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Scientific Note

A systemic fungicide might reduce the male genitalia of a stingless bee species by one-third

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Abstract. Bees are essential pollinating insects that significantly contribute to crop production. However, the use of pesticides in modern agriculture has resulted in bees being exposed to a plethora of harmful substances. Larvae of bees are particularly susceptible to exposure, as they can consume contaminated larval food during development. This study opportunely examined the effects of fungicides and insecticides on the size and shape of the genitalia of *Scaptotrigona bipunctata* (Lepeletier, 1836) males (Hymenoptera: Apidae: Meliponini) during larval development when experimental design was structured to obtain female bees (workers). Therefore, the geometric morphometric analyses were based on male bees that opportunistically emerged. Our findings showed a significant difference in the size of the genitalia of *S. bipunctata* males exposed to a systemic fungicide during larval development, while the genital shape remained similar. Although the exact impact of these morphological changes on the reproductive success of *S. bipunctata* males is uncertain, they suggest an adverse effect of pesticides on bees.

Keywords: bees, geometric morphometrics, larval development, male reproductive system, toxicological risk assessment.

Stingless bees are eusocial insects that play a critical role in angiosperm reproduction by pollinating plants. This essential ecosystem service is crucial for maintaining biodiversity (Klein et al. 2007) and contributes significantly to food production in terms of both quality and quantity (Klatt et al. 2014). Unfortunately, the intensification of modern agriculture has led to a significant increase in pesticide use worldwide (Chakrabarti et al. 2014), which can have toxic effects on non-target organisms, including pollinators (European Commission 2015).

In this context, Brazil has become one of the world's largest consumers of pesticides, with a 152% increase in usage over the last 15 years, while the cultivated area has only increased by 8% during the same period (Santos et al. 2018). Although the mechanism of action of fungicides is specific, studies have reported their impact on pollinators (Sanchez-Bayo & Goka 2014; Simon-Delso et al. 2017; Carneiro et al. 2020). In addition, there is evidence of increased toxicity when fungicides and insecticides are used in combination (*e.g.*, Papaefthimiou & Theophilidis 2001; Malaspina et al. 2008).

To investigate the toxicity risk of a fungicide (Carbendazim) associated with an insecticide (Chlorpyrifos) to larvae of *Scaptotrigona bipunctata* (Lepeletier, 1836) (Hymenoptera: Apidae: Meliponini) reared *in vitro*, we conducted experiments in a non-*Apis* bee species expecting female emergence. Stingless bee larvae have not shown male emergence in artificially reared experiments (Baptistella et al. 2012; Menezes et al. 2013; Santos et al. 2015). This may be due to the fact that stingless bee males are typically produced in small clusters in combs for short periods, and often in quantities below 10% (Velthuis et al. 2005), making their observation rather rare.

However, in our study, we unexpectedly observed a significant number of males in both the control and pesticide exposure treatments during larval development and emergence of bees. Thus, considering that: (i) stimulating enlarged male production within queen-right colonies is not possible to date, and (ii) the male production per comb is typically low, we used the emerged individuals to make the first-ever evaluation of males chronically exposed to a combination of pesticides throughout their larval development.

We conducted a triplicate bioassay using 20 larvae *per* experiment (3×20 = 60 larvae), totaling 240 larvae at the end of experiment from March to May 2019. One-to-three-day-old bee larvae of *S. bipunctata* were reared *in vitro* by transferring them to rearing plates and placing them in airtight plastic containers (7×11×17 cm) containing saline solution to control internal relative humidity. These plates were maintained in an incubator at a constant temperature of 25°C (model Luca-161/04, LUCADEMA®, São Paulo, Brazil), in complete darkness (0L:24D). The larvae were chronically exposed to commercial formulations of the fungicide Carbendazim Nortox (Methyl benzimidazol-2-ylcarbamate[CARBENDAZIM]), with 50% active ingredient in combination with the insecticide Chlorpyrifos (0,0-diethyl-0-3,5,6-trichloro-2-pyridyl phosphorothioate) - Lorsban® 480BR, with 48% active ingredient, through a contaminated diet (Barbosa et al. 2015). These substances were diluted into 35 µL of the larval food provided to each larva all at once.

