Queen bee acceptance under threat: Neurotoxic insecticides provoke deep damage in queen-worker relationships

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ABSTRACT

Virgin queens (gynes) exhibit a range of behaviors in order to be accepted as the leader of colony. However, environmental neurotoxic insecticides as neonicotinoids may affect the social performance of the bees. Here, we evaluated the sublethal effects of neonicotinoid imidacloprid on the larval food of queens from *Plebeia droryana*, a species of neotropical stingless bee. Several behaviors were analyzed as multivariate response variables in a Hotelling test, as well as generalized additive mixed models. Our findings demonstrate that treated queens perform less wing vibration and trophallaxis with their workers. Furthermore, the treated gynes encounter more harassment (aggression) from their workers, suggesting that workers can differentiate nontreated queens from treated queens most likely by chemical signals. Our data indicate that the behavioral repertoire underlying the queen selection process by the stingless bee *P. droryana* may be seriously affected by residual doses of imidacloprid in larval food. As a result, such queens are rather undernourished and aggressed by workers, which most likely compromises the viability and permanence of colonies in the long term.

1. Introduction

Queen selection is a very common phenomenon in insect societies, being frequently observed in ants (Holzer et al., 2008; Meunier et al., 2011; Sorvari, 2017) and bees (Imperatriz-Fonseca and Zucchi, 1995; Pérez-Sato et al., 2007; Santos et al., 2015; Veiga et al., 2017). Queen selection by workers is fundamental to future generations of colonies in social insects and may involve genetic relatedness, chemical cues or behavior mechanisms (Holzer et al., 2008; Imperatriz-Fonseca et al., 1995; Meunier et al., 2011; Sorvari, 2017; van Zweden, 2010). The final listed feature (i.e., behavior) displays a key role in this process because if new queens accurately exhibit their behaviors to colony members that are directly responsible for their selection, then the communication is successful, and their chances of being accepted are greater (Holzer et al., 2008; Imperatriz-Fonseca and Zucchi, 1995; Meunier et al., 2011; Sorvari, 2017; van Zweden, 2010). Consequently, it is not enough to emerge as being of a certain caste: a queen needs to be selected.

Among stingless bees, which are closely related to honeybees and bumblebees (Cardinal and Packer, 2007), virgin queens must exhibit a range of behaviors to workers before being accepted as leaders of their nests (da Silva et al., 1972; Imperatriz-Fonseca and Zucchi, 1995; Nogueira-Ferreira et al., 2009; Santos et al., 2015; Araújo et al., 2017; Veiga et al., 2017). During queen selection among stingless bees, the

putative new queens (hereafter, gynes) usually make significant indirect or direct contact with workers (da Silva et al., 1972; Nogueira-Ferreira et al., 2009; Pinho et al., 2010; Santos et al., 2015; Veiga et al., 2017). For example, in an individual context when contact with workers are indirect, the gynes may (1) run into nests, displaying themselves to colony members, (2) clean themselves and (3) vibrate their wings (da Silva et al., 1972; Imperatriz-Fonseca and Zucchi, 1995; Nogueira-Ferreira et al., 2009; Santos et al., 2015; Araújo et al., 2017; Veiga et al., 2017). However, in a social context when direct contact with workers is necessary, the gynes may (1) antennate the workers, which often is followed by (2) trophallaxis (mouth-to-mouth liquid food exchange). The gynes may also be (3) harassed by workers and (4) defend themselves against such aggression (Wenseleers et al., 2004; Jarau et al., 2009a, 2009b; Santos et al., 2015; Araújo et al., 2017; Veiga et al., 2017).

There is no pre-established sequence for gynes to display such behaviors, and these behaviors decrease after a queen's acceptance (da Silva et al., 1972; Imperatriz-Fonseca and Zucchi, 1995; Nogueira-Ferreira et al., 2009; Santos et al., 2015; Araújo et al., 2017; Veiga et al., 2017). Nevertheless, it is believed that the first week after the queen's emergence is crucial for the workers' decision because during this period, the gynes acquire the glandular development needed for pheromone production (Cruz-Landim et al., 2006) and the sexual

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maturity for mating (da Silva et al., 1972; Imperatriz-Fonseca and Zucchi, 1995; Nogueira-Ferreira et al., 2009; Santos et al., 2015; Araújo et al., 2017; Veiga et al., 2017).

