No evidence of effects or interaction between the widely used herbicide, glyphosate, and a common parasite in bumble bees

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Background. Glyphosate is the world's most used pesticide and it is used without the mitigation measures that could reduce the exposure of pollinators to it. However, studies are starting to suggest negative impacts of this pesticide on bees, an essential group of pollinators. Accordingly, whether glyphosate, alone or alongside other stressors, is detrimental to bee health is a vital question. Bees are suffering declines across the globe, and pesticides, including glyphosate, have been suggested as being factors in these declines.

Methods. Here we test, across a range of experimental paradigms, whether glyphosate impacts a wild bumble bee species, *Bombus terrestris*. In addition, we build upon existing work with honey bees testing glyphosate-parasite interactions by conducting fully crossed experiments with glyphosate and a common bumble bee trypanosome gut parasite, *Crithidia bombi*. We utilised regulatory acute toxicity testing protocols, modified to allow for exposure to multiple stressors. These protocols are expanded upon to test for effects on long term survival (20 days). Microcolony testing, using unmated workers, was employed to measure the impacts of either stressor on a proxy of reproductive success. This microcolony testing was conducted with both acute and chronic exposure to cover a range of exposure scenarios.

Results. We found no effects of acute or chronic exposure to glyphosate, over a range of timespans post-exposure, on mortality or a range of sublethal metrics. We also found no interaction between glyphosate and *Crithidia bombi* in any metric, although there was conflicting evidence of increased parasite intensity after an acute exposure to glyphosate. In contrast to published literature, we found no direct impacts of this parasite on bee health. Our testing focussed on mortality and worker reproduction, so impacts of either or both of these stressors on other sublethal metrics could still exist.

Conclusions. Our results expand the current knowledge on glyphosate by testing a previously untested species, *Bombus terrestris*, using acute exposure, and by incorporating a parasite never before tested alongside glyphosate. In conclusion our results find that glyphosate, as an active ingredient, is unlikely to be harmful to bumble bees either alone, or alongside *Crithidia bombi*.

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2 herbicide, glyphosate, and a common parasite in bumble bees.

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Abstract

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33 contrast to published literature, we found no direct impacts of this parasite on bee health. Our

34 testing focussed on mortality and worker reproduction, so impacts of either or both of these

35 stressors on other sublethal metrics could still exist.

36 **Conclusions.** Our results expand the current knowledge on glyphosate by testing a previously

37 untested species, *Bombus terrestris*, using acute exposure, and by incorporating a parasite

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39 active ingredient, is unlikely to be harmful to bumble bees either alone, or alongside *Crithidia*
40 *bombi*.
41
42 **Introduction**
43 Glyphosate is the world's most used pesticide (Duke and Powles, 2008, Benbrook 2016). It is a
44 herbicide used to suppress weeds in agricultural and amenity settings (Duke and Powles, 2008,
45 Duke, 2018). Glyphosate helps reduce the need for tilling and mechanical weeding, which helps
46 protect against soil erosion and boosts farmers yields and profits (Becki, Flower and Ashworth
47 2020). Bees are exposed to glyphosate frequently in nature through spraying of weeds,
48 contamination of water, and application onto glyphosate resistant flowering crops (Odemer et
49 al. 2020, Straw, Carpentier and Brown, 2021). Glyphosate-based herbicide products typically do
50 not carry any mitigation measures aimed at reducing bees exposure to them. Research into
51 herbicides, and glyphosate specifically, has grown considerably in recent years, with just 15
52 papers found in a systematic review of literature up to 2018 (Cullen et al. 2019), the first of
53 which was published in 2011, while five were published in the final year searched. Several more
54 publications have emerged since then (e.g. Odemer et al. 2020, Motta et al. 2020, Motta et al.
55 2020). To date most of these studies have used honey bees, *Apis mellifera*, with only a few
56 testing the impacts on other bee species (Abraham et al. 2018, Seide et al. 2018, Ruiz-Toledo
57 and Sánchez-Guillén, 2014). Glyphosate has also undergone regulatory testing for
58 governmental authorities worldwide to determine its effects on bees (EFSA, 2015, Duke 2018).
59 Glyphosate is currently approved in all major territories (Duke 2018), and where it is not
60 approved (Mexico, for example) this is for human health reasons, not bee health reasons
61 (Alcántara-de la Cruz et al. 2021).
62
63 In the European Union (EU) and North America pesticide regulation uses a tiered approach,
64 with initial toxicity testing focussing solely on mortality (lower tier), and, if toxicity thresholds
65 are met in the lower tier tests, then more complex experiments are conducted (higher tier)
66 (EFSA, 2013, EPA, 2014). In the EU specifically this initial testing comprises two tests, acute oral
67 exposure and acute contact exposure, both performed with the pure active ingredient and the
68 representative formulation (EFSA, 2013). It has been suggested that this mortality-focussed
69 approach is inadequate to properly assess the toxicity of a substance, and that there should be
70 a move towards a fitness-based approach that also considers sublethal and reproductive effects
71 at lower tiers (Straub, Strobl and Neumann, 2020). In the EU, glyphosate did not meet the
72 toxicity thresholds required to trigger higher tier testing, so was approved for use with only
73 very minimal bee testing (EFSA, 2015). Alongside regulatory testing a number of academic
74 experiments have found that oral exposure to glyphosate does not cause mortality in adult
75 bees (Herbert et al. 2014, Goñalons and Farina 2018, Motta, Raymann and Moran 2019, Blot et
76 al. 2019, Faita et al. 2020, Almasri et al. 2021), although there is mixed evidence, with Almasri

77 et al. (2020) and Motta and Moran (2020) finding mortality at doses considerably lower than
78 doses found to be non-lethal in other work.
79
80 While there is little strong evidence that glyphosate causes mortality in adult bees, it has been
81 found to cause a range of sublethal effects in honey bees (reviewed in Farina et al. 2019).
82 Chronic exposure to field realistic doses has been found to impair learning (Herbert et al. 2014,
83 Balbuena et al. 2015) and increase the length of time taken to return to a colony (Balbuena et
84 al. 2015). Chronic exposure has also been linked to larval mortality, reduced body mass, and a
85 reduction in successful moulting (Vazquez et al. 2018), although the evidence here is mixed
86 with conflicting results across years and colonies. At a molecular level, glyphosate has been
87 found to impair antioxidant and acetylcholinesterase production (Helmer et al. 2015, Boily et al.
88 2013). While these results are limited in their scope, and derive only from honey bees, they
89 represent clear evidence that the herbicide glyphosate can be biologically active in bees, and
90 that examining the mortality effects of glyphosate in isolation are insufficient to understand its
91 impacts on bees.
92
93 Motta, Raymann and Moran (2018) and Motta et al. (2020) found that in honey bees chronic
94 exposure to glyphosate does not typically cause significant mortality, but that glyphosate can
95 synergise with parasites to cause mortality. Exposure to the opportunistic parasite *Serratia*
96 *marcescens* caused some mortality, around 20-30% more than the control, while glyphosate
97 caused no more mortality than the control. However, when both stressors were applied
98 simultaneously the mortality increased by almost 80% compared to the control. This result was
99 replicated with a glyphosate-based formulation, Roundup® ProMAX, in Motta et al. (2020),
100 showing that the formulation also causes the synergism. Glyphosate induced a knockdown of
101 protective gut bacteria that allowed the parasite to be more deadly, thus explaining how an
102 otherwise non-lethal pesticide synergises to cause substantial mortality. This result highlights
103 the importance of testing multiple stressors on bees, as even individually non-lethal pesticides
104 can cause considerable synergism alongside common parasites.
105
106 In addition to this synergism, there is mixed evidence for the interaction between another bee
107 parasite group, *Nosema spp.,* and glyphosate in honey bees. Both Blot et al. (2019) and Faita et
108 al. (2020) found no effect of chronic exposure to glyphosate on mortality and a significant effect
109 of *Nosema spp.*. However, only Faita et al. (2020) observed a significant interaction between
110 the two stressors, with a 17% increase in mortality compared to the *Nosema spp.* alone. This
111 difference may be attributable to the use of a formulation by Faita et al. (2020), rather than just
112 the active ingredient used by Blot et al. (2020), or the mix of *Nosema apis* and *Nosema ceranae*
113 used by Faita et al. (2020), rather than just *Nosema ceranae* used by Blot et al. (2020). In fact,

114   the use of a formulation in Faita et al. (2020) does prevent the effect observed being
115   attributable to glyphosate as the other ingredients may have driven the effect.
116
117   The studies described above focus on honey bees and common parasites. Here we extend this
118   approach to bumble bees (*Bombus* spp.) and their common trypanosome gut parasite *Crithidia*
119   *bombi*, which has been found at prevalences of up to 82% in the wild (Gillespie 2010), although
120   this level of infection is not found in all studies, with large variation between years, sites and
121   species (Shykoff and Schmid-Hempel 1999, Korner and Schmid-Hempel, 2005, Rutrecht and
122   Brown, 2008, Gillespie 2010, Jones and Brown, 2014, Hicks et al. 2018). *C. bombi* is likely less
123   damaging of a parasite to *Bombus terrestris* than either *Nosema spp.* or *S. marcescens* are to
124   honey bees, with no individual effect on mortality in otherwise unstressed bees (Brown, Loosli,
125   and Schmid-Hempel 2000, Fauser-Misslin et al. 2014, Baron, Raine and Brown 2014). At the
126   colony level, uncontrolled or post-founding infections have no impact on growth or production
127   of sexuals (Shykoff and Schmid-Hempel, 1991, Fauser-Misslin et al. 2014). In contrast, when
128   experimentally infected bees are starved, worker mortality rates increase by 50% (Brown,
129   Loosli, and Schmid-Hempel, 2000), and when infections are experimentally controlled and occur
130   before the stressful hibernation period, the parasite has dramatic negative impacts of up to
131   40% on host fitness (Brown, Schmid-Hempel, and Schmid-Hempel 2003, Yourth, Brown and
132   Schmid-Hempel, 2008). Thus, this parasite is most likely to have impacts on bumble bees when
133   combined with other stressors.
134
135   Finally, *C. bombi* infection is strongly related to the host gut microbiome, with specific bacterial
136   groups like *Apibacter, Lactobacillus* Firm-5 and *Gilliamella* conferring increased resistance in *B.*
137   *terrestris* (Koch and Schmid-Hempel, 2011, Mockler et al. 2018). Interestingly, a range of studies
138   have found an effect of glyphosate on the honey bee microbiome (Dai et al. 2018, Motta,
139   Raymann and Moran, 2018, Motta and Moran, 2020, Blot et al. 2019, Motta et al. 2020),
140   consistently finding that it changes the microbiome composition. This suggests that, despite
141   differences between *A. mellifera* and *B. terrestris* and their microbiomes, glyphosate might
142   impact *C. bombi* indirectly through modifications of the gut microbiome.
143
144   In this study, we test whether glyphosate has direct impacts on worker mortality or
145   reproduction, whether it interacts with *C. bombi* to impact these metrics of bee health, and
146   whether infected bumble bees that are exposed to glyphosate, either acutely or chronically, will
147   have increased *C. bombi* intensities.

Materials & Methods

General

Bombus terrestris audax colonies were ordered from Agralan Ltd, Swindon, UK. Colonies were maintained on *ad libitum* sucrose and honey bee collected pollen from Thorne, Windsor, UK and Agralan Ltd, Swindon, UK respectively. 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 in the experiment. Further molecular screening may have revealed viral presence (Graystock et al. 2013) undetectable by visual screening, but this was beyond the scope of our study. Random allocation of colonies to experiments, and the use of internal colony controls, mitigated against any such infections having impacts on our results. The number of bees or microcolonies included in each treatment group is presented in Supplementary Tables 1-5. Bees were removed from their colonies without regard for their age, hence bee age is not controlled between treatments, however it was randomly distributed between treatments. Pesticides were applied as pure active ingredient, glyphosate (Sigma-Aldrich) CAS-no: 1071-83-6 and dimethoate (Sigma-Aldrich) CAS-no: 60-51-5.

