CLEITON GUEDES RODRIGUES

LEAF FERTILIZERS AFFECT SURVIVAL AND BEHAVIOR OF THE NEOTROPICAL STINGLESS BEE *Friesella schrottkyi* (HYMENOPTERA: APIDAE: MELIPONINI)

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-graduação em Entomologia, para obtenção do título de *Magister Scientiae*.

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APROVADA: 12 de agosto de 2016.

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SUMÁRIO

RESUMO

RODRIGUES, Cleiton Guedes, M.Sc., Universidade Federal de Viçosa, agosto de 2016. **Fertilizantes foliares afetam sobrevivência e comportamento da abelha Neotropical sem ferrão *Friesella schrottkyi* (Hymenoptera: Apidae: Meliponini)**. Orientador: Raul Narciso Carvalho Guedes. Coorientadores: Maria Augusta Lima Siqueira e Wagner Barbosa Faria.

Os cientistas ao redor do mundo estão preocupados com a mortalidade das abelhas, insetos que são muito importantes na economia agrícola. E maior conhecimento é necessário a respeito dos fatores envolvidos. Os metais pesados (Zn, Cu, Mn) são parte de vários agroquímicos utilizados para aumentar a produção. Porém, os potenciais efeitos deles em polinizadores são raramente considerados, especialmente em abelhas sem ferrão. As abelhas sem ferrão são ecológica e economicamente relevantes na região Neotropical. Nós buscamos entender com este estudo se os fertilizantes foliares contendo metais pesados afetam a sobrevivência, comportamento e fisiologia de abelhas forrageiras *Friesella schrottkyi*. Assim, nos testamos dois fertilizantes foliares: sulfato de cobre (Cu=24%) e uma mistura de micronutrientes (Cu=0.6%, Mn=3%, Zn=5%). Nós também usamos abelhas não expostas como controle negative e o bioinseticida spinosad como controle positivo. As abelhas foram expostas via ingestão (solução contaminada) e contato por 72 h. Nossos resultados demonstram que o sulfato de cobre causou 100% mortalidade em abelhas sob exposição oral. Spinosad seguiu da mesma forma, mas com 6% de sobrevivência no final do período de exposição. Os insetos que sobreviveram após 72h de exposição foram investigados em aspectos de comportamento (atividade de caminhamento e voo) e fisiologia (taxa de respiração). Exposição subletal a ambos fertilizantes em suas respectivas concentrações de uso também afetou significativamente as operárias. Sulfato de cobre aumentou a decolagem para voo nas operárias, contrastando com a mistura de micronutriente e o controle negativo. A atividade geral e de caminhamento não foi afetada pelos fertilizantes foliares. A taxa respiratória mostrou-se semelhante em operárias expostas por contato, enquanto operárias expostas oralmente a mistura de micronutrientes tiverem atividade respiratória reduzida. Portanto, fertilizantes foliares afetam *F. schrottkyi* e podem também afetar outras abelhas sem ferrão potencialmente afetando polinização e merecendo atenção.

ABSTRACT

RODRIGUES, Cleiton Guedes, M.Sc, Universidade Federal de Viçosa, August, 2016. **Leaf fertilizers affect survival and behavior of the Neotropical stingless bee *Friesella schrottkyi* (Hymenoptera: Apidae: Meliponini)**. Adviser: Raul Narciso Carvalho Guedes. Co-advisers: Maria Augusta Lima Siqueira and Wagner Barbosa Faria.

Scientists all over the world have been concerned about the mortality of bees, which represent a great deal in agricultural economy. More knowledge is necessary regarding the factors involved. Heavy metals (Zn, Cu, Mn) are part of several agricultural products market for aiming yield increase. However, their potential effects on pollinators are seldom considered. The native stingless bees are pollinator species ecologically and economically relevant in the Neotropical region. We aimed to study whether foliar fertilizers containing heavy metals would affect the survival, behavior and physiology of *Friesella schrottkyi* foraging bees. Thus, we tested two leaf fertilizers: copper sulfate (Cu=24%) and micronutrient mix (Cu=0.6%, Mn=3%, Zn=5%). We also used unexposed bees as negative control and the biopesticide spinosad as positive control. Therefore, bees were exposed by ingestion (contaminated sugar solution) or contact during 72h. We have found that copper sulfate under oral exposure caused 100% mortality of bees in less than 72h. Spinosad followed the same trend with only 6% of survival. Those surviving insects were then investigated for effects on their behavior (walking and flight activity) and physiology (respiration rate). Copper sulfate enhanced flight take-off on stingless bee workers, unlike workers exposed to the micronutrient mix. There was no significant effect of leaf fertilizers on the overall activity and walking behavior of worker bees. No significant effect was observed for the respiration rate of worker bees under contact exposure, but workers orally exposed to the micronutrient mix exhibited a reduced respiration rate. Therefore, leaf fertilizers do affect *F. schrottkyi*, what may also occur with other stingless bees, potentially compromising their pollination activity deserving attention.

PREÂMBULO

A mortalidade das abelhas no mundo tem causado preocupação em vários meios da sociedade, desde o científico ao popular. Várias pesquisas, em sua maioria com abelhas melíferas, reportam que as causas dessa mortalidade são multifatoriais, caracterizadas por parasitas e patógenos, má nutrição, introdução de espécies exóticas, fragmentação do ambiente, mudanças climáticas e uso de pesticidas (vanEngelsdorp et al. 2008; Vanbergen and Initiative 2013; Freitas et al. 2009; Klein et al. 2007). Porém, pouco tem sido levantado sobre efeitos de outros agroquímicos que não sejam inseticidas, especialmente em abelhas sem ferrão. Desta forma, visando preencher essa lacuna, nosso objetivo foi avaliar se os fertilizantes foliares que possuem metais pesados em sua constituição poderiam afetar a sobrevivência e o comportamento da abelha nativa sem ferrão *Friesella schrottkyi*.

