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Larvae of stingless bee *Scaptotrigona bipunctata* exposed to organophosphorus pesticide develop into lighter, smaller and deformed adult workers☆

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ABSTRACT

Organophosphorus pesticides such as chlorpyrifos are often used in agriculture due to their broad spectrum of action. However, this insecticide and acaricide is considered highly toxic to the environment and can cause toxicity in nontarget insects such as bees. In addition to adult individuals, immature can also be exposed to residues of this insecticide by larval food. Thus, we investigated the effects of chlorpyrifos concentrations on the larval development of stingless bee *Scaptotrigona bipunctata* workers reared *in vitro*. We evaluated four different biomarkers: a) survival, b) development time, c) body mass and d) morphological characteristics (head width, intertegular distance, wing area and proportion of deformed bees). The exposure of the larvae to different doses of chlorpyrifos significantly reduced survival probability but did not cause changes in the development time. Regarding morphometric analysis, bees exposed to chlorpyrifos showed a reduction in body mass and size, and 28% of the emerged adults showed a reduction in wing area and deformations. Therefore, this work shows that *S. bipunctata* larvae exposed to the sublethal effects of chlorpyrifos are likely to have reduced chances of survival. However, if they emerge, they will be lighter, smaller and less able than equivalent but not exposed workers. These impaired attributes have the potential to compromise the future workforce in colonies exposed to this pesticide.

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1. Introduction

Bees are considered important pollinating agents not only in agricultural crops but also in wild plants (Potts et al., 2010; IPBES, 2016; Woodcock et al., 2019). The pollination services performed by these insects are essential for maintaining the diversity of plants, production of food, seeds and pastures (Garibaldi et al., 2011; Giannini et al., 2015; Kremen, 2018). However, the bee populations loss reported in recent years is worrying, as it may lead to an imminent crisis in pollination services (Johnson et al., 2010; Lebuhn et al., 2013; Sánchez-Bayo and Wyckhuysb, 2019). This decline is attributed to multiple factors, but the overuse of pesticides has been implicated as a major cause of the loss of bee populations (Brown et al., 2016; Azpiazu et al., 2019; Sgolastra et al., 2020).

Organophosphorus insecticides are among the most widely used pesticides worldwide (Dai et al., 2019). These products are highly toxic to the environment (Iupac, 2020), and due to their broad spectrum of action, they are considered toxic to any insect, including nontarget ones (Nicholls and Altieri, 2013; Stanley et al., 2015). These compounds act on the nervous system of insects by inhibiting acetylcholinesterase (AChE). This enzyme regulates the level of the neurotransmitter acetylcholine, which acts on the transmission mechanism of nerve impulses (Thompson, 1999).

In this group of organophosphorus pesticides, the insecticide and acaricide chlorpyrifos is often used due to its greater persistence and effectiveness against a wide range of pests (Rehman et al., 2012; Cutler et al., 2014). In Brazil, chlorpyrifos is used in crops that occupy extensive areas, such as apples, citrus, coffee, corn, cotton and soybeans (MAPA, 2019). Although these agricultural crops present different levels of dependence on bees for pollination, these insects frequently visit them to collect floral resources such as nectar and/or pollen (Milfont et al., 2013; Földesi et al., 2016; Hipólito et al., 2018). Thus, it is likely that this insecticide will come

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into contact with bees at different levels of exposure, since residues of chlorpyrifos have been detected in pollen, honey and brood combs of honey bees (DeGrandi-Hoffman et al., 2013; Naggar et al., 2015; Calatayud-Vernich et al., 2018; Tosi et al., 2018).

