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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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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. In this paper, 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. By 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.

1. Introduction

The last decades have seen significant declines in wild bee diversity at local and regional scales [1–3], together with abnormal honeybee 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, inasmuch 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 honeybees 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 honeybee, *Apis mellifera* [16,22]. However, there is increasing evidence that solitary bees (*Osmia bicornis*) are more sensitive to certain pesticide treatments than honeybees 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 approximately 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 haemolymph increase in parallel for up to 6 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 lifespan 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

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

(b) Test solution preparation

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

We tested a propiconazole concentration of 62.5 mg l^{-1}. This concentration corresponds to the field application rate of the commercial formulation Protil ® EC (250 g l^{-1} of a.i.) in orchards (25 ml hl^{-1} or 0.25 l ha^{-1}). To obtain this concentration, we prepared a stock solution with a propiconazole concentration of 25 g l^{-1} by dissolving Protil® EC in distilled water. The stock solution was then diluted with 38% w : v (33% w : w) sugar solution to achieve the desired concentration.

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

(c) Exposure phase

Previous studies have shown that upon emergence out of the cocoon, *Osmia* females take about 1 day to come out of their natal nest [38]. Therefore, 24 h after emergence, meconium-free females were individually housed in small plastic cylinders (width: 3.5 cm; height: 5.5 cm) with a transparent plastic lid through which a feeder made with a 1 ml syringe was inserted. Each feeder contained approximately 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 h. 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 was 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; height: 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×. Head size is strongly correlated to body weight in *Osmia* [30]. Sample sizes were approximately 30 bees per treatment.

(e) Experiment 2

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

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

All statistical analyses are described in the electronic supplementary material.

3. Results

(a) Exposure phase feeding

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

(b) Experiment 1

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

Cumulative survival curves differed significantly among treatments (d.f. = 3, $\chi^2 = 12.99$, $p = 0.005$) (figure 3). Throughout the first days following exposure, mortality in the MIX treatment was much 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 (figure 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, figure S2).

(c) Experiment 2

Nectar feeding rate during the 3-day post-exposure phase significantly differed among treatments (table 1). As in

Table 1. Best selected ($\Delta AICc < 2$) 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 ($p < 0.05$) are in bold, marginally significant predictors ($p = 0.05–0.1$) are in italics. Positive and negative signs in parentheses denote the direction of the relationship.

	Response variable		model components	AIC_c	ΔAIC_c	w_i	R^2 (%)
exposure phase	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
experiment 1	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	1	**HS (+)**	123.1	0.00	0.467	27
	(sqrt-transformed)	2	ET (−) + **HS (+)**	123.1	0.03	0.460	31

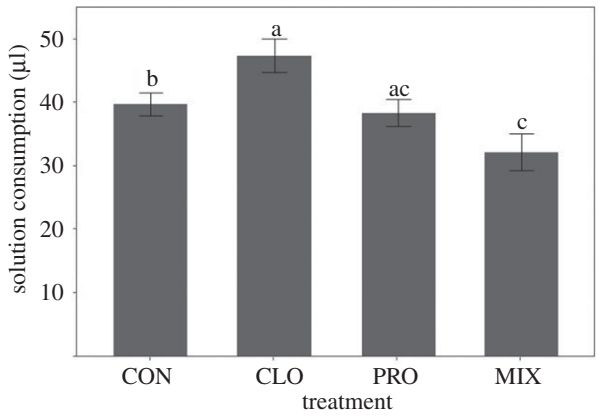

Figure 1. Mean + s.e. test solution ingested during the 4 h exposure phase in *O. bicornis* females orally exposed to four treatments (CON, control; CLO, clothianidin; PRO, propiconazole; MIX, clothianidin + propiconazole mixture). Different letters denote significant differences (Fisher's LSD *post hoc*, $p < 0.05$).

