflects output better than larval number. By using weight, the greater investment required to rear an L4 larva, versus an L1 larva, is reflected, whereas number of larvae would not reflect this investment disparity. As such, larval weight per worker was chosen as the quantitative metric used for analysis.

1.8. Experiment five: microcolony chronic exposure

This protocol is derived from the OECD 245 honey bee chronic oral toxicity test, with modification to account for the different test species.

Workers used in the experiment were age controlled. To achieve this, 8 workers were taken from a source colony, tagged and moved into a microcolony box. Pupae and enclosed larvae from the same colony were added, with the 8 tagged workers acting as nurses for them. Newly emerged workers were identified by their lack of a tag, and 10 days after the start of emergence they were moved to Nicot® cages for parasite inoculation. Inoculation followed the Modified Ecotoxicological Protocol OECD 247, with treatment groups detailed in Table 1. After excluding non-feeders, bees were then allocated to microcolonies in groups of six based on treatment, with all workers within a microcolony originating from the same source colony. Because the allocation to microcolonies occurred after non-feeders were excluded, there is no by treatment exclusion effect. By selecting newly emerged workers over a 10-day period, workers were age controlled to be within 10 days of one another. Workers were given *ad libitum* sucrose and pollen for a week while the parasite developed. After seven days the workers were moved to a fresh microcolony to reset their reproductive effort.

Data from Thompson et al. (2014) were used to inform the chronic exposure scenario. Thompson et al. (2014) measured glyphosate concentration in returning nectar and pollen from honey bees foraging on *Phacelia tanacetifolia* sprayed with a glyphosate-based herbicide formulation (MON 52276) according to full label restrictions. Using WebPlotDigitiser (Rohatgi, 2020), the values from Thompson et al. (2014)'s graphs were extracted. An inverse relationship model was used to model

the declining residue concentration: $Glyphosate\ Concentration = Intercept + \frac{Constant}{Time}$. As the data from Thompson et al. (2014) had missing days and no data after 7 days, missing data were either interpolated or extrapolated. These modelled concentrations of returning nectar and pollen were then used to generate an exposure regime. Sucrose was fed to the bees *ad libitum* and was spiked with pesticides in concentrations shown in Figure 1. In all treatments 50% w/w sucrose was changed daily, and the previous day's consumption was recorded. The glyphosate concentration provided decreased over time with the modelled values. Degradation of the glyphosate will have occurred in the sucrose; however, this is largely insignificant given glyphosate's long half-life of 47-267 days (as measured in seawater) (Mercurio et al., 2014). 5g of pollen was provided and in glyphosate treatments this was spiked with an average concentration of glyphosate over the 10 days exposure (110mg/kg). This was done as changing pollen daily was not feasible, and 5g was used as this amount was rarely wholly consumed by a group of workers in 14 days. In the positive control, the dimethoate concentration was maintained at a constant 1mg/L, and pollen was not spiked in this treatment. As in OECD 245 for honey bees (OECD, 2017a), exposure ended on day 10, and all bumble bees were fed unspiked sucrose for another four days. On day 14 bumble bees were frozen and reproductive output measured, as described above. Mortality was recorded daily.

As the dataset used to calculate our chronic exposure regime was from a semi-field exposure study conducted on honey bees (Thompson et al., 2014), the use of these data for *B. terrestris* may be problematic. There are no comparable data from honey bees and bumble bees to be able to see if the same spraying regime leads to similar returning nectar concentrations. However, as the only available dataset, it is the best choice to inform the chronic exposure regime.

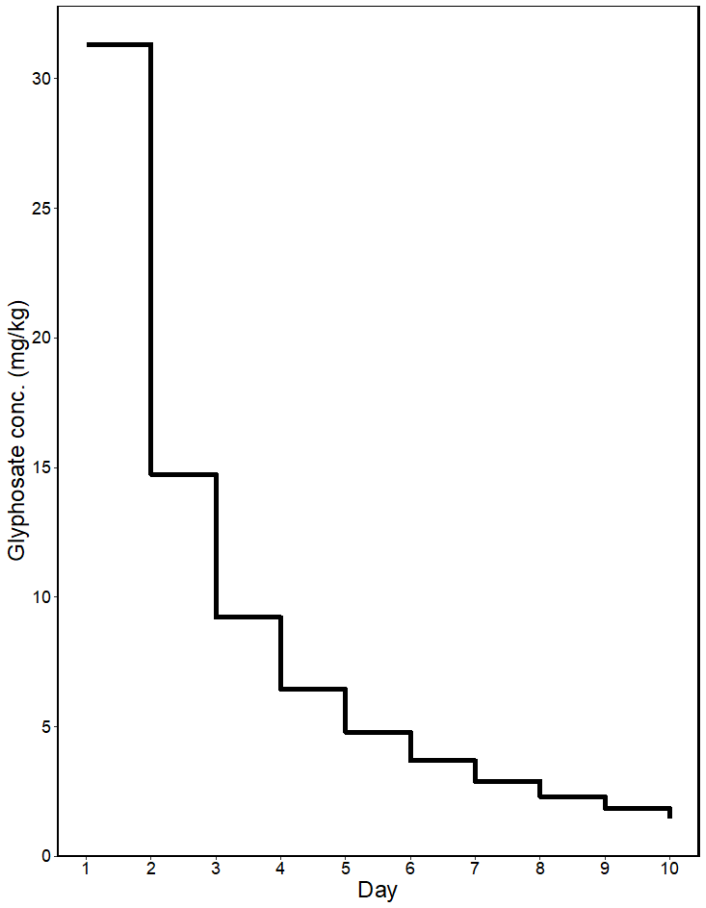

Figure 1. The stepwise chronic exposure profile generated from Thompson et al. (2014), with glyphosate concentration (in mg/kg) on the Y axis and time in days on the X axis.

1.9. Testing for interactions between azoxystrobin, sulfoxaflor, and *Crithidia bombi*: modified ecotoxicological protocol OECD 247

Pesticides were applied as pure active ingredient, except Azoxystrobin, which was applied as part of the formulation Amistar (MAPP 18039). The experiment otherwise followed the methods as detailed in Section 1.5 above, using the doses shown in Table 2.

Table 2. Cells giving treatments for testing interactions between azoxystrobin, sulfoxaflor, and *Crithidia bombi*. Each experiment was conducted with its own positive and negative control.

Control	***C. bombi* only** 10,000 cells per worker	**Positive control** 4µg dimethoate per worker
Sulfoxaflor only 0.06µg per worker	**Sulfoxaflor and *C. bombi*** 10,000 cells per worker 0.06µg per worker	
Azoxystrobin only 200µg per worker (equivalent dose of formulation)	**Azoxystrobin and *C. bombi*** 10,000 cells per worker 200µg per worker (equivalent dose of formulation)	

1.10. Statistical testing for agrochemical-parasite interactions in workers

Statistical analyses were carried out in 'R', and all plots were made using 'ggplot2' and 'survminer'. AIC model simplification was used, with conditional model averaging where no single model had >95% AIC support. The candidate set of models was chosen by adding the next best supported model until a cumulative >95% AIC support was reached using 'MuMIn' Parameter estimates and 95% confidence intervals are reported. 'lme4' was used for Linear Mixed Effects models and 'coxme' was used for Mixed Effects Cox Proportional Hazards models. Confidence intervals not crossing zero indicate a significant effect, so a confidence interval of -1.00 to 1.00 would not be significant, but a confidence interval of -2.00 to -1.00 would be. Model assumptions were checked graphically and using statistical testing. Where found to be non-normal, a Kruskal Wallis test was used. In experiments with an absence of mortality, no statistical testing was conducted.

1.11. Azoystrobin, glyphosate, and *C. bombi* in male bumble bees: experimental methods

We examined the impacts of chronic exposure to two agrochemicals and a trypanosome parasite on male bumble bees. For these experiments, azoxystrobin was applied as Amistar formulation, diluted to a concentration of 576 ppb to match the average across four days of degradation in nectar post-application (Schatz and Wallner, 2009), while glyphosate was diluted to a concentration of 18,000 ppb to match the average over three days of degradation in nectar post-application (Thompson et al., 2014). *C. bombi* inocula were prepared as described above (Section 1.3). Two full-factorial experiments, each with four treatment groups (control, agrochemical, parasite, agrochemical X parasite), and starting with 40 bees in each treatment group, were conducted.

Males were weighed, allocated to treatment groups (following the same method as used for workers, see Section 1.3 above), and then housed individually in Nicot cages (OECD, 2017). The following day, bees in the parasite treatment groups were inoculated via syringes containing 40ul of parasite inoculum, while the remaining bees received a 50% sugar water solution of the same

volume. Post-treatment, *ad libitum* sugar water was returned. The parasitaemia was allowed to develop for 8 days, prior to application of agrochemicals to the relevant treatment groups. In the azoxystrobin treatment, exposure occurred for four days, whilst for glyphosate exposure occurred for 3 days, to match degradation dynamics in the field (see above). Mortality was checked on every day of the experiment, and sugar consumption was measured across the agrochemical exposure period. At the end of the experiment, bees were frozen, dissected, and checked for parasites. Parasite intensity was measured for those animals positive for *Crithidia* (see Section 1.3 above for further methods for parasite quantification).

1.12. Azoystrobin, glyphosate, and *C. bombi* in male bumble bees: statistical analysis

Mortality was analysed using Kaplan-Meier tests, while sugar consumption and parasite intensity were assessed using GLMs. Again, all analyses were conducted in R.

1.13. Impacts of Sulfoxaflor and *C. bombi* on learning in worker bumble bees: methodology

Three colonies were evenly divided into two queenless sub-colonies to produce six sub-colonies. Each sub-colony was placed into a colony box (27x20x13 cm) connected by a transparent plastic tube (25 cm long, 1 cm in diameter) to another empty colony box which served as a foraging arena containing an *ad libitum* supply of 50% w/w sucrose solution and pollen. The boxes containing the sub-colonies were covered to keep them in darkness, while the foraging arenas were left uncovered. These were set up in a room with natural light to facilitate the development of normal foraging behaviour. Three days after splitting the colonies, one sub-colony from each of the three pairs was inoculated with *C. bombi*. *C. bombi* inoculum was produced from the purified faeces of 75 individuals from two infected colonies, following Cole (1970). Inoculum was diluted in 4ml of 50% w/w sucrose solution for each of the three sub-colonies to drink from. The number of *C. bombi* cells fed corresponded to the number of individuals in each sub-colony multiplied by 10,000, for an average dose of 10,000 *C. bombi* cells per bee. Control sub-colonies were given 4ml of 50% w/w sucrose solution without any parasite. After 24 hours the inocula had been totally consumed and the sub-colonies were returned to *ad libitum* sucrose. All sub-colonies were screened at 7 and 10 days post-inoculation to confirm infection, and its absence in the control sub-colonies.

Over the course of 14 days, the sub-colonies were placed daily under red light and five foragers were collected from each one for harnessing (30 bees in total). Once harnessed, bees were held horizontally with modelling clay and prompted to extend their proboscis by touching their antennae with a droplet of 50% w/w sucrose solution. Bees that extended their proboscis were fed 50% w/w sucrose solution until satiated. Bees that did not feed were not used for PER. To avoid dehydration, the bees had damp paper roll put in their harnesses. They were then left overnight in a dark room at 24°C, because leaving bumblebees to starve for 18 hours is necessary to increase their responsiveness (Riveros & Gronenberg, 2009).

Bees in the sulfoxaflor treatment were prompted to extend their proboscis and fed 12ng of sulfoxaflor in 10µl of a 25% w/w sucrose solution, prepared each morning. Since Siviter et al. (2019) did not find any effect for low field realistic doses of sulfoxaflor on bumble bee olfactory learning, we chose to test a worst-case scenario whilst limiting our experimentation to a sublethal dose. As such, our dose was almost four times lower than the non-observable adverse effect level in bumblebees of 44 ng (Azpiazu et al., 2021). We chose a dose of 12ng as a range finder test we performed resulted in some mortality at higher doses. This dose was still conservatively low as field exposure studies performed for regulatory testing found that when strict mitigation methods for

sulfoxaflor application are not applied, nectar concentrations could reach lethal levels (Linguadoca et al., 2021). Such worse case scenarios arise when farmers spray their crops when flower buds are present or already in bloom, which is too late to avoid pollinator contact with high doses of the insecticide. Control bees were fed 10µl of 25% w/w sucrose solution in the same manner as those exposed to sulfoxaflor. Bees that did not drink the whole treatment droplet were excluded from the experiment. PER was conducted 30 minutes after the last bee drank its given solution.

We used the Proboscis Extension Reflex protocol using lavender scent as a conditioned stimulus and a 50% w/w sucrose solution as an unconditioned stimulus. 4µl of lavender oil was pipetted onto a strip of filter paper that was replaced every 24 trials. A stream of unscented air was constantly blown through a different tube to avoid bees misidentifying a change in air flow as a stimulus. Trials were conducted in a clear box connected to a vent duct hose that ensured air circulation. During trials, bees were placed 3cm from the odour tube. They were exposed to unscented air for 5 seconds, then to scented air for 10 seconds. 6 seconds after the start of the scented airflow the antennae were stimulated with 0.8µl sucrose solution from a pipette, and an unconditioned response was recorded if a bee extended its proboscis. A conditioned response was recorded if a bee extended its proboscis during the first 6 seconds of scented airflow, thereby demonstrating learning in the form of classical conditioning. Bees that showed either response were fed the 0.8µl sucrose solution as a reward to reinforce the behaviour. Each bee underwent 15 trials, and 3 unscented probe trials that were randomly placed between the 1st and 5th, 5th and 10th and the 10th and 15th scented trials. The purpose of these probes was to ensure that bees would develop a conditioned stimulus solely in response to the lavender scent. Bees that would have extended their proboscis during a probe trial would not have been included in the analysis, but none did. Therefore, each bee underwent a total of 18 trials with an interval of approximately 12 minutes between each one. Bees that did not extend their proboscis in at least 5 trials when their antennae were stimulated with sucrose solution were excluded from the analysis. Bees that died whilst undergoing PER were also excluded from the analysis but recorded as deaths. Tested bees were then frozen at -80°C. Three intertegular measurements were taken with a Mitutoyo digital calliper to obtain mean intertegular values (a measure of bee size), since the absorption rate of the insecticide and its effects on bee cognition may be affected by a bee's size (Fournier et al., 2014; Samuelson et al., 2016). The 162 bees that were part of the final sample were dissected and their hindgut screened for the presence or absence of *C. bombi*. All 100 bees from the uninfected treatments tested negative for *C. bombi* and all 62 bees from the infected treatments tested positive. Parasite load of individual bees was not recorded or considered in the analysis as Martin et al. (2018) found no relationship between parasite intensity and bumblebee olfactory learning.