The acceptance or execution of the queen among stingless bees is a common process for these social insects. Therefore, stingless bee gynes must necessarily and accurately exhibit the entire behavioral repertoire to their workers to be accepted into the nest (da Silva et al., 1972; Imperatriz-Fonseca and Zucchi, 1995; Jarau et al., 2009a; Pinho et al., 2010; Santos et al., 2015; Veiga et al., 2017). Accordingly, queen bee acceptance portrays strong selective pressure on stingless bee gynes early in their lives.

Currently, there is significant worldwide concern about environmental contamination caused by the insecticides and its consequences for nontarget organisms (Blacquière et al., 2012; Goulson, 2013; van Lexmond et al., 2014). One of the greatest puzzles for scientists is understanding how chemical substances with neurological action may affect animal behavior. For example, currently the neonicotinoids are one of the most commonly insecticides employed in global agriculture (Blacquière et al., 2012; van der Sluijs et al., 2013; Simon-Delso et al., 2014). Neonicotinoids mimic the neurotransmitter acetylcholine, ie., they interact with the nicotinic acetylcholine receptors (nAChRs) of the insect central nervous system acting agonistically on nAChRs on the post-synaptic membrane (van der Sluijs et al., 2013; Simon-Delso et al., 2014). Consequently, this induces a neuronal hyper-excitation, which can lead to the insect's death within minutes (Matsuda et al., 2001; van der Sluijs et al., 2013). However, on residual concentrations the neonicotinoids may negatively affect nontarget insects like bees impairing, for example, their foraging behavior, colony growth and reproduction (Mommaerts et al., 2010; Sandrock et al., 2014; Rundlöf et al., 2015; Wu-Smart and Spivak, 2016).

The most likely pathway in which insects are exposed to neonicotinoids may be due such insecticide to entry in the plant and to spread itself throughout all plant tissues making them toxic to any beneficial insects like bees that feed upon the plant (Simon-Delso et al., 2014). As a result, the neonicotinoids have been found as residue in pollen grains and nectar (Bonmatin et al., 2005; Dively and Kamel, 2012; Maus et al., 2003; Morandin and Winston, 2003). This consequently contaminates the food and intoxicates the bees inside the nest (Desneux et al., 2007; Lundin et al., 2015; Amulen et al., 2017; Pisa et al., 2017; Rondeau et al., 2014; Rosa et al., 2015b; Zhu et al., 2014).

Imidacloprid is a neonicotinoid insecticide that acts, therefore, on the nicotinic acetylcholine receptors of insects (Matsuda et al., 2001). As such, it has been successfully used on nontreated pests for agricultural applications in Brazil, where it is broadly applied to more than 70 nontreated agricultural pests that damage approximately 45 Brazilian crops (MAPA, 2017). However, the continuous use of imidacloprid should be rethought given that it is highly toxic to nontarget organisms such as bees (Rondeau et al., 2014; Scholer and Krischik, 2014; Wang et al., 2015; Whitehorn et al., 2012; Wu-Smart and Spivak, 2016; Wu-Smart and Spivak, 2018), which are insects that cross-pollinate most crops worldwide (Carvalheiro et al., 2010; Klein et al., 2007; Kremen et al., 2002).

Many studies have demonstrated that honeybee, bumblebee, and fire ant queens, for example, have been exposed to sublethal doses of imidacloprid, consequently having adverse effects on survival and causing behavior impairment (Rondeau et al., 2014; Scholer and Krischik, 2014; Wang et al., 2015; Whitehorn et al., 2012; Wu-Smart and Spivak, 2016; Wu-Smart and Spivak, 2018). However, to date, we do not have any evidence as to whether the behavioral repertoire of stingless bee gynes is also affected by imidacloprid. Therefore, taking into account its mode of neurosystemic action in insects, we hypothesized that stingless bee gynes treated with sublethal doses of imidacloprid may have their behavioral repertoire altered.

