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Original article

Azadirachtin-induced antifeeding in Neotropical stingless bees

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Abstract – The ongoing debate regarding the role of pesticides in the global decline of bee populations is increasing the demand for use of biopesticides, compounds generally believed to be less harmful to pollinators. However, there is lack of evidence justifying such perceptions, particularly regarding native pollinator species like Neotropical stingless bees. Here, we investigated whether azadirachtin, a neem-based biopesticide, causes significant lethal and sublethal effects on adult workers of the Neotropical stingless bee species *Melipona quadrifasciata* and *Partamona helleri*. Susceptibility to azadirachtin varied with several factors, including the route of exposure, the concentration of the biopesticide, and the bee species. We found that although azadirachtin did not affect worker bee mortality, flight, or respiration rate, it did, however, induce a significant antifeeding effect on the stingless bee species.

biopesticides / wild pollinators / pesticide toxicity / sublethal effects / risk assessment

1. INTRODUCTION

Many cultivated plants depend on bee pollination for fruit and seed production, as reviewed by Klein et al. (2007), and as such, bees are particularly important in light of the increasing demand for pollination services for agriculture food production (Chauzat et al. 2013; Breeze et al. 2014). Pollination deficits can lead to reduced yields, in turn leading to economic loss in agriculture systems; moreover, the estimated loss in ecosystem services provided by pollinators is valued at around €153 million annually (Gallai et al. 2009). The conspicuous and ever-growing use of agriculture pesticides is a recognized threat to bee populations and the ecological services they provide, particularly that of pollination (Sanchez-Bayo and Goka 2014; Barbosa et al. 2015b; Guedes et al. 2016). Thus, risk analyses of pesticides are needed in order to assess such potential threats, encompassing not only lethal but also sublethal exposure via contact and ingestion, in both honeybee and native bee pollinator populations (Van der Sluijs et al. 2013; Fairbrother et al. 2014; Barbosa et al. 2015b; Lima et al. 2016).

Many of the lethal and sublethal effects of synthetic pesticides on honeybees are broadly recognized and have been extensively reported in the scientific literature (e.g., Arena and Sgolastra 2014; Fairbrother et al. 2014; Staveley et al. 2014). However, such studies have largely focused on honeybees and neonicotinoid insecticides (Barbosa et al. 2015b), and a handful of other synthetic compounds (Suri and Singh 2011; Valk and Koomen 2012; Godfray et al. 2014; Roubik 2014). One of the consequences of such emphasis is a growing interest in the use of alternative pesticidal compounds, primarily those of natural origin, which are commonly referred to as biopesticides (Isman 2006; Gerwick

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and Sparks 2014; Villaverde et al. 2014; Guedes et al. 2016). Nonetheless, given that the source of origin is not a determinant of biological activity, the common perception that biopesticides are safer to pollinators is misguided (Coats 1994; Bahlai et al. 2010; Barbosa et al. 2015b; Guedes et al. 2016). This prevailing notion, however, has led to a knowledge gap regarding the potential impacts of biopesticides on pollinators, although recent studies indicate that the impacts of biopesticide compounds on some bee pollinators may be significant (Barbosa et al. 2015a; Barbosa et al. 2015b; Barbosa et al. 2015c; Tomé et al. 2015a; Tomé et al. 2015b).

The botanical biopesticide azadirachtin, obtained from the Indian neem tree (*Azadirachta indica* A. Juss. [Meliaceae]), is the most widely used natural pesticide today. The initial optimism regarding its safety on non-target species has, however, subsequently been replaced by a more cautious posture (e.g., Barbosa et al. 2015a; Barbosa et al. 2015b; Barbosa et al. 2015c; Cordeiro et al. 2010; Lima et al. 2015). The use of azadirachtin and derived products is still increasing (Isman 2006; Isman and Grieneisen 2014), likely due to its appeal as an efficacious biopesticide with a rapid environmental breakdown (Boeke et al. 2004; Isman and Grieneisen 2014). As such, the lack of research on the potential impacts of azadirachtin on bee pollinator species remains a concern worthy of greater attention.

Another serious bias of studies of insecticide-pollinator interactions is the overwhelming focus on honeybees, often at the expense of native bee fauna (Valk and Koomen 2012; Barbosa et al. 2015b; Lima et al. 2016). The stingless bee fauna of megadiverse tropical countries illustrates the importance of this group of native pollinators, not only for cultivated crops but also for native plant species (Kremen et al. 2002; Del Sarto et al. 2005; Barbosa et al. 2015b). The scarcity of information available regarding the decline of this group of pollinators, and the threat imposed by pesticides, biopesticides included, to these species, justifies the concern, as does research indicating that stingless bees are generally more vulnerable to these compounds than are honeybees (Tomé et al. 2012; Arena and Sgolastra 2014; Del Sarto et al. 2014; Barbosa et al. 2015a, 2015c; Tomé et al. 2015a; Tomé et al. 2015b). Our goal here was to redress this knowledge gap somewhat by assessing the potential impacts of azadirachtin on two species of stingless bee pollinator species, *Melipona quadrifasciata* Lepeletier and *Partamona helleri* Friese.

In this experiment, adult foraging workers of the two stingless bee species were exposed to field rates of the biopesticide azadirachtin via contact and oral exposure, and their lethal and sublethal effects were recorded. The relative species-dependent toxicity usually associated with azadirachtin makes predictions of its toxicity difficult, but, based on the few studies available with pollinators, this insecticide is unlikely to cause high acute mortality in stingless bees, although significant sublethal effects are likely to occur (Barbosa et al. 2015a, 2015c; Lima et al. 2016).

2. MATERIAL AND METHODS

2.1. Bees and insecticide

Bee specimens of both species were collected from five colonies each of *M. quadrifasciata* and *P. helleri* in Viçosa county, in the state of Minas Gerais, Brazil (20° 45′ S and 42° 52′ W), and established in the experimental apiary of the Federal University of Viçosa. The adult foraging bees used in the experiment were collected at the colony entrance of each hive using glass jars and were subsequently taken to the laboratory. The insects were maintained without food in wooden cages (35 cm × 35 cm × 35 cm) covered with organza and kept under environmentally controlled conditions (25 ± 2 °C, 70 ± 10 % relative humidity, 24-h scotophase) for 1 h, following which they were subjected to the bioassays.