A total of 326 bumblebees underwent PER (81 control, 81 sulfoxaflor, 82 *C. bombi*, and 82 sulfoxaflor + *C. bombi*). Of these, 161 bees did not extend their proboscis in at least 5 trials when their antennae were stimulated with sucrose solution and so were excluded from the analysis (35 control, 25 sulfoxaflor, 58 *C. bombi*, and 43 sulfoxaflor + *C. bombi*). 3 bees died during the trials, 2 of the control treatment and one of the sulfoxaflor + *C. bombi* treatment. Overall, 164 bees were excluded from the statistical analysis, for a final sample size of 162 bumblebees (44 control, 56 sulfoxaflor, 24 *C. bombi*, and 38 sulfoxaflor + *C. bombi*).

1.14. Impacts of sulfoxaflor and *C. bombi* on learning in worker bumble bees: statistical analysis

All analyses were conducted in R. We analysed three measures of learning: Responsiveness (if a bee displayed at least one conditioned response to the stimulus), Learning Level (the number of conditioned responses for bees that displayed at least one conditioned response) and Learning Speed (the trial number in which a bee displayed its first conditioned response). The first two were analysed using GLMs, while Learning Speed was analysed using a Cox Proportional-Hazards model.

1.15. Impacts of sulfoxaflor and *C. bombi* on colony founding in bumble bee queens: general methods

Gynes (virgin female sexuals) and males were reared from colonies of *B. terrestris audax*. Once gynes were at least 7 days old they were mated with males in communal mating cages (60 X 50 X 50 cm, with netting on all sides). Queens were then allocated to the four treatment groups (control, sulfoxaflor, *C. bombi*, sulfoxaflor X *C. bombi*) using a stratified approach based on the natal colony of the queen and the male they mated with. Queens in the parasite groups were then inoculated with a minimum of 100ul inoculum containing 30,000 parasite cells. Two days post-mating all queens were placed individually into large falcon tubes (3cm X 11.5cm) and hibernated for 12 weeks at 4°C in a climate chamber. Post-hibernation queens were placed in a dedicated bee room for colony rearing (red light, 28°C, 50-60% relative humidity) in individual plastic rearing boxes (13cm X 8cm X 5.6cm) and provided with *ad libitum* pollen. Queens in the sulfoxaflor treatment groups were exposed to sulfoxaflor in their sugar water over 4 days following Linguadoca et al. (2021) – Day 1: 161ppb, Day 2: 47ppb, Day 3: 14ppb, Day 4: 4ppb. Queens were observed for the next 8 weeks, during which mortality, egg-laying, and offspring-emergence were recorded.

1.16. Impacts of sulfoxaflor and *C. bombi* on colony founding in bumble bee queens: statistical analysis

All analyses were conducted in R. The proportion of queens that died, that laid eggs, and from whose colony workers emerged were all analysed using chi-squared tests. The timing of these events was analysed using Cox Regression, with best models chosen following the Akaike Information Criterion approach.

2. Results

2.1. Modified ecotoxicological protocol OECD 247 in workers: small scale

2.1.1. Parasite intensity

The Glyphosate + *C. bombi* treatment had a significantly higher parasite intensity than the *C. bombi* only treatment (Kruskal-Wallis $X^2(1)= 7.885$, $p = 0.005$). Glyphosate + *C. bombi* treated bees ($n = 21$) had an average parasite intensity of 14,519± 10,462 (SD) cells per µL compared to 6,946± 5,682 cells per µL in the *C. bombi* only treatment ($n = 23$) (Figure 2).

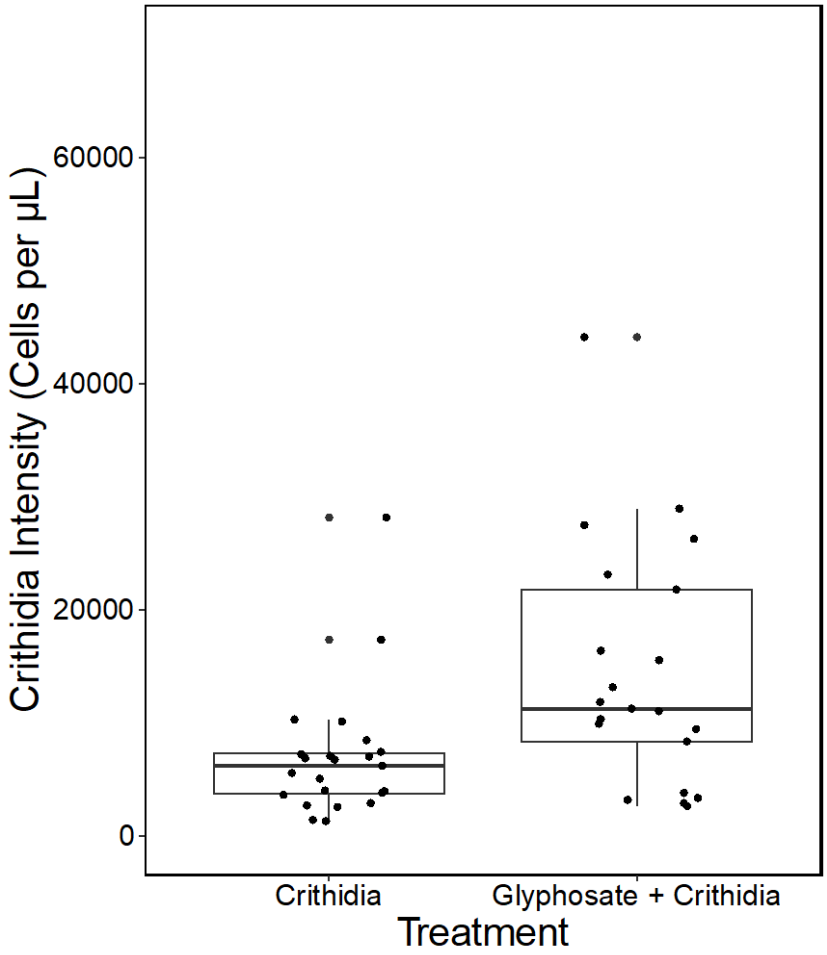

Figure 2. A boxplot with overlaid jittered data points showing the parasite intensity by treatment.

2.1.2. Mortality

No mortality was observed in either the *C. bombi*, or the Glyphosate + *C. bombi* treatment.

2.2. Modified ecotoxicological protocol OECD 247 in workers: full scale

2.2.1. Parasite intensity

In contrast to the first experiment, bees in the Glyphosate + *C. bombi* treatment did not have a significantly different parasite intensity to the *C. bombi* only treatment (Kruskal-Wallis $X^2(1)$= 0.42818, p = 0.5129). Glyphosate + *C. bombi* treated bees (n = 34) had an average parasite intensity of 24,124± 14,664 cells per µL, compared to the 20,756± 14,473 cells per µL in the *C. bombi* only treatment (n = 32) (Figure 3). Neither body weight nor batch had a significant effect on parasite intensity (Linear Mixed Effect model: parameter estimate (PE) = 66,940.7, 95% CI [-19,878.3 to 152,664.5] and (PE) = 897.3, 95% CI [-6,843.0 to 8,512.8] respectively).

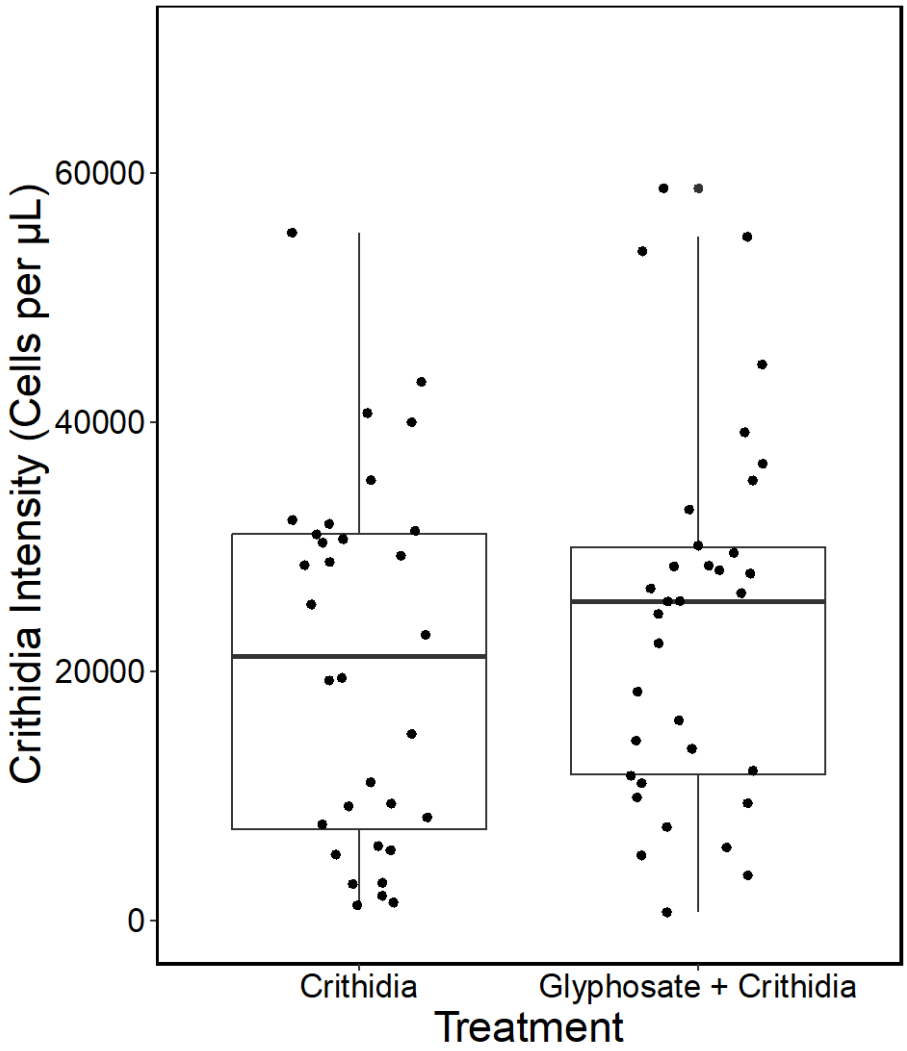

Figure 3. A boxplot with overlaid jittered data points showing the parasite intensity by treatment.

2.2.2. Mortality

No mortality was observed in any treatment except the positive control, where all bees died within 24 hours.

2.3. Modified ecotoxicological protocol OECD 247 in workers: long term survival

2.3.1. Mortality

All bees in the positive control treatment, bar one, died within two days, while all other treatments experienced mortality over the 20-day period.

C. bombi only, Glyphosate only, and Glyphosate + *C. bombi* treatments did not have significantly different mortality compared to the negative control (Cox proportional hazards mixed effects model: parameter estimate (PE) = 0.728, 95% CI [-0.81 to 0.96], (PE) = 1.27, 95% CI [-0.92 to 1.18], and PE = 1.19, 95% CI [-0.89 to 1.14], respectively). *C. bombi* only, Glyphosate only, and Glyphosate + *C. bombi* had 4%, 7% and 6% mortality respectively, while the control had 2% mortality (see Figure 4), a difference in real terms of one to two bees.

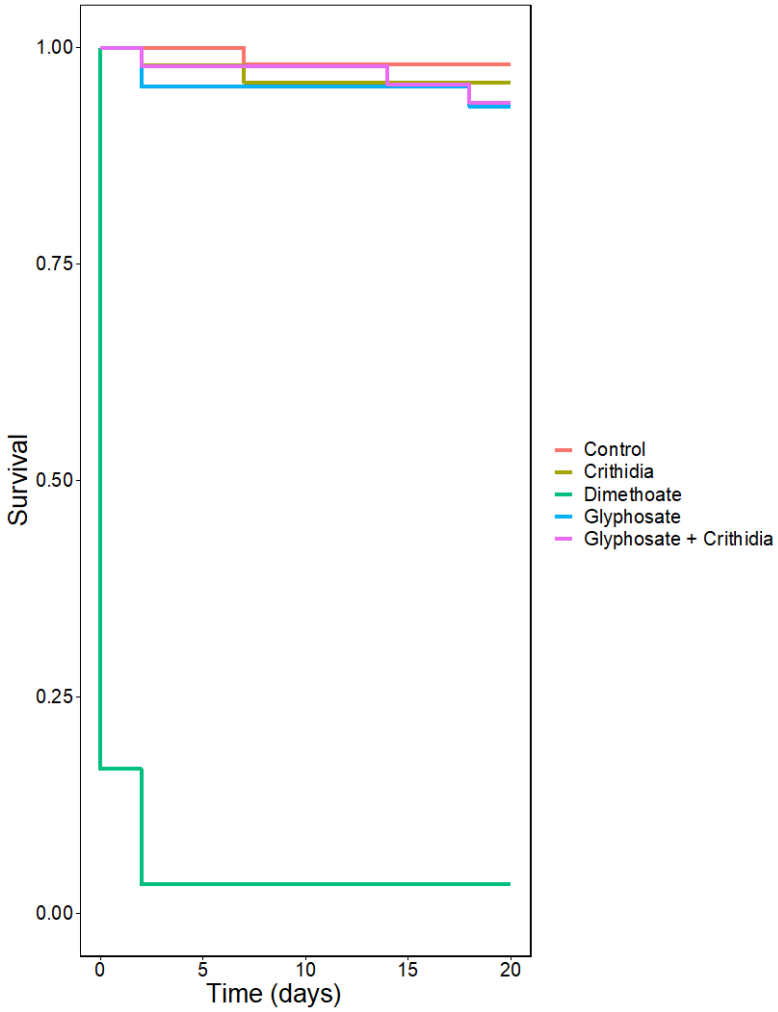

Figure 4. A Kaplan-Meier plot showing the survival over time by treatment.

2.4. Microcolony acute exposure

2.4.1. Reproduction

There was no significant difference in reproductive output between treatments. While the mean larval weight per worker (±SD and number of microcolonies) varied between treatments (0.510g ± 0.224, *n* = 8 in the control, 0.458g ± 0.349, *n* = 11 in the *C. bombi* only treatment, 0.405 ± 0.141, *n* = 9 in the Glyphosate only treatment and 0.339g ± 0.224, *n* = 10 in the Glyphosate + *C. bombi* treatment (see Figure 5)), a null model, which contained the response variable, the covariate of initial worker weight and the random colony variable, but not the treatment variable, was the best supported model with ≥95% AIC support. This model found a significant effect of original weight of nurse workers on reproductive output (Linear mixed effects model (LMER) =0.26, 95% CI [0.14 to 0.37]), with heavier workers being more successful at rearing offspring.

