DECODING COLONY SIZE OF AMAZONIAN STINGLESS BEES THROUGH INTRINSIC PARAMETERS

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Abstract

1. Stingless bees live in populous colonies that vary in size from a few hundred individuals to tens of thousands, although information on actual colony size is limited. Determining colony sizes using easily measurable biological parameters are important steps to understanding their life histories and ease their utilization and keeping. The objectives of this study were to determine the colony size of five Amazonian stingless bee species (*Melipona flavolineat*a, *Melipona fasciculata*, *Scaptotrigona* aff. *postica*, *Frieseomelitta longipes* and *Plebeia minima*), and to identify biological parameters that covary with colony size. 2. The number of brood cells, adult bees, and food stocks were counted under laboratory conditions, alongside field assessments of egg-laying rate and external activity of adult workers. To identify covariates of colony size, the number of adult bees was regressed against the number of brood cells, egg-laying rate, external activity of adult workers, and food stocks, and the best Candidate models were ranked using the Akaike Information Criterion. 3. Mean (± s.d.) adult population sizes were: 1,046 ± 185 in *M. flavolineata*; 593 ± 300 in *M. fasciculata*; 7,404 ± 1,391 in *S.* aff. *postica*; 2,425 ± 1,000 in *F. longipes*; and 405 ± 254 in *P. minima*. We showed that the external activity is the biological parameter, after the number of brood cells, that presents the best relationship with the number of adult bees, which can be easily evaluated in the field.

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Data availability statement

The data that support the findings of this study are openly available in Open Science Foundation - OSF at https://osf.io/, reference number

https://osf.io/s32gm/**.**

Conflict of interest

All authors declare no conflict of interest.

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NUMBERED ABSTRACT

1. Stingless bees live in populous colonies that vary in size from a few hundred individuals to tens of thousands, although information on actual colony size is limited. Determining colony sizes using easily measurable biological parameters are important steps to understanding their life histories and ease their utilization and keeping. The objectives of this study were to determine the colony size of five Amazonian stingless bee species (*Melipona flavolineata* , *Melipona fasciculata* ,*Scaptotrigona* aff. *postica* , *Frieseomelitta longipes*and *Plebeia minima*), and to identify biological parameters that covary with colony size.

2. The number of brood cells, adult bees, and food stocks were counted under laboratory conditions, alongside field assessments of egg-laying rate and external activity of adult workers. To identify covariates of colony size, the number of adult bees was regressed against the number of brood cells, egg-laying rate, external activity of adult workers, and food stocks, and the best Candidate models were ranked using the Akaike Information Criterion.

3. Mean (± s.d.) adult population sizes were: 1,046 ± 185 in *M. flavolineata* ; 593 ± 300 in *M. fasciculata* ; 7,404 ± 1,391 in*S.* aff. *postica* ; 2,425 ± 1,000 in *F. longipes* ; and 405 ± 254 in *P. minima* . We showed that the external activity is the biological parameter, after the number of brood cells, that presents the best relationship with the number of adult bees, which can be easily evaluated in the field.

Keywords : Meliponini, Population size, External activity, Egg-laying rate, Food stocks.

INTRODUCTION

Colony size is related to several aspects of life history strategies in social insects, such as colony efficiency, division of labor, social interactions, task partitioning and reproduction (Oster and Wilson 1978; Anderson &

Ratnieks 1999; Bourke 1999; Strohm and Bordon-Hauser 2003; Dornhaus et al. 2006; Hou et al. 2010; Fewell & Harrison 2016). Eusocial hymenopterans (bees, ants, wasps) live in colonies of variable size that are considered as "superorganisms" (Holldobler and Wilson 2009), in which each individual (i.e., unit, "cell") cooperates for the survival and reproductive success of the major unit (i.e., colony, the "organism"). Therefore, colony size is linked to their life history and influences several aspects of collective organization (Gillooly et al. 2010; Dornhaus et al. 2012).