Pollen and nectar with pesticide residues taken to the colony can result in a cascade of systemic exposures (Rortais et al., 2005; Chauzat et al., 2006; Krupke et al., 2012). This is because the resources collected will be processed by the nurse bees and supplied to developing larvae through larval food (Rosa et al., 2016; Santos et al., 2016; Dai et al., 2019). The immature exposure to pesticides may seem subtle and indirect, but the larvae can be exposed to the insecticide via two routes, orally and topically (Zhu et al., 2014). Therefore, chronic exposure to pesticides during development can negatively affect bees, leading to inadequate larval nutrition, developmental delays or even impacting the survival of adult individuals (Zhu et al., 2014; Rosa et al., 2016; Santos et al., 2016). Conceivably, these impacts during the larval phase can affect the functional integrity of these bees with serious consequences for the maintenance of colonies (Carvalho et al., 2013; Pettis et al., 2013).

Through the *in vitro* larval rearing method, we can evaluate the lethal and sublethal effects of xenobiotics on the development of the immature. Due to controlled laboratory conditions it is possible to quantify the dose ingested by each individual and to assess larval and pupal mortality (Aupinel et al., 2007; Dorigo et al., 2019). Moreover, biologically relevant parameters such as the emergence rate and weight of individuals can be directly monitored (Hendriksma et al., 2011).

In ecotoxicological studies, *Apis mellifera* L. is considered a relevant model organism due to its economic value as a pollinator (Tavares et al., 2015). However, it is known that the susceptibility of bees to a certain pesticide may differ from one species to another due to nutritional, behavioral and physiological differences (Del Sarto et al., 2014; Dorneles et al., 2017). Currently in Brazil work is underway to determine whether it is safe to use the honeybees as a model species as a substitute for other native bee species in the pesticide risk assessment (Dorigo et al., 2019; Rosa-Fontana et al., 2020). As such, stingless bees are prominent but still poorly investigated in risk assessment (Prado-Silva et al., 2018). They are an important group of eusocial bees, pollinators of tropical regions in natural and agricultural ecosystems (Slaa et al., 2006; Venturieri et al., 2011; Witter et al., 2015).

In the case of stingless bees, the evaluation of the possible effects of pesticides on larval development is particularly relevant. Unlike honey bees, stingless bees do not progressively feed their brood (Menezes et al., 2013). The larval nutrition of this group consists of mass food deposition in brood cells. In this way, the queen lays her eggs on the larval food and then the workers close the cell (Sakagami, 1982). Thus, the brood cells remain operculated from oviposition to the emergence of adults (Nogueira-Neto, 1997).

Among stingless bees, *Scaptotrigona bipunctata* (Lepeletier) has a wide geographical distribution in the Neotropical Region, including Bolivia, Brazil, Paraguay and Peru (Camargo and Pedro, 2013). It is a managed species that has populous nests (Nogueira-Neto, 1997) whose individuals are easily adapted to experimental conditions. For these reasons, we selected *S. bipunctata* as an experimental model to evaluate the potential effects of chronic exposure to the insecticide chlorpyrifos on the development of immature, survival and morphometric parameters of newly emerged adults.

2. Material and methods

2.1. Bees and study area

Brood combs of *S. bipunctata* were collected from three different colonies kept at meliponary of the Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), Porto Alegre, Brazil.

2.2. Preparation of larval food with insecticide

The active ingredient chlorpyrifos (Pestanal, 250 mg: ≤100%, analytical standard, Sigma Aldrich, Brazil) was used to prepare a stock solution of 1 μg a.i./μL of acetone. This solution was then diluted in the larval food at established concentrations.

The highest reported concentration of chlorpyrifos found in pollen was 0.967 ng/mg (DeGrandi-Hoffman et al., 2013), and the amount of pollen in *S. bipunctata* larval food is 1.9 mg (Rosa et al., 2015). With these data, we can estimate that a larva would consume 1.8 ng of the insecticide. However, the larvae were overdosed 35 fold (volume of the diet, 35 μL/bee). Thus, we established the following doses to proceed with the tests: 16.1 ng a.i./larva (T1), 32.2 ng a.i./larva (T2) and 63.0 ng a.i./larva (T3).