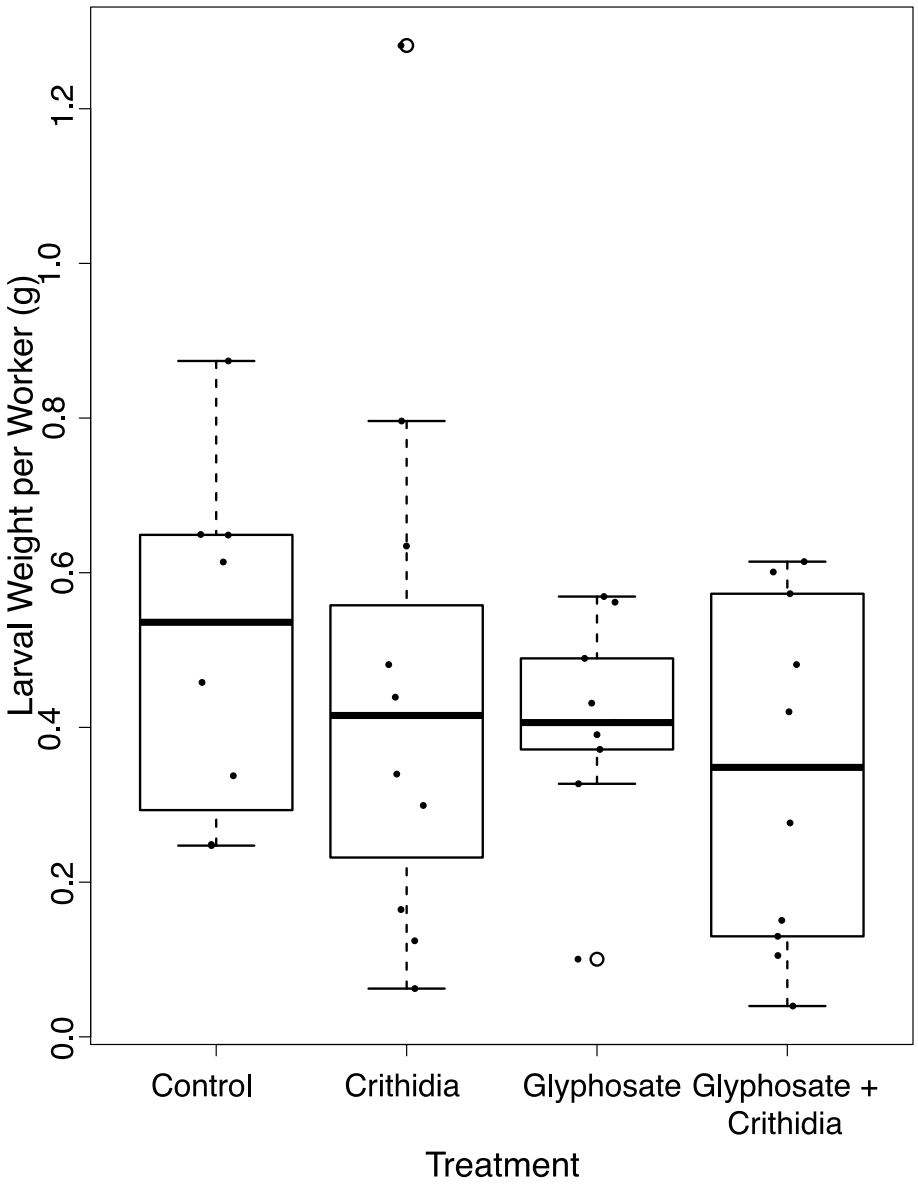

Figure 5. A boxplot showing the larval weight per microcolony standardised by the number of workers, presented by treatment with overlaid jittered data points.

2.4.2. Parasite intensity

Glyphosate + *C. bombi* exposed bees did not have a significantly different parasite intensity to the *C. bombi* only treatment (Linear Mixed Effect model: parameter estimate (PE) = -314.6, 95% CI [-2,865.81 to 2,236.55]). Glyphosate + *C. bombi* treated bees (*n* = 64) had an average parasite intensity of 18,362± 7,704 cells per µL, compared to the 18,635± 5,884 cells per µL in the *C. bombi* only treatment (*n* = 74) (see Figure 6).

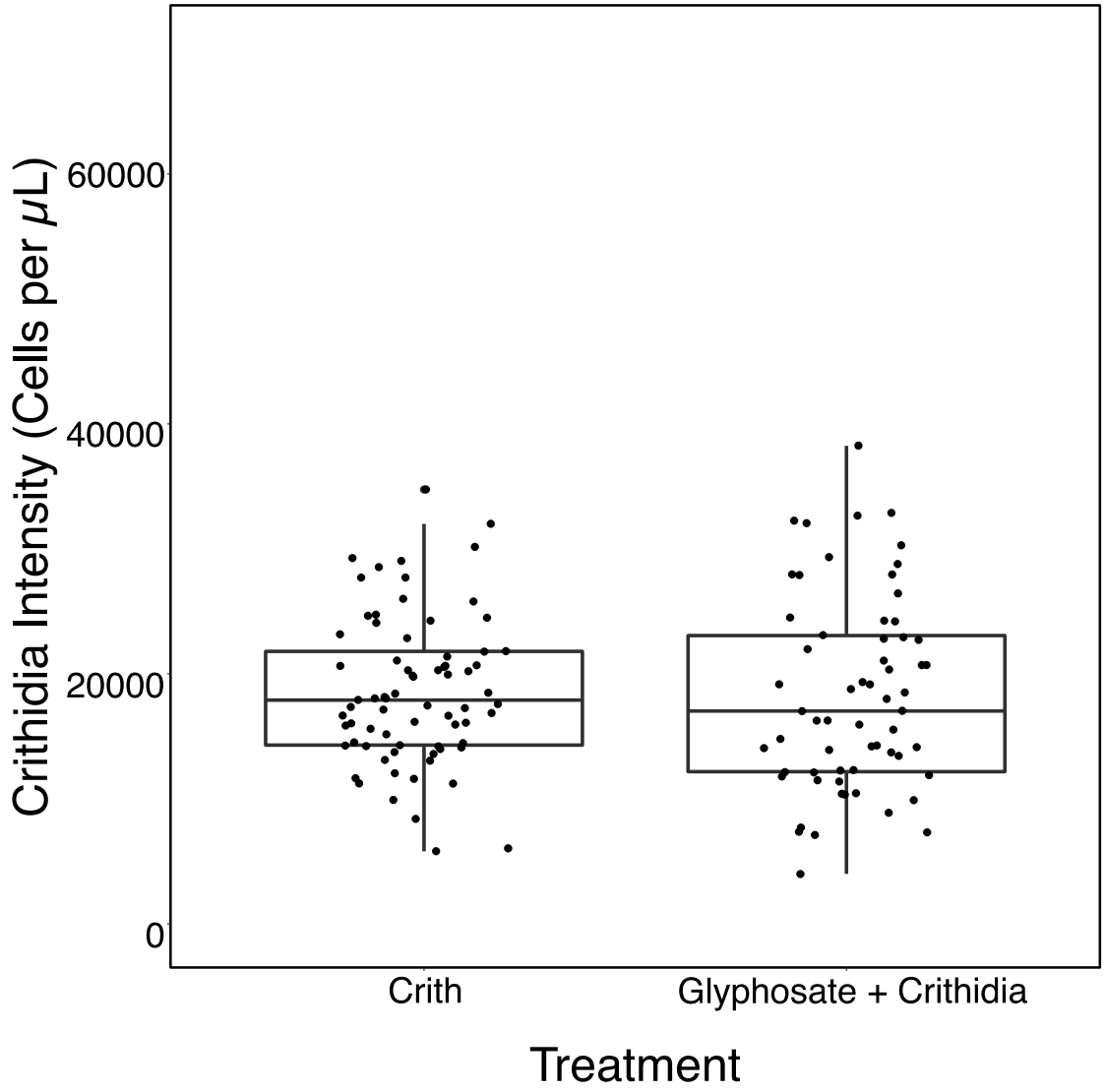

Figure 6. A boxplot with overlaid jittered data points showing the parasite intensity by treatment.

2.4.3. Mortality

There was no significant difference in mortality by treatment (Fisher Exact test (two sided) *p*= 0.679). *C. bombi* only, Glyphosate only and Glyphosate + *C. bombi* had 1%, 0% and 3% mortality respectively, while the control had 2% mortality, a real terms difference of one bee.

2.5. Microcolony chronic exposure

2.5.1. Reproduction

There was no significant difference in reproductive output between treatments. The mean larval weight per worker (±SD and number of microcolonies) varied between treatments, with 0.106g ±

0.077, *n* = 8 in the control, 0.053g ± 0.054, *n* = 8 in the *C. bombi* only treatment, 0.143g ± 0.139, *n* = 8 in the Glyphosate only treatment and 0.124g ± 0.103, *n* = 8 in the Glyphosate + *C. bombi* treatment (see Figure 7). The model average with a cumulative ≥95% AIC support did not include the treatment term. The two models included were both null models, one with the co-variate of initial worker weight and random colony variable, and the second with just the random colony variable. This model found no significant effect of original weight of nurse workers on reproductive output (Linear mixed effects model (LMER) =0.20, 95% CI [-0.15 to 0.27]).

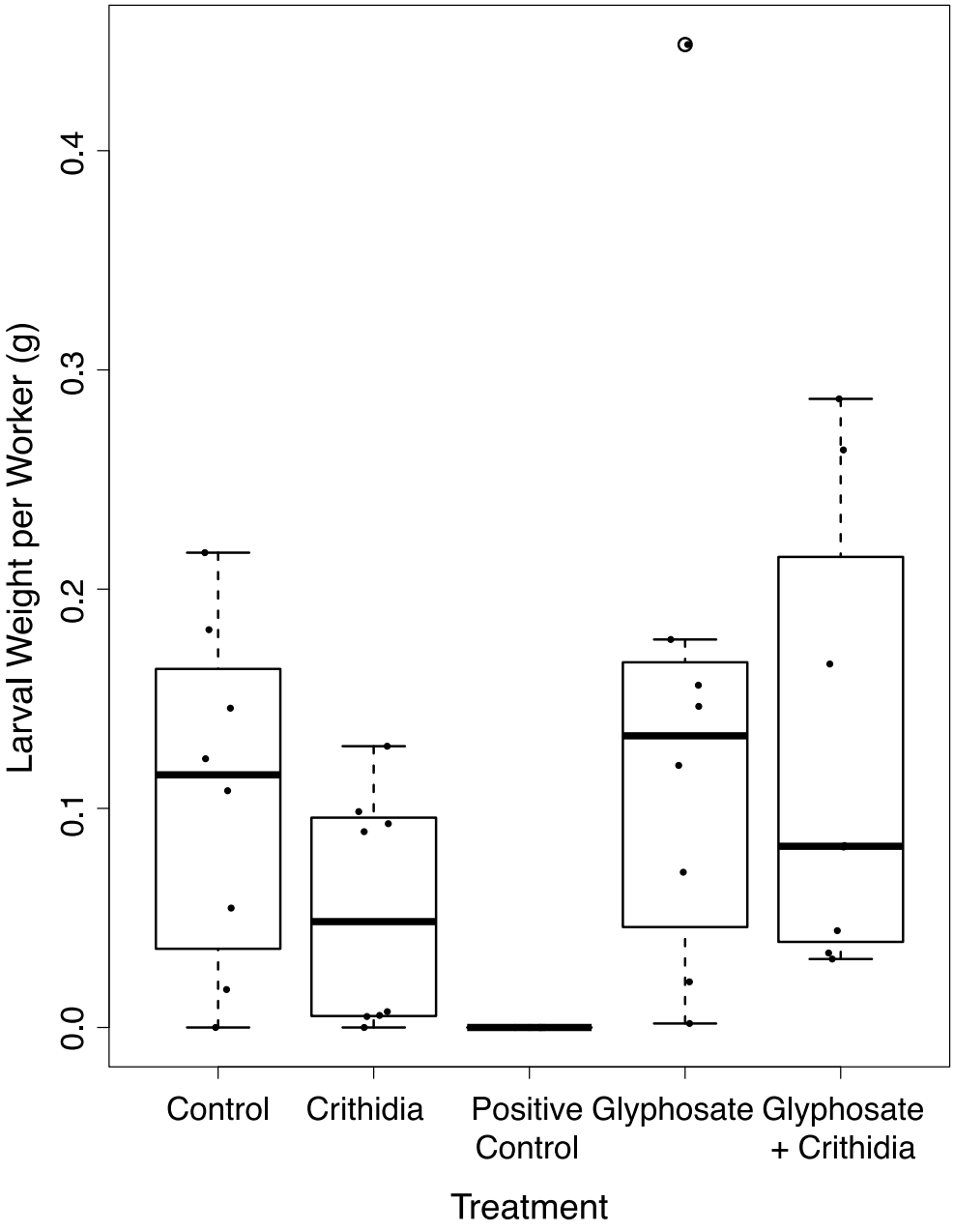

Figure 7. A boxplot showing the larval weight per microcolony standardised by the number of workers, presented by treatment with overlaid jittered data points. All bees in the positive control died; accordingly, they produced no larvae.

2.5.2. Sucrose/Glyphosate consumption

Over the 10-day exposure period the average consumption of sucrose per worker was 5.890± 0.676mL in the control, 5.880± 0.865mL in the *C. bombi* only treatment, 5.947± 0.875mL in the Glyphosate only treatment, and 6.271± 0.746mL in the Glyphosate + *C. bombi* treatment.

The model average that contained models with a cumulative ≥95% AIC support did not include the Treatment term. As such Treatment had no effect on sucrose consumption. The weight of the bees at the start of exposure also did not affect sucrose consumption, (Linear Mixed Effect model: parameter estimate (PE) = 0.062, 95% CI [-0.052 to 0.069]).

Over the 10-day exposure period the average consumption of glyphosate per worker was 38.7± 5.4µg in the Glyphosate only treatment, and 41.4± 4.3µg in the Glyphosate + *C. bombi* treatment. The majority of this consumption was in the initial few days, as the concentration decreased markedly over time. Figure 8 shows the sharp decline in glyphosate consumption over time.

