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The reduced-risk insecticide azadirachtin poses a toxicological hazard to stingless bee *Partamona helleri* (Friese, 1900) queens

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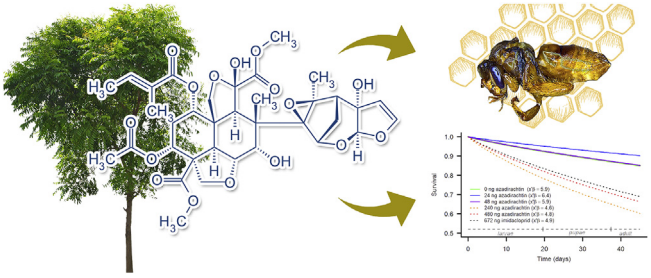
HIGHLIGHTS

- Stingless bee conservation depends on the fitness of the queens.
- The biopesticide azadirachtin reduced the survival of queens reared *in vitro*.
- The reproductive system and morphology of queens were impaired by azadirachtin.
- Azadirachtin can compromise the maintenance of stingless bees populations.

GRAPHICAL ABSTRACT



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ABSTRACT

Large-scale pesticide application poses a major threat to bee biodiversity by causing a decline in bee populations that, in turn, compromises ecosystem maintenance and agricultural productivity. Biopesticides are considered an alternative to synthetic pesticides with a focus on reducing potential detrimental effects to beneficial organisms such as bees. The production of healthy queen stingless bees is essential for the survival and reproduction of hives, although it remains unknown whether biopesticides influence stingless bee reproduction. In the present study, we investigated the effects of the biopesticide azadirachtin on the survival, behavior, morphology, development, and reproduction of queens of the stingless bee *Partamona helleri* (Friese, 1900). The neonicotinoid imidacloprid was used as a toxic reference standard. Queens were orally exposed *in vitro* to a contaminated diet (containing azadirachtin and imidacloprid) during development. Azadirachtin resulted in reduced survival, similarly to imidacloprid, altered development time, caused deformations, and reduced the size of the queens' reproductive organs. All of these factors could potentially compromise colony survival. Results from the present study showed azadirachtin posed a toxicological hazard to *P. helleri* queens.

1. Introduction

The utilization of synthetic organic pesticides has accelerated since their invention, with increased pest control application promoting improved agricultural productivity (Casida and Quistad, 1998; Nauen and Bretschneider, 2002; Popp et al., 2013).

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However, the effects of these pesticides on non-target beneficial organisms such as pollinators (e.g., bees) represent one of the major problems associated with pesticide (Godfray et al., 2015; Guedes et al., 2016; Imperatriz-Fonseca et al., 2016; Sánchez-Bayo et al., 2016).

Brazil is one of the world's largest users of pesticides (Faostat, 2018) and hosts the greatest biodiversity of stingless bees (Apidae: Meliponini) (Camargo and Pedro, 2013; Roubik, 2014). These bees maintain the genetic variability of native and cultivated flora, and contribute to increased productivity of many crops (Flach et al., 2006; Johnson and Hubbell, 1975; Slaa et al., 2006). Therefore, it is necessary to apply parsimonious strategies in pest management via the use of reduced-risk pesticides that will reduce the effects on the health and reproductive capacity of stingless bees (Lima et al., 2016).

Biopesticides, which are derived from naturally occurring substances, are considered a reduced-risk alternative to synthetic pesticides for controlling pests and potentially reducing the negative effects on beneficial organisms (Barbosa et al., 2015b). However, recent studies have demonstrated lethal and sublethal effects of biopesticides on bees, casting serious doubts on their non-target effects (Barbosa et al., 2015a, 2015c; Lopes et al., 2017; Scott-Dupree et al., 2009; Tomé et al., 2015a, 2015b).

Azadirachtin, a biopesticide obtained from neem (*Azadirachta indica*), is considered safe for organic agriculture and is therefore widely applied in the agricultural industry (Bernardes et al., 2017; Isman, 2006; Zehnder et al., 2007). However, studies have shown that azadirachtin interferes with the endocrine system of insects, affecting their development (Mordue (Luntz) and Blackwell, 1993; Mordue (Luntz) and Nisbet, 2000). It inhibits the release of neuropeptides, which act on the prothoracic gland and corpora allatum, changing the levels of ecdysteroids and juvenile hormone titers, and resulting in abnormal development (Martinez and van Emden, 2001; Mordue (Luntz) and Blackwell, 1993; Mordue (Luntz) and Nisbet, 2000). Azadirachtin may also affect reproduction by inhibiting cell division and protein synthesis, consequently preventing oogenesis and vitellogenesis (Barbosa et al., 2015a; Sayah et al., 1996).

