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Melina Paola Michlig, Adriana Cecilia Pacini, Julieta Merke, Emanuel Matías Orellano, Romina Brasca, et al.. Sublethal exposure to imidacloprid in commercial *Apis mellifera* colonies in early spring: performance of honey bees and insecticide transference between in-hive products. *Apidologie*, 2023, 54 (2), pp.15. 10.1007/s13592-023-00993-2 . hal-04476999

HAL Id: hal-04476999

<https://hal.science/hal-04476999>

Submitted on 26 Feb 2024

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Sublethal exposure to imidacloprid in commercial *Apis mellifera* colonies in early spring: performance of honey bees and insecticide transference between in-hive products

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Received 3 May 2022 – Revised 5 December 2022 – Accepted 9 January 2023

Abstract – Honey bees have an important role in ecosystems as pollinators. However, in recent years, bee populations have declined, with habitat destruction, pesticide use, and climate change contributing to the decline. One of the most important risk factors is the use of neurotoxic pesticides, such as neonicotinoids. The aim of this work was to study the effects of imidacloprid in commercial *Apis mellifera* L. colonies artificially fed with syrups spiked with this insecticide and its possible transfer among in-hive products such as honey and larvae. For this purpose, 30 colonies were placed in the same apiary; once a week for 7 weeks in early spring, each colony was fed with 0.5 L of syrup with the following doses of imidacloprid: 0, 15, 30, 120, and 240 $\mu\text{g kg}^{-1}$. The colony strength was evaluated by monitoring: the number of adult bees and brood combs, queenlessness, unhealthy colonies (by detection of *Nosema* spp. spores and European foulbrood), as well as pollen and honey storage. Worker bees, larvae, honey, and beeswax were sampled to evaluate imidacloprid transfer within the hive. Trends in the persistence of the compound showed that up to 60% of the parent (not metabolized) was stored in honey, and the absence of residues in the larvae suggests that they were not exposed. Another result showed a certain impact in the honey reserves and honey yield with a reduction of this resource in the colonies exposed to imidacloprid.

Imidacloprid / *Apis mellifera* L. / Sublethal doses / Colony strength / Residues

1. INTRODUCTION

In the last four decades, Argentina has undergone an agricultural expansion process promoted by the increase of transgenic crops, no-tillage,

and a higher use of fertilizers and agrochemicals (Viglizzo and Jobbágy 2010). One of the main problems related to the use of pesticides is the detrimental effects on non-target organisms, such as bees.

Honey bees, *Apis mellifera* L. (Hymenoptera: Apidae), have an important ecological and economic value around the world. Apart from the contribution to global food supply, humans also benefit from a wide range of beehive products such as honey and pollen (Klein et al. 2007;

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Manuscript editor: Peter Rosenkranz.

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Steele and Shardlow 2019). In the mid-1990s, Argentina ranked third in the world honey market (FAOSTAT 2021). During the 2005/2006 season, a peak volume of honey production of 104,000 tons was reached, but in the subsequent years, a notable reduction in honey production was observed, with decreases of up to 50% in 2015 compared to the 2005/2006 season (FAOSTAT 2021).

This decrease in honey production correlates with a phenomenon that has been happening since the beginning of the twenty-first century; significant honeybee colony losses have been reported mainly not only in the northern hemisphere (Ellis et al. 2010; Neumann and Carreck 2010; Potts et al. 2010; Kulhanek et al. 2017; Gray et al. 2020), but also in South America (Vandame and Palacio 2010; Maggi et al. 2016; Antúnez et al. 2017; Requier et al. 2018). The decline in bee population has promoted several studies about the factors influencing the vitality of the colonies (Potts et al. 2010; Vanbergen 2013). The combined effects of stressors, such as biological, environmental, chemical, and nutritional factors, are reported as having weakened and caused the death of bee colonies (Nazzi and Pennacchio 2014, Di Pascuale et al. 2016, Tong et al. 2019).

One of the most important risk factors is the use of neurotoxic pesticides, such as neonicotinoids (Godfray et al. 2015). These pesticides (e.g., acetamiprid, clothianidin, imidacloprid, nitenpyram, thiacloprid, and thiamethoxam) are widely used for systemic protection of crops against pests. They are relatively small molecules and are highly water-soluble, allowing them to translocate, i.e., they are distributed throughout the plant through sap, which makes them versatile. Neonicotinoids are applied to soil, seed, and leaves for the control of sucking insects (Jeschke et al. 2011). Sixty percent of all global neonicotinoid applications are delivered as seed and soil treatments because these modes reduce drift and are considered “safer” (Bonmatin et al. 2015).

After application of neonicotinoids, their concentrations in soils, waterways, and non-target plants decline rapidly (Bonmatin et al. 2005). However, under some soil conditions, their

persistence might be prolonged, half-lives can exceed a thousand days, and, notably, they can accumulate when used repeatedly (Bonmatin et al. 2005).

As the pollen and nectar collected by bees become contaminated with neonicotinoid residues, so are the bee bread, honey, and beeswax in the beehives (Blacquièrre et al. 2012). This is the main route of exposure for bees to these insecticides, although residues in contaminated water may be an additional pathway of exposure, as bees take water to cool down and prepare liquid food for the brood (Bonmatin et al. 2015).