Hence, since behaviors exhibited by the gynes of stingless bees are a crucial step to their future acceptance into colonies, but assuming that insecticides may impair it, here, we evaluated whether imidacloprid may affect this process in *Plebeia droryana*.

2. Material and methods

2.1. Queen rearing and toxicological analysis

We obtained larval food and larvae of *P. droryana* from five colonies in a stingless bee apiary located at the Pontifical Catholic University of Rio Grande do Sul (PUCRS), Brazil 30° 3´38.108" S 51° 10´23.604" W. Larvae destined to become queens in *P. droryana* need 0.660 μL of larval food to change into this caste (Santos et al., 2015; dos Santos et al., 2016). To detect any effect of imidacloprid residues on queens of *P droryana* we performed two bioassays, one without adding imidacloprid into the larval food (nontreated treatment) and another by adding residual dose of imidacloprid, i.e., approximately 6.5 ppm of a.i. (insecticide treatment).

The residual amount of insecticide mentioned above was incorporated in our analyses after consulting the toxicological literature for the amount of residue detected in pollen grains in natural (or field) conditions (Bonmatin et al., 2005; Dively and Kamel, 2012; Maus et al., 2003; Morandin and Winston, 2003). As a result, we chose using the reference indicating ca. 0.005 μg/g of a.i. in pollen grains (Bonmatin et al., 2005). By considering that the weight of pollen grains inside brood cells of *P. droryana* (Rosa et al., 2015a, 2015b) reaches ca. 1.3 μg, we added 0.0065 μg/g of a.i. to the larval food offered to queen larvae of *P. droryana*. The queen rearing protocol and aliquot preparations are already described elsewhere (Santos et al., 2015; dos Santos et al., 2016).

We performed three replicates each for nontreated and insecticide treatments by transferring 30 larvae to three rearing plates, respectively, totaling 90 larvae for every treatment. After that, the first 10 gynes that emerged from both nontreated and insecticide treatments, despite their respective triplicate, were used for behavioral analysis.

2.2. Behavioral records by videotaping

The ten *P. droryana* gynes had their thoraxes painted using a nontoxic pen and were classified according to their respective treatment. After that, we built small boxes in which we introduced approximately 30 (whitish) callow workers and two to three combs containing approximately 50 well-developed pupae. We fed these bees with pollen and syrup ad libitum on a daily basis (for details, see Santos et al., 2015).

Data recording started as soon as the small boxes were ready and when the *P. droryana* gynes were one day old. Additionally, our videotaping was always done for nine consecutive days for each gyne, in the afternoon beginning around 2 p.m., because it is a period of activity of bees, and for 10 min, that is enough time to have a great perspective of their all-day behaviors. This time period was chosen because less than one week from emergence is sufficient to observe the keystone behaviors during the acceptance process of stingless bee gynes (da Silva et al., 1972; Imperatriz-Fonseca and Zucchi, 1995; Santos et al., 2015).

2.3. Selected behaviors

As previously described, stingless bee gynes may exhibit indirect (individual context) and direct (social context) contact with their workers during the queen selection process. Hence, in the individual context, the following behaviors were observed: (a) autogrooming (the queen cleans herself), (b) run into the colony, and (c) wing vibration. For the social context, the following behaviors were analyzed: (d) trophallaxis with workers, (e) antennation (touching her antennae with other bees), (f) harassment (aggression via workers biting her wings, legs, antennae, and abdominal region) and, (g) defending herself against worker aggression (i.e., turning her body with an abrupt abdomen movement). The duration time of every gyne behavior was not

evaluated, i.e., only its occurrence was registered. All these seven behaviors were recorded altogether by videotaping and then after choosing one of them (from *a* to *g*), for example, autogrooming, it was counted how many times queens performed such behavior within that 10 min.

2.4. Data analysis

We performed a discriminant analysis for applying a T^2 Hotelling test. This analysis is a type of multivariate *t*-test incorporating more than one response variable and is suitable for discriminating two groups (Hotelling, 1931; Curran, 2017). This test was carried out to investigate whether nontreated versus treated gynes would be different from each other. For this, we used the function *hotelling.test* (with 9999 permutations) from the package "Hotelling" (Curran, 2017). Therefore, the above gyne behaviors (a-g) were incorporated as multivariate response variables in the model, while both populations (nontreated vs. treated gynes) were ascribed as predictor variables.