Caste determination in stingless bees belonging to the tribe Meliponini is trophic, except for the genus *Melipona*. During larval development, queens ingest a greater quantity of food than workers (Hartfelder et al., 2006). Thus, by consuming larger amounts of pollen and nectar that could potentially contain pesticides, immature queens become more sensitive to xenobiotics than immature workers (Lima et al., 2013; Velthuis et al., 2003). The reproductive capacity of queens determines the survival of the colonies and, consequently, the viability of local populations (Baron et al., 2017a; Imperatriz-Fonseca et al., 1995; VanEngelsdorp et al., 2013). However, little is known about the effects of pesticides on queen stingless bees (Dos Santos et al., 2016; Lima et al., 2016).

In the present study, we investigated the effects of azadirachtin on the survival, development, external morphology, body mass, and locomotory behavior of *P. helleri* queens. Moreover, we analyzed the influence of azadirachtin on caste determination and the reproductive system of *P. helleri* queens. This stingless bee species pollinates countless plant species in Brazil and, therefore, is ecologically important for the maintenance of tropical landscapes (Camargo and Pedro, 2003; Lopes De Carvalho et al., 1999).

2. Material and methods

2.1. Ethical guidelines and collection of starter colony

We performed the present study with permission from the Chico Mendes Institute for Biodiversity Conservation (SISBIO permit no. 46746-1) of the Brazilian Ministry of the Environment, and in accordance with the country's legislation. Several stingless bee species are included on the Brazilian list of endangered species; however, *P. helleri* is neither a protected nor endangered species, and is commonly found in degraded and urban areas in Brazil. We transferred the collected colonies to an area similar to the natural areas where the species is endemic, one year before the beginning of the experiments. We kept the colonies in an area far away 2 km from areas where pesticides are used. We collected individual bees from the colonies without causing harm to the original colonies and transferred them to the laboratory where the experiments were performed.

2.2. In vitro rearing and chronic exposure to insecticides

Five colonies of *P. helleri* were collected from the rural area of Viçosa county (MG, Brazil; 20°45′S, 42°52′W) and were established in the campus of the Federal University of Viçosa (Viçosa, MG, Brazil) to facilitate sample collection. We applied two commercial insecticide formulations for the experiments: Cursor (emulsifiable concentrate at 10 g of azadirachtin kg^{-1}; BIO CARB, Curitiba, PR, Brazil) and Evidence 700 WG (water-dispersible granules at 700 g imidacloprid L^{-1}; Bayer CropScience, São Paulo, SP, Brazil). We used the neonicotinoid imidacloprid as a parameter for high toxicity in bees (Lima et al., 2016; Tomé et al., 2017, 2015b, 2012). In *P. helleri*, imidacloprid caused 100% of mortality after 3 h of oral exposure, while azadirachtin caused less than 20% of mortality after 24 h of oral exposure (Tomé et al., 2015b). Based on the recommended concentrations for field control of whitefly (*Bemisia tabaci*) (Ministério da Agricultura, Pecuária e Abastecimento, 2017), we chose insecticide doses as follows: azadirachtin at 30 ng of active ingredient (a.i.) μL^{-1} (30000 ppb) and imidacloprid at 42 ng a. i. μL^{-1} (42000 ppb). *Bemisia tabaci* causes significant losses to tomato crops, which are potentially visited by *P. helleri* (Ministério da Agricultura, Pecuária e Abastecimento, 2017; Lopes De Carvalho et al., 1999).

We chronically exposed *P. helleri* queens for, approximately, 40 days during post-embryonic development (i.e., after eclosion until adult emergence) to increasing doses of azadirachtin (24, 48, 240, and 480 ng a. i. $queen^{-1}$) following the dilutions of 1/100, 1/50, 1/10, and 1/5 of the recommended dose for field control of *B. tabaci* pests. The maximum dose of azadirachtin that represents the dilution of 1/1 was not used because it prevents eggs from hatching (Barbosa et al., 2015c). Imidacloprid was used as a toxic reference standard; therefore, we applied the dilution of 1/5 (672 ng a. i. $queen^{-1}$) to compare it to the highest dose of azadirachtin used in the present study. The control consisted of exposure to distilled water to determine the natural mortality of *P. helleri* immature queens.

