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*Research Article*

# The Worker Flow at the Hive Entrance Predicts When Nest Cleaning is Intensified in Stingless Bees

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Waste produced by living organisms is commonly disposed of as a prophylactic measure to avoid the spread of diseases and parasite infestation. For social insects, a proportion of workers is allocated to dispose the waste material outside the colony's nest. However, most nests of social insects have a single entrance, where a high flow of individuals may create congestion, potentially compromising normal foraging activities and colony growth/health. Here, we investigated how two species of stingless bees (*Tetragonisca fiebrigi* and *Plebeia droryana*) deal with waste disposal and regular foraging activities, and the impacts of these activities on traffic flow at nest entrances. First, we compared the average traffic of bees assigned to waste removal activities. Following this, we investigated probabilities for waste removal as bee traffic increases. Then, we estimated a cutoff value to predict the likelihood that waste removal activities will be intensified over foraging trips. We found that, on average, the number of bees performing waste removal activities was lower than those undertaking foraging trips for both species. In addition, we observed that as overall bee traffic increases, the number of workers engaged in waste removal reduces or even ceases. Our models indicate that bee traffic of approximately 15 individuals/time is a cutoff score, below which, colonies invest in waste removal and above which, foraging trips are increased. It suggests that both species use the entrances of their colonies in an optimized way by adjusting which tasks should be intensified as the traffic of individuals increases.

## 1. Introduction

Animals employ diverse waste disposal methods, including actively performed ones such as dung beetles rolling and burying waste [1] and birds of prey regurgitating indigestible parts [2], as well as passively achieved ones like termites storing waste within intricate nests [3]. It would be expected that for organisms living either solitarily or in small groups; such materials are readily and safely eliminated due to the small overall production. However, individuals that are members of large societies may face harmful effects at a colony level as organic waste accumulates in large quantities, awaiting effective elimination [4–7]. Social insects, such as ants, bees, termites, and wasps, have developed multiple strategies for overcoming these hazards inside colonies [4–7]. Such strategies integrate collective defense mechanisms known as social immunity which may include behavioral, physiological, and organizational components [4, 5, 7].

One of the issues that must be dealt with is the removal of waste from nests [4–7]. Such activity plays a prophylactic role within colonies and may have evolved to reduce or prevent risks associated with disease spread or parasite infestation [4, 7–10]. Waste removal is particularly important for social insects inhabiting populous colonies, as these societies generate and store substantial quantities of organic material within their nests, encompassing both food and materials for nest construction [8–12]. In this context, colony refers to a group of social insects living together, often with cooperative efforts, but without a highly organized social structure, while society describes a more structured organization among social insects, with distinct roles, complex communication, and a hierarchical system. Bee societies, for instance, involve workers and a queen with specific functions [13].

Stingless bees (Hymenoptera: Apidae: Meliponini) are a highly diverse group of social bees that thrive in tropical and subtropical regions [14]. This monophyletic taxon comprises over 500 species [15]. Their colonies may comprise hundreds or even thousands of individuals [16]. Since they live in dense societies, most stingless bee species produce large amounts of waste that is stored and disposed of by specialized workers at particular places (waste-dump areas) within colonies [12, 17–19]. Waste materials include faeces, old brood cells, cocoons, dead adults, and dead brood [11, 12, 19]. Small waste pellets are prepared by workers who then often transfer and carry them out of the nest using their mandibles [12, 17–20].

Given that waste removal entails both entering and exiting colonies, the effects on bee traffic must be taken into account, as these activities could potentially restrict the allocation of resources to other crucial tasks, such as foraging for floral resources (nectar and pollen) and plant resin [20–22] or colony defense [23–26]. The traffic of bees flowing through the entrance of colonies may provide relevant information about their growth or health. For instance, it is frequently employed as a proxy for both colony size (interspecies) [21, 27] and colony health/strength (intraspecies) [28, 29]. However, an elevated influx of bees involved in waste removal might indicate vulnerability, which contrasts with colony growth or well-being.

