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Glyphosate commercial formulation negatively affects the reproductive success of solitary wild bees in a Pampean agroecosystem

Sofia GRAFFIGNA[1,2,3], Hugo J. MARRERO[2,3], Juan P. TORRETTA[4,5]

[1]Departamento de Biología, Bioquímica y Farmacia, Universidad Nacional del Sur, San Juan 670, 8000, Bahía Blanca, Argentina
[2]Laboratorio de Interacciones Bióticas en Agroecosistemas, (LIBA), Bahía Blanca, Argentina
[3]Centro de Recursos Naturales Renovables de las Zonas Semiáridas, CONICET,, Camino de la Carrindanga Km. 7, 8000, Bahía Blanca, Argentina
[4]Facultad de Agronomía, Cátedra de Botánica General, Universidad de Buenos Aires, Av. San Martín 4453, C1417DSE, Buenos Aires, Argentina
[5]Consejo Nacional de Investigaciones Científicas y Técnicas, Buenos Aires, Argentina

Abstract – Glyphosate is the most widely used herbicide worldwide and it could have negative effects on wild bees. We study the effect of glyphosate commercial formulation on the nesting behavior of wild solitary bees (*Megachile* spp.) and the survival of immature stages in a Pampean agroecosystem. In four plots without agricultural management located in an agricultural field, we placed 480 wooden trap-nests. The traps were sprayed with two different concentrations of glyphosate commercial formulation and only with water. The number of cells per nest was significantly lower in glyphosate treated traps compared with the water treatment. The probability of finding breeding cells was two times higher in nests without glyphosate commercial formulation compared with treated nests. Larvae completed their development and emerged as adults approximately four times more in nests without glyphosate commercial formulation, relative to those with glyphosate. Our results indicate that glyphosate commercial formulation could be conditioning the behavior of the nesting females and it is affecting their reproduction.

Agrochemical effects / *Megachile* / Bee survival / Trap nests / Agricultural management

1. INTRODUCTION

The use of land for agricultural purposes transformed nearly 50% of land surface in only 300 years (Ellis et al. 2010). This transformation, product of deterministic factors (such as agricultural expansion and agrochemicals) and stochastic factors (such as natural habitats loss and fragmentation), is the main cause of biodiversity change and loss worldwide (Sala et al. 2000; Tscharntke et al. 2005). Pollination, an important ecosystem service, is affected by these changes (Tscharntke et al. 2005; Potts et al. 2010) and some strategies are used to counteract these effects. For example, the management of crop edges was proposed to increase abundance and diversity of pollinators and thus improve pollination quality (Sáez et al. 2014). Although crops edges have a limited extension, these places are important biodiversity reservoirs (Hendrickx et al. 2007; Billeter et al.

2008; Torretta and Poggio 2013). Crop edges usually have destructive management (Torretta and Poggio 2013), where both herbicides and insecticides are used to control spontaneous plants and non-beneficial arthropods (Gill and Garg 2014; Bartomeus and Bosch 2018). Therefore, it is necessary to establish how current agricultural management can affect the development of pollinator communities, and establish suitable management strategies for crop edges.

The current model of agricultural production uses large amounts of agrochemicals that might directly or indirectly affect pollinator communities and other organisms in agroecosystem such as honeybee (*Apis mellifera* L.) or wild bees (Gary et al. 1972; Kopit and Pitts-Singer 2018; Rosanigo et al. 2020); larvae and adults of toads (Cauble and Wagner 2005, Bernal et al. 2009); and fishes (Marques et al. 2014). Particularly in pollinators, direct effects are associated with the reduction of population abundance, cognitive changes, decreased in longevity and in visual abilities, among others (Boily et al. 2013; Herbert et al. 2014). On the other hand, indirect effects are associated with reduction of availability of floral and nesting resources (Devillers et al. 2002). Although several studies reported these effects, the majority were conducted in the laboratory and with managed pollinators such as honeybees or different species of *Bombus* (Mullin 2015) with few examples including wild bees. However, the recommended agrochemical dose may not have effects on managed bees (Thompson et al. 2014), but may be lethal in wild bees which are less adapted to agricultural management. Recently, Rosanigo et al. (2020) reported a diminished reproductive success of *Megachile* (*Pseudocentron*) *gomprenoides* Vachal in built nests in agricultural lots (principally: soybean) compared with built nests in semi-natural areas paired. For this reason, studies *in situ* and with wild bees become indispensable.

