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Effects of imidacloprid on survival and nest development in the neo-tropical bumblebee *Bombus ephippiatus*

Alejandra MARTÍNEZ DE CASTRO DUBERNARD¹ , Dave GOULSON²,
Lislie SOLÍS-MONTERO^{1,3}, Rémy VANDAME¹

¹ Departamento Agricultura Sociedad y Ambiente, El Colegio de La Frontera Sur, Chiapas, Mexico

² School of Life Sciences, University of Sussex, Brighton, UK

³ El Consejo Nacional de Ciencia Y Tecnología, Mexico city, Mexico

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Abstract – The use of pesticides is one of the causes that contribute to the decline of bumblebees worldwide. Some effects of neonicotinoids are known on bumblebees from temperate climates; however, there are no studies carried out with species from tropical climates, in which there could be different effects since they have different ecological traits. We demonstrated that in a neo-tropical bumblebee, *B. ephippiatus*, exposed to field-realistic levels of imidacloprid, bee survival, and colony growth, were significantly reduced. The most important conclusion of our study is that tropical bumblebees are affected in a similar way to temperate species.

Agriculture / Bees / *Bombus* / Pesticides / Toxicology

1. INTRODUCTION

Whether pesticides have the same effects on the little-known tropical bumblebee species colonies as they do on the temperate ones is a question to be addressed. Many differences in the ecology and physiology of tropical bee species could explain a differential susceptibility of temperate and tropical species of bumblebees to pesticides, in particular to the neonicotinoid ones. Our aim in the present was therefore to compare the effects of imidacloprid on a tropical bumblebee species, when comparing to published results obtained with temperate species. In order to address this topic with bumblebees of

Mexico, it is necessary to synthesize the state of knowledge, first of pesticide effects on bees and then of bumblebees in Mexico.

Bees are considered the most important insect pollinator (Greenleaf et al. 2007) and their pollination services are crucial for food production (Wagner 2020). Currently, there is evidence of insect decline around the world (Dirzo et al. 2014; Wagner 2020) and wild bees are not the exception. Several factors are driving declines in bee diversity and abundance, including climate change, monocultures, deforestation, introduction of non-native bees, and habitat fragmentation. In addition, pesticides are considered to be one of the main causes of wild bee decline, and they have been the subject of considerable research (IPBES 2016).

As an integral part of the intensification of agriculture, pesticide use has increased significantly, leading to a chronic exposure of

Corresponding author: A. Martínez de Castro Dubernard,
ale.mcdcd@gmail.com

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bumblebees to these chemicals during foraging in arable landscapes (Goulson et al. 2015; Mommaerts et al. 2010). Pesticides could affect populations of bumblebees directly or indirectly; while insecticides directly harm the bees, herbicides suppress the food they get from weeds (Williams and Osborne 2009). Neonicotinoids are a relatively new family of insecticides, introduced into the market in the mid-nineties, and being today the most widely used group of insecticides worldwide (Blake and Copping 2017; Wood and Goulson 2017).

The active ingredients that make up the neonicotinoids group are acetamiprid, imidacloprid, clothianidin, dinotefuran, nitenpyram, thiacloprid, and thiamethoxam (Simon-Delso et al. 2015). They are considered neuroactive molecules due to their mode of action, which affects the nicotinic cholinergic receptors of the nervous system, not only in pest insects but also in beneficial insects and other animals (Blake and Copping 2017; Taillebois et al. 2018). These pesticides are frequently applied as seed treatments, and their solubility in water, as well as the potential for leaching, causes contamination of soils, where residues can be found up to 3 years after treatment (Botías et al. 2015). Because these insecticides are systemic, they are absorbed by plants from the soil and transported throughout their tissues via the vascular system, being bioavailable to bees and other pollinators in sub-lethal concentrations through pollen and nectar from crops and wild plants (Alkassab and Kirchner 2017; Blake and Copping 2017; Botías et al. 2016). Furthermore, this group of pesticides is considered to be highly toxic for bees (Suchail et al. 2000).