Among neonicotinoids, imidacloprid was the first compound with widespread use (Jeschke et al. 2011), and actually, it is the most commonly used neonicotinoid in South America (Mitchell et al. 2017), even combined with other pesticides, mostly pyrethroids Camara de Sanidad Agropecuaria y Fertilizantes (CASAFE) 2015. Imidacloprid acts as a cholinergic-nicotinic agonist and affects neuronal processes in the brain, such as olfactory learning of bees (Decourtye et al. 2004; Simon-Delso et al. 2015). It has been shown that the olfactory learning was significantly impaired after chronic oral exposure of bees to imidacloprid ($0.02 \text{ ng } \mu\text{L}^{-1}$) for 11 days (Li et al. 2019). Also, the genes encoding major royal jelly proteins, which play critical and multifunctional roles in the physiology, development, and colonial extension of honey bees, were strongly downregulated after the sublethal exposition of bees to this insecticide (Wu et al. 2017). Based on a report of risk assessment for bees issued by the European Food Safety Authority (EFSA) (EFSA 2018), in 2018, the European Union prohibited imidacloprid application to outdoor crops, to minimize the exposure of bees and other pollinators in the field. However, despite Europe banning three neonicotinoids (imidacloprid, clothianidin and thiamethoxam) in the field, no positive impacts on long-term changes in colony numbers were found with respect to those countries which continued to use them (Moritz and Erler 2016). Although agriculture (both production and export) is the most important economic activity in Argentina, the environmental consequences of the use of neonicotinoids such as imidacloprid are poorly known.

At that time, laboratory-based studies concluded that imidacloprid cause disorder in individual honey bees; however, the effect of imidacloprid at the whole-colony level in the field is uncertain (Blacquiere et al. 2012, Cresswell et al. 2014; Godfray et al. 2015). In social species such as honey bees, the efficient division of labor and coordination of tasks provide a buffering effect against environmental stressors; however, when stressors are frequent, this buffering capacity could have a limit (Klein et al. 2017). Reinforcing these suggestions, Di Noi et al. (2021) conducted a review of the existing literature regarding the type of effects evaluated in *A. mellifera*, collecting information about regions, methodological approaches, the type of contaminants, and honey bees' life stages. They conclude that great majority of examined papers were about adult honey bees, and few papers have investigated the sublethal effects on honey bees in their natural conditions and habitats.

In this context, an open-field feeding study was started in one of the apiaries belonging to the Experimental Station of the National Institute of Agricultural Technology (INTA) placed in Rafaela, Santa Fe, Argentina, to evaluate the impact of chronic exposure of bees to imidacloprid. For this purpose, *A. mellifera* L. colonies were fed with sucrose syrups containing different imidacloprid doses for 7 weeks, from September to October 2014. The effects on the health of the colonies were evaluated with the manifestation of some infections like nosemosis and queen problems. In addition to the assessment of the effects on colony health, less addressed aspects, such as the bioavailability and transfer between in-hive matrices (Benuszak et al. 2017), were also studied. Thus, colony assessment was complemented with the analysis of imidacloprid residues in the different colony components, such as bees, larvae, honey, and beeswax, to explore, for example, if bees stored the pesticide in the honey and wax differentially and/or fed larvae with the contaminant.

2. MATERIALS AND METHODS

2.1. Apiary

Field trials were conducted at the beginning of the honey yield season, during September–October 2014, so as to emulate the exposure of the bees by foraging contaminated pollen and nectar. Therefore, 30 *A. mellifera* colonies located at INTA Rafaela Experimental Station (Santa Fe Province, Argentina, 31°11'49"S 61°29'45"W) were randomly distributed in two parallel lines, two hives per stand and stands spaced 1 m apart, and were submitted to five different treatments: control and T1, T2, T3, and T4, comprising 6 colonies per treatment. The experimental colonies were surrounded by alfalfa fields. At the beginning of the trial, each colony was made up of 10 frames per brood chamber, with 15,000 worker bees and a 1-year-old queen of the same genetic origin. The *A. mellifera* colonies were free of *Varroa* because an organic treatment with oxalic acid was applied before the beginning of the study.

2.2. Imidacloprid dosages

To define the exposure doses for the treatment, we used the concentrations found in nectar and pollen published by several authors (Sanchez-Bayo and Goka 2014; Bonmatin et al. 2015; Lu et al. 2016), previous toxicity laboratory-based assays (EFSA 2013; Laurino et al. 2013) and protocols provided by the Environmental Protection Agency (EPA 2014). The selected doses were 15, 30, 120, and 240 μg of imidacloprid per kg of syrup.

The solid standard of imidacloprid (98.9% purity) used for the experiment was obtained from Sigma-Aldrich (Darmstadt, Germany). Firstly, two primary solutions were prepared in distilled water, one at 200 mg L^{-1} and the other at 20 mg L^{-1} , and were stored in the freezer at $-20\text{ }^{\circ}\text{C}$ until the moment of preparing the sucrose syrups. The highest concentration solution was used to spike the sucrose syrups for T3 and T4 (120 and 240 $\mu\text{g kg}^{-1}$) and the lowest for T1 and T2 (15 and 30 $\mu\text{g kg}^{-1}$). The final

syrups consisted of 2:1 sugar/distilled water (density = 1.326 g mL⁻¹ at 25 °C) and the suitable doses of imidacloprid to achieve the final dosage levels.

From September–October 2014, each colony (6 colonies per treatment) was supplied once a week for seven weeks with sucrose syrup using internal hive feeders. A batch of fresh syrup per concentration was prepared, and a specified volume was weekly supplied to each colony according to its population. Thus, in the first week, the colonies of 15,000 workers were supplied with 500 mL (665 g) of sucrose solution, 0.03 mL/bee/week = 0.04 g/bee/week or 6.3 mg/bee/day. That is 1/15 of the daily consumption of a forager, 1/7 the daily consumption of a brood attendant, and 1/5 the daily consumption of a worker larvae, according to Rortais et al. (2005). The following weeks, the volumes were adjusted according to the development of the colonies to guarantee the doses of 0.03 mL per individual. To verify the correct dosage, imidacloprid concentrations in all the syrups administered weekly were measured by UHPLC-MS/MS analysis.