Nest sanitation is a daily activity in colonies of most stingless bee species [12, 17–20, 24]. However, it may conflict with foraging activities by competing for space (nest entrance) and the proportion of bees allocated for different tasks. For example, colonies of stingless bees frequently have a single entrance that serves multiple purposes (e.g., waste removal, forage, defense, and passage of reproductive castes, such as queens and males). Therefore, an optimization system for its use is expected. Guard bees often curtail forager access in numerous instances. There are cases, such as with *Partamona* spp., where the entrances are generously sized, accommodating multiple guard bees simultaneously. Moreover, within these societies, members are allocated asymmetrically to numerous tasks within and outside colonies. This dynamic allocation of individuals (workers) becomes imperative to ensure not only the optimal execution of these tasks but also, on a broader spectrum, to uphold colony health and facilitate growth [30, 31].

The study of waste removal in stingless bee colonies may provide valuable insights into how large insect societies allocate individuals among beneficial but potentially conflicting activities (nest sanitation vs. foraging trips) [12, 17–20, 24]. Here, we hypothesize that as bee traffic increases, workers may modify their external activities by engaging most colony members in foraging trips. We predict that as waste removal has a binomial nature (performed; not performed) and since it relates to the number of bees flowing through nest entrance, there should be a cutoff score, below which, nest sanitation activities overtake foraging trips as a more demanding task.

## 2. Materials and Methods

*2.1. Study Area and Species.* The study was carried out at the Pontifical Catholic University of Rio Grande do Sul's Science and Technological Park (TECNOPUC), in the municipality of Viamão (−30.097462, −51.1551126), Rio Grande do Sul, southern Brazil. At this site, there were 34 hives of *Tetragonisca fiebrigi* (Schwarz, 1938) and 13 hives of *Plebeia droryana* (Friese, 1900) on which we performed our bee traffic experiments (Figure 1). *Tetragonisca fiebrigi* is found in Argentina, Bolivia, Brazil, and Paraguay [32]. Its nest entrance, elongated and crafted from wax, is overseen by several vigilant bee guards who either monitor or hover nearby. On the other hand, *P. droryana* also inhabits the same countries as *T. fiebrigi* [32]. Nevertheless, it possesses a smaller nest entrance constructed from wax blended with propolis but, unlike *T. fiebrigi*, the bee guards of *P. droryana* do not hover over their entrance.

*2.2. Experimental Design.* To assess the correlation between worker bee waste removal and increasing bee traffic within colonies, we monitored the ingress and egress of all bees from the nests. During these observations, we also noted whether departing individuals carried waste in their mandibles (Figure 1). Our observations were conducted on sunny days with temperatures exceeding 21°C, relative humidity surpassing 50%, spanning from 10:00 am to 04:00 pm, and were conducted by two researchers (ABF and JTZ) over four consecutive days in October 2021. For the purpose of recording both bee traffic and waste disposal, we placed a camera on a tripod positioned 30 cm away from the nest entrance, roughly at the same height. Each beehive's activities were recorded for three-minute intervals. Through careful analysis of the recorded video footage, we accurately gauged bee traffic and waste removal by documenting every entry and exit event of bees from the colonies.

It has been proposed that waste removal flights in stingless bees tend to last much less time than foraging flights. For example, while waste removal usually lasts few seconds [10], foraging trips may last several minutes [33–35]. Therefore, to avoid double counting of returning bees involved in waste removal or foraging, the number of the former was subtracted from the latter to provide an accurate value of the bees performing foraging trips. Thus, for example, if we counted 10 bees arriving at or leaving a colony and three of them were assigned (seen) removing waste, the inferred number of foraging trips was seven bees.

Figure 1: Two stingless bee species (Hymenoptera: Apidae: Meliponini) studied in this work. Left panel: *Tetragonisca fiebrigi* workers with two hovering guards, two standing guards (outside and inside nest's entrance tube). The remaining worker (the subject of this study) is shown removing a waste pellet between their mandibles. Right panel: *Plebeia droryana* workers. Similar situation as the left panel. However, *P. droryana* does not possess hovering guards, while those standing guards (one observable) are located only in the border of the nest's entrance. Behaviors illustrated by Júlia Zuch.

