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Fabio Sgolastra[1*], Xavier Arnan[2], Riccardo Cabbri[3], Gloria Isani[3], Piotr Medrzycki[4], Dariusz Teper[5], Jordi Bosch[2]

[1]Dipartimento di Scienze e Tecnologie Agro-Alimentari, *Alma Mater Studiorum* Università di Bologna

[2]CREAF, Universitat Autònoma de Barcelona, Bellaterra, Spain

[3]Dipartimento di Scienze Mediche Veterinarie, *Alma Mater Studiorum* Università di Bologna

[4]CREA-Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agrari, Centro di Ricerca Agricoltura ed Ambiente, Bologna, Italy

[5]Research Institute of Horticulture, Apiculture Division, Puławy, Poland

Abstract

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

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

1. Introduction

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

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

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

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

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

2. Material and Methods

(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 Protil ® 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 Protil ® 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*

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

(d) Experiment 1

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

(e) Experiment 2

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

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

Experiment 2

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

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

4. Discussion

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

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

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

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

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

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

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

Our study shows that a single meal with a cocktail of pesticides at sublethal doses and realistic concentrations during the pre-nesting period affects feeding behavior, ovary maturation and longevity in a solitary bee. Importantly, none of these effects were observed when bees were 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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Figure 1. Mean + SE test solution ingested during the 4-hour exposure phase in *O. bicornis* females orally exposed to four treatments (CON: control; CLO: clothianidin; PRO: propiconazole; MIX: clothianidin + propiconazole mixture). Different letters denote significant differences (Fisher LSD Post-hoc, $P<0.05$).

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

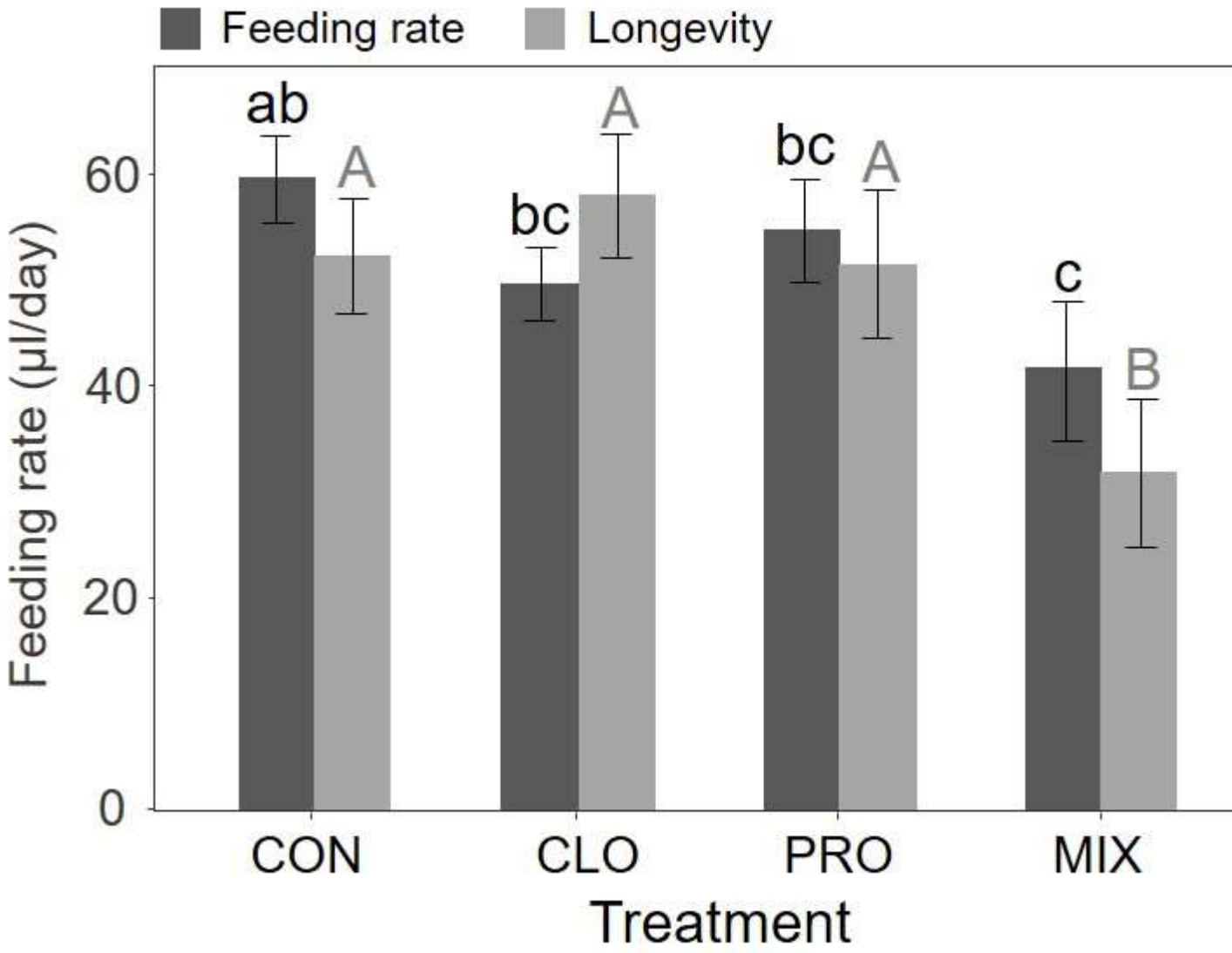
Figure 3. Experiment 1 - Cumulative survival probability of *O. bicornis* females orally exposed to four treatments (CON: control; CLO: clothianidin; PRO: propiconazole; MIX: clothianidin + propiconazole mixture). Synergistic interactions between CLO and PRO treatments ($P <0.05$; one-tailed binomial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