Each week was verified the total consumption of sucrose solution by colonies. At the end of this 7-week period, honey supers (9 frames per super) were placed on top of a queen excluder to limit queen access and prevent egg-laying activity in the honey supers.

2.3. Sample collection

To evaluate the distribution of imidacloprid between in-hive matrices, a sampling plan was designed taking care to avoid being invasive and disruptive to bees during manual operations. Thus, samples of larvae and worker adult bees were taken from two colonies per treatment during the feeding period of seven weeks. Each week, before the addition of sugar syrups, a total of 100 worker adult bees per sampled colony were removed for further analysis. Additionally, in weeks 1, 3, and 7 (beginning, middle and end of the feeding period), bee samplings were repeated 24 and 48 h after sucrose supplementation to test the capability of honey bees

to metabolize imidacloprid. So, bees from two colonies per treatment were sampled for residue analysis once a week before the addition of syrups and 24 and 48 h after. In the same weeks (1, 3, and 7), 100 brood cells with larvae were also extracted. Two months after the end of the feeding period (December 2014), samples of honey and beeswax were taken from the brood chamber and honey super of all colonies. All samples were stored at -20 °C until analysis.

2.4. Imidacloprid residue analysis

The methodology employed for imidacloprid residue determination was based on QuEChERS method (Anastassiades et al. 2003) and was already developed in a previous analytical work (Michlig et al. 2018). Briefly, 5 g of each sample (honey or bees) was soaked for 1 h with 10 mL of acid water (2% formic acid). Soaking was performed at room temperature for bees and at 50 °C for honey. Then, 10 mL of acetonitrile was incorporated and, to favor the extraction of the analyte, the aqueous phase was saturated by adding MgSO₄ and NaCl salts. Melted beeswax samples (2 g at 80 °C) were also extracted with 10 mL of acetonitrile and subjected to a freeze-out step at -20 °C overnight.

To reduce matrix interferences, all the extracts were subjected to dispersive solid phase extraction through the addition of MgSO₄, PSA, and C18. The purified extracts were taken to dryness and reconstituted in an aqueous phase. All instrumental analyses were conducted on an ultraperformance liquid chromatograph (UPLC) Waters Acquity (Milford, MA, USA) coupled to a triple quadrupole mass spectrometer Waters Micro-mass TQD (Manchester, England). The limits of quantitation (LOQ) were 0.25, 0.5, and 1 µg kg⁻¹ for honey, bees, and beeswax, respectively. Prior to the analysis of samples, the methods were validated in accordance with the guidance document SANTE 11,955/2015 (SANTE 2015).

In addition, to evaluate the percentage of imidacloprid finally stored in the honey, an estimate mass balance was carried out to measure the amount (mg) of imidacloprid that entered

each of the colonies and was finally found in the honey. For that, the mass of imidacloprid that entered was calculated as the concentration of the syrup multiplied by the total kilograms (or mL) supplied during the 7 weeks (Eq. 1). This calculation was done disregarding the imidacloprid coming from the surrounding environment because it is a variable that could not be controlled. The mass stored in honey was calculated as the weight of honey (kg) accumulated in the brood chamber multiplied by its concentration of imidacloprid plus the weight of honey (kg) from super multiplied by its concentration (Eq. 2). These two values, expressed in mg of imidacloprid, allowed us to evaluate the percentage finally deposited in the honey (Eq. 3).

$$m_1 = \sum_{i=1}^7 m_i * C_i \quad (1)$$

$$m_2 = m_{hr} * C_{hr} + m_{hs} * C_{hs} \quad (2)$$

$$\text{Imidacloprid in honey}(\%) = \frac{m_2}{m_1} * 100 \quad (3)$$

where m_1 (mg) is the initial mass of imidacloprid, and m_i (kg) and C_i (mg kg⁻¹) are the amount of syrup supplied per week and the concentration of this syrup, respectively.

m_2 (mg) is the mass of imidacloprid in the honey, m_{hr} and m_{hs} (kg) are the weight of honey from reserves and supers, and C_{hr} and C_{hs} (mg kg⁻¹) are the concentrations of imidacloprid in the honey of these compartments.

2.5. Colony assessment

The possible impact of imidacloprid on colonies dynamics was estimated at the end of the feeding period (October 2014) through the number of worker bees (CCBe), the number of cells with sealed brood (CBRA), and the number of cells with pollen (CCP) and honey reserves (CCH) according to the Liebefeld method (Dainat et al. 2020). The honey supers were

added after the feeding period ended, in October 2014. Each honey super was weighed before being added in the hive and prior to the honey harvest (February 2015); the difference in weight exposed the yield of honey per colony.

During the colony management, complementary inspections were carried out to detect queen replacement and pathogen challenge. *Nosema* spp. spores were counted in adult bees from two colonies per treatment at the beginning, middle, and end of the feeding period. For this purpose, worker honey bees were manually collected at the hive entrance. A minimum of 60 bees were gathered and placed in labeled plastic flasks containing 60 mL of 96° ethyl alcohol. Spore suspensions were prepared by adding 60 mL of distilled water to crushed abdomens of the 60 randomly selected individuals of each colony. *Nosema* spp. spores per bee were determined using light microscopy (40×) (Nikon, China) and hemocytometer. For each sample, the number of spores was counted in 80 hemocytometer squares (5 groups of 16 squares) (Cantwell 1970; Fries et al. 2013). This is the most frequently used sampling method, since it provides information about the number of spores per bee, and can detect 5% of infected bees with 95% of confidence (Fries 1988).