The method for *in vitro* rearing of *P. helleri* queens was adapted from method described by Lima et al. (2013). We used polyethylene 24-well microplates with honey bee wax-coated wells as artificial breeding combs. Larval food was collected from natural brood combs with a surgical aspirator (MA520 Aspiramax, NS group, São Paulo, Brazil) and was mixed with the insecticides using the dilutions mentioned above and mass provided in the wax-coated cells. Each artificial breeding cell received 80 μL of treated larval food (75 μL of pure larval food + 5 μL of insecticide solution), which is sufficient to produce *P. helleri* queens (Campos and Coelho, 1993). Each individual artificial breeding cell hosted one egg, which was placed vertically on the treated larval food. This method allowed the exposure of larvae to the contaminated diet from their first larval instar (Lima et al., 2013). We maintained the artificial breeding combs containing eggs inside glass chambers at 28 ± 2 °C under dark conditions during larval development. Relative

humidity in the glass chambers was adjusted to 97 ± 3% during larval feeding and 80 ± 3% until the emergence of the queens. We used a saturated solution of NaCl to control humidity after larval feeding. After emergence, we maintained the queens individually in Petri dishes (7 cm diameter × 1.5 cm high) at 28 ± 2 °C and 75 ± 5% relative humidity. Adult queens were fed *ad libitum* a water-based solution of *Apis mellifera* honey (80% honey, 20% distilled water) for 3 days until the remainder of the bioassays were completed. At Day 3, there was a low natural mortality and the queens were actively walking. Therefore, we standardized this age to perform remainder bioassays.

2.3. Survival

Survival of larvae was assessed daily by monitoring larval development. Individuals without spiracle movement or with dark tegument were considered dead, and unhatched eggs were discarded to avoid fungal contamination. The number of individuals sampled per treatment (i.e., insecticide dose, including the control) ranged from 89 to 105 (from 5 colonies), totaling 577 queens sampled.

2.4. Development, external morphology, caste determination, and body mass

Insects were counted as deformed when any change in their normal external morphology (i.e., deformed antennae, wings, legs, or mouth parts) was observed (Barbosa et al., 2015c). We noted caste determination when individuals reached their pupal stage (approximately 24 days after egg hatching) by analyzing each pupa under a stereomicroscope (SZ2-ILST, Olympus corporation, Tokyo, Japan). Queens were identified by the presence of 10 flagella, absence of gonopods and corbiculae (Michener, 1944). Three-day-old adult queens were fed *ad libitum* on a water-based solution of *Apis mellifera* honey prior being weighted in analytical scale (model XS3DU, Mettler Toledo, Columbus, OH) for body mass measurements.

2.5. Walking behavior

We used a computerized video tracking system (ViewPoint LifeSciences) to record each 3-day-old adult queen walking in an arena that consisted of an open Petri dish 9 cm diameter × 2 cm high for 10 min. This method allowed us to evaluate possible sublethal effects on locomotion by measuring parameters such as walking velocity (cm s-1), walking distance (cm), resting time (s), and number of stops for insects that had survived the pesticide exposure (Tomé et al., 2012). The minimum number of queens sampled per colony was 18 and the maximum was 32 per treatment, totaling 157 queens sampled within 3 colonies. It was not possible to use the original number of colonies (5) due to the high mortality of individuals in some colonies. We did not use deformed queens (i.e., queens with any change in their normal external morphology) in this bioassay.

2.6. Morphometry of the reproductive system

We dissected the reproductive system of 3-day-old adult queens (exposed to different concentrations of azadirachtin, imidacloprid, and the control) in insect physiological solution (0.1 M phosphate buffer at pH 7.4). Ovaries with their lateral oviducts were then preserved in 70% ethanol and photographed using a digital camera (AxioCam ERc 5s; Zeiss, Göttingen, Germany) coupled to a stereomicroscope (Stemi 2000-C; Zeiss, Göttingen, Germany). We measured the reproductive system area (mm^2) that encompassed the ovaries, lateral oviducts, and common oviduct using the software Image-Pro Plus™ (MediaCybernetics) (Lisboa et al., 2005). We also dissected 3-day-old adult workers from the control treatment to compare them to queens exposed to insecticides. In total, 21 individuals were dissected, accounting for 3 individuals per treatment over all 6 treatments (i.e., 18 individuals), which were randomly sampled among the colonies, and 3 workers from the control.