2.3. In vitro rearing of Scaptotrigona bipunctata workers

First, larval food was collected from newly operculated cells. The food was homogenized and mixed with the insecticide using the dilutions mentioned above and then transferred to the rearing plate cavity with an automatic pipette. Each cavity received 35 μL of larval food (protocol previously established by the research group) over which a 1–3 days old larva was transferred.

Throughout the period of larval development until the emergence of adults, the rearing plates were packed in airtight containers and kept inside B.O.D. (Biochemical oxygen demand) at 28 °C. The relative humidity inside the containers was controlled using distilled water (98%) during the feeding period, which was from the 1st to the 5th day, saturated sodium chloride solution (75%) until the 28th day and with no solution (65%) until the emergence of adults.

2.4. Chronic exposure to insecticide

The bioassay was based on the Teste Guideline 239 to honey bee larval toxicity test using repeated exposure (Oecd, 2016). The larvae were divided into five experimental groups as follows: three treatments with different doses of chlorpyrifos (16.1 ng a.i./larva (T1), 32.2 ng a.i./larva (T2) and 63.0 ng a.i./larva (T3)), a control group (without insecticide) and a solvent control (with acetone). Each group was three replicates, and for each replicate, the test unit consisted of a rearing plate with 30 larvae, totaling the transfer of 90 larvae per treatment. The larvae tested in each replicate came from a single colony.

Immatures were monitored daily, and the mortality rate was recorded from the transfer of the larvae to the emergence of adults. The development time (days) until the emergence of adults was also recorded. Dead individuals were identified both by the darkened tegument and the absence of spiracle movement and were then removed from the rearing plates.

2.5. Body mass and morphometric analysis

The fresh body mass of the newly emerged adult workers was obtained using a precision analytical balance. Head width and intertegular distance were measured using a stereoscopic microscope (Leica M205 A) connected to a digital camera (Leica DMC2900) and Leica Application Suite (software LAS V4.8). Males were identified in the imago stage and excluded from morphometric analysis.

Finally, adult bees were visually inspected for external

morphological deformities. In addition, the right anterior wing of 30 individuals per treatment was removed and mounted on slides to acquire its image and measure the area using ImageJ software (Schneider et al., 2012). A tutorial on using ImageJ software was added as supplementary material.

2.6. Statistical analysis

To assess the adult mortality and emergency rate, we analyzed the survival probability of *S. bipunctata* larvae exposed to different doses of insecticide. For this purpose, the data were analyzed by the survival curves obtained through Kaplan-Meier estimators using the 'Surv' function of the *survival* package (Therneau, 2015). As the workers emerged at different times (40–52 days), the survival curves were standardized by censoring the data on the 52nd day (counted from the transfer of the larvae). The difference between the survival curves was analyzed using the log-rank test with the 'survdiff' function of the *survival* package. If significant, multiple pairwise comparisons between treatments were conducted using the 'pairwise_survdiff' function of the *survminer* package (Kassambara and Kosinski, 2018) with adjustment of the *p*-value by Benjamini-Hochberg (BH).

To investigate whether the developmental time of bees exposed to different treatments was affected by the intake of chlorpyrifos, we used a generalized linear model (GLM) with a Poisson distribution. On the other hand, to compare body mass (in grams) and morphometric data (head width and intertegular distance in mm; wing area in mm^2) of newly emerged adults, we used GLM with Gaussian distribution. Both GLMs were performed using the function 'glm'. Since there could be a variation between evaluated colonies concerning to particular sensitivity to the tested substance, we performed a generalized linear mixed model (GLMM, function 'glmer') using the colonies as random effects. Then, we employed a model selection based on the Akaike's Information Criterion (AIC) using the function 'AICctab'. The selection of the GLM model was based on the lower AIC value, assuming a negligible variation between the colonies. The potential capability of flying emerged bees from each treatment was estimated according to their expected flight range and, consequently, their inferred coverage area of flight. Thus, since intertegular span in bees is highly correlated with their own foraging distance (Greenleaf et al., 2007), we calculated their expected flight range using such morphological measurements in the function 'foragedist' of the *pollimetry* package (Kendall et al., 2019). After obtaining their expected flight radius, we used such data to calculate the area of the circle ($A = \pi.r2$) as a proxy to infer their coverage area. All analyses were carried out in R software (Ihaka and Gentleman, 1996; R Core Team, 2018).