Modified Ecotoxicological Protocol OECD 247: General Methods

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 based on Siviter, Matthews and Brown (In Preparation) 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 rank allocated to treatments based on weight, with an even distribution of source colonies by treatment. 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. Pilot work had demonstrated that

182   this method leads to very high infection rates (>95%). At dissection any bees with a parasite
183   intensity of 0 cells per µL were deemed to have a failed infection, and were excluded from the
184   experiment. A further single worker with an intensity of 100 cells per µl, which is more likely to
185   have resulted from contamination of the slide than an infection, was also excluded.
186
187   Sucrose syringes were removed for 2-4 hours prior to exposure to the inoculum, starving the
188   bees. Then 40µL of solution was pipetted into a fresh syringe and this was added to each cage.
189   The bees were left to feed on the inoculum for a further four hours, at which point the syringe
190   was removed and consumption visually verified. Bees that did not consume >80% of the
191   solution were excluded from the experiment. Bees were returned to *ad libitum* sucrose with a
192   syringe of 50% (w/w) sucrose and had a small ball of pollen added (~1g).
193
194   Bees were left for 7 days for the parasite infection to develop, at which point they entered the
195   pesticide exposure phase. Here the above steps for parasite exposure were repeated, but with
196   pesticide-laced treatment solutions replacing the parasite treatment solutions. The treatment
197   doses used in all acute exposure experiment are listed in Table 1. below.
198
199   After exposure to the pesticide, mortality was recorded at four hours, 24 hours and 48 hours.
200   Mortality was defined as a lack of response to physical agitation. Dead bees were discarded as
201   their corpses degrade too quickly to be dissected.
202
203   Any bees who survived the full 48 hours were weighed (Scout SKX, Ohaus, Switzerland,
204   accuracy limit of 0.001g), then transferred to a 2mL Eppendorf tube and frozen at -80C° for
205   later dissection. Bees in the *C. bombi* or *C. bombi* + Glyphosate treatment groups were later
206   dissected. Bees were removed from the freezer and placed on ice. The abdomen was cut off
207   and was pinned to a black wax plate. The abdomen was cut open on one side, and pinned open.
208   100µL of 0.8% Ringers solution was pipetted directly onto the gut to prevent desiccation and
209   another 100µL onto the wax to the side of the body. The honey crop was cut, and the gut
210   transferred to the droplet on the wax. The ileum was isolated and cut at both ends, with care to
211   remove any Malpighian tubules and tracheal tissue. The ileum was moved to a 1.5ml Eppendorf
212   with 100µL of 0.8% Ringers solution and ground using a pestle for five seconds in a set pattern
213   of movements. The ground gut was then vortexed for a single second and 10µL pipetted onto a
214   Neubauer haemocytometer slide and the *C. bombi* concentration counted. All endpoints are
215   presented as mean ± one standard deviation.

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, 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.

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 analysed as part of a different project. This manipulation did not affect the mortality metric as mortality was recorded prior to the manipulation. Further, it would not affect the parasite intensity measure as there is no by treatment differences, and the timescale of the extraction is too short to influence *C. bombi* levels. This experiment was conducted in two batches with just a single day stagger between them.

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 protocol 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.

Experiment four: Microcolony Exposure- 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 per microcolony box were added.

247

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

251

252 Due to time constraints only bumble bees receiving a treatment were moved to Nicot cages and
253 exposed. Bees in the control treatment were never moved to Nicot cages, bees in the *C. bombi*
254 only treatment and the glyphosate only treatment were moved to Nicot cages just once, and
255 those in the Glyphosate + *C. bombi* treatment were moved to Nicot cages twice. This had the
256 potential to cause a by treatment effect as being moved to a Nicot cage is a potentially stressful
257 experience. However, the day prior to the *C. bombi* exposure day all bees were manipulated as
258 they were moved from their source colony to a microcolony box. Similarly, on the glyphosate
259 exposure day all bees not moved into Nicot cages were manipulated as they were moved into a
260 fresh microcolony box. As such it is only the marginal additional level of stress from the time in
261 the Nicot cages that could produce a by treatment effect. Bees in the Nicot cages were also
262 kept in their microcolony box adjacent to nest-mates to reduce stress.

263

264 Non-feeders were excluded from the experiment at each of the exposure steps, which
265 alongside mortality led to slightly lower worker numbers in the micro-colonies (Glyphosate: 6.9
266 ± 1.2, *C. bombi*: 6.7 ± 1.1, Glyphosate + *C. bombi*: 6.4 ± 1.1 (SD)), versus the control (7.8 ± 0.4
267 (SD)). Workers who died (n = 4) or escaped (n = 5) during the experiment were recorded, but
268 not replaced. This was accounted for in the analysis, however, with reproductive output
269 expressed per worker present at end of experiment. Given that worker reproduction is highly
270 dependent on the laying individual (Blacquière et al. 2012), this should robustly account for
271 differing worker numbers.

272

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

277

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

Experiment five: Microcolony Exposure- Chronic Exposure

285

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

288

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

302

303 Data from Thompson et al. (2014) were used to inform the chronic exposure scenario.
304 Thompson et al. (2014) measured glyphosate concentration in returning nectar and pollen from
305 honey bees foraging on *Phacelia tanacetifolia* sprayed with a glyphosate-based herbicide
306 formulation (MON 52276) according to full label restrictions. Using WebPlotDigitiser (Rohatgi,
307 2020), the values from Thompson et al. (2014)'s graphs were extracted. An inverse relationship
308 model was used to model the declining residue concentration:

309 $Glyphosate\ Concentration = Intercept + \frac{Constant}{Time}$. As the data from Thompson et al. (2014)

310 has missing days and no data after 7 days, missing data were either interpolated or
311 extrapolated. These modelled concentrations of returning nectar and pollen were then used to
312 generate an exposure regime. Sucrose was fed to the bees *ad libitum* and was spiked with
313 pesticides in concentrations shown in Figure 1. In all treatments 50% w/w sucrose was changed
314 daily, and the previous day's consumption was recorded. The glyphosate concentration
315 provided decreased over time with the modelled values. Degradation of the glyphosate will
316 have occurred in the sucrose; however, this is largely insignificant given glyphosate's long half-
317 life of 47-267 days (as measured in seawater) (Mercurio et al. 2014). 5g of pollen was provided
318 and in glyphosate treatments this was spiked with an average concentration of glyphosate over
319 the 10 days exposure (110mg/kg). This was done as changing pollen daily was not feasible, and
320 5g was used as this amount was rarely wholly consumed by a group of workers in 14 days. In
321 the positive control, the dimethoate concentration was maintained at a constant 1mg/L, and

322   pollen was not spiked in this treatment. Following OECD 245 for honey bees (OECD, 2017a),
323   exposure ended on day 10, and all bumble bees were fed unspiked sucrose for another four
324   days. On day 14 bumble bees were frozen and reproductive output measured, as described
325   above. Mortality was recorded daily.
326
327   As the dataset used to calculate our chronic exposure regime was from a semi-field exposure
328   studied conducted in honey bees (Thompson et al. 2014), the use of these data for *B. terrestris*
329   may be problematic. There are no comparable data from honey bees and bumble bees to be
330   able to see if the same spraying regime leads to similar returning nectar concentrations.
331   However, as the only available dataset it is the best choice to inform the chronic exposure
332   regime.

Statistical testing

334   Statistical analyses were carried out in 'R' programming software version 3.6.2 (R Core Team
335   2019) following the same analysis scheme as Straw, Carpentier and Brown, (2021). All plots
336   were made using 'ggplot2' version 3.2.1 (Wickham, 2016) and 'survminer' version 0.4.6
337   (Kassambara, Kosinski & Biecek, 2019). AIC model simplification was used, with conditional
338   model averaging where no single model had >95% AIC support. The candidate set of models
339   was chosen by adding the next best supported model until a cumulative >95% AIC support was
340   reached. 'MuMIn' version 1.43.17 was used for model averaging (Bartoń 2020). Parameter
341   estimates and 95% confidence intervals are reported. 'lme4' version 1.1-23 was used for Linear
342   Mixed Effects models (Bates et al. 2020) and 'coxme' version 2.2-16 was used for Mixed Effects
343   Cox Proportional Hazards models (Therneau, 2020). Confidence intervals not crossing zero
344   indicate a significant effect, so a confidence interval of -1.00 to 1.00 would not be significant,
345   but a confidence interval of -2.00 to -1.00 would be. Model assumptions were checked
346   graphically and using statistical testing, including using 'e1071' version 1.7-4 (Mayer et al.
347   2021). Model parameters, AIC weights and final models are presented in Supplementary Tables
348   6-11.
349
350   **Experiment one: Modified Ecotoxicological Protocol OECD 247 Small Scale**
351   **Parasite intensity:** Data were found to be non-normal using a Shapiro-Wilks test, so a Kruskal
352   Wallis test was used with the model (Parasite Intensity ~ Treatment).
353   **Mortality:** Due to an absence of mortality in the experiment no statistical testing was
354   conducted.
355
356   **Experiment two: Modified Ecotoxicological Protocol OECD 247 Full Scale**

357   **Parasite intensity:** Data were found to be non-normal using a Shapiro-Wilks test, so a Kruskal
358   Wallis test was used with the model (Parasite Intensity ~ Treatment).
359   **Mortality:** Due to an absence of mortality in the experiment, except in the positive control
360   where all bees died, no statistical testing was conducted.
361

362   **Experiment three: Modified Ecotoxicological Protocol OECD 247: Long Term Survival**
363   **Mortality:** A Cox Proportional Hazards model was used to analyse the mortality data. Due to
364   the near complete mortality in the positive control treatment, this treatment was excluded
365   from the mortality analysis as it violates the proportionality of hazards assumption. The full
366   model used was (Mortality ~ Treatment + Body Weight + (1|Colony)). Proportionality of hazards
367   was checked graphically.
368

369   **Experiments four and five: Microcolony Exposure- Acute Exposure and Chronic Exposure**
370   **Reproduction:** Larval weight, adjusted to the number of workers present at the end of the
371   experiment, was found to be non-normal using a Shapiro-Wilks test. It was accordingly square
372   root transformed, and confirmed to be normal using a further Shapiro-Wilks test. The full
373   model used was (Larval Weight per Worker ~ Treatment + Body Weight of Initial Workers +
374   Number of Workers Alive at the End of the Experiment + (1|Colony)).
375   **Parasite intensity:** A Linear Mixed Effect model was used to analyse the parasite intensity data.
376   The full model used was (Parasite Intensity ~ Treatment + (1|Micro Colony ID) + (1|Colony)).
377

378   **Acute Exposure Only:**
379   **Mortality:** Mortality was too low to allow a Linear model, Linear Mixed Effects models, or Chi-
380   Square test. Accordingly, a Fishers Exact test was used with the model (Survival ~ Treatment).
381

382   **Chronic Exposure Only:**
383   **Sucrose/Glyphosate consumption:** A Linear Mixed Effect model was used to analyse the
384   Sucrose Consumption data. The full model used was (Sucrose Consumption ~ Treatment * Time
385   + Weight of Bees at Start of Exposure + (1|Micro Colony ID) + (1|Colony)).
386   **Mortality:** Mortality was too low to allow a Linear model, Linear Mixed Effects models, or Chi-
387   Square test. Accordingly, a Fishers Exact test was used with the model (Survival ~ Treatment).

388 **Results**

Modified Ecotoxicological Protocol OECD 247: Small Scale

390   Parasite intensity

391 The Glyphosate + *C. bombi* treatment had a significantly higher parasite intensity than the *C.*
392 *bombi* only treatment (Kruskal-Wallis $X^2(1)= 7.885$, $p = 0.005$). Glyphosate + *C. bombi* treated
393 bees (*n* = 21) had an average parasite intensity of 14,519± 10,462 (SD) cells per μL compared to
394 6,946± 5,682 cells per μL in the *C. bombi* only treatment (*n* = 23) (Figure 2).

395   Mortality

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

Modified Ecotoxicological Protocol OECD 247: Full Scale

398   Parasite intensity

399 In contrast to the first experiment, Glyphosate + *C. bombi* did not have a significantly different
400 parasite intensity to the *C. bombi* only treatment (Kruskal-Wallis $X^2(1)= 0.42818$, $p = 0.5129$).
401 Glyphosate + *C. bombi* treated bees (*n* = 34) had an average parasite intensity of 24,124±
402 14,664 cells per μL, compared to the 20,756± 14,473 cells per μL in the *C. bombi* only treatment
403 (*n* = 32) (see Figure 3). Neither body weight or batch had a significant effect on parasite
404 intensity (Linear Mixed Effect model: parameter estimate (PE) = 66,940.7, 95% CI [-19,878.3 to
405 152,664.5] and (PE) = 897.3, 95% CI [-6,843.0 to 8,512.8] respectively).