2.7. Statistical analysis

We compared larval mortality in each treatment via a survival analysis with a Weibull distribution and colonies inserted as a random effect. Curves were compared by contrasts resulting from aggregation of non-significant factor levels (Crawley, 2012). We fitted generalized linear models (GLMs) to external morphology and caste determination data with a binomial distribution (link = logit). We checked for overdispersion in the models and, when necessary, used GLMs with a quasi-binomial to correct for overdispersion. We applied a regression analysis to evaluate the development time, body mass, walking behavior, and reproductive system morphometry data, considering azadirachtin doses as the explanatory variable. Student's *t* tests were used to compare control and imidacloprid treatments. Colonies were considered as replicates in all models, except for survival and morphometry data. To estimate survival data, individuals were considered as replicates, whereas colonies were adjusted as a random effect due to spatial pseudoreplication. Morphometry data were obtained by measuring randomly sampled individuals among colonies. Residuals were checked in all models to verify the adequacy of the distributions. All analyses were performed using R software (version 3.3.1; R Core Team, 2016).

3. Results

3.1. Pesticides effects on survival

The survival of *P. helleri* after ingestion of contaminated diet exhibited a significant difference among the treatments ($\chi^2 = 55.1$, df = 7, $p < 0.001$; Fig. 1). Azadirachtin at 24 and 48 ng did not cause

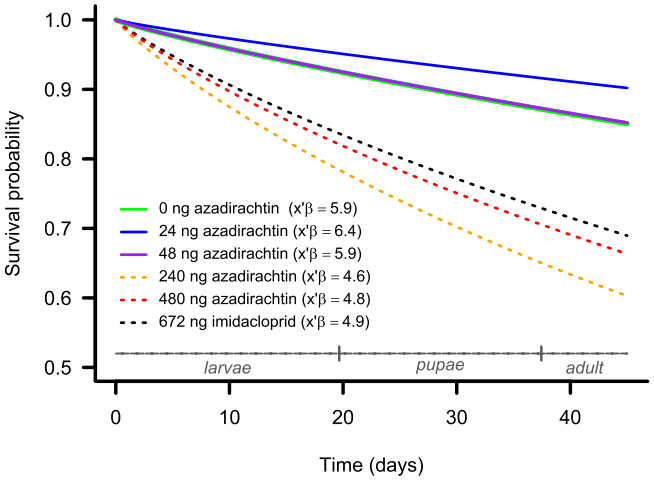

Fig. 1. Survival of *Partamona helleri* queens reared *in vitro* with azadirachtin-contaminated or imidacloprid-contaminated diets. Curves coded with different lines were significantly different, according to the contrasts by gradual simplification of the model a posteriori ($p < 0.05$). Each dose is followed by $x'\beta$ value, according to the Weibull survival function $S(t \mid x) = \exp\left[-\left(\frac{t}{exp(x'\beta)}\right)\right]^{\frac{1}{1+\beta}}$, where *S* is survival probability, *t* is time (days) and *x* is pesticide dose (ng a. i. · bee^{-1}).

high mortality, resembling the untreated control ($\chi^2 = 0.3$, df = 1, $p = 0.6$), but higher doses of azadirachtin (240 and 480 ng) and imidacloprid exhibited higher mortality ($\chi^2 = 35.2$, df = 1, $p < 0.001$).

3.2. Development, external morphology, caste determination, and body mass

Developmental time in *P. helleri* queens was significantly delayed after ingestion of the azadirachtin-treated diet ($F_{1, 22} = 4.45$, $p = 0.046$; Fig. 2A). No significant difference was found between imidacloprid treatment and the control ($t_6 = 2.16$, $p = 0.075$).

A significant increase in the number of deformed individuals following increasing doses of azadirachtin was observed ($\chi^2 = 23$, df = 23, $p = 0.003$; Fig. 2B), whereas imidacloprid treatment did not affect external morphology ($F_{1, 8} = 0.52$, $p = 0.49$). Deformed queens displayed contorted antennae, mandibles, legs, and wings (Fig. 3). More than half—58.5% (±standard error [SE] 4.4%) —of deformed pupae emerged; however, most of them were unable to walk or feed.