As abelhas nativas sem ferrão, *Friesella schrottkyi*, comum no Sul e Sudeste brasileiro pertencem a um grupo de polinizadores relevantes na região Neotropical, os Meliponinios, que visitam também plantas cultivadas contribuindo para sua produção (Camargo, 1989). As colônias de *Friesella schrottkyi* são formadas por cerca de 300 indivíduos pequenos (ca 3,0 mm e 3,0 mg) que geralmente vivem em caules ocos de madeira. Além disso, exibirem entrada críptica, a qual é fechada no final da tarde.

Os fertilizantes foliares possuem em sua formulação certas quantidades de metais pesados que são essenciais para desenvolvimento das plantas (Singh et al. 2011), mas que podem ser tóxicos aos insetos. No entanto, suas toxicidades para os insetos dependem de vários fatores tais como dose, rota de exposição, espécie química, bem como idade, sexo e genética dos indivíduos expostos (Tchounwou et al. 2012). Os metais pesados zinco (Zn), cobre (Cu) e Manganês (Mn) são utilizados em significativa quantidade na agricultura. Esses elementos em altas concentrações, sozinhos ou misturados podem ser letais ou proporcionar efeitos subletais aos insetos (Bayley et al. 1995; Søvik et al. 2015). Em nosso experimento testamos uma mistura de micronutrientes e sulfato de cobre que é fonte de Cu e comparamos os resultados com o bioinseticida spinosad, o controle positivo, e água destilada, o controle negativo.

Nossos resultados nos surpreenderam porque não esperávamos que o sulfato de cobre fosse mais danoso as abelhas que o bioinseticida spinosad. Nós esperamos que esse estudo possa incentivar mais investigações neste contexto, inclusive com outras espécies de abelhas sem ferrão, bem como outros elementos e assim contribuir para soluções mais sustentáveis na produção agrícola.

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Leaf Fertilizers Affect Survival and Behavior of the Neotropical Stingless Bee *Friesella schrottkyi* (Meliponini: Apidae: Hymenoptera)

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Abstract

The ongoing concern about bee decline has largely focused on honey bees and neonicotinoid insecticides, while native pollinators such as Neotropical stingless bees and agrochemicals such as other insecticide groups, pesticides in general, and fertilizers—especially leaf fertilizers—remain neglected as potential contributors to pollination decline. In an effort to explore this knowledge gap, we assessed the lethal and sublethal behavioral impact of heavy metal-containing leaf fertilizers in a native pollinator of ecological importance in the Neotropics: the stingless bee *Friesella schrottkyi* (Friese). Two leaf fertilizers—copper sulfate (24% Cu) and a micronutrient mix (Arrank L: 5% S, 5% Zn, 3% Mn, 0.6% Cu, 0.5% B, and 0.06% Mo)—were used in oral and contact exposure bioassays. The biopesticide spinosad and water were used as positive and negative controls, respectively. Copper sulfate compromised the survival of stingless bee workers, particularly with oral exposure, although less than spinosad under contact exposure. Sublethal exposure to both leaf fertilizers at their field rates also caused significant effects in exposed workers. Copper sulfate enhanced flight take-off on stingless bee workers, unlike workers exposed to the micronutrient mix. There was no significant effect of leaf fertilizers on the overall activity and walking behavior of worker bees. No significant effect was observed for the respiration rate of worker bees under contact exposure, but workers orally exposed to the micronutrient mix exhibited a reduced respiration rate. Therefore, leaf fertilizers do affect *F. schrottkyi*, what may also occur with other stingless bees, potentially compromising their pollination activity deserving attention.

RESUMO

A preocupação contínua com o declínio de abelhas persiste grandemente centrada na abelha melífera e em inseticidas neonicotinoides, enquanto polinizadores silvestres, como abelhas Neotropicais sem ferrão, e outros agroquímicos incluindo outros inseticidas, pesticidas em geral e fertilizantes, especialmente fertilizantes foliares, persistem negligenciados como potenciais contribuidores para o declínio de polinizadores. Na tentativa de explorar esta deficiência de conhecimento, nós avaliamos o impacto letal e subletal de fertilizantes foliares contendo metais pesados em uma espécie polinizadora nativa de importância ecológica nos Neotrópicos, a abelha sem ferrão *Friesella schrottkyi* (Friese). Dois fertilizantes foliares, sulfato de cobre (24% Cu) e uma mistura micronutriente (5% S, 5% Zn, 3% Mn, 0.6% Cu, 0.5% B e 0.06% Mo), foram usados em bioensaios de exposição oral e por contato, além do biopesticida spinosad e água desionizada como controles positivo e negativo, respectivamente. Sulfato de cobre comprometeu a sobrevivência de operárias de abelhas sem ferrão especialmente sob exposição oral, mas não tão drasticamente quanto spinosad em exposição por contato. Exposição subletal a ambos fertilizantes em suas respectivas concentrações de uso também afetou significativamente as operárias. Sulfato de cobre aumentou a decolagem para voo nas operárias, contrastando com a mistura de micronutriente e o controle negativo. A atividade geral e de caminhamento não foi afetada pelos fertilizantes foliares. A taxa respiratória mostrou-se semelhante em operárias expostas por contato, enquanto operárias expostas oralmente à mistura micronutriente tiverem atividade respiratória reduzida. Portanto, fertilizantes foliares afetam *F. schrottkyi* e podem também afetar outras abelhas sem ferrão potencialmente afetando polinização e merecendo atenção.

Key words: heavy metal, native pollinator, fertilizer toxicity, risk assessment, nontarget effect

The currently increasing worldwide demand for pollination services to enhance food quality and production sustainability brings the decline of populations of the honey bee (*Apis mellifera* L.) to the forefront of standing regulatory and popular concerns (Chauzat et al. 2013, Breeze et al. 2014). This issue is not novel, and heated debate around the topic continues, involving a rather diverse set of participants ranging from laymen to politicians, beekeepers, and researchers, as illustrated by the varied venues of communication used (e.g., Rollins 2009, Schacker 2009, Gross 2013, Kleinman and Suryanarayanan 2013, Roubik 2014).