3. Results

3.1. Survival and development of the immature

The survival of immature *S. bipunctata* was significantly impaired after exposure to a diet with increasing doses of chlorpyrifos (Log-rank: $\chi^2 = 22.2$, $d.f. = 3$, $p < 0.001$; Fig. 1).

The survival probability in the control was 90% (95% CI: 84–96%), and in insecticide treatments, there was a significant reduction in the survival probability: T1 = 59% (95% CI: 50–70%), T2 = 67% (95% CI: 58–77%) and T3 = 69% (95% CI: 60–80%). Bioassays performed with the solvent control (with acetone) the survival probability of the immature was not impacted (Log-rank: $\chi^2 = 0.1$, $d.f. = 1$, $p = 0.8$).

Exposure to chlorpyrifos induced a higher mortality between the eye-pigmented pupae and imago stages. The development time of workers was not affected by exposure to the insecticide (GLM Poisson, z-value = −0.10, $p = 0.91$). The average time of emergence of bees (±standard deviation) was 44 ± 2.6 days (period from the transfer of the larvae until adult emergence).

3.2. Body mass and morphometric analysis of newly emerged adults

Exposure to chlorpyrifos during the larval stage significantly reduced the body mass of newly emerged adults when compared to the control (GLM Gaussian, $\chi^2 = 52.09$, $p < 0.001$; Fig. 2). The bees exposed to this insecticide were 21% lighter overall than bees not exposed. There was no significant difference among treatments with the addition of the insecticide.

The head width of newly emerged adults was not significantly affected by exposure to chlorpyrifos (GLM Gaussian, $\chi^2 = 3.49$, $p = 0.06$). However, there was a significant reduction in the intertegular distance (GLM Gaussian, $\chi^2 = 18.12$, $p < 0.001$; Table 1). The

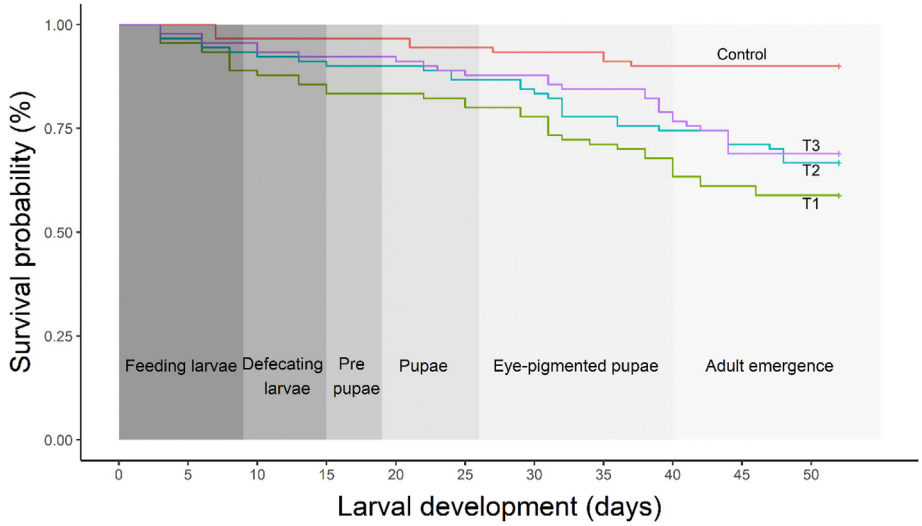

Fig. 1. Survival probability of *Scaptotrigona bipunctata* workers reared *in vitro* and exposed to chlorpyrifos during the larval period. Control = food without insecticide; T1 = 16.1 ng a.i./larva; T2 = 32.2 ng a.i./larva; T3 = 63.0 ng a.i./larva. Gray shading indicates the mean duration of each phase of development until the emergence of adults.