A commercial azadirachtin-based insecticide formulation was used in the study (Azamax, 12 g a.i./L, emulsifiable concentrate; DVA Agro Brasil, Campinas, SP, Brazil) and diluted either in deionized and distilled water (for contact exposure bioassays) or in 50 % (*w*/*w*) aqueous sucrose solution (for oral exposure bioassays). The azadirachtin concentrations used in the bioassay exposures consisted of the maximum field rate (30 mg a.i./L) and twice the maximum field rate (60 mg a.i./L); controls were exposed only to the solvent (i.e., either water or 50 % aqueous sucrose solution). The maximum field rate used was based on the

recommended concentration for control of the whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Sternorrhyncha: Aleyrodidae) in open tomato fields in Brazil (Ministério da Agricultura, Pecuária e Abastecimento 2015).

2.2. Time-mortality (survival) bioassays

Contact and oral exposure time-mortality bioassays were performed with adult foraging workers of both stingless bee species, with identical methods adopted for both species. We followed the procedures described by Tomé et al. (2015b), in which the contact bioassays were performed using 250-mL transparent containers with inner walls impregnated with 500 μL insecticide solution (or water for controls) using an artist's air brush (Sagyma SW440A, Yamar Brasil, São Paulo, SP, Brazil) coupled with an air pump (Prismatex 131A Tipo 2 VC, Itu, SP, Brazil) at a pressure of 6.9×10^4 Pa. The sprayed containers were dried for 2 h in a dark exhausting chamber kept at 25 ± 3 °C. Then, 10 adult non-starved worker bees were released into each container, which were covered by organza in the upper part to prevent the insects from escaping. Each container corresponded to an experimental unit, and five containers, each with insects from a single colony (i.e., five colonies, or replicates) were used for each species and insecticide treatment (and control). Uncontaminated sucrose solution was provided ad libitum to the bees via a feeder extending through a hole in the containers, regardless of the treatment; the feeder allowed only a single insect to feed at a time and the feeding was brief with the other nine insects maintaining contact with the contaminated surface of the container. After 3 h of exposure, the treated insects were transferred to uncontaminated containers and fed uncontaminated sucrose solution. Survival was assessed at 1-h intervals for 24 h, with mortality defined as those insects unable to walk the length of their body.

The same experimental units of the contact bioassay were also used for the oral exposure bioassays, including the same number of insects and replicates. However, insecticide exposure in this case was achieved by providing 500 μL of azadirachtin-contaminated sucrose solution (except for untreated controls) in drilled Eppendorf® tubes used as feeders and inserted through a hole in each of the 250-mL transparent containers, each one with 10 bees of each species. The bees were kept in uncontaminated containers throughout this experiment. Food was withheld from the adult foragers for 1 h, after which they were allowed to feed for 3 h either in azadirachtin-contaminated or uncontaminated (i.e., control) diet; following this, the bees were provided with uncontaminated sucrose solution. The insecticide dose ingested was obtained by weighing the feeders before and after the experiment and dividing by the number of bees in the container. Mortality was recorded at 6, 9, 12, 24, 48, and 72 h after the beginning of the exposure. The worker was considered as dead if unable to walk the length of their body.

2.3. Feeding bioassays

Food consumption and potential antifeedant effects caused by azadirachtin exposure were evaluated through two bioassays. Feeding avoidance (i.e., antifeeding effect) was assessed in individual bees, while food consumption was assessed in groups of 10 worker bees of the same colony to allow for the group effect on these social organisms; otherwise, consumption is likely compromised. Preliminary investigation with both species indicated an average consumption of 10 ± 1 μL sucrose per bee within a 1-min period. Therefore, for the feeding avoidance bioassay, each individual worker bee was provided with 4 μL of sucrose solution (azadirachtin-contaminated, excepted with the control treatment) as a drop at the bottom of the horizontal glass vials (15 cm length × 2 cm diameter) where the individual bees were maintained for 5 min ensuring the ingestion of the whole solution after 1 h of starvation. The bioassay was carried out with 10 foraging workers from each of the same five colonies previously used, which were tested in each azadirachtin treatment (0, 30, and 60 mg a.i./L). The adult bees were attracted by a light source at the outside of the bottom of the feeder, following the procedure outlined by Barbosa et al. (2015a). After this period of 5-min exposure, the bees were transferred to identical containers maintained at the same conditions and with provision of 4 μL of uncontaminated sucrose diet and allowed to feed for an additional 5-min period. All of the bees ingested the contaminated diet (except the controls, which fed on uncontaminated diet). The number of bees feeding on the uncontaminated diet after the initial 5-min exposure to the azadirachtin-contaminated diet (except for the control) was recorded to assess whether azadirachtin ingestion impairs subsequent feeding.

The intake of diet after azadirachtin exposure was assessed in a complementary food consumption bioassay where adult workers were maintained in 250 mL containers, as in the time-mortality bioassays for oral exposure. Each container had a group of 10 bees of a given colony (for a total of five colonies, or replicates, per treatment) provisioned with diet (i.e., sucrose solution with either 0, 30, or 60 mg azadirachtin/L) for 3 h. These workers were subsequently transferred to 250-mL containers provisioned with uncontaminated diet, as previously described. The feeders were replenished with uncontaminated sucrose diet in each treatment, and the diet consumption was recorded at 6, 9, 12, 24, 48, and 72 h by weighing the food provided before and after these periods of ingestion. Thus, the frequency of bees exhibiting food avoidance following azadirachtin exposure was recorded in the first consumption bioassay and food ingestion was recorded in the second, also after azadirachtin exposure.