Alongside with honeybees (Apini), stingless bees (Meliponini) are advanced eusocial bees, which live in perennial colonies and possess a great diversity of nesting habits and life-history traits (Roubik 2006; Grüter 2020). They have a pantropical distribution (Michener 2013), and their colonies are generally composed of a single queen, hundreds to thousands of workers, and dozens to hundreds of males (Roubik 2006), with populations ranging from a few hundred to over a hundred thousand individuals (Wille and Michener 1973; Wille 1983; Grüter 2020). However, for most stingless bee species (417 species in the Neotropical region and 244 in Brazil; Pedro 2014), empirical data on colony size is scarce or absent, with the notable exception of *Trigona spinipes*(Fabricius, 1793), for which a recent study (Valadares et al. 2021) provided robust measurements and estimates and for *Melipona rufiventris* and *M. seminigra* (Roubik & Peralta, 1983). Where authors have provided estimates, in most cases, there is often no mention about the methods used (Lindauer and Kerr 1960; Wille and Michener 1973; Michener 1974; Wille 1983; Kerr et al. 2001), and in the handful of studies that present formulae for estimating colony size, there is no attempt to validate estimates using empirical data (i.e. counting number of individuals in studied colonies) (Ihering 1930; Aidar 1996). Moreover, these formulae are often based on single species, leaving uncertainty over their applicability to other species.

Several biological parameters (e.g., the number of immature bees, external activity, life expectancy of individuals, egg laying rate, size of brood combs, colony weight) are related to the number of individuals present in a colony. Therefore, they can be used as proxies to estimate the size of the colonies (DeGrandi-Hoffman et al. 1989; Malham et al. 2013; Duarte et al. 2016; Roldão -Bordoni et al. 2018). However, some of these parameters involve highly invasive sampling methods, in some cases provoking the death of sampled colonies (Delaplane et al. 2013). For this reason, the main aim of this study was to understand how some of these parameters relate to colony size and their viability as proxy measures of colony size.

Stingless bees are essential pollinators of many crops (Giannini et al. 2020). Therefore, knowledge on colony size is important for their use in crop pollination and for better management practices in meliponiculture (Jaffé et al. 2015). Thus, the main objectives of this study were: (i) to measure the colony size (number of adult bees) of five Amazonian stingless bee species and (ii) to determine biological parameters of colonies (number of immature bees, egg laying rate, external activity, food stocks) that covary with colony size.

MATERIAL AND METHODS

Study site and species

This study was performed in the meliponary housed at the Botany Department of Embrapa Amazônia Oriental (1º26'11.52"S, 48deg26'35.50"W), Belem, Para, Brazil, during the dry season of 2016 (September and October), and the rainy seasons of 2017 and 2018 (May). Five species of stingless bees were studied (Fig. S1): *Melipona flavolineata*Friese, 1900, *Melipona fasciculata* Smith, 1854,*Scaptotrigona* aff. *postica* Latreille, 1807,*Frieseomelitta longipes* (Smith, 1854) and *Plebeia minima*(Gribodo, 1893). These species were chosen because they are well adapted to housing in wooden hives, resistant to handling and manipulation, and can be multiplied for use in crop pollination (Contrera et al. 2011; Jaffe et al. 2015; Leao et al. 2016). *Melipona flavolineata*occurs in the Brazilian states of Ceara, Maranhao, Para and Tocantins;*M. fasciculata* occurs in the states of Maranhao, Mato Grosso, Para, Piaui and Tocantins; *S.* aff. *postica* and *F. longipes* occurs in the state of Para; and *P. minima* in the states of Acre, Amapa, Amazonas, Maranhao, Mato Grosso and Para, besides Peru, Bolivia, and Suriname (Pedro 2014).

For the experiment, eight colonies of *M. flavolineata* , eight colonies of *M. fasciculata* , nine colonies of *F. longipes* , 12 colonies of *P. minima* and 13 colonies of *S.* aff.*postica* were used. These colonies were left

undisturbed and without supplemental feeding during the three months prior to the experiment and were kept in individual wooden shelters (Contrera and Venturieri 2008), distanced at least 2 meters from each other. To reduce the possibility of drifting (i.e., foragers returning to wrong colonies; see Oliveira et al. 2021), the entrances of neighboring colonies were arranged in opposing directions.