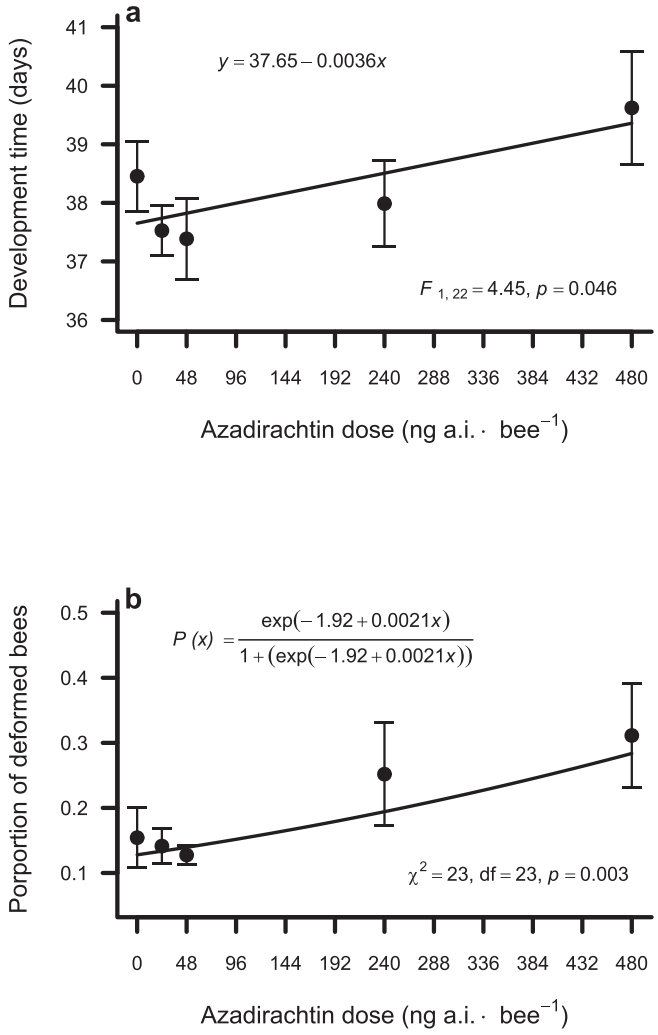

Fig. 2. Development of queens (**a**) and proportion of deformed pupae among alive pupae (**b**) of *Partamona helleri* reared *in vitro* with azadirachtin-contaminated diet. The circles represent the means and vertical bars are standard errors (SE). Imidacloprid did not cause significant effect on these variables therefore, it was not included in the figure.

There was no significant difference in the proportion of individuals that emerged into queens (azadirachtin: $F_{1, 23} = 0.15$, $p = 0.7$; imidacloprid: $F_{1, 8} = 0.51$, $p = 0.5$), with only a small proportion of individuals emerging into workers (3.3%) or males (1.6%) in each treatment.

Body mass was not affected by the ingestion of increasing doses of azadirachtin ($F_{1, 17} = 0.12$, $p = 0.73$). The mean (±SE) body mass was 23.9 (±0.5) and 24.1 (±0.5) mg for queens from the control and azadirachtin (ranging from 24 to 480 ng queen^{-1}) treatments, respectively. Conversely, the ingestion of imidacloprid at 672 ng queen^{-1} reduced the body mass of queens to 18.7 (±0.5) mg ($t_3 = 4.34$, $p = 0.023$).

3.3. Walking behavior

Azadirachtin did not affect any of the locomotory parameters measured for the non-deformed queens that survived the azadirachtin exposure (walking velocity $F_{1, 13} = 0.1$, $p = 0.74$; walking distance: $F_{1, 13} = 0.9$, $p = 0.36$; resting time: $F_{1, 13} = 3.95$, $p = 0.07$; and number of stops: $F_{1, 13} = 1.96$, $p = 0.19$). Similar to azadirachtin imidacloprid did not affect the locomotory parameters (walking velocity: $t_2 = 1.4$, $p = 0.3$; walking distance: $t_2 = 3.5$, $p = 0.07$; resting time: $t_2 = 0.5$, $p = 0.66$; and number of stops: $t_2 = 0.27$, $p = 0.81$). The means (±SE) of the locomotory parameters for all queens measured during the experiment were 1.3 (±0.1) cm s^{-1} (walking velocity), 617.2 (±62.7) cm (walking distance), 168 (±28) s (resting time), and 656.6 (±65) (number of stops).