2.4. Walking bioassays

Twelve adult worker bees (3-day old) were collected from individualized brood combs taken from the five different colonies of each species upon progeny pupation (Tomé et al. 2012). Therefore, in this experiment, we used 120 bees (12 bees × 5 colonies × 2 treatments), which were individually subjected to walking bioassays on azadirachtin half-treated arenas, in accordance with previously described methods (Guedes et al. 2009; Cordeiro et al. 2010). Briefly, filter paper discs (9 cm in diameter with porosity of 3 μm, 0.5 % ash content, and 80 g/m^2 density; Nalgon Equip. Científicos, Itupeva, SP, Brazil) were treated with 1 mL of azadirachtin (or water) solution at the same concentration of the previous bioassays. Each arena was composed of a water-treated filter paper fixed to the bottom of a Petri dish, with half of the azadirachtin-treated disc fixed over the control disc using water-based white (synthetic) glue resin, such that half of the arena was treated with azadirachtin and the other half left untreated; both halves were left untreated in the control treatment. The inner walls of the Petri dish arenas were coated with Teflon TPFE® (DuPont, Wilmington, DE, USA) to prevent the bees from escaping. A single 3-day-old adult worker was released into the center of each arena, and the arena then placed under a video tracking system (ViewPoint LifeSciences, Montreal, Quebec, Canada). The walking bees were recorded for 10 min, after which the videos were digitally transferred to a computer. This system allowed calculation of the time spent in each half of the arena (azadirachtin-treated and untreated) and thus indication of potential repellence (i.e., number of bees spending less than 1 s on the azadirachtin-treated half of the arena) and irritability (i.e., number of bees remained for longer on the untreated half of the arena) triggered by azadirachtin.

2.5. Flight takeoff bioassays

Flight takeoff bioassays were performed with adult foraging workers following exposure to azadirachtin either by contact or ingestion, as previously detailed in the time-mortality (survival) bioassays. After a 3-h period of exposure to azadirachtin (0, 30, and 60 mg of azadirachtin/L) and a 24-h period under uncontaminated conditions, the insects were individually subjected to the flight takeoff bioassays, following the procedure described by Tomé et al. (2015b). For this bioassay, a three-staked wooden cage tower was used (35 cm × 35 cm × 35 cm, each forming a 105-cm tall tower) that allowed for unrestricted movement and flight. A fluorescent lamp was placed 5 cm above the tower in a dark room to attract the flying worker bees. An individual foraging worker was then released from a Petri dish at the center bottom of the tower after a 1-min acclimation and cover removal allowing the insect to take off for flight. Vertical flight takeoff and activity was observed for 2 min and recorded as (a) no flight, (b) flight up to a height of 35 cm, (c) flight between heights of 35 and 70 cm, (d) flight between heights of 71 and 105 cm, and (e) flight reaching the light source at a height of 110 cm. Fifty bees from different colonies (i.e., 10 from each of the five colonies) were used for each insecticidal treatment and mode of exposure. The flight activity was recorded for each individual bee and colony. The worker bees treated with water only (control) were from the same colonies as the azadirachtin-exposed bees and they were all maintained under the same conditions.

2.6. Respirometry bioassays

Bees exposed to azadirachtin both by contact and oral ingestion were also subjected to respirometry bioassays, as respiratory rhythms can indicate behavioral and ontogeny alterations in stingless bees (Teixeira et al. 2011). Thus, following azadirachtin exposure as

previously described for the survival bioassays, five individual insects from each colony (i.e., independent biological replicates) and subjected to each azadirachtin treatment in both types of exposure (i.e., contact and oral exposure) were individually enclosed in 25-mL glass containers connected to a completely closed system (TR3C respirometer equipped with a CO_2 analyzer; Sable Systems International, Las Vegas, NV, USA). The CO_2 production (μL CO_2/h/bee) was determined after a 3-h period by injecting CO_2-free air into the chamber for 2 min at a flow rate of 600 mL/min. The air current was directed to an infrared reader connected to the system, allowing for the determination of the CO_2 produced per bee.

2.7. Statistical analyses

Kaplan-Meier estimators were used to calculate survival curves and estimates of median survival times (LT_{50}'s), when appropriate (PROC LIFETEST; SAS Institute 2008). Normality and homoscedasticity assumptions were tested for food consumption, walking, flight takeoff, and respirometry results (PROC UNIVARIATE; SAS Institute 2008). A paired *t* test was used to analyze walking data, whereas a Wilcoxon rank sum test was used to analyze food avoidance results (PROC FREQ; SAS Institute 2008). Food consumption results were tested via repeated measures analyses of variance, with time as a pseudoreplicate (PROC ANOVA with PROFILE statement; SAS Institute 2008). Finally, flight takeoff data were subjected to a (nonparametric) Kruskal-Wallis test ($P < 0.05$) (PROC NPAR1WAY; SAS Institute 2008).

3. RESULTS

3.1. Time-mortality (survival) response

Azadirachtin did not cause any bee mortality up to 24 h of contact exposure for *M. quadrifasciata* ($\chi^2 = 0.098$, $df = 2$, $P = 0.952$) and *P. helleri* ($\chi^2 = 0.200$, $df = 2$, $P = 0.905$). Similarly, no mortality was observed for either *M. quadrifasciata* ($\chi^2 = 0.202$, $df = 2$, $P = 0.904$) or *P. helleri* ($\chi^2 = 1.333$, $df = 2$, $P = 0.513$) in any treatment (azadirachtin and control) up to 72 h following oral exposure. Therefore, no lethal effect of azadirachtin was detected after neither via of exposure for the species studied, and as such, median lethal times could not be estimated for any treatment.