Colony size

We used the number of adult bees to represent the colony size, like previous studies on other eusocial bees (Dornhaus et al. 2012). To count the number of adult bees in individual colonies, hives were closed at night on the day prior to the experiment using a fine metal gauze (5 x 10 cm) to cover hive entrances, which prevented adult bees from leaving the colony while allowing adequate ventilation. The following day, hives were transferred to a small laboratory and enclosed in a 1.5 x 1 m fine mesh cage to aid collection of adult bees using a manual aspirator. For the more populous species (*F. longipes* and *S. aff. postica*), carbon dioxide (glass cylinder, 95% concentration) was given to the colony for up to two minutes to anesthetize the individuals (Tustain and Faulke 1979) prior to their capture. Males and female workers were not discriminated during counting, while the mated queen and the gynes were counted and separated from other adult bees. At the end of the bee counting, all brood combs, food pots and adult bees were carefully returned to the hive and colonies received supplemental food (sugar syrup and pollen) to aid their recovery post assessment. By following these procedures, no colonies died due to our handling.

Biological parameters

We investigated the relationships between colony size and four biological parameters of stingless bee colonies: (i) external activity (in the field, before lab counts - non-invasive), (ii) egg-laying rate (in the field, before lab counts - moderately invasive –hives were opened to paint the brood cells), (iii) food stores (in the lab – invasive –all pollen/nectar pots were weighed), (iv) brood cells (immature bees; in lab - extremely invasive –all brood cells were removed and counted) (Figure S2 and S3). These parameters were chosen since they reflect the intensity of resource gathering by colonies and their reproductive rates, factors likely linked to population size. Moreover, they are easily measurable, allowing their replication in other species.

Measurement of external activity and egg-laying rates were done *in situ* , seven days before the counting of adult bees in the laboratory, whereas measurement of brood cells and food stocks were obtained during counts of adult bees (see previous section), to avoid excessive handling of the colonies.

To estimate (i) external activity of colonies, we counted the number of workers returning to the colony over 5 min per hour (Hilario et al. 2000). Data were collected during the peak activity period for all species (0900 and 1100; two survey hours per day), for five consecutive days, to obtain the average number of bees returning to the nest. On rainy days, data were not collected. To evaluate (ii) the egg-laying rate, the edge of the newest brood comb (in *Melipona* and*S.* aff. *postica*) was marked with water-based acrylic paints, and after 24 h the new cells were counted. For *F. longipes* and *P. minima* , all constructed cells were painted and after 24 h, all new cells were counted. This procedure was repeated for all species during three consecutive days. From this data we calculated the average number of new cells constructed over a three-day period.

For the evaluation of (iii) amount of food stocks, we weighed all the food pots of the colonies (honey and pollen) using a precision balance (Toledo Prix 3; minimal load: 5g) for all species, except *P. minima* , for which we used a precision balance Master; minimal load: 0.02g). To obtain the (iv) number of brood cells (i.e., immature bees) in species that construct brood combs in horizontal layers (*Melipona* spp. and *S.* aff*, postica*), brood combs were individually measured and photographed for counting of cells. In*F. longipes* and *P. minima* , in which brood cells are built in loose bunches of cells (Roubik 2006), brood cells were marked with water-based acrylic paints and manually counted (Figure S3).

Data analyses

To analyze relationships between colony size and biological parameters of the colonies we used linear mixed models (LMMs) using the R package 'nlme' (Bates et al. 2015). Prior to model construction, data from individual species (adult bee counts and biological parameters) were standardized by using z-scores, to allow

meaningful comparisons among the different bee species, and effect sizes. The response variable was the number of adult bees per colony, with the external activity, egg-laying rate, food stocks and number of brood cells, and included as fixed effects, and species (five levels) held as random grouping factor.