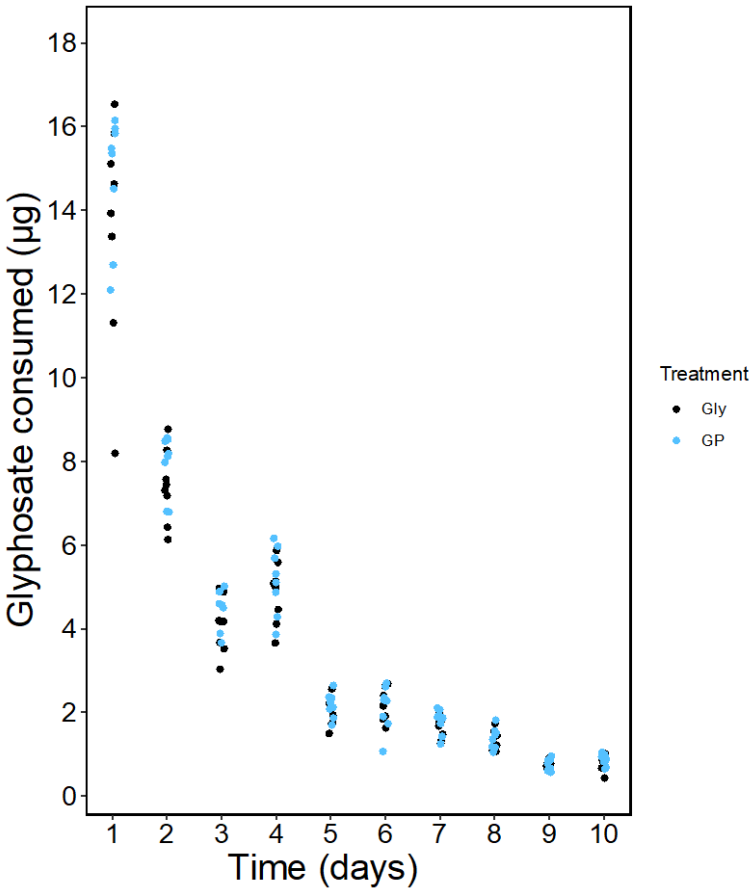

Figure 8. A scatter plot showing the daily consumption of the active ingredient glyphosate over time, presented by treatment. Data points have been horizontally jittered for clarity. Bees in the Control and *C. bombi* only treatments had glyphosate exposures of zero, and have been omitted from the graph.

2.5.3. Parasite intensity

The Glyphosate + *C. bombi* treated bees did not have a significantly different parasite intensity to the *C. bombi* only treatment (Linear Mixed Effect model: parameter estimate (PE) = 1649.0, 95% CI [-3251.24 to 6529.72]). Glyphosate + *C. bombi* treated bees (*n* = 42) had an average parasite intensity of 20,562± 7065 cells per µL compared to 18,759± 9403 cells per µL for the *C. bombi* only treatment (*n* = 44) (see Figure 9).

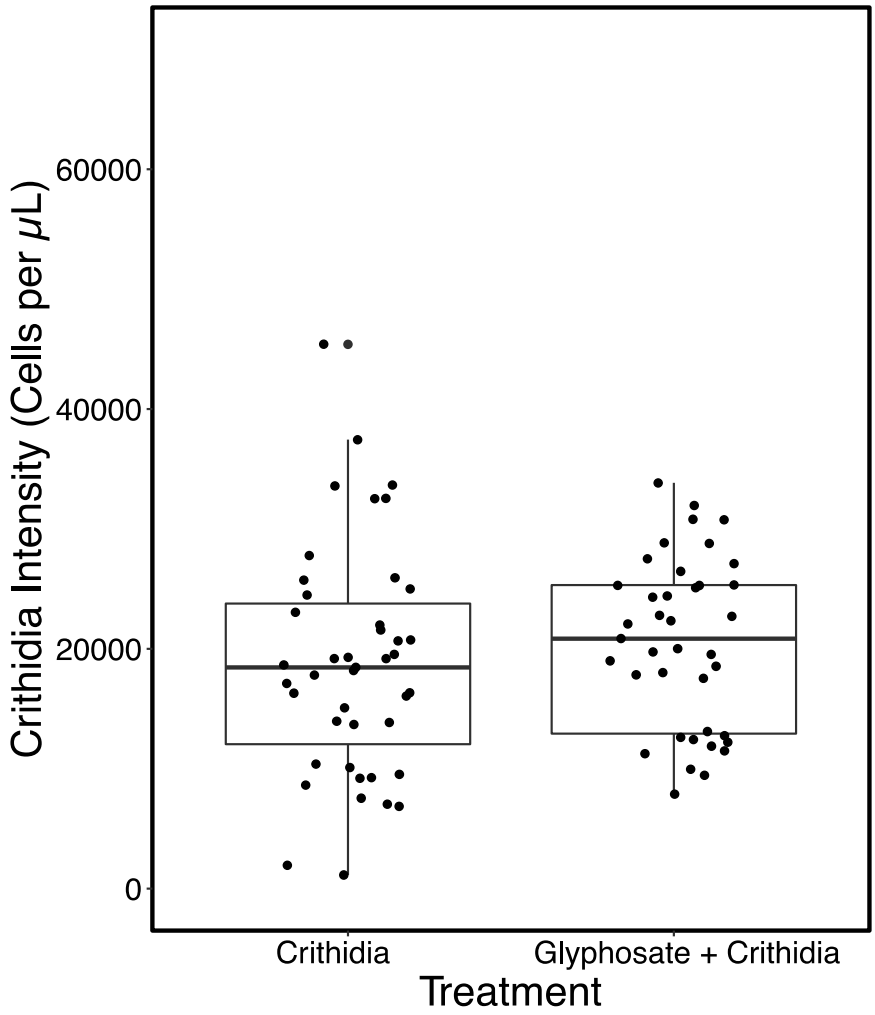

Figure 9. A boxplot with overlaid jittered data points showing the parasite intensity by treatment.

2.5.4. Mortality

All bees in the positive control treatment died. There was no significant difference in mortality between the remaining treatments (Fisher Exact test (two sided) *p*= 0.903). *C. bombi* only, Glyphosate only and Glyphosate + *C. bombi* had 0%, 2% and 2% mortality respectively, while the control had 4% mortality, a real terms difference of one to two bees.

2.6. Azoxystrobin and *C. bombi* in workers

No bees in the control or *C. bombi* treatment died. To permit statistical mortality analysis, a single artificial mortality event was added to the Control data.

Azoxystrobin exposure significantly increased mortality (44% mortality; Cox-Proportional Hazards, $p = 0.007$) versus the control, but Azoxystrobin + *C. bombi* exposure did not (16%; Cox-Proportional Hazards, $p = 0.132$). All bees in the positive control died, as expected.

There was no statistically significant difference in parasite intensity between the *C. bombi* and Azoxystrobin + *C. bombi* treatment (Kruskal-Wallace, $X^2 = 1.201$, $p = 0.272$).

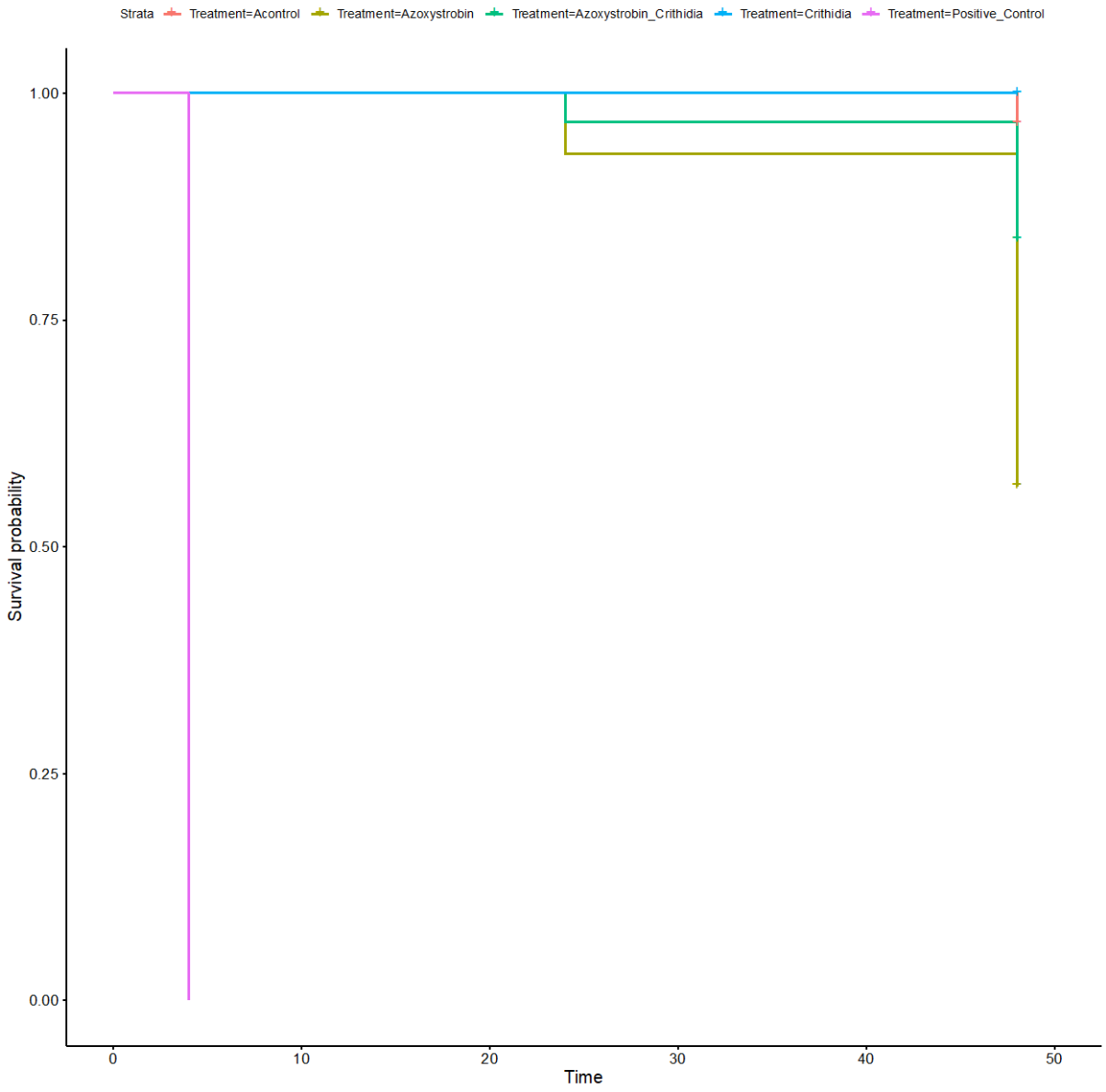

Figure 10. A Kaplan-Meier plot showing mortality (y axis) against time in hours (x axis) by treatment.

2.7. Sulfoxaflor and *C. bombi* in workers

There was no effect of any treatment on mortality. While there was 4% mortality in the sulfoxaflor treatment, and 9% in the sulfoxaflor + *C. bombi* treatment, this only represented 2 and 4 bees respectively. As such, there was insufficient statistical variation between treatments for an informative statistical test to be run.

Similarly, there was no statistically significant difference in parasite intensity between the *C. bombi* and sulfoxaflor + *C. bombi* treatment (Kruskal-Wallace, $X^2 = 0.407$, $p = 0.523$).

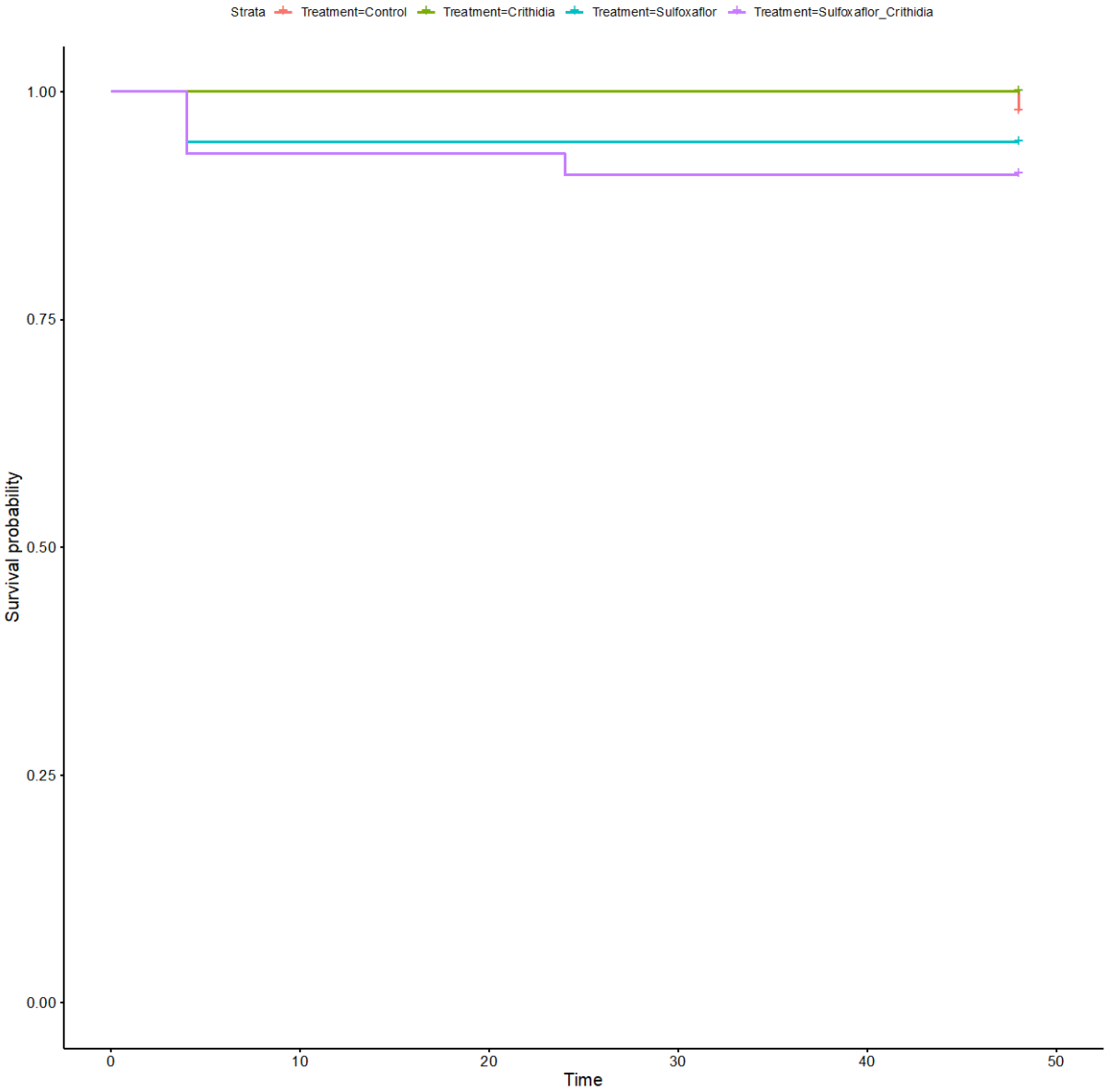

Figure 11. A Kaplan-Meier plot showing mortality (y axis) against time in hours (x axis) by treatment.

2.8. Azoystrobin, glyphosate, and *C. bombi* in male bumble bees

2.8.1. Azoxystrobin, glyphosate, *C. bombi*, and mortality

There were no significant impacts of any of the treatments on mortality, with most animals surviving until the end of the experiments (Azoxystrobin Experiment: Control: 32/40, Azoxystrobin: 33/40, *C. bombi*: 37/40, Azoxyystrobin X *C. bombi*: 36/40; Glyphosate Experiment: Control: 30/40, Glyphosate: 33/40, *C. bombi*: 34/40, Glyphosate X *C. bombi*: 32/40).