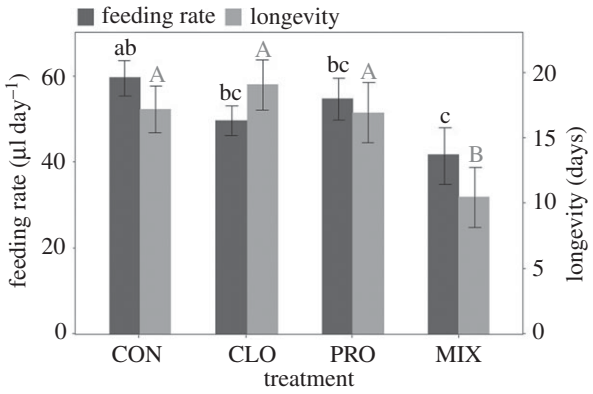

Figure 2. Experiment 1—mean + s.e. post-exposure feeding rate and longevity in *O. bicornis* females orally exposed to four treatments (CON, control; CLO, clothianidin; PRO, propiconazole; MIX, clothianidin + propiconazole mixture). Different letters denote significant differences (Fisher's LSD *post hoc*, $p < 0.05$).

experiment 1, it was highest in the CON treatment and lowest in the MIX treatment (figure 4). In contrast with 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 3 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 (d.f. = 3, $\chi^2 = 45.72$, $p < 0.001$). Survival was again lowest in the MIX treatment (figure 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 (figure 4). Oocyte length 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 honeybee-collected pollen and on bee body surfaces [41,46,47]. In a previous study [18], we showed

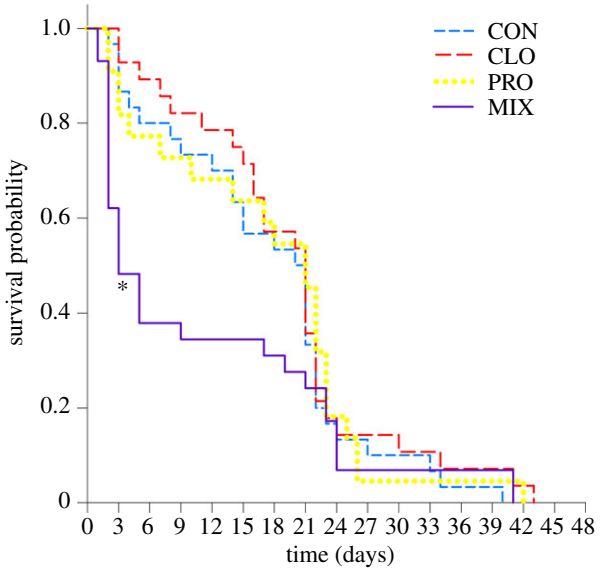

Figure 3. Experiment 1—cumulative survival probability of *O. bicornis* females orally exposed to four treatments (CON, control; CLO, clothianidin; PRO, propiconazole; MIX, clothianidin + propiconazole mixture). Synergistic interactions between CLO and PRO treatments ($p < 0.05$; one-tailed binomial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

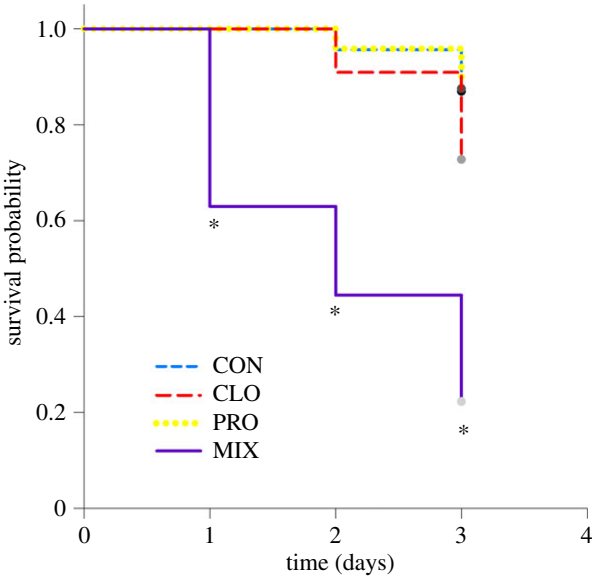

Figure 5. Experiment 2—cumulative survival probability of *O. bicornis* females orally exposed to four treatments (CON, control; CLO, clothianidin; PRO, propiconazole; MIX, clothianidin + propiconazole mixture). Synergistic interactions between CLO and PRO treatments ($p < 0.05$; one-tailed binomial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