We considered only simple models (single terms, four competing models). Candidate models were ranked using the *dredge* function in the R package "*MuMIn* " (AICc values – Akaike Information Criterion – corrected for small sample sizes) (Barton, 2019). Models within 2 delta AICc of the model with the lowest AICc value were considered statistically equivalent. Parameter estimates and confidence intervals (95%) of models were constructed using restricted maximum likelihood (REML) and model fit was assessed using marginal R^2values (Nakagawa and Schielzeth 2013). In addition to analyses with standardized data (z-scores), simple regression models of the raw data from individual species were used to test relationships between colony size and predictors, using the *lm* function in the R "*car* " package (Fox and Weisberg 2011). Residuals from selected models were visually checked for assumptions of homogeneity of variance and normality using plots.

For obtaining the equations for estimating the colonial sizes according to the measured parameters, a simple regression was used. All analyses were performed in the R software (R Core Team 2018).

RESULTS

Colony size

Adult bee counts in 50 colonies of five Amazonian stingless bees revealed those species with small (<1,000 adult bees: *P. minima* [12 colonies], *M. fasciculata* [8] *and M. flavolineata* [8]), medium (between 1,000 and 5,000 adult bees;*F. longipes* [9]), and large-sized colonies (>5,000 adult bees; *S.* aff. *postica* [13]) (Table 1).

Relationships between colony size and biological parameters

The number of brood cells was the biological parameter that best explained colony size, followed by the external activity and egg-laying rate (Table 2 and Figure 1). As brood counts involved highly invasive sampling (complete removal of brood comb), which would likely lead to eventual death of the colony, we also considered the second candidate model as the most viable proxy for colony size.

The number of brood cells was the biological parameter that best explained colony size, followed by the external activity and egg-laying rate (Table 2 and Figure 1). The linear regression of each biological parameter tested, and the number of adult bees showed a strong relationship with the external activity for two of the species evaluated (*M. flavolineata* : $R^2 = 0.193$; *M. fasciculata* : $R^2 = 0.752$; *S.*aff. *postica* : $R^2 = 0.313$*; F. longipes* : $R^2 = 0.221$; *P. minima* : $R^2 = 0.559$), while there was a strong relationship with the laying rate in four species (*M. flavolineata* : $R^2 = 0.654$*; M. fasciculata* : $R^2 = 0.879$; *S.*aff. *postica* : $R^2 = 0.055$; *F. longipes* : $R^2 = 0.575$; *P. minima* : $R^2 = 0.726$). In only one species there was a strong relationship with the food stocks (*M. flavolineata* : $R^2 = 0.0003$;*M. fasciculata* : $R^2 = 0.872$*; S. aff. postica* : $R^2 = 0.014$; *F. longipes* : $R^2 = 0.109$; *P. minima* : $R^2 = 0.104$), and there was a strong relationship with the number of brood cells in all species evaluated (*M. flavolineata* : $R^2 = 0.596$*; M. fasciculata* : $R^2 = 0.908$*; S.* aff. *postica* : $R^2 = 0.562$; *F. longipes* : $R^2 = 0.784$; *P. minima* : $R^2 = 0.752$) (Figure 2).

The formulae that provided an estimation of population size for all species, by using a simple regression, are: (1) External activity: Y=45.9*X + 448.9; p<0.001; R^2=0.913; (2) Brood cells: Y=0.905*X - 21.6; p<0.001, R^2=0.964; (3) Egg-laying rate: Y=25.9*X + 372.5; p<0.001; R^2=0.906. The number of food stocks was not significant in the simple regression (p<0.108; R^2=0.052).

DISCUSSION

By studying 50 colonies from five species of stingless bees, we showed that the number of brood cells and external activity are the biological parameters that best relate to colony size, regardless of the striking differences in their size and life history traits. To measure bee colony size and relate it to biological parameters closes important knowledge gaps in the life histories of several widely distributed Amazonian stingless bees, but also identifies useful proxy measures of colony size for colony management and multiplication.

The number of brood cells was the best predictor of colony size; however, its measurement involves an intensive handling of colonies, and it is impossible to obtain in natural nests. External activity was also positively correlated with population size, and it is feasible to obtain in managed or natural colonies; we hypothesize this relationship occurs because it is linked with the number of foragers (the last task in the life of workers; Michener 1974; Sakagami 1982; Wille 1983), which are responsible for keeping the food stocks in optimal levels, and thus in capacity to nourish the immatures and adult bees.

