

Original Article

Pollination efficiency by bee guilds (Hymenoptera: Apoidea) to annatto flowers, *Bixa orellana* L.1753 (Bixaceae)

Eficiência de polinização por guildas de abelhas (Hymenoptera: Apoidea) para flores de urucum, *Bixa orellana* L.1753 (Bixaceae)

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Abstract

Insect's pollination is essential in the maintaining of species abundance and diversity in the biomes. Bees are the most common pollinators of flowering plants, being their services very important in the conservation of biotope and biodiversity. The aims of this paper, are: to know the bee diversity and their population patterns over annatto flowers in the orchard; evaluate seed production in annatto plants with and without entomophilous pollination; to compare whether body size and intertegular distance of bees visiting annatto flowers correlates, and whether size of floral visitor bees in annatto influence in the pollination and productivity. During a flowering season in Dourados, Mato Grosso do Sul state, Brazil, 240 flower buds were chosen from five different plants and divided into six groups. In each group had 40 flower buds in the pre-anthesis phase (treatments): T1 (control), T2 (natural self-pollination), T3 (Anemophilous pollination), T4 (Big bees), T5 (Medium bees) and T6 (Small bees). Bee species were identified and measured by two metrics: body size (BS) and intertegular distance (ITD) to categorize their guilds. The treatments considered in the population's analyses were the following: species richness, constancy, frequency, dominance of each bee species, and if bee size would influence in the process of annatto pollination. The analysis of variance was determined by the Tukey and Kruskal-Wallis tests ($P < 0.05$) for the population parameters. Floral visitor bee species in *B. orellana* were divided into two groups according to the periods of their activity: 6h-9h and 14h-18h, and in four guilds, based in their body size. Bees recorded from 6h to 9h were responsible for higher efficiency in the pollination rate; represented by the following species: *Bombus morio*, *Centris sponsa*, *Centris fuscata*; *Epicharis flava*, *Epicharis affinis*, *Epicharis analis*, *Euglossa* sp., *Eulaema nigrita*, *Geotrigona mombuca*, *Melipona quadrifasciata*, *Hylaesus* sp., and *Pseudaugochlora graminea*. Seed production was higher in the control (free access to all combined floral visitor), followed by big bees, and medium bees. The two metrics: (BS and ITD) has significant positive correlation. Big body species were more successful in the pollination of *B. orellana*.

Keywords: Apidae, body size, reproductive success, entomophily, seed production.

Resumo

A polinização por insetos é essencial na manutenção da abundância e diversidade de espécies nos biomas. As abelhas são os polinizadores mais comuns de plantas com flores, sendo seus serviços muito importantes na conservação do biótopo e da biodiversidade. Os objetivos deste trabalho são: conhecer a diversidade de abelhas e seus padrões populacionais sobre flores de urucum no pomar; avaliar a produção de sementes em plantas de urucum com e sem polinização entomófila, comparar se o tamanho corporal e a distância intertegular das abelhas que visitam flores de urucum se correlacionam, e se o tamanho das espécies de abelhas visitantes florais do urucum influencia na polinização e produtividade. Durante uma estação de floração em Dourados, MS, Brasil, 240 botões florais foram escolhidos de cinco plantas diferentes e divididos em seis grupos. Cada grupo teve 40 botões florais na fase de pré-antese (tratamentos): T1 (controle), T2 (autopolinização natural), T3 (anemofilia), T4 (abelhas grandes), T5 (abelhas médias) e T6 (abelhas pequenas). As espécies de abelhas foram identificadas e medidas por duas métricas: tamanho corporal (BS) e distância intertegular (ITD) para categorizar suas guildas. Os tratamentos foram considerados em uma análise com os seguintes parâmetros: riqueza de espécies, constância, frequência, dominância de cada espécie de abelha e, se o tamanho das abelhas influenciaria no processo de polinização. A análise de variância foi determinada pelos testes de Tukey e Kruskal-Wallis ($P < 0,05$) para os parâmetros populacionais. Abelhas visitantes florais em *B. orellana* foram divididas em dois grupos, de acordo com os períodos de visitação: 6h-9h e 14h-18h e,

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em quatro guildas, com base em seus tamanhos corporais. As abelhas registradas das 6h às 9h foram responsáveis pela maior eficiência na taxa de polinização; representadas pelas seguintes espécie: *Bombus morio*, *Centris sponsa*, *Centris fuscata*, *Epicharis flava*, *Epicharis affinis*, *Epicharis analis*, *Euglossa* sp., *Eulaema nigrita*, *Geotrigona mombuca*, *Melipona quadrifasciata*, *Hylaeus* sp. e *Pseudaugochlora graminea*. A produção de sementes foi maior no controle, seguido por abelhas grandes e médias. As duas métricas (BS e ITD) apresentam correlação positiva e significativa. Espécies de grande porte foram mais bem-sucedidas na polinização de *B. orellana*.