3.4. Morphometry of reproductive system

There was a significant reduction in 3-day-old virgin queens reproductive system area after the ingestion of increasing doses of azadirachtin during post-embryonic development ($F_{1, 14} = 34.2$, $p < 0.001$; Fig. 4). The reproductive system area (±SE) of queens treated with imidacloprid was similar to the control ($t_2 = 3.7$, $p = 0.07$).

4. Discussion

According to our results, the bioinsecticide azadirachtin decreases longevity and causes other sublethal effects on *P. helleri* queens. Azadirachtin at higher doses (240–480 ng queen^{-1}) and imidacloprid at 672 ng queen^{-1} were observed to cause similar decreases in survival, although the greatest range of sublethal effects was associated with azadirachtin oral exposure. Sublethal effects, including developmental delay, deformations, and atrophy of *P. helleri* reproductive system were probably due to the toxic effects of azadirachtin (Mordue, 2004; Mordue (Luntz) and Nisbet, 2000). The most common effects of azadirachtin includes disturbances in the ecdysteroid and juvenile hormone titers that cause abnormal development and affect reproduction via impairment of oogenesis and vitellogenesis (Barbosa et al., 2015a; Martinez and van Emden, 2001; Mordue (Luntz) and Blackwell, 1993; Mordue (Luntz) and Nisbet, 2000; Sayah et al., 1996).

Partamona helleri queens exposed to imidacloprid showed a higher survival rate, which was in contrast to the high toxicity of this insecticide reported in other bee species (Soares et al., 2015; Tomé et al., 2017, 2012; Valdovinos-Núñez et al., 2009; van der Sluijs et al., 2013; Williams et al., 2015). Despite the low mortality of imidacloprid in *P. helleri* queens, the exposure to this insecticide caused a decrease in body mass. This decrease might be due to energy deprivation caused by the high energy demand during the metabolism of the insecticide or the detoxification process (Gong and Diao, 2017; Rand et al., 2015).

Unlike the effects observed in the stingless bee *Plebeia droryana*

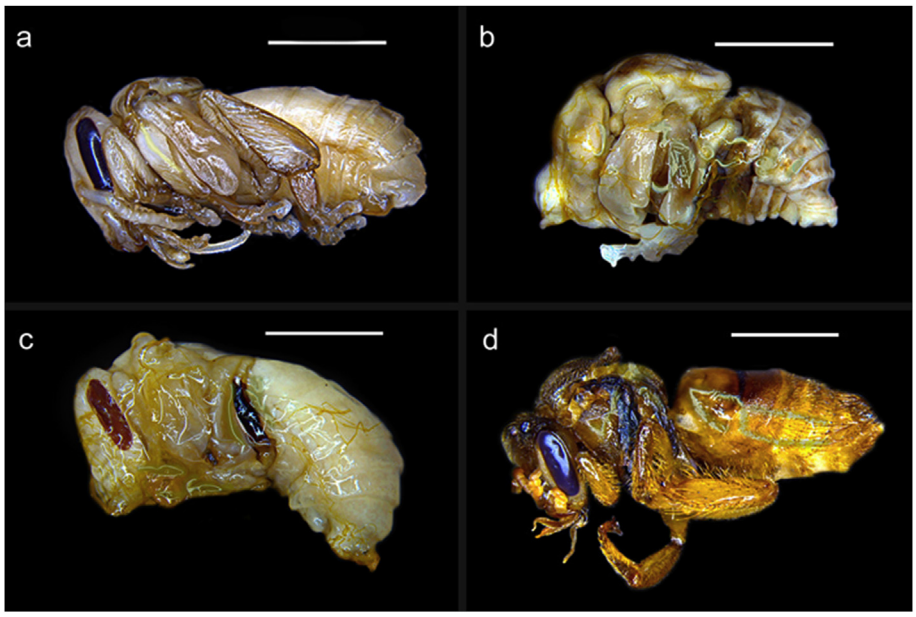

Fig. 3. Pupae of queens of *Partamona helleri* that ingested the uncontaminated diet (control) or those that ingested the azadirachtin-contaminated diet during the post-embryonic development. (a) Thirty-three-day old pupa of control with normal appendages. (b) Twenty-day old pupa exposed to azadirachtin at 480 ng a. i. bee^{-1}, without the head. (c) Twenty-seven day old pupa exposed to azadirachtin at 240 ng a. i. bee^{-1}, with deformed appendages (antennae, legs and mouth parts) and with wound cuticle (pigmentation) on the silk produced in the early pupal stage. (d) Newly-emerged queen (0 days old) exposed to azadirachtin at 240 ng a. i. bee^{-1} with deformed appendages. Bars = 2 mm.