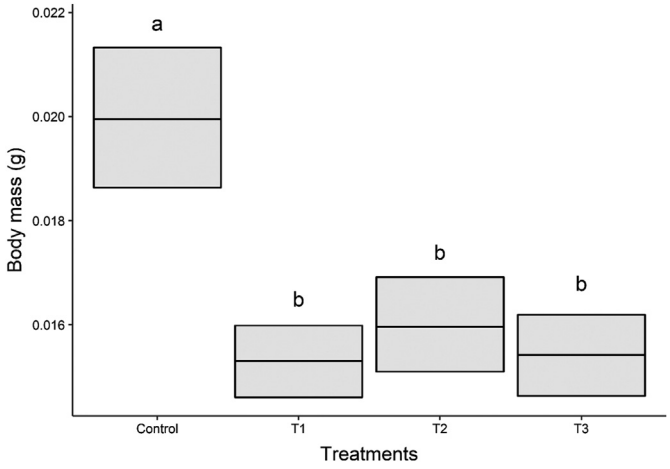

Fig. 2. Body mass (means and 95% confidence interval) of newly emerged *Scaptotrigona bipunctata* workers reared *in vitro* and exposed to chlorpyrifos during the larval period. Control = food without insecticide; T1 = 16.1 ng a.i./larva; T2 = 32.2 ng a.i./larva; T3 = 63.0 ng a.i./larva. Different letters indicate significant differences.

bees exposed to higher chlorpyrifos dose were approximately 5% smaller than the control.

Finally, we showed that the smallest bees most likely will be worst foragers. For example, when we compared the flight range of T3 versus controls, we estimated that bees exposed to chlorpyrifos would have a 14% smaller flight radius (average: 302 m) than control bees (average: 352 m). Consequently, their corresponding flight coverage area would be 26% smaller than the inferred area for the control group (Table 1).

Of the bees exposed to chlorpyrifos during the larval stage, 28% had deformed appendages (antennae and legs) and reduced wing area (Fig. 3).

Of the individuals who had changes in wing development, the reduction in wing area was significantly less than that of the control (GLM Gaussian, $\chi^2 = 1995$, $p < 0.001$; Fig. 4). The bees exposed to chlorpyrifos doses showed a reduction of up to 83% of the wing area in relation to the control group.

4. Discussion

Considering the risk assessment of pesticides for bees, we performed the *in vitro* rearing of *S. bipunctata* workers and analyzed the chronic toxicity of chlorpyrifos concentrations during the development of the immature. It is important to note that our bioassays were carried out at 28 °C, and in this temperature there may be a reduction in the lethal and sublethal effects of the insecticide chlorpyrifos, leading to an increase in its degradation and consequently less accumulation. In a study by Beeck et al. (2017) the authors evaluated the effect of chlorpyrifos at different temperatures (20 and 24 °C) in the damselfly *Ischnura elegans* (Odonata, Zygoptera). The results indicated that individuals exposed to a higher temperature had lower mortality and less oxidative damage.

Exposure to this insecticide had a significant impact on the survival of *S. bipunctata* workers. As a similar result, Santos et al. (2016) also observed an increase in the mortality of queen larvae of stingless bee *Plebeia droryana* (Friese) after exposure to the insecticide chlorpyrifos (8.8–88 ng a.i./larva). Zhu et al. (2014) reported that chronic food exposure to residual doses of chlorpyrifos (1.5 ng a.i./μL) also had significant impacts on the survival of honey bee larvae.