Palavras-chave: Apidae, tamanho corporal, sucesso reprodutivo, entomofilia, produção de sementes.

1. Introduction

Pollinating agents, such as the insects are essential for conservation of the ecosystems, as well as to enhance agricultural production. They play a crucial role in maintaining the diversity, abundance and activities of other organisms, resulting in environmental and biodiversity conservation (Silva-Neto et al., 2016; Magalhães et al., 2024). The pollination services provided by insects, especially native bees, are of vital significance for flowering plants (Phanerogams), increasing their rates of reproduction and productivity, both in natural forests and agroecosystems (Thakur, 2012; Rocha et al., 2023; Magalhães et al., 2024). Pollination is based on a largely mutualistic relationship between plants and pollinators, in which, plants provide resources for bees (mainly pollen and nectar) and receive the benefits of pollen transfer (Proctor et al., 1996; Solís-Montero et al., 2015; Mesquita-Neto et al., 2021). In addition to food resources and the offer of substances such as volatile oils, resins, or even places for nesting, resting, and mating (Roubik, 1989), they are also part of the counterpart of Phanerogamae for their pollinators.

Bixa orellana is native to the Amazon Biome, probably having Brazil as origin center. Widely cultivated in tropical regions of the world, this evergreen tree can reach 9m in height, with white or pink flowers. Fruits are ovoid-globular in shape (capsule), attached to a cluster that can contain just over 30 capsules; each of these can contain between 30 and 45 ovoid or conical seeds, covered by a red-orange pigment or viscous aril. The bixin (carotenoid) is the main component that serves as a dye and to other uses. Native (indigenous) South American peoples for long time known its value as a dye for food, popular medicines, and in ornamental body paintings in their cultural ceremonies. In recent decades, science discovered that several chemical-synthetic dyes are potentially carcinogenic, so annatto nowadays is recommended in Europe and North America to replace them, and as a result, it has gained great economic importance in the world (Raddatz-Mota et al., 2017). Because this plant became economically important worldwide, it began to demand several studies, including those aimed at optimizing its large-scale production system around the world, such as pollination and seed production.

In order for the annatto (*Bixa orellana* Linnaeus, 1753) to fructify in an economically viable production system, biotic pollinating agents are needed. Its flowers attract some guilds of Apidae, such as the species of the genera: *Bombus*, *Xylocopa*, and *Epicharis* (Venturieri and Venturieri, 1992; Venturieri and Fernandes, 2003). Guild here mean groups of bees that apply mechanisms

of resources partitioning in a competitive way by species not necessarily related phylogenetically, but with similar life, evolution and adaptation histories to ensure their survival in nature (Blondel, 2003).

Flower of some plant species, to be pollinated, depend of some bee species that can “buzz” (vibrate) the anthers of the flower. This vibration allows the release of their pollen grains (Rocha and Polatto, 2017; Vallejo-Marín, 2019). The vibration of the flight muscles from the thorax, jaws, abdomen and legs of these bees creates a cloud of pollen in the air, which is later stored in the scopae and/or corbiculae of the hind legs (Buchmann and Hurley, 1978; Woodrow et al., 2024). Pollination by vibration occurs typically in flowers with poricidal anthers, in which case the pollen is offered as the only floral resource, therefore called ‘pollen flowers’ (Vogel, 1990).

As pointed out by Buchmann and Hurley (1978), female bees that vibrate and outline the flower's corolla, recline around or on the tip of the anthers and grip the stamens tightly. This behavior results from contracting and relaxing the thoracic flight muscles and culminates in expelling pollen from the anthers. Wille (1963), stated that stingless bees (Apidae: Meliponini) are frequent visitors of flowers with large amounts of pollen. According to the behavioral pattern of pollen collection, three groups of bees that visit flowers, are recognized: vibrators, collectors and biters. Vibrating bees transmit vibration to anthers to collect pollen; collectors gather pollen from the flower surface, after it has been vibrated by other bees, and biting bees are those that chew the flower anthers to collect pollen (Buchmann and Hurley, 1978; Inouye 1980; Vallejo-Marín, 2019). Portman et al. (2019) review and classified the pollen collection by bees in two mainly different modalities: active and incidental. The first is divided in six different subtypes of behaviors: scraping with the extremities, buzzing, rubbing with the body and/or scopae, rubbing with the face, tapping, and rasping. Portman et al. (2019) highlights that beyond active and incidental pollen gathering behaviors, there are many intermediate steps in which bees temporarily accumulate pollen on a discrete patch of specialized hairs.

