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Combined exposure to sublethal concentrations of an insecticide and a fungicide affect feeding, ovary development and longevity in a solitary bee

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1 **Combined exposure to sublethal concentrations of an insecticide and a fungicide affect**
2 **feeding, ovary development and longevity in a solitary bee**

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27 **Abstract**

28 Pollinators in agroecosystems are often exposed to pesticide mixtures. Even at low concentrations,
29 the effects of these mixtures on bee populations are difficult to predict due to potential synergistic
30 interactions. We orally exposed newly-emerged females of the solitary bee *Osmia bicornis* to
31 environmentally-realistic levels of clothianidin (neonicotinoid insecticide) and propiconazole
32 (fungicide), singly and in combination. The amount of feeding solution consumed was highest in
33 bees exposed to the neonicotinoid, and lowest in bees exposed to the pesticide mixture. Ovary
34 maturation and longevity of bees of the neonicotinoid and the fungicide treatments did not differ
35 from those of control bees. In contrast, bees exposed to the pesticide mixture showed slow ovary
36 maturation and decreased longevity. We found a synergistic interaction between the neonicotinoid
37 and the fungicide on survival probability. We also found an interaction between treatment and
38 emergence time (an indicator of physiological condition) on longevity. Longevity was negatively
39 correlated to physiological condition only in the fungicide and the mixture treatments. Delayed
40 ovary maturation and premature death imply a shortened nesting period (highly correlated to
41 fecundity in *Osmia*). Our findings provide a mechanism to explain the observed dynamics of
42 solitary bee populations exposed to multiple chemical residues in agricultural environments.

43 **Key words:** neonicotinoids, insecticide, ergosterol-biosynthesis-inhibiting fungicide, synergism,
44 ecotoxicology, *Osmia bicornis*

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55 1. Introduction

56 The last decades have seen significant declines in wild bee diversity at local and regional scales [1-
57 3], together with abnormal honey bee colony losses in various parts of the world [4,5]. Although
58 these declines are undoubtedly caused by a combination of factors, pesticides in general, and
59 neonicotinoid insecticides in particular, have often been signalled as one of the main drivers of the
60 population declines experienced by both wild and managed species. For this reason, the use of
61 neonicotinoids has been recently restricted in the European Union [6]. Nonetheless, neonicotinoids
62 are still used on a wide range of crops and account for more than 30% of the global insecticide
63 market [7]. Neonicotinoids are highly toxic to insects [8-10]. However, studies testing lethal and
64 sublethal effects of neonicotinoids on bees often yield inconsistent results [11-14]. There are several
65 important challenges when assessing the potential hazards of pesticides on bees. First, in as much as
66 possible, bees should be subjected to realistic exposure conditions, likely to be experienced in field
67 situations. In relation to this, some studies have been criticized based on allegedly overestimated
68 exposure in terms of concentration and duration (e.g., studies testing acute exposure to high doses
69 rather than chronic exposure to low doses) [15]. Second, in agricultural environments bees are often
70 exposed to combinations of chemicals [16]. This is important because certain pesticide mixtures
71 have been shown to produce synergistic effects [17-19]. Yet, with some exceptions [e.g., 17-20],
72 ecotoxicological studies usually test single compounds. Third, sensitivity to pesticides may be
73 highly influenced by the physiological condition of the bee. A recent review [21] shows that
74 response to pesticide exposure in honey bees is highly variable at the individual level and
75 dependent on several endogenous factors such as genetic background, body size and age. Fourth,
76 the effects of pesticides may be species-dependent. Most bee ecotoxicological studies have been
77 conducted on a single species, the western honey bee, *Apis mellifera* [16,22]. However, there is
78 increasing evidence that solitary bees (*Osmia bicornis*) are more sensitive to certain pesticide
79 treatments than honey bees and bumblebees [12,13,18,23].

80

81 In this study, we tested the effects of environmentally-realistic oral exposure to clothianidin (a
82 neonicotinoid insecticide) and propiconazole (an ergosterol-biosynthesis-inhibiting (EBI)
83 fungicide), singly and in combination, in the solitary bee *O. bicornis*. In agricultural environments,
84 bees are likely to be exposed simultaneously to both compounds because these two groups of
85 agrochemicals are commonly applied to various crops [24,25].

86

87 A key question in ecotoxicological studies is whether the test doses applied in the laboratory can be
88 considered to be field realistic. However, estimating field realistic pesticide doses is not easy. The

89 amount of nectar collected in a foraging bout by a nesting *Osmia* female can be estimated from the
90 literature [26], and concentrations of pesticides in nectar can be measured (e.g., [27,28]). However,
91 it is difficult to establish how much of the nectar collected is actually ingested by the foraging
92 female *versus* regurgitated onto the larval food provision. Nonetheless, we know that upon
93 emergence out of the natal nest, and prior to engaging in nesting activities, *Osmia* females collect
94 nectar exclusively for their own consumption [29]. Therefore, we provided newly-emerged *Osmia*
95 females in the laboratory with *ad libitum* feeding solution to simulate this “first nectar meal”. To
96 account for the physiological condition of the bees, we measured body size and emergence time.
97 Adult body size in *Osmia* is strongly correlated to the amount of food ingested during the larval
98 period [30]. Large bees have higher lipid content [31], and are more likely to survive the winter
99 [32]. As for emergence time, *Osmia* females lose ~7.5% of their body weight during the process of
100 emerging out of the cocoon [31]. Previous studies have shown that the probability to start a nest and
101 reproduce decreases with emergence time [33], indicating that females that take longer to emerge
102 are less vigorous than females that emerge promptly.

103
104 Upon feeding at the flowers, newly-emerged *Osmia* females undergo a short period (2-4 days)
105 during which they complete ovary maturation prior to initiating nesting activities [33,34]. During
106 this period ovary size and vitellogenin concentration in the hemolymph increase in parallel for up to
107 six days [35]. On average, individual *Osmia* females live for about 20 days, and their fecundity is
108 low (10-20 eggs) and highly correlated to the duration of the nesting period [33,34]. Therefore, any
109 effects on ovary maturation during this pre-nesting phase may significantly delay the onset of
110 nesting activities, with important consequences on reproductive success. Consequently, we
111 measured vitellogenin levels, ovary maturation and longevity in females exposed to the
112 neonicotinoid insecticide and the EBI fungicide, singly and in combination. Based on previous
113 studies showing synergistic mortality effects between clothianidin and propiconazole [18], we
114 hypothesize lower vitellogenin levels, slower ovary maturation and shorter life span in newly-
115 emerged *O. bicornis* females taking their first meal on the neonicotinoid-fungicide mixture. We
116 also hypothesize that these effects will be stronger on bees in poor physiological condition (smaller
117 bees and/or bees taking longer to emerge).

118

119 **2. Material and Methods**

120 *(a) Bee population and treatments*

121 *Osmia bicornis* cocoons were obtained from a population nesting in a pesticide free area in
122 Kazimierz Landscape Park, Poland. In January 2016, wintering adults within their cocoons were
123 shipped to the CREA-AA in Bologna, Italy, where they were transferred to a 3 °C cabinet. In early
124 April 2016, cocoons were taken to the laboratory of Agricultural Entomology at the University of
125 Bologna. In mid-April 2016, cocoons presumed to contain females (generally larger than those
126 containing males) were incubated at 21±2 °C and 55±10% RH under natural light. Emergence was
127 checked daily. Since most males emerge a few days before females, any emerging males were
128 discarded. We recorded the days each female took to emerge out of the cocoon following
129 incubation (henceforth emergence time). Upon emergence, females were transferred to a Plexiglas
130 laboratory cage (50 x 50 x 50 cm) to allow them to deposit the meconium. Females emerging on
131 any given day were equally distributed among four treatments: control (feeding solution with 1%-
132 acetone, CON), propiconazole (PRO), clothianidin (CLO) and mixture (propiconazole +
133 clothianidin, MIX). Throughout the study bees were maintained at 21-23 °C, 40-50 % RH under
134 natural light.

135 *(b) Test solution preparation*

136 We used clothianidin active ingredient (purity 99%) from Dr Ehrenstorfer GmbH. A stock solution
137 was prepared by dissolving technical grade clothianidin (99% pure) in acetone at a nominal
138 concentration of 1000 mg/L (actual concentration: 1090 mg/L), which was then diluted to 1 mg/L
139 (actual concentration: 0.983 mg/L). The stock solution was then diluted in a 38% w:v (33% w:w)
140 sugar + distilled water solution to achieve the desired concentration of 10 µg/L (corresponding to
141 8.6 µg/Kg). This concentration is within the range of clothianidin residues found in nectar collected
142 from flowers of oilseed rape grown from clothianidin-coated seeds (6.7-16 µg/L [12]; 5-16 µg/Kg
143 [24]; 2.3-10.1 µg/Kg [36]; <0.7-13.2 µg/Kg [37]);

144 We tested a propiconazole concentration of 62.5 mg/L. This concentration corresponds to the field
145 application rate of the commercial formulation Protill ® EC (250 g/L of a.i.) in orchards (25 mL/hL
146 or 0.25 L/ha). To obtain this concentration we prepared a stock solution with a propiconazole
147 concentration of 25 g/L by dissolving Protill ® EC in distilled water. The stock solution was then
148 diluted with 38% w:v (33% w:w) sugar solution to achieve the desired concentration.

149 The final concentration of acetone in the feeding solution was adjusted to 1% (v:v) with pure
150 acetone in all treatments.

151 *(c) Exposure phase*

152 Previous studies have shown that upon emergence out of the cocoon, *Osmia* females take about one
153 day to come out of their natal nest [38]. Therefore, 24 hours after emergence, meconium-free
154 females were individually housed in small plastic cylinders (width: 3.5 cm; high: 5.5 cm) with a
155 transparent plastic lid through which a feeder made with a 1-mL syringe was inserted. Each feeder
156 contained ~150 μ L of feeding solution (33% sucrose concentration w:w) with or without pesticides.
157 A flower petal (*Euryops*, Asteraceae) was attached to the tip of the syringe to ensure the bees
158 located the feeder quickly (see [18,39] for details). To simulate a first nectar meal, bees were
159 maintained in these cylinders for 4 hours. Preliminary trials showed that extending this exposure
160 phase up to 8 h did not result in increased solution consumption. To measure the amount of solution
161 ingested by each bee, syringes were weighed before and after the exposure phase. Three cages
162 without bees served as controls to account for potential evaporation. Only bees that fed were
163 included in the statistical analyses. In natural conditions, newly-emerged bees have to fly to reach
164 flowers on which to sip nectar. In our laboratory set-up bees only had to walk a very short distance
165 to have access to a feeding solution source. Therefore, if anything, our method can be assumed to
166 underestimate the amount of nectar and chemical residue ingested by a newly-emerged bee in her
167 first nectar meal. Sample size were 35-50 bees per treatment.

168 (d) Experiment 1

169 After the exposure phase, each bee was individually transferred to a plastic ice cream cup (width:
170 5.5-8 cm; high: 7 cm) with a transparent lid through which a 2.5 mL syringe filled with sucrose
171 solution (33% sugar concentration, w:w) was inserted. Again, a flower petal was attached to the tip
172 of the feeder to ensure the bees located the feeder quickly. Bees were allowed to feed *ad libitum* and
173 the sucrose solution in the feeder was renewed every 3 days. Solution consumption was visually
174 assessed every day. Mortality was monitored daily until all bees died. Upon death, the head width
175 of each bee was measured under a stereomicroscope at 32 X. Head size is strongly correlated to
176 body weight in *Osmia* [30]. Sample sizes were ~30 bees per treatment.

177 (e) Experiment 2

178 We followed the same procedure as the experiment 1 with two modifications. First, because pollen
179 consumption enhances ovary maturation in *Osmia* [40], bees of this experiment were provided with
180 a source of pollen throughout the post-exposure phase. In each ice cream cup we provided ~55 mg
181 of pollen in a 1.5 ml Eppendorf tube cap. Pollen was obtained from nests of an *O. bicornis*
182 population nesting in a pear/apple orchard near Bologna. Several provision masses (pollen mixed
183 with nectar) from various nests were mixed to obtain a common homogeneous pollen source from

184 which 55 mg portions were taken. Samples of this pollen source were subjected to palynological
185 and chemical multiresidue analyses (see details in the electronic supplementary material). Chemical
186 analyses revealed that the provisions contained several pesticide residues, including insecticides,
187 fungicides and herbicides at very low concentrations (electronic supplementary material, Table S1).
188 Although unplanned, the presence of these residues resulted in a more realistic exposure, congruent
189 with the co-occurrence of multiple compounds in pollen-nectar matrices in agricultural
190 environments [41,42]. Importantly, no obvious negative effects were observed in the nesting *O.*
191 *bicornis* population from which the provisions were taken or its progeny.

192 Second, in this experiment the post exposure phase was interrupted after 3 days to measure
193 vitellogenin levels in the haemolymph and ovary maturation. Details on vitellogenin and ovary
194 maturation measurements are available in the electronic supplementary material.

195 All statistical analyses are described in the electronic supplementary material.

196

197 **3. Results**

198 *(a) Exposure phase feeding*

199 The amount of feeding solution ingested during the 4-hour exposure phase differed among
200 treatments (Table 1). Bees of the CLO treatment fed significantly more than bees of the other
201 treatments, and feeding levels were lowest in the MIX treatment (Fig. 1). Solution ingestion during
202 this phase also depended on body size (larger bees ingested more syrup), but not on emergence time
203 (Table 1). However, the interaction between treatment and emergence time was significant. As
204 emergence time increased, feeding increased in CLO bees, whereas it decreased in PRO and MIX
205 bees, and did not change in CON bees (electronic supplementary material, figure S1).

206 *Experiment 1*

207 Differences among treatments in feeding rate (μL of syrup per day) during the post-exposure phase
208 approached significance (Table 1), again with bees of the MIX treatment tending to feed less (Fig.
209 2). Both body size and emergence time affected post-exposure feeding (Table 1). Feeding rates
210 were higher in larger bees and lower in bees that took longer to emerge.

211 Cumulative survival curves differed significantly among treatments ($df = 3$, $\chi^2 = 12.99$, $P = 0.005$)
212 (Fig. 3). Throughout the first days following exposure, mortality in the MIX treatment was much
213 greater than mortality in the other treatments, yielding a significant synergistic interaction between

214 clothianidin and propiconazole on day 4 (day 4: $p = 0.045$; day 8: $p = 0.075$; day 17: $p = 0.44$). That
215 is, the CLO–PRO combination was significantly more toxic than the sum of the toxicity of the two
216 compounds separately. Consequently, longevity differed significantly across treatments (Table 1),
217 and was shortest in the MIX treatment (Fig. 2). Body size had no effect on longevity, but bees that
218 took longer to emerge tended to have shorter longevity (Table 1). In addition, there was a
219 significant interaction between treatment and emergence time. As emergence time increased,
220 longevity decreased in PRO and MIX bees, but did not change in CON and CLO bees (Table 1,
221 electronic supplementary material, Fig. S2).

222 *Experiment 2*

223 Nectar feeding rate during the three-day post-exposure phase significantly differed among
224 treatments (Table 1). As in experiment 1, it was highest in the CON treatment and lowest in the
225 MIX treatment (Fig. 4). In contrast to experiment 1, body size and emergence time did not affect
226 post-exposure feeding (Table 1), but it is important to note that the post-exposure phase lasted only
227 three days in this experiment. We repeatedly observed *O. bicornis* females feeding on the pollen
228 provided. However, the amount of pollen consumed could not be measured because bees spread the
229 pollen all over the hoarding cage.

230 Three-day cumulative survival curves differed among treatments ($df = 3$, $\chi^2 = 45.72$, $P < 0.001$).
231 Survival was again lowest in the MIX treatment (Fig. 5), and there was a significant synergistic
232 interaction between clothianidin and propiconazole on all three assessment time points (day 1: $p <$
233 0.001 ; day 2: $p < 0.001$; day 3: $p = 0.002$). Oocyte length and vitellogenin concentration were
234 measured in all the bees that survived the 3-day post-exposure period ($n=55$). We found significant
235 differences among treatments in basal oocyte mean length (Table 1), with bees of the MIX
236 treatment having shorter oocytes than bees of the other treatments (Fig. 4). Ovary size was
237 positively related to head size, but was not related to emergence time (Table 1). We found no
238 differences among treatments in vitellogenin concentration (Table 1). Larger bees had higher
239 vitellogenin concentrations, but emergence time did not affect vitellogenin levels (Table 1). No
240 interactions between treatment and head size or emergence time were apparent in this experiment
241 (Table 1).

242

243 **4. Discussion**

244 Wild and managed bees are exposed to pesticide mixtures in agricultural and urban areas [41,43-
245 45]. Neonicotinoids and EBI fungicides, in particular, are routinely used on many crops [24,25],
246 and have often been found together in the nectar and pollen of both cultivated and wild flowers
247 [37,41], in honey bee-collected pollen and on bee body surfaces [41,46,47]. In a previous study [18]
248 we showed synergistic mortality effects in honey bees, bumblebees and solitary bees (*Osmia*
249 *bicornis*) acutely exposed to sublethal doses of CLO (0.63 ng/bee) and PRO (7 µg/bee) in a fixed
250 amount of syrup (10 µL). The amount of CLO ingested by bees in that study was within the range
251 of CLO potentially ingested in a foraging bout. However, the tested concentration (63 µg/L of
252 CLO) was higher than concentrations likely to be found in nectar (<0.7-16 µg/L) [12,24,36,37,48].
253 On the other hand, considering the honey stomach capacity of honey bees (~ 30 µL) and
254 bumblebees (80 µL) [49,50] it is conceivable that a bee could ingest more than 10 µL of nectar in a
255 single foraging bout. At any rate, given the difficulty to estimate what proportion of the nectar
256 collected by a nesting female bee is ingested versus regurgitated in the nest, in this study we worked
257 with pre-nesting females, which consume all the nectar they collect. Our study provides first-time-
258 evidence that oral exposure to field-relevant concentrations of an insecticide and a fungicide
259 mixture affect feeding behavior, ovary maturation and longevity in a solitary bee.

260

261 Results of syrup consumption during the exposure phase show that *O. bicornis* females not only did
262 not avoid but even preferred neonicotinoid-laced syrup. This behavior has also been observed in
263 bumblebees and honey bees [51,52]. Interestingly, syrup consumption during this phase was lowest
264 in bees of the MIX treatment, indicating that the attractiveness of clothianidin was lost when
265 propiconazole was added. Post-exposure feeding rate (ml of syrup consumed per day) was also
266 lowest in the MIX treatment in both experiments (although differences among treatments narrowly
267 failed significance in Experiment 1), suggesting that the clothianidin-propiconazole combination
268 alters the feeding behavior of *O. bicornis*.

269 Vitellogenin is a fat-body-synthesized glycolipophosphoprotein that constitutes a significant part of
270 the yolk protein of insect eggs [53]. In *Osmia*, vitellogenin concentration in the hemolymph
271 increases with ovary maturation, reaching maximum levels 3-6 days after adult emergence and
272 gradually declining thereafter [35]. Studies on honey bee and bumblebee queens have reported a
273 strong up-regulation of vitellogenin genes [54] but slower ovary maturation following experimental
274 neonicotinoid exposure [55,52]. Because pollen feeding enhances ovary development in
275 bumblebees [56], Baron *et al.* [52] hypothesized a reduction in pollen consumption in bees exposed
276 to neonicotinoids. *Osmia* females also require pollen to mature their oocytes [40]. Our bees clearly

277 fed on the pollen supplied in experiment 2, but we could not establish whether pollen consumption
278 differed among treatments because bees spread the pollen over the hoarding cages. At any rate, we
279 did not find differences in vitellogenin concentration or ovary maturation between clothianidin-
280 exposed and control bees. On the other hand, we found that ovary maturation was slowest in bees of
281 the MIX treatment, even if this reduction was not accompanied by increased levels of vitellogenin
282 concentration.

283 In experiment 1, longevity of propiconazole- and clothianidin-exposed bees (mean: 17 and 19 days,
284 respectively) did not differ from that of control bees (mean: 17.5 days). These life spans are similar
285 to those recorded in field and greenhouse populations (17.5 - 24 days [33,34,57]; though mean
286 longevity can be extended up to 30.5 days under bad weather conditions; [34]). Bees of the CLO
287 treatment consumed larger amounts of feeding solution, thus ingesting greater amounts of sugar,
288 which could have buffered any negative effect of clothianidin [58]. By contrast, exposure to the
289 MIX treatment resulted in significantly reduced longevity. Life span of bees of the MIX treatment
290 in experiment 1 was 10 days, that is 0.5-0.6 times shorter than that of control bees (17.5 days) and
291 bees exposed to single compounds (17 and 19 days, respectively). The negative effect of the
292 pesticide mixture was further evidenced by the comparison of the survival curves of the various
293 treatments, revealing a synergistic interaction between clothianidin and propiconazole on survival
294 probability in both experiments. Three days after exposure, mortality in the MIX treatment of
295 experiment 2 was 78%, more than twice higher than expected under additive (non-synergistic)
296 effects (36%). Bees of experiment 2 were fed pollen during the post-exposure phase whereas bees
297 of the experiment 1 were not, and the pollen supplied was contaminated with pesticide residues
298 (electronic supplementary material, Table S1). This pollen was obtained from *O. bicornis*
299 provisions from a population nesting in a pear/apple orchard that was sprayed during bloom with
300 boscalid. This fungicide was the main chemical residue found in the pollen, but four other
301 chemicals that were not sprayed in the orchard were also found. Pollen analysis of the provisions
302 revealed that *O. bicornis* females foraged mostly on wild plants (*Quercus robur* (39%), *Ranunculus*
303 spp. (27%), *Cercis* spp. (25%), apple/pear (2 %)). Thus, our study provides further evidence of
304 pesticide exposure affecting not only bees foraging on sprayed crops, but also those foraging on the
305 accompanying flora [13,59,60].

306 Differences between experiments 1 and 2 in survival probability at day 3 were very small for the
307 CON (87% vs 87%) and PRO (82% vs 88%) treatments. By contrast, these differences were very
308 pronounced for the CLO (93% vs 73 %) and the MIX treatments (48% and 22%), suggesting that,

309 even at the low concentrations recorded, the presence of additional pesticides in the pollen supplied
310 in experiment 2 interacted with the clothianidin ingested during the exposure phase.

311 We used body size and timing of emergence as proxies of physiological condition. Not surprisingly,
312 large bees consumed more feeding solution during the exposure phase and during the post-exposure
313 phase of experiment 1. No such relationship was found in experiment 2, but the post-exposure
314 phase of this experiment lasted only three days. Larger bees also had higher levels of vitellogenin in
315 the hemolymph and, in agreement with previous studies [33], produced larger oocytes. However,
316 large bees did not live longer than small bees. Studies on *Osmia* populations nesting in field and
317 greenhouse conditions have also failed to find a relationship between female body size and
318 longevity (or nesting period) [33,34,61-63].

319 Emergence time affected post-exposure feeding solution consumption rate and longevity in
320 experiment 1, both of which were lower in females with long emergence periods. These results are
321 congruent with the reduced ability of bees that take long to emerge to start nesting activities [33].
322 As with body size, such a relationship was not apparent in experiment 2, possibly due to the short
323 post-exposure phase of this experiment. Despite their lower feeding solution consumption, we did
324 not find lower vitellogenin levels or slower ovary maturation in bees with long emergence times.

325 Physiological condition may influence sensitivity to pesticides [21]. Our results show that the
326 negative effects of emergence time on longevity occurred only in the MIX and PRO treatments. The
327 suboptimal physiological condition of bees with long pre-emergence periods could have reduced
328 their detoxification capacity making them more vulnerable to these two treatments. To our
329 knowledge, this is the first time an effect of physiological condition on sensitivity to pesticides is
330 shown for a solitary bee. Ecotoxicological studies are often carried out under conditions that are
331 assumed to be optimal for the test organisms (e.g., healthy individuals kept at adequate
332 temperatures with *ad libitum* feeding). In the field, however, bees may be exposed to various stress
333 factors, such as parasites, diseases, and limiting food resources, which could magnify the negative
334 effects of pesticides. In their review, Holmstrup *et al.* [64] argue that synergistic interactions
335 between toxic compounds and natural stressors are frequent and should be considered in risk
336 assessment schemes.