The benign perception of the honey bee also helps in understanding the ongoing contention and controversy regarding honey bee decline (Keller 1993, Horvath et al. 2013, Barbosa et al. 2015b). Curiously though, the use of honey bees as surrogate pollinators is disputable because they may not be as representative of other bee pollinators as is commonly accepted (Hardstone and Scott 2010, Barua et al. 2012, Arena and Sgolastra 2014, Godfray et al. 2014, Gómez-Escobar et al. 2014, Barbosa et al. 2015b). Furthermore, honey bees are an invasive species in the Americas. The prevailing Africanized honey bee phenotype of Neotropical America is very aggressive regarding both human handling and the displacement of native species (Wilms et al. 1996, Goulson 2003, Barbosa et al. 2015b), which is altogether inconsistent with the prevalent benign view of honey bees that dominates in North America and Europe (Keller 1993, Barua et al. 2012).

Achieving recognition of the underlying causes of pollinator decline, initially attributed to intensive neonicotinoid insecticide use, has proven to be challenging. There is an apparent converging perception of the multifactorial nature of the phenomenon (Chauzat et al. 2013, Vanbergen et al. 2013, Godfray et al. 2014). Insecticides seem to potentiate pollination decline, although the bulk of evidence comes from studies that focused mainly on honey bees (sometimes bumble bees) in combination with one insecticide group, the neonicotinoids (vanEngelsdorp and Meixner 2010, Barbosa et al. 2015b), leaving a knowledge gap regarding other possible contributing causes. This knowledge gap has been drawing attention, and studies are now expanding to include other pollinator species and other pesticides including biopesticides, fungicides, and others (Valk and Koomen 2013; Johnson et al. 2013; Sanchez-Bayo and Goka 2014; Barbosa et al. 2015a,b,c; Tomé et al. 2012, 2015a,b). Nonetheless, studies with native bees and agrochemicals other than pesticides lag far behind.

Stingless bee species are the prevailing wild pollinators in Neotropical America (Valk and Koomen 2013). They exhibit higher pollination efficiency than the honey bee in several native and cultivated plant species (Kremen et al. 2002). In addition, some stingless bees are also economically important in the production of specialty honeys (Slaa et al. 2006, Roubik 2014, Barbosa et al. 2015b). Although some species are formally recognized as endangered (MAPA Normative Instruction No. 3, May 27th 2003), they retain their ecological and economic importance at present, despite the risks imposed by habitat fragmentation, intensive agriculture production, and competition with Africanized honey bees (Roubik 2014, Barbosa et al. 2015b).

When considering agricultural production as a potential threat to Neotropical stingless bees, the problems likely go beyond pesticide use. Fertilizers—particularly leaf fertilizers—are used heavily in intensive agricultural production systems of Neotropical America (Yamada 2004). Leaf fertilizers are a source of micronutrients for growing plants which, although essential for both plant and animal species, are harmful at higher concentrations, particularly for the latter (Peña et al. 1999, Deng et al. 2007, Borowska and Pyza 2011, Tchounwou et al. 2012, Baghban et al. 2014). Their use at different phases of the plant phenology, including the vegetative–reproductive phase transition and the reproductive phase (Yamada 2004, Fageria et al. 2009, Haytova 2013), favor their interaction with pollinators. Such interactions have not been studied.

Copper (Cu), manganese (Mn), and zinc (Zn) are among the most frequently used heavy metals present in leaf fertilizers, and these are in broad use in agriculture, including organic production systems, as micronutrients and fungicides (Yamada 2004, Deng et al. 2007, Andrews and Baker 2015). Furthermore, the toxic effects of heavy metals have been recognized in some insect species (Bayley et al. 1995, Toft and Jensen 1998, Malakar et al. 2009, Mogren and Trumble 2010, Borowska and Pyza 2011, Baghban et al. 2014, Sovik et al. 2015). Nonetheless, the potential impact of leaf fertilizers rich in heavy metals on native pollinators, particularly Neotropical stingless bees, represents a knowledge gap that remains unfilled, justifying the present study.

Here we assess the potential lethal and sublethal (behavioral) effects of two leaf fertilizers in the stingless bee pollinator species *Friesella schrottkyi* (Friese) (Hymenoptera: Apidae: Meliponini), a common species in southern and southeastern Brazil (Camargo 1989). This is a species of small individuals (ca. 3.0 mm and 3.0 mg) with colonies of about 300 workers nesting in pre-existing wooden logs exhibiting a cryptic entrance, which is closed at night. We expected potential sublethal effects based on the results of a previous heavy metal study on honey bees (Sovik et al. 2015), but not significant short-term lethal effects such as are usually reported for insecticides (Tomé et al. 2012, 2015a,b; Barbosa et al. 2015a,c).

Materials and Methods

Insects and Agrochemicals

Four hives of the stingless bee species *F. schrottkyi* were field-collected between September and October 2014 and established in wood boxes at the Botanical Garden of the Federal University of Viçosa (Viçosa, MG, Brazil). Each colony was considered a biological replicate to allow representative genetic variability in the experiments. Adult foraging workers were collected from each colony using a mouth aspirator during the time of peak foraging activity in the hives (i.e., between 1100 and 1300 hours). The worker bees thus obtained were transferred to a transparent plastic container (250 ml) and taken to the laboratory where they were starved for 2 h before being used in the bioassays. The insects were maintained under the same conditions in which the bioassays were sequentially performed, i.e., in an environment-controlled room at 25 ± 2°C and 70 ± 10% relative humidity until the bioassays started. The body masses of the individual workers were determined by quickly chilling the insects and weighing them on an electronic scale (model XS3DU, Mettler Toledo, Columbus, OH). The average body mass of the worker bees of *F. schrottkyi* was 2.95 ± 0.05 mg.