Researches have shown that some pollinators are more effective than others and the study of floral biology is essential to understand the plant's reproductive system and identify which floral visitors are really pollinators, based on their visiting behavior, as pointed out by Blochtein et al. (2014). In general, is accepted that the most efficient pollinators to flowers very rich in pollen, such as those of *B. orellana*, are bees capable of buzzing movements, or “buzz-pollination”, typically robust, big body bees (e.g. De Luca et al., 2019; Mesquita-Neto et al., 2021). In bee-flower interactions, stigma contact was

positively correlated with bee size and anther-stigma distance. Adequately sized and large bees contacted the stigma with high frequencies. Thus, a visitor is most likely to come into contact with the anthers and stigma when its size matches or exceeds the stigma-anther separation space of that flower. The morphological fit between bee size and stigma-anther separation is a predictor of pollination effectiveness. Research results did not provide evidence of a strict size correspondence between bees and flowers, but rather a minimum size threshold for pollen transfer to the anther to occur. Not only bee equivalent in size to the stigma-anther separation, but also larger bees, had foraging behaviors that promoted cross-pollination. Bee size categories can be linked to flower size, specifically the distance of separation between stigma and anther (Mesquita-Neto et al., 2021).

The frequencies used to generate floral vibrations are not closely linked to the body size of the bee, and this may allow an individual bee to use different sonication frequencies when visiting flowers of different plant species that require the vibration behavior. Then, the bee species can have a wider range of botanical species available in that habitat (De Luca et al., 2019). These authors highlight that this mutualism can be advantageous in the plant-pollinator relationship with this interdependence of pollination by vibration, since the species can adjust their vibration pattern to the plant species that requires buzz-pollination available in the habitat. They also pointed out that the relationship between the body size of the bee species and the vibration rate, which reveals that bee species with large body sizes may have a greater advantage when visiting flowering plant species that require vibration behavior. Due to the ability of big bee species to maximize the amplitude of their vibrations, they are able to extract a greater quantity of pollen grains in their greater foraging efforts (De Luca et al., 2019).

In this research, considering the great diversity of bee species visiting the flowers of *B. orellana*, its floral morphology and their large amount of pollen, with poricidal anthers, our hypothesis is that large bees will be more efficient for its pollination, increasing the production of viable seeds. In this perspective, the aims of this paper, are: to know the bee diversity and their population patterns over annatto flowers in the orchard; evaluate seed production in annatto plants with and without entomophilous pollination; to compare whether body size and intertegular distance of bees visiting annatto flowers correlates, and whether size of floral visitor bees in annatto influence in the pollination and productivity.

2. Materials and Methods

2.1. Place

The annatto orchard consisted of plants with three years old. It is located in the *Estância Biocenose* (22°20'88.90" S, 54°92'70.26" N), *Fazenda Coqueiro*, municipality of Dourados, Mato Grosso do Sul state, Brazil. The plants of *Bixa orellana* were kept under an organic cultivation system, without irrigation. On the nearby of the orchard (200 m to 300 m apart) were detected two apiaries of the

Africanized bee *Apis mellifera* L. 1758, located at the south of *Estância Biocenose*.

This orchard is located about 500m apart a native forest fragment with 35 ha of total area (22° 12' 44.36" S, 54° 55'13.83" W), 430 m of altitude, known as *Reserva Florestal Fazenda Coqueiro*, located two kilometers from the Highway MS-162, and nine km from Dourados downtown, state of Mato Grosso do Sul (MS), Brazil. That forest fragment, connects with Cerrado Bioma, but have phytophysiognomy of tropical semi-deciduous forest (Atlantic Forest domain). The native vegetation of the forest fragment on the nearby of the orchard is composed mainly by plants from the families: Annonaceae, Apocynaceae, Araliaceae, Aquifoliaceae, Boraginaceae, Burseraceae, Caricaceae, Cecropiaceae, Clusiaceae, Euphorbiaceae, