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Spinosad in the native stingless bee *Melipona quadrifasciata*: Regrettable non-target toxicity of a bioinsecticide

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HIGHLIGHTS

- Imidacloprid and spinosad are highly toxic to adult stingless bee workers.
- The bioinsecticide spinosad was more toxic to the bee workers than imidacloprid.
- Imidacloprid impaired worker respiration, overall group activity and flight.
- Spinosad impaired flight, but not respiration and overall group activity.
- Both insecticides were highly hazardous to the stingless bee *Melipona quadrifasciata.*

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ABSTRACT

The risks imposed by novel insecticides, mainly bioinsecticides, are largely unknown despite their increased use and their perceived environmental safety, which is based on their natural origin. Furthermore, unlike honeybees, native pollinator species have received little attention. In the present study, the lethal and sublethal effects of the neonicotinoid imidacloprid and the bioinsecticide spinosad were assessed in the stingless bee species *Melipona quadrifasciata*, an important native pollinator in the Neotropical region. The adult stingless bee workers exhibited high oral insecticide susceptibility, with LD_{50}s of 23.54 and 12.07 ng a.i./bee for imidacloprid and spinosad, respectively. Imidacloprid also impaired worker respiration and overall group activity and flight, while spinosad significantly impaired only worker flight despite exhibiting higher oral toxicity to adult workers than imidacloprid. These findings indicate the hazardous nature not only of imidacloprid but also the bioinsecticide spinosad to adult workers of the native pollinator *M. quadrifasciata.* Therefore, bioinsecticides should not be exempted from risk assessment analysis due to their lethal and sublethal components.

1. Introduction

Reductions in pollinator populations, such as honeybees and bumblebees, have been drawing continuous attention and have been linked to diverse stressors, including climate change, habitat fragmentation, exotic species introductions, parasites, pathogens, malnutrition, and pesticide use (Goulson et al., 2008; Freitas et al., 2009; Potts et al., 2010a,b; Vanbergen, 2013). The prevailing general consensus is of a multifactorial effect, leading to the decay and loss of bee colonies (Potts et al., 2010a,b; Vanbergen, 2013). However, exposure to pesticides, mainly insecticides, seems to potentiate the decline of bee colonies (vanEngelsdorp and Meixner, 2010; Becher et al., 2013).

Insecticide use is routine and even indispensable in several agricultural systems (Oerke and Dehne, 2004; Cooper and Dobson, 2007). Pollinators can physically contact and ingest insecticides, and acute lethal toxicity testing of insecticides to honeybee workers is a longstanding requirement for insecticide registration in several countries (Lewis et al., 1998; Whitford et al., 2002). However, there has been an apparent shift in attention to the sublethal effects of insecticides, which may compromise individual fitness and contribute to colony decline (Valdovinos-Núñez et al., 2009; Brittain et al., 2010; Johnson et al., 2010; Bryden et al., 2013). Such effects include developmental alterations, longevity and queen production declines, neural disturbances, memory and learning impairments, and walking and foraging disabilities (Decourtye et al., 2004a,b; Yang et al., 2008; Belzunces et al., 2012; Henry et al., 2012; Tomé et al., 2012). Pyrethroids, the phenylpyrazole insecticide fipronil, and neonicotinoids in particular have been the main focus of these studies, instigating intense debates and

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calls for banning their use in Europe (Blacquière et al., 2012; European Food Safety Authority, 2013; Gross, 2013) and restricting their use in other countries, such as in Brazil (Instituto Nacional do Meio Ambiente e Recursos Naturais Renováveis, 2012).

The use restriction and even banning of synthetic insecticides, in addition to the growing demand for organically produced crops, has increased the pressure and necessity for bioinsecticides (i.e., insecticidal molecules of biological origin) (Isman, 2006; Villaverde et al., 2014). Natural products are valuable for crop protection as stand-alone insecticides, or as templates for development of more efficacious synthetic insecticides. Nonetheless, the common perception that bioinsecticides are safer for humans and the environment due to their (natural) origin and consequently should benefit from "fast-track" registration is disputable (Coats, 1994; Kidd, 2000; Bahlai et al., 2010).