2.8.2. Azoxystrobin, glyphosate, *C. bombi*, and sugar consumption

There were no significant effects of treatment group on sugar consumption during the agrochemical exposure period in the Azoxystrobin experiment (p = 0.332; Figure 12).

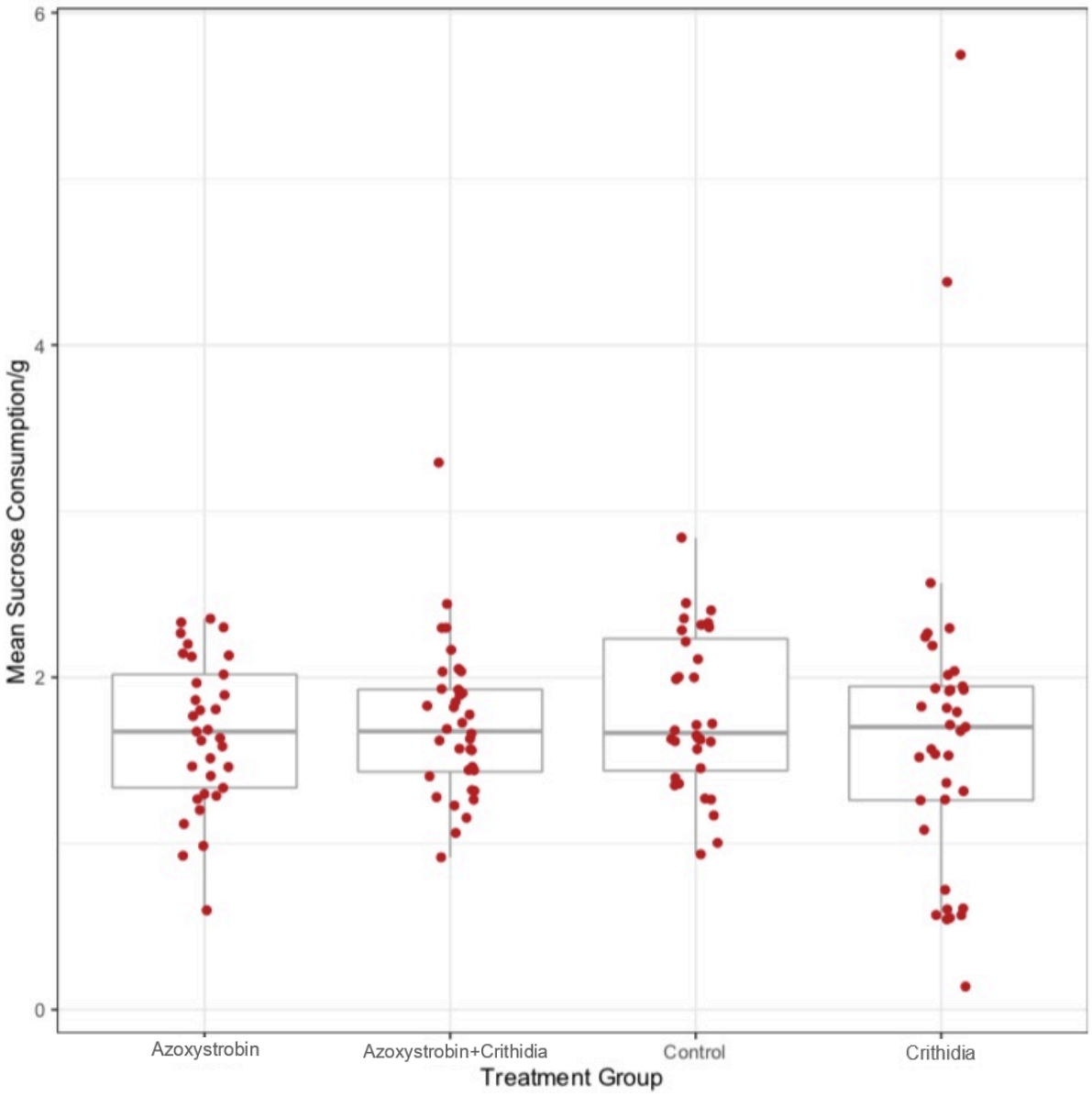

Figure 12. No effect of treatment on sucrose consumption during chronic Amistar (azoxystrobin**) exposure.**

In contrast, there was a significant effect of treatment on sucrose consumption during chronic exposure to glyphosate ($p = 0.025$), with animals exposed to glyphosate consuming more sucrose than animals in the two unexposed treatment groups (Figure 13).

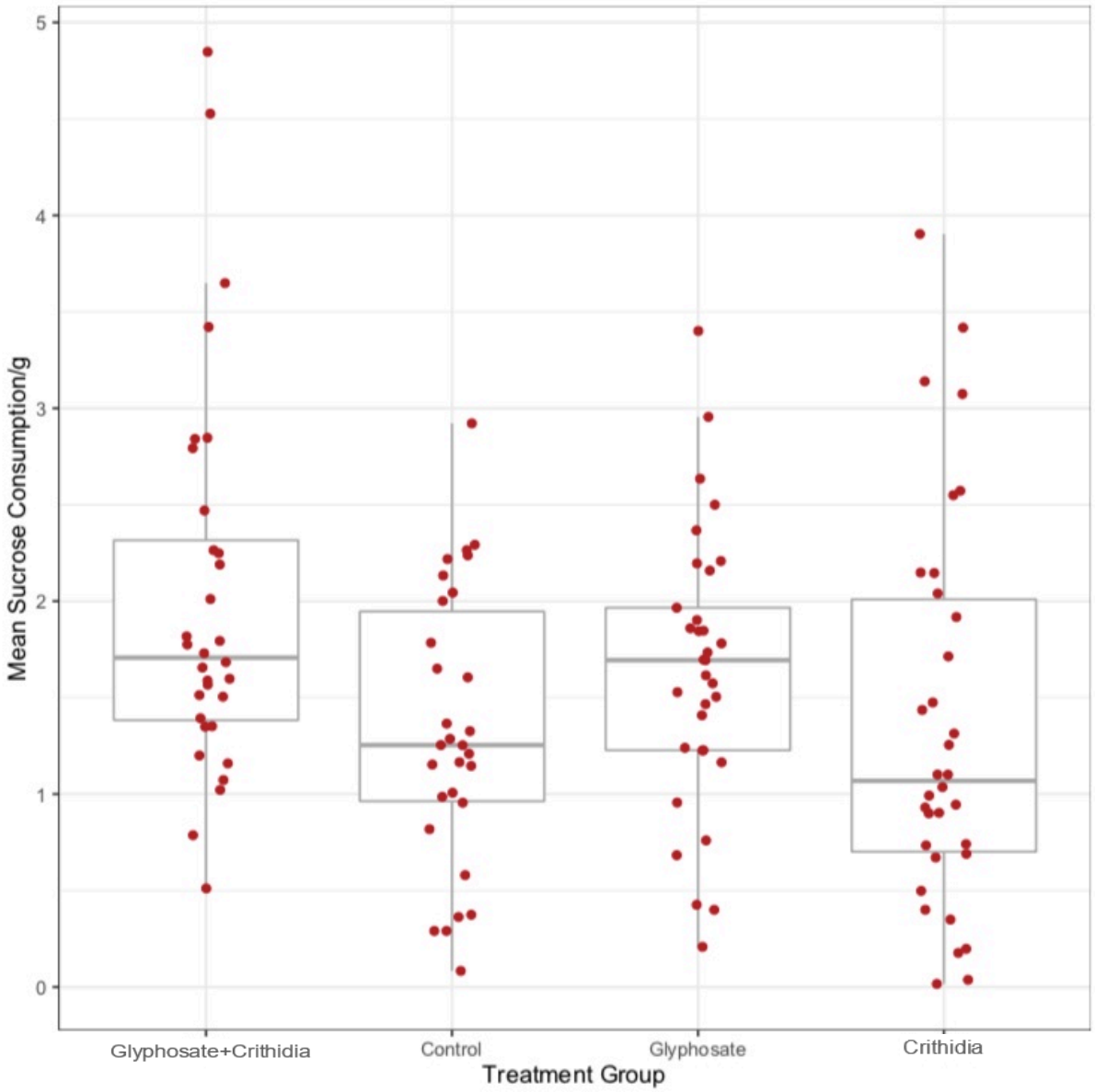

Figure 13. Treatment significantly affects sucrose consumption during chronic glyphosate exposure

2.8.3. Azoxystrobin, glyphosate, *C. bombi*, and parasite intensity

Exposure to azoxystrobin significantly reduced the intensity of parasite infections in male bees that survived the exposure period ($p < 0.001$; Figure 14). Similarly, exposure to glyphosate also significantly reduced the intensity of parasite infections in male bees who survived the exposure period ($p = 0.001$; Figure 15).

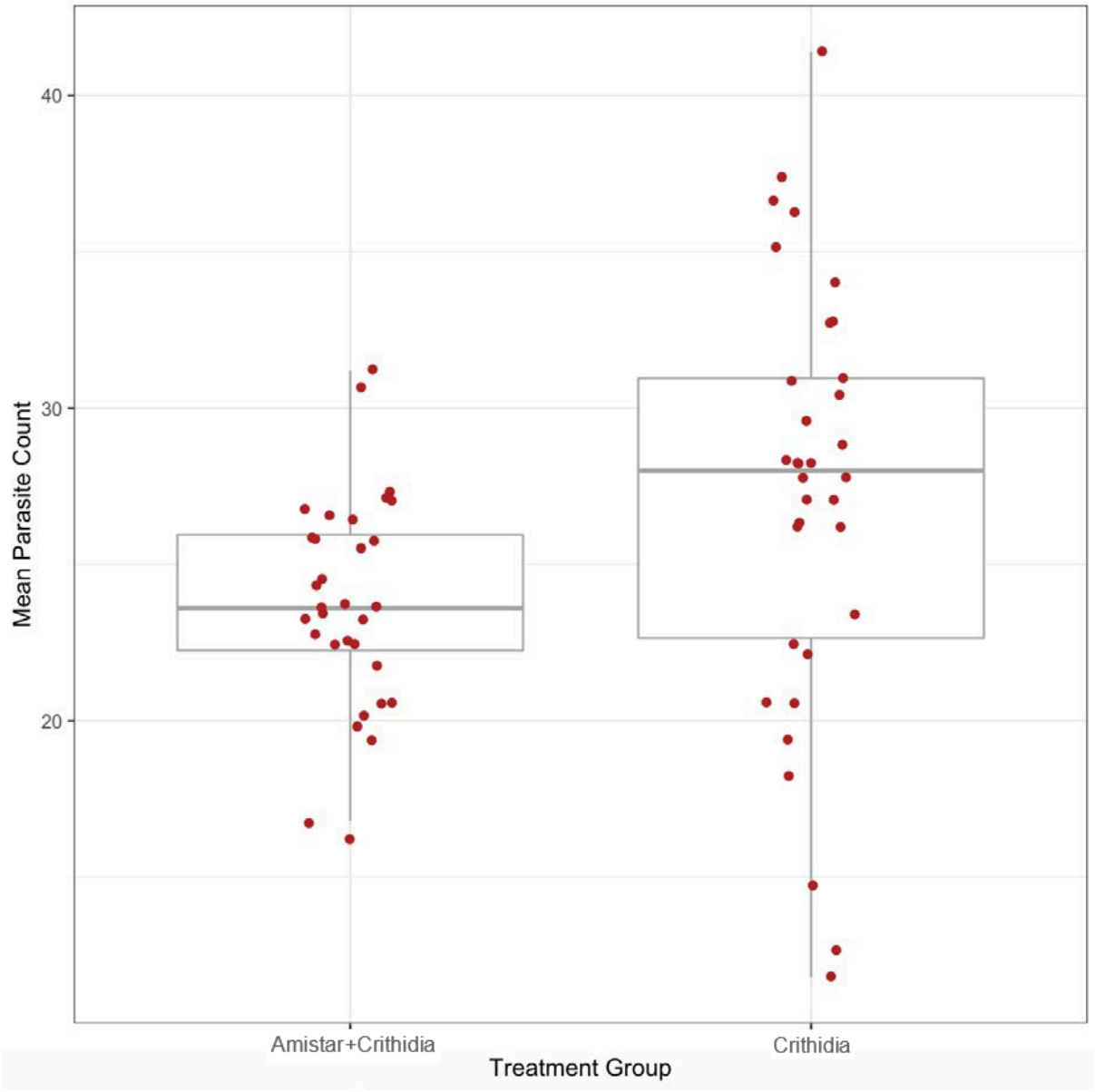

Figure 14. Exposure to azoxystrobin in its Amistar formulation reduces parasite intensity.

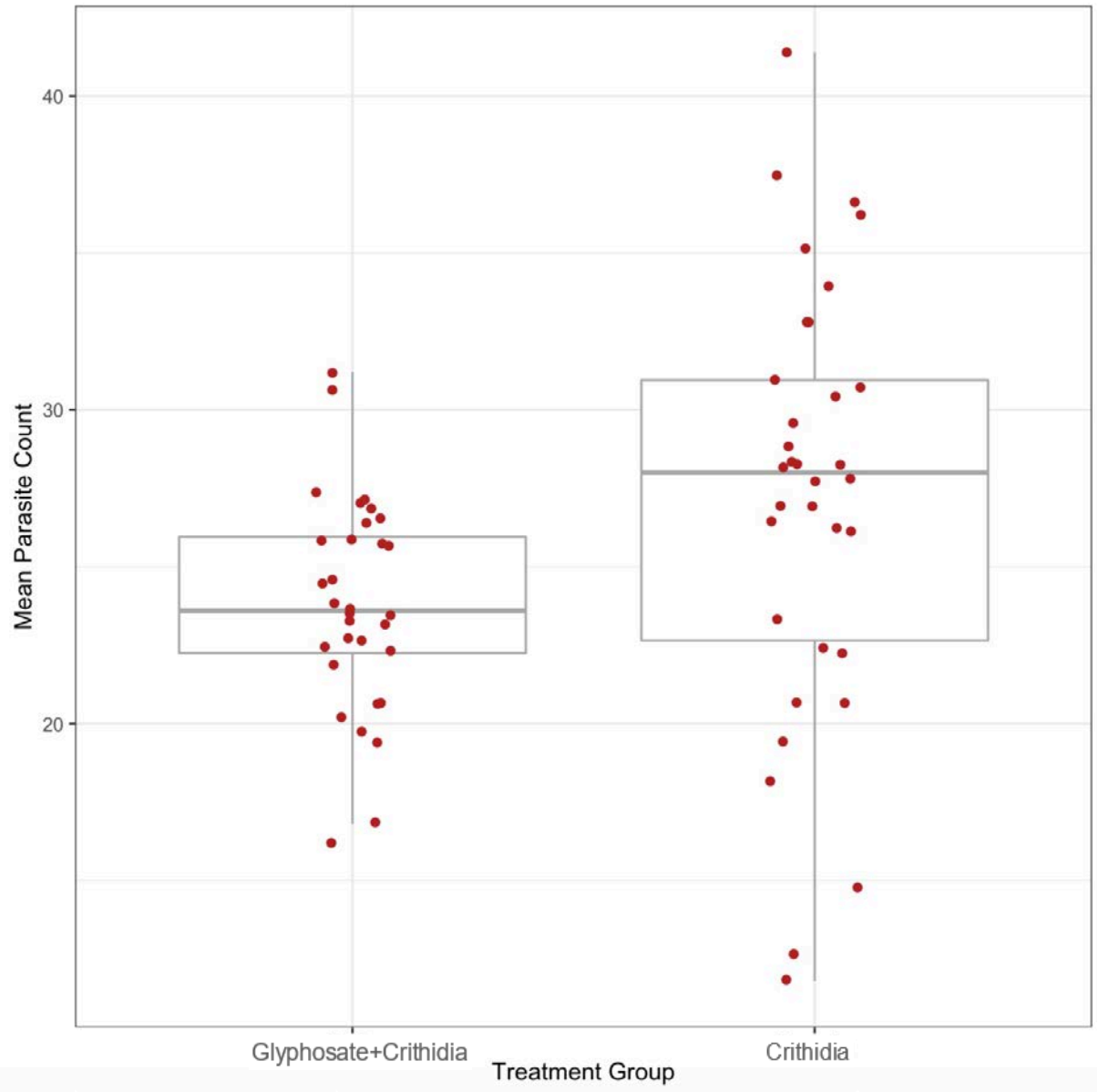

Figure 15. Exposure to Glyphosate reduces parasite intensity.