### *2.3. Statistical Analysis*

*2.3.1. Waste Removal Comparison.* To analyze bee traffic, we fitted two generalized linear mixed models (GLMMs) for each stingless bee species. In addition, we conducted a model selection for the best final GLMM. Thus, we ran GLMMs with Poisson distribution family ("glmer" function) and negative binomial ("glmer.nb" function); functions of package lme4 [36]. They were compared according to their corresponding Akaike information criteria and model's weight. This was done by using the function "AICctab" of package bbmle [37].

The best GLMMs for both *T. fiebrigi* and *P. droryana* were selected for the next stage of the analysis (see Results section). The GLMMs for both stingless bee species were structured as follows: response variable: bee traffic ascribed as the number of bees arriving/leaving colonies over three minutes; predictor, grouping variable (fixed effect): waste removal behavior (yes = 1, no = 0); random crossed effects: beehive identification, observation day, and observation time.

*2.3.2. Waste Removal Probability.* Since the nature of waste removal variable was binary, we performed a logistic regression. Such an analysis may assist in the prediction of likelihood of bees performing, or not, waste removal (response variable) related to the number of individuals arriving at/leaving colonies, i.e., bee traffic (predictor variable). Therefore, we fitted two mixed effects logistic regressions (binomial family), for each stingless bee species, using the same random effects structure as described above. These analyses were performed with "glmer" function of package lme4 [36].

*2.3.3. Accuracy for Logistic Regression and Cutoff Scores.* The accuracy of mixed effects logistic regressions was evaluated with receiver operating characteristic (ROC) curves that consider two possible types of error (false positives, and false negatives). In the first case, it evaluates the proportion of events (code 1) that are correctly predicted by the model as actual events (known as sensitivity). In the second case, it considers the proportion of the nonevents (code 0) which the model correctly predicts as nonevents (known as specificity). Both of these parameters are computed based on a designated prediction probability threshold. The interpretation of the ROC analysis was facilitated by examining the area under the curve (AUC). Elevated AUC values are indicative of a more desirable measure, suggesting the model's capability to effectively differentiate between the positive and negative classes.

In addition, we calculated the misclassification error rate for each model. Finally, we estimated a cutoff score for each species that would optimally identify both false positive rate and false negative rate simultaneously. Such an estimate provides a threshold for prediction probability suggesting a value (here, number of bees) in which waste removal is preferably occurring (above cut off score) or foraging is being intensified (below cut off score). The ROC, AUC, and cutoff scores were generated with functions provided by the package *InformationValue* [38]. All analyses were performed in R programming language [39, 40].