3.2. Food avoidance and consumption

Azadirachtin did not elicit food avoidance in the stingless bee species *M. quadrifasciata* ($\chi^2 = 2.45$, $df = 2$, $P = 0.30$, Figure 1a). In contrast, azadirachtin did elicit food avoidance (of uncontaminated diet) among workers of *P. helleri*, particularly at the highest azadirachtin concentration, which corresponded to twice the registered label rate ($\chi^2 = 18.91$, $df = 2$, $P < 0.001$, Figure 1b). Food consumption, however, was more distinctly affected by the presence of azadirachtin. Such differences, although significant for *M. quadrifasciata*, particularly after shifting to the uncontaminated diet ($F_{1,2} = 5.98$, $P = 0.01$), were soon recovered (Figure 2a), with little effect on cumulative food consumption observed after 6 h of feeding on the uncontaminated diet (Figure 2b). *P. helleri* was more drastically affected by early ingestion of the azadirachtin-contaminated diet ($F_{1,2} = 7.88$, $P = 0.006$), which impaired subsequent food consumption through time and retaining lower levels of food intake through to the end of the observation period, at 72 h (Figure 2c, d).

3.3. Walking response

Representative walking tracks of adult worker bees of both species when released on half-treated arenas are exhibited in Figure 3. Although azadirachtin did not elicit repellence at the field rate in either bee species (*M. quadrifasciata*: $t_4 = 0.91$, $P = 0.41$; *P. helleri*: $t_4 = 1.27$, $P = 0.27$), this biopesticide elicited significant irritability at twice the field rate for *M. quadrifasciata* ($t_4 = 3.15$, $P = 0.03$; Figure 4a), but not for *P. helleri* ($t_4 = 1.75$, $P = 0.15$; Figure 4b), in response to contact exposure.

3.4. Flight takeoff response

Flight takeoff performance was assessed for azadirachtin-exposed and unexposed adult foraging workers, but no significant effect was detected for either ingestion or contact exposure regardless

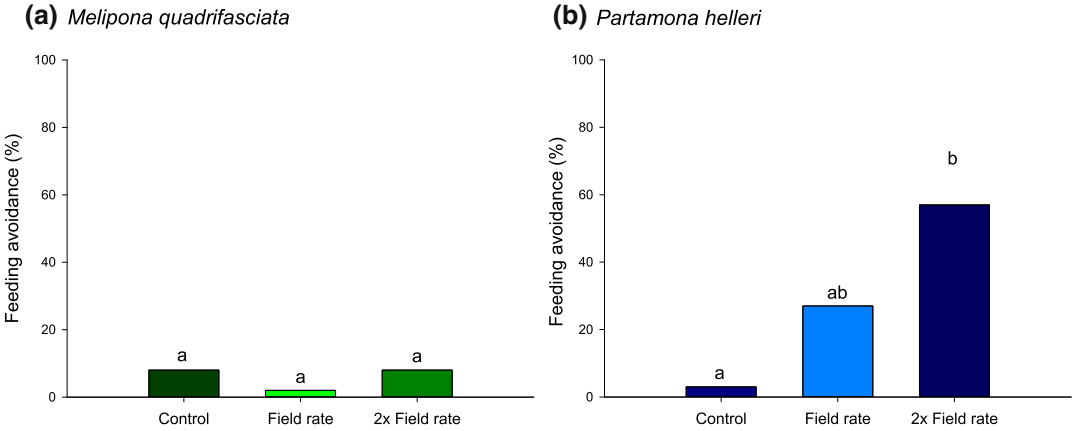

Figure 1. Feeding avoidance (primary antifeedancy) of an uncontaminated diet by foraging workers of two stingless bee species, *M. quadrifasciata* (**a**) and *P. helleri* (**b**), orally exposed to azadirachtin for 5 min. *Histogram bars with the same letters* are not significantly different by the Wilcoxon rank sum test ($P < 0.05$).

of the species ($H < 1.82$, $df = 2$, $P > 0.40$). For ingestion exposure, the average timed response for *M. quadrifasciata* was 13.59 ± 3.74 s and for *P. helleri* 11.80 ± 4 s; for contact exposure, the average timed response for *M. quadrifasciata* was 10.34 ± 3.4 s and for *P. helleri* 12.12 ± 4.5 s.

3.5. Respiration rate

Azadirachtin did not affect the respiration rate of bees of either species exposed by contact or exposure ($P > 0.05$). Contact-exposed worker bees of *M. quadrifasciata* exhibited an average respiration rate of 0.28 ± 0.03 μL CO_2/h/bee ($F_{1,2} = 0.43$, $P = 0.65$), whereas worker bees of *P. helleri* exhibited an average respiration rate of 0.10 ± 0.02 μL CO_2/h/bee ($F_{1,2} = 0.04$, $P = 0.95$). Orally exposed worker bees of *M. quadrifasciata* exhibited an average respiration rate of 0.32 ± 0.04 μL CO_2/h/bee ($F_{1,2} = 1.07$, $P = 0.37$), whereas worker bees of *P. helleri* exhibited an average respiration rate of 0.10 ± 0.02 μL CO_2/h/bee ($F_{1,2} = 0.10$, $P = 0.90$).

4. DISCUSSION

In this study, we assessed the potential impacts of the biopesticide azadirachtin on adult workers of two stingless bee species, *M. quadrifasciata* and *P. helleri* , through both contact and oral exposure routes. Based on the results of the few previous studies available, we did not expect azadirachtin to induce high acute mortality, but considered sublethal effects to be more likely, although azadirachtin toxicity appears to be species dependent even among pollinators (Barbosa et al. 2015a, Barbosa et al. 2015c). Indeed, our results were congruent with such expectations, as we observed negligible acute mortality following contact and oral exposure to azadirachtin. However, sublethal effects on walking activity and food consumption were detected varying with the stingless bee species.

The lack of azadirachtin repellence to both pollinator species indicates that they are unlikely to avoid field exposure to azadirachtin-contaminated flowers and will collect contaminated resources. The irritability to azadirachtin-contaminated surfaces exhibited by workers of *M. quadrifasciata* will likely reduce the collection of large amounts of contaminated resources. Nonetheless, such irritability occurred only at high azadirachtin concentrations, a scenario unlikely to take place under field conditions. In contrast, *P. helleri* did not exhibit irritability to azadirachtin-contaminated surfaces. This finding reinforces the risk of exposure to azadirachtin for both stingless bees under field conditions, unlike the reports with other insect and mite species that exhibit azadirachtin irritability and even repellence (Cordeiro et al. 2010; Lima et al. 2013).