Afterward, we analyzed the behavioral pattern (a-g) exhibited by nontreated queens versus treated queens (response variable) on a case-by-case basis. For this, we fitted a generalized additive mixed model (GAMM) with Poisson distribution. GAMM is a flexible regression model that alternatively incorporates nonparametric functions and correlated data, allowing the simultaneous inference on the smoothing parameters and the variance components (Bolker et al., 2009; Lin and Zhang, 1999). Therefore, we assumed both treatments (nontreated vs. treated gynes) and the elapsed time as fixed effects, whereas colonies and the period of observation were considered random effects. This analysis was performed using the function *gamm* from the package "mgcv" (Wood, 2011). All analyses were carried out in R (R Core Team, 2016).

3. Results

The videotape recording totaled 1170 min, with 760 min for nontreated treatment and 410 for insecticide treatment. Overall, running ($n = 1020$) and vibrating wings ($n = 990$) were the behaviors more commonly observed, followed by cleaning (autogrooming, $n = 353$), Fig. 1. On the other hand, socially, antennating the workers ($n = 689$) and harassment by them were observed often ($n = 277$), while trophallaxis ($n = 114$) and defense against worker aggression ($n = 104$) were less often observed (Fig. 1). As a whole, the treated queens are less

active than nontreated ones (Fig. 1). Furthermore, when all queen behaviors were analyzed as a unique multivariate response variable, we demonstrated that treated gynes were different from nontreated gynes (Hotelling $T^2 = 22.84$, d.f. = 8, 94, $p = 0.003$).

We observed that nearly all seven behaviors analyzed in gynes of *P. droryana* showed a similar trend during the beginning of the observations where the majority differed in frequency. Thus, except cleaning herself and running, all other behaviors had a curve pattern similar each other, varying according to the height of the plateau (Figs. 2 and 3). Another general indication comes from a period in which nontreated queens exhibit most of their behaviors: overall, they reach a plateau around five to six days after emergence, suggesting ceasing or reduced interest in conflict with their workers.

When the results were evaluated in detail, we did not find evidence for differences between the nontreated vs. treated gynes related to behaviors such as cleaning themselves ($p = 0.26$), to running ($p = 0.87$), antennation ($p = 0.40$) and defending themselves against worker aggression ($p = 0.29$) (Table 1, Figs. 2 and 3). On the other hand, we saw statistical significance for wing vibrating ($p = 0.01$), trophallaxis ($p = 0.01$) and harassment by workers ($p < 0.001$) (Table 1, Figs. 2 and 3). Finally, concerning the period of observation, we found an effect on running, wing vibration and defense against worker aggression ($p < 0.01$, respectively), suggesting that irrespective of queen treatments, the time of observation may have some effect on behaviors exhibited by them (Table 1, Figs. 2 and 3).