Two commercial (ready to use) leaf fertilizers in common use in Brazilian agriculture were tested: copper sulfate (Sulfato de Cobre Penta 24; a salt formulation containing 240 g/Kg Cu and 110 g/Kg S; Multitécnica Industrial, Sete Lagoas, MG, Brazil) and a micronutrient mix (Arrank L; homogeneous suspension containing (w/v) 4.00% S, 0.50% B, 0.60% Cu, 3.00% Mn, 0.06% Mo, and 5.00% Zn, corresponding to 50.80, 6.35, 7.62, 38.10, 0.76, and 63.50 g/liter, respectively, in the formulation; Quimifol, São Paulo, SP, Brazil). The bioinsecticide spinosad (Tracer; suspension concentrate; 480 g/liter, Dow AgroSciences, Santo Amaro, SP, Brazil) was used as a positive control because of its recognized high lethal and sublethal toxicity to stingless bees and its pattern of use by spraying (Barbosa et al. 2015c; Tomé et al. 2015a,b), which resembles the application of leaf fertilizers. Spinosad is generated as a fermentation product from the actynomycete *Saccharopolyspora spinosa* (Mertz & Yo) (Sparks et al. 2001, Salgado and Sparks 2010). Deionized water was used as negative control.

The leaf fertilizers were used at their respective label rates, 400 liter/ha for Arrank L and 100 liter/ha for Sulfato de Cobre Penta 24. Spinosad was used at its maximum field recommended label rate for control of the tomato pinworm *Tuta absoluta* (Meyrick) (17 ml or 8.16 g a.i./100 liter at 1,000 liter/ha; Ministério da Agricultura, Pecuária e Abastecimento 2015). This insect species is a major pest of tomato plants, which are a frequent target of visitation and pollination by native stingless bees (Kremen et al. 2002, Del Sarto et al. 2014). The respective concentrations of each agrochemical were prepared using deionized water for contact exposure and in 50% (w/w, final concentration) aqueous sucrose solution for the oral exposure bioassays. Unexposed bees used in the negative control were subjected only to distilled water and uncontaminated sucrose solution for the contact and oral bioassays, respectively.

Time–Mortality (Survival) Bioassay

Two survival bioassays were carried out to explore two distinct modes of exposure: tarsal contact and oral ingestion. The former was performed by applying 2 ml of either the leaf fertilizers or bioinsecticide solutions to transparent glass containers (250 ml; EME, Paulicéia, SP, Brazil), which then remained under rotation in a heavy-duty rotator (Roto-Torque 7637, ColeParmer, Vernon Hills, IL) for 2 h within a fume hood until dry to coat their inner walls. The top portion of each container was subsequently coated with Teflon PTFE (DuPont, Wilmington, DE) to prevent the insects from escaping the treated area. The containers were closed with a piece of organza and a rubber band. Four replicates were used in each bioassay of each treatment (including the control treatments). Each replicate consisted of a container into which 12 adult foraging worker bees from each colony (i.e., replicate) were released and maintained with food provision to assess food consumption, as described in the next subsection. The insects were subjected to up to 72 h of exposure and recognized as dead if they were unable to stand upright and walk the length of their bodies. Mortality was checked every half hour during the first 6 h-expose and then at every hour until 12-h exposure and subsequently at 12 h-intervals up to 72 h.

The time–mortality bioassay of oral ingestion was performed in 250-ml polyethylene containers following the same conditions as the contact exposure bioassays and with the same number of replicates, but 10 adult foraging workers were used per replicate. These adult worker bees were fed on sucrose solution (treated or not, depending on the treatment) using drilled Eppendorf tubes (Eppendorf, São Paulo, SP, Brazil) as feeders that were inserted through a hole in the container (Tomé et al. 2015b). The leaf fertilizers and the bioinsecticide spinosad (positive control) were diluted with aqueous sucrose solution (50% w/w) and used at the following (final) concentrations: copper sulfate at 5.0 g/liter, micronutrient mix at 2 ml/liter, and spinosad at 81.6 mg a.i./liter. Uncontaminated distilled water was used as a negative control. Mortality was checked as previously described for the contact bioassays.

Food Consumption

The average food consumption per worker bee was determined by weighing the food vials on electronic scales at 24-h intervals until the end of the survival experiment for oral exposure. For the contact exposure, food consumption was determined by weighing the food vials only at the start of the experiment and at its end. Food vials were maintained at the same environmental conditions, but without bees, to allow determination of diet loss by evaporation to correct for such a loss in the other diet vials (provided to the bees for feeding).

Overall Activity and Walking Bioassay

Both group and individual activity bioassays were performed with adult foraging workers of *F. schrottkyi*. Due to the higher short-term mortality of workers exposed to spinosad (and also copper sulfate under oral exposure), these treatments were not considered in the overall activity and walking bioassays, which used only (live) insects after 72 h of exposure to leaf fertilizers or unexposed; food was not provided during the determinations of walking activity. A group of eight foraging worker bees from each hive, which were obtained using mouth aspirators, were used to assess the overall group activity of each treatment. The insects of each group were simultaneously placed into a Petri dish (9.0 cm diameter), the bottom of which was covered with filter paper (porosity of 3 microns, 0.5% ash content, 9 cm diameter, 80 g/m^2 density; Nalgon Equipamentos Científicos Ltda, Itupeva, SP, Brazil). The Petri dishes were covered with a transparent glass cover to prevent the insects from escaping, and after a 20-min acclimation, the overall group activity was digitally recorded for 10 min using an automated video-tracking system equipped with a CCD camera (ViewPoint LifeSciences, Montreal, QC, Canada; Tomé et al. 2015a,b). Overall group insect activity was recorded as changes in pixels between two consecutive pictures registered at 10^{-2} s intervals. All forms of movement from the group of insects were recorded including walking, body part movements, and conspecific interactions.

The individual walking activity of worker bees was recorded using the same containers and equipment used to record the overall group activity. However, single adult workers were placed within the Petri dish, rather than a set of eight (as in the group activity), following the methods detailed elsewhere (Tomé et al. 2012, Barbosa et al. 2015c). A minimum of seven bees were used from each of the four colonies. The exact number varied by treatment depending on the survival obtained. The walking activity of each individual foraging worker was recorded for 10 min using the ViewPoint tracking system and the following parameters were assessed: walking distance (cm), resting time (s), and walking velocity (cm/s). The determinations for individual insects were averaged per colony, which was considered the biological replicate of the bioassays.