2.6. Statistical analyses

Imidacloprid in honey and beeswax were analyzed with Kruskal–Wallis (K-W) statistic test, because the data were not normal. For imidacloprid analysis in adult bees and larvae, we described the concentration in each week for each treatment. The K-W statistic test was performed to assess the effects of treatments, by comparing the results of each parameter (*Nosema* spp. abundance, CCBe, CBRA, CCP, CCH) with those of the control. Chi-square test was used to compare mortality and queen problems for treatments. All analyses were done using INFostat software version 2011 (<http://www.infostat.com.ar>).

3. RESULTS

3.1. Sample collection

3.1.1. Dietary syrups

The doses of imidacloprid in the dietary syrups were checked by measuring their concentrations in the syrups of all treatments during each of the 7 weeks of feeding. Imidacloprid concentrations remained stable throughout the 7 weeks and were within the expected values of 16 ± 3 , 32 ± 7 , 123 ± 4 , and $244 \pm 35 \mu\text{g kg}^{-1}$ for T1, T2, T3, and T4, respectively.

3.1.2. Adults' bees and larvae

The results indicated that initial adult bees (Sect. 2.1) and those belonging to the control treatment were not exposed to imidacloprid (Table I). As expected, the highest concentrations of imidacloprid were obtained in adult bee bodies from T3 and T4. Anyway, taking into account that adult bees weigh approximately 100 mg, in none of the cases would the concentrations found exceed 3 ng per bee ($30 \mu\text{g kg}^{-1}$). Therefore, the bees would have evidently been exposed to sublethal doses since the reported LD50 varies between 4 and 120 ng per bee

Table I Imidacloprid concentration (μg) per kilogram of adult bee throughout 7 weeks (W) of feeding. Samplings were performed before (t0) and 24 and 48 h after syrup supplementation. The highest for T3 and T4 (120 and $240 \mu\text{g kg}^{-1}$) and the lowest for T1 and T2 (15 and $30 \mu\text{g kg}^{-1}$)

Treatment	Hive	W1			W3			W4	W5	W6	W7		
		t0	24 h	48 h	t0	24 h	48 h	t0	t0	t0	t0	24 h	48 h
control	3	ND ^a	ND	ND	<LOQ ^b	ND	ND	ND	ND	-	-	ND	ND
	19	ND	ND	ND	ND	ND	ND	ND	-	<LOQ	-	ND	ND
T1 (15 $\mu\text{g kg}^{-1}$)	7 ^c	ND	<LOQ	<LOQ	-	-	-	-	-	-	<LOQ	-	-
	11	ND	-	-	ND	1.03	<LOQ	0.83	<LOQ	<LOQ	ND	1.58	ND
	25	ND	ND	ND	ND	ND	<LOQ	ND	-	1.00	ND	<LOQ	ND
T2 (30 $\mu\text{g kg}^{-1}$)	10	ND	<LOQ	1.24	<LOQ	1.11	0.71	0.56	-	<LOQ	ND	1.17	<LOQ
	18	ND	<LOQ	<LOQ	0.65	0.98	<LOQ	0.89	0.57	<LOQ	<LOQ	8.41	<LOQ
T3 (120 $\mu\text{g kg}^{-1}$)	6	ND	-	-	-	-	-	-	-	ND	1.12	3.95	1.91
	28	ND	1.17	0.83	1.19	5.04	1.21	1.25	6.57	2.29	1.90	5.58	2.84
	29 ^d	ND	1.28	3.11	10.08	15.18	1.85	2.20	2.26	5.45	5.51	-	-
T4 (240 $\mu\text{g kg}^{-1}$)	4	ND	2.82	10.12	8.10	28.90	4.26	ND	3.55	3.68	3.26	2.76	7.98
	21	ND	7.58	3.03	1.38	6.96	5.79	<LOQ	2.58	1.37	6.41	4.87	3.03

^a not detected

^b LOQ (limit of quantitation) = $0.50 \mu\text{g kg}^{-1}$, was established as the minimum level of fortification of the recovery assays [M.P. Michlig et al. 2018]

^{c,d} Sampling was replaced in hive 7 by 25, and in hive 29 by 6.

□ ND: Not detected or below LOQ

□ Between 0.50 to $3.00 \mu\text{g kg}^{-1}$

▤ Between 3.01 to $5.00 \mu\text{g kg}^{-1}$

▥ Between 5.01 to $10.00 \mu\text{g kg}^{-1}$

▧ Between 10.01 to $30.00 \mu\text{g kg}^{-1}$

(EFSA 2013, Laurino et al. 2013; Sanchez-Bayo and Goka 2014). At this point, it is important to remember also that in order to ensure that the colonies consumed all of the syrup, the average consumption of treated syrup per day was low compared to the typical feeding patterns of workers and larvae.

Even though imidacloprid concentrations in the tissues of worker bees were dispersive but correlated with treatment levels ($R^2=0.6414$), only 10% of the larvae sampled had residues of this insecticide, and none of them exceeded $1 \mu\text{g kg}^{-1}$ (Table II).

3.1.3. Honey and beeswax

Because imidacloprid is a relatively small and highly water-soluble molecule, honey was expected to be more contaminated than wax. In fact, honey had the highest concentrations of imidacloprid, with 87% of positive samples,

with residues of imidacloprid being detected within a range between LOQ of $0.25 \mu\text{g kg}^{-1}$ and $91 \mu\text{g kg}^{-1}$ in the reserve honey from the brood chamber and from LOQ to $53 \mu\text{g kg}^{-1}$ in the honey from supers (Figure 1A). For reserve honey and honey from supers, we found statistical differences between treatments ($p < 0.001$ and $p = 0.009$); control showed differences with all other treatments, and T1 and T2 indicated differences with T3 and T4 (Figure 1A).

The mass balance performed between the milligrams of imidacloprid introduced to the colonies through the sugar syrups and those finally obtained in the honey (Eqs. 1, 2, and 3) showed that up to 60% of the total of imidacloprid supplied during the 7 weeks was stored in the honey.