406   Mortality

407 No mortality was observed in any treatment bar the positive control, where all bees died within
408 24 hours.

Modified Ecotoxicological Protocol OECD 247: Long Term Survival

410   Mortality

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

413

414   *C. bombi* only, Glyphosate only, and Glyphosate + *C. bombi* did not have significantly different
415   mortality compared to the negative control (Cox proportional hazards mixed effects model:
416   parameter estimate (PE) = 0.728, 95% CI [-0.81 to 0.96], (PE) = 1.27, 95% CI [-0.92 to 1.18], and
417   PE = 1.19, 95% CI [-0.89 to 1.14], respectively). *C. bombi* only, Glyphosate only, and Glyphosate
418   + *C. bombi* had 4%, 7% and 6% mortality respectively, while the control had 2% mortality (see
419   Figure 4), a real terms difference of one to two bees.
420

Experiment four: Microcolony Exposure- Acute Exposure

Reproduction

423   There was no significant difference in reproductive output between treatments. While the
424   mean larval weight per worker (±SD and number of microcolonies) varied between treatments
425   (0.510g ± 0.224, *n* = 8 in the control, 0.458g ± 0.349, *n* = 11 in the *C. bombi* only treatment,
426   0.405 ± 0.141, *n* = 9 in the Glyphosate only treatment and 0.339g ± 0.224, *n* = 10 in the
427   Glyphosate + *C. bombi* treatment (see Figure 5)), a null model, which contained the response
428   variable, the co-variate of initial worker weight and the random colony variable, but not the
429   treatment variable, was the best supported model with ≥95% AIC support. This model found a
430   significant effect of Original Weight of Nurse Workers on reproductive output (Linear mixed
431   effects model (LMER) =0.26, 95% CI [0.14 to 0.37]), with heavier workers being more successful
432   at rearing offspring.

Parasite intensity

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

Mortality

440   There was no significant difference in mortality by treatment (Fisher Exact test (two sided) *p*=
441   0.679). *C. bombi* only, Glyphosate only and Glyphosate + *C. bombi* had 1%, 0% and 3% mortality
442   respectively, while the control had 2% mortality, a real terms difference of one bee.

443 ## Experiment five: Microcolony Exposure- Chronic Exposure

444 ### Reproduction

445 There was no significant difference in reproductive output between treatments. The mean
446 larval weight per worker (±SD and number of microcolonies) varied between treatments, with
447 0.106g ± 0.077, n = 8 in the control, 0.053g ± 0.054, n = 8 in the *C. bombi* only treatment, 0.143g
448 ± 0.139, n = 8 in the Glyphosate only treatment and 0.124g ± 0.103, n = 8 in the Glyphosate + *C.*
449 *bombi* treatment (see Figure 7). The model average with a cumulative ≥95% AIC support did not
450 include the treatment term. The two models included were both null models, one with the co-
451 variate of initial worker weight and random colony variable, and the second with just the
452 random colony variable. This model found no significant effect of Original Weight of Nurse
453 Workers on reproductive output (Linear mixed effects model (LMER) =0.20, 95% CI [-0.15 to
454 0.27]).

455 ### Sucrose/Glyphosate consumption

456 Over the 10-day exposure period the average consumption of sucrose per worker was 5.890±
457 0.676mL in the control, 5.880± 0.865mL in the *C. bombi* only treatment, 5.947± 0.875mL in the
458 Glyphosate only treatment, and 6.271± 0.746mL in the Glyphosate + *C. bombi* treatment.
459
460 The model average that contained models with a cumulative ≥95% AIC support did not include
461 the Treatment term. As such Treatment had no effect on sucrose consumption. The weight of
462 the bees at the start of exposure also did not affect sucrose consumption, (Linear Mixed Effect
463 model: parameter estimate (PE) = 0.062, 95% CI [-0.052 to 0.069]).
464
465 Over the 10-day exposure period the average consumption of glyphosate per worker was 38.7±
466 5.4μg in the Glyphosate only treatment, and 41.4± 4.3μg in the Glyphosate + *C. bombi*
467 treatment. The majority of this consumption was in the initial few days, as the concentration
468 decreased markedly over time. Figure 8 shows the sharp decline in glyphosate consumption
469 over time.

470 ### Parasite intensity

471 Glyphosate + *C. bombi* did not have a significantly different parasite intensity to the *C. bombi*
472 only treatment (Linear Mixed Effect model: parameter estimate (PE) = 1649.0, 95% CI [-3251.24
473 to 6529.72]). Glyphosate + *C. bombi* treated bees (n = 42) had an average parasite intensity of
474 20,562± 7065 cells per μL compared to 18,759± 9403 cells per μL for the *C. bombi* only
475 treatment (n = 44) (see Figure 9).
476

477   Mortality

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

Discussion

483 Through a series of experiments, we show no robust evidence for the effects of either
484 glyphosate, *C. bombi*, or their combination, on mortality or a range of sublethal effects (sucrose
485 consumption, parasite intensity and reproduction) in bumble bees. Acute exposure to either
486 stressor or their combination over a range of timescales representing the majority of a bee's
487 lifespan did not cause mortality, nor did chronic exposure over a 10-day period. While an initial
488 experiment found an acute dose of 200µg of glyphosate caused a considerable increase in the
489 intensity of the parasite *C. bombi*, this effect was not seen in any of the follow up experiments.
490 We found no evidence to suggest glyphosate affects reproduction among workers, and,
491 contrary to predictions from previous studies (Shykoff & Schmid-Hempel 1991, Brown, Loosli
492 and Schmid-Hempel 2000), no evidence that *C. bombi* does either.

Mortality

494 The most basic metric of bee health is mortality. A dead bee can contribute nothing further to
495 its fitness, as it is unable to contribute to the provisioning of brood or production of sexuals.
496 Most regulatory systems use mortality as the initial metric to assess toxicity (EFSA 2012, EFSA
497 2013, EPA, 2014). In the EU, lower tier testing considers just acute contact and oral toxicity in
498 honey bees (EFSA 2012, EFSA 2013), and bumble bees (including OECD 247 studies), although
499 the addition of bumble bee data has not yet been fully implemented (EFSA 2015). In the case of
500 glyphosate, the LD_{50}s derived were found to be above the threshold value of 200µg active
501 ingredient per bee (or equivalent highest possible tested dose)(EFSA, 2015), although this was
502 only done with honey bees, as bumble bee data are not due to be submitted until the 2025 EU
503 renewal of glyphosate. As such, glyphosate was not entered into higher tier testing, meaning
504 that from a regulatory testing standpoint only short-term mortality was considered (EFSA,
505 2015). This was used to justify the current lack of any mitigation measures for exposure of bees
506 to glyphosate or glyphosate-based herbicides.
507
508 The data presented here supports the regulatory conclusion that glyphosate does not cause
509 mortality in the short term (EFSA, 2015). These data also expand the species upon which we
510 have evidence of the mortality effects of glyphosate, with the addition of a bumble bee to the
511 previously studied honey bee. Our results show no mortality over a range of exposures and
512 time periods from 2-20 days, going well beyond the two-day test regulators will conduct on
513 bumble bees using OECD 247. Additionally, there were no mortality effects from the interaction
514 between glyphosate, with either acute or chronic exposure, and *C. bombi* in worker bumble
515 bees. It is important to clarify that our experiments used glyphosate as an active ingredient, not
516 as a formulation.
517

518   Several experiments have tested glyphosate-based herbicide formulations, as opposed to the
519   active ingredient glyphosate, on honey bees (Abraham et al. 2018, Faita et al. 2020, Odemer et
520   al. 2020 and Motta et al. 2020) and non-*Apis* bees (Ruiz-Toledo and Sánchez-Guillén, 2014,
521   Abraham et al. 2018, Seide et al. 2018, Straw, Carpentier and Brown, 2021). However, co-
522   formulants in glyphosate-based herbicides can have significant effects on toxicity (Motta et al.
523   2020, Straw, Carpentier and Brown, 2021), making these studies difficult to interpret from the
524   perspective of the active ingredient. Consequently, the following discussion of existing
525   academic literature will be limited to experiments that solely test the active ingredient
526   glyphosate.
527
528   In line with our results, the academic literature has largely found no evidence for effects of
529   glyphosate on adult honey bee worker survival. Over a range of concentrations up to 210mg/kg,
530   and across a range of timelines, no significant mortality has been observed in multiple studies
531   (Herbert et al. 2014, Goñalons and Farina 2018, Motta, Raymann and Moran 2019, Blot et al.
532   2019). Yet, despite these results, Almasri et al. (2020) found that just 0.00083mg/kg, a
533   concentration approximately 2 million times lower than 210mg/kg, significantly reduced
534   survival over 20 days. It is not clear from Almasri et al.'s (2020) methods if the solvent dimethyl
535   sulfoxide was present in the control treatment, which could potentially have confounded the
536   results. Interestingly, Almasri et al. (2021) failed to replicate this result using the same
537   concentration. Further, Motta and Moran, (2020) found that concentrations as low as
538   9.625mg/kg caused significant mortality over 20-40 days. However, neither Almasri et al. (2020)
539   or Motta and Moran, (2020) report screening their honey bees for parasites prior to the trial.
540   While a recent meta-analysis of the mortality effects of glyphosate on bees suggested a
541   significant effect of glyphosate on mortality (Battisti et al. 2021), the methods used heavily
542   predisposed the results to confirm the mortality hypothesis (Straw 2021), In addition, errors in
543   the data extraction process and analysis mean that the conclusions drawn in this meta-analysis
544   lack support (Straw 2021).
545
546   Interestingly, work on honey bees has not been limited to adult workers, honey bee larvae have
547   also been tested. In honey bees, evidence for mortality in larvae is heavily mixed. Tomé et al.
548   (2020) found that six days of exposure to 0.054mg/kg, but not 0.0008mg/kg, caused significant
549   mortality at 18 days after treatment started, although the authors note that the 16% mortality
550   is 'considered incidental because [their methodology] accepts up to 30% control mortality'.
551   Vazquez et al. (2018) had very mixed results over five days exposure, with their highest
552   treatment group 5mg/kg causing significant mortality in one colony, but no change in four
553   colonies, and significantly reduced mortality in one colony. Dai et al. (2018) found that over 21
554   days exposure to 4mg/kg or 20mg/kg caused significant mortality, but that 0.8mg/kg did not.
555

556 These mixed results, for both adults and larvae, heavily indicate strong colony effects, or that
557 some bees were infected with a parasite, like *Serratia marcescens*, which synergises with
558 glyphosate to cause mortality (Motta, Raymann and Moran, 2018, Motta et al. 2020). Notably,
559 none of these studies, in adults or larvae, explicitly reported screening their bees for signs of
560 disease. Thompson et al. (2014) used verifiably healthy bees making it the most robust study to
561 date. They found that over 15 days of exposure neither 75mg/L, 150mg/L or 301 mg/L caused
562 any larval mortality. This highlights the importance of screening bees for diseases prior to
563 experiments, as well as the need for more work to understand the effects of pesticides on
564 parasite exposed bees. Odemer et al. (2020) also found no evidence of mortality in a range of
565 experiments (adults and larvae) using parasite-free honey bees, but, as noted above, these
566 results are not directly comparable because of the use of a glyphosate-based formulation
567 (although glyphosate co-formulants are linked to increased, not reduced, toxicity (Mesnage,
568 Bernay and Séralini 2012, Nagy et al. 2021)).
569
570 The larval mortality literature relates to the experiments presented here because the larvae in
571 the chronic exposure experiment will also have been fed glyphosate by the nurse workers.
572 However, in our experimental paradigm the peak exposure for micro-colonies would have
573 occurred while new offspring were still in the egg stage. Our results did not explicitly consider
574 larval mortality, but no effect was seen on larval number or weight (consistent with Thompson
575 et al. (2014)), which indicates that if any mortality occurred it was below the level required to
576 reduce reproductive success. Further experiments, where peak exposure occurs at the larval
577 feeding stage, are required to understand whether results from honey bee larvae extrapolate
578 to bumble bee larvae. Larvae were not the primary subjects of this study, adult workers were,
579 and as such the evidence collected on their mortality is more substantial.
580
581 In the short term (two days) and long term (20 days) after exposure to a relatively high acute
582 dose of glyphosate, no mortality was seen in individually housed bees in three separate
583 experiments (Modified Ecotoxicological Protocol OECD 247: Small Scale, Full Scale and Long-
584 Term Mortality). As 20 days is representative of a considerable proportion of a bumble bee
585 worker's lifespan (Brian, 1952, Rodd, Plowright and Owen, 1980, Goldblatt and Fell, 1987), this
586 indicates that there is no delayed mortality response and no meaningful shortening of
587 longevity. All the academic studies cited above have used chronic exposure to glyphosate, not
588 acute exposure. As such, there is presently no non-regulatory data on acute exposure to
589 glyphosate in any bee species, nor any data on glyphosate exposure in bumble bees, so our
590 results represent a substantive contribution to the understanding of glyphosate's effects on bee
591 mortality.
592

593 In the microcolony experiments no significant mortality was seen with either adult workers
594 acutely exposed, or age controlled young adult workers with chronic exposure. This
595 demonstrates that even while the bees are housed collectively under more natural conditions,
596 and exerting themselves rearing young, any potential stress was insufficient to cause mortality.
597 The finding of no mortality with a fully field realistic chronic exposure regime in parasite free
598 bumble bees supports the evidence that chronic glyphosate exposure is non-lethal to healthy
599 worker bees (Herbert et al. 2014, Goñalons and Farina 2018, Motta, Raymann and Moran 2019,
600 Blot et al. 2019). The lack of increased mortality alongside *C. bombi* infection also aids our
601 understanding of which parasites can synergise with glyphosate to cause mortality in bees.
602 Mortality, however, is not the only metric of bee health, and other sublethal metrics like
603 parasite intensity are important to consider for a more complete picture of bee health.

604 ## Parasite intensity.

605 The initial experiment found a 109% increase in *C. bombi* intensity. As a preliminary experiment
606 the methods were less robust than later experiments, with a smaller sample size and no
607 tracking of colony of origin or body weight through the experiment. However, the balanced
608 experimental design accounts for this variation and as such it is unlikely to be confounded.
609 Further the sample size of *C. bombi* n = 21 and Glyphosate + *C. bombi* n = 23 is appropriately
610 powered (Logan, Ruis-González and Brown, 2005).
611
612 The follow up experiment to this, found a 16% increase in *C. bombi* intensity, although this
613 effect was not statistically significant. In this trial the sample size was larger, and the co-variates
614 of colony of origin and body weight were tracked throughout.
615
616 These opposing results can be explained in several ways. Principally either of the two
617 experiment could have delivered a false positive or a false negative result, which is the simplest
618 solution, and there is no evidence to confirm or contradict this. Alternatively, it is possible that
619 some of the other variables in the experiment such as the parasite, the colonies used, or other
620 unknown effects are acting individually or in combination to alter the parasite intensity.
621
622 As with all bumble bee toxicity testing the colonies used differed between experiments.
623 Because of this, there could be a parasite by host genotype interaction (Baer and Schmid-
624 Hempel, 2003), or a parasite by host microbiome interaction (Koch and Schmid-Hempel, 2011,
625 Mockler et al. 2018) as has been observed in experiments previously. However, we believe that
626 this is unlikely as in each experiment three or more colonies were used to account for inter-
627 colony variation, these were evenly distributed to treatment groups, and colonies were sourced
628 from the same supplier.

629

630 More interestingly, the two experiments differed heavily in the average parasite intensity in the
631 *C. bombi* only treatments. While the first experiment had a parasite intensity of 6,946± 5,682
632 cells per µL (SD), the follow up experiment had a mean parasite intensity of 20,756± 14,473
633 cells per µL, which is considerably higher. It is possible that this increase in parasite intensity
634 reached a plateau, meaning any increase in parasite intensity caused by glyphosate could no
635 longer occur, as there was no further scope for intensity to rise. To assess the evidence for this
636 hypothesis we can look to prior *C. bombi* literature.

637

638 The majority of the literature on *C. bombi* in *B. terrestris* uses faecal counts, which are not
639 directly comparable to homogenised gut counts. Further, there are currently no comparable
640 data on peak parasite intensity using homogenised gut counts. As such it is not possible to
641 know if the levels seen in our second experiment do represent a plateau. However, our
642 methods were based on unpublished work (Siviter, Matthews and Brown, In Preparation), that
643 found a mean parasite intensity of 1,849± 1,966 cells per µL, comparable to levels in our first
644 experiment, but more than ten times lower than intensity levels in our second experiment. This
645 is indicative that a plateau may have been reached. Similarly, in our microcolony experiments,
646 which took place in between the two experiments on individual bumblebees, a high parasite
647 intensity (Acute: 18,635± 5,884 cells per µL and Chronic: 18,759± 9403 cells per µL) was
648 recorded.

649

650 Faecal parasite counts from Logan, Ruiz-González and Brown, (2005) found parasite intensity to
651 rise to a peak at around 13 days post inoculation. So, at 9 days post-exposure the parasite
652 intensity should not have plateaued. However, in all experiments after our first small scale
653 experiment, the parasite intensities at either 9 days or 21 days were in the 20,000 cells per µL
654 range. This again supports the plateau hypothesis because there is a consistent and high
655 parasite intensity across a range of experiments and conditions.

656

657 If the *C. bombi* intensity had reached a plateau, that such high parasite intensities do not cause
658 any measurable impacts on the other metrics recorded under the conditions tested here does
659 indicate that even if glyphosate does increase parasite intensity, this is not likely to lead to any
660 reduction in fitness. As such any effect that might exist is unlikely to be environmentally
661 relevant or robust.

662

663 A final explanation for these conflicting results may come from the parasite source used. The *C.
664 bombi* used in the experiments was from the same original source, wild caught infected *B.
665 terrestris* spring queens. Faeces collected from infected queens were used to infect a
666 commercial colony which were kept as a parasite source. As each commercial colony neared

667   the end of its lifespan, faeces was collected from workers in it and used to infect a new
668   commercial colony. Theoretically, within a year the serial passage of the parasite could lead to
669   selection for higher infection levels, and if this were the case it could explain our experimental
670   results. However, previous work with *C. bombi* suggests that the opposite occurs, with serial
671   passage within a colony reducing infectivity to non-colony members (Yourth and Schmid-
672   Hempel, 2006), which would result in lower prevalence and intensity of infections in our
673   experimental paradigm, a pattern we did not see. Consequently, it seems unlikely that an
674   increase in transmissibility or growth in *C. bombi* across the course of experiments can explain
675   our results. While parasite intensity is an important factor in bee health, reproductive success is
676   much more important to a bee's fitness.

677   ## Reproduction

678   Reproductive success is the ultimate metric of bee health, directly representing bee fitness.
679   Drone production by unmated workers in a microcolony set up is designed to function as a
680   proxy of this, and itself does not directly represent a field realistic measure of whole colony
681   sexual production. There is even some evidence that microcolonies can give contradictory
682   results to queenright laboratory or full field experiments (Oystaeyen et al. 2020). As such our
683   results should be interpreted with caution, and are not a field realistic measure of reproductive
684   success.

685

686   No significant effect on reproduction was found in any experiment, despite at times large
687   differences between treatments (up to a 33.5% difference in reproductive success versus the
688   control), which is potentially indicative of power limitation. Indeed, it is possible that both
689   microcolony experiments were power limited, with ~10 microcolonies per treatment (a total of
690   38 and 36 microcolonies in each experiment). This is less than other microcolony experiments
691   like Oystaeyen et al. (2020) which used 20 per treatment, and Siviter et al. (2019) which used 30
692   per treatment. The power limitation hypothesis is supported by the lack of a significant effect
693   of *C. bombi* on reproductive success in both experiments, which contrasts with a range of
694   published literature (Yourth, Brown and Schmid-Hempel 2008, Shykoff and Schmid-Hempel,
695   1991, Brown, Schmid-Hempel, and Schmid-Hempel 2003). Interestingly, while not significant, *C.*
696   *bombi* reduced reproductive success by 10.2% and 50.0% in the Acute and Chronic experiments
697   respectively. This is a similar scale of reduction to previously published data (Brown, Schmid-
698   Hempel, and Schmid-Hempel 2003). The data presented here also indicate that acute exposure
699   to glyphosate is more likely to impact reproductive success than chronic exposure, with a 20.6%
700   decline in reproductive success after acute exposure, versus a 34.9% increase after chronic
701   exposure. Overall, we would suggest that this evidence be used to guide future studies,

702 conducted ideally in field conditions with larger sample sizes to provide more high quality and
703 definitive evidence for any potential effects.
704
705 There was a considerably lower reproductive output overall in the Chronic experiment than in
706 the Acute exposure experiment. This is likely because the workers in the Chronic exposure
707 experiment were age controlled, and thus likely to be much younger on average. This could
708 have led to a delay in ovary development retarding reproductive output. In the Chronic
709 exposure experiment, sucrose consumption was also tracked to allow for the total glyphosate
710 exposure to be measured.

711 ## Sucrose

712 Sucrose consumption can be an indicator of bee health (Straw and Brown, In Review). While in
713 isolation this metric has no clear relation to fitness, the ultimate measure of bee health, it can
714 be useful in indicating that a bee is acting abnormally. In the case of exposure to the co-
715 formulant alcohol ethoxylates, reduced sucrose consumption went hand in hand with weight
716 loss and gut melanisation (Straw and Brown, In Review). Further, sucrose consumption could be
717 a corollary of pollination services, as bees with lower appetites might forage less, although in
718 social bees nectar foraging is a response to both individual and colony-level nectar needs
719 (Hendriksma, Toth and Shafir, 2019). Under chronic exposure, no treatment affected sucrose
720 consumption, indicating that glyphosate did not significantly affect the bees dietary
721 consumption.
722
723 Under microcolony conditions worker bees consumed an average of 38.7 or 41.4μg of
724 glyphosate (Glyphosate and Glyphosate + *C. bombi* treatments respectively) under a field
725 realistic, degrading concentration exposure regime. This can be used to inform future research
726 as to the cumulative exposure bees would experience in the wild. The majority of this
727 glyphosate was consumed within the first few days of exposure, with the rapidly declining
728 residues causing the consumption from day five onwards to contribute little to overall
729 exposure. Consequently, future studies could truncate the glyphosate exposure to five days
730 with little reduction in exposure. However, it is also worth noting that there is no limit on the
731 number of sprays of a glyphosate-based herbicide per year, or a mandated time gap between
732 them (Roundup ProActive Label), so repeat exposure could occur. As such, the 38.7 or 41.4μg
733 dose does not necessarily represent the total dose a bee could be exposed to over their
734 lifetime.
735
736 The stepwise degradation method of exposure, as developed for bees in Linguadoca et al.
737 (2021), is the most field realistic existing method of simulating real pesticide exposure in a

738 laboratory setting. By mimicking the degradation of the substance the exposure profile is
739 accurately portrayed, whereas a flat exposure, even using a time-weighted average dose, would
740 lack the nuance of the initial peak followed by a lengthy tail. As such, the lack of mortality
741 resulting from this chronic exposure can be seen as a very rigorous result, representing the best
742 approximation of the effects of a field realistic exposure possible.
743
744 The research presented here principally used acute oral exposure to 200μg of glyphosate as an
745 active ingredient. None of the research into the effects of glyphosate on the honey bee
746 microbiome has used acute exposure, instead using chronic exposure at a range of
747 concentrations from 0.8mg/kg (Dai et al. 2018) to 210mg/kg (Blot et al. 2019). It is possible that
748 sustained exposure to glyphosate is more impactful than a single more concentrated instance
749 of exposure because the gut microbial community is not afforded opportunity to recover.
750 Alternatively, exposure to the considerably higher acute concentration may also have a more
751 severe impact, potentially acting to cull sensitive species and strains. Given that bees are
752 exposed to both acute and chronic exposure to glyphosate in the wild, if future research
753 considered acute exposure our understanding of how glyphosate affects bee health would be
754 more complete.
755
756 How the acute exposure to 200μg of glyphosate used in this study relates to in-field exposure is
757 unknown. There is no data, even from honey bees, to be able to accurately predict acute
758 exposure to herbicides that lack any mitigation measures. Given that flowering weeds can be
759 sprayed while bees are foraging on them, and glyphosate is typically sprayed in very
760 concentrated sprays (compared with insecticides), for a bee to consume 200μg in a short period
761 of time immediately after a spray application is not implausible, although lower doses are more
762 likely. More work on acute exposure of bees to agrochemicals without bee specific mitigation
763 measures is needed to inform future research. However, with no effects on a range of metrics
764 seen at this potentially high-end dose, it is likely that more field realistic acute exposures would
765 also not have an effect on bumble bees.

766 **Conclusions**

767 As the world's most used pesticide (Duke and Powles, 2008, Benbrook, 2016), the application of
768 glyphosate is a hotly debated topic, largely due to its human carcinogenicity (Alcántara-de la
769 Cruz et al. 2021), but increasingly regarding its potential toxicity to bees (Cullen et al. 2019).
770 Given its wide usage, the implications for changing its regulatory status would substantively
771 reshape conventional farming practices (Beckie, Flower and Ashworth, 2020), and thus need to
772 be made using robust and environmentally sound science. As such it is imperative that evidence
773 for or against its impacts on bees is of the highest of standards.

774

775 With that in mind, the findings presented here provide robust evidence that oral exposure to
776 the active ingredient glyphosate does not induce mortality in the bumble bee *B. terrestris*. We
777 report mixed evidence for the effect of glyphosate on *C. bombi* parasite intensity, with
778 insufficient evidence to describe the effect as environmentally robust. While future research
779 could elucidate the impacts of glyphosate on *C. bombi* intensity, as we found no effects in any
780 metric of their combination, research efforts are best focussed on other pesticide-parasite
781 combinations. Further we report no effects of glyphosate, *C. bombi* or their combination on
782 worker reproductive output, but this conclusion is potentially limited by the power of the study.
783 Our results thus do not indicate any requirement to change the regulatory status of the active
784 ingredient glyphosate as it pertains to bumble bees. As glyphosate has been found to impact
785 honey bees as measured by a range of sublethal metrics (Boily et al. 2013, Herbert et al. 2014,
786 Balbuena et al. 2015, Helmer et al. 2015, Vazquez et al. 2018), further research using wild bee
787 species and sublethal metrics would help resolve whether this widely used chemical is safe for
788 bees.

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References

Abraham, J., Benhotons, G.S., Krampah, I., Tagba, J., Amissah, C., and Abraham, J.D. (2018). Commercially formulated glyphosate can kill non-target pollinator bees under laboratory conditions. Entomologia Experimentalis et Applicata, *166*, 695-702.

Adler, L.S., Michaud, K.M., Ellner, S.P., McArt, S.H., Stevenson, P.C. and Irwin, R.E. 2018. Disease where you dine: plant species and floral traits associated with pathogen transmission in bumblebees. Ecology, *99*, 2535–2545.

Alcántara-de la Cruz, R., Cruz-Hipolito, H.E. Alfredo Domínguez-Valenzuela, J., and De Prado, R. (2021). Glyphosate ban in Mexico: potential impacts on agriculture and weed management. Pest Management Science, *77*, 3820-3831.

Almasri, H., Tavares, D.A., Diogon, M., Pioz, M., Alamil, M., Sené, D., Tchamitchian, S., Cousin, M., Brunet, J-C., and Belzunces, L.P. (2021). Physiological effects of the interaction between Nosema ceranae and sequential and overlapping exposure to glyphosate and difenoconazole in the honey bee *Apis mellifera*. Ecotoxicology and Environmental Safety, *217*, 112258.

Almasri, H., Tavares, D.A., Pioz, M., Sené, D., Tchamitchian, S., Cousin, M., Brunet, J.L., and Belzunces, L.P. (2020). Mixtures of an insecticide, a fungicide and a herbicide induce high toxicities and systemic physiological disturbances in winter *Apis mellifera* honey bees. Ecotoxicology and Environmental Safety. *203*, 111013.

Baer, B., and Schmid-Hempel, P. (2003). Bumblebee workers from different sire groups vary in susceptibility to parasite infection. Ecology Letters*, 6*, 106–10.

Balbuena, M.S., Tison, L., Hahn, M.L., Greggers, U., Menzel, R., and Farina, W.M. (2015). Effects of sublethal doses of glyphosate on honeybee navigation. Journal of Experimental Biology, *218*, 2799–2805.

Baron, G.L., Raine, N.E., and Brown, M.J.F. (2014). Impact of chronic exposure to a pyrethroid pesticide on bumblebees and interactions with a trypanosome parasite. Journal of Applied Ecology, *51,* 460–69.

Bartoń, K. MuMIn: multi-model inference. R package version 1.0.0. https://CRAN.R-project.org/package=MuMIn (2020).

Bates, D., Mächler, M., Bolker, B., and Walker, S. (2015) Fitting linear mixed-effects models using lme4. Journal of Statistical Software, *67*, 1–48.

Battisti, L., Potrich, M., Sampaio, A.R., de Castilhos Ghisi, N., Costa-Maia, M., Abati, R., dos Reis Martinez, C.B., and Sofia, S.H., (2021). Is glyphosate toxic to bees? A meta- analytical review. Science of the Total Environment, *767*, 145397.

Beckie. H.J., Flower, K.C., and Ashworth, M.B. (2020). Farming without glyphosate? Plants, *9*, 1-15.

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

829   Blacquière, T., Smagghe, G., van Gestel, C.A.M., and Mommaerts, V. (2012). Neonicotinoids in bees: a
830   review on concentrations, side-effects and risk assessment. Ecotoxicology, *21*, 973–992.
831   Blot, N., Veillat, L., Rouzé, R., and Delatte, H. (2019). Glyphosate, but not its metabolite AMPA, alters
832   the honeybee gut microbiota. PLoS One, *14*, e0215466.
833   Boily, M., Sarrasin, B., DeBlois, C., Aras, P., and Chagnon, M. (2013). Acetylcholinesterase in
834   honey bees (*Apis mellifera*) exposed to neonicotinoids, atrazine and glyphosate: lab-
835   oratory and field experiments. Environmental Science and Pollution Research, *20*, 5603–
836   5614.
837   Brian, A. D. (1952). Division of labour and foraging in *Bombus agrorum* Fabricius. Journal of
838   Animal Ecology, *21*, 223–240.
839   Brown, M.J.F., Loosli, R. and Schmid-Hempel, P. (2000). Condition-dependant expression of virulence in
840   a trypanosome infecting bumblebees. Oikos*, 91*, 421–27.
841   Brown, M.J.F., Schmid-Hempel, R., and Schmid-Hempel, P. (2003). Strong context-dependent virulence
842   in a host-parasite system: reconciling genetic evidence with theory. Journal of Animal Ecology, *72*,
843   994–1002.
844   Cisarovsky, G., and Schmid-Hempel, P. (2014). Combining laboratory and field approaches to
845   investigate the importance of flower nectar in the horizontal transmission of a bumblebee
846   parasite. Entomologia Experimentalis et Applicata, *152,* 209–15.
847   Cullen, M.G., Thompson, L.J., Carolan, L.C., Stout, J.C. and Stanley, D.A. (2019). Fungicides, herbicides
848   and bees: A systematic review of existing research and methods. PLoS ONE, *14*, e0225743.
849   Dai, P., Yan, Z., Ma, S., Yang, Y., Wang, Q., Hou, C., Wu, Y., Liu, Y., and Diao, Q. (2018). The herbicide
850   glyphosate negatively affects midgut bacterial communities and survival of honey bee during
851   larvae reared *in vitro*. Journal of Agricultural and Food Chemistry, *66,* 7786–93.
852   Duke, S.O., and Powles, S.B. 2008. Glyphosate: A Once-in-a-Century Herbicide. Pest Management
853   Science, *64*, 319–25.
854   Duke, S.O. (2018). The history and current status of glyphosate. Pest Management Science, *74*, 1027-
855   1034.
856   Durrer, S., and Schmid-Hempel, P. (1994). Shared use of flowers leads to horizontal pathogen
857   transmission. Proceedings of the Royal Society of London B: Biological Sciences, *258*, 299-
858   302.
859   EFSA. (2012). Scientific Opinion on the science behind the development of a risk assessment of Plant
860   Protection Products on bees (*Apis mellifera*, *Bombus* spp. and solitary bees). EFSA Journal, *10*,
861   2668.
862   EFSA. (2013). EFSA Guidance document on the risk assessment of plant protection products on bees
863   (*Apis mellifera*, *Bombus spp*. and solitary bees). EFSA Journal*, 11*, 3295.
864   EFSA. (2015). Conclusion on the peer review of the pesticide risk assessment of the active
865   substance glyphosate. EFSA Journal*, 13*, 4302.

866 EPA. (2014). Guidance for assessing pesticide risks to bees. Office of Pesticide Programs,
867   Washington DC.
868 Faita, M.R., Cardozo, M.M., Amandio, D.T.T., Orth, A.I., and Nodari, R.O., 2020. Glyphosate-
869   based herbicides and Nosema sp. microsporidia reduce honey bee (*Apis mellifera* L.)
870   survivability under laboratory conditions. Journal of Apicultural Research, *59*, 332–342.
871 Farina, W.M., Balbuena, M.S., Herbert, L.T., Megoni Goñalons, C., and Vázquez, D.E. (2019).
872   Effects of the herbicide glyphosate on honey bee sensory and cognitive abilities: individual
873   impairments with implications for the hive. Insects, 354, 1–16.
874 Fauser-Misslin, A., Sadd, B.M., Neumann, P., and Sandrock, C. (2014). Influence of combined pesticide
875   and parasite exposure on bumblebee colony traits in the laboratory. Journal of Applied Ecology,
876   51, 450–59.
877 Figueroa, L.L., Blinder, M., Grincavitch, C., Jelinek, A., Mann, E.K., Merva, L.A. Metz, L.E., Zhao,
878   A.Y., Irwin, R.E., McArt, S.H., and Adler, L.S. (2019). Bee pathogen transmission dynamics:
879   deposition, persistence and acquisition on flowers. Proceedings of the Royal Society B.,
880   *286*, 20190603.
881 Folly, A.J., Koch, H., Stevenson, P.C., and Brown, M.J.F. (2017). Larvae act as a transient transmission
882   hub for the prevalent bumblebee parasite *Crithidia bombi*. Journal of Invertebrate Pathology, *148*,
883   81–85.
884 Gillespie, S. 2010. Factors affecting parasite prevalence among wild bumblebees. Ecological
885   Entomology, *35*, 737–47.
886 Goldblatt J.W., and Fell, R.D. (1987). Adult longevity of workers of the bumble bees *Bombus
887   fervidus* (F.) and *Bombus pennsylvanicus* (De Geer) (Hymenoptera: Apidae). Canadian
888   Journal of Zoology, *65*, 2349-2353.
889 Gonalons, C.M., and Farina, W.M. (2018). Impaired associative learning after chronic exposure
890   to pesticides in young adult honey bees. Journal of Experimental Biology, *221*, 176644.
891 Graystock, P., Yates, K., Evison, S.E.F., Darvill, B., Goulson, D., and Hughes, W.O (2013). The
892   Trojan hives: pollinator pathogens, imported and distributed in bumblebee colonies.
893   Journal of Applied Ecology, *50*, 1207-1215.
894 Helmer, S.H., Kerbaol, A., Aras, P., Jumarie, C., and Boily, M., (2015). Effects of realistic doses of
895   atrazine, metolachlor, and glyphosate on lipid peroxidation and diet-derived antiox- idants
896   in caged honey bees (*Apis mellifera*). Environmental Science and Pollution Research, *22*,
897   8010–8021.
898 Hendriksma, H.P., Toth, A.L., and Shafir, S. (2019). Individual and colony level foraging decisions of
899   bumble bees and honey bees in relation to balancing of nutrient needs. Frontiers in Ecology and
900   Evolution, *7*, 177.
901 Herbert, L.T., Vázquez, D.E., Arenas, A., and Farina, W.M. (2014). Effects offield-realistic doses
902   of glyphosate on honeybee appetitive behaviour. Journal of Experimental Biology, *217*,
903   3457–3464.

904   Hicks, B. J., Pilgrim, B.L., Perry, E., and Marshall, H.D. (2018). Observations of native bumble bees inside
905   of commercial colonies of *Bombus impatiens* (Hymenoptera: Apidae) and the potential for
906   pathogen spillover. Canadian Entomologist, *150,* 520–31.
907   Jones, C.M. and Brown, M.J.F. (2014). Parasites and genetic diversity in an invasive bumble bee. Journal
908   of Animal Ecology, *83*, 1428-1440.
909   Kassambara, A., Kosinski, M. and Biecek, P. survminer: drawing survival curves using 'ggplot2'. R
910   package version 0.4.6. https://CRAN.R-project.org/package=survminer (2019).
911   Koch, H., and Schmid-Hempel, P. (2011). Socially transmitted gut microbiota protect bumble bees
912   against an intestinal parasite. Proceedings of the National Academy of Sciences, *108*, 19288–92.
913   Koch, H., Woodward, J., Langat, M.K., Brown, M.J.F., and Stevenson, P.C. (2019) Flagellum
914   removal by a nectar metabolite inhibits infectivity of a bumblebee parasite. Current
915   Biology, *29*, 3494-3500.
916   Korner, P., and Schmid-Hempel, P. (2005). Correlates of parasite load in bumblebees in an
917   Alpine habitat. Entomological Science, *8*, 151–160.
918   Linguadoca, A., Rizzi C., Villa, S., and Brown, M.J.F. (2021). Sulfoxaflor and nutritional deficiency
919   synergistically reduce survival and fecundity in bumblebees. Science of the total environment,
920   *795*, 148680.
921   Logan, A., Ruiz-González and Brown, M.J.F. (2005). The impact of host starvation on parasite
922   development and population dynamics in an intestinal trypanosome parasite of bumble
923   bees. Parasitology, *130*, 637-642.
924   Mayer, D. Dimitriadou, E., Hornik, K., Weingessel, A., Leisch, F., Chang, C.-C., and Lin, C.-C.
925   (2021). Misc Functions of the Department of Statistics, Probability Theory Group (Formerly:
926   E1071), TU Wien. R package version 1.7-4. https://cran.r-
927   project.org/web/packages/e1071/index.html
928   Mercurio, P., Flores, F., Mueller, J.F., Carter, S., and Negri, A.P. (2014). Glyphosate persistence
929   in seawater. Marine Pollution Bulletin, *85*, 385-390.
930   Mesnage, R., Bernay, B., and Séralini, G-E. (2013). Ethoxylated adjuvants of glyphosate-based
931   herbicides are active principles of human cell toxicity. Toxicology, *313*, 122-128.
932   Mockler, B.K., Kwong, W.K., Moran, N.A., and Koch, H. (2018). Microbiome structure influences
933   infection by the parasite *Crithidia bombi* in bumble bees. Applied and Environmental
934   Microbiology, *83,* e02335-17.
935   Motta, E.V.S., and Moran, N.A. (2020). Impact of glyphosate on the honey bee gut microbiota: effects
936   of intensity, duration, and timing of exposure. mSystems, *5*, e00268-20.
937   Motta, E.V.S., Mak, M., De Jong, T.K., Powell, J.E., O'Donnell, A., Suhr, K.J., Riddington, I.M., and Moran,
938   N.A. (2020). Oral or topical exposure to glyphosate in herbicide formulation impacts the gut
939   microbiota and survival rates of honey bees. Applied Environmental Microbiology, *86*, se01150-
940   20.

941 Motta, E.V.S., Raymann, K., and Moran, N.A. (2018). Glyphosate perturbs the gut microbiota of honey
942   bees. Proceedings of the National Academy of Sciences, *115*, 10305–10310.
943 Nagy, K., Duca, R. C., Lovas, S., Creta, M., Scheepers, P. T. J., Godderis, L., and Ádám, B. (2020).
944   Systematic review of comparative studies assessing the toxicity of pesticide active ingredients and
945   their product formulations. Environmental Research, *181*, 108926.
946 Odemer, R., Alkassab, A.T., Bischoff, G., Frommberger, M., Wernecke, A., Wirtz, I.P., Pistorius,
947   J., and Odemer, F. (2020). Chronic high glyphosate exposure delays individual worker bee
948   (*Apis mellifera* l.) development under field conditions. Insects, *11*, 1-20.
949 OECD. (2017a). Honey Bee (*Apis mellifera* L.), Chronic oral toxicity test (10-day feeding). OECD
950   Guidelines for the Testing of Chemicals, *245*, 1–9
951 OECD. (2017b). Bumblebee, acute oral toxicity test. OECD Guidelines for the Testing of Chemicals, *247*,
952   1–12
953 Oystaeyen, A.V., Klatt, B.K., Petit, C., Lenaerts, N., and Wäckers, F. (2020). Short-term lab
954   assessments and microcolonies are insufficient for the risk assessment of insecticides for
955   bees. Chemosphere, *273*, 128518.
956 R Development Core Team. (2019). R: a language and environment for statistical computing. R
957   Foundation for Statistical Computing. https://www.R-project.org.
958 Rodd, F.H., Plowright, R.C., & Owen, R.E. (1980). Mortality rates of adult bumble bee workers
959   (Hymenoptera: Apidae). Canadian Journal of Zoology, 58, 1718-1721.
960 Rohatgi, A. (2020). WebPlotDigitizer. Version 4.4. https://automeris.io/WebPlotDigitizer
961 Roundup® ProActive Label. (2019). Retrieved from https://www.green-
962   tech.co.uk/media/shared/chemical-
963   labels/Roundup%20Pro%20Active%20360%20Product%20Label-min.pdf
964 Ruiz-Toledo, J., and Sánchez-Guillén, D. (2014). Effect of the concentration of glyphosate
965   present in bodywaters near transgenic soybean fields on the honeybee *Apis mellifera*,and
966   the stingless bee *Tetragonisca angustula*. Acta zoológica Mexico, *30*, 408–413.
967 Rutrecht, S.T., and Brown, M.J.F. (2008). The life-history impact and implications of multiple parasites
968   for bumble bee queens. International Journal for Parasitology, *38*, 799–808.
969 Schmid-Hempel, P., Puhr, K., Kruger, N., Reber, C., and Schmid-Hempel, R. (1999). Dynamic and genetic
970   consequences of variation in horizontal transmission for a microparasitic infection. Evolution, *53*,
971   426– 34.
972 Seide, V.E., Bernardes, R.C., Pereira, E.J.G., and Lima, M.A.P. (2018). Glyphosate is lethal and cry
973   toxins alter the development of the stingless bee *Melipona quadrifasciata*. Environmental
974   Pollution, *243*, 1854–1860.
975 Shykoff, J. A., and Schmid-Hempel, P. 1991. Incidence and effects of four parasites in natural
976   populations of bumble bees in Switzerland. Apidologie, *22,* 117–25.
977 Siviter, H., Horner, J., Brown, M.J.F., and Leadbeater, E. (2020) Sulfoxaflor exposure reduces egg
978   laying in bumblebees *Bombus terrestris*. Journal of Applied Ecology, *57*, 160–169.

979 Straub, L., Strobl, V., and Neumann, P. (2020). The need for an evolutionary approach to ecotoxicology.
980   Nature Ecology & Evolution, *4*, 895.
981 Straw, E.A., Carpentier, E.N., Brown, M.J.F. (2021). Roundup causes high levels of mortality following
982   contact exposure in bumble bees. Journal of Applied Ecology, *58*, 1167-1176.
983 Straw, E.A. (2021). Letter to the editors regarding Battisti et al (2021): Is glyphosate toxic to bees? A
984   meta-analytical review. Science of the Total Environment, *790*, 147556.
985 Therneau, T.M. A package for survival analysis in R. Version 3.1-11. https://CRAN.R-
986   project.org/package=survival (2020).
987 Thompson, H.M., Levine, S.L., Doering, J., Norman, S., Manson, P., Sutton, P., and von Mérey, G. (2014).
988   Evaluating exposure and potential effects on honeybee brood (*Apis mellifera*) development using
989   glyphosate as an example. Integrated Environmental Assessment and Management*, 10, 463–470.
990 Tomé, H.V.V., Schmehl, D.R., Wedde, A.E., Godoy, R.S.M., Ravaiano, S.V., Guedes, R.N.C.,
991   Martins, G.F., and Ellis, J.D. (2020). Frequently encountered pesticides can cause multiple
992   disorders in developing worker honey bees. Environmental Pollution, *256*, 113420.
993 Vázquez, D.E., Ilina, N., Pagano, E.A., Zavala, J.A., and Farina, W.M. (2018). Glyphosate affects the larval
994   development of honey bees depending on the susceptibility of colonies. PLoS One, *13*, 1-19.
995 Wickham, W. ggplot2: elegant graphics for data analysis. Springer-Verlag New York. https://CRAN.R-
996   project.org/package=ggplot2 (2016).
997 Yourth, C. P., and Schmid-Hempel, P. (2006). Serial passage of the parasite *Crithidia bombi* within a
998   colony of its host, *Bombus terrestris*, reduces success in unrelated hosts. *Proceedings of the Royal*
999   *Society B: Biological Sciences* 273 (1587): 655–59.
1000 Yourth, C.P., Brown, M.J.F., and Schmid-Hempel, P. (2008). Effects of natal and novel *Crithidia bombi*
1001   (Trypanosomatidae) infections on *Bombus terrestris* hosts. Insectes Sociaux, *55*, 86– 90.

Table 1. Showing the doses of parasite or pesticide given to each worker in a given treatment.

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   Table 1. Showing the doses of parasite or pesticide given to each worker in a given treatment.

Figure 1

Chronic exposure profile

Figure 1. Showing a stepwise chronic exposure profile for nectar generated from Thompson et al. (2014). With glyphosate concentration (in mg/kg) presented on the Y axis and time in days on the X axis.
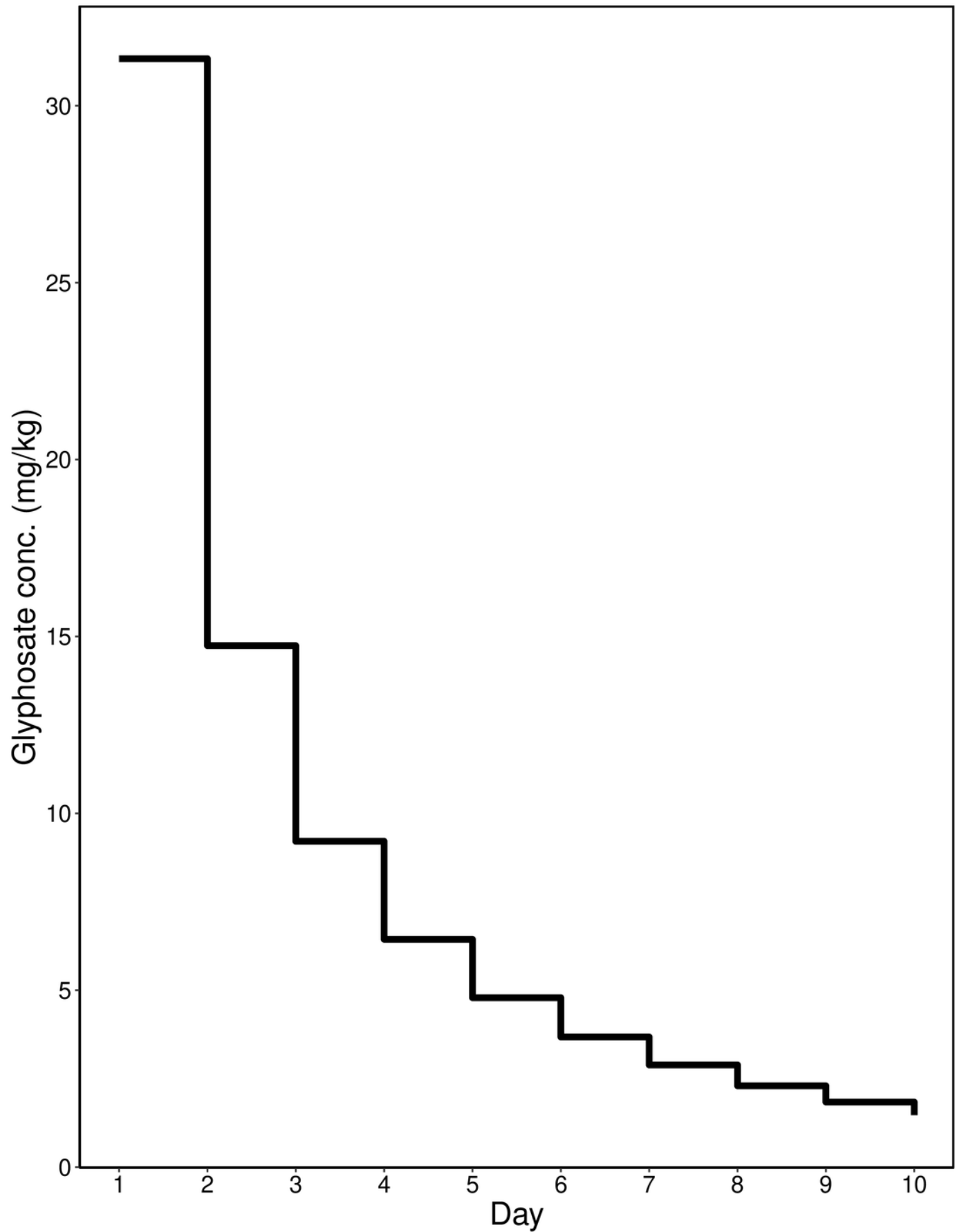
Figure 2

Modified Ecotoxicological Protocol OECD 247: Small Scale- Parasite Intensity

Figure 2. A boxplot with overlaid jittered data points showing the parasite intensity by treatment.
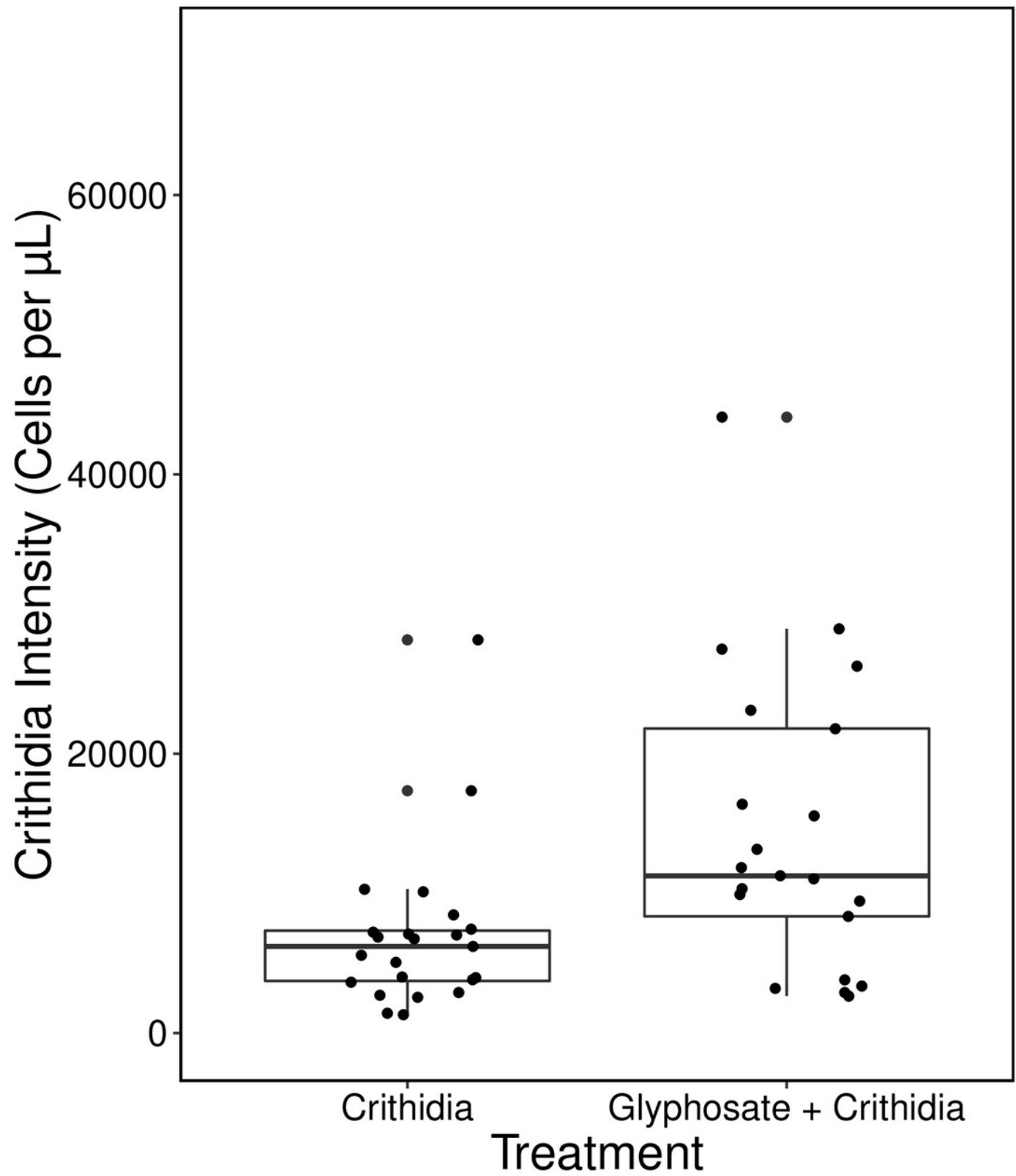
Figure 3

Modified Ecotoxicological Protocol OECD 247: Full Scale - Parasite Intensity

Figure 3. A boxplot with overlaid jittered data points showing the parasite intensity by treatment.
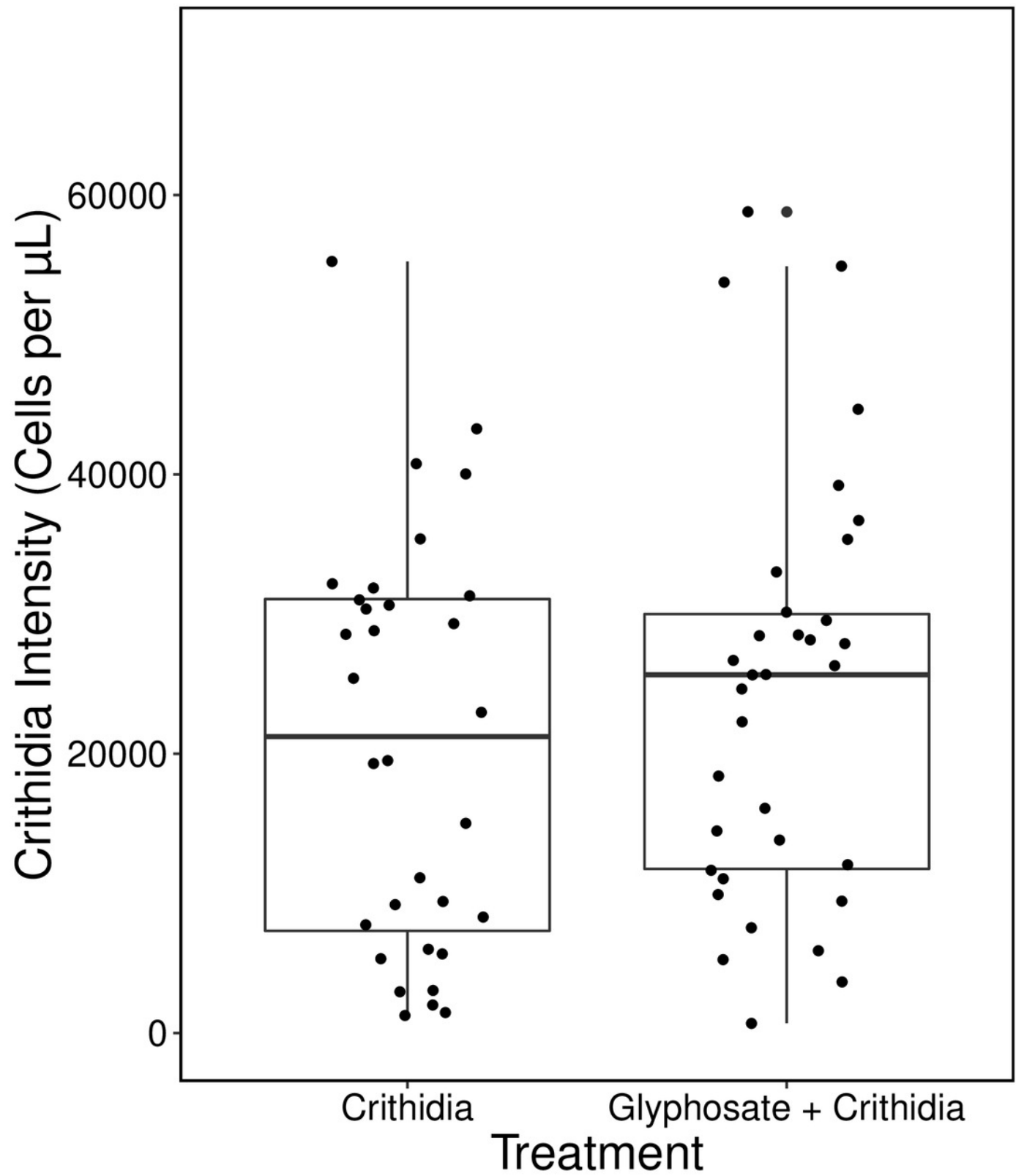
Figure 4

Long term survival after acute exposure

Figure 4. A Kaplan-Meier plot showing the survival over time by treatment.
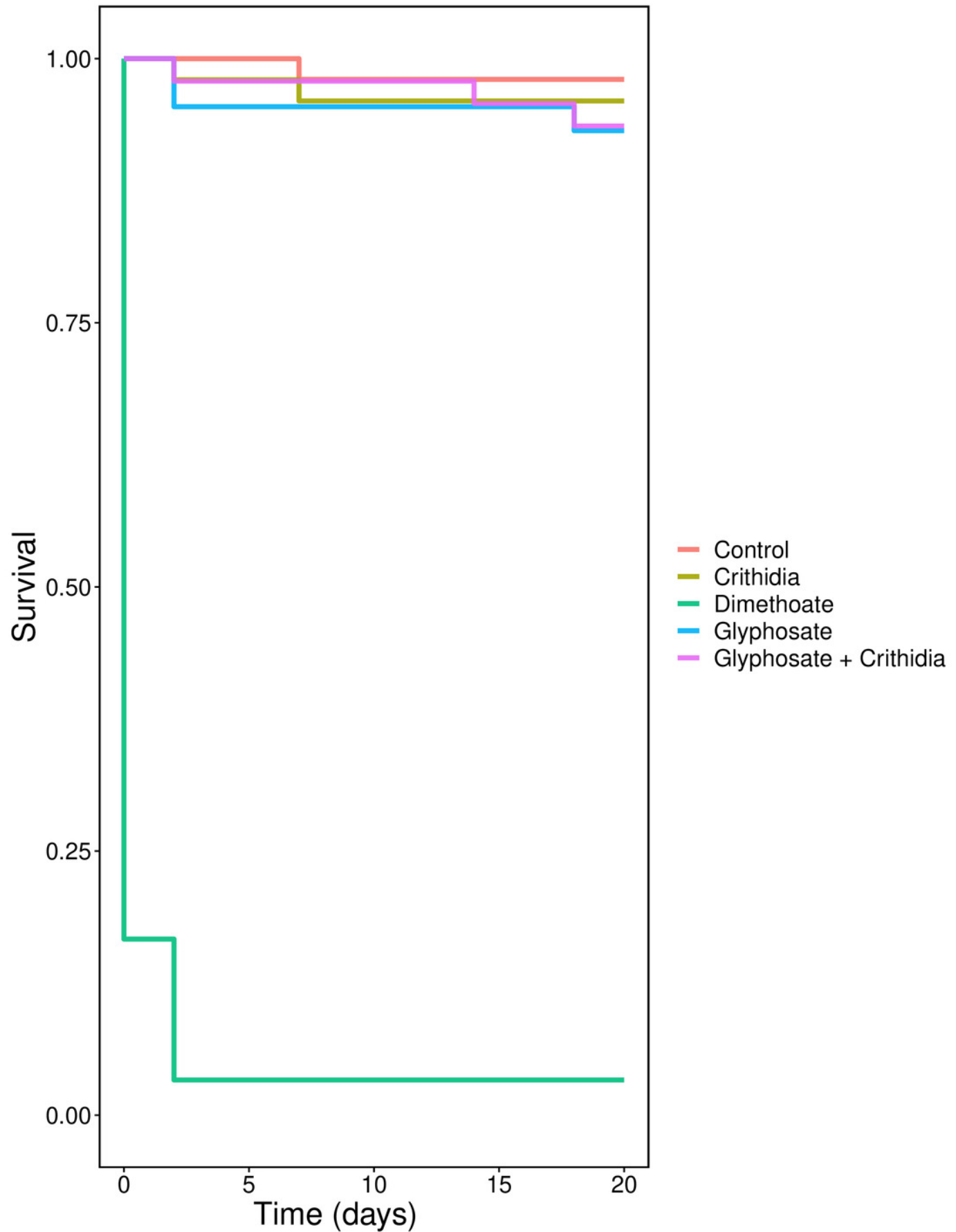
Figure 5

Larval weight adjusted for worker number after acute exposure

Figure 5. A boxplot showing the larval weight per microcolony standardised by the number of workers, presented by treatment with overlaid jittered data points.
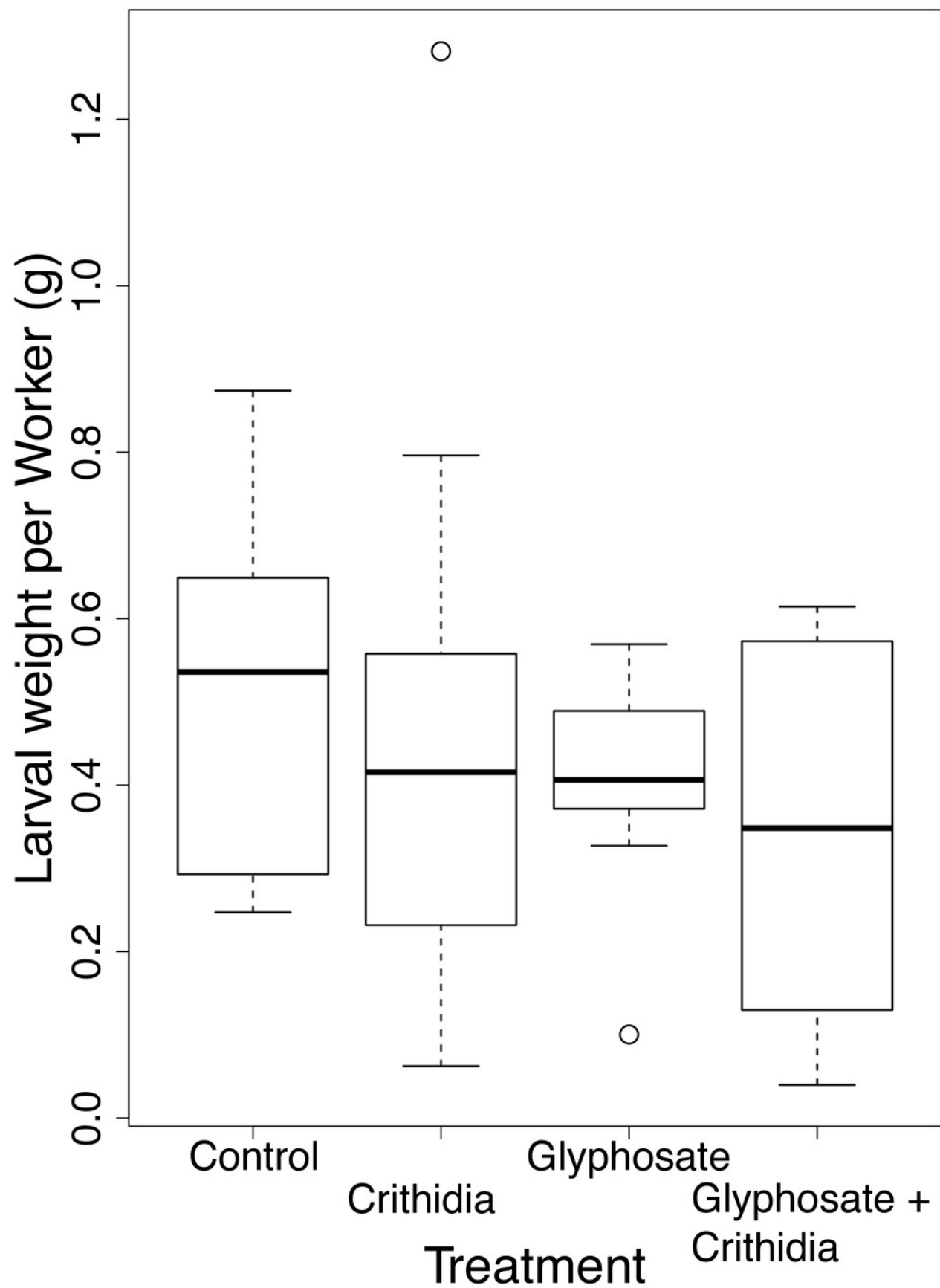
Figure 6