4. Discussion

The behavior exhibited by any organism is a mechanism used to send information from one individual to another, which must accurately interpret it as true communication. Here, we demonstrated that if *P. droryana* gynes are fed with residual doses of imidacloprid, the behavioral pattern exhibited to workers required for her acceptance is seriously impaired. As a result, workers fed much less the imidacloprid-treated gynes as compared to control gynes. Additionally, imidacloprid-treated gynes are also much more harassed by her workers on the same situation. In other words, imidacloprid-treated gynes may remain undernourished for a long time and encounter considerable hostility from their workers, which may have contributed to the reduced observation time of imidacloprid-treated queens (410 min) compared to nontreated queens (760 min) because some queens who received the former treatment died before the experiment ended.
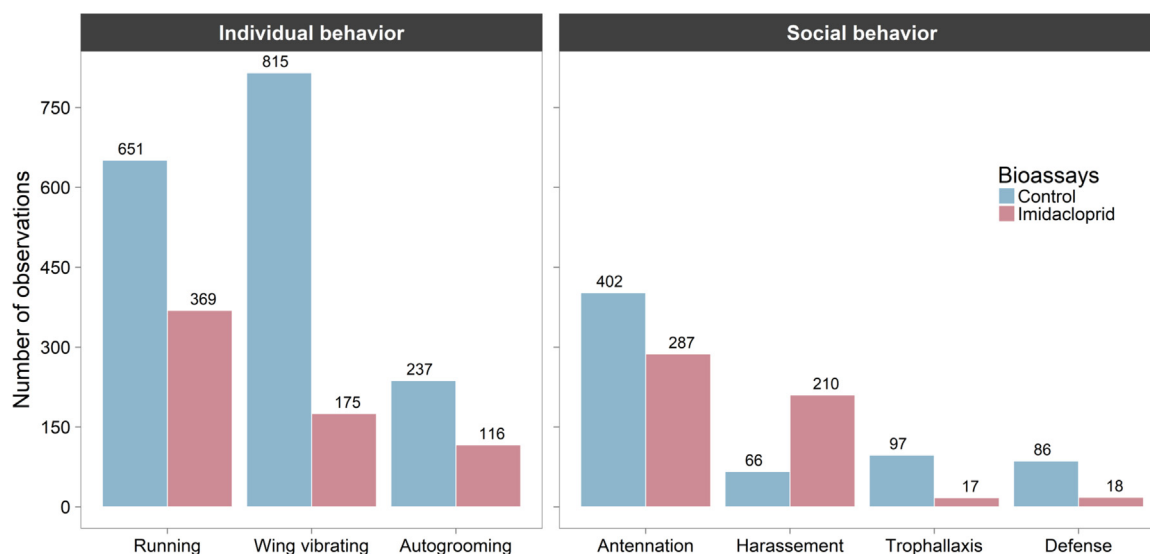