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

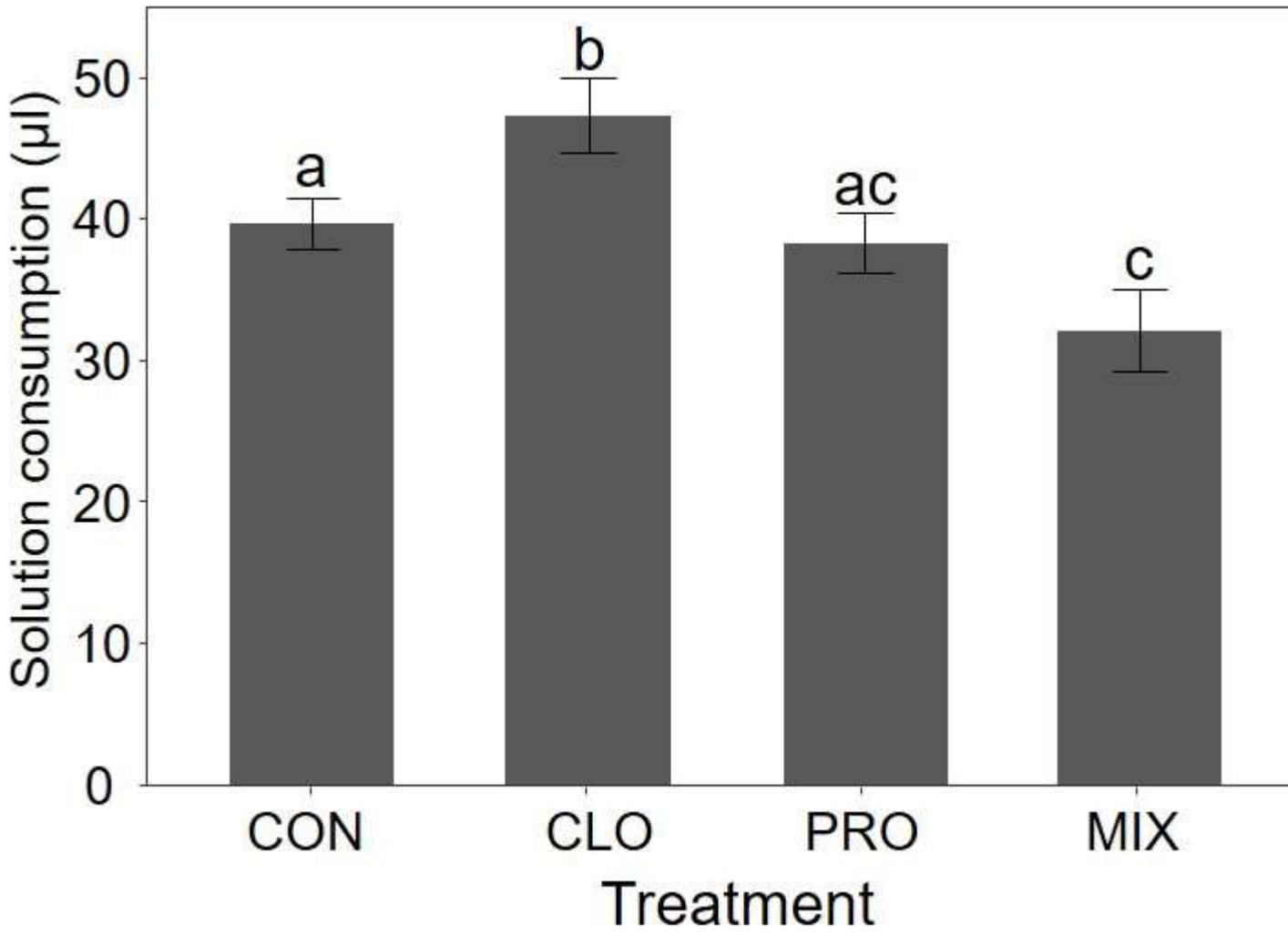
Figure 5. Experiment 2 - Cumulative survival probability of *O. bicornis* females orally exposed to four treatments (CON: control; CLO: clothianidin; PRO: propiconazole; MIX: clothianidin + propiconazole mixture). Synergistic interactions between CLO and PRO treatments ($P <0.05$; one-tailed binomial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