Flight Take-off Bioassay

Surviving workers exposed for 72 h to either contact or oral exposure to the leaf fertilizers and to walking bioassays were subsequently subjected to flight take-off bioassays, using the methods earlier described by Tomé et al. (2015b). Again spinosad (and also copper sulfate under oral exposure) were not used in the flight take-off bioassays due to their higher short-term mortality to stingless bee workers. Briefly, a 105-cm tower was formed by three stacked wooden cages (35 by 35 by 35 cm each) opened to allow insect flight between them. A fluorescent lamp was placed 5 cm above the tower in a dark room to attract the flying insects. An individual foraging worker was released from a Petri dish at the center bottom of the tower, whose cover was removed after 1 min to allow the insect to take flight toward the light source. The bees' vertical flight take-off and flight activity was observed for 2 min and recorded as follows: 1) no flight, 2) flight up to 35 cm high, 3) flight between 35 and

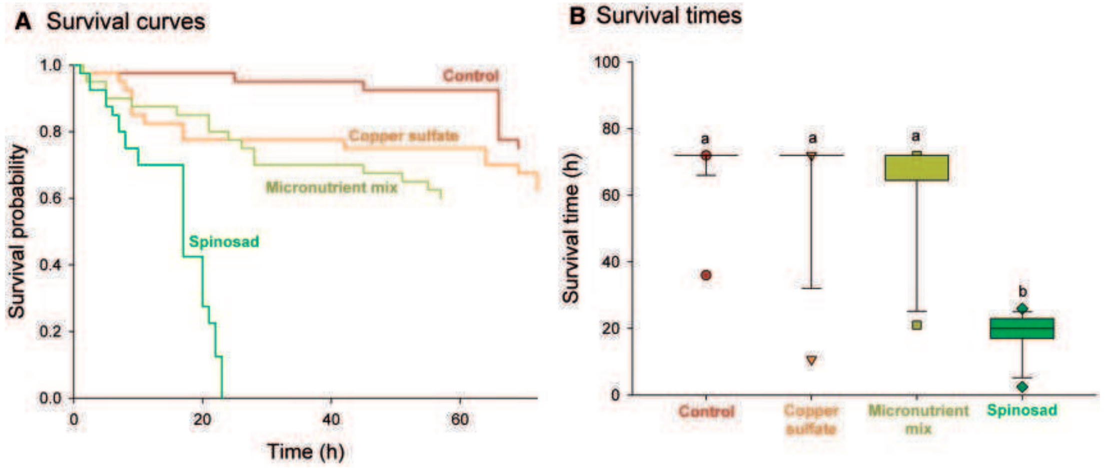

Fig. 1. Survival curves (**A**) and box plots of the median survival times (LT_{50}'s) (**B**) of adult workers of *F. schrottkyi* contact-exposed to leaf fertilizers at field rates. Box plots indicate the median and range of dispersion (lower and upper quartiles and outliers) of the LT_{50}'s. The box plots are significantly different based on Holm–Sidak's test ($P < 0.05$) (online figure in color.).

70 cm high, 4) flight between 71 and 105 cm high, and 5) flight reaching the light source at 110 cm high. A minimum number of seven bees was used per replicate. The exact number varied with treatment depending on the survival obtained.

Respirometry Bioassay

As gas exchange, and thus respiration rate, is a measure of individual level stress, a respirometry bioassay was carried out with the same individual insects used on the walking and flight take-off bioassays after 72-h exposure (contact and oral) to leaf fertilizers (or for unexposed insects, water). The determinations for individual insects were averaged per colony, which was considered the biological replicate of the bioassays. Each insect was placed into a glass tube (25 ml), and the whole set of tubes was connected to a completely closed system. The CO_2 production (µl CO_2/h/bee) was determined after a 3-h period by injecting CO_2-free air into the chambers for 2 min at a flow of 600 ml/min using a TR3C respirometer equipped with a CO_2 analyzer (Sable Systems International, Las Vegas, NV; Tomé et al. 2015a).

Statistical Analyses

The survival results were subjected to survival analyses using Kaplan–Meier estimators to obtain the survival curves and estimates of median survival time (LT_{50}'s; PROC LIFETEST; SAS Institute 2008). Overall similarity among survival curves was tested using the χ^2 Log-Rank test ($P < 0.05$), and pairwise comparisons were performed using Holm–Sidak's method ($P < 0.05$). The overall group activity, individual walking activity parameters, food consumption, and respiration rate were subjected to analysis of variance and Tukey's HSD test ($P < 0.05$) when appropriate. The body mass of the individual workers was used as covariate in the analyses of individual walking activity and respiration rate to adjust for the effects of body mass on these traits. The assumptions of normality and homoscedasticity were checked, but no data transformations were necessary (PROC UNIVARIATE; SAS Institute 2008). The results of flight take-off were subjected to the Kruskal–Wallis (nonparametric) test ($P < 0.05$).

Results

Time–Mortality (Survival) Responses

The survival curves of worker bees exposed either to leaf fertilizers, spinosad, or water by surface contact were significantly different ($\chi^2 = 75.18$, df = 3, $P < 0.01$; Fig. 1A). The survival curves obtained via oral exposure of worker stingless bees were also significantly different ($\chi^2 = 35.05$, df = 3, $P < 0.01$; Fig. 2A). Although unexposed insects exhibited higher survival in both exposure bioassays, the results of exposed insects differed with the agrochemical and mode of exposure. While spinosad led to quick bee mortality under contact exposure (LT_{50} [95% CL] = 17.00 [13.67–20.33] h) significantly differing from the unexposed and fertilizer-exposed bees (Fig. 1B), copper sulfate led to faster 100% mortality under oral exposure (LT_{50} [95% CL] = 24.00 [20.24–27.75] h), followed by spinosad (LT_{50} [95% CL] = 56.00 [50.36–61.63] h; Fig. 2B). Because the mortality of unexposed and micronutrient mix-exposed bees did not reach the 50% level even after 72 h of exposure, their respective LT_{50}'s were not estimated for orally exposed insects. Spinosad was not subjected to the sublethal bioassays due to its high (acute) short-term mortality, which prevented such studies at the label rate used, as was also the case for copper sulfate under oral exposure.