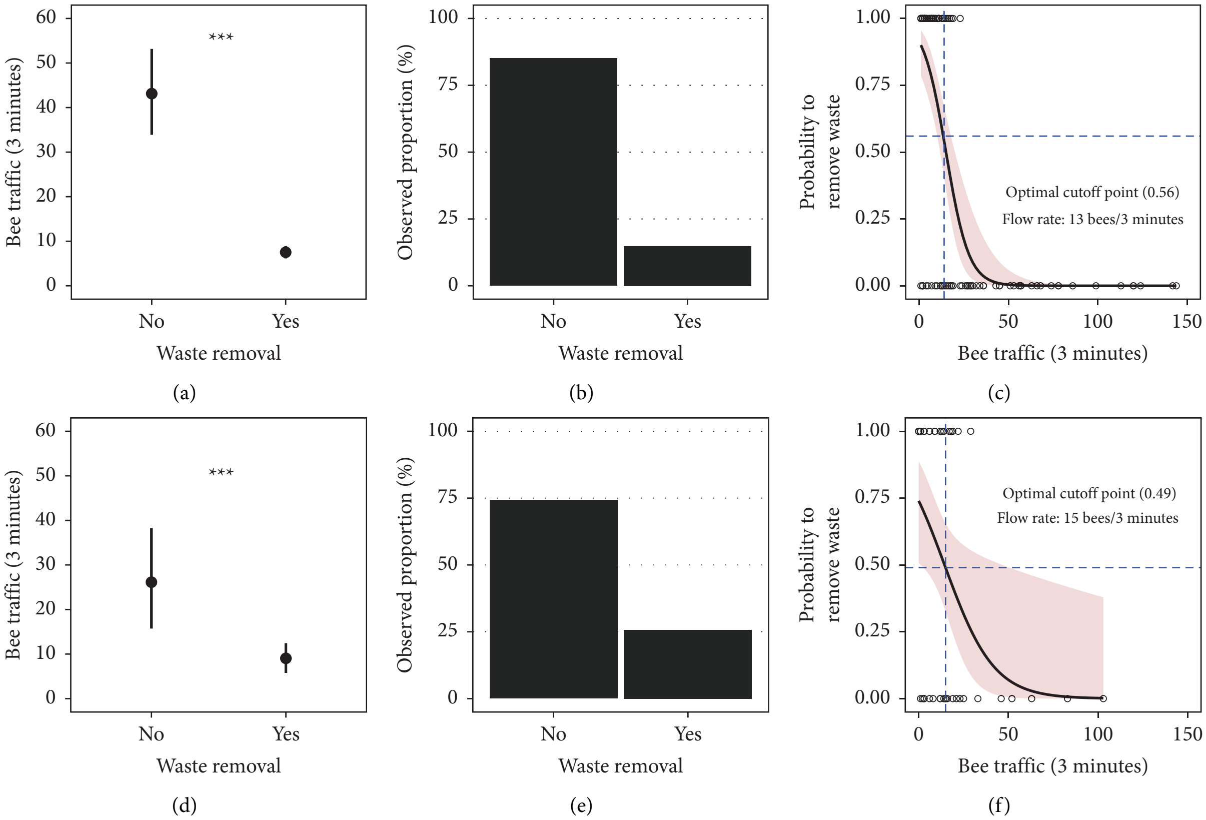
## 3. Results

Overall, we recorded 3,148 bee workers of *T. fiebrigi* at the colony entrance (i.e., leaving or returning). For this species, we found a significant difference in groups of bees performing, or not performing, waste removal (Table 1, Figure 2(a)). Thus, the average bee traffic equated to 43.1 foragers (CI 95%: 32.8–53.4) and 7.5 waste removers (CI 95%: 6.1–9.0) per observation period (three minutes). The overall proportion of workers at colony entrances assigned to each task was 85.1% for foraging and 14.9% for removing waste (Figure 2(b)).

Table 1: Outputs of the generalized linear mixed model (GLMM) showing the difference between two groups of bee workers of *Tetragonisca fiebrigi* (Hymenoptera: Apidae: Meliponini) removing, or not removing, waste from colonies.

| Fixed effects | Estimate | S.E. | *z* value | *p* value |
|---|---|---|---|---|
| Intercept | 3.59 | 0.34 | 10.45 | <0.001 |
| Waste removal (yes) | −1.78 | 0.07 | −25.20 | <0.001 |
| Random effect | Variance | Std. Dev. | *N* | |
| Beehive identification | 0.30 | 0.54 | 34 | |
| Observation day | 0.40 | 0.63 | 4 | |
| Observation time | 0.36 | 0.60 | 54 | |
| Model selection | AICc[1] | dAICc[2] | df[3] | Weight[4] |
| GLMM negative binomial (selected model) | 965.6 | 0.0 | 5 | 0.51 |
| GLMM Poisson | 965.7 | 0.1 | 6 | 0.49 |

S.E: standard Error; *z* value: standard score, i.e., standard deviations from their means. Negative values when the raw score is below the mean, positive when above; *p* value: probability to find *z* scores by chance. [1]Computation of AIC; [2]differences among AICs; [3]degree of freedom; [4]weight of AICs.



(a) (b) (c)

(d) (e) (f)

Figure 2: Comparisons and probabilities for bee workers of *Tetragonisca fiebrigi* (a–c) and *Plebeia droryana* (d–f) removing waste from colonies. (a) Average bee traffic per three minutes. (b) Proportion of bees performing one or the other behavior. (c) Relationship between the number of bee workers leaving colonies for waste removal flights (y-axis: 1.00) or foraging flights (y-axis: 0.00). Notes: points in (a) and (d) are mean values; the vertical lines show the confidence intervals at 95%, and asterisks indicate significant differences ($p < 0.001$); dashed, horizontal lines in (b) and (e) were provided just to aid visualization; in (c) and (f), the solid lines show the predicted model, while red shadows inform the confidence intervals at 95%. Open points are the observed data, whereas the dashed, blue lines exhibit the cut-off scores (horizontal) and expected bee traffic (vertical) as indicated by the sentences inserted in panels.