To induce sublethal effects on bees, we administered residual doses of Carbendazim (0.00875 µg a.i./bee; Ramos et al. *in prep.*) in association with Chlorpyrifos (0.0088 µg a.i./bee; Santos et al. 2016). Subsequently, emerged males (N = 32 or 13.3% of *in vitro* rearing) were pick up and preserved in Eppendorf tubes filled with a 70% alcohol solution. Their genitalia were then dissected using tweezers and captured using a digital camera attached to a stereomicroscope (Leica DMC 2900, Singapore).

Since geometric morphometrics is a reliable tool for evaluating subtle variations in the shape and size of bee structures (Lima et al. 2016; Galaschi-Teixeira et al. 2018; Maia et al. 2022), here, we selected 16 landmarks (LMs) for geometric morphometrics (Fig. 1), and manually digitized the images twice using tpsdig2 (Rohlf 2005). To ensure consistency and precision in the positioning of the genital capsule, we utilized a modeling clay mold as a substrate to better accommodate it. This supportive medium was handled to be stiffer, and prior to image capture, we calibrated it using a genital capsule from an adult male from one of our colonies. This provided a firm and precise

support, minimizing any potential movement during image capture, thereby enhancing stability and, consequently, increasing accuracy in the positioning of each sample. Subsequent analyses were conducted using the R programming language (Ihaka & Gentleman 1996; R Core Team 2018). The TPS file containing the coordinates of the LMs of the male genitals, generated in tpsdig2, was used to perform a generalized Procrustes analysis (GPA) using the 'gpagen' function. We then used this GPA within the 'bilat.symmetry' function (see Supplementary Data 1, Tab. S1) to extract the symmetric component of the genital shape with 'symm.shape'. To assess the presence of any possible outliers, a graphic was plotted, considering the limited number of bees available for analysis. However, no outliers were identified (see Supplementary Data 1, Fig. S1).

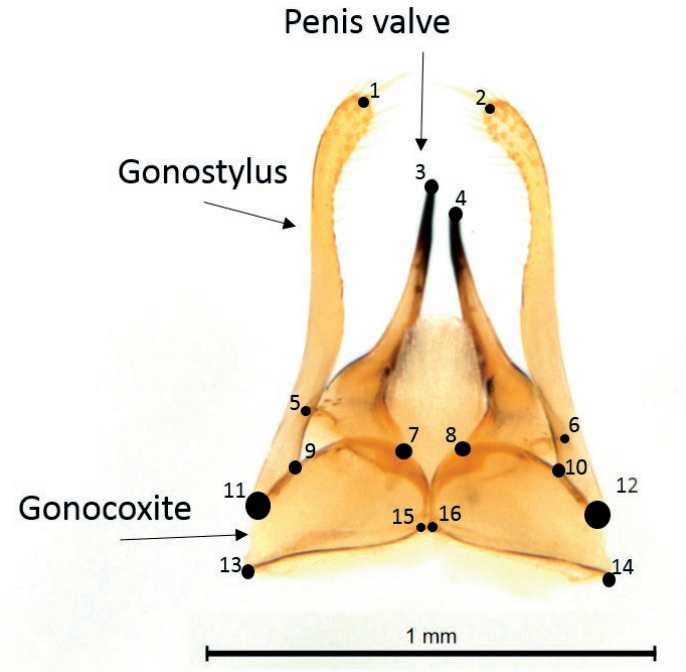

Figure 1. Landmarks (n = 16) were used to analyze the variation in shape and size of the male genitalia of the stingless bee *Scaptotrigona bipunctata*.

After that, we tested the covariation of the shape and size (allometry) of the male genitals using the function 'procD.lm'. Moreover, we investigated whether the male groups (control vs. exposed) had different genital sizes (centroid log) and shapes (Procrustes multivariate ANOVA) using the function 'procD.lm'. All of these analyses were conducted using the geomorph package (Adams et al. 2017).

Finally, we performed a canonical variation analysis of the shape, adjusted with cross-validation using the Jackknife (leave-one-out) method to assess data accuracy and estimate the error rate between the male groups, using the CVA function in the Morpho package (Schlager 2017). We used the 'plotRefToTarget' function (Adams et al. 2017) to plot the symmetrized mean shape of the male genitals against the mean shape of the control and exposed males. All the analyses conducted here were permuted 1,999 times to obtain reliable statistical results.

Our data indicated no allometry in the genitals of *S. bipunctata* males regarding logsize (F = 0.48, P = 0.76; groups, F = 0.44, P = 0.95, Supplementary Data 1, Tab. S2). We observed that the systemic fungicide affected the size of the genitals of *S. bipunctata* males, but neither the insecticide nor the combined action between them (synergic effect) showed any effect on such a structure, as well as the control group (Procrustes regression, $F_{(3,28)} = 3.69$, P = 0.03), as shown in Fig. 2 and Supplementary Data 1, Tab. S3). There was a large variation in the size of the genitalia, except for Carbendazim exposure, which had lower variation but consistently smaller sizes.

On the other hand, we did not detect any significant difference in the genital shapes between the control and exposed males (Procrustes MANOVA, $F_{(3,28)} = 1.06$, P > 0.05), as shown in Supplementary Data 1 (Fig. S2 and Tabs. S4-S5). Our cross-validation analysis demonstrated low global accuracy for the discrimination between males (18.75%), with control males being poorly assigned to their correct group (9%). Therefore, in contrast to size, the shape of the male genitalia may not be affected by external agents, although further research is needed to address this issue. Based on our morphological analysis of the genitals of male *S. bipunctata*, both exposed and non-exposed to the associated action of the fungicide and insecticide, we can conclude that the size of the reproductive structure was affected, but by a single toxic substance rather than a combined effect with other pesticides.

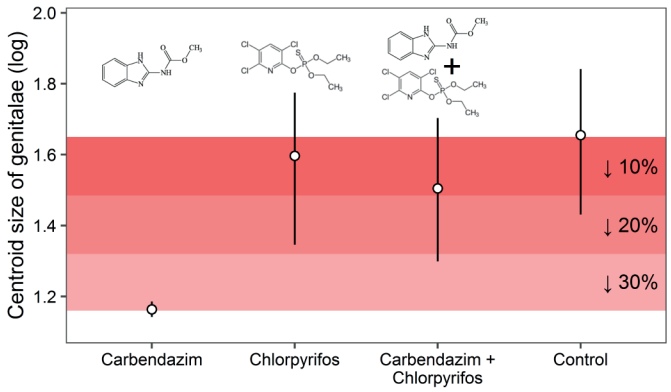

Figure 2. Comparison of the centroid size (log) of the genitalia of male stingless bees *Scaptotrigona bipunctata*. Notes: The centroid indicates the mean (center) of a shape. Points show the average size, and vertical lines exhibit the 95% confidence intervals: Carbendazim = 1.16 (CI 0.03), Chlorpyrifos = 1.59 (CI 0.37), Carbendazim + Chlorpyrifos (synergic effect) = 1.50 (CI 0.24), Control = 1.65 (CI 0.25). The red gradient exhibits a reduction of genitalia size by 10%, with the average of the control as the baseline. Average values whose confidence intervals do not overlap can be inferred as statistically different from each other (Sim & Reid 1999).

Male production is a costly investment for stingless bees because they do not participate in regular colony activities like their sisters, the workers (Velthuis et al. 2005). Furthermore, the few males produced by the queen mother are breeding individuals that essentially live to mate with virgin queens (Velthuis et al. 2005). Therefore, given its crucial role in mating, it can be inferred that male genitals are under strong selective pressure to maintain both their size and shape patterns, as they act as mating plugs in stingless bees, preventing new copulation by newly-mated queens (Kerr et al. 1962).

We are currently working to determine how to experimentally produce a large number of males of *S. bipunctata* and other stingless bee species in laboratory conditions to overcome the limitations imposed by their low natural production. If we succeed in developing such a procedure, it will enable us to conduct systematic experiments to test not only the effects of exposing males to associated agrochemicals but also the implications of modified genitalia shapes on the reproductive success of *S. bipunctata* males.

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Authors' Contributions

JDR: Conceptualization, Methodology, Investigation, Writing - original draft. LC: Data preparation, Data analysis. CFS: Data analysis, Writing. BB: Review, Supervision, Project administration.

Conflict of Interest Statement

The authors declare that they have no conflict of interests.

Supplementary Material

Supplementary data 1.

Supplementary data to this article can be found online at https://doi.org/10.37486/2675-1305.ec05043.

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