337 Our study shows that a single meal with a cocktail of pesticides at sublethal doses and realistic
338 concentrations during the pre-nesting period affects feeding behavior, ovary maturation and
339 longevity in a solitary bee. Importantly, none of these effects were observed when bees were
340 exposed to either compound singly. The pre-nesting period is a critical stage in the life cycle of

341 solitary bees for two reasons. First, females in poor physiological condition are less likely to start
342 nesting activities and reproduce [33]. Our results show that nesting success of these weakened
343 females may be further compromised by exposure to pesticide mixtures at realistic field
344 concentrations. Second, fecundity of females that do successfully nest is highly correlated to the
345 duration of the nesting period [33,34], which is constrained by ovary maturation at one end [33,35]
346 and by death at the other end. Our insecticide-fungicide mixture had negative effects on both ovary
347 maturation and longevity, thus affecting the duration of the nesting period at both ends. Under field
348 conditions, *Osmia* females live ~ 20 days on average [34]. Of this time, ~ 5 days are spent maturing
349 the ovaries [35], prior to the initiation of nesting activities (pre-nesting period) [33,34]. During the
350 rest of their life time (nesting period), females build and provision nest cells and lay eggs at a rate
351 of ~ 0.7 per day [34]. If we assume that mean longevities recorded in our study are representative of
352 longevities under field conditions, females of our MIX treatment would have laid a mean of 3.5
353 eggs compared to 8.4 in control bees. We conclude that our findings have direct repercussions on
354 the reproductive success of solitary bees, and provide a potential mechanism to explain observed
355 negative dynamics of *Osmia* populations in agricultural environments [12,13,65]. Our study has
356 also important implications for pesticide regulation. Current risk assessment schemes rely on tests
357 of single compounds [27,28]. Our results underscore the need to consider pesticide combinations
358 likely to occur in agricultural environments.

359 **Data accessibility.** Data available from the Dryad Digital Repository:

360 <https://doi.org/10.5061/dryad.895pn6p> [66].

361 **Author contributions.** F.S and J.B. conceived the experiments. F.S, J.B., R.C., G.I., D.T. and P.M.
362 designed the experiments. F.S. and R.C. collected the data. X.A. analysed the data. F.S. and J.B.
363 took the lead in writing the manuscript.

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569 **Figure 1.** Mean + SE test solution ingested during the 4-hour exposure phase in *O. bicornis* females
570 orally exposed to four treatments (CON: control; CLO: clothianidin; PRO: propiconazole; MIX:
571 clothianidin + propiconazole mixture). Different letters denote significant differences (Fisher LSD
572 Post-hoc, P<0.05).

573 **Figure 2.** Experiment 1 - Mean + SE post-exposure feeding rate (μ l of feeding solution ingested per
574 day) and longevity in *O. bicornis* females orally exposed to four treatments (CON: control; CLO:
575 clothianidin; PRO: propiconazole; MIX: clothianidin + propiconazole mixture). Different letters
576 denote significant differences (Fisher LSD Post-hoc, P<0.05).

577 **Figure 3.** Experiment 1 - Cumulative survival probability of *O. bicornis* females orally exposed to
578 four treatments (CON: control; CLO: clothianidin; PRO: propiconazole; MIX: clothianidin +
579 propiconazole mixture). Synergistic interactions between CLO and PRO treatments (P <0.05; one-
580 tailed binomial proportion test; assessment times: 4, 8, and 17 days) are marked with an asterisk.

581 **Figure 4.** Experiment 2 - Mean + SE post-exposure feeding rate and basal oocyte length in *O.*
582 *bicornis* females orally exposed to four different treatments (CON: control; CLO: clothianidin;
583 PRO: propiconazole; MIX: mixture). Different letters denote significant differences (Fisher LSD
584 Post-hoc, P<0.05).

585 **Figure 5.** Experiment 2 - Cumulative survival probability of *O. bicornis* females orally exposed to
586 four treatments (CON: control; CLO: clothianidin; PRO: propiconazole; MIX: clothianidin +
587 propiconazole mixture). Synergistic interactions between CLO and PRO treatments (P <0.05; one-
588 tailed binomial proportion test; assessment times: 1, 2, 3 days) are marked with an asterisk.

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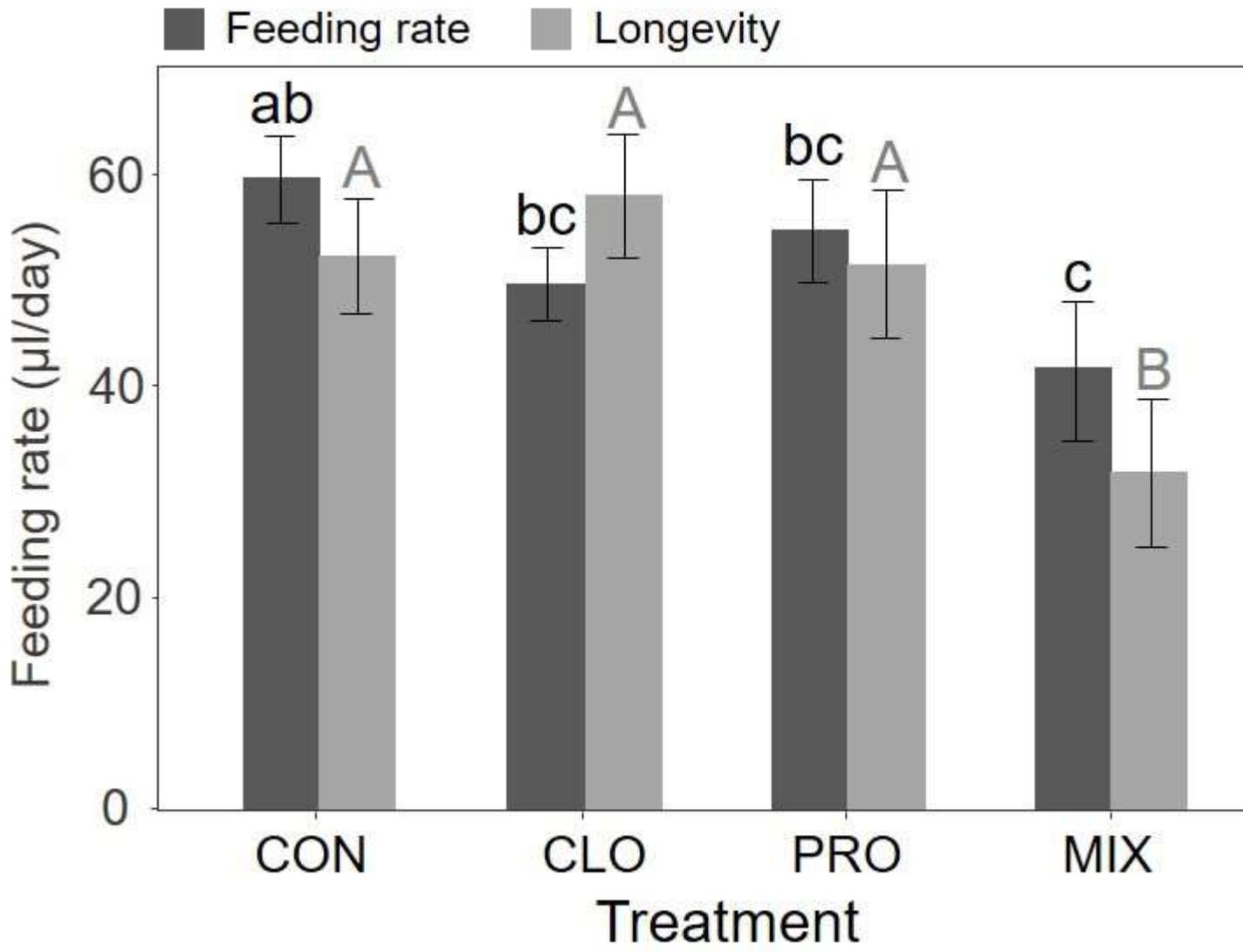
596 **Table 1.** Best selected ($\Delta AIC_c < 2$) general linear models explaining the effects of treatment (Tr),
 597 emergence time (ET), head size (HS) and the interactions between treatment and emergence time
 598 and treatment and head size on each response variable. Significant predictors ($p < 0.05$) in bold,
 599 marginally significant predictors ($p=0.05 - 0.1$) in italics. Positive and negative signs in brackets
 600 denote the direction of the relationship.