Spinosad is a bioinsecticide made from spinosyns, which is generated as a fermentation product from the actynomycete species *Saccharopolyspora spinosa* (Mertz & Yao) (Sparks et al., 2001). This compound is an agonist of nicotinic acethylcholine receptors and also interferes with receptors of γ-aminobutiric acid (GABA) in the nervous system (Salgado, 1998; Sparks et al., 2001). Spinosad was initially considered safe for several non-target arthropods, and its use was allowed and expanded for crop protection (Miles, 2003; Sarfraz et al., 2005), especially in organic production, but such perceived selectivity has been challenged (Morandin et al., 2005; Biondi et al., 2012).

The assessment of the ecological risk of insecticides to pollinators has largely centered on the honeybee *Apis mellifera* L., which is the bioindicator of choice for other arthropod pollinators (Lewis et al., 1998; Whitford et al., 2002). There have been efforts to encompass other species, and at least some of this research challenges the common use and extrapolation of honeybee results to other pollinators (Thompson and Hunt, 1999; Besard et al., 2011; Tomé et al., 2012; Del Sarto et al., 2014). The native Neotropical stingless bee *Melipona quadrifasciata* (Lepeletier) produces highly valued honey and is an important pollinator for both wild and cultivated plant species (Slaa et al., 2006; Bispo dos Santos et al., 2009). Furthermore, *M. quadrifasciata* is a more suitable species for an insecticide impact assessment on pollinators in tropical America due to its greater ecological relevance than the exotic Africanized *A. mellifera* (Kremen et al., 2002; Tomé et al., 2012). The stingless bee *M. quadrifasciata* is also closely related to *Melipona capixaba* (Moure & Camargo), another Neotropical stingless bee species formally recognized as an endangered species by the Brazilian Ministry of Environment (MAPA Normative Instruction No. 3, May 27th 2003) and the International Union for the Conservation of Nature and Natural Resources (IUCN, 2013). These factors emphasize the importance of *M. quadrifasciata* as a surrogate species in insecticide risk assessments.

Here, we assessed the acute toxicity of spinosad (and imidacloprid as a positive control) to *M. quadrifasciata* adult workers that were orally exposed to the insecticide. The sublethal impact of the bioinsecticide spinosad (and the neonicotinoid imidacloprid) was also assessed with regard to respiration rate, overall group activity and the flight behavior of adult workers. Highly deleterious effects of imidacloprid were expected on orally exposed workers of *M. quadrifasciata* based on recent results with this species (Tomé et al., 2012). In contrast, only a mild effect was expected for spinosad exposure because of its presumed non-target safety (Miles, 2003; Sarfraz et al., 2005).

2. Material and methods

2.1. Insects and insecticides

Three colonies of *M. quadrifasciata* were initially collected at the edge of an area of secondary subtropical forest in Viçosa county (state of Minas Gerais, Brazil; 20°45′S and 42°52′W). They were subsequently established in the experimental apiary of the Federal University of Viçosa, where they are permanently maintained. The colonies of this species are small (300–600 individuals), and the workers performed nest activities for longer than the honeybee workers starting to fly when older, between 28 and 30 d after emergence. These colonies were later used for the bioassays. The adult workers were collected in the hive entrance using glass jars, which were subsequently transferred to the laboratory and maintained for 1 h in wooden cages covered with organza (35 × 35 × 35 cm) in the dark without food and under 25 ± 2 °C and 70 ± 10% relative humidity until the bioassays were established.

The two insecticides were used in their respective commercial formulations as follows: imidacloprid (700 g a.i. L^{-1}, water dispersible granules; Bayer CropScience, São Paulo, SP, Brazil), and spinosad (480 g a.i. L^{-1}, suspension concentrate; Dow AgroScience, Santo Amaro, SP, Brazil). The insecticides were diluted in a 50% sucrose solution using deionized and distilled water to obtain the final insecticide concentrations.