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

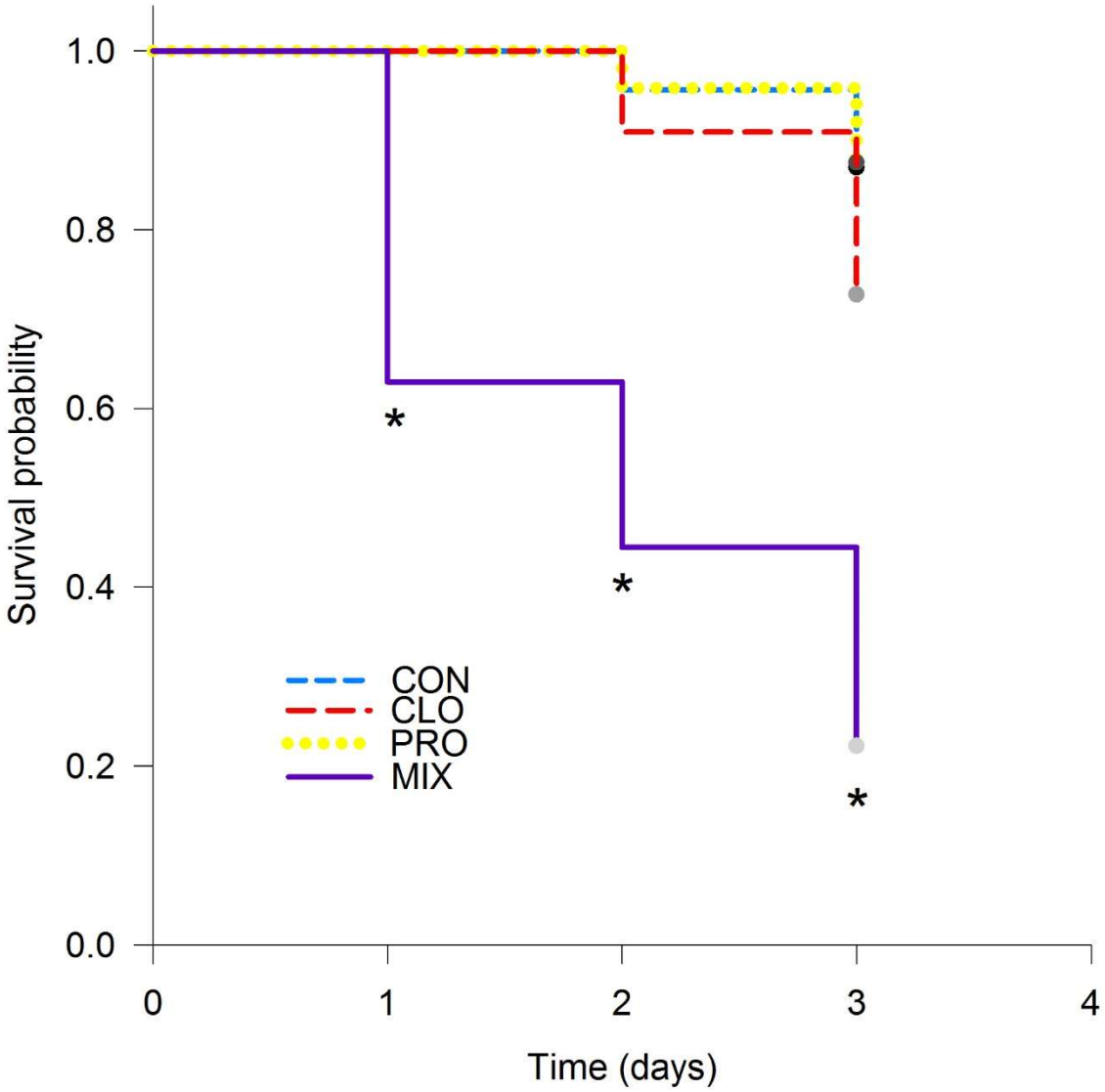
	Response variable		***Model components***	***AIC_c***	***ΔAIC_c***	***w_i***	***R^2 (%)***
Experiment 1	Exposure feeding	1	**Tr** + ET (+) + **HS (+)** + **Tr:ET**	1376.7	0.00	0.592	22
		2	**Tr** + **HS (+)**	1378.4	1.73	0.249	17
	Post-exposure feeding rate	1	*Tr* + **ET (-)** + **HS (+)**	707.1	0.00	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