Imidacloprid was also detected in 60% of the collected beeswax samples, with levels ranging from 1 (LOQ) to $35 \mu\text{g kg}^{-1}$ in the brood chamber and from LOQ up to $12 \mu\text{g kg}^{-1}$ in the wax taken from the supers (Figure 1B).

Table II Concentration of imidacloprid ($\mu\text{g kg}^{-1}$) in the larvae of each week (W) in relation to the treatments

Treatment	Hive	W1	W3	W7
		t0	t0	t0
Control	3	ND	ND	ND
	19	<LOQ*	ND	ND
T1 ($15 \mu\text{g kg}^{-1}$)	7	<LOQ	-	-
	11	-	ND	1.24
	25	ND	ND	<LOQ
T2 ($30 \mu\text{g kg}^{-1}$)	10	ND	ND	ND
	18	<LOQ	ND	<LOQ
T3 ($120 \mu\text{g kg}^{-1}$)	6	-	-	-
	28	0.63	0.57	ND
	29	ND	ND	ND
T4 ($240 \mu\text{g kg}^{-1}$)	4	ND	<LOQ	ND
	21	ND	<LOQ	ND

* LOQ (limit of quantitation) = $0.50 \mu\text{g kg}^{-1}$, was established as the minimum level of fortification of the recovery assays [M.P. Michlig et al. 2018].

□ ND: Not detected or below LOQ

■ Between 0.50 to $3.00 \mu\text{g kg}^{-1}$

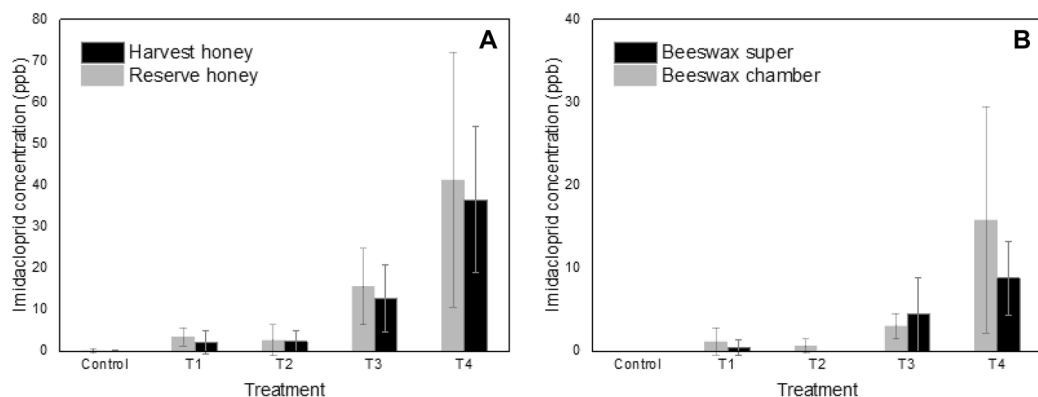


Figure 1. Imidacloprid in honey **A** and beeswax **B**. Mean concentration (ppb or $\mu\text{g kg}^{-1}$) and standard deviation ($n=6$). T1 = $15 \mu\text{g kg}^{-1}$; T2 = $30 \mu\text{g kg}^{-1}$; T3 = $120 \mu\text{g kg}^{-1}$; T4 = $240 \mu\text{g kg}^{-1}$.

3.2. Colony assessment

3.2.1. Colony strength and honey yield

The colonies fed repeatedly with syrup supplemented with imidacloprid did not show changes in their development; actually, neither the adult bee population level (CCBe) nor the CBrA showed significant differences ($p=0.89$ and $p=0.86$, respectively) between treatments and the control at the end of the supplementation

(Table III). By contrast, honey reserves (CCH) showed differences ($p=0.037$), with half of the combs being complete in T4 compared to other treatments. This pattern of behavior was also observed in T2 ($p=0.0002$). The data show reduced honey stores and yield in a dose-dependent manner. Table III shows that T2 had 40% less CCH and T4 had 50% less than the control, which indicates a dose-dependent decrease inversely related to the treatments. During harvest, no differences in honey yield from supers

Table III Means of number of combs covered by adult bees (CCBe), capped brood area (CBrA), and combs covered with pollen (CCP) and honey reserves (CCH). T1 = $15 \mu\text{g kg}^{-1}$; T2 = $30 \mu\text{g kg}^{-1}$; T3 = $120 \mu\text{g kg}^{-1}$; T4 = $240 \mu\text{g kg}^{-1}$

Treatment ^a	CCBe		CBrA		CCP		CCH		Honey yield ^d (kg)
	Initial ^b	Final ^c	Initial ^b	Final ^c	Initial ^b	Final ^c	Initial ^b	Final ^c	
Control	5.8	10.3	3.6	6.2	0.3	0.5	0.9	1.8 ^e	23 ± 17
T1	5.8	9.8	3.2	5.7	0.8	0.5	1.0	1.9	21 ± 17
T2	5.8	9.8	3.3	6.0	0.3	0.3	0.9	1.1 ^e	22 ± 19
T3	5.8	10.2	3.2	5.2	0.3	0.5	0.8	1.8	17 ± 18
T4	5.6	9.0	3.5	5.6	0.3	0.5	0.8	0.9 ^e	20 ± 17

^a $n=6$ (six colonies per treatment)

^bBeginning of the feeding period: September 10, 2014

^cEnd of the feeding period: October 22, 2014

^dHoney yield (mean and standard deviation) at harvest on February 12, 2015

^eStatistically significant differences between honey reserves (CCH) of some treatments and control, verified by multiple comparisons

were recorded among treatments ($p=0.96$). Nevertheless, all treatments have less honey yield than control, average honey yields in T3 were 27% lower than in the control or any other treatment, which indicates that T3 was also affected (Table III).