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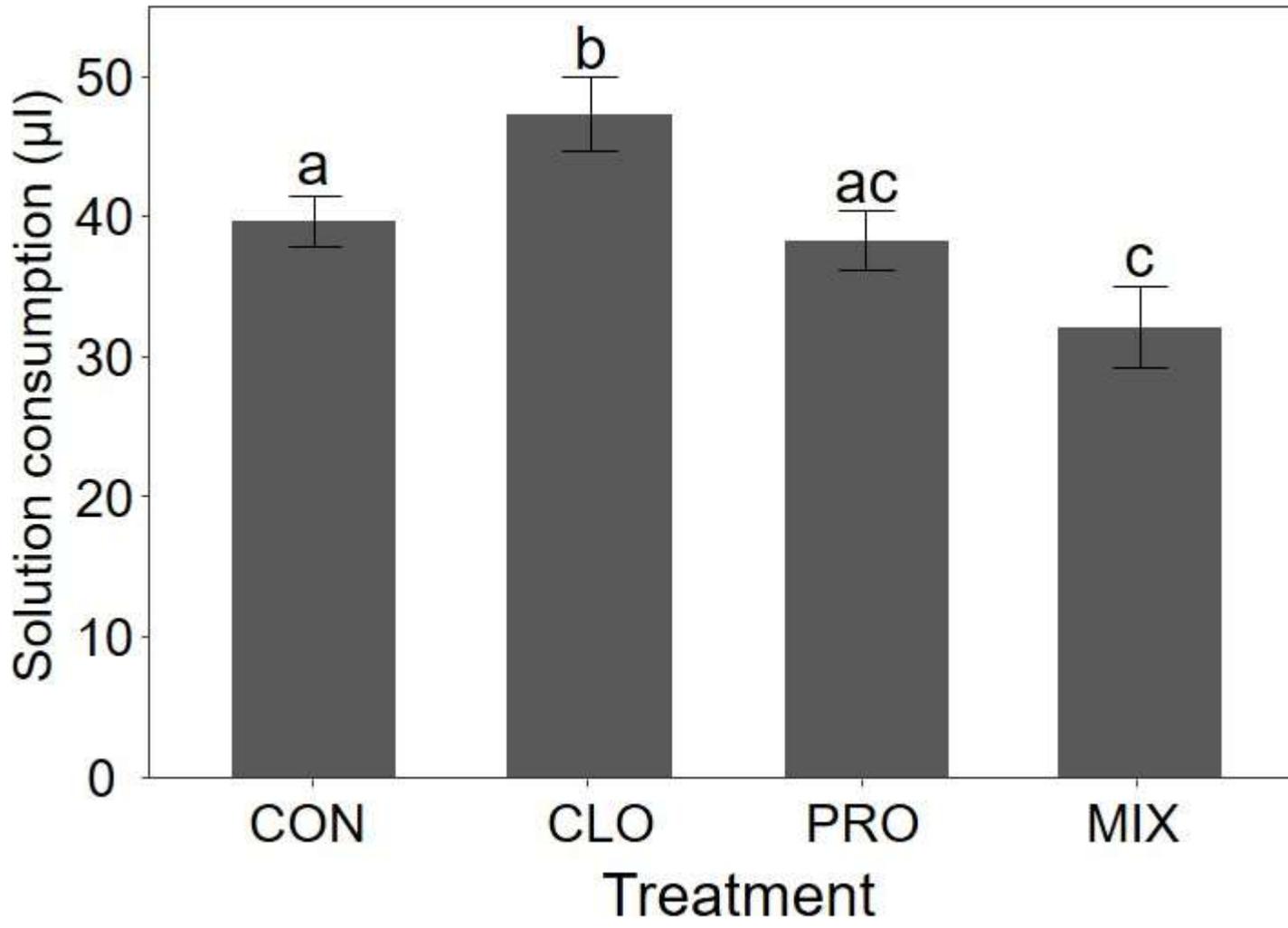
	<i>Response variable</i>		<i>Model components</i>	<i>AIC_c</i>	<i>ΔAIC_c</i>	<i>w_i</i>	<i>R² (%)</i>	
Experiment 1	Exposure feeding	1	Tr + ET (+) + HS (+) + Tr:ET	1376.7	0.00	0.592	22	
		2	Tr + HS (+)	1378.4	1.73	0.249	17	
	Post-exposure feeding rate	1	<i>Tr</i> + ET (-) + HS (+)	707.1	0.00	0.463	21	
		2	ET (-) + HS (+)	707.5	0.44	0.371	14	
	Longevity (sqrt-transformed)	1	Tr + ET (+) + Tr:ET	380.3	0.00	0.358	26	
		2	Tr + ET (-) + HS (+) + Tr:ET	381.3	0.99	0.218	27	
		3	Tr + ET (-) + HS (+)	381.9	1.62	0.159	21	
		4	Tr + ET (-)	382.2	1.89	0.139	19	
	Experiment 2	Post-exposure feeding rate	1	Tr	647.5	0.00	0.562	22
		Oocyte length	1	Tr + HS (+)	-51.0	0.00	0.667	37
2			Tr + ET (+) + HS (+)	-49.3	1.78	0.273	38	
Vitellogenin concentration (sqrt-transformed)		1	HS (+)	123.1	0.00	0.467	27	
		2	ET (-) + HS (+)	123.1	0.03	0.460	31	