In Mexico, the introduction and use of chemical pesticides result from the technological paradigm of capitalist modernization, known as the Green Revolution and adopted since the 1940s (Bejarano González 2017). According to records of the COFEPRIS (Federal Commission for the Protection of Sanitary Risks), most of the permits granted in Mexico these days regarding neonicotinoids are for imidacloprid. The permissions are registered for their application in more than 70 crops, and 62% are foliar applications, followed

by 14% of seed coating application (COFEPRIS 2020), so there is considerable potential for bumblebees to be exposed to imidacloprid in Mexico.

Due to their role as pollinators, bumblebees are important species in ecosystems, being eusocial bees with an annual life cycle. They are considered generalists, for visiting a large number of plant species, and they have an important role in the pollination of native plants in temperate and mountainous regions (Plowright 1984; Williams et al. 2009, 2014; Goulson 2010; Hatfield et al. 2012).

Bombus ephippiatus is a tropical species distributed in Mexico and Central America. It is the species of bumblebees with the greatest distribution in Mexico since it is present in almost the entire territory, from Chihuahua to Chiapas and between altitudes ranging from 800 to 3400 m above sea level (Labougle 1990; Chavarría-Villaseñor 1996). Unlike temperate species, colonies of neo-tropical bumblebees are not seasonal and have active colonies year-round, meaning that queens do not hibernate and start a new nest after having mated (Chavarría-Villaseñor 1996). Under laboratory conditions, this bumblebee has two life cycles in a year, each of which consists of a period of 6 months. It is also important to add that this is a species with great potential for management and use in agriculture (Torres-Ruiz and Jones 2012).

Physiological functions such as immune responses, thermal strategies, detoxification response, susceptibility to disease, and pesticides can vary across bumblebee species and could plausibly be different in neo-tropical bumblebees compared to the better-studied temperate species (Arena and Sgolastra 2014; Woodard 2017). Moreover, the energy budget of warm-climate bees is likely to be different from temperate bees. In particular, they are likely to burn less energy on generating heat for brood incubation and thus might consume less nectar. Metabolism and genetic mechanisms could also be affected depending on temperature and environmental conditions (Pimslser et al. 2020), which could lead to different effects of pesticides on tropical and temperate species of bumblebees.

Bumblebee research in Mesoamerica has been focused in the field of taxonomy, rearing, climate

change, and recommendations to policy-makers about bumblebee commercialization (Williams et al. 2020; Martínez-López et al. 2021), and little is known about the effect of pesticides in tropical bumblebees (Alkassab and Kirchner 2017; Siviter et al. 2021). For this reason, in this work, we intend to inquire about the consequences that imidacloprid has in tropical bumblebees when exposed to field-realistic levels of imidacloprid in nectar and pollen. Many studies have been made in temperate regions regarding the effect of neonicotinoids in bumblebees; however, this is the first study made in Mesoamerica and specifically, with a neo-tropical species.

Because bumblebees are considered important pollinators, as well as considering the important use of pesticides in agriculture in tropical countries such as Mexico, particularly imidacloprid, it is important to determine the effects of a diet contaminated with imidacloprid in tropical bumblebees. Their effect on nest development, which is a key aspect of the survival of colonies, is of particular interest. *Bombus ephippiatus* is widely distributed in the Mesoamerican region and imidacloprid is a highly used pesticide in Mexico, so studying its effects on this species can provide an indication as to what is happening with bumblebees in the entire region.

2. MATERIALS AND METHODS

2.1. Bumblebees

We studied the neo-tropical bumblebee species *B. ephippiatus*. Colonies to be used were raised in the laboratory and came from queens collected at different sites in San Cristóbal de Las Casas, Chiapas, Mexico. The bumblebees used in this experiment are the fifth generation reared in captivity. In the case of the experiments starting with mated queens, the queens used were mated days before in the El Colegio de la Frontera Sur bumblebee mating room. Queens and males were chosen from different colonies to avoid inbreeding. Bumblebee colonies were maintained in the laboratory at 27 °C, with a relative humidity of 60%, and under red light.