2.2. Dose-mortality bioassays

The dose-mortality bioassays were performed by exposing adult worker bees to different concentrations of each insecticide. A control without insecticide exposure was used to assess natural mortality and subsequently correct the mortality data. The insects that were initially maintained for 1 h in cages were subsequently individualized in cylindrical glass tubes (15 cm length and 1.5 cm diameter) sealed with parafilm (Parafilm M, PPP Co., Chicago, IL, USA) and provided with 10 μL of insecticide-contaminated 50% sucrose solution (bees in the control received uncontaminated sucrose solution). The bees that completely depleted the sucrose solution were transferred to 200 mL plastic containers with an *ad libitum* provision of uncontaminated 50% sucrose solution for 24 h, after which mortality was recorded. The adult workers were considered dead if they were unable to respond when prodded with a fine hair brush. Preliminary experiments allowed the determination of dose ranges for the subsequent dose-mortality bioassays. Therefore, six doses of imidacloprid were used (5.0, 10.0, 30.0, 50.0, 70.0, and 90.0 ng a.i./bee), and five doses of spinosad were used (5.0, 10.0, 17.5, 25.0, and 42.5 ng a.i./bee) for the respective dose-mortality bioassays, in addition to the control without insecticide exposure. Dose-mortality bioassays were blocked by colony using 10 workers per dose of each insecticide per block (i.e., colony); three colonies were used for each bioassay encompassing 30 workers for each dose of each insecticide tested.

2.3. Overall group activity

Bioassays of the overall group activity of *M. quadrifasciata* workers were performed 3 and 24 h after exposure to the respective LD_5s for imidacloprid and spinosad and the non-exposed controls, as previously described. The exposed and unexposed insects were transferred to Petri dishes (9.0 cm diameter and 2 cm high) in groups of 10 individuals from the same colony, and three replicates (i.e., colonies) were used for each determination. The bottom of each Petri dish was covered with filter paper (Whatman No. 1), and the dish was covered with transparent plastic film to prevent insect escape. The overall insect activity, including walking behavior, insect interactions, and the movement of body parts (e.g., trophallaxy, grooming), was recorded for 10 min and digitally transferred to a computer using a video tracking system equipped with a digital CCD camera (ViewPoint LifeSciences, Montreal, QC, Canada). The overall insect activity was recorded as spatial movement of bees over time and registered as pixels $^{-1} \times 10^{-2}$. After the test, the bees were returned to their 200 mL plastic containers with

an uncontaminated sucrose solution *ad libitum* until the 24 h bioassay. An acclimation period of 2 h in the Petri dish was used before the activity recording. The bioassays were performed under 25 ± 2 °C and artificial fluorescent light between 14:00 and 18:00 h in the afternoon.

2.4. Flight bioassays

The worker bees were subjected to two flight bioassays comparing each insecticide effect on worker flight with the control (untreated workers). The same number of colonies (three with 10 workers from each) used in the overall activity bioassays were used in both flight bioassays after 24 h of the insecticide exposure. A 105 cm tower was formed with three stacked wooden cages covered with organza fabric at their sides (35 × 35 × 35 cm each) and opened in their top and bottom parts to allow free insect flight through them. A fluorescent lamp (60 W, 800 lumens) was placed 10 cm above the tower in a dark room. The 1st bioassay explored the vertical bee flight towards the light source after the insect was released in the center bottom of the tower, and the flight take-off (or lack thereof) was recorded within 1 min of worker release. The flight activity was stratified as follows: (I) no flight (i.e., bee remained on the base of the tower), (II) flight up to 35 cm high, (III) flight between 35 and 70 cm high, (IV) flight between 70 and 105 cm high, and (V) flight reaching the light source at 120 cm high.

The 2nd flight bioassay explored the free-fall flight of the workers by individually releasing the bees 5 cm below the light source within the wooden tower and recording their site of landing. The free-fall flight was stratified as follows: (I) free-fall without flight, landing directly on the tower base; (II) initial fall followed by flight, landing up to 35 cm high; (III) initial free-fall followed flight, landing between 35 and 70 cm high; (IV) initial free-fall followed by flight, landing between 70 and 105 cm high; and (V) no-fall and flight towards the light source.

2.5. Respirometry bioassays

Because respiration rate is a measure of the individual level of stress (Kestler, 1991), respirometry bioassays were conducted 3 and 24 h after the workers were exposed or not exposed to an insecticide-contaminated sucrose solution, as previously detailed. The insecticide doses used corresponded to the recorded LD_5 of each insecticide. The respiration rate was determined in three batches (i.e., replicates) of six insects from the same colony. CO_2 production was recorded using a TR3C respirometer equipped with a CO_2 analyzer (Sable Systems International, Las Vegas, NV, USA). Each adult bee was individualized in 25 mL glass chambers connected to a completely closed system. CO_2 production (μmol 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^{-1}. The air current directed the bee-produced CO_2 to an infrared reader connected to the system. CO_2 production was also determined in a control chamber without any insect.