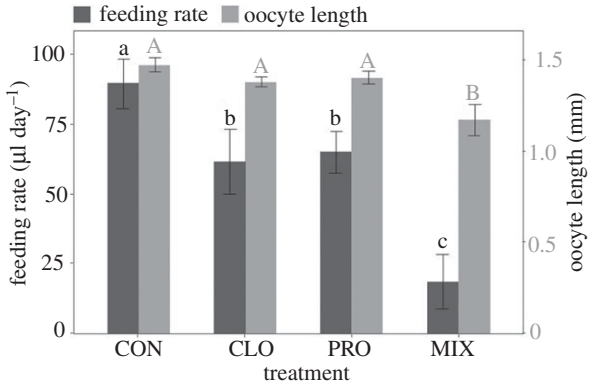

Figure 4. Experiment 2—mean + s.e. post-exposure feeding rate and basal oocyte length in *O. bicornis* females orally exposed to four different treatments (CON, control; CLO, clothianidin; PRO, propiconazole; MIX, clothianidin + propiconazole mixture). Different letters denote significant differences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

synergistic mortality effects in honeybees, bumblebees and solitary bees (*O. bicornis)* acutely exposed to sublethal doses of CLO (0.63 ng bee^{-1}) and PRO (7 µg bee^{-1}) 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^{-1} of CLO) was higher than concentrations likely to be found in nectar (< 0.7–16 µg l^{-1}) [12,24,36,37,48]. On the other hand, considering the honey stomach capacity of honeybees (approximately 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 behaviour, 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 behaviour has also been observed in bumblebees and honeybees [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 (microlitres 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 behaviour 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 haemolymph increases with ovary maturation, reaching maximum levels 3–6 days after adult emergence and gradually declining thereafter [35]. Studies on honeybee and bumblebee queens have reported a strong upregulation of vitellogenin genes [54] but slower ovary maturation following experimental neonicotinoid exposure [52,55]. 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 fed on the pollen supplied in experiment 2, but we could not establish whether pollen consumption differed among treatments because bees spread the pollen over the hoarding cages. At any rate, we did not find differences in vitellogenin concentration or ovary maturation between clothianidin-exposed and control bees. On the other hand, we found that ovary maturation was slowest in bees of the MIX treatment, even if this reduction was not accompanied by increased levels of vitellogenin concentration.

In experiment 1, the longevity of propiconazole- and clothianidin-exposed bees (mean: 17 and 19 days, respectively)

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

The differences between experiments 1 and 2 in survival probability at day 3 were very small for the CON (87 versus 87%) and PRO (82 versus 88%) treatments. By contrast, these differences were very pronounced for the CLO (93 versus 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 3 days. Larger bees also had higher levels of vitellogenin in the haemolymph 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 longer 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 behaviour, 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 solitary bees for two reasons. First, females in poor physiological condition are less likely to start nesting activities and reproduce [33]. Our results show that the nesting success of these weakened females may be further compromised by exposure to pesticide mixtures at realistic field concentrations. Second, fecundity of females that do successfully nest is highly correlated to the duration of the nesting period [33,34], which is constrained by ovary maturation at one end [33,35] and by death at the other end. Our insecticide–fungicide mixture had negative effects on both ovary maturation and longevity, thus affecting the duration of the nesting period at both ends. Under field conditions, *Osmia* females live approximately 20 days on average [34]. Of this time, approximately 5 days are spent maturing the ovaries [35], prior to the initiation of nesting activities (pre-nesting period) [33,34]. During the rest of their lifetime (nesting period), females build and provision nest cells and lay eggs at a rate of approximately 0.7 day^{-1} [34]. If we assume that mean longevities recorded in our study are representative of longevities under field conditions, females of our MIX treatment would have laid a mean of 3.5 eggs compared to 8.4 in control bees. We conclude that our findings have direct repercussions on the reproductive success of solitary bees, and provide a potential mechanism to explain observed negative dynamics of *Osmia* populations in agricultural environments [12,13,65]. Our study has also important implications for pesticide regulation. Current risk assessment schemes rely on tests of single compounds [27,28]. Our results underscore the need to consider pesticide combinations likely to occur in agricultural environments.

Data accessibility. Data available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.895pn6p [66].

Authors' contributions. F.S and J.B. conceived the experiments. F.S, J.B., R.C., G.I., D.T. and P.M. designed the experiments. F.S. and R.C. collected the data. X.A. analysed the data. F.S. and J.B. took the lead in writing the manuscript.

Competing interests. We have no competing interests.

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