External activity is commonly used for measuring the "strength" of the colony; together with the number of adult bees, the number of brood cells and the number of food pots (Hilario et al. 2000; Gostinski et al. 2017). External activity is a metric that can be easily assessed even in natural colonies, where the measurement of brood combs and food stocks is impossible to obtain without destroying the colony (Hilario 2007). The relationship of colony size with the other measured biological parameters varied among the different species. The food stocks only presented a positive relationship with the number of adult bees in*M. fasciculata* . For the other species, there was no relationship, as shown in the general model, including all species. Regarding the egg-laying rate, in *S.* aff. *postica* we observed high levels of variation in the relationship with the adult population, probably due to the high mortality of immature bees observed in this species (Figure S4).

Among the five species studied, *S.* aff. *postica* had the largest population, corroborating previous estimates made by Lindauer and Kerr (1960; around 15,000 adult workers), while *P. minima*presented the smallest population (mean colony size around 400), much higher than the estimate of 175 bees made by Wille and Michener (1973). For *M. fasciculata* , our measures presented a smaller population than the estimate of 776 adult bees, which may be due to methodological differences between studies (Kerr et al. 2001). Another possibility is that the since the study of Kerr et al. (2001) was made with other populations of *M. fasciculata* , in a region approximately 600 km from our study site and with different vegetation physiognomies. Thus, differences in the estimates made for *M. fasciculata* (and for*P. minima* ; Wille and Michener 1973) may be due to populational variation and the resources available to colonies in the different areas. For *F. longipes* and *M. flavolineata* , there were no previous estimates of their population size.

The size of a colony in social insects is linked with several aspects of their life-history, such as foraging strategies and reproduction rates (Oster and Wilson 1978; Planque et al. 2010). Species with large populations, like we found in *S.* aff. *postica* and *F. longipes* potentially have large numbers of workers involved in defense and resource gathering, and therefore the colony's consumption of resources may increase proportionally. In contrast, small populations, such as *P. minima* , although potentially not able to collect large amounts of resources, may have smaller resource demands, thus compensating for the small number of available foragers. However, relationships between consumption/gathering of resources are yet to be studied in stingless bees, with one of the reasons being the perceived difficulty in estimating colony size. As such, our results will help future studies investigate this relationship.

From a practical perspective, knowledge on stingless bee colony sizes is highly relevant for their use as crop pollinators (Giannini et al., 2020). For example, in the Amazon region, *Scaptotrigona* aff.*postica* visit crops of economic importance, such as Rambutan (*Nephelium lappaceum* L.) and pollinate others, such as Acai palm (*Euterpe oleracea* Mart) (Ricon-Rabanales 2015; Campbell et al. 2018). However, for crop pollination, clear recommendations on colony stocking densities are necessary for the development of effective managed pollinator programs, as already defined for *Apis mellifera* (Vaudo et al. 2012). This number depends on the foraging range of the colonies, the number of foragers, and the number of flowers that need to be pollinated within croplands (Kuhn-Neto et al. 2009; Rands and Whitney 2011). Thus, our data can be combined with existing information on species' foraging ranges to develop novel managed pollinator protocols using native bee species for several important regional crops (Campbell et al. 2019; Araujo et al. 2004).

Finding an efficient method for estimating population size is important for commercial rearing of stingless bee colonies, for monitoring colony health, and for future scientific studies. Our study is the first to count the total number of bees (adult and immature) and correlate it with intrinsic factors in meliponine colonies. Previous studies provided formulae to estimate colony size but did not validate these formulae with biological data (Ihering 1930; Aidar 1996), and a recent study showed that colony size of stingless bees has been

overestimated (Valadares et al. 2021). Since the direct measure of the number of adult bees involves invasive sampling methods (Valadares et al. 2021), our study provided a reliable approach to estimate the number of adult bees by using the external activity. On the other hand, our study shows that estimating population size will not be possible with a general formula that works for all Meliponini.