Glyphosate (N-phosphonomethyl-glycine, $C_3H_8NO_5P$, CAS 1071-83-6) is a broad-spectrum herbicide. In the case of glyphosate (and other agrochemicals), active ingredients are combined with other ingredients (e.g., adjuvants) to create the commercial formulae (Chaufan et al. 2014). An adjuvant is generally broadly defined as any substance separately added to a pesticide product

that will improve the performance of the product (Coalova et al. 2014). Glyphosate commercial formulation (hereafter: GLY-CF) is mainly used to keep crops edges free of spontaneous weeds and to clear land for crop production. In addition, GLY-CF is used in urban and peri-urban areas for the same purposes since it is a chemical that is economically cheap, easy to use and find in agrochemical markets (Abraham et al. 2018). This herbicide affects honeybees and could affect pollinators that use these semi-natural habitats as refugia and/or nesting sites (Kopit and Pitts-Singer 2018, but see Thompson et al. 2014). Sublethal doses of glyphosate deteriorate cognitive functions in honeybees, leading for example a taste decrease (Eiri and Nieh 2012), and infield-realistic doses damage the recovery of memory acquired during exploratory orientation flights (Balbuena et al. 2015). Ingestion of glyphosate at lower concentrations than those recommended by the manufacturer produced disorientation and deterioration of associative learning in workers of *A. mellifera* (Herbert et al. 2014; Balbuena et al. 2015). On the other hand, larvae of *A. mellifera* exposed to sublethal doses of glyphosate showed a delay in their development (Vázquez et al. 2018). Also, a recent study showed an increase in worker mortality of two species of social bees *A. mellifera* and *Hypotrigona ruspolii* (Magretti) (Apidae, Meliponini) after contact with plants sprayed with GLY-CF (Abraham et al. 2018). Despite the information available on the effects of glyphosate on managed bees, there are no studies that demonstrate the consequences of the use of this herbicide on solitary wild bees, which are relevant in crop pollination (Garibaldi et al. 2013).

The present study focused on trap nesting wild bees belonging to the genus *Megachile* (Megachilidae). All *Megachile* species are solitary and some of them build their nests in preexisting cavities in wood and hollow stems, among others (Michener 2007). Some studied species in the Pampean region use plant materials such as pieces of leaves, petals, and even chewed leaves for construction of brood cells or nest conditioning (Torretta and Durante 2011, Torretta et al. 2012, 2014). The number of brood cell per nest is variable and in each of them the females place a mass of pollen and one egg (Michener 2007). The aim of our study was to evaluate the

effect of GLY-CF on the nesting behavior of solitary bees in a Pampean agroecosystem. In addition, we estimated the survival of immature stages (larvae, prepupae, and pupae) reared in trap nests exposed to different concentrations of GLY-CF. Our hypothesis is that GLY-CF negatively affects the reproductive success of wild bees in agroecosystems through a reduction in the survival rate of immature stages. Our predictions are as follows: (i) female bees will indistinctly trap nests sprayed and not sprayed with GLY-CF (i.e., the females do not recognize the GLY-CF–impregnated trap nests); (ii) fertile females will build fewer brood cells per nests sprayed with GLY-CF than those not sprayed; and (iii) there will be a negative relationship between survival of immature stages and the GLY-CF concentration applied to each trap nest.

2. MATERIALS AND METHODS

2.1. Study area

The study was carried out in an agroecosystem located in the Pampean region (S 35° 56′, W 61° 11′, Buenos Aires province, Argentina). The Pampean region is a grassland turned into cultivated fields at rapid rate, affecting both landscape structure and land use patterns (Medan et al. 2011). The landscape is a mosaic of crop fields, and in lesser proportion sown pastures and semi-natural grasslands used for livestock grazing (Tognetti et al. 2010; Marrero et al. 2014) with scarce and small (agriculturally) unmanaged areas (henceforth restored fragments). The use of herbicides and pesticides is frequent in agricultural fragments which are predominant in this agroecosystem. Restored fragments (size range: 0.5–3 ha) consisted of permanently fenced enclosures which had not been cultivated or grazed for the last 20 years and were therefore considered noncropped fragments (Tognetti et al. 2010).

2.2. Sampling design

The survey was carried out during monthly visits from December 2017 to April 2018. Four restored fragments were selected based on the great abundance of pollinators present in these sites, considering data obtained from a previous study (Rosanigo 2017; Rosanigo et al. 2020). Fragments whose areas vary between 0.5 and 1 ha (Pompozzi et al. 2019) were immersed in an agricultural matrix. In these semi-natural areas, vegetation is a grassland dominated by exotics grasses and some entomophilous plant species (Marrero et al. 2014; Rosanigo 2017). The restored fragments were separated by more than 1000 m in order to enhance their independence. At each restored fragment, 120 trap nests (40 by treatment, totaling 480 in all fragments) were placed in packages of eight traps each, containing two traps of four different diameters (6, 8, 10, and 12 mm). Each trap nest consisted of a $2 \times 2 \times 12$ cm block of wood with a 9 cm deep hole (Figure 1) (for a comparable approach see Dorado et al. 2011). Fifteen trap nest packages were placed per restored fragment and five of them were used in each treatment (normal, saturated, and control). The packages of trap nests of each treatment were separated by approximately 25 m within each restored fragment, and inside each treatment, packages were separated by a distance of two meters (Figure 1).

The treatments consisted of trap nests sprayed with two different concentrations of glyphosate commercial formulation Roundup® ControlMax: the "normal" treatment consisted of trap nests sprayed with the manufacturer recommended concentration (8 g GLY-CF /l distilled water), "saturated" treatment in which the trap nests were sprayed with a solution with twice the recommended concentration (16 g GLY-CF /l distilled water), and the "control" treatment consisted of trap nests sprayed only with distilled water. We used distilled water to eliminate the possible effects of the chemical components of tap water, as arsenic, which is present in water at our study site (Minaverry and Cáceres 2016). GLY-CF's doses were calculated based on normal field rates (CASAFE 2020). We used the saturated dose as a treatment in the present study, because untrained and/or poorly trained people usually use doses that largely exceed the normal dose (Abraham et al. 2018). Although it is not the recommended dose, it could be realistic.

In December 2017, the trap nest packages were installed and sprayed with distilled water,
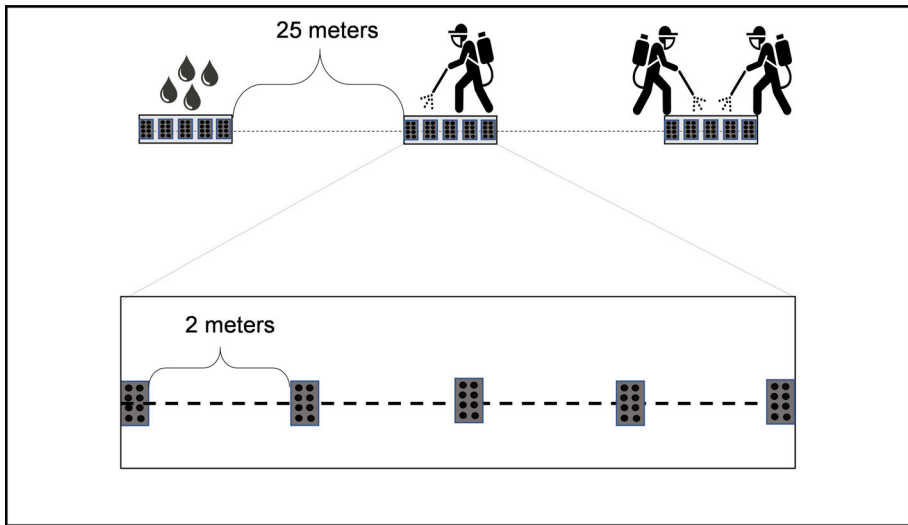
Figure 1. Scheme of disposition of traps nest in each restored fragment. The packages of trap nests of each treatment were separated by approximately 25 m within each restored fragment, and inside each treatment, packages were separated by a distance of two meters.