2.9. No impact of Sulfoxaflor and *C. bombi* on learning in worker bumble bees

There were no significant effects of sulfoxaflor, *C. bombi*, or their interaction, on any measure of learning. Bees exposed to sulfoxaflor gave a conditioned response 48% of the time, those exposed to *C. bombi* 42% of the time, and to both sulfoxaflor and *C. bombi* 26% of the time, in comparison to the control at 55% of the time (Responsiveness: GLM Parameter Estimate (PE) = -0.09, 95% CI [-0.62 to 0.43]; *C. bombi* PE = -0.14, 95% CI [-0.84 to 0.56]; sulfoxaflor and *C. bombi* PE = -0.29, 95% CI [-1.37 to 0.79]; Figure 16). Similarly, bees that had conditioned responses and were exposed to sulfoxaflor showed on average 3 conditioned responses, those exposed to *C. bombi* showed 5, those exposed to both sulfoxaflor and *C. bombi* showed 3, in comparison to the control which showed 3 (quasi-Poisson model: sulfoxaflor PE = 0.15, 95% CI [-0.28 to 0.60]; *C. bombi* PE = 0.48, 95% CI [-0.06 to 1.00]; sulfoxaflor and *C. bombi* PE = 0.07, 95% CI [-0.55 to 0.65]; Figure 17). Finally, for learning speed, bees that had conditioned responses and were exposed to sulfoxaflor, *C. bombi*, or were part of the control showed their first conditioned response, on average, on the 13th trial, whilst those

exposed to both sulfoxaflor and *C. bombi* showed theirs on the 14th trial (Cox Proportional Hazards sulfoxaflor PE = -0.02, 95% CI [-1.15 to 1.11]; *C. bombi* PE = 0.00, 95% CI [-1.78 to 1.78]; sulfoxaflor and *C. bombi* PE = -0.33, 95% CI [-3.23 to 2.56]; Figure 18).

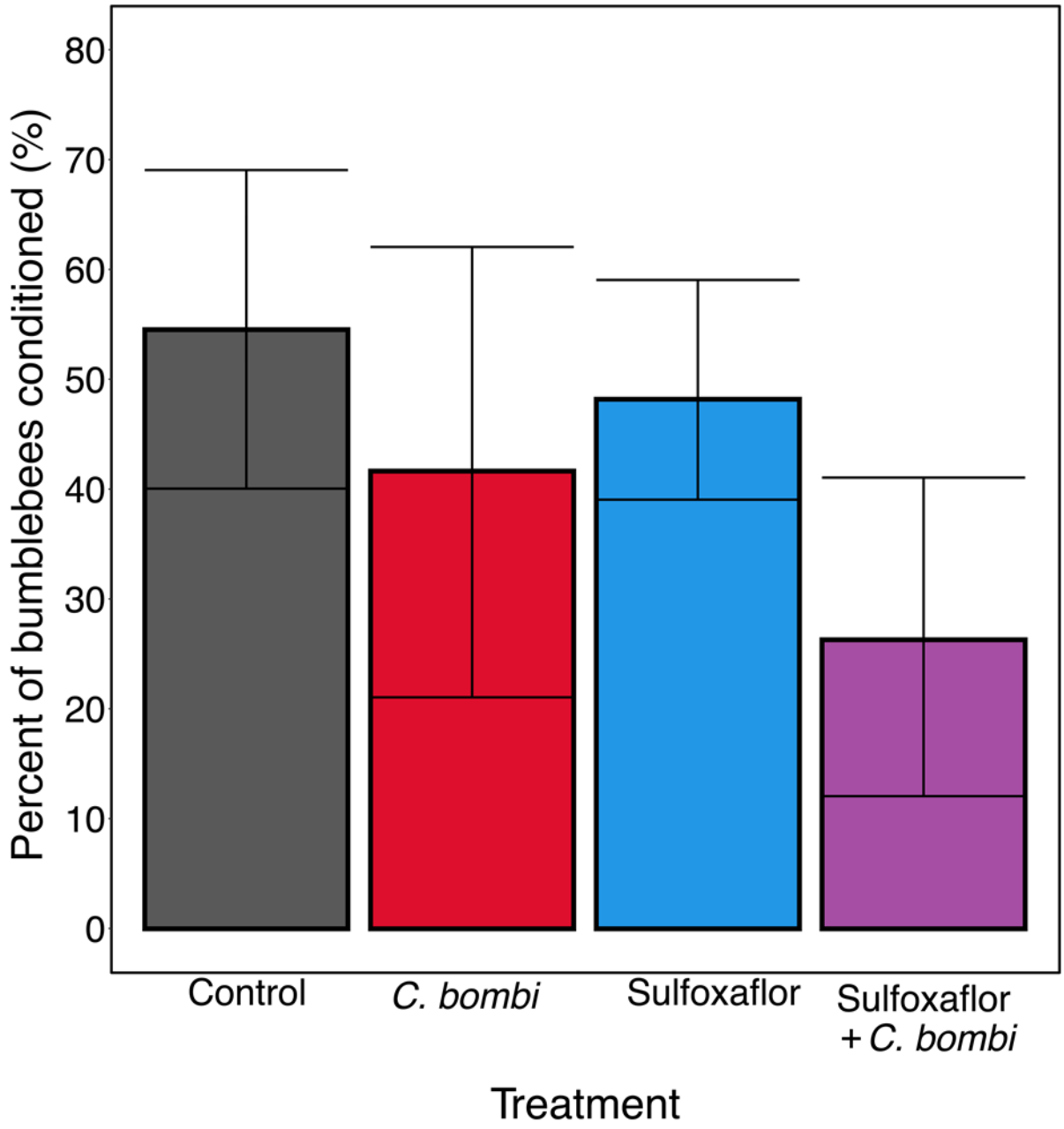

Figure 16. Responsiveness. The proportion of workers that showed a conditioned response in each treatment ±95% confidence interval (control n=44; sulfoxaflor n=56; *C. bombi* n=24; sulfoxaflor + *C. bombi* n=38).

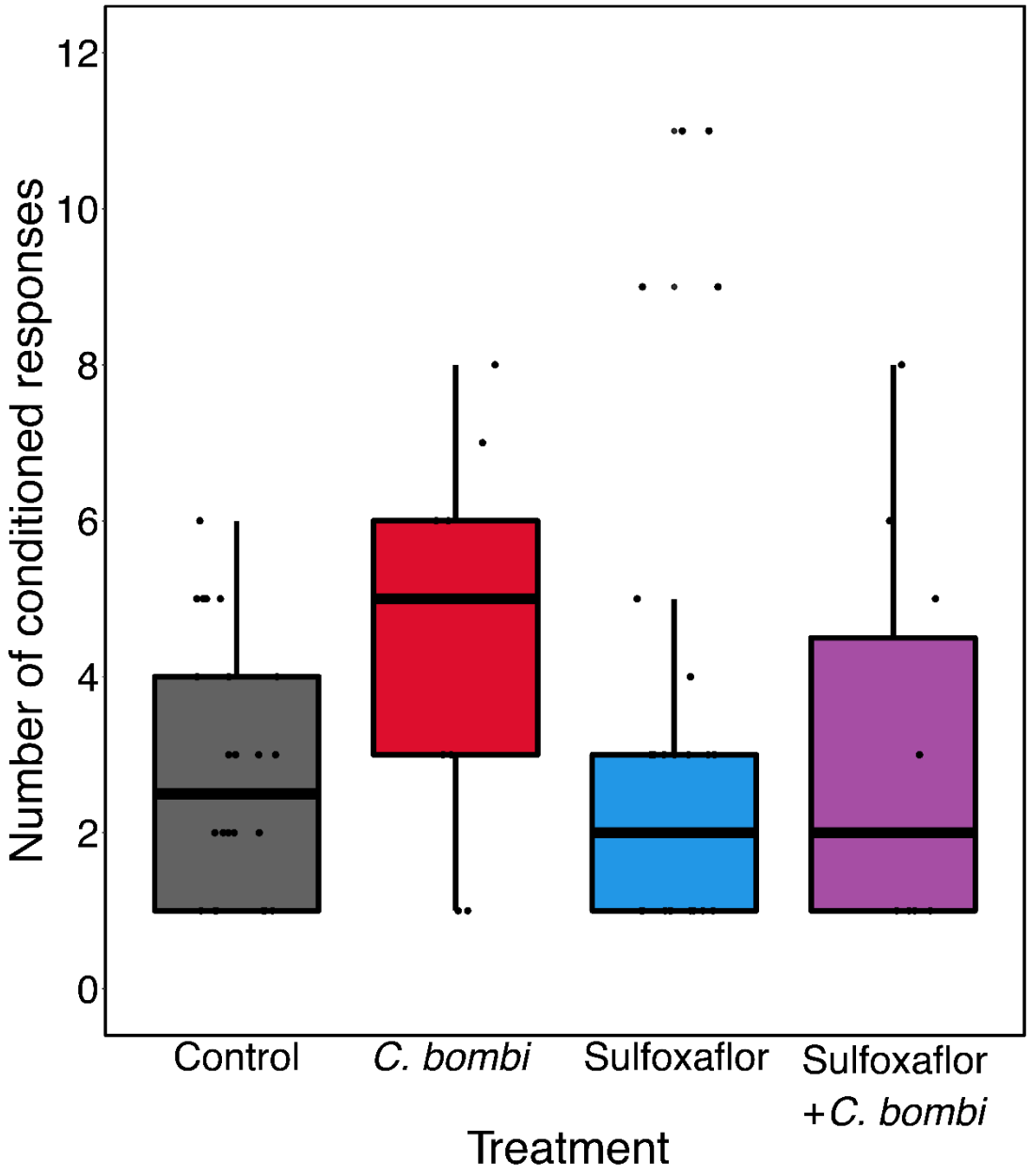

Figure 16. Learning Level. Average number of conditioned responses a responsive worker in each treatment group exhibited ±95% confidence interval (control n=24; sulfoxaflor n=27; *C. bombi* n=10; sulfoxaflor + *C. bombi* n=10). The number of bees differs from the other figures as this analysis only took responsive bees into account.

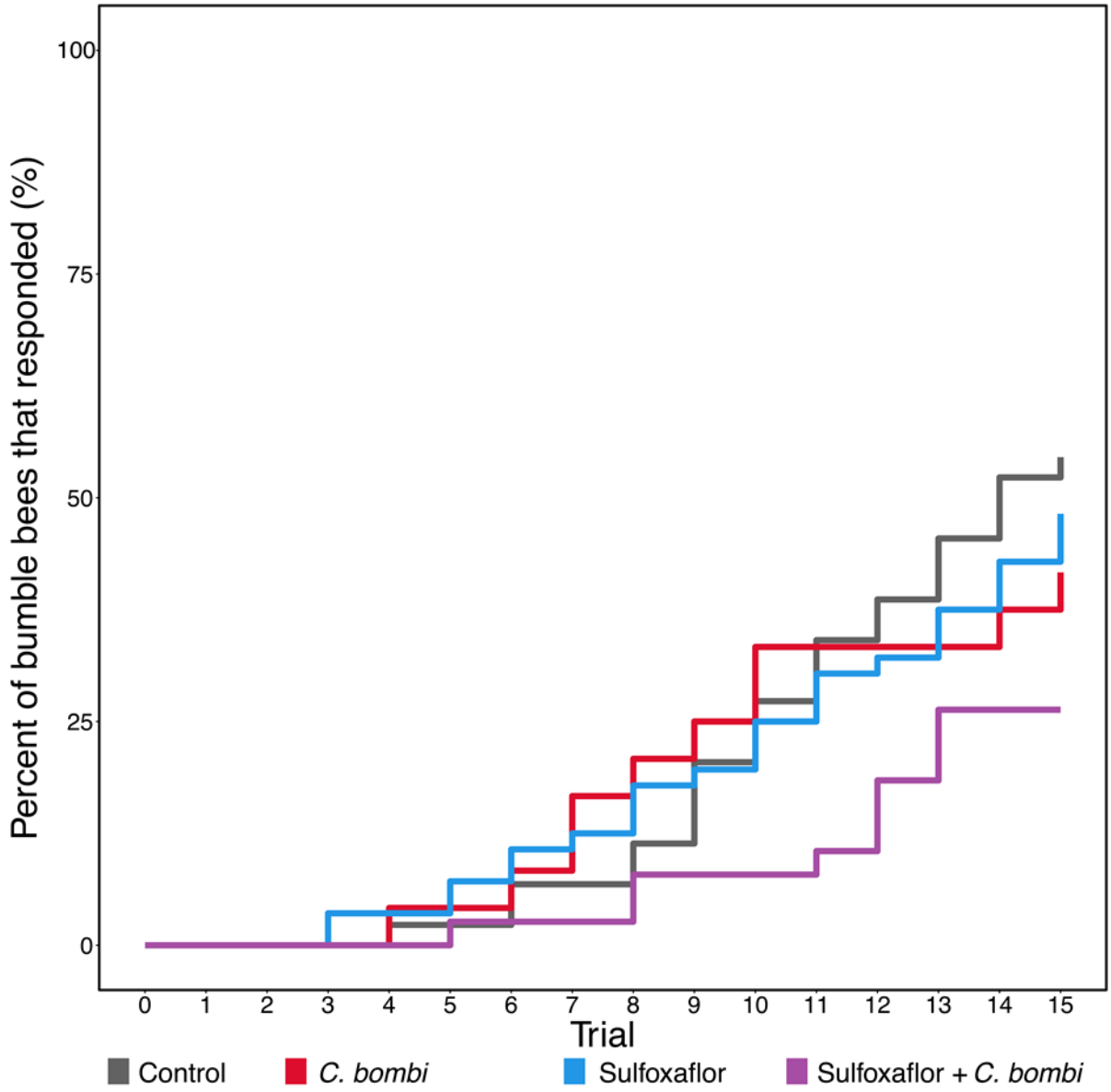

Figure 18. Learning Speed. Trial at which on average a responsive worker would show its first conditioned response (control n=44; sulfoxaflor n=56; *C. bombi* n=24; sulfoxaflor + *C. bombi* n=38).