For building a reliable and feasible estimation method for population size, future studies should focus on single species or genus. It is also important to collect larger and broader samples to deal with disturbances caused by natural variation and thus understand the error range of the method. External activity is a good parameter to start developing a formula, because it is a variable that is easy to obtain in natural or managed colonies, and it is highly correlated with population size in most species. Future studies also must focus on other bee genera and verify possible variation in interspecific population size related with different life-history traits, as well as with temporal fluctuations.

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AUTHOR'S CONTRIBUTIONS

Kamila Leao : Conceptualization; Data Curation; Formal Analysis, Investigation, Methodology, Validation, Visualization, Writing – Original Draft Preparation, Writing – Review and Editing.**Alistair Cambell** : Formal Analysis, Methodology, Writing – Review and Editing. **Jamille Veiga** : Formal Analysis, Methodology, Validation. **Cristiano Menezes** : Conceptualization, Funding Acquisition, Project Administration, Resources, Supervision.**Felipe Contrera** : Conceptualization, Funding Acquisition, Project Administration, Resources, Supervision, Writing – Original Draft Preparation, Writing – Review and Editing.

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Table 1. Mean and standard deviation of colony sizes in five species of stingless bees (*Melipona flavolineata* , *Melipona fasciculata* , *Scaptotrigona* aff. *postica* ,*Frieseomelitta longipes* , *Plebeia minima*). n = Number of colonies counted, Adult bees = Number of adult bees counted in each colony (males + workers), Sample size = colony size considering (small <1,000 adult bees; medium = between 1,000 and 5,000 adult bees and large-sized colonies >5,000 adult bees), Total population = Adult bees + Brood cells, Brood cells= Number of brood cells (bees in the egg, larva or pupa phase) counted in each colony, Range of adult bees = Minimum and maximum the number of adult bees for each species studied.

Species	**n**	**Colony size**	**Total population**	**Brood cells**	**Brood cells**	**D Adult bees**	**Range of adult bees**	**Range adult b**

M. flavolineata	8	small	2,111.13 + 404.71	2,111.13 + 404.71	1,065.13 + 244.25	1,046.00 + 185.17	768 – 1,353
M. fasciculata	8	medium	1,340.38 + 730.39	1,340.38 + 730.39	747.63 + 438.76	592.75 + 300.06	294 – 1,008
S. aff. *postica*	13	large	15,429.46 + 2,781.99	15,429.46 + 2,781.99	8,025.46 + 1,582.63	7,404.00 + 1,390.96	5,898 – 10,036
F. longipes	9	medium	5,415.77 + 2,167.08	5,415.77 + 2,167.08	2,990.44 + 1,230.91	2,425.33 + 1,000.16	1,051 – 4,393
P. minima	12	small	880.75 + 471.74	880.75 + 471.74	476.0 + 233.49	404.75 + 254.42	208 -981

Table 2. Results from LMM analyses of standardized (z-scores) colony sizes (number of adult bees) and four biological parameters (fixed effects): number of external activity of workers, egg-laying rate, food stocks (total weight) and brood cells (for details on parameter estimation, see *Materials and Methods*). Parameter estimates (coefficients) are presented with 95% confidence intervals, model AICc values, and delta AICc.

Model	**Intercept**	**External activity**	**Egg-laying**	**Food stocks**	**Brood cells**	**AICc**	**Δ**
1	0.00				**0.84 (0.68 - 1.00)**	84.99	0
2	0.00	**0.62 (0.39 - 0.85)**				121.17	3
3	0.00		**0.58 (0.34 - 0.82)**			124.95	3
4 (NULL)	0.00					143.15	5
5	0.00			0.17 (-0.11 - 0.46)		144.00	5