"normal" and "saturated" GLY-CF. For this purpose, we used different sprayers, one for each treatment, taking all precautions required to handle the herbicide. The trap nests packages were sprayed completely, covering the entire external surface with the solution. Approximately 1 l of solution was used for the packages of each treatment with glyphosate (1000 ml/20 packs = 50 ml/pack and 50 ml/8 nest traps = 6.25 ml/trap nest) and the same volume of distilled water for the traps nest control treatment. This was repeated in January, February, and March 2018.

In each visit, each trap nest was checked to register the presence/absence of bee-constructed nests (Table S1). Subsequently, each trap nest was sprayed again, in the same way as in the first visit and regardless of whether it contained bee-constructed nests or not, in order to simulate what actually happens in a field under agricultural management (Figure 2). Currently, GLY-CF could be sprayed 1–3 times or more after the crop has emerged, leaving the crop unharmed but controlling all actively growing weeds (Benbrook 2016). Finally, during the last visit to the study site (April 2018), all nest traps were collected and taken to the laboratory. In the lab, the traps were opened to record the number of bee-constructed nests per treatment and the number of brood cells present in each

constructed nest. The brood cells of each bee-constructed nest were individually placed in plastic tubes with a cotton plug inside boxes under laboratory conditions until adult emergence. Each plastic tube was labeled with the nest number, position of the cell within the nest (from 1 to n, with 1 being the first constructed cell), and "control," "normal," and "saturated" treatment. When the adults emerged, they were sacrificed and mounted on entomological pins for further determination. Eighteen months later, we opened the remaining cells and confirmed that larvae were dead.

2.3. Analysis

Bee-constructed nests were evaluated using data from all the traps. The probability of finding a nest (0 = without nest, or 1 = with nests) was considered. A nest was considered to be bee-constructed if it contained at least one brood cell. For the brood cells, the data of cells quantity in all traps (with and without nests) were used, considering a value of 0 for traps without nest. Finally, survival was estimated using only data from traps with nest, such as the number of cells that completed their development and resulted in emerged adults per nest. We obtained a bee survival value in each trap that contained at least one cell.
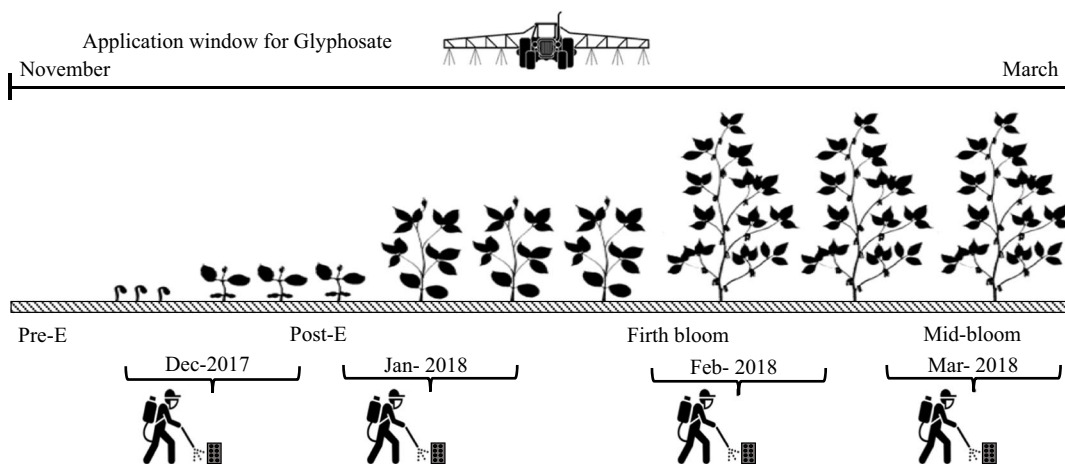
Figure 2. Scheme of application of GLY-CF on trap nest. The application window for GLY-CF in summer season crops in Pampas extends from the pre-emerged (pre-E) until the mid-bloom. To simulate the applications that occur in agricultural management, we sprayed traps-nest at four different times: the first two during the post-emerged (post-E) and the others during firth-bloom and mid-bloom. Our applications were intentionally synchronized with the fumigation of the associated crops.