2.10. Impacts of Sulfoxaflor and *C. bombi* on colony founding in bumble bee queens

A total of 197 queens were placed into hibernation. Of these, 32 died during hibernation or within the 4-day exposure period post-hibernation. Here we report analyses of the remaining queens by their initial treatment group.

While there was a trend for all 3 treatment groups to have a lower proportion of queens founding colonies than in the control group, there was no significant difference in colony founding success (X^2 = 2.383, p= 0.123; Figure 19). In contrast, infection by *C. bombi* significantly delayed colony founding (by 1.6 days when compared to the control; PE = -1.554, 95%CI = -2.750 to -0.359), and the interaction between sulfoxaflor exposure and *C. bombi* inoculation was also a significant predictor of the rate of colony founding by queens (PE= 1.7652, 95% CI =0.325 to 3.205; Figure 20).

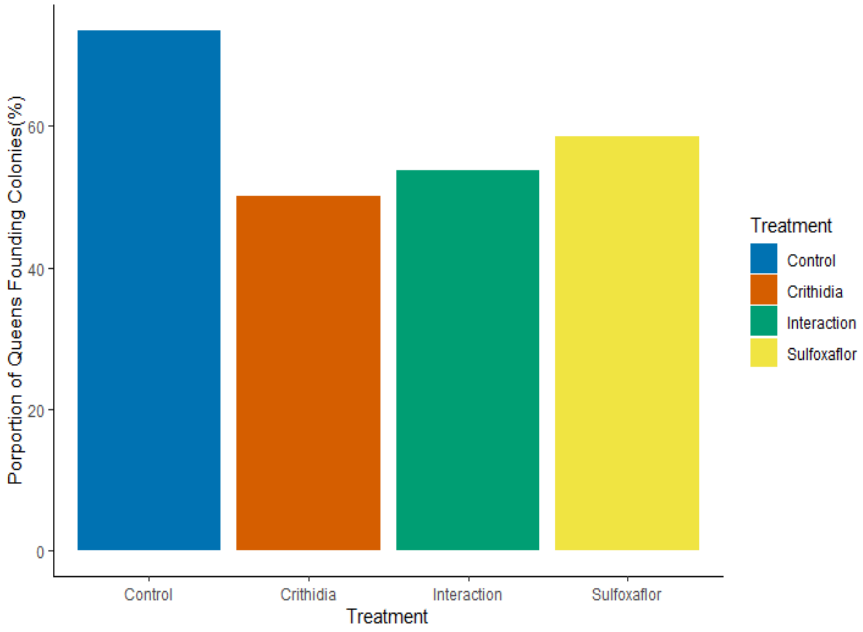

Figure 19. The proportion of queens that initiated a colony within the 8-week post-hibernation period. There was no significant effect of treatment group on colony founding.

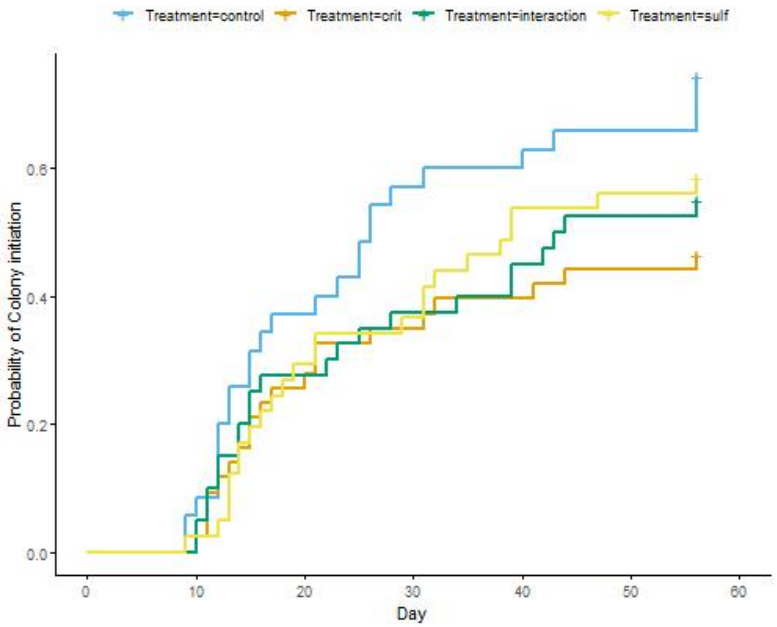

Figure 20. The Kaplan-Meier curves for the cumulative probability of colony founding over time for queens in the four treatment groups.

Again, though there was a trend for all 3 treatment groups to have a lower proportion of queens with emerged workers than in the control group, there was no significant difference in worker emergence across groups (X^2 = 3.827, p= 0.281; Figure 21). In contrast, infection by *C. bombi* (PE:-

1.937, 95% CI: -3.994 to 0.120), sulfoxaflor exposure (PE: -0.395 CI: -1.048 to 0.258), and their interaction (PE: 2.646 CI: 0.195 to 5.098) were all included in the final model of the date of worker emergence (Figure 22), with all 3 treatments associated with a delay in worker emergence (2 days for *C. bombi*, 2.5 days for sulfoxaflor, and 1 day for the interaction). But despite this, none of them were significant predictors of worker emergence (all CI cross zero).

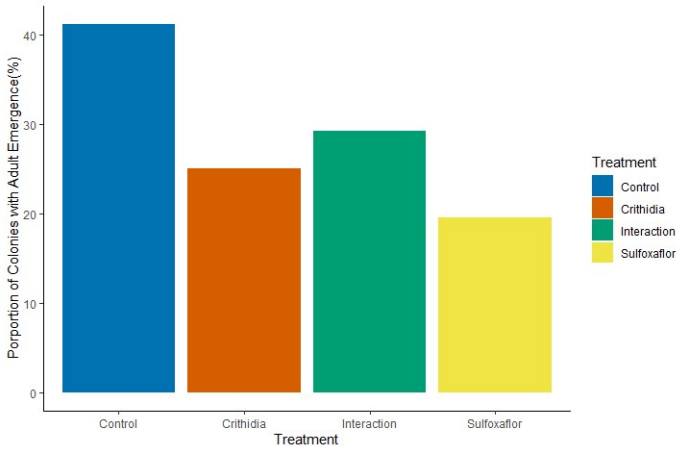

Figure 21. The proportion of queens where workers emerged within the 8-week post-hibernation period. There was no significant effect of treatment group on the probability of worker emergence.

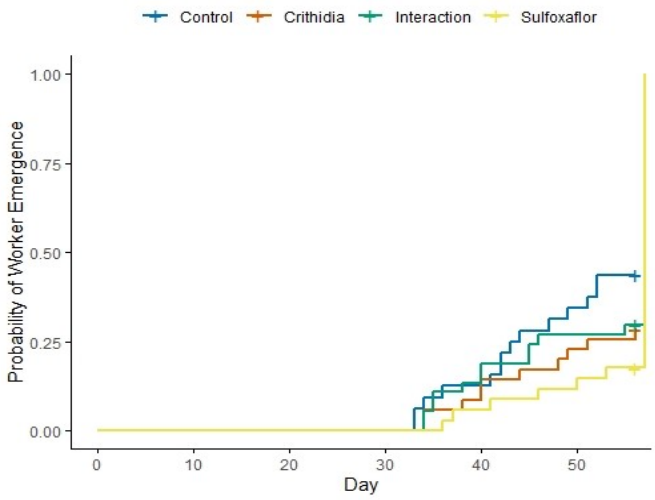

Figure 22. The Kaplan-Meier curves for the cumulative probability of worker emergence over time for queens in the four treatment groups.

40 queens died during the 8-week colony-founding period, but mortality was distributed approximately evenly across treatments (X^2 = 2.159, p= 0.142; Figure 23). Similarly, there was no effect of treatment on the rate at which queens died (only sulfaxoflor exposure was included in the final model, but it was a non-significant effect; PE: 0.893, 95% CI: -0.590 to 2.377; Figure 24).

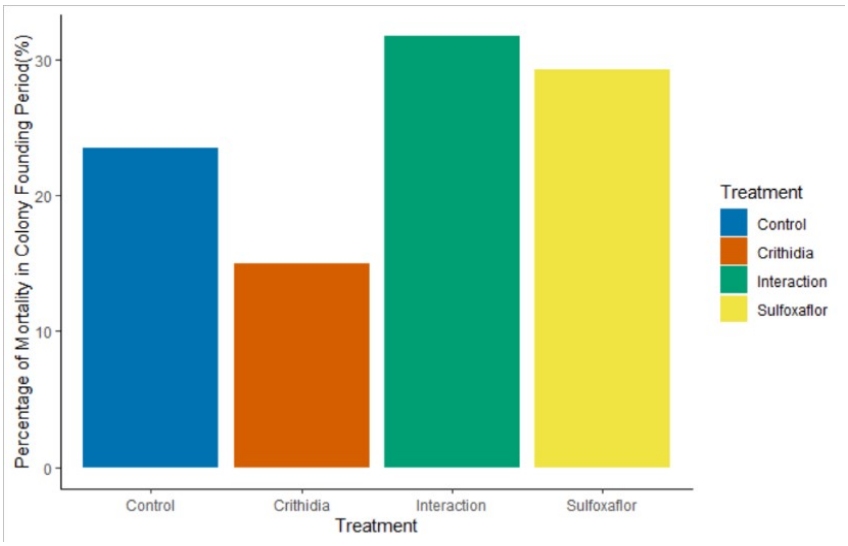

Figure 23. The proportion of queens who died within the 8-week post-hibernation period. There was no significant effect of treatment group on the probability of death.

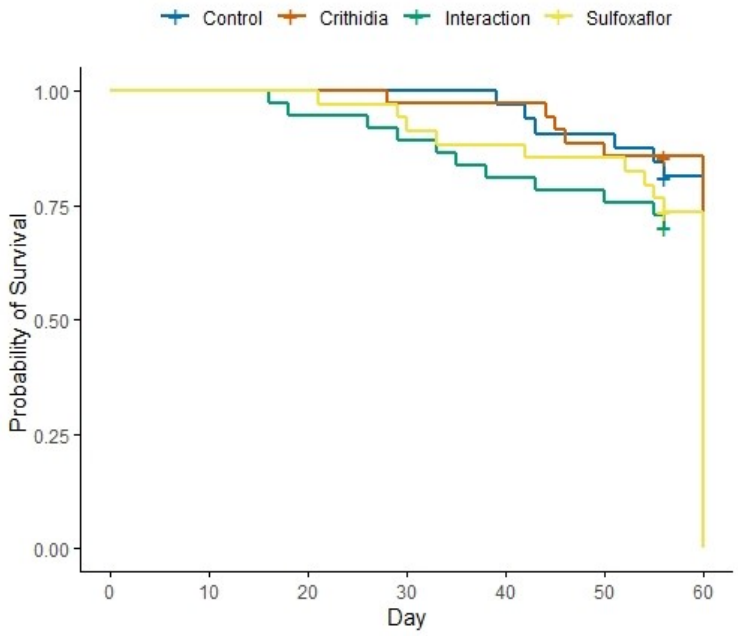

Figure 24. The Kaplan-Meier curves for the cumulative probability of queen death over time in the four treatment groups.

3. Discussion

3.1. Glyphosate X *C. bombi*: Mortality

The most basic metric of bee health is mortality. A dead bee can contribute nothing further to its fitness, as it is unable to contribute to the provisioning of brood or production of sexuals. Most regulatory systems use mortality as the initial metric to assess toxicity. In the EU, lower tier testing considers just acute contact and oral toxicity in honey bees and bumble bees (including OECD 247 studies), although the addition of bumble bee data has not yet been fully implemented. In the case of glyphosate, the LD_{50}s derived were found to be above the threshold value of 200µg active ingredient per bee (or equivalent highest possible tested dose), although this was only done with honey bees, as bumble bee data are not due to be submitted until the 2025 EU renewal of glyphosate.

The data presented here support the regulatory conclusion that glyphosate does not cause mortality in the short term. These data also expand the species upon which we have evidence of the mortality effects of glyphosate, with the addition of a bumble bee to the previously studied honey bee. Our results show no mortality over a range of exposures and time periods from 2-20 days, going well beyond the two-day test that regulators will be obliged to conduct on bumble bees using OECD 247. Additionally, there were no mortality effects from the interaction between glyphosate, with either acute or chronic exposure, and the parasite *C. bombi* in worker bumble bees. It is important to clarify that our experiments used glyphosate as an active ingredient, not as a formulation.

In the short term (two days) and long term (20 days) after exposure to a relatively high acute dose of glyphosate, no mortality was seen in individually housed bees in three separate experiments. As 20 days is representative of a considerable proportion of a bumble bee worker's lifespan, this indicates that there is no delayed mortality response and no meaningful shortening of longevity. All other academic studies have used chronic exposure to glyphosate, not acute exposure. As such, there is presently no non-regulatory data on acute exposure to glyphosate in any bee species, nor any data on glyphosate exposure in bumble bees, so our results represent a substantive contribution to the understanding of glyphosate's effects on bee mortality.

In the microcolony experiments no significant mortality was seen with either adult workers acutely exposed, or age controlled young adult workers through chronic exposure. This demonstrates that, even while the bees are housed collectively under more natural conditions and exerting themselves rearing young, any potential stress brought on by glyphosate was insufficient to cause additional mortality. The finding of no mortality with a fully field realistic chronic exposure regime in parasite free bumble bees supports the evidence that chronic glyphosate exposure is non-lethal to healthy worker bees

3.2. Glyphosate X *C. bombi*: Parasite intensity.

The initial experiment found an approximately two-fold increase in *C. bombi* intensity. As a preliminary experiment, the methods were less robust than later experiments because of its smaller sample size and no tracking of colony of origin or body weight through the experiment. However, the balanced experimental design accounts for this variation and as such it is unlikely to be confounded.