2.6. Statistical analyses

The data from the dose-mortality bioassays were subjected to probit analyses to estimate the toxicological parameters LD_5 and LD_{50} (PROC PROBIT; SAS Institute, 2008). The overall group activity and the respiration rate were subjected to two-way analyses of variance (time × insecticide treatment) and Tukey's HSD test ($p < 0.05$) when appropriate (PROC GLM; SAS Institute, 2008). As the time interval was assessed in different insect samples, they are not pseudoreplicates in time and therefore subject to regular two-way analyses of variance instead of repeated measures analyses of variance. Normality and homoscedasticity assumptions of analysis of variance were ascertained before such analyses (PROC UNIVARIATE; SAS Institute, 2008), and the overall group activity and respiration rate were transformed to $\log(x + 1)$ to satisfy these assumptions. Neither of the flight bioassay results satisfied the assumptions for the analysis of variance, so they were subjected to the (non-parametric) Kruskal–Wallis test at $p < 0.05$ (PROC NPAR1WAY; SAS Institute, 2008).

3. Results

3.1. Acute insecticide toxicity

The probit model was suitable to the results of the dose-mortality bioassays for both insecticides, imidacloprid and spinosad, based on the low χ^2 and high p-values obtained in the goodness-of-fit tests (Fig. 1). The LD_{50} estimates obtained with the probit model were 23.54 and 12.07 ng a.i./bee for imidacloprid and spinosad, respectively (Fig. 1). Therefore, spinosad was nearly two times more toxic to the *M. quadrifasciata* workers than imidacloprid. The estimated LD_5s for imidacloprid and spinosad (5.38 and 5.29 ng a.i./bee, respectively) were similar, and each was used in the subsequent bioassays.

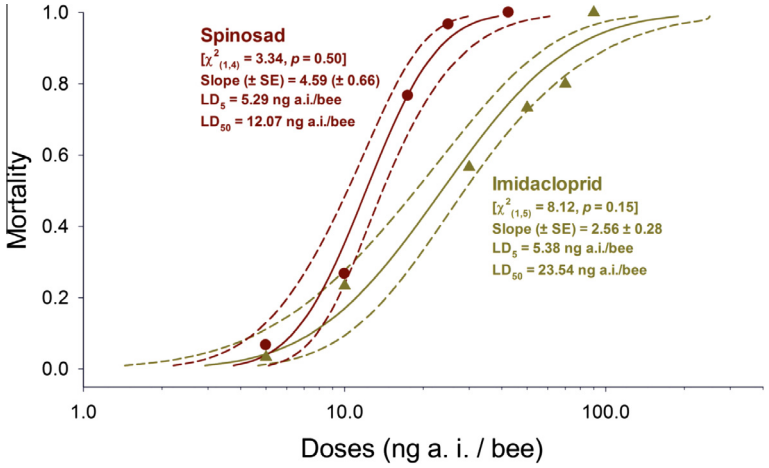

Fig. 1. Dose–mortality curves of adult workers of the stingless bee *Melipona quadrifasciata* orally exposed to imidacloprid and spinosad. The slopes, LD_{50}s and LD_5s are indicated. The dotted lines represent the 95% fiducial limits of each curve.