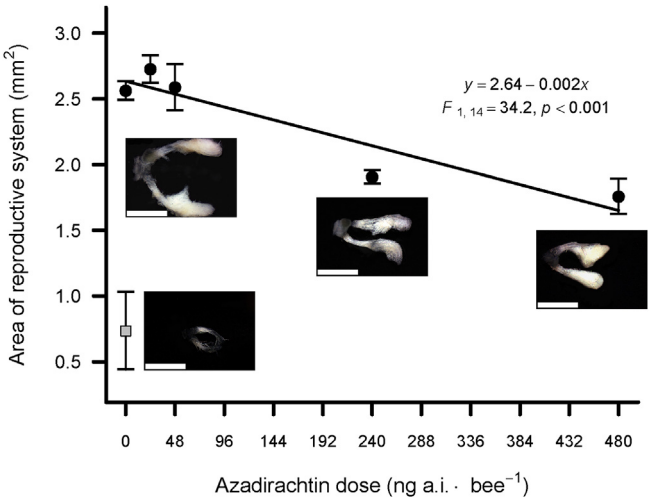

Fig. 4. Morphometry of the reproductive system of queens of *Partamona helleri* reared *in vitro* with azadirachtin-contaminated diets. The black circles represent the means and the grey square represents the data of workers that were used as a proxy for atrophied ovaries. The graph illustrates the aspect of the reproductive system of different treatments. Vertical bars = standard errors; white bars = 2 mm. Imidacloprid did not cause significant effect on these variables therefore, it was not included in the figure.

after larval exposure to the organophosphorus insecticide chlorpyrifos (Dos Santos et al., 2016), neither azadirachtin nor imidacloprid affected the caste differentiation in *P. helleri*. The low rate of queen emergence in *P. droryana* was related to an impairment of larval feeding behavior, since caste differentiation in this bee species, as well as in *P. helleri*, is related to the amount of larval food intake (Dos Santos et al., 2016; Hartfelder et al., 2006). However, in the present study, insecticides added to the bees' food did not inhibit food intake by larvae and did not change the rate of emerged queens in any of the treatments. Moreover, azadirachtin did not affect the walking activity of the non-deformed queens, as previously reported (Barbosa et al., 2015c). Imidacloprid was expected to affect the locomotion of the queens due to its neurotoxic action on brain regions responsible for motor information processing and integration (Buckingham et al., 1997; Déglise et al., 2002; Tomé et al., 2012). However, this insecticide did not impair the walking activity of the queens in the present study, which could be explained by insecticide clearing during larval development (Rand et al., 2015).

The azadirachtin treatment impaired development of the reproductive system, even in non-deformed exposed queens, which might be critical for colony maintenance and reproduction (Wu-Smart and Spivak, 2018). The maintenance of bee populations is highly dependent on the fitness of the queen since the colony population depends on their reproductive output, which stimulates the swarm (Imperatriz-Fonseca, 1977; Imperatriz-Fonseca et al., 1995). In contrast, when queen bees emerge that have reproductive deficits, a mass killing of young queens might occur, which would compromise the creation of new nests or supersede the dominant queen. This would affect the maintenance of bee populations and the survival of the colony itself (Baron et al., 2017a, 2017b; Dixon et al., 2014; VanEngelsdorp et al., 2013).

5. Conclusions

To the best of our knowledge, the present study is one of the pioneer studies on the effects of bioinsecticides on queen stingless bees. When these queens ingest large quantities of pollen and nectar, which might be contaminated, they are more exposed to pesticide intake than queens larvae of honey bees, which are nourished with royal jelly, a glandular food produced by the workers (Hartfelder et al., 2006; Hartfelder and Engels, 1989; Haydak, 1970). This reinforces the idea that the use of honey bees as a model species for pesticide risk assessments cannot be representative of the overall susceptibility of native bees and, therefore, toxicological studies should also extend to other non-*Apis* bees. This might contribute to generating appropriate information to subsidize conservation strategies for pollinators (Bahlai et al., 2010;

Barbosa et al., 2015b; Guedes et al., 2016). We suggest that, in view of the potential biopesticide effects, these substances should be used in a way to minimize bee contamination and their use should be associated with alternative pest control methods.

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