The follow up experiment to this preliminary experiment found a 16% increase in *C. bombi* intensity, although this effect was not statistically significant. In this expanded trial, the sample size was larger and the co-variates of colony of origin and body weight were tracked throughout.

These opposing results can be explained in several ways. Principally either of the two experiments (the preliminary and the follow-up experiments) could have delivered a false positive or a false negative result, which is the simplest solution, and there is no evidence to confirm or contradict this.

Alternatively, it is possible that some of the other variables in the experiment, such as the parasite, the colonies used or other unknown effects, are acting individually or in combination to alter the parasite intensity.

3.3. Glyphosate X *C. bombi*: Reproduction

Reproductive success is the ultimate metric of bee health, directly representing bee fitness. Drone production by unmated workers in a microcolony set up is designed to function as a proxy of this, and itself does not directly represent a field realistic measure of whole colony sexual production. There is even some evidence that microcolonies can give contradictory results to queenright laboratory or full field experiments (Van Oystaeyen et al., 2021). As such, our results should be interpreted with caution, and are not field realistic measures of reproductive success.

No significant effect on reproduction was found in any experiment, despite at times large differences between treatments (up to a 33.5% difference in reproductive success versus the control), which is potentially indicative of statistical power limitation. Indeed, it is possible that both microcolony experiments were power limited, with ~10 microcolonies per treatment (a total of 38 and 36 microcolonies in each experiment).

Interestingly, while not significant, *C. bombi* reduced reproductive success by 10.2% and 50.0% in the Acute and Chronic experiments respectively. This is a similar scale of reduction to previously published data (Brown et al., 2003). The data presented here also indicate that acute exposure to glyphosate is more likely to impact reproductive success than chronic exposure, with a 20.6% decline in reproductive success after acute exposure, versus a 34.9% decline after chronic exposure. Overall, we suggest that this evidence be used to guide future studies, conducted ideally in field conditions with larger sample sizes to provide more high quality and definitive evidence for any potential effects.

There was a considerably lower reproductive output overall in the Chronic experiment than in the Acute exposure experiment. This is likely because the workers in the Chronic exposure experiment were age controlled, and thus likely to be much younger on average. This could have led to a delay in ovary development retarding reproductive output. In the Chronic exposure experiment, sucrose consumption was also tracked to allow for the total glyphosate exposure to be measured, though it also varied little across treatments.

3.4. Glyphosate X *C. bombi*: Sucrose

Sucrose consumption can be an indicator of bee health. While in isolation this metric has no clear relation to fitness, the ultimate measure of bee health, it can be useful in indicating that a bee is acting abnormally. In the case of exposure to the co-formulant alcohol ethoxylates (as found in Amistar), reduced sucrose consumption went hand in hand with weight loss and gut melanisation (Straw and Brown, 2021). Further, sucrose consumption could be a corollary of pollination services, as bees with lower appetites might forage less, although in social bees nectar foraging is a response to both individual and colony-level nectar needs. Under chronic exposure, no treatment affected sucrose consumption, indicating that glyphosate did not significantly affect the bees' dietary consumption.

Under microcolony conditions, worker bees consumed an average of 38.7 or 41.4µg of glyphosate (Glyphosate and Glyphosate + *C. bombi* treatments respectively) under a field realistic, degrading concentration exposure regime. This can be used to inform future research into the consequences of cumulative exposure of bees in the wild. The majority of this glyphosate was consumed within the first few days of exposure, with the rapidly declining residues causing the consumption from day five onwards to contribute little to overall exposure. Consequently, future studies could truncate glyphosate exposure to five days with little reduction in exposure. However, it is also worth noting that there is no limit on the number of sprays of a glyphosate-based herbicide per year that can be

undertaken, or a mandated time gap between them (Roundup ProActive Label), so repeat exposure could occur. As such, the 38.7 or 41.4µg dose does not necessarily represent the total dose a bee could be exposed to over its lifetime.

The research presented here principally used an acute oral exposure of 200µg of glyphosate as an active ingredient. None of the research into the effects of glyphosate on the honey bee microbiome has used acute exposure, instead using chronic exposure at a range of concentrations from 0.8mg/kg (Dai et al., 2018) to 210mg/kg (Blot et al., 2019). It is possible that sustained exposure to glyphosate is more impactful than a single more concentrated instance of exposure because the gut microbial community is not afforded opportunity to recover. Alternatively, exposure to the considerably higher acute concentration may also have a more severe impact, potentially acting to cull sensitive microbiome species and strains. Given that bees are exposed to both acute and chronic exposure to glyphosate in the wild, if future research considered acute exposure our understanding of how glyphosate affects bee health would be more complete.

How the acute exposure to 200µg of glyphosate used in this study relates to in-field exposure is unknown. There are no data, even from honey bees, to enable accurate predictions of acute exposure to herbicides (herbicides lack bee mitigation measures). Given that flowering weeds can be sprayed while bees are foraging on them, and glyphosate is typically sprayed in very concentrated sprays (compared with insecticides), for a bee to consume 200µg in a short period of time immediately after a spray application is not implausible, although lower doses are more likely. More work on acute exposure of bees to agrochemicals lacking bee-specific mitigation measures is needed to inform future research. However, with no effects on a range of metrics seen at this potentially high-end dose, it is likely that more field-realistic acute exposures would also not have an effect on bumble bees.

3.5. Azoxystrobin X *C. bombi*

The Azoxystrobin treatment caused unexpected mortality, which in turn reduced the sample size below the intended number. Further work (Straw and Brown, 2021) isolated the co-formulant alcohol ethoxylates as the cause of the mortality of the formulation Amistar, with azoxystrobin (the active ingredient) being cleared of causing any toxicity. Interestingly, infection with *C. bombi* may have alleviated this mortality, although there was no statistically significant interaction. Future work should investigate this potential interaction further to determine whether it is a real biological relationship or an experimental anomaly.

3.6. Sulfoxaflor X *C. bombi*

The sulfoxaflor treatment was chosen at the NOEL (No observable effect level) dose, so the lack of mortality was expected. As an insecticide, it is fully capable of causing mortality. We chose a non-lethal dose at the edge of causing mortality to see if *C. bombi* causes mortality when co-exposed. No mortality was observed. This indicates no apparent interactions between the agrochemical and the parasite.

3.7. Azoystrobin, glyphosate, and *C. bombi* in male bumble bees

There were no direct or interactive impacts of the agrochemical and parasite exposure on mortality in male bumble bees, indicating that these agrochemicals would likely pass risk assessment if male bumble bees were incorporated as an additional test model. These results match what has been shown in workers (this Deliverable). Interestingly, our results suggest that both agrochemicals inhibit parasite growth in their bumble bee hosts. Such reductions in parasite intensity could be driven by direct impacts on the parasite population itself – although a mechanism for such effects is unclear – or by impacts on the host gut microbiome, which is known to modulate infection by *C. bombi* (Koch

& Schmid-Hempel, 2011). This reduction in the intensity of infections could act to reduce transmission of the parasite from males to new gynes, which could have important positive implications for bumble bee population success. Experimental work to explore these questions is unfortunately well beyond the scope of PoshBee.

3.8. Learning

We found no significant impact of either sulfoxaflor, the parasite *C. bombi*, or their combination, on three different measures of learning in worker bumble bees. This suggests that learning, which has often been blamed for the impacts of agrochemicals on bee health, may not be influenced by either of these stressors. Again, this is an important result as it provides added confidence for the use of sulfoxaflor in agricultural systems, particularly against the background of high parasite prevalence. Importantly, we have modified a learning methodology to incorporate two stressors, opening it up for future studies of the impacts of interactive stressors on bee health.

3.9. Colony founding

In contrast to previous studies with neonicotinoid insecticides (Baron et al., 2017), we found very little impact of a field-realistic exposure of sulfoxaflor on queen colony founding or mortality. In addition, there was only one significant effect associated with the interaction between sulfoxaflor and the parasite *C. bombi*, and this was an apparent amelioration of the impact of the parasite on egg-laying. This suggests that, at current field realistic levels, there is no evidence to suggest that sulfoxaflor, or its interaction with parasites, is likely to impact the colony-founding success of bumble bee queens as they emerge from hibernation in spring.

3.10. Conclusion

In general, our experiments found few direct or interactive effects of agrochemicals and parasites on measures of bumble bee health. Interestingly, the effects we did find suggest positive impacts on bee health, through a reduction in parasite intensity in males, and a reduction in the impacts of a common parasite on colony founding success in queens. This is important as it provides potential evidence for the safe use of these agrochemicals in agricultural systems. Further experimental work, looking at interactions with other, more virulent parasites, is warranted.

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5. References

Azpiazu, C., Bosch, J., Bortolotti, L., Medrzycki, P., Teper, D., Molowny-Horas, R., Sgolastra, F. (2021) Toxicity of the insecticide sulfoxaflor alone and in combination with the fungicide fluxapyroxad in three bee species. *Scientific Reports* **11**, 1-9.

Baron G.L., Jansen, V.A.A., Brown, M.J.F., Raine, N.E. (2017) Pesticide reduces bumblebee colony initiation and increases probability of population extinction. *Nature Ecology and Evolution* **1**, 1308–1316.

Benbrook, C.M. (2016). Trends in glyphosate herbicide use in the United States and globally. *Environmental Science Europe*, **28,** 1-15.

Blot, N., Veillat, L., Rouzé, R., and Delatte, H. (2019). Glyphosate, but not its metabolite AMPA, alters the honeybee gut microbiota. *PLoS One*, **14**, e0215466.

Brown, M.J.F., Loosli, R., Schmid-Hempel, P. (2000) Condition-dependent expression of virulence in a trypanosome infecting bumblebees. *Oikos* **91**, 421-427.

Brown, M.J.F., Schmid-Hempel, R., Schmid-Hempel, P. (2003) Strong context-dependent virulence in a host-parasite system: reconciling genetic evidence with theory. *Journal of Animal Ecology* **72**, 994-1002.

Cole, R. J. (1970) Application of triangulation method to purification of *Nosema* spores from insect tissues. *Journal of Invertebrate Pathology* **15**, 193-195.

Dai, P., Yan, Z., Ma, S., Yang, Y., Wang, Q., Hou, C., Wu, Y., Liu, Y., and Diao, Q. (2018). The herbicide glyphosate negatively affects midgut bacterial communities and survival of honey bee during larvae reared *in vitro*. *Journal of Agricultural and Food Chemistry*, **66***,* 7786–93.

Duke, S.O., & Powles, S.B. 2008. Glyphosate: A Once-in-a-Century Herbicide. *Pest Management Science*, **64,** 319–25.

Fournier, A., Rollin, O., Le Féon, V., Decourtye, A., Henry, M. (2014) Crop-emptying rate and the design of pesticide risk assessment schemes in the honey bee and wild bees (*Hymenoptera: Apidae*). *Journal of Economic Entomology* **107**, 38-46.

Garibaldi, L.A., Steffan-Dewenter, I., Winfree, R., Aizen, M.A., Bommarco, R., Cunningham, S.A., Kremen, C., Carvalheiro, L.G., Harder, L.D., Afik, O., et al. (2013) Wild pollinators enhance fruit set of crops regardless of honey bee abundance. *Science* **339**, 1608-1611.

Goulson, D., Nicholls, E., Botías, C., and Rotheray, E.L. (2015). Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science*, **347,** 1255957.

Koch, H., Schmid-Hempel, P. (2011) Socially transmitted gut microbiota protect bumble bees against an intestinal parasite. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 19288-19292.

Linguadoca, A., Rizzi, C., Villa, S., & Brown, M.J.F. (2021) Sulfoxaflor and nutritional deficiency synergistically reduce survival and fecundity in bumblebees. *Science of The Total Environment* **795**, 148680.

Martin, C.D., Fountain, M.T., & Brown, M.J.F. (2018) Bumblebee olfactory learning affected by task allocation but not by a trypanosome parasite. *Scientific Reports* **8**, 1-8.

Mercurio, P. Flores, F., Mueller, J.F., Carter, S., Negri, A.P. (2014) Glyphosate persistence in seawater. *Marine Pollution Bulletin*, **85**, 385-390.

OECD (2017) Bumblebee, acute oral toxicity test. *OECD Guidelines for the Testing of Chemicals*, **247**, 1-12

OECD (2017a) Honey bee (*Apis mellifera* L.), chronic oral toxicity test (10-day feeding). *OECD Guidelines for the Testing of Chemicals*, **245**, 1-9

Riveros, A. J., Gronenberg, W. (2009) Olfactory learning and memory in the bumblebee *Bombus occidentalis*. *Die Naturwissenschaften* **96**, 851-856.

Rohatgi, A. (2020) WebPlotDigitizer. Version 4.4.

Rutrecht, S.T., Brown, M.J.F. (2008) The life history impact and implications of multiple parasites for bumble bee queens. *International Journal of Parasitology* **38**, 799-808.

Samuelson, E.E.W., Chen-Wishart, Z.P., Gill, R.J., Leadbeater, E. (2016) Effect of acute pesticide exposure on bee spatial working memory using an analogue of the radial-arm maze. *Scientific Reports* **6**, 1-11.

Schatz, K., Wallner, F. (2009) *Pflanzenschutz mittelapplikation in blühendenraps (Brassicanapus) und deren auswirkungen auf dierück-stands situationen in honig, nektar und pollen der honigbeine (*Apis mellifera). PhD Thesis Universität Hohenheim.

Schmid-Hempel, P. (1998) Parasites in Social Insects. Princeton University Press, Princeton, New Jersey.

Shykoff, J.A., Schmid-Hempel, P. (1991) Incidence and effects of four parasites in natural populations of bumble bees in Switzerland. *Apidologie* **22**, 117-125.

Siviter, H., Bailes, E.J., Martin, C.D., Oliver, T.R., Koricheva, J., Leadbeater, E., et al. (2021). Agrochemicals interact synergistically to increase bee mortality. *Nature* **596**, 389-392

Straw, E.A., Brown, M.J.F. (2021) Co-formulant in a commercial fungicide product causes lethal and sub-lethal effects in bumble bees. *Scientific Reports* **11**, 21653.

Siviter, H., Scott, A., Pasquier, G., Pull, C.D., Brown, M.J.F., Leadbeater, E. (2019) No evidence for negative impacts of acute sulfoxaflor exposure on bee olfactory conditioning or working memory. *PeerJ **7***, e7208.

Thompson, H.M., Levine, S.L., Doering, J., Norman, S., Manson, P., Sutton, P., von Mérey, G. (2014) Evaluating exposure and potential effects on honeybee brood (*Apis mellifera*) development using glyphosate as an example. *Integrated Environmental Assessment and Management* **10**, 463-470

Van Oystaeyen, A., Klatt, B.K., Petic, C., Lenaerts, N., Wäckers, F. (2021) Short-term lab assessments and microcolonies are insufficient for the risk assessment of insecticides for bees. *Chemosphere*, **273**, 128518