In addition, our findings demonstrate that the larger the bee traffic, the lower the likelihood of workers being assigned to remove waste from inside colonies (Table 2, Figure 2(c)). On such a negative relationship, we found that the cutoff value was 0.56, which represents a bee traffic value of 13 bees per observation period ($y$-axis and $x$-axis, respectively, in Figure 2(c)). Therefore, if the traffic of bees leaving or entering the nest for any purpose (waste removal and foraging) within three minutes is less than 13 individuals, then it most likely means that nest cleanliness will be intensified. However, when the value is greater than 13 individuals, there is a lower chance for waste removal and, thus, we expect foraging trips to be augmented (Figure 2(c)).

Table 2: Outputs of mixed effect logistic regression displaying the probabilities for bee workers of *Tetragonisca fiebrigi* (Hymenoptera: Apidae: Meliponini) to remove waste as a function of the number of individuals departing colonies.

| Fixed effects | Estimate | S.E. | *z* value | *p* value |
|---|---|---|---|---|
| Intercept | 3.35 | 1.10 | 3.04 | 0.002 |
| Number of bees | −0.19 | 0.05 | −3.78 | 0.001 |
| Random effect | Variance | Std. Dev. | *N* | |
| Beehive identification | 0.18 | 0.43 | 34 | |
| Observation day | 1.43 | 1.19 | 4 | |
| Observation time | 0.00 | 0.00 | 54 | |

S.E: standard error; *z* value: standard score, i.e., standard deviations from their means. Negative values when raw score is below the mean, positive when above; *p* value: probability to find *z* scores by chance.

For *P. droryana*, we recorded 973 bees leaving or entering colonies. Once again, we found significant difference in bees removing waste from their colonies (Table 3 and Figure 2(d)). As a result, the average bee traffic for *P. droyana* was 26.1 foragers (CI 95%: 14.0–38.2) and 9.1 waste removers (CI 95%: 5.3–12.8) per observation period (Figure 2(d)). The overall proportion of bees involved in foraging was slightly lower than that observed for *T. fiebrigi*, with 74.3% of bee workers engaged in foraging and 25.7% of bees involved in waste removal (Figure 2(e)). Finally, we observed a similar pattern as before in the probability for *P. droryana* workers to remove waste from colonies. In other words, we found a negative relationship indicating that as the bee traffic intensifies, the chance that workers remove waste from their colonies reduces (Table 4, Figure 2(f)). The cut-off score was estimated as 0.49 (Figure 2(f)). This implies that waste removal will occur if bee traffic is lower than 15 individuals per three minutes, while foraging trips will be intensified at bee traffic rates above this value (Figure 2(f)).

The goodness of fit of the mixed effect logistic regression model for *T. fibrigi* achieved a high AUC score (0.93; specificity = 0.80; sensitivity = 0.90) and a low misclassification error (0.13) (Figure 3(a)). The same deduction applies for *P. droryana*, moreover, less accurately since the AUC scored 0.72 (specificity = 0.59; sensitivity = 0.77), and the misclassification error rate had also a moderate reliability (0.31) (Figure 3(b)).

### 3.1. Additional Features of Waste Removal Videotaping.

We observed workers of *P. droryana* flying out their colonies with a white substance in their corbiculae. The origin of that substance was unknown. However, it was like resin or pure wax.

## 4. Discussion

Our findings demonstrate a sophisticated system where colonies seem to self-regulate the number of individuals allocated to waste removal activities based on the intensity of bee traffic in nest entrances. Our data reveal that as bee traffic remains low (13–15 individuals per three minute observation period), we should expect waste removal to be intensified over foraging trips. Therefore, we have evidence that under low bee traffic, the chance for waste removal occurring is greater and apparently preferred. However, as bee traffic increases, the colonies invert their task allocations, and nest sanitation is discontinued in favor of foraging trips. In addition, on excessive flow of bees in the colony entrances (dozens of individuals), nest hygiene activities are no longer observed at all, as bees focus exclusively on resource collection.