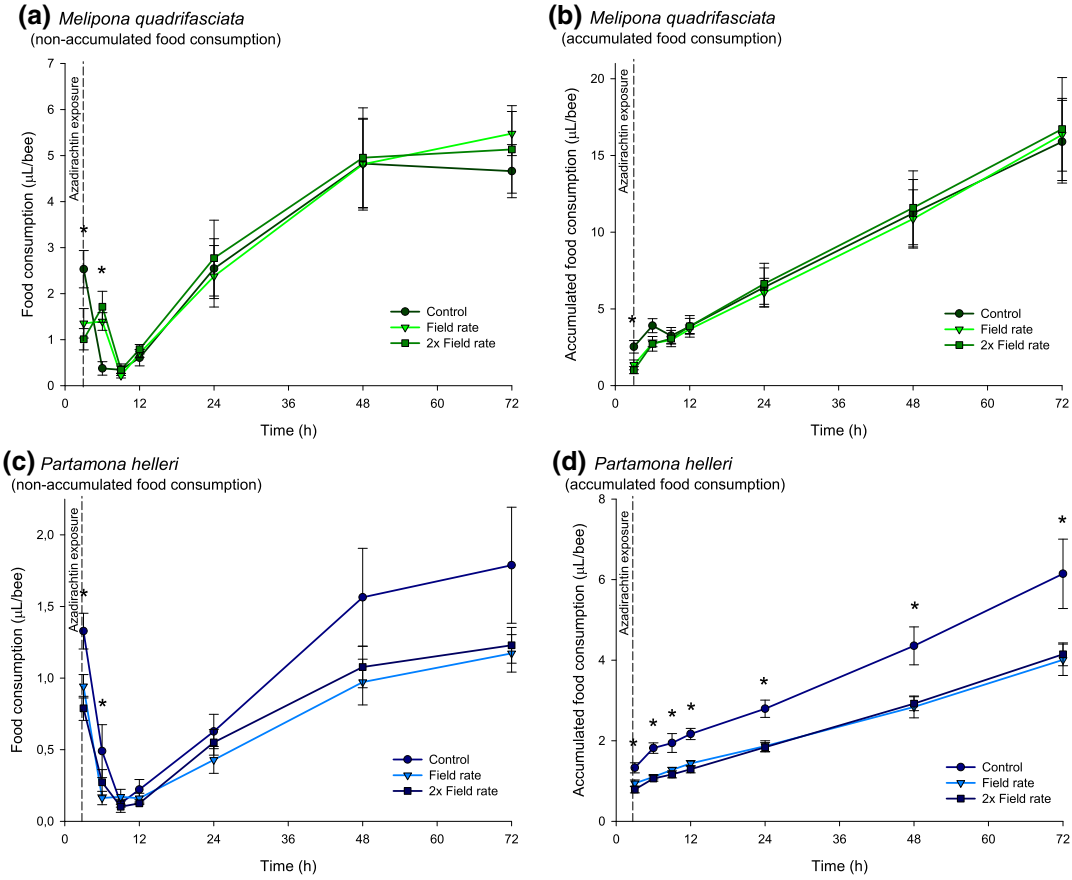

Figure 2. Consumption (mean ± SE) of uncontaminated feed by foraging workers of two stingless bee species, *M. quadrifasciata* (**a**, **b**) and *P. helleri* (**c**, **d**), orally exposed to azadirachtin. *Asterisks* indicate significant differences between treatments by Fisher's *F* test ($P < 0.05$).

The differences between the feeding behavior of both stingless bees after azadirachtin ingestion also deserve attention. Because workers of *P. helleri* exhibited concentration-dependent azadirachtin feeding avoidance and also reduced food consumption, colonies of this species are more likely to be harmed by azadirachtin than *M. quadrifasciata*. Azadirachtin is characterized as a growth regulator and sterilant insecticide but has also been shown to induce antifeeding behavior in insects (Mordue (Luntz) et al. 1998; Mordue (Luntz) and Nisbet 2000). The former two effects are irrelevant for adult worker bees, given that they are already fully developed and sterile, but larval exposure to azadirachtin may result in a different outcome (Tomé et al. 2015a), and insufficient feeding may impair worker foraging. However, the antifeeding behavior observed in *P. helleri*, but not *M. quadrifasciata*, suggests that the former will have a smaller risk of contamination than the latter.

In addition to azadirachtin, several other compounds in the plant biosynthetic pathway leading to its production have antifeedant properties (Aerts and Mordue (Luntz) 1997). The early feeding avoidance (i.e., primary antifeedancy) seems to be mediated by (gustatory) contact chemoreception, but internal feedback mechanisms may also take place that lead to toxic consequences to the insect (i.e., secondary antifeedancy) (Trumm and Dorn 2000; Mordue (Luntz) et al. 2010). Such effects are known to vary with insect species (Mordue (Luntz) et al. 2010), as observed here for