Fig. 1. Number of behaviors exhibited by queens of *Plebeia droryana*. Nontreated queens did not receive any residue of neonicotinoid imidacloprid; Imidacloprid: queens had 0.0065 μg/g of a.i. of neonicotinoid imidacloprid added to her larval food.
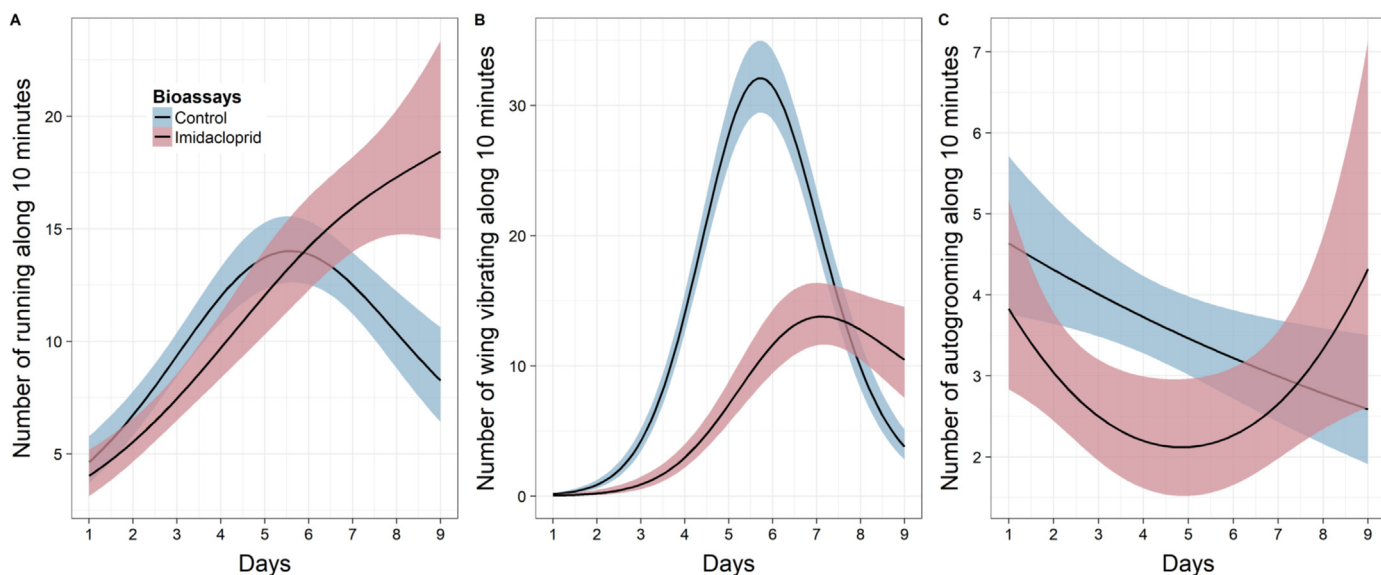
Fig. 2. Individual context – Behavioral pattern of virgin queens of stingless bee *Plebeia droryana* (Hymenoptera: Apidae, Meliponini) in the first days after emergence. Nontreated queens did not receive any residue of neonicotinoid imidacloprid; Imidacloprid queens had 0.0065 µg/g of a.i. of neonicotinoid imidacloprid added to her larval food.
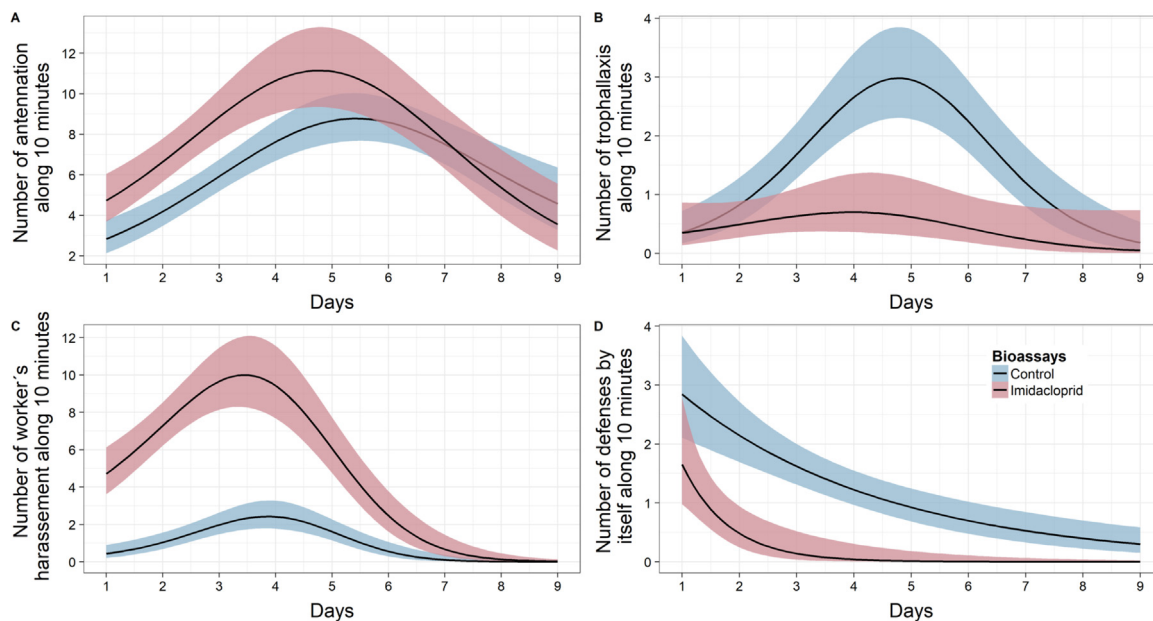

Fig. 3. Social context – Behavioral pattern of virgin queens of stingless bee *Plebeia droryana* (Hymenoptera: Apidae, Meliponini) in the first days after emergence. Nontreated queens did not receive any residue of neonicotinoid imidacloprid; Imidacloprid queens had 0.0065 µg/g of a.i. of neonicotinoid imidacloprid added to her larval food.

When considering the seven queen behaviors analyzed, the fact that treated queens performed much less trophallaxis than their counterparts while encountering much more aggression was significant. We assume that if such treated queens performed more antennation with their workers, it could be a first attempt to obtain trophallaxis given that this behavior is often preceded by antennation (Contrera et al., 2009). However, even though treated queens performed substantial antennation, the workers did not reciprocate with trophallaxis. This indicates that workers most likely recognized something peculiar with treated queens and therefore harassed the treated queens much more than nontreated ones. Therefore, it may help explain why treated queens showed an increase in individual behaviors at the end of the observations such as cleaning herself, running into the colonies and vibrating her wings. Since these behaviors are relevant to spread

cuticular hydrocarbon compounds (non-volatile pheromones) on body, as well as exhibit herself to nestmates or still to spread volatile pheromones inside nests, respectively, then such treated queens may have tried to call more attention (and less harassment) of her workers.