Generalized linear and mixed models (GLMMs) were used to evaluate the bee-constructed nests, the number of brood cells, and the survival of immature stages (response variables). The R statistical software (R Development Core Team 2013) was used, with the glmer function of the lme4 package, with a binomial distribution for nest and survival and negative binomial distribution for number of cell (Table I). In the models, the concentrations of herbicide (normal, saturated, and control) were used as fixed factor (categorical variable), while the identity of the restored fragments, the package, and the diameter of the trap were used as nested random factors (Table I). *Megachile* species were not used as a random factor because one of the two species was very abundant and explained most of the variance. Overdispersion test was estimated with the dispersion glmer function of package blmeco, while the goodness of the models fit was performed using the DHARMa packages in R (R Development Core Team 2013).

3. RESULTS

A total of 43 bee-constructed nests were found (occupation of approximately 9% of the total traps), of which 20 corresponded to control, 11 to normal concentration, and 12 to saturated concentration of GLY-CF (Table S1). In total, 140 brood cells were found, 84 of which corresponded to the control, 30 to the normal, and 26 to the saturated treatment. The nests were occupied by two species of bees *Megachile* (*Pesudocentron*) *gomphrenoides* and *Megachile* (*Dactylomegachile*) sp. The most abundant species was *M. gomphrenoides* with more than 90% of the nests occupied by this species.

Models did not show overdispersion and presented an appropriate goodness of fit (Table I; Supplementary material, Fig. S1). No significant differences were found in the probability that a trap nest was occupied among the different treatments (ChiSquare = 4.502; p = 0.105) (Table II). However, significant differences were found in the number of built brood cells control vs. normal and saturated treatments (ChiSquare = 6.8693; p = 0.03224). The number of brood cells in a nest without GLY-CF is more than twice in relation to the treated nests (Figure 3). Finally, survival of immatures stages also showed significant differences between treatments with GLY-CF and the control (ChiSquare = 11.248; p = 0.0036). The survival of the immature stages in the control nests was 52%, while the nests treated with GLY-CF had a survival of 14% for the normal concentration and approximately 4% for the saturated one (Figure 4).

Table I. Models used for the analysis. Response variables: nests, cells, and survival. Random factors: identity of the restored fragments (site), the group of traps (package), and the hole trap diameter (diameter). These variables were nested (1|site/package/diameter). Fixed factors: herbicide concentration (treatment). The overdispersion values (dispersion_glmer function of package blmeco) were computed as the square root of the penalized residual sum of squares divided by n, the number of observations. This quantity may be interpreted as the dispersion factor of a binomial and Poisson mixed model

Model	Formula	Distribution	Overdispersion
M1	nests~ (1|site/package/diameter) + treatment	Binomial	0.658
M2	cells ~ (1|site/package/diameter) + treatment	Negative binomial	0.505
M3	survival ~ (1|site/package/diameter) + treatment	Binomial	0.831

4. DISCUSSION

Results show that our hypothesis related to the negative effects of GLY-CF on nesting biology of *Megachile gomphrenoides* and *Megachile* sp. was plausible. In accordance with our predictions, the probability of a female bee building a nest was not related to the use of GLY-CF, and both the number of brood cells per nest and the survival of the immature stages (larvae, prepupae, or pupae) were greater in nests constructed in GLY-CF free traps. Our results suggest that females of these bees select their nesting sites without considering the presence/absence of GLY-CF. However, the total number of brood cells in the nests varies depending on GLY-CF presence in nesting site. This result could be related to a cognitive failure of nesting females that, exposed to GLY-CF, fail to integrate the spatial information, and consequently make erroneous foraging trips. Therefore, it is difficult for these females to return to their nest, as it was reported for workers of honeybees (Boily et al. 2013; Balbuena et al. 2015). In this way, females of *Megachile* spp. could have established a nest and built one or few brood cells and then abandoned it, because they did not manage to return to the nest as a consequence of erroneous forage trips. Another possible explanation (non-mutually exclusive) is that adults in