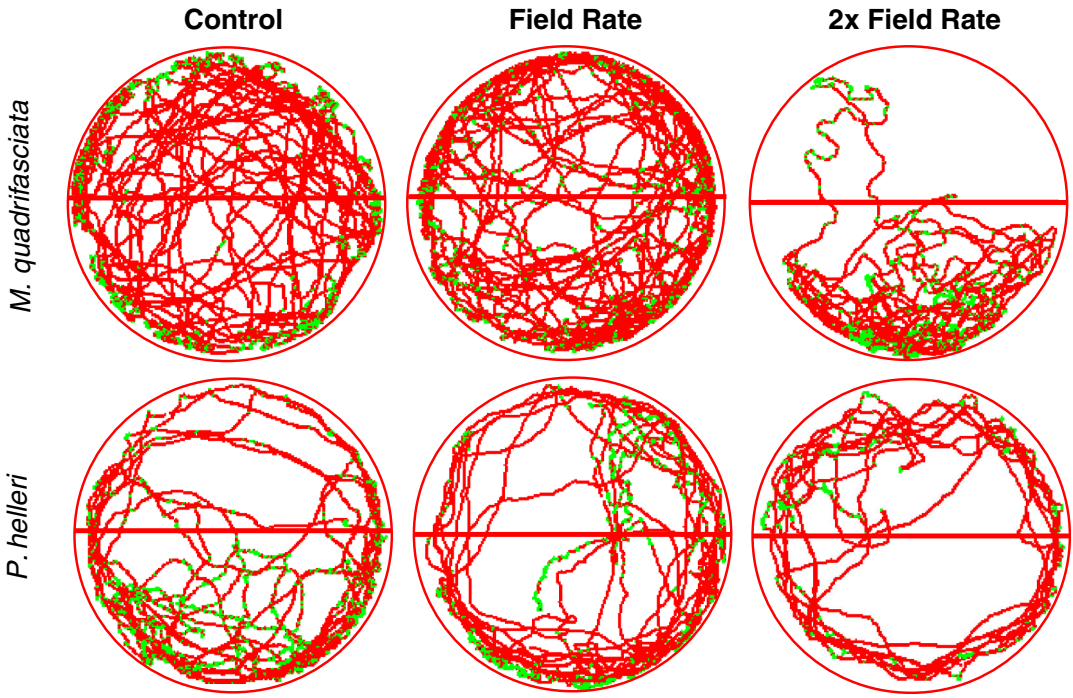

Figure 3. Representative tracks showing the walking activity of workers of two stingless bee species, *M. quadrifasciata* and *P. helleri*, over a 10-min period on paper-filter arenas (9 cm diameter) half impregnated with dried azadirachtin residues (*upper half of each arena*). *Red tracks* indicate high walking velocity; *green tracks* indicate low (initial) velocity.

stingless bees. The primary antifeeding response to azadirachtin is a result of the stimulation of deterrent (gustatory) chemoreceptors by azadirachtin, which also is believed

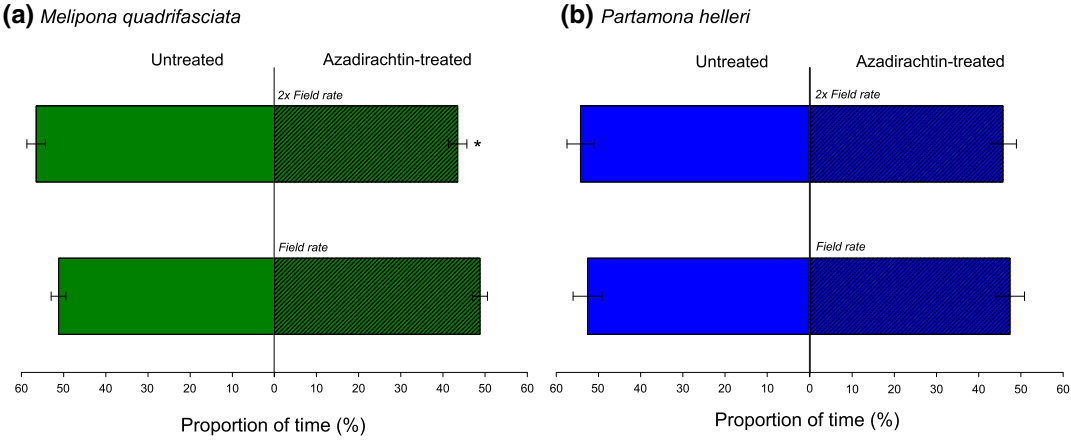

Figure 4. Insecticide irritability of workers of two stingless bee species, *M. quadrifasciata* (**a**) and *P. helleri* (**b**), exposed for a 10-min period on paper-filter arenas (9 cm diameter) half impregnated with dried azadirachtin residues. *Asterisks on histogram bars* indicate significant differences between the treated and untreated halves of the arena (*t* test; $P < 0.05$).

to have inhibitory effects on the firing of sucrose-sensitive cells of the gustatory chemoreceptors (Simmonds and Blaney 1984, 1996).

Several species have been shown to be extremely sensitive to the primary antifeedant properties of azadirachtin, thus, starving to death rather than ingesting the biopesticide (Mordue (Luntz) et al. 2010). Such an effect on *P. helleri* was significant only at azadirachtin concentrations twice that of the label rate. However, this feeding avoidance behavior remained even when the bees were provided with an uncontaminated diet, and their food consumption was also compromised, what may also compromise future foraging. Secondary antifeedancy effects were not assessed in our experiments, but likely occur and may explain the subsequent feeding avoidance and reduced diet consumption behaviors exhibited by *P. helleri* following exposure to azadirachtin.

Biopesticides, of which azadirachtin is one of the most prominent examples under commercial worldwide use (Isman 2006; Isman and Grieneisen 2014), are generally regarded as safer to non-target arthropod species, despite the lack of a proven relationship between origin (either natural or synthetic) and toxicity (Coats 1994; Barbosa et al. 2015b; Guedes et al. 2016). Such a notion, although misguided in many cases, does appear to be valid for azadirachtin and stingless bees, with only one of the two tested species exhibiting mild susceptibility to the antifeedant effects of the compound. Yet, these effects may be important, however, since they appear to compromise subsequent foraging and reduce feeding by foraging workers. Moreover, we focused solely on the impacts of azadirachtin exposure on adult bees, whereas larval exposure to azadirachtin may be of even greater importance given that the developmental stages are more vulnerable to the growth regulator properties of this biopesticide.

Another point of concern is that, as reported in this manuscript, the impacts of azadirachtin exposure can vary quite considerably even between the two species of stingless bees (Meliponini). Therefore, although only one of the tested species (*P. helleri*) was significantly affected at a sublethal level by azadirachtin, other species of stingless bees may prove to be more susceptible. This topic deserves future attention, especially in light of the fact that honeybees are questionable surrogates for stingless bees in pesticide impact assessments (Arena and Sgolastra 2014; Barbosa et al. 2015b).