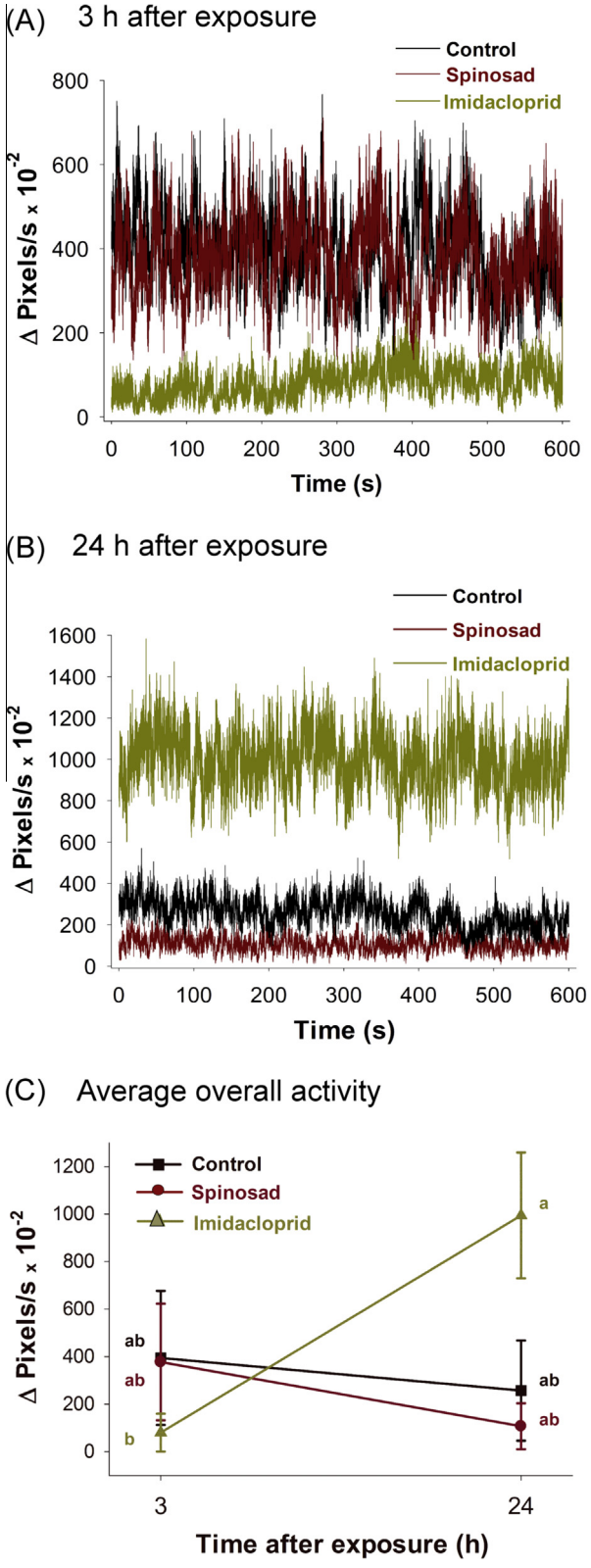

Fig. 2. Overall group activity of groups of ten adult workers of the stingless bee *Melipona quadrifasciata* three and 24 h after being orally exposed to imidacloprid and spinosad. The two upper plots represent the overall activity during 10 min (A and B), while the bottom plot represent the average (±SE) overall activity expressed as changes in registered pixels per second ($\times 10^{-2}$). The The different letters indicate significant differences among insecticide treatments based on Tukey's HSD test ($p < 0.05$).

3.2. Overall insect group activity

The overall worker bee activity was significantly different for insecticide treatments ($F_{2,12} = 6.56$, $p = 0.01$), time ($F_{1,12} = 7.95$, $p = 0.01$) and interaction between treatment and time ($F_{2,12} = 23.88$, $p < 0.001$). The imidacloprid-exposed workers exhibited an average group activity about four times lower than the spinosad-exposed and unexposed workers 3 h after exposure (Fig. 2AC). In contrast, a reversion in overall group activity subsequently took place, and the imidacloprid-exposed workers exhibited nearly five times higher activity than the spinosad-exposed and unexposed workers 24 h after exposure (Fig. 2BC). The group activity of spinosad-exposed and unexposed workers did not change significantly between 3 and 24 h after exposure (Fig. 2).

3.3. Flight activity

Both imidacloprid and spinosad significantly affected the flight performance of workers (Fig. 3). In contrast to the unexposed bees, which were largely able to reach the light source, the flight of the imidacloprid-exposed bees was greatly compromised, with the bees not reaching heights above 35 cm (Fig. 3A). Imidacloprid also significantly impaired the free-fall flight of the workers, which were unable to recover from the initial free-fall after being released, unlike the unexposed workers (Fig. 3C). Spinosad also impaired both vertical and free-fall flights compared to unexposed workers, but the observed impairment was not as drastic as with imidacloprid exposure (Fig. 3BD).

3.4. Respiration rate

The respiration rate of worker bees was significantly different for insecticide treatments ($F_{2,102} = 13.28$, $p < 0.001$), and interaction between treatments and time ($F_{2,102} = 3.50$, $p = 0.03$), but was not different between 3 and 24 h ($F_{1,102} = 0.25$, $p = 0.62$). Imidacloprid significantly reduced the respiration rate of workers both at 3 and 24 h after exposure compared with spinosad-exposed and unexposed workers (Fig. 4). Furthermore, the respiration rate of workers exposed to imidacloprid increased between 3 and 24 h after exposure. Spinosad did not significantly alter the worker respiration rate 3 h after exposure, but the bees exhibited reduction on respiration rates 24 h after exposure (Fig. 4). The respiration rate in the unexposed workers remained at approximately the same levels at the 3 h and 24 h recordings (Fig. 4).