2.2. Rearing of tropical bumblebee *Bombus ephippiatus*

Bombus ephippiatus breeding in captivity was achieved under controlled conditions. To raise this bumblebee, it is necessary to have an environmental temperature of 27 °C and a relative humidity of 60%. Mating takes place in a 1.5 × 1.5 m flight cage where males and queens are placed at a ratio of 2:1. Once the females are fertilized, they are placed into an initiation cage, where they are fed with pollen and fructose syrup, until reaching a population of 15 ± 3 workers. They are then moved to a development cage, in which colonies receive pollen and fructose syrup ad libitum. Once they reach the end of their cycle, there is a production of males and queens inside the colonies. Newly emerged queens are then separated from their colonies and introduced in the mating cage to get fertilized and start a new cycle.

2.3. Food

All colonies of bumblebees in this study were fed with pollen from the region collected by the bee *Apis mellifera*. The pollen came from a pesticide-free zone from a coffee plantation in Chiapas. A colony-feeding test was also done in 10 colonies to detect possible acute intoxication by detecting any behavioral responses in bees. However, we did not screen the pollen for pesticides and hence cannot be certain that there were traces of pesticides, which could be a potential limitation of our study. To obtain a single uniform pollen source, the pollen grains were liquefied and mixed with distilled water (pollen: water, 3:1). As a substitute for nectar, bees were given 50% fructose syrup ad libitum.

2.4. Preparation of pesticide treatments

Imidacloprid was mixed in its pure form with the fructose syrup and with the pollen for the colonies that received treatment. For this, imidacloprid stock solutions were prepared with

purified distilled water. To achieve the desired concentrations for both treatments, these solutions were first diluted in the water that would be used for pollen and syrup preparation. We used a low dose of 2 ng/g and a high dose of 10 ng/g, administrated with pollen and sugar syrup. Doses were chosen based on a literature review of imidacloprid residues in pollen and nectar (Bonmatin et al. 2015; David et al. 2016; Wood and Goulson 2017). The dose of 2 ng/g is close to the lowest found value, and the dose of 10 ng/g is slightly above the average. The effects of imidacloprid on worker survival rate in microcolonies and nest development in queenright colonies were evaluated. Two experiments were carried out.

2.5. Experiment 1: effects of imidacloprid in microcolonies

Newly emerged workers from five *B. ephippium* queenright source colonies were randomly selected and five of them were placed in each of the 75 queenless microcolony cages. Each microcolony contained workers from the same queenright colony. Cages consist of three sidewalls (14 cm × 6.3 cm × 6.3 cm) and a 1-cm-thick wooden lid. The front face is a sliding glass plate 2 mm thick (12.6 cm × 5 cm), and

as a base, they have a rack located 2 cm above ground to ventilate the nest. A plastic perforated tube was used as a syrup feeder and a Petri dish lid was put inside to provide a solid base for nest building and as a feeder to administrate pollen (Figure 1). Microcolonies were divided and randomly assigned into three treatment groups of 25 microcolonies each: control (without pesticides), low dose (2 ng/g), and high dose (10 ng/g) of pesticide. Before the exposure to imidacloprid, workers were fed ad libitum with control (no pesticide) syrup for 24 h to acclimatize them to the experimental conditions. Dead bees during this acclimation time were replaced with workers from a queenright colony. Microcolonies were maintained for 40 days; during this period, first day of egg laying, number of males produced, male weight, and mortality were measured. Sucrose and pollen were changed every two days.

2.6. Experiment 2: queenright colonies development

For measuring nest development, five queenright colonies per treatment were exposed to the previously described concentrations (control, 2 ng/g, 10 ng/g). Colonies had one queen and 50 ± 5 workers when treatment began. The boxes used in this experiment are much larger and are



Figure 1. Microcolony box.

divided into two parts: the brood chamber, where the nest is located (23 cm × 30 cm × 18 cm) and a mesh space on the floor that simulates the outside for bee waste (11 cm × 30 cm × 18 cm). The box has side holes to place drinkers and be able to supply all the necessary syrup and a transparent roof to be able to carry out routine checks without disturbing them (Figure 2). The growth of the colony was measured by counting the brood (number of cells with eggs, larvae, and pupae), number of workers, and mortality every two days. Dead bees were removed from the nest. Colonies were exposed to treatments for 40 days. Given that neonicotinoids are found in both crop flowers and wildflowers growing nearby (Botías et al. 2016), they can persist in plants for many months, and routinely turn up in honey samples from arable areas, we would argue that chronic exposure of this sort is very likely to occur in the wild. Sucrose and pollen were changed every two days and nests were fed ad libitum.