3.2.2. Failures and health status of the colonies

Throughout the study, few mortality events were observed in all groups, with no difference ($p=0.58$) between imidacloprid-fed and control colonies, but all groups had some problem with the queen. For instance, during the feeding period, four colony deaths occurred, one from each colony fed imidacloprid (T1, T2, T3, and T4). Queenlessness is one of the most common ways to lose a colony. In this case, there were two colonies with laying workers and only drone brood cells: one from control treatment and the other from T3. Besides, two colonies from T1 replaced the queen in the course of the feeding period (Figure 2).

The presence of signs of European foulbrood in control treatment and a T2 colony caused the decline of honey production, among other effects. The abundance of *Nosema* spp. spores (Table IV) showed no significant differences among treatments either at the beginning or at the end of the feeding period ($p=0.40$ and $p=0.27$, respectively). The observations of health status showed no relationship between failures in the colonies and any of the treatments, either control or fed with different doses of imidacloprid ($p=0.31$).

4. DISCUSSION

Honey bee colonies were fed with four different concentrations of imidacloprid in sucrose syrup during spring. Their development and survival were evaluated simultaneously with control hives (fed with sucrose syrup). The tiered approach incorporated a wide range of treatments (four levels), in which dosages were chosen to mimic a realistic field situation, following the protocols provided by the Environmental Protection Agency for a better assessment of risk to bees (EPA 2014). Thus, two more

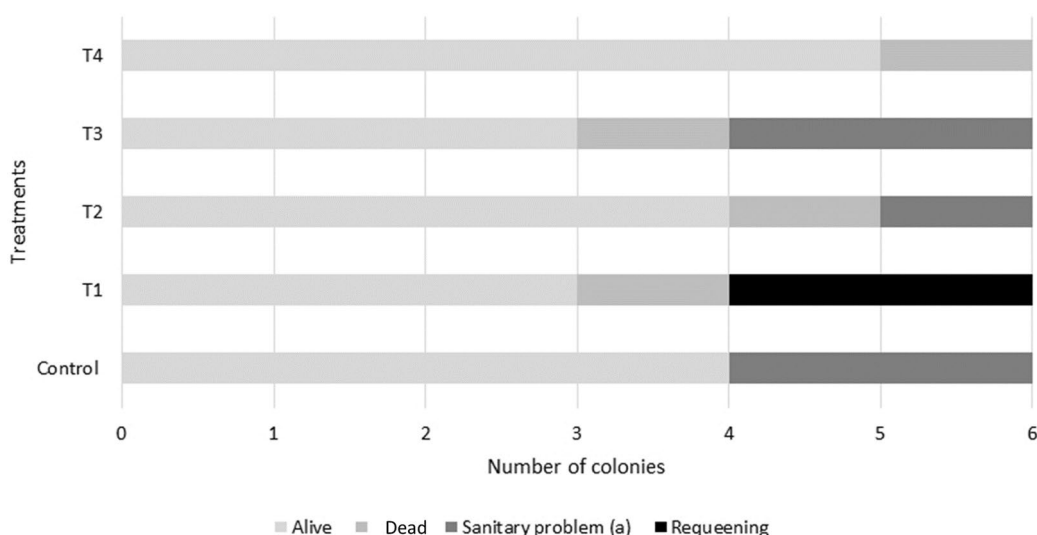


Figure 2. Number of colonies alive, dead or with any problem in function of each treatment. a Sanitary problem: Orphan or European foulbrood. T1 = 15 $\mu\text{g kg}^{-1}$; T2 = 30 $\mu\text{g kg}^{-1}$; T3 = 120 $\mu\text{g kg}^{-1}$; T4 = 240 $\mu\text{g kg}^{-1}$.

Table IV Mean and standard deviation (SD) of *Nosema* spp. per treatment at the beginning, middle, and end of the feeding period. T1 = 15 $\mu\text{g kg}^{-1}$; T2 = 30 $\mu\text{g kg}^{-1}$; T3 = 120 $\mu\text{g kg}^{-1}$; T4 = 240 $\mu\text{g kg}^{-1}$

<i>Nosema</i> spp.	Mean \pm SD at the beginning	Mean \pm SD at the middle	Mean \pm SD at the end
Control	90,000 \pm 113,137	167,500 \pm 236,880	22,500 \pm 10,606
T1	490,000 \pm 593,969	140,000 \pm 197,989	591,666 \pm 82,496
T2	132,500 \pm 187,383	130,000 \pm 183,847	845,000 \pm 77,781
T3	405,000 \pm 56,568	45,000 \pm 63,639	585,000 \pm 14,142
T4	92,500 \pm 130,814	0 \pm 0	395,000 \pm 558,614
<i>p</i> -value	0.40	0.78	0.27

conservative levels at lower tiers (15, 30 $\mu\text{g kg}^{-1}$) and two more realistic ones at higher tiers (120 and 240 $\mu\text{g kg}^{-1}$) were selected. The study simulated a long-term exposure with sublethal imidacloprid concentrations.

Chronic sublethal exposures to neonicotinoids occur frequently in nature, so the doses were chosen to mimic a realistic field situation. In an attempt to integrate the results of food stored in honey bee colonies found across the globe, Bonmatin et al. (2015) indicated that bees are chronically exposed to neonicotinoids and metabolites in a general range from 1 to 100 ppb. In turn, Lu et al. (2016) analyzed eight neonicotinoids in pollen and honey from Massachusetts and found that imidacloprid was the most abundant, with concentrations < 1 up to 43 ppb in pollen and up to 15 ppb in honey.