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Authors' contributions Designed the experiments: RCB, HVVT, WFB, RNCG, and MAPL
Performed the experiments: RCB, HVVT, and WFB
Contributed analysis tools and materials: RCB, RNCG, HVVT, WFB, and MAPL
Wrote the paper: RCB, MAPL, and RNCG

Compliance with ethical standards

Ethics statement The study was performed with the permission of the Chico Mendes Institute for Biodiversity Conservation [ICMBio] (SISBIO permit no. 46746-1) of the Brazilian Ministry of the Environment and in accordance with the country's legislation. The bee specimens were collected in the field without depleting the original colonies and the experiments were carried out in the laboratory. Although some stingless bee species are included on the Brazilian list of endangered species, the species that were the focus of this study are neither protected nor endangered species.

Effet antiappétant de l'azadirachtine chez les abeilles sans aiguillon néo-tropicales

biopesticide / pollinisateur sauvage / toxicité / pesticide / effet sublétal / évaluation des risques

Durch Azadirachtin verursachte Antifraß-Effekte bei neotropischen stachellosen Bienen

biopestizide / natürliche bestäuber / pestizidtoxizität / subletale effekte / risikoabschätzung

REFERENCES

Aert, R.J., Mordue (Luntz), A.J. (1997) Feeding deterrence and toxicity of neem triterpenoids. J. Chem. Ecol. 23:2117–2132.

Arena, M., Sgolastra, F. (2014) A meta-analysis comparing the sensitivity of bees to pesticides. Ecotoxicology 23:324–334.

Bahlai, C.A., Xue, Y., McCreary, C.M, Schaafsma, A.W., Hallett, R.H. (2010) Choosing organic pesticides over synthetic pesticides may not effectively mitigate environmental risk in soybeans. PLoS One 5:e11250. doi: 10.1371/journal.pone.0011250

Barbosa, W.F., De Meyer, L., Guedes, R.N.C., Smagghe, G. (2015a) Lethal and sublethal effects of azadirachtin on the bumblebee *Bombus terrestris* (Hymenoptera: Apidae). Ecotoxicology 24:130–142.

Barbosa, W.F., Smagghe, G., Guedes, R.N.C. (2015b) Pesticides and reduced-risk insecticides, native bees and pantropical stingless bees: pitfalls and perspectives. Pest Manag. Sci. 71:1049–1053.

Barbosa, W.F., Tomé, H.V.V., Bernardes, R.C., Siqueira, M.A.L., Smagghe, G., Guedes, R.N.C. (2015c) Biopesticide-induced behavioral and morphological alterations in the stingless bee *Melipona quadrifasciata*. Environ. Toxicol. Chem. 34:2149–2158.

Boeke, S.J., Boersma, M.G., Alink, G.M., van Loon, J.J., van Huis, A., Dicke, M., Rietjens, I.M. (2004) Safety evalutation of neem (*Azadirachta indica*) derived pesticides. J. Ethnopharmacol. 94:25–41.

Breeze, T.D., Vaissière, B.E., Bommarco, R., Petanidou, T., Seraphides, N., Kozák, L., Moretti, M. (2014) Agricultural policies exacerbate honeybee pollination service supply-demand mismatches across Europe. PLoS One 9:e82996. doi: 10.1371/journal.pone.0082996

Chauzat, P., Laurent, M., Pierre, M., Hendrikx, P., Ribière-Chabert, M. (2013) A pan-European epidemiological study on honey bee colony losses. Epilobee - A Pan-European epidemiological study on honeybee colony lossses 2012-2013. European Union Reference Laboratory for Honeybee Health (EURL), Sophia Antipolis, France.

Coats, J.R. (1994) Risks from natural versus synthetic insecticides. Annu. Rev. Entomol. 39:489–515.

Cordeiro, E.M.G., Corrêa, A.S., Venzon, M., Guedes, R.N.C. (2010) Insecticide survival and behavioral avoidance in the lacewings *Chrysoperla externa* and *Ceraeochrysa cubana*. Chemosphere 81:1352–1357.

Del Sarto, M.C.L., Peruquetti, R.C., Campos, L.A.O. (2005) Evaluation of the neotropical stingless bee *Melipona quadrifasciata* (Hymenoptera: Apidae) as pollinator of greenhouse tomatoes. J. Econ. Entomol. 98:260–266.

Del Sarto, M.C.L., Oliveira, E.E., Guedes, R.N.C., Campos, L.A.O. (2014) Differential insecticide susceptibility of the Neotropical stingless bee *Melipona quadrifasciata* and the honey bee *Apis mellifera*. Apidologie 45:626–636.

Fairbrother, A., Purdy, J., Anderson, T., Fell, R. (2014) Risks of neonicotinoid insecticides to honeybees. Environ. Toxicol. Chem. 33:719–731.

Gallai, N., Salles, J.M., Settele, J., Vaissière, B.E. (2009) Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. Ecol. Econ. 68:810–821.

Gerwick, B.C., Sparks, T.C. (2014) Natural products for pest control: an analysis of their role, value and future. Pest Manag. Sci. 70:1169–1185.

Godfray, H.C.J., Blacquière, T., Field, L.M., Hails, R.S., Petrokofsky, G., Potts, S.G., McLean, A.R. (2014) A restatement of the natural science evidence base concerning neonicotinoid insecticides and insect pollinators. Proc. R. Soc. B Biol. Sci. 281:20140558.

Guedes, N.M.P., Guedes, R.N.C., Ferreira, G.H., Silva, L.B. (2009) Flight take-off and walking behavior of insecticide-susceptible and - resistant strains of *Sitophilus zeamais* exposed to deltamethrin. Bull. Entomol. Res. 99:393–400.

Guedes, R.N.C., Smagghe, G., Stark, J.D., Desneux, N. (2016) Pesticide-induced stress in arthropod pests for optimized integrated pest management programs. Annu. Rev. Entomol. 61: 1–20.

Isman, M. (2006) Botanical insecticides, deterrents, and repellents in modern agriculture and na increasingly regulated world. Annu. Rev. Entomol. 51:45–66.