Table 2. GLMM outputs. The table shows the outputs of generalized linear mixed-effects models with binomial (nests and survival) and negative binomial (cell) errors for the different response variables. There was one fixed factor: "treatment" (control, normal, and saturated); and three nested random factors: "site" (identity of the restored fragments), "package" (the group of traps) and "diameter" (the hole trap diameter)

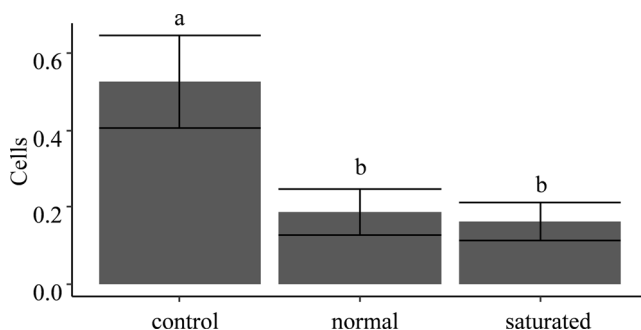|	Estimate	Standard error	Z value	Probability ($> |z|$)	Significance
M1: nests					
Intercept	− 2.9608	0.8272	− 3.579	0.000345	***
Normal	− 0.8395	0.4459	− 1.883	0.059715	
Saturated	− 0.7247	0.4359	− 1.663	0.096372	
M2: cells					
Intercept	− 1.3712	0.8236	− 1.665	0.0959	
Normal	− 1.1779	0.5418	− 2.174	0.0297	*
Saturated	− 1.3829	0.5742	− 2.408	0.0160	*
M3: survival					
Intercept	− 0.6696	0.1875	− 3.571	0.000356	***
Normal	− 1.3453	0.5644	− 2.384	0.017136	*
Saturated	− 2.5885	1.0362	− 2.498	0.012484	*

* 0.01, ** 0.001, *** 0.0001

Figure 3. Number of built brood cells in nest sprayed with distilled water (control) or with different concentrations of GLY-CF (normal and saturated). The figure shows the mean (± SD) number of brood cells per nest under different treatments.

contact with GLY-CF die before finishing their nests, as reported for social species (Abraham et al. 2018). It is possible that GLY-CF could be impregnated in the wood and affects females by direct contact. However, no dead adult bees were found inside the nests (although they might have died outside them). Also, numerous studies suggest that glyphosate and other agrochemicals intervene directly in the intestinal microbiota of bees, causing a reduction in bee survival (Dai et al. 2018; Motta et al. 2018; Vázquez et al. 2018; Blot et al. 2019).

The survival of immatures stages (i.e., the probability of emerging as adult) was four times higher in the GLY-CF free nests than ones treated with GLY-CF. This result could be explained by many studies carried out for other bee species. A similar trend of mortality was observed in a recent study under lab conditions in which Abraham et al. (2018) recorded about 25% of mortality in *A. mellifera* during the

first 24 h of contact with plants sprayed with GLY-CF. Mortality increased to 75% as so did the GLY-CF concentration (Abraham et al. 2018). In another study, Dai et al. (2018) showed that the survival of the immature stages was lower for individuals fed diets with GLY-CF, also causing the decrease in larval weight. Vázquez et al. (2018) found that immature stages of *A. mellifera* that ate food with traces of glyphosate had a higher proportion of larvae with delayed molt and weight reduction, which could lead to a possible reduction in bee survival. However, contrary to these results, Thompson et al. (2014) found no significant effects on the survival, development, and average weight of larvae of *A. mellifera*, nor did they observe significant mortality in adult bees under different concentrations of GLY-CF.