4. Discussion

Both imidacloprid and spinosad were highly toxic to the adult workers of *M. quadrifasciata*, with LD_{50}s in the range of 12.07 and 23.54 ng ingested per bee for spinosad and imidacloprid, respectively. Although imidacloprid is broadly recognized as very toxic to bees, usually with LD_{50}s in the range of 3.8 to over 81.0 ng/bee (Decourtye et al., 2004a,b; Cresswell, 2011; Blacquière et al., 2012), the results with spinosad provide some evidence of deleterious effects on bees (Miles, 2003; Morandin et al., 2005; Besard et al., 2011; Biondi et al., 2012; Gradish et al., 2012a,b). Surprisingly, spinosad exhibited higher acute toxicity than imidacloprid, suggesting its potential impact on *M. quadrifasciata*. The apparently higher susceptibility of stingless bees to spinosad, compared with the honeybee and bumblebee (Mayes et al., 2003; Bailey et al., 2005; Morandin et al., 2005), should also be a matter of concern in future insecticide impact assessments in warmer climates. Lethality, however, is a simplistic indicator of environmental

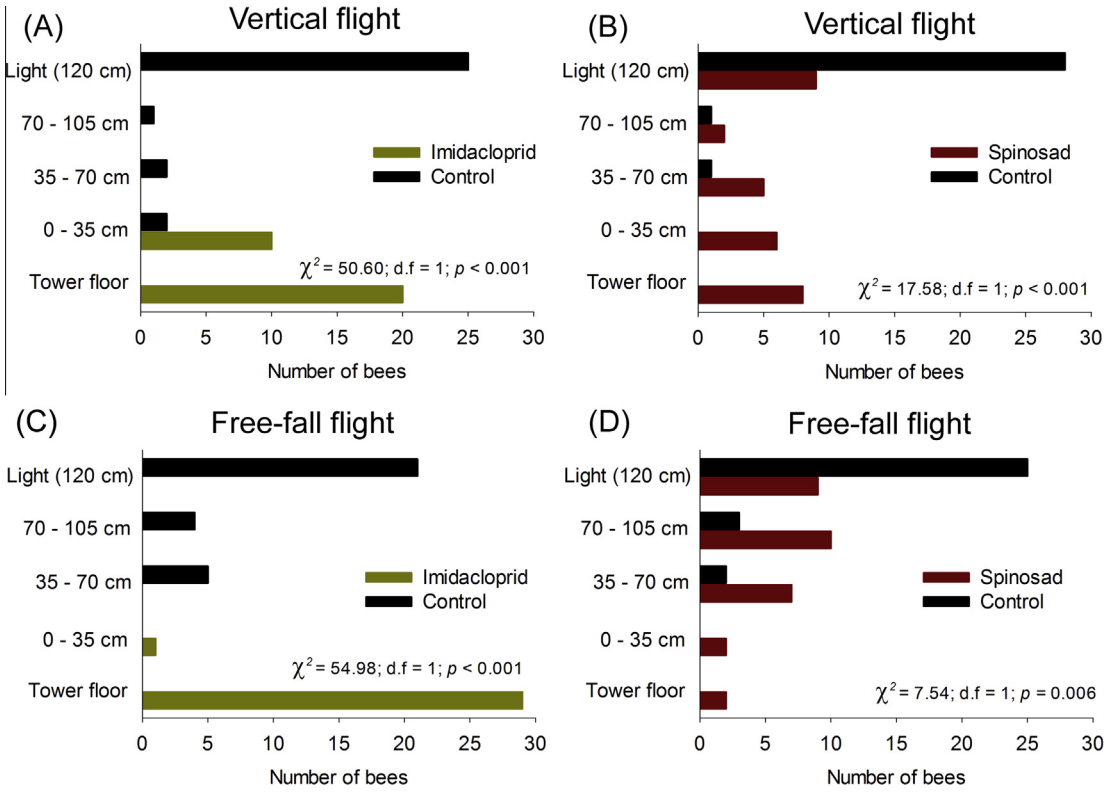

Fig. 3. Flight activity (vertical and free-fall flight) of adult workers of the stingless bee *Melipona quadrifasciata* 24 h after being orally exposed to imidacloprid (A and C) and spinosad (B and D).