Isman, M., Grieneisen, M.L. (2014) Botanical insecticide research: many publications, limited useful data. Trends Plant Sci 19:140–145.

Klein, A.M., Vaissière, B.E., Cane, J.H., Steffan-Dewenter, I., Cunningham, S.A., Kremen, C., Tscharntke, T. (2007) Importance of pollinators in changing landscapes for world crops. Proc. R. Soc. B 274:303–313.

Kremen, C., Williams, N.M., Thorp, R.W. (2002) Crop pollinations from native bees at risk from agricultural intensification. Proc. Natl. Acad. Sci. USA 99:16812–16816.

Lima, D.B., Melo, J.W., Guedes, R.N.C., Siqueira, H.A., Pallini, A., Gondim Jr, M.G. (2013) Survival and behavioural response to acaricides of the coconut mite predator *Neoseiulus baraki*. Exp. Appl. Acarol. 60:381–393.

Lima, D.B., Melo, J.W.S., Guedes, N.M.P., Gontijo, L.M., Guedes, R.N.C., Gondim Jr, M. G.C. (2015) Bioinsecticide-predator interactions: azadirachtin behavioral and reproductive impairment of the coconut mite predator *Neoseiulus baraki*. PLoS One 10:e0118343. doi: 10.1371/journal.pone.0118343

Lima, M.A.P., Martins, G.F, Oliveira, E.E., Guedes, R.N.C. (2016) Agrochemical-induced stress in stingless bees: peculiarities, underlying basis, and challenges. J. Comp. Physiol. A doi:10.1007/s00359-016-1110-3.

Ministério da Agricultura, Pecuária e Abastecimento (2015) AGROFIT: Sistema de Agrotóxicos Fitossanitários/ MAPA/CGAF/DFIA/DAS, Brasília, Brazil. [online] http://extranet.agricutlura.gov.br/agrofit_cons/principal_agrofit_cons) (accessed 08 March 15)

Mordeu (Luntz), A.J., Morgan, E.D., Nisbet, A.J. (2010) Azadirachtin, a natural product in insect control. In: Gilbert LI, Gill SS (eds) Insect Control - Biological and Synthetic Agents. Academic, London, pp. 185–205.

Mordue (Luntz), A.J., Nisbet, A.J. (2000) Azadirachtin from the neem tree (*Azadirachta indica*): its actions against insects. Ann. Soc Entomol. Brasil 29:615–6332.

Mordue (Luntz), A.J., Simmonds, M.S., Ley, S.V., Blaney, W.M., Mordue, W., Nasiruddin, M., Nisbet, A.J. (1998) Actions of azadirachtin, a plant allelochemical, against insects. Pest Sci. 54: 277–284.

Roubik, D. (2014) Pollinator Safety in Agriculture. Pollination Services for Sustainable Agriculture - Field Manuals. FAO, Rome.

Sanchez-Bayo, F., Goka, K. (2014) Pesticide Residues and Bees – A Risk Assessment. PLoS One 9:e94482. doi: 10.1371/journal.pone.0094482

SAS Institute (2008) SAS/STAT User's Guide. SAS, Cary, NC, USA.

Simmonds, M.S.J., Blaney, W.M. (1984) Some effects of azadirachtin on lepidopterous larvae. In: Proceedings of the 2nd International Neem Conferences, pp. 163–180.

Simmonds, M.S.J., Blaney, W.M. (1996) Azadirachtin: advances in understanding its activity as an antifeedant. Entomol. Exp. Appl. 80:23–26.

Staveley, J.P., Law, S.A., Fairbrother, A., Menzie, C.A. (2014) A causal analysis of observed declines in managed honey bees (*Apis mellifera*). Hum Ecol Risk Assess. 20:566–591.

Suri, K.S., Singh, G. (2011) Insecticide-induced resurgence of the whitebacked planthopper *Sogatella furcifera* (Horvath) (Hemiptera: Delphacidae) on rice varieties with different levels of resistance. Crop Prot. 30:118–124.

Teixeira, L.V., Waterhouse, J.M., Marques, M.D. (2011) Respiratory rhythms in stingless bee workers: circadian and ultradian components throughout adult development. J. Comp. Physiol. A 197:361–372.

Tomé, H.V.V., Martins, G.F., Lima, M.A.P., Campos, L.A.O., Guedes, R.N.C. (2012) Imidacloprid-Induced impairment of mushroom bodies and behavior of the native stingless bee *Melipona quadrifasciata anthidioides*. PLoS One 7:e38406.

Tomé, H.V.V., Barbosa, W.F., Corrêa, A.S., Gontijo, L.M., Martins, G.F., Guedes, R.N.C. (2015a) Reduced-risk insecticides in Neotropical stingless bee species: impact on survival and activity. Ann. Appl. Biol. 167:186–196.

Tomé, H.V.V., Barbosa, W.F., Martins, G.F., Guedes, R.N.C. (2015b) Spinosad in the native stingless bee *Melipona quadrifasciata*: Regrettable non-target toxicity of a bioinsecticide. Chemosphere 124:103–109.

Trumm, P., Dorn, A. (2000) Effects of azadirachtin on the regulation of midgut peristalsis by the stomatogastric nervous system in *Locusta migratoria.* Phytoparasitica 28:7–26.

Valk, H., Koomen, I. (2012) Aspects determining the risk of pesticides to wild bees: risk profiles for focal crops on three continents. Pollination Services for SUstainable Agriculture - Field Manuals. FAO, Rome

Van der Sluijs, J.P., Simon-Delso, N., Goulson, D., Maxim, L., Bonmatin, J.M., Belzunces, L.P. (2013) Neonicotinoids, bee disorders and the sustainability of pollinator services. Curr. Opin. Environ. Sustain. 5:293–305.

Villaverde, J.J., Sevilla-Morán, B., Sandín-España, P., López-Goti, C., Alonso-Prados, J.L. (2014) Biopesticides in the framework of the European Pesticide Regulation (EC) No. 1107/2009. Pest Manag. Sci. 70:2–5.