Food Consumption

The food consumption by worker bees varied with treatments both under contact exposure ($F_{3,12} = 6.58$, $P < 0.01$) and under oral exposure ($F_{3,12} = 4.92$, $P < 0.01$). Copper sulfate and spinosad led to nearly twice the food consumption compared with the micronutrient mix in contact-exposed bees, while unexposed bees exhibited intermediary results (Fig. 3A). Copper sulfate also led to higher food consumption under oral exposure, but spinosad did not differ between unexposed bees and bees provided with the micronutrient mix (Fig. 3B).

Overall Group Activity and Walking Activity

The leaf fertilizers did not affect the overall group activity of stingless bee workers under contact exposure (43.60 ± 0.13 Δ pixels/s $\times 10^{-2}$; $F_{2,8} = 0.61$, $P = 0.57$), neither did the micronutrient mix under oral exposure (37.71 ± 0.08 Δ pixels/s $\times 10^{-2}$; $F_{1,4} = 1.83$, $P = 0.25$). In the individual activity bioassays, the leaf fertilizers also did not lead to significant differences in the distance walked

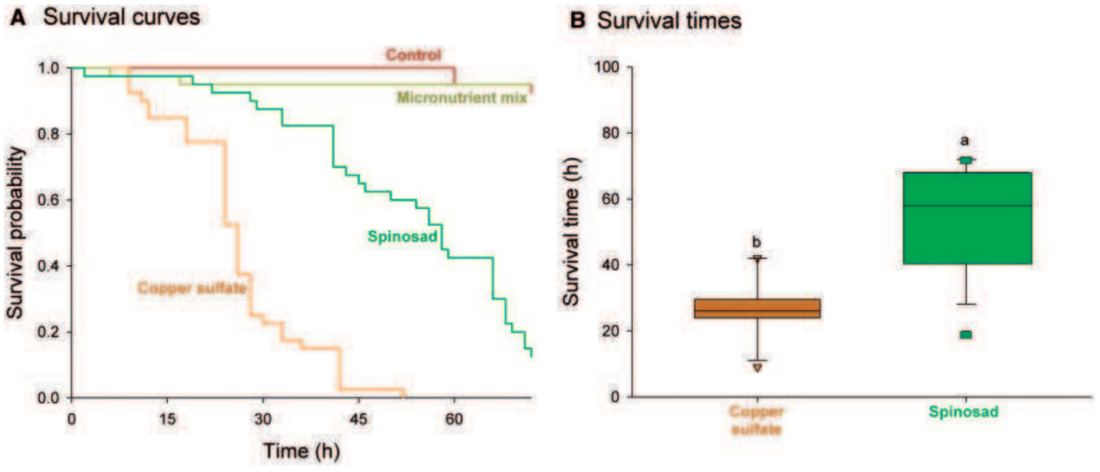

Fig. 2. Survival curves (**A**) and box plots of the median survival times (LT_{50}'s) (**B**) of adult workers of *F. schrottkyi* orally exposed to leaf fertilizers at field rates. Box plots indicate the median and range of dispersion (lower and upper quartiles and outliers) of the LT_{50}'s. The box plots are significantly different based on Holm–Sidak's test ($P < 0.05$) (online figure in color.).

(contact: 579.37 ± 69.65 cm [$F_{2,8} = 1.64$, $P = 0.25$]; oral: 491.67 ± 92.34 cm [$F_{1,6} = 2.22$, $P = 0.19$]), or resting time (contact: 173.45 ± 11.93 s [$F_{2,8} = 3.29$, $P = 0.09$]; oral: 170.92 ± 20.56 s [$F_{1,6} = 3.66$, $P = 0.10$]), and did not affect walking velocity (contact: 1.24 ± 0.12 cm/s [$F_{2,8} = 1.39$, $P = 0.30$]; oral: 1.14 ± 0.14 cm/s [$F_{1,6} = 1.42$, $P = 0.28$]), regardless of the exposure.

Flight Take-off

Take-off and vertical flight activity were significantly higher for worker bees contact-exposed to copper sulfate compared with those exposed to the micronutrient mix or unexposed ($H = 13.66$, df = 2, $P = 0.01$; Fig. 4). There was no difference in take-off and vertical flight activity of unexposed worker bees and those orally exposed to the micronutrient mix ($H = 2.08$, df = 1, $P = 0.15$).

Respiration Rate

Copper sulfate led to a higher respiration rate in contact-exposed worker bees that differed from unexposed and micronutrient mix-exposed worker bees ($F_{2,8} = 6.56$, $P = 0.02$; Fig. 5). In contrast, the micronutrient mix significantly compromised the respiration rate of orally exposed worker bees ($F_{1,5} = 66.29$, $P < 0.01$; Fig. 5).

Discussion

The aim of our study was to assess the potential effects of commonly used leaf fertilizers on the Neotropical stingless bee species *F. schrottkyi* via contact and oral exposure of adult worker bees at the respective field use rates. The bioinsecticide spinosad was used as a positive control because this compound has been reported as highly toxic to stingless bees (Barbosa et al. 2015b,c; Tomé et al. 2015a,b), and water without any agrochemicals was used as a negative control. What we were not expecting was the high toxicity of copper sulfate, used as a foliar fertilizer, to workers of *F. schrottkyi* under oral exposure, leading to their complete demise in less than 72 h ($LT_{50} = 17.00$ hs), which was twice as fast as the mortality caused by spinosad under the same treatment.