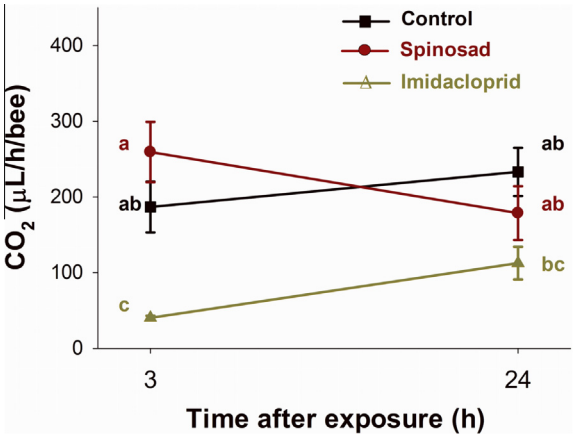

Fig. 4. Respiration rate of individual (±SE) adult workers of the stingless bee *Melipona quadrifasciata* three and 24 h after being orally exposed to imidacloprid and spinosad. The different letters indicate significant differences among insecticide treatments based on Tukey's HSD test (p < 0.05).

impact, and sublethal effects of imidacloprid and spinosad in *M. quadrifasciata* were also assessed.

The toxicity of imidacloprid and even more so of spinosad to *M. quadrifasciata*, illustrates the high (acute) mortality caused by insecticide ingestion. However, the sublethal effects of insecticides are even more important than their lethal effect. Imidacloprid, for instance, depressed the overall group activity soon after exposure, with a subsequent increase in activity above normal levels. The reason for this result is unclear because a short-term increase in activity is expected under exposure to this neurotoxic compound. Imidacloprid agonistically interacts with nicotinic acetylcholine receptors, with a subsequent reduction in activity due to the eventual collapse of the excitatory stimulus transmission (Salgado, 1998; Sparks et al., 2001). The initial depression of activity with subsequent hyperactivity may result from secondary neural interactions of imidacloprid within the individual, or lower agonist affinity that may promote excitatory symptoms in insects, what deserves future attention (Salgado, 1998; Tan et al., 2007).

Spinosad does not seem to interfere with the overall group activity of *M. quadrifasciata* workers. However, this insecticide does impair flight activity, which is likely to compromise foraging activity, eventually compromising colony maintenance and ultimate survival (Yang et al., 2008; Blacquière et al., 2012; Henry et al., 2012). This finding is consistent with previous laboratory studies with spinosad reporting its negative impact in honeybees and bumblebees (Mayes et al., 2003; Bailey et al., 2005; Morandin et al., 2005), but such an effect was not evident in field studies (Scott-Dupree et al., 2009; Biondi et al., 2012). Regardless that, the perceived notion of environmental safety of bioinsecticides, and particularly of spinosad, deserves caution.

Respiration rate is an indicator of physiological stress, and insecticides can compromise insect respiration by impairing muscle activity, leading to paralysis (Kestler, 1991; Zafeiridou and Theophilidis, 2006). Indeed, imidacloprid compromised the respiration rate of *M. quadrifasciata* up to 24 h after exposure, likely reflecting in-flight and group activity interference. The insects however seem to undergo some recovery around 24 h after exposure, a likely consequence of the rapid breakdown and excretion of imidacloprid, as recently reported in honeybees and bumblebees (Cresswell et al., 2014). Spinosad represented a distinct contrast at a sublethal exposure in the same range of imidacloprid (5 ng a.i./bee), eliciting only a marginal reduction in respiration rate 24 h after exposure and not affecting the overall group activity. However, spinosad also impaired worker flight, albeit at a lower degree than imidacloprid. The reason for this result may be the high

demands of energy and muscle synchronization and activity required for flight, leading to its more drastic impairment even under low exposure (Candy et al., 1997). However, the impairment of overall group activity in eusocial bees may also have important consequences for the individuals and the colony (Tomé et al., 2014).

The acute toxicity of spinosad in adult workers of the stingless bee *M. quadrifasciata* was even higher than that of imidacloprid, and both compounds elicited deleterious sublethal effects on group and/or flight activities in this species. These findings further challenge the common extrapolation of toxicity assessments with *A. mellifera* to all (native) bee pollinators, which have been recognized as about 10-fold more tolerant to pesticides than stingless bees based on a recent meta-analysis study (Arena and Sgolastra, 2014).

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