In our work, during the developmental stages of *S. bipunctata*, the highest mortality rates were observed between the eye-pigmented pupae and the imago. Whereas chlorpyrifos is lipophilic (water solubility is 1.05 mg/L at 20 °C), this late response to toxicity may be a consequence of the accumulation of the insecticide in the fat body. This organ, in addition to playing an important role in detoxification, also stores toxins (Yu et al., 1984; Feng et al., 2018). During metamorphosis, the fat body mobilizes energy reserves to supply the pupae metabolic needs, and at that moment, the insecticide that was stored can be released by contacting the hemolymph until it reaches the target organ (Tadei et al., 2019). The fat body is rich in cytochrome P450 (Tavares et al., 2017), an enzyme that acts in the biotransformation of chlorpyrifos to its reactive metabolite chlorpyrifos-oxon, a potent AChE inhibitor (Hodgson, 2010). According to Shi et al. (2013) and Zhu et al. (2016) the greatest expression of genes related to cytochrome P450 are during the eye-pigmented pupae and adult stages, which corresponds to the period of greatest mortality in our study. Furthermore, the lower mortality during the larval period may be related to the fact that brain structures are in formation. Tavares et al. (2017) measured the expression of AChE during the development of honey bees and observed that the enzyme activity is lower in the larvae but begins to intensify in the pupal stage, increasing progressively until the imago phase.

The addition of chlorpyrifos to the larval diet did not alter the development time of *S. bipunctata* workers. This result was similar to that found by Dai et al. (2019), in which the total development time of immature honey bees fed chlorpyrifos (0.5–8 ng a.i./μL) did not differ from that of the control group. However, these results contrast with the effects observed in the stingless bee *P. droryana*, in which individuals exposed to the same insecticide took 1.2 times longer to develop when compared to the control (Santos et al., 2016). Tomé et al. (2020) also observed a delay in the total development time of larvae of honey bees exposed to chlorpyrifos (20 ng a.i./bee). This dissimilarity in the results can be explained because the susceptibility to a product can vary between bee species because of their physiological differences and detoxification capacity.

In addition to the lethal effects, exposure to different doses of chlorpyrifos during the larval stage caused a decrease in body mass in newly emerged adults compared to the control. This reduction was not the result of the rejection of the diet because the larvae of *S. bipunctata* ingested all the larval food provided (35 μL/larva). Therefore, chlorpyrifos did not interrupt larval feeding behavior.

Table 1
Relationship between body size (average intertegular distances), expected flight radius and inferred flight coverage area of *Scaptotrigona bipunctata* workers.

Treatments	Intertegular distances (mm)	95% confidence interval	Flight range estimation (m)[a]	Coverage area inferring (ha)[b]
Control	1.69	1.73–1.65	352	38.9
T1	1.65	1.68–1.61	330	34.2
T2	1.62	1.66–1.58	313	30.7
T3	1.60	1.64–1.56	302	28.6

[a] Based on Bayesian predictive models using the intertegular distances of bees in the function 'foragedist' (pollimetry package; Kendall et al. 2019);
[b] Area of the circle: $A = \pi.r^2$, where pi = 3.1415 …, r = radius or flight range; hectare (ha) = 10,000 m^2.