Among the different castes that integrate the colonies, nurse workers and nectar foragers are the most exposed to contaminants because they consume the greatest rates of pollen and nectar, respectively. While nurse workers consume on average 6.5 mg/day of pollen, nectar foragers collect 80.2 mg/day of nectar (Lu et al. 2016; Rortais et al. 2017). According to this daily consumption and the imidacloprid residues found in pollen and nectar, adult worker bees could be ingesting on average < 0.5 ng/bee a day with maximum daily doses close to 6 ng/bee (Sanchez-Bayo and Goka 2014; Rortais et al. 2017). Indeed, only the two highest treatments of 0.7 ng/bee/day (T3) and 1.4 ng/bee/day (T4) were consistent with natural exposures ($0.5 < 0.7$

and $1.4 < 6$ ng/day), whereas the other two treatments were below the average exposure.

The colony behaves like a “superorganism”; indeed, the functional unit of the honey bee is the colony itself, and the number of organisms involved in the diverse tasks to keep such colony is critical. A colony of honey bees is typically composed of 10,000 to 60,000 individuals, with variations between summer and winter; it works as a cooperative unit, keeping food storage, hygiene of cells, defense of the hive, care of the young, etc. (Van der Sluijs et al. 2013). Hence, a sublethal effect that alters the number of individuals performing specific tasks may influence the functioning of the entire colony.

At low concentrations of neonicotinoids, sublethal effects can be found in the colonies that do not directly kill individuals but can become lethal over time. In this study were evaluated the entire composition of the colonies exposed to each treatment. At the beginning of the trial, each colony had an average of 15,000 workers and a queen of the same age (1 year old) and genetic origin and controlled health conditions. However, when the study ended, the colonies did not show differences except for honey stores, despite the fact that they were supplemented with different doses of imidacloprid. Most of the parameters surveyed (CCBe, CBrA, CCP, *Nosema* spore numbers) during this experiment followed normal seasonal patterns; the colonies did not show any immediate effect, and all groups have some problem in relation with queenlees, death, orphan, or sanitary problems. One of the

possible reasons for these results could be the open-field feeding, where bees are free to forage on sources other than the spiked diet, which dilutes the effects of the studied contaminant (Di Noi et al. 2021).

Nevertheless, some differences were found in relation with honey reserves and honey yield. In T2 and T4 were 40% and 50% less honey reserves than control, respectively, which indicates a dose-dependent decrease inversely related to the treatments. Besides, average honey yields in T3 were 27% lower than in the control or any other treatment, which indicates that T3 was also affected even if this was not reflected in the honey store. Overall, the data show reduced honey stores and yield in a dose-dependent manner.

Some similar studies report different results, where the authors showed increased pollen transport during the feeding period and the number of brood cells in winter (Faucon et al. 2005), a long-term mortality even 8 months after exposure (Colin et al. 2019), elevation of *Nosema* spore numbers (Pettis et al. 2013), failures of the queen (Sandrock et al. 2014; Dively et al. 2015; Wu-Smart and Spivak 2016; Tsvetkov et al. 2017; Hernández López et al. 2017), lower adult bee populations, brood surface areas and average frame weights, and reduced temperature control (Meikle et al. 2016). Nevertheless, they were aware that it is difficult to determine the effects of sublethal exposure at the colony-level, largely due to natural variation among colonies and to uncontrolled factors, such as differential exposure of experimental colonies to exogenous agrochemicals (Meikle et al. 2016). Even were informed an effects at colony level, since bee mortality was substantially lower compared to in vitro experiments exposed to neonicotinoids such as clothianidin (Odemer et al. 2018).

The fact that the test was conducted during the spring–summer period, when bees are more active and consume and metabolize food more quickly, could have mitigated the effects of imidacloprid. In winter, bees have longer life cycles and, considering time as an exposure factor, lower concentrations of imidacloprid could

be lethal to older bees. Extrapolation of the honey bee toxicity scale to the lifespan of winter bees suggests that imidacloprid in honey at $0.25 \mu\text{g kg}^{-1}$ or ppb would be lethal for a large proportion of bees approaching the end of their life (Rondeau et al. 2014). In particular and as already mentioned, given that syrup consumption is mainly carried out by foraging bees and they are the ones that also find diverse sources in the field, imidacloprid concentrations and thus its effects may have been diluted by other dietary sources.

Another reason that may help explain the lack of clear colony impacts in this experiment is the fact that being honey bees that are social insects, with large size of their colonies and high reproductive rate, they may have generated resistance, like many other insect pest species, to insecticides such as neonicotinoids that have been in use for over 20 years in Argentina and most countries (Bass et al. 2015). Therefore, concentration levels that were previously toxic for bees may not be now, i.e., current toxic levels of imidacloprid to bees are likely to be higher than when the chemical was first introduced to agriculture. Consequently, any impacts on their colonies are expected to be lower than before.

In the current research, it was observed that at the instant before feeding (t_0), the imidacloprid concentrations were high, principally in T3 and T4. For example, in one colony of T4 and week 7, the concentration of imidacloprid decreased from 6.4 in t_0 to 3 ppb at 48 h after artificial feeding. It could be interpreted that those t_0 concentrations probably represent mostly undigested syrup in the gut or that, despite the supplementation being done only once a week, the bees were in contact with the insecticide every day of the exposure period either by direct consumption of the sucrose syrup or by the one they stored in the cells. Anyway, the trends in the persistence of the compound after 24 and 48 h of exposure showed some differences between T3 and T4. While in T3, the concentrations at 24 h were twice as high as those found at 48 h, and in T4, differences between days were smaller. These results suggest that there was a decreased detoxification capacity in bees exposed to the highest doses.

The lack of complete elimination of imidacloprid in honey bees exposed for ten days was already observed by Sanchez-Bayo et al. (2017). Exposing honey bees to imidacloprid through syrup, as the days went by, they note an increase in the relative proportion of imidacloprid residues vs. the intake for the day, suggesting a slowdown of its metabolism with exposure time.

