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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