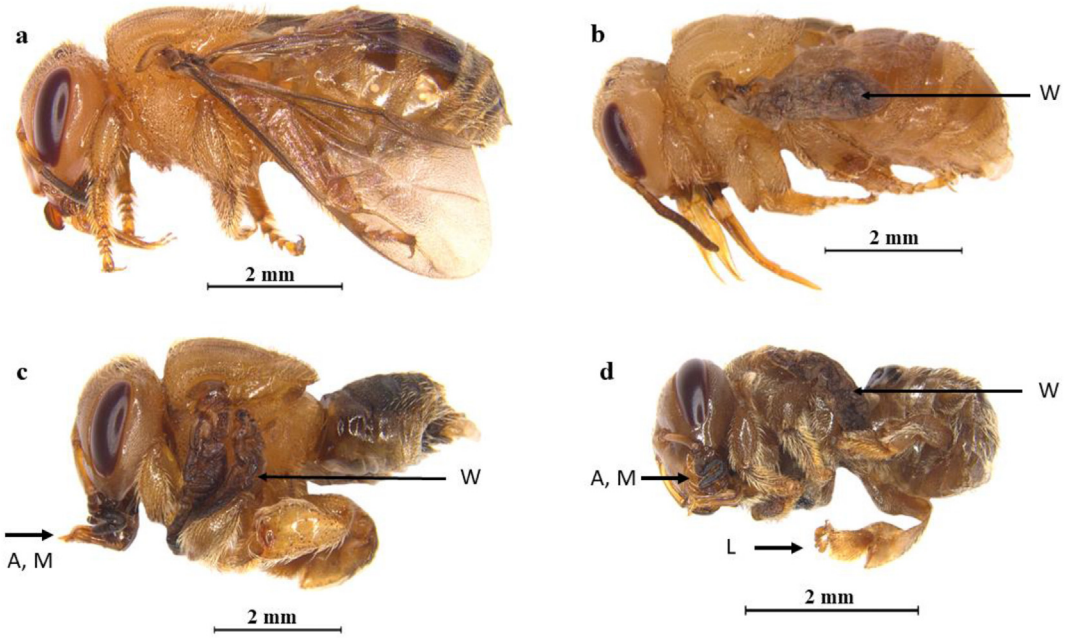

Fig. 3. Lateral view of newly emerged *Scaptotrigona bipunctata* workers exposed during the larval period to the control diet (food without insecticide) or diet with chlorpyrifos (16.1 ng a.i./larva (T1), 32.2 ng a.i./larva (T2) and 63.0 ng a.i./larva (T3)). (a) Adult in the control group with normal appendages. (b) Adult exposed to chlorpyrifos (T1) with reduced wing area. (c) Adult exposed to chlorpyrifos (T2) with deformed appendages and reduced wing area. (d) Adult exposed to chlorpyrifos (T3) with deformed appendages and reduced wing area. Arrows indicate malformations in the antennae (A), regions of the mouthparts (M), legs (L) and reduction of the wing area (W).

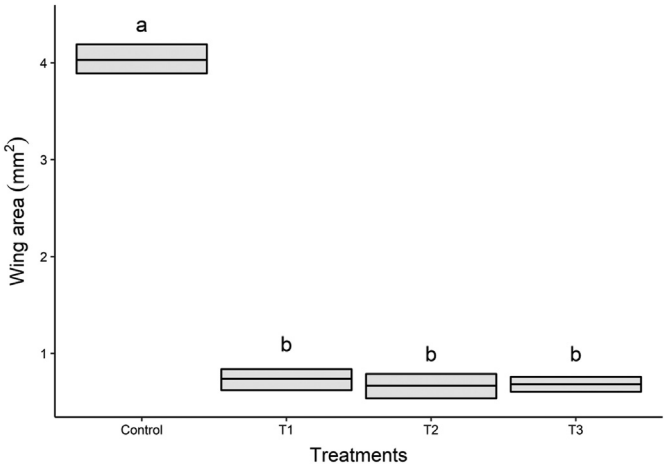

Fig. 4. Morphometry of the anterior wings (means and 95% confidence interval) of newly emerged *Scaptotrigona bipunctata* workers reared *in vitro* and exposed to chlorpyrifos during the larval period. Control = food without insecticide; T1 = 16.1 ng a.i./larva; T2 = 32.2 ng a.i./larva; T3 = 63.0 ng a.i./larva. Different letters indicate significant differences.

The decrease in mass is expected due to the increase in energy metabolism in response to activation of detoxification and defense mechanisms (Rand et al., 2015).