Larval exposure to imidacloprid may cause greater problems to the colonies because it affects their proper growth; larvae could be affected by imidacloprid contamination as low as 0.04 ng/larva (Yang et al. 2012). For example, delayed development was observed in the early stages (days 4 and 8) when combs were contaminated with pesticides, among them imidacloprid, and then the developed adults showed a shorter lifespan and low survival rate (Wu et al. 2011). Our results on the analysis of imidacloprid in the larvae suggest that the larval food was not exposed to the insecticide; larvae consume a composite of pollen and honey, so the proportion of honey in their diet is minimal. The experiment here used imidacloprid only in syrup, not in pollen, so larval exposure was minimal, as evidenced by the lack of residues in most of samples (Table II). As processed pollen is the source of protein for the brood might represent most of the potential risk for them. In fact, since pollen contains on average higher residues of imidacloprid than nectar, the results obtained from field trials that use pollen are very different (Sandrock et al. 2014; Dively et al. 2015), as this impacts mostly on larvae, their nurse bees, and queens rather than forager bees. Therefore, our research should be complemented with the study of the effects generated in colonies exposed to contaminated pollen.

As shown by the residue levels, a high proportion of imidacloprid was found in honey reserves and honey supers. Since honey supers were added to the hives when the feeding period had finished, it is noticeable that bees were exposed and in contact with the contaminant even after the end of the feeding period. Meikle et al. (2016) also used contaminated syrups to study the performance of honey bee colonies exposed to imidacloprid. They detected imidacloprid, even almost 7 months after the end of the artificial feeding period, in honey reserves.

Since honey is in contact with wax from the honeycomb, if either honey or wax becomes contaminated, the other is expected to be contaminated as well. However, the distribution of a pollutant between matrices depends on different variables, such as physicochemical properties of the contaminant and the host matrix, time and surface of contact between the matrices, and temperature. Due to the beeswax composition, a complex mixture of hydrocarbons, fatty acids, esters, and other substances of low polarity, a low incidence of contaminated wax samples by imidacloprid might be expected. However, as in our work, the detection of imidacloprid and other neonicotinoids in beeswax has already been reported in the literature (Yáñez et al. 2013). Capturing the wax, a fraction of the residues presents in honey or in the beebread; it represents a loss of residues available to the worker bees. However, such residues may affect the larvae (Wu et al. 2017), as they are in continuous contact with the cell walls.

Traces of imidacloprid were found even in witness control colonies, suggesting that the colonies possibly encountered other sources of contamination or that they presented robbing behavior, i.e., bees of the most populated colonies enter weaker nearby colonies and rob their stores. So, part of the reason for the fewer than expected differences in imidacloprid concentration among treatment groups, and its presence in the control group, was likely due to robbing and drifting, as well as to dilution due to the addition of nectar from the spring nectar flow.

Overall, our results showed an effect but it is not clear and indicates the need to conduct longer studies involving contrasting environments in terms of food availability and with or without pesticides. The information obtained will allow us to understand the unresolved questions of the present work.

5. CONCLUSIONS

During the study period and under the conditions applied in our trial, no direct impacts on colony strength (CCBe, CBRa, CCP) were observed in treatments supplied with sugar syrups containing different doses of pure (not

formulated) imidacloprid. Nevertheless, there were no deaths in the control colonies and one or two colonies per treatment fed with syrups supplied with imidacloprid did die; we concluded that concentrations of imidacloprid in the syrups used for this evaluation could have caused the immediate death of the colonies. The colonies that received some dose of imidacloprid also reflected some decrease in either the concentration of honey stored and honey yields compared to the colonies of control treatment.

The lack of imidacloprid in the larvae suggests that the food supplied by nurses was not in contact with the pesticide.

Furthermore, the highest level of imidacloprid residue found (3 ng per bee), which is lower than the LD50 values reported in the literature, confirms the sublethal conditions of this assay. Diverse levels of imidacloprid residues were found in bees, honey, and wax, but they were closely correlated with the dose supplied in each treatment. A significant percentage of the parent (not metabolized) molecule was stored in honey.

However, additional research is needed to further evaluate possible chronic and long-term effects of exposure of colonies to imidacloprid at different times of the year.

ACKNOWLEDGEMENTS

We are especially grateful to Prof. Horacio R. Beldoménico and Agr. Eng. César Salto for their crucial contributions to the design of the experiment.

AUTHOR CONTRIBUTION

Melina Paola Michlig, Adriana Cecilia Pacini, Julieta Merke, Emanuel Matías Orellano, and María Rosa Repetti conceived this research and designed experiments; Melina Paola Michlig and Adriana Cecilia Pacini, Julieta Merke, and María Rosa Repetti participated in the design and interpretation of the data; Melina Paola Michlig, Adriana Cecilia Pacini, Romina Brasca, and Emanuel Matías Orellano performed experiments and analysis; Melina Paola Michlig, Adriana Cecilia Pacini, Julieta Merke, and María Rosa Repetti wrote the paper and participated in the revisions of it. All authors read and approved the final manuscript.

FUNDING

This work was supported by the National Agency for Scientific and Technological Promotion (ANPCyT), under PICT 2011 Res. 98/11, Buenos Aires, Argentina; the Projects of the CAI + D research program at National University of Littoral (UNL), Santa Fe, Argentina; and the project of the National Institute of Agricultural Technology (INTA), PNAPI Project No. 1112042 and Specific Project “Estrategias multidisciplinares para mitigar el efecto del nuevo contexto ambiental y productivo sobre la colmena.”

DATA AVAILABILITY

Data will be made available on reasonable request.

CODE AVAILABILITY

Not applicable.

DECLARATIONS

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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