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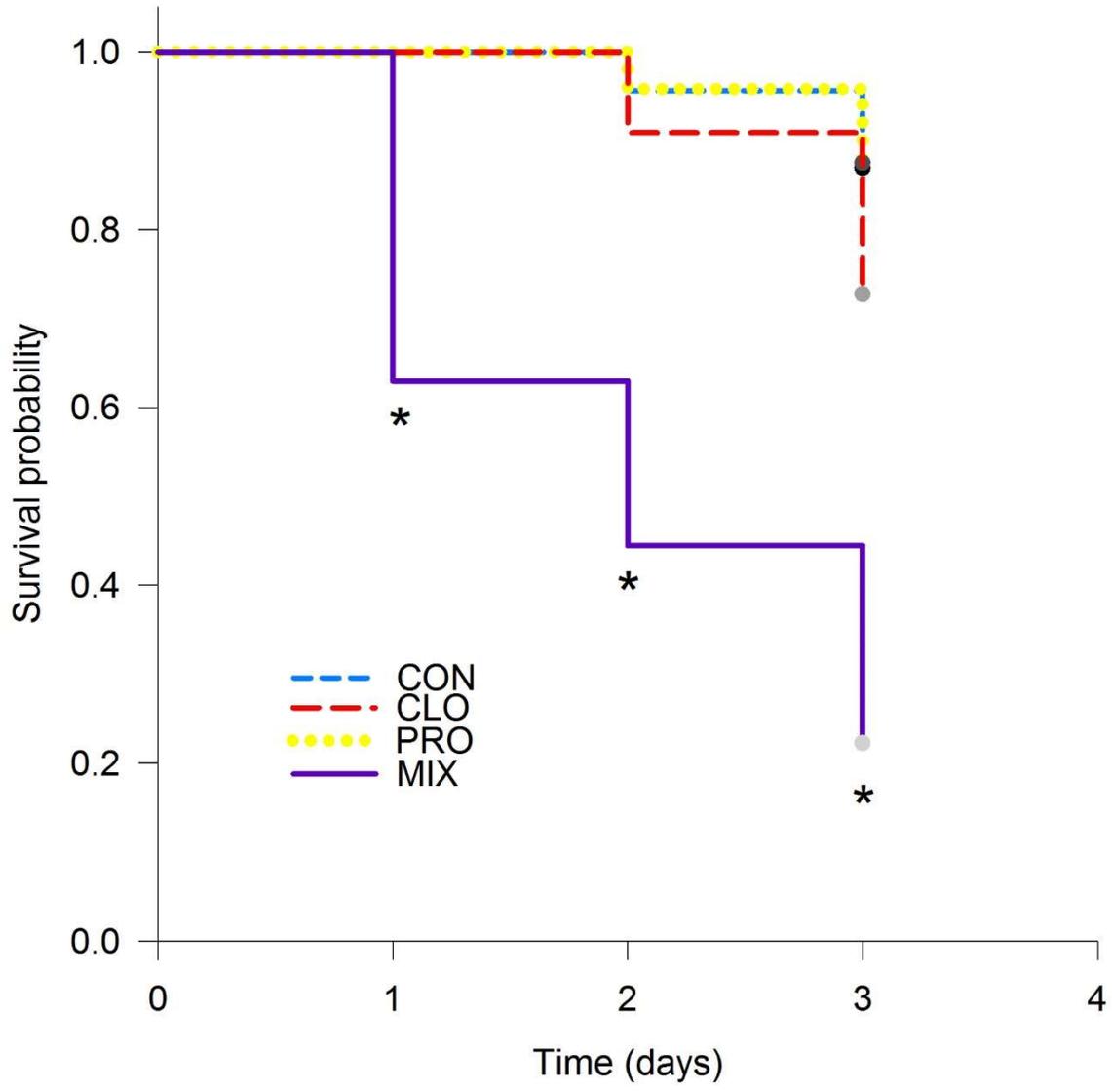
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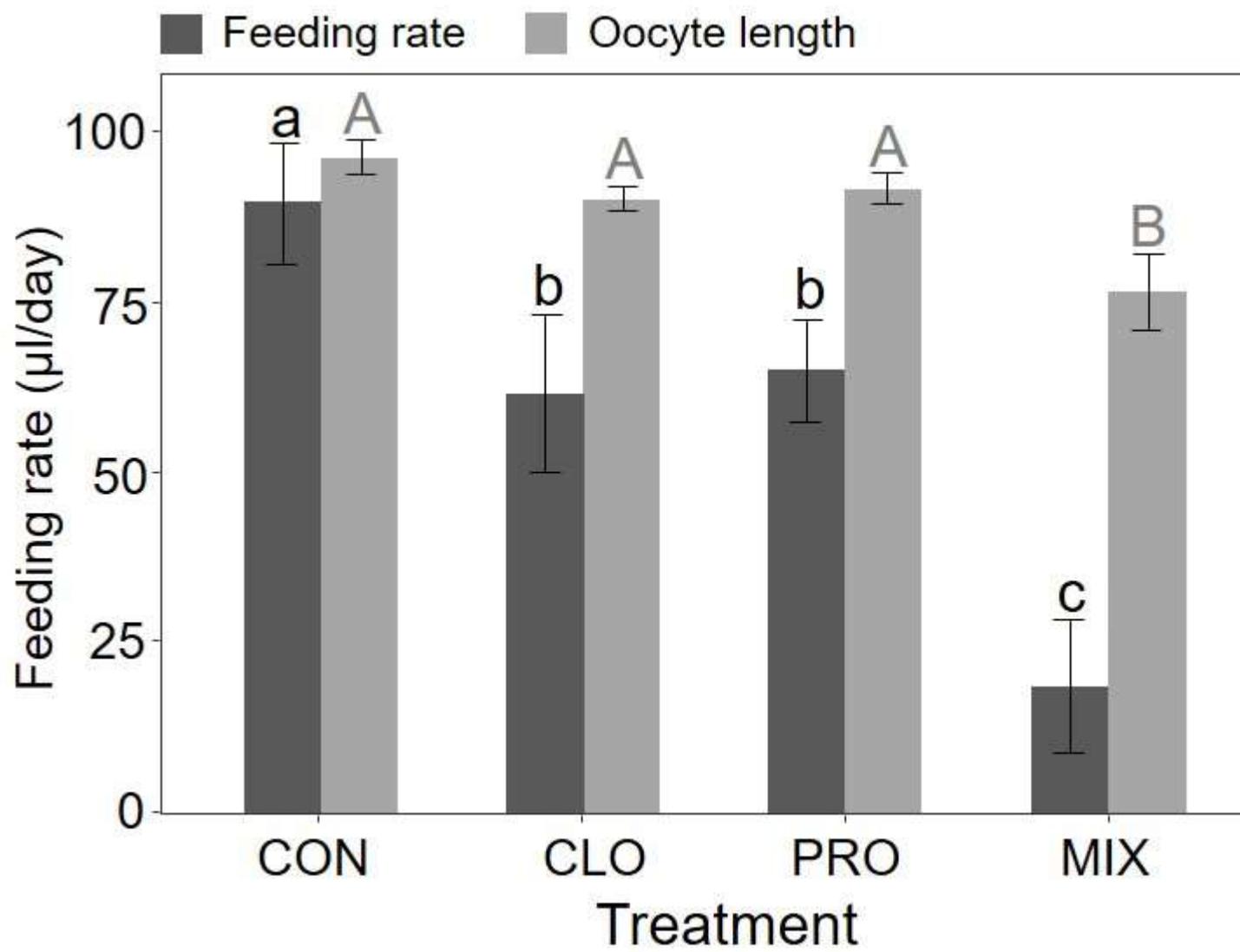