The head width of newly emerged adults was not affected by the intake of chlorpyrifos. However, the intertegular distance of exposed individuals decreases significantly when compared to the control. The reduced size of the workers caused by the ingestion of chlorpyrifos during the larval phase can subsequently affect the foraging activity (Wu et al., 2011). As the size of bees is correlated with foraging distance (Greenleaf et al., 2007), our data indicate that bees exposed to chlorpyrifos will be smaller and most likely poorly foragers since their flight range and coverage area are expected to be substantially reduced. As a consequence, this deficit in flight capacity may reflect a decrease in the collection of food resources leading to the impairment of the colony (Banaszak-Cibicka et al., 2018).

After exposure to chlorpyrifos during the larval stage, 28% of newly emerged adults (that is, almost $^1/_3$ of the bees) had deformed appendages and reduced wing area. Another study with chlorpyrifos also reported malformations during development. Dinh et al. (2016) exposed *Coenagrion scitulum* (Odonata, Zygoptera) during the larval phase, and their results indicated a high incidence of malformations in the wings. Similar results with other organophosphorus insecticides have also been described by Atkins and Kellum (1986). After exposure of *A. mellifera* immature to dimethoate and malathion, the newly emerged adults showed a reduction in the wing area or absence of wings. Silva (2014) also evaluated the effect of dimethoate on the ontogenetic development of honey bees. In that study, individuals exposed to the insecticide did not form the head and thoracic appendages. This result was a consequence of the absence of development of the imaginal discs (structures that originate the antennae, legs and wings).

The development and differentiation of imaginal discs during metamorphosis is controlled by the levels of juvenile hormone and 20-hydroxyecdysone (20E) (Cruz-Landim, 2009). Therefore, any disorder that can deregulate the balance of these hormones during the period of metamorphosis can cause physiological responses that culminate in the malformation of individuals (Chen et al., 2016). Although chlorpyrifos is a neurotoxic insecticide, a secondary action could trigger hormonal disorders and consequently lead to deformities. Boncristiani et al. (2012) evaluated the action of acaricide coumaphos (organophosphorus) in adult workers of *A. mellifera*. The authors observed that coumaphos was able to downregulate a gene associated with the synthesis of 20E, the hormone that is related to neuromuscular morphogenesis and the body structures of adults (Mello et al., 2014).

For Dinh et al. (2016), the malformations resulting from exposure to chlorpyrifos can be explained by the action of the insecticide

on the muscles. The authors observed that chlorpyrifos negatively affected the flying muscle mass of *C. scitulum*. Silva (2014) also reported changes in the segmental musculature of the group of bees exposed to dimethoate. During metamorphosis, the muscular system undergoes several modifications to adapt it to the adult body anatomy, and at that moment, the formation of the legs, antennae and flight muscles occurs (Cruz-Landim, 2009). Thus, the malformations observed in our study could be the result of the cytotoxic effect of chlorpyrifos on the muscles of the appendages. Therefore, further studies are needed to define the mechanism responsible for the toxicity of chlorpyrifos that leads to morphological changes in the immature.

5. Conclusions

In view of the results presented here, we conclude that the effects of chronic exposure at chlorpyrifos concentrations, such as reduced survival, reduced body size and deformities in newly emerged adults, can contribute to the colony decline over time. And that due to the reduction in the body size of the workers, the foraging activity will be compromised since the flight range will be substantially reduced. Finally, we consider that the biomarkers used in this study can be useful for monitoring bees to elucidate the action of pesticides on these pollinators. Moreover, we suggest that *S. bipunctata* can be used as an experimental model for risk assessment, contributing to the generation of adequate information to support conservation strategies for native species.

Author contributions

Andressa Linhares Dorneles: Conceptualization, Methodology, Validation, Formal analysis, editing, writing and review. Annelise de Souza Rosa-Fontana: writing and review. Charles Fernando dos Santos: Formal analysis, writing and review. Betina Blochtein: Supervision and review.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.116414.

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