Copper sulfate under contact exposure exhibited milder effects than spinosad, resembling the foliar micronutrient mix, in contrast with oral exposure. Therefore, copper (Cu) penetration via cuticle does not seem as important as uptake via diet (i.e., contaminated pollen and nectar), which, for instance, is able to accumulate in

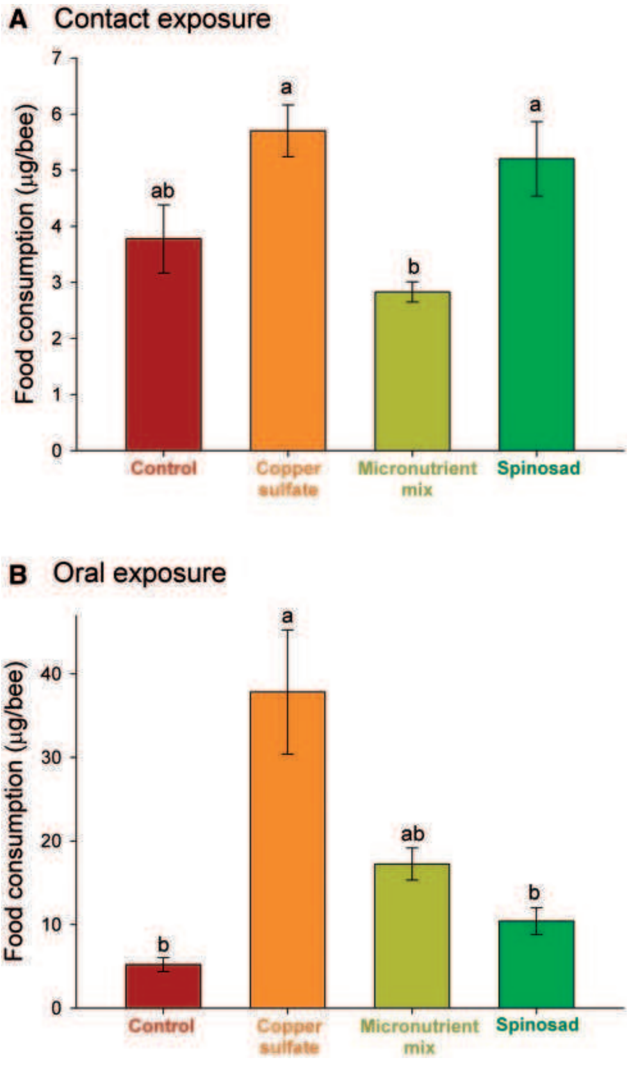

Fig. 3. Food consumption by adult workers of *F. schrottkyi* contact-exposed (**A**) and orally exposed (**B**) to leaf fertilizers at field rates. The histogram bars with different letters indicate significant differences among treatments based on Tukey's HSD test ($P < 0.05$) (online figure in color.).

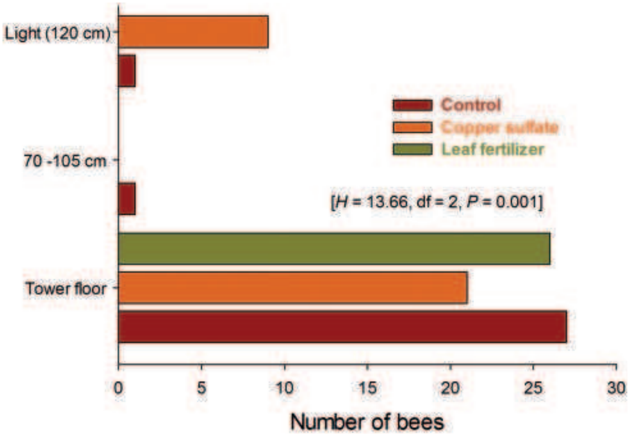

Fig. 4. Flight take-off activity of adult workers of *F. schrottkyi* contact-exposed to leaf fertilizers at field rates. The results of the (nonparametric) Kruskal–Wallis test ($P < 0.05$) used to test the differences between treatments are indicated (online figure in color.).

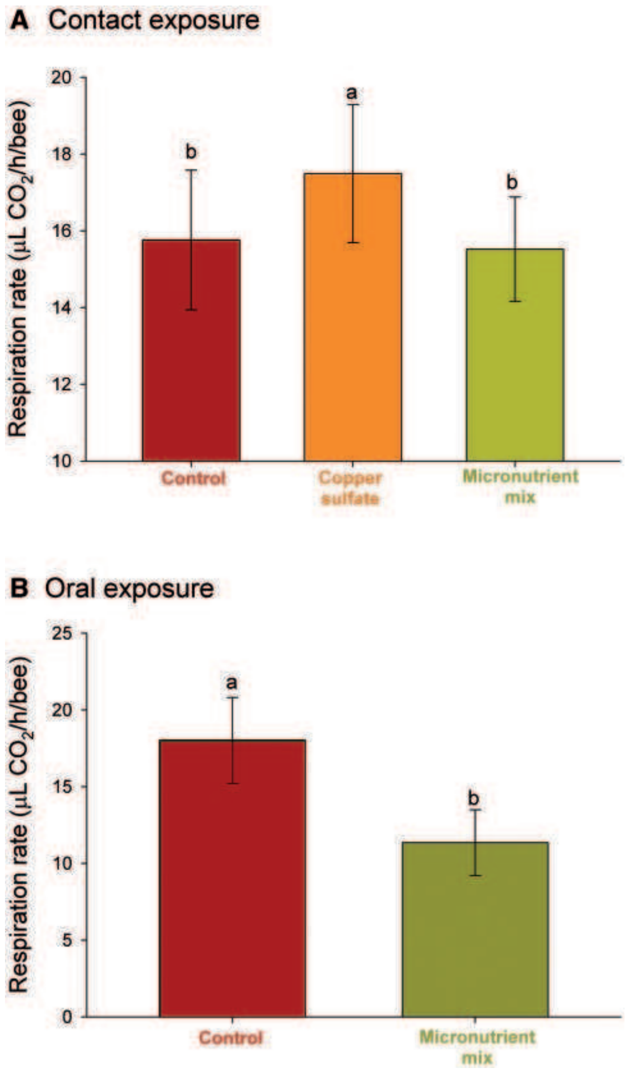

Fig. 5. Respiration rate of individual (± SE) adult workers of *F. schrottkyi* either contact-exposed (**A**) or orally exposed (**B**) to leaf fertilizers at field rates. The histogram bars with different letters indicate significant differences among treatments based on Tukey's HSD test ($P < 0.05$) (online figure in color.).

housefly larvae fed on contaminated diet, compromising their immune cells at concentrations between 0.005 and 1.0 g/liter (Borowska et al. 2004, Borowska and Pyza 2011). The latter concentration is five times lower than the concentration in copper sulfate as leaf fertilizer used in our experiments. However, copper sulfate did not reduce, but rather significantly increased food ingestion by the worker bees, which is likely to maximize the oral exposure (and uptake) of this leaf fertilizer by foraging stingless bee workers and its harmful consequences. Lower rather than higher Cu concentrations (0.025–0.050 g/liter) were reported as favoring efficiency of food conversion and growth rate in the cotton bollworm (Baghban et al. 2014), suggesting that higher concentrations in the range used in our study may impair food conversion, potentially requiring a higher feeding rate to minimize the consequent reduction in growth.

The micronutrient mix did not compromise survival, unlike copper sulfate, even though it contained not only Cu but also a series of other heavy metals that may either synergize the Cu toxicity or exhibit significant toxicity by themselves. The risk of potentiation by chemical—and particularly agrochemical—mixtures is frequently neglected, although their use is rather common (Yang 1994, Guedes et al. 2016). The joint (potentiating) effect of mercury (Hg; 1.2 µg/g diet) and selenium (Se; 1.0 µg/g diet) reported in the phorid fly *Megasilia scalaris* Loew is an example of such a possibility (Jensen et al. 2006), as are the lethal effects of Cu (20.0–200.00 µg/g diet) and zinc (Zn; 62–1,740 µg/g diet), another heavy metal, as has been reported in other insect species (Popham and Shelby 2006, Noret et al. 2007, Baghban et al. 2014). However, the levels of micronutrients present in the mix used in our experiment were not large enough to affect stingless bee mortality, although their ranges overlap the concentrations used in the other reported studies.

The effects of leaf fertilizers containing heavy metals are not limited solely to reduced survival. Sublethal effects, which are currently receiving increasing attention in pesticide (eco)toxicology (Guedes et al. 2016), have not been the object of much research attention regarding leaf fertilizers so far, although some heavy metals were reported to lead to sublethal effects in insects, particularly Cu, Se, and Zn (e.g., Bayley et al. 1995, Mogren and Trumble 2010, Baghban et al. 2014). We did not detect a significant interference from leaf fertilizers on the walking behavior and activity of worker bees of *F. schrottkyi*, but copper sulfate enhanced flight take-off and vertical flight activity, while the micronutrient mix reduced the respiration rate of orally exposed insects. Such responses, as well as the lethal effects observed, are likely due to the heavy metals constituting the leaf fertilizers studied—Cu in the case of copper sulfate, and Cu again along with Mn, Mo, and Zn in the case of the micronutrient mix—potentially aided by sulfur (S; also present in both leaf fertilizers) and boron (B, a light metalloid present in the micronutrient mix), which also exhibits biological activity.

Heavy metals are relatively dense metals or metalloids (i.e., density>3.5 g/cm^3, atomic weight>23, and atomic number>20), some of which are essential for life in small amounts, including Cu, Mn, Mo, and Zn, which is the reason for their presence in leaf fertilizers applied to cultivated plants. Their presence is beneficial to plants and animals at such amounts. At low levels, these heavy metals act as cofactors of several enzymes (e.g., Linder and Hazegh-Azam 1996, Cox 1999), but they are toxic at higher concentrations, leading to biphasic dose–response relationships (Calabrese 2008, Cutler 2013, Guedes and Cutler 2014).

The toxic actions of heavy metals usually stem from their binding activities to vital cellular components including structural proteins, enzymes, and nucleic acids. This activity compromises cell functions by generating extremely toxic reactive forms of oxygen (or oxidative free radicals; Halliwell and Gutteridget 1984, Phillips and Hilliker 1990, Cox 1999). Therefore, the range of effects from heavy metal exposure may vary from beneficial, to mildly harmful, to very harmful. The range of heavy metal concentrations in the leaf fertilizers tested here against Neotropical stingless bees were in the harmful range, particularly for oral exposure when compromising survival, and resulting in significant sublethal effects in food consumption, flight, and respiration rate—traits likely to affect foraging in worker bees and thus, colony sustainability. However, field exposure needs to be assessed for reliable risk assessment.

S and B also exhibit biological activity, particularly among arthropods. S is a known acaricide and fungicide still in commercial use (e.g., Ministério da Agricultura, Pecuária e Abastecimento 2015), while B has antimicrobial and insecticidal activity and is also still used today for such purposes (e.g., Ministério da Agricultura, Pecuária e Abastecimento 2015). Therefore, both these compounds may also be involved in the observed toxicity of leaf fertilizers, although their respective activities may be indirectly expressed by compromising insect (and bee) symbionts (Guedes et al. 2016). Regardless, leaf fertilizers seem to deserve attention and concern regarding their potential impact on native pollinators, notably Neotropical stingless bees such as *F. schrottkyi*. Their heavy metal content is above the safety threshold for the stingless bee species studied, which may also be the case for related species. Furthermore, the mix of heavy metals in some leaf fertilizers and the presence of S and sometimes B may increase their risks. In sum, leaf fertilizers deserve proper risk assessment because of the isolated and mixed use of heavy metals in such fertilizers.

Acknowledgments

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CONCLUSÕES

O fertilizante foliar sulfato de cobre, fonte do metal pesado Cu, afetou a sobrevivência de abelhas operarias de *Friesella schrottkyi* levando a mortalidade de 100% dos indivíduos sob exposição oral e com uma TL_{50} de apenas 24h. O consumo das abelhas sob exposição oral com sulfato de cobre proporcionou maior consumo da solução contaminada, mesmo em exposição por contato o sulfato de cobre induziu as abelhas operárias ao maior consumo. Os fertilizantes foliares não afetaram a atividade de grupo ou individual das abelhas operárias expostas tanto via contato como oral. Porém, o levantamento de voo foi maior para aquelas abelhas sob contato com sulfato de cobre, bem como a taxa respiratória. Em contraste a mistura de micronutrientes também comprometeu a taxa respiratória das abelhas operárias oralmente expostas. Embora nosso estudo nos permitam tirar algumas conclusões ainda são necessárias mais pesquisas sobre o efeito dos fertilizantes foliares em abelhas já que são produtos amplamente utilizado na agricultura.