Along their evolutionary histories, social insects have had to deal with problems associated with living in groups; in particular, how to deal with waste accumulation and disposal [4–7]. The solutions developed by most of them may involve defecating outside the nests—honey bees *Apis mellifera* [41] or disposing their refuses outside the colonies—leafcutter ants *Messor barbarous* and *Atta colombica* [9, 42] and several stingless bee species [12, 17–19, 24]. Nevertheless, social insects like stingless bees inhabit nests that commonly have just a single entrance to the colony [21, 27, 43]. As a result, these structures appear to be built in a way that facilitates incoming and outgoing traffic of foraging bees [21, 27]. Furthermore, the shape and size of nest entrances in stingless bees appear to adapt appropriately to the demands of homing nestmates, serving as a structure that facilitates a substantial flow of bees while enabling "crash-landing" behavior, possibly as a defense strategy against predators, as observed in *Partamona helleri* [44].

It has been observed that workers of most stingless bee species may land or take off on different sides (center vs. edge) of entrances or they may adopt distinctive velocities (acceleration and deceleration) when arriving from the field [21, 27, 44, 45]. It has been suggested also that such strategies evolved to optimize flow of bees in/out colonies by reducing or avoiding collisions and traffic jams [21, 27, 44, 45].

In our study, we provide information that colonies of stingless bees (e.g., *T. fiebrigi* and *P. droryana*) may amend the usage of their entrances by adjusting which tasks will be favored based on the intensity of bee traffic occurring therein. Even though the models for both species were slightly different, overall, they showed a similar pattern. Our results indicate that species are able to perceive small changes in the number of bees leaving colonies and respond by allocating workers to the most urgent task (nest sanitation or foraging).

Even though the task (nest sanitation vs. foraging) observed at the entrance of beehives shifts according to the traffic of bees, we are unable to say definitively which activities triggered this duty to be stopped. It is also unclear whether individuals can shift between foraging and waste removal in the same day or in different moments. Some studies argue that as traffic of bees (forager workers) increases, the number of guards also increases [21, 27]. Conversely, other authors also have noted that there is an age gradient in workers performing waste disposal and foraging flights [12, 19, 20, 24]. Therefore, there might be any possibility that they compose the defense system or foraging initiatives for their colonies [24, 46]. Given that forager bees and waste removal bees are exposed to very different substances (nectar and pollen vs. waste), it is likely that waste removal bees are exposed to a greater number of pathogens. Further studies could evaluate whether waste removal

Table 3: Outputs of the generalized linear mixed model (GLMM) showing the difference between two groups of bee workers of *Plebeia droryana* (Hymenoptera: Apidae: Meliponini) removing, or not removing, waste from colonies.

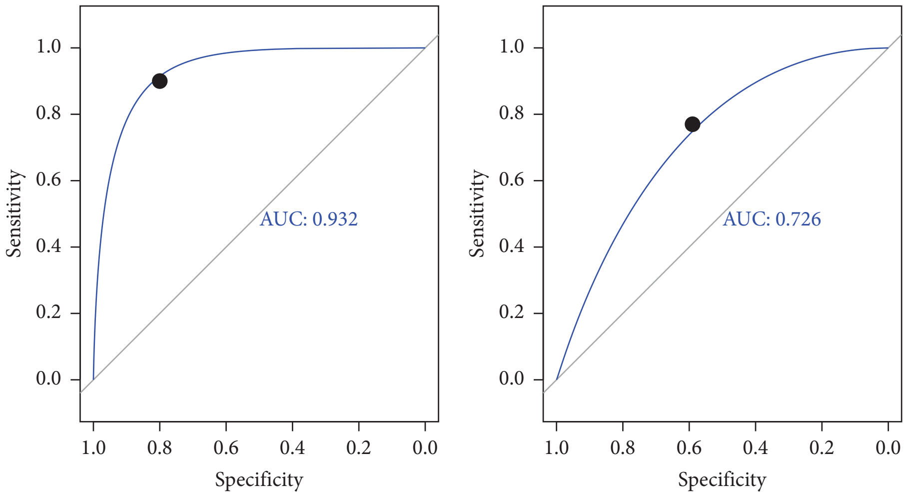
| Fixed effects | Estimate | S.E. | $z$ value | $p$ value |
|---|---|---|---|---|
| Intercept | 2.93 | 0.39 | 7.49 | <0.001 |
| Waste removal (yes) | −0.93 | 0.31 | −2.93 | 0.003 |
| **Random effect** | **Variance** | **Std. Dev.** | $N$ | |
| Beehive identification | 0.00 | 0.00 | 14 | |
| Observation day | $2.479e-01$ | $4.979e-01$ | 3 | |
| Observation time | $4.871e-15$ | $6.979e-08$ | 22 | |
| **Model selection** | **AICc[1]** | **dAICc[2]** | **df[3]** | **Weight[4]** |
| GLMM negative binomial (selected model) | 344.4 | 0.0 | 6 | 1.00 |
| GLMM Poisson | 471.2 | 126.8 | 5 | <0.001 |