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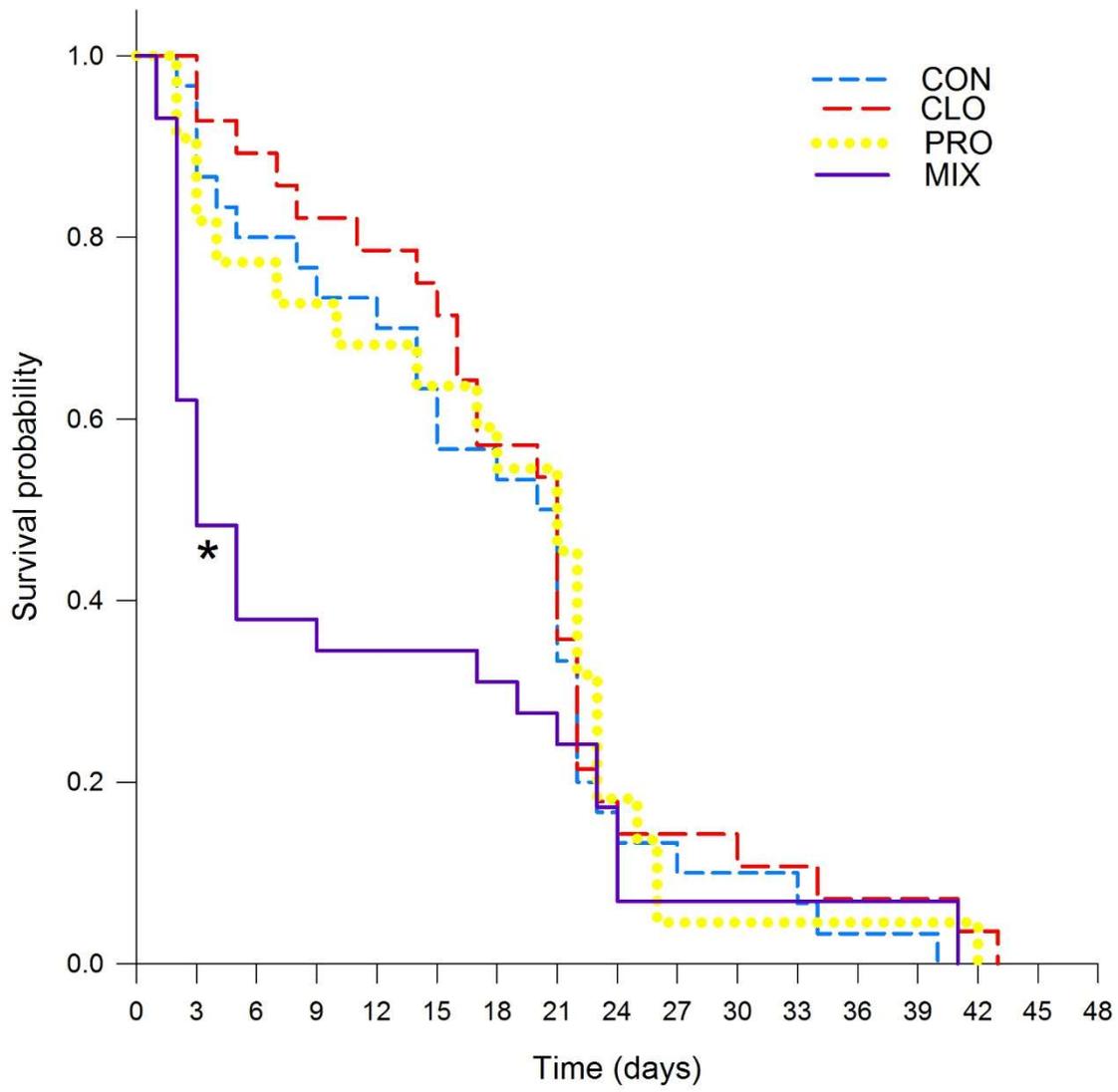
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