Feeding rate
Longevity
ab
A
bc
A
bc
A
c
B
60
40
20
0
Feeding rate (µl/day)
CON
CLO
PRO
MIX
Treatment

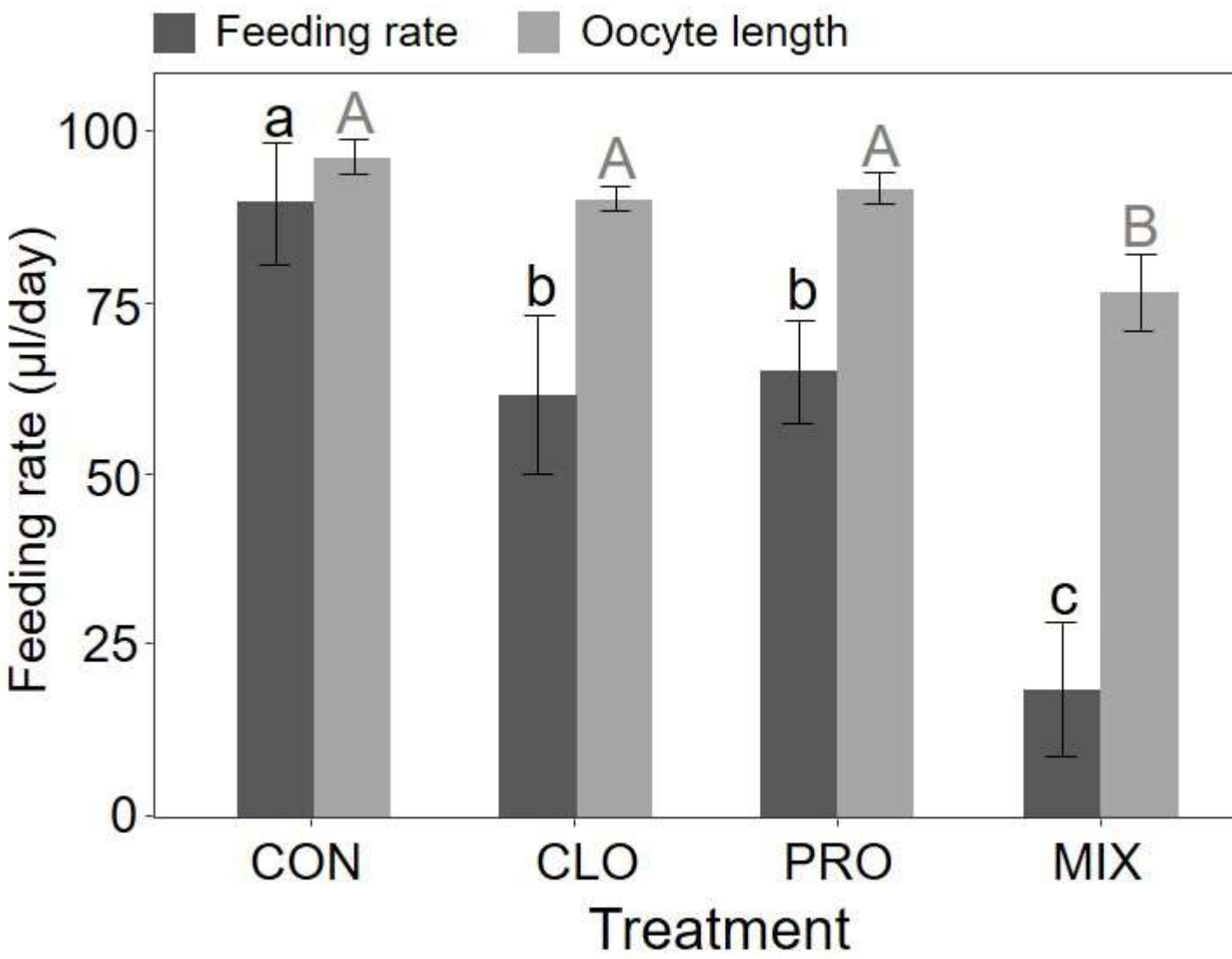
604


Solution consumption (µl)
0
10
20
30
40
50
a
b
ac
c
CON
CLO
PRO
MIX
Treatment

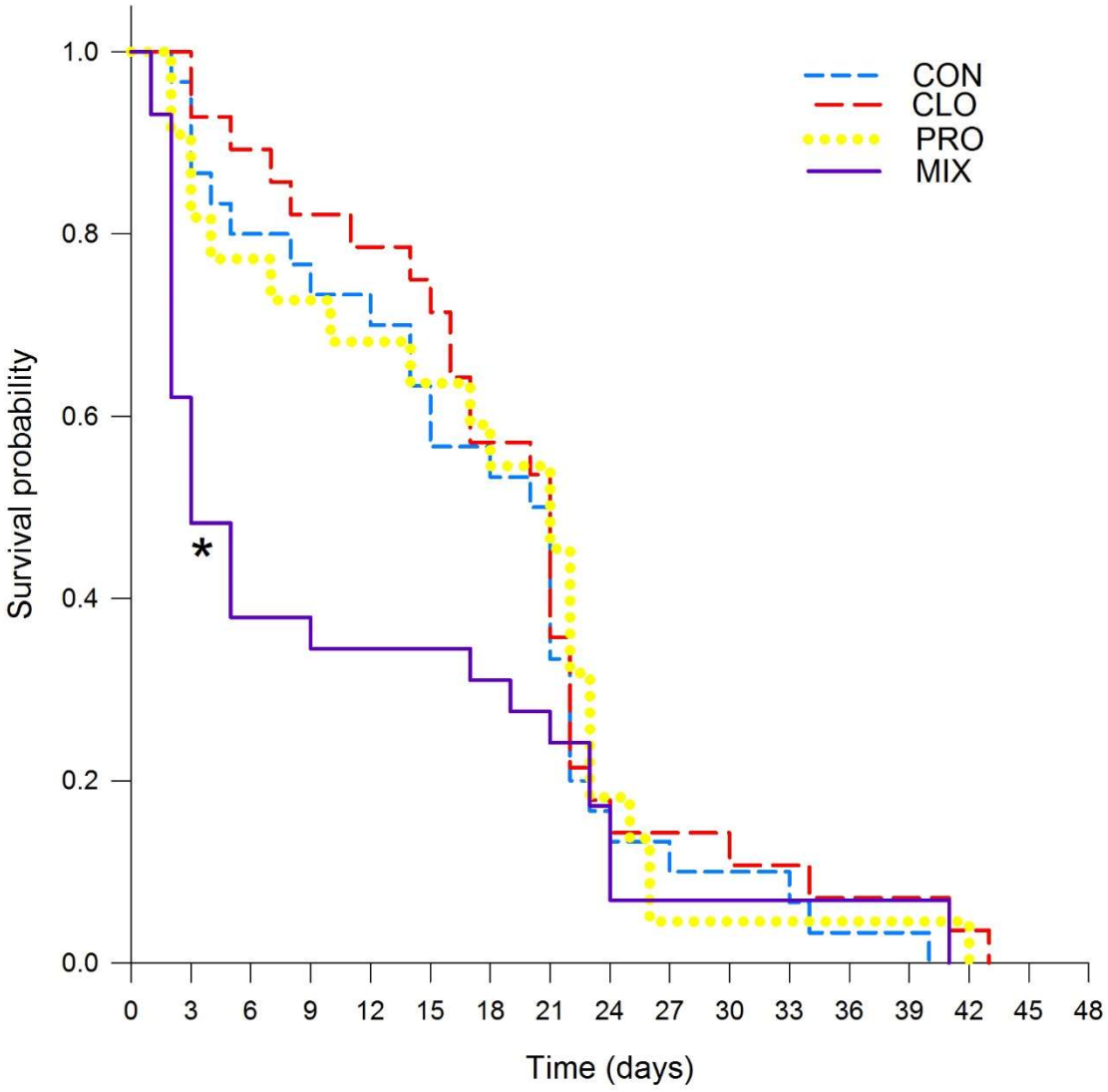
605


1.0
0.8
0.6
0.4
0.2
0.0
Survival probability
0
1
2
3
4
Time (days)
CON
CLO
PRO
MIX
*
*
*

606


Feeding rate
Oocyte length
Feeding rate (µl/day)
100
75
50
25
0
a
A
b
A
b
A
c
B
CON
CLO
PRO
MIX
Treatment

607


CON
CLO
PRO
MIX
Survival probability
1.0
0.8
0.6
0.4
0.2
0.0
*
0
3
6
9
12
15
18
21
24
27
30
33
36
39
42
45
48
Time (days)

608