S.E: standard error; $z$ value: standard score, i.e., standard deviations from their means. Negative values when the raw score is below the mean, positive when above; $p$ value: probability to find $z$ scores by chance. [1]Computation of AIC; [2]differences among AICs; [3]degree of freedom; [4]weight of AICs.

Table 4: Outputs of mixed effect logistic regression displaying the probabilities for bee workers of *Plebeia droryana* (Hymenoptera: Apidae: Meliponini) to remove waste as a function of the number of individuals departing colonies.

| Fixed effects | Estimate | S.E. | $z$ value | $p$ value |
|---|---|---|---|---|
| Intercept | 1.04 | 0.51 | 2.01 | 0.04 |
| Number of bees | −0.07 | 0.03 | −2.19 | 0.02 |
| **Random effect** | **Variance** | **Std. Dev.** | $N$ | |
| Beehive identification | 0.00 | 0.00 | 14 | |
| Observation day | 0.00 | 0.00 | 3 | |
| Observation time | 0.00 | 0.00 | 22 | |

S.E: standard error; $z$ value: standard score, i.e., standard deviations from their means. Negative values when the raw score is below the mean, positive when above; $p$ value: probability to find $z$ scores by chance.



(a) (b)

Figure 3: Some parameters evaluating the goodness of fit of mixed effect logistic regressions for *Tetragonisca fiebrigi* (a) and *Plebeia droryana* (b). Here, the diagnostics of predicted probability scores are exhibited as ROC curves (blue lines) showing the AUC values and cutoff points (black points).

workers, in these and in other stingless bees, preferably become guards rather than foragers to avoid contamination of food supplies.

## 5. Conclusion

Sanitation is a prophylactic behavior that most animals adopt to reduce or avoid chances of disease and parasitism. Here, we found that two social bee species readily maximize this activity during periods when other colony demands such as foraging are not urgently required. Therefore, a potential conflict could emerge as nest sanitation and foraging trips have dichotomous solutions (i.e., a zero sum game). Furthermore, as waste removal is associated with low bee traffic rates, then observation of this activity at high bee traffic rates could be a useful early indicator of colony contamination (disease, intoxication, and poisoning) or parasitism (e.g., phorid flies).

In short, several organisms with different lifestyles (social, gregarious, or solitary) face the same problems of nest traffic flow and decisions relating to the intensification of different activities [47]. Our research demonstrates that these stingless bee species have developed a sophisticated mechanism for controlling traffic in which their members cooperate to intensify some activities under given conditions. In most cities, sanitary activities such as garbage collection are performed during periods when vehicle traffic is reduced. This is likely to increase the efficiency of waste removal, enabling garbage trucks to move faster and more smoothly along road. In this study, a similar behavior was observed in stingless bees, whereby waste disposal activities were performed during periods when colony traffic is low.

## Data Availability

The datasets generated and analyzed during the current study are deposited in GitHub repository in Zenodo (https://doi.org/10.5281/zenodo.11204240).

## Ethical Approval

This work complied with the laws governing animal research in Brazil.

## Disclosure

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

## Authors' Contributions

KD and CFS planned the biological questions and the sampling design. ABF and JTZ collected the biological data and organized the dataset for statistical analysis. BPM validated the dataset and revised the statistical analyses. KD and CFS executed the data analysis. BB contributed to conceptual developing of this work and they wrote the original draft. All the authors discussed the main topics and reviewed the last version of the manuscript.

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