Our findings suggest that worker aggression on treated gynes may be trigged by both behavioral and chemical cues. For example, the delay of starting wing vibrating or vibrating less often may have been a signal to workers, indicating that the treated queens were not viable given that the wing vibrating of virgin queens in stingless bees is a very common behavior (Imperatriz-Fonseca and Zucchi, 1995; Nogueira-Ferreira et al., 2009; Santos et al., 2015; Veiga et al., 2017). Another possible reason that treated queens are harassed more is that their cuticular hydrocarbon profile (CHC) differ from those of nontreated queens. It is well known that virgin queens in many social insect species

Table 1
Parameters and estimated regression coefficients from the generalized additive mixed models (GAMM) for behaviors of queens of *Plebeia droryana*.

Context	Fixed effects	Estimate	F	P
INDIVIDUAL	Running	− 0.02	0.02	0.87
	Days	0.16	27.04	< 0.001***
	Autogrooming	− 0.22	1.23	0.26
	Days	− 0.05	1.73	0.19
	Wing vibrating	− 0.9	6	0.01**
	Days	0.53	50.91	< 0.001***
SOCIAL	Antennation	0.19	0.7	0.4
	Days	0.08	3.52	0.06
	Trophallaxis	− 0.98	6.32	0.01**
	Days	0.03	0.24	0.62
	Harassment	1.63	15.36	< 0.001***
	Days	− 0.13	2.29	0.13
	Defense	− 0.71	1.09	0.29
	Days	− 0.43	6.34	0.01**

possess a particular CHC profile, i.e., the "queen signal" (Araújo et al., 2017; Ferreira-Caliman et al., 2013; Nunes et al., 2009). If treated queens smell different than nontreated queens, this may be a genuine signal to workers because they can accurately discriminate and eliminate not healthy nestmates – social immunity (Cremer et al., 2018). To date, no study has addressed this issue in bees. However, studies in cockroaches, for example, have found that individuals exposed to insecticides modify their CHC profiles (Morakchi et al., 2006, 2005).

Our findings are the first to show the adverse effects on the behavior of queens in stingless bees caused by residual doses of insecticides — in this case, imidacloprid. As such, our data demonstrate how vulnerable the queens in *P. droryana* may be if they are contaminated with a residual quantity of imidacloprid, supporting other studies on other social insects such as honeybees, bumblebees, and fire ants (Scholer and Krischik, 2014; Wang et al., 2015; Whitehorn et al., 2012; Wu-Smart and Spivak, 2016; Wu-Smart and Spivak, 2018).

Although neonicotinoids are thought for pest insects, the human beings are also being exposed to such insecticides because some vegetables and fruits seem to contain residues of these chemical compounds in its tissues (Chen et al., 2014). It demonstrates that not only arthropods, but also vertebrates may suffer any damage as exposed to neonicotinoids once them seem to be closely related to brain disorder causing behavioral alteration as autism in children exposed to imidacloprid (Keil et al., 2014). Furthermore, neurological problems as anencephaly have been observed in human population living near agricultural areas employing neonicotinoids as chemical defensives (Yang et al., 2014). Therefore, these data evidence how negative may be the neonicotinoids to living organisms nontarget of chemical control as are beneficial arthropods and human beings. Consequently, we need to rethink its broad usage on global agriculture.

5. Conclusions

The continuous use of agricultural defenses such as imidacloprid may cause negative effects in nontarget organisms that would otherwise benefit the crops through pollination. Furthermore, agriculture expansion often fragments most landscapes, which affects wild bees by restraining their nesting substrates and provision of floral resources. Nectar and pollen from crop blossoms could be an alternative to feeding bees. However, flowers containing residues of neurotoxic insecticides may seriously compromise the diet, development, behavioral repertoire and consequently the survival of bees in the long term.

Acknowledgments

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