2.7. Statistical analysis

We conducted a survival analysis (Kaplan–Meier method) using the survival package (Therneau and

Grambsch 2000) in R statistical software ver. 3.6.0 (R Core Development Team 2019) to compare survivorship curves across treatments in microcolonies. We represented graphically the survival probability, using the survminer package (Alboukadel and Kosinski 2019). The amount of brood and number of workers in queenright colonies were each compared across treatments using two separate generalized linear mixed-effects models (glmer function in the lme4 package in R; Bates et al. 2014) using Poisson distribution. Both analyses included fixed effects of the treatment, the day, and the interaction between both factors. The colony of origin was included as a random effect. The variance and covariance of the random effects were obtained using the Ranef function (package lme4). Later, we calculated an ANOVA of the model using car package (Fox and Weisberg 2019).

3. RESULTS

3.1. Experiment 1: effects of imidacloprid in microcolonies

Workers exposed to 10 ng/g of imidacloprid never started a nest. Of the untreated



Figure 2. Queenright colony box.

microcolonies and the microcolonies exposed to 2 ng/g of imidacloprid, 64% and 56% microcolonies started a nest respectively ($p > 0.01$). Figure 3 shows that during the first 12 days of the experiment, there was a 100% survival rate for the control microcolonies. In contrast, for microcolonies exposed to 2 ng/g and 10 ng/g of imidacloprid, the mortality started to increase on day 6 and day 4 of the experiment, respectively. All microcolonies exposed to 10 ng/g of imidacloprid were dead by day 31 of exposure. All microcolonies exposed to 2 ng/g of imidacloprid were dead by day 35 of exposure ($p < 0.01$).

Five males were born from untreated microcolonies, and their average weight was 0.1300 ± 0.0067 g. Only one male was born in a microcolony exposed to the lowest concentration of imidacloprid and its weight was 0.1233 g. No males were born from microcolonies exposed to 10 ng/g of imidacloprid since the bees never started a nest. These emerged males were removed straight away. Data on brood was not collected in addition to emerged

males. However, some of the brood could potentially have emerged after the 40 days window in the control microcolonies and microcolonies exposed to 2 ng/g.

3.2. Experiment 2: queenright colonies development

Birth and death rates were measured separately (Figure 4a). For colonies exposed to 10 ng/g of imidacloprid, the number of workers decreased over the time of exposure. Similar to the results for the microcolonies, all workers of queenright colonies exposed to the highest pesticide concentration were dead by day 31 of the experiment; this means that there was a decrease in the emergence of bees and there was an increase in the mortality of bees. In contrast, in the control colonies, which did not receive treatment, the number of workers increased through the study. On the other hand, queenright colonies exposed to 2 ng/g of imidacloprid