Microcolony Exposure - Acute Exposure - Parasite Intensity

Figure 6. A boxplot with overlaid jittered data points showing the parasite intensity by treatment.
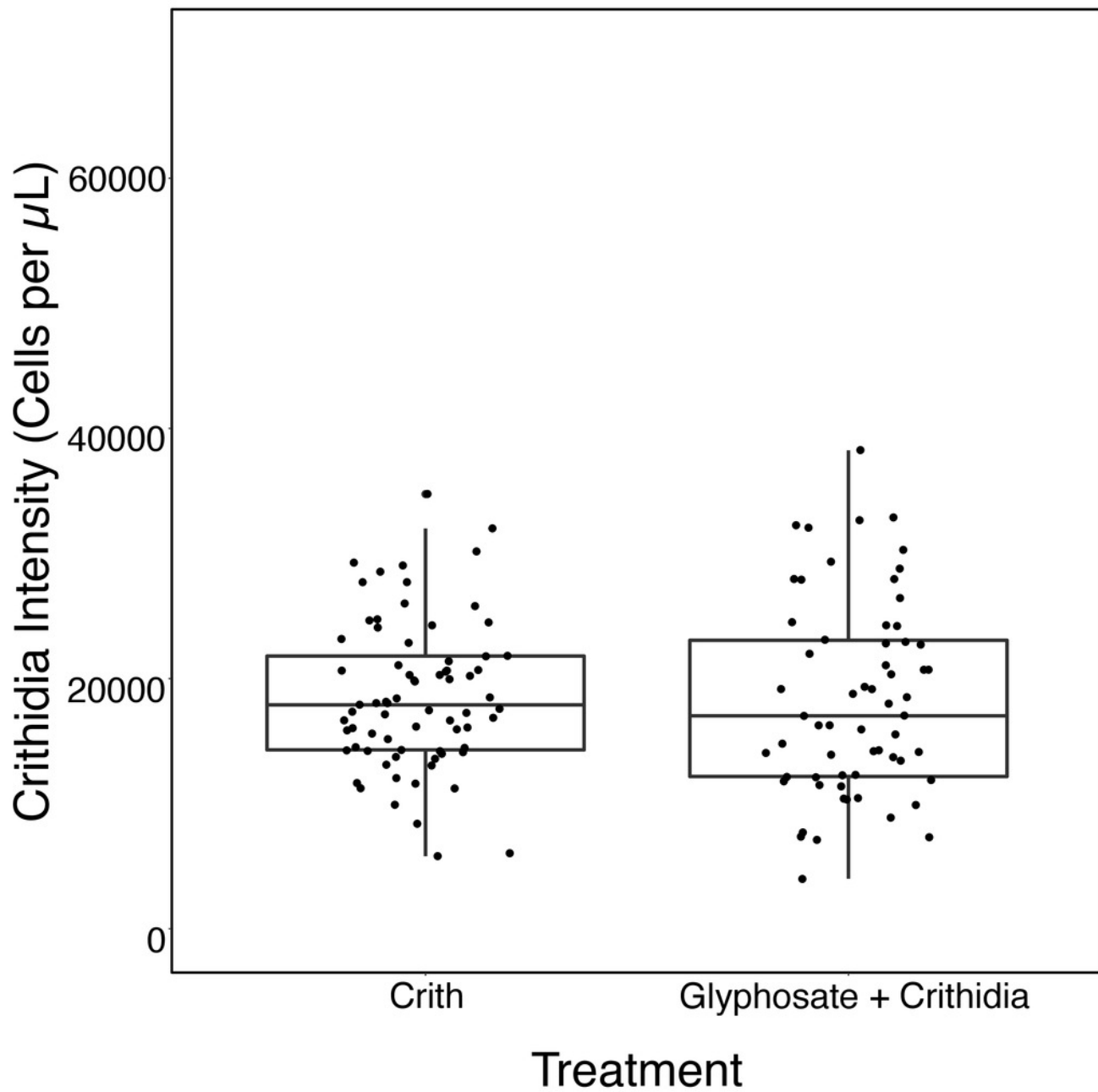
Figure 7

Larval weight adjusted by worker number after chronic exposure

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.
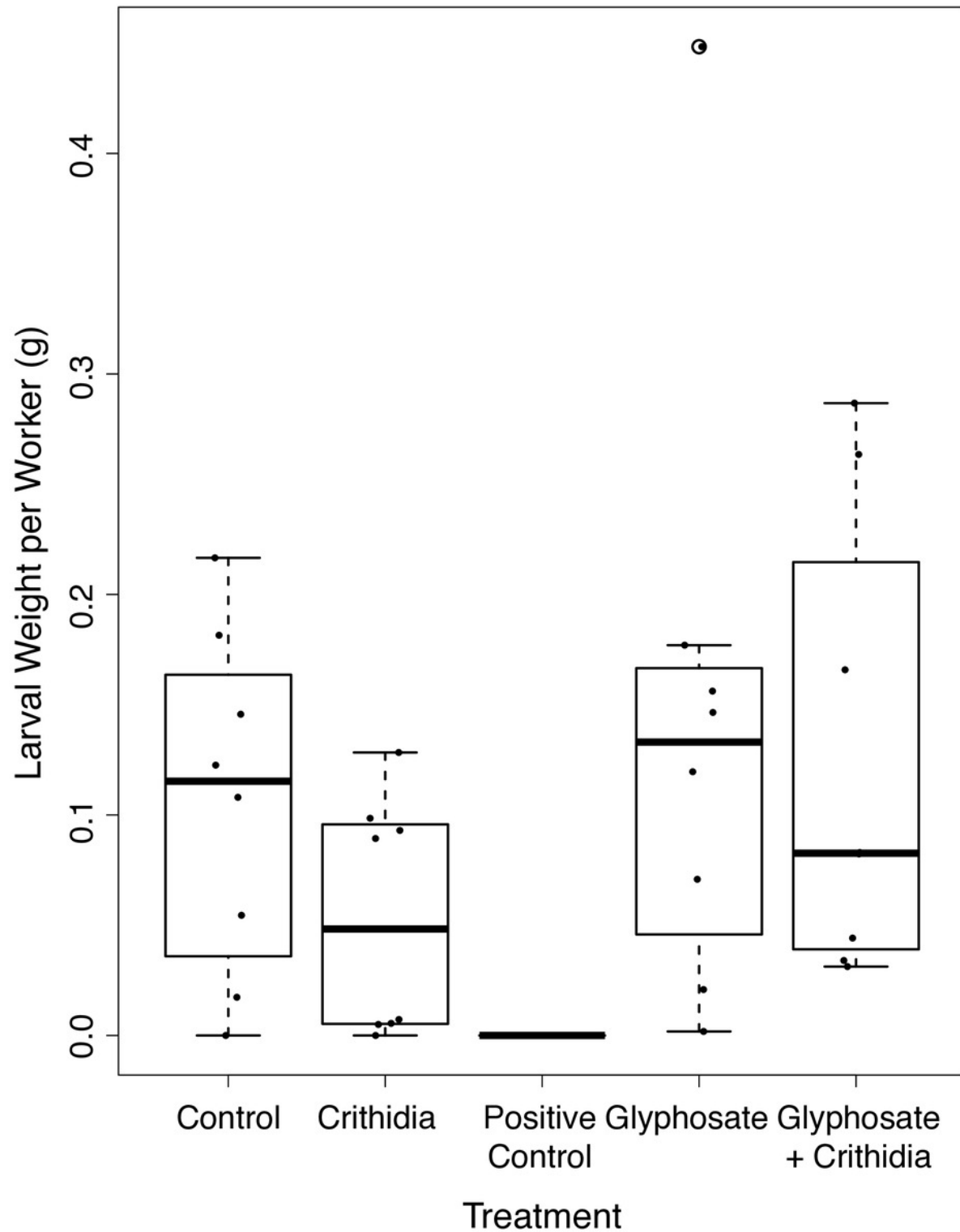
Figure 8

Glyphosate consumption over chronic exposure period

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.
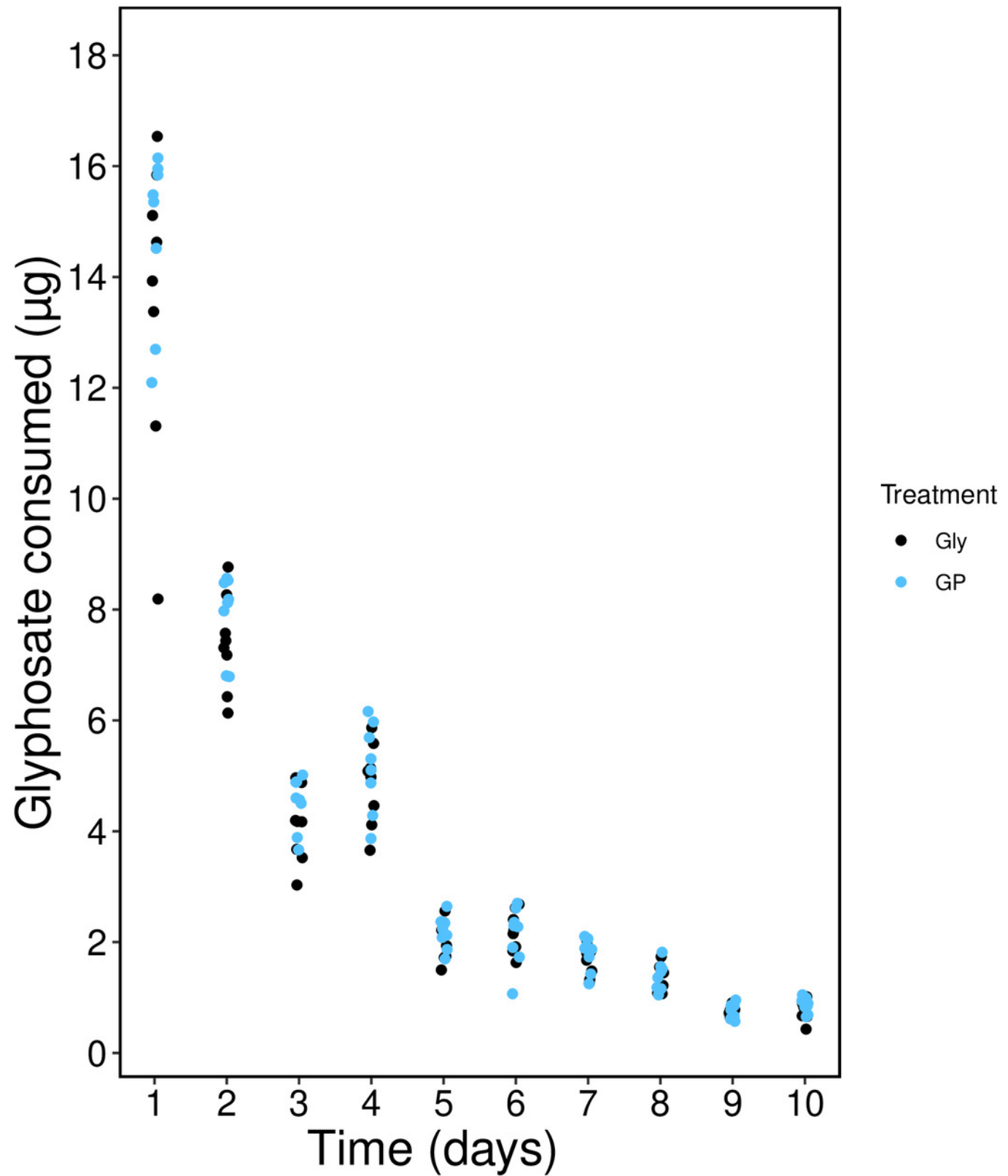
Figure 9

Microcolony Exposure - Chronic Exposure - Parasite Intensity

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