Although all the studies cited here were carried out under laboratory conditions and using different
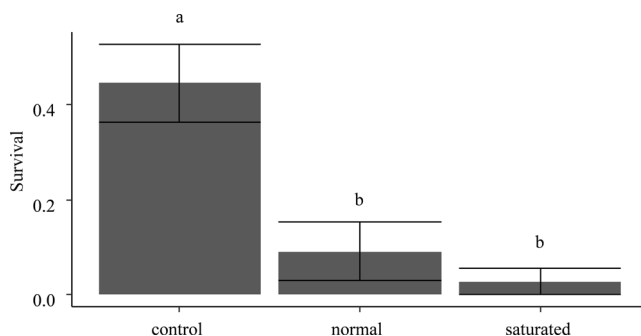

Figure 4. Survival (measured as emerged adults/constructed cells) of immature stages in treatments without (control) and with GLY-CF (normal and saturated). The figure shows the mean (± SD) survival of immature stages per nest under different treatments.

concentrations of agrochemicals, some of these recommended by the manufacturer, it is believed that in agricultural management recommendations are not (always) followed. The GLY-CF applications are made in many crops in excess, besides being applied by persons not trained in its use (Abraham et al. 2018). For this reason, we suggest field experimentation to evaluate in more realistic way agrochemicals effects on wild bees. It is possible that the combination of glyphosate with other agrochemicals may have negative synergistic effects on wild bees, as well as vary according to the entry route (Boily et al. 2013; Kopit and Pitts-Singer 2018). Moreover, the toxicity of the glyphosate formulation could be given not only by the active component but also by the presence of adjuvants, as observed by Chaufan et al. (2014) in their study with human cells. Coalova et al. (2014) demonstrated that the addition of an adjuvant to glyphosate formulation increases the toxicity of the mixture in cell human culture. On the other hand, in agroecosystems, the GLY-CF is prepared in tap water (in a different way to what was done here, where we used distilled water). Therefore, if the chemical components of tap water had any harmful effect on the bees, we would not be registering it. However, our results were similar to those reported by Rosanigo et al. (2020), who registered a higher mortality rate by unknown causes (i.e., not parasitism) of *Megachile gomphrenoides* in nests built in agricultural lots fumigated with GLY-CF prepared in tap water compared with semi-natural areas. These authors suggest that such results could be due to the use of agrochemicals (Rosanigo et al. 2020).

As a conclusion, it is important to emphasize studies like this one, since it is possible to use the results as a model to compare with other insects (Sgolastra et al. 2018). Our results, based only on obtained data in one study site and 1 year, suggest that the effects of GLY-CF are negative for these leaf cutter bee species. These results indicate an even greater risk compared with social species such as *A. mellifera*, because solitary bees in temperate areas exhibit uni- or bivoltine life cycles (Torretta et al. 2012, 2014) and the number of eggs laid by female bees is never more than two per day (Sgolastra et al. 2018). In addition, solitary bees have prolonged larval development and require

different sites and materials for nesting (Torretta and Durante 2011, Torretta et al. 2012, 2014, Sgolastra et al. 2018). Likewise, the loss of a solitary female bee involves the loss of her whole offspring (Kopit and Pitts-Singer 2018). Finally, it is important to highlight the importance of species of native pollinators over the exotic managed species. Several studies suggest that, under favorable circumstances, native bees provide a service equivalent to honey bees for crops with high pollination requirements (Kremen et al. 2002; Garibaldi et al. 2013; Sponsler et al. 2019). In turn, native bees better cushion environmental fluctuations and are less vulnerable to outbreaks of different diseases (Kremen et al. 2002). On the other hand, they promote the persistence of native plants, which would allow slightly reducing the homogenization of landscapes (Garibaldi et al. 2013).

AUTHORS' CONTRIBUTIONS

SG, HJM, and JPT conceived this research and designed experiments; HJM and JPT wrote the paper and participated in the revisions of it. All authors read and approved the final manuscript.

La formulation commerciale du glyphosate affecte négativement le succès de la reproduction des abeilles sauvages solitaires dans un agroécosystème pampéen.

Effets agrochimiques / *Megachile* / survie des abeilles / nids-pièges / agriculture.

Eine kommerzielle Glyphosphat-Formulierung hat einen negativen Einfluss auf den Reproduktionserfolg solitärer Bienen in einem Pampa-Ökosystem.

Agrochemie-Effekte / *Megachile* **/ Überleben von Bienen / Nistfallen / Landwirtschaft.**

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