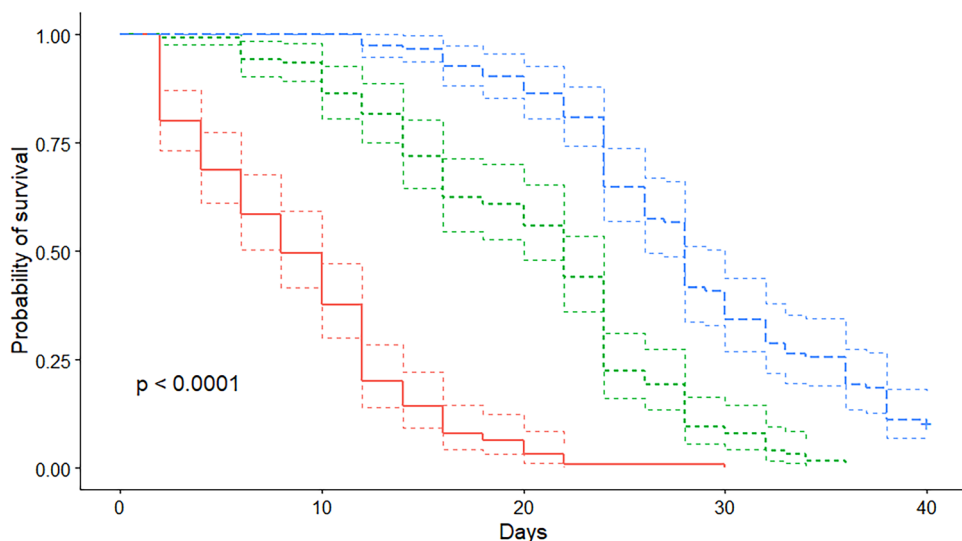


Figure 3. Probability of survival and intervals of confidence of *Bombus ephippiatus* workers exposed to contaminated food with imidacloprid at different concentrations (high dose in red color; low dose in green color) and a control without pesticide (in blue color).

had an approximately constant number of workers, where the mortality and the emergence of bees were similar. Mortality was higher than in the control colonies, but lower than colonies

exposed to 10 ng/g of imidacloprid. The number of workers emerging in the colonies exposed to 2 ng/g was less than in control colonies, but higher than in the colonies exposed to 10 ng/g