Figure 1

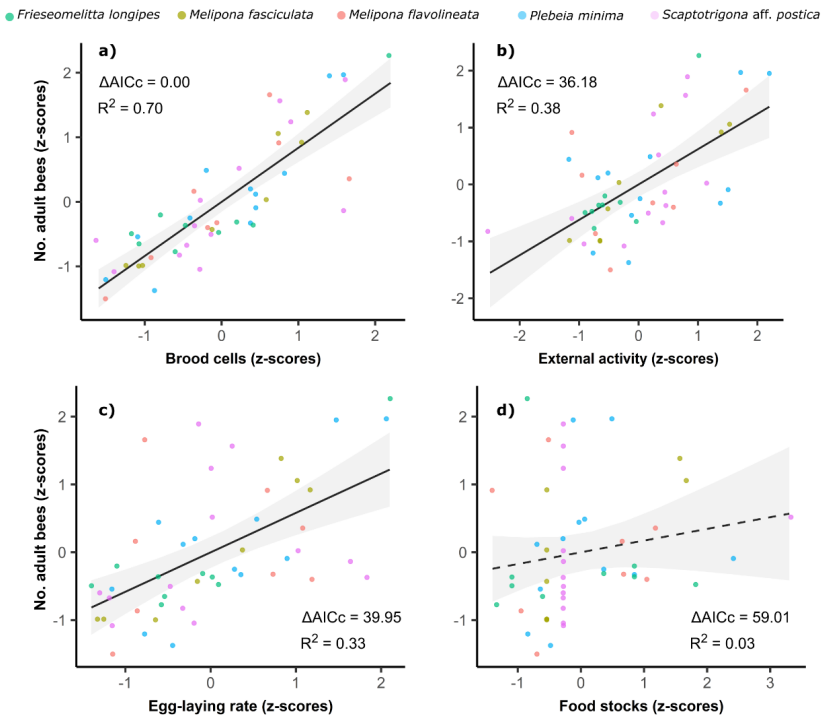

Figure 1. Effect of biological parameters (number of external activity of workers, egg-laying rate, food stocks (total weight) and brood cells (for details on parameter estimation, see *Materials and Methods*) on colony size (number of adult bees) considering all species. Points represent individual colonies, and the black line is the general estimate of the model. The gray shaded area defines the 95% confidence intervals for the model. The predictor and response variables for individual species were standardized using Z-scores. a) - Relationship between brood cells and size of the adult population, b) - Relationship between external activity and size of the adult population, c) - Relationship between egg-laying rate and size of the adult population, d) - Relationship between food stocks and size of the adult population. Colors represent colonies of five stingless bee species: pink = *Melipona flavolineata* , gold = *Melipona fasciculata* , green = *Frieseomelitta longipes* , lilac = *Scaptotrigona*aff. *postica* , blue = *Plebeia minima* .

Figure 2

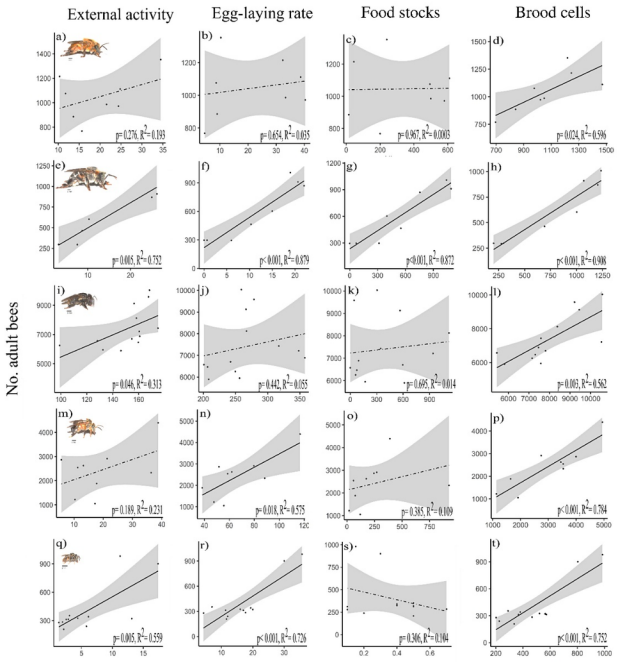

Figure 2. Linear regressions between biological parameters (number of external activity of workers, egg-laying rate, food stocks (total weight) and brood cells (for details on parameter estimation, see *Materials and Methods*) and colony size (adult bees) of each bee species studied (*Melipona flavolineata* [8 colonies], *Melipona fasciculata* [9], S*captotrigona* aff.*postica* [13], *Frieseomelitta longipes* [9] and *Plebeia minima* [12]).