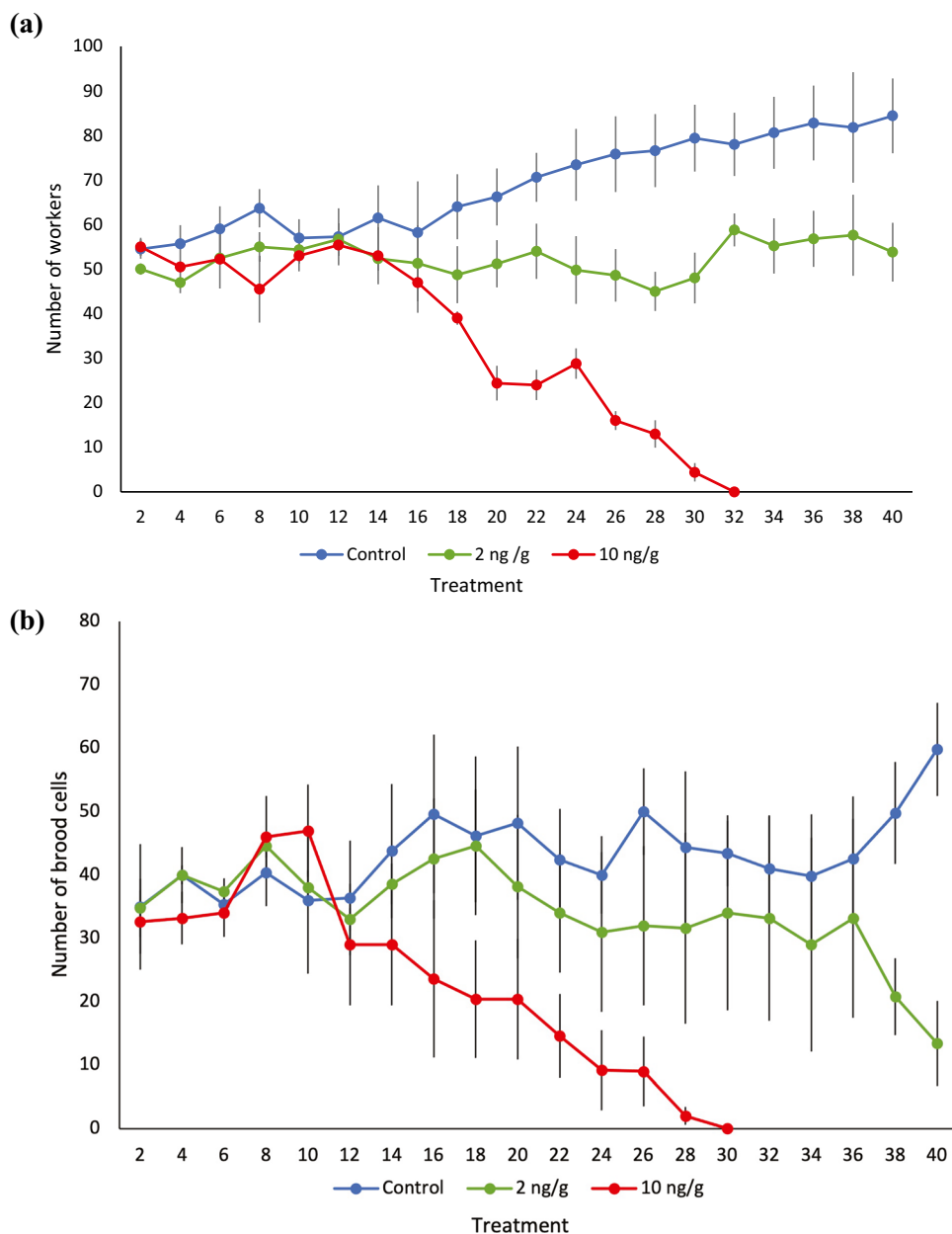


Figure 4. Nest development: number of workers **a** and brood cells **b** in *Bombus ephippiatus* colonies exposed to pollen and nectar contaminated with different concentrations of imidacloprid and untreated colonies.

of imidacloprid. The amount of brood in nests exposed to imidacloprid had a similar pattern to the number of workers. Nests exposed to 10 ng/g had a decreasing amount of brood over the days of exposure. Nests exposed to 2 ng/g had an approximately constant amount of brood until a sudden decrease occurred on day 36. For the case of untreated colonies, amount of brood showed a constant number and an abrupt increase by day 36 (Figure 4b). The statistical analysis showed that number of workers and amount of brood differed according to the treatment, the day, and the interaction between both variables (Table 1).

4. DISCUSSION

We demonstrated that, in the neo-tropical bumblebee *B. ephippiatus*, bee survival was significantly affected when microcolonies were exposed to low and high doses of imidacloprid. In addition, we showed a negative relationship between the amount of imidacloprid in food and the amount of workers and brood in queenright colonies. This suggests a chronic lethal effect in *B. ephippiatus* through constant exposure to imidacloprid.

Our study shows that the use of microcolonies of five workers is useful to compare worker survival across the exposure of different treatments, as this lets us standardize small and homogenous colonies. However, with our experimental design, the use of microcolonies is not useful to determine the effects of imidacloprid on the brood because there is an early mortality of workers, which does not allow time for egg laying and care of the nest. It is possible that starting treatments 10 days after setting up the microcolonies would let us evaluate the effect on the brood since this would allow the establishment of the nest and egg laying before the workers are affected by the pesticide (Krueger et al. 2021). A recent study by Van Oystaeyen et al. (2021) suggested that microcolonies are not fully representative of queenright colonies with regard to the effects of pesticides so it may be necessary to carry out more work on queenright colonies regarding the effect of pesticides in *B. ephippiatus*.

Table 1 Summarized statistics of two generalized linear mixed-effects models. The values in parentheses are the standard deviations of variance for random effects

Variable	Estimate (SE)	Chisq	P value
Number of workers			
<i>Fixed effect</i>			
Treatment		157.19	<0.001
Day		148.95	<0.001
Treatment*Day		1459.64	<0.001
<i>Random effect</i>			
Colony	0.002672 (0.05169)		
Amount of brood			
<i>Fixed effect</i>			
Treatment		37.047	<0.001
Day		30.507	<0.001
Treatment*Day		976.458	<0.001
<i>Random effect</i>			
Colony	0.01857 (0.1363)		

Nest development was affected in a negative way in the case of queenright colonies exposed to imidacloprid, showing again a negative relationship between the amount of workers and brood, and the concentration of imidacloprid in pollen and nectar consumed. The sudden shift in brood in control queenright colonies on day 36 of the experiment could be because of the growth of the colony. Since more workers are emerged in the nest, more brood care (e.g., feeding and incubating) is provided. The sudden brood decrease at day 36 on queenright colonies exposed to 2 ng/g could be attributed to the lack of workers in the nest and a cumulative effect of the toxin. However, this could be due to a lag effect that remains to be explored.

A reason on why there was a decrease in the amount of brood and workers could also be attributed to behavioral responses, which have been observed in *B. impatiens* when exposed to this insecticide (Crall et al. 2018). A decrease in the brood could also be because of a lack of nest

care due to the mortality of workers or to reduced fecundity, as it has been found that there is a significant reduction in ovarian development and fecundity of workers of *B. terrestris* after ingesting environmentally realistic levels of imidacloprid (Laycock et al. 2012). Imidacloprid could also be affecting brood development directly. Further studies are needed in this species to test these hypotheses.

The most important finding in our research is to show that the effects of imidacloprid on colonies of tropical bumblebee species are similar to those reported for bumblebees from temperate climates. For example, survival rates reported in our study were similar to survival rates found in the temperate bumblebees *B. terrestris* and *B. impatiens* when exposed to imidacloprid (Tasei et al. 2000; Mommaerts et al. 2010; Wu and Spivak 2017; Gill et al. 2012). Colony growth was also affected in *B. terrestris* colonies exposed to this neonicotinoid (Laycock et al. 2012; Whitehorn et al. 2012). These studies used neonicotinoid concentrations between 0.7 and 25 ng/g. While our study uses concentrations between this interval, results suggest a possible extrapolation for pesticide effects on tropical bumblebees, including effects on foraging and studies carried out in the field.

Our results demonstrate that the ingestion by *B. ephippiatus* of field realistic concentrations of imidacloprid causes a decrease in survival rates and affects nest development negatively. This could lead to significant losses in wild populations since the production of males and queens occurs at the end of the life cycle and exposed nests would never reach this point, as the inputs brought to the nest by workers are essential to reach this stage.

This is the first work involving the effect of imidacloprid in a neo-tropical bumblebee species. Given the physiological and ecological adaptations of tropical bees to tropical climates, it was possible to expect a different susceptibility of these species, compared to species in temperate climates. However, the effects of imidacloprid were surprisingly similar to the ones described in temperate species, suggesting a somewhat universal effect of this neonicotinoid

insecticide on bumblebees. Further studies with other species of tropical bees and other insecticides should be carried out, in order to show whether these pesticides actually act in a similar way across the diversity of the bee superfamily.

5. CONCLUSIONS

The most important conclusion of our study is that tropical bumblebees, at least in the case of *B. ephippiatus*, are affected by the insecticide imidacloprid in a similar way as temperate species. Because imidacloprid is applied in more than 70 crops in Mexico, we suggest these effects could be commonly occurring in wild populations of native bumblebees. The transition to a more sustainable agriculture and the reversal of agricultural landscape simplification offer strategic responses to the risks associated with the exposure of pesticides in wild pollinators. Bumblebees are considered important pollinators for wildflowers and crops, and we urge measures to reduce pesticide use, in which it is worth mentioning the promotion of biological pest management, complemented by farmer education, organic agriculture, and policies to reduce pesticide use globally.

AUTHOR CONTRIBUTION

Study conception and design were made by all authors. Material preparations, data collection, and analysis were performed by Alejandra Martínez de Castro Dubernard. Lislie Solis Montero also performed data analysis. Alejandra Martínez de Castro Dubernard wrote the first draft of the manuscript and other authors participated in the review and editing. Funding acquisition was made by Alejandra Martínez de Castro Dubernard and Rémy Vandame. Resources were provided by Rémy Vandame.

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AVAILABILITY OF DATA AND MATERIAL

Data will be made available on a data repository.

CODE AVAILABILITY

Analyses are standard procedure, but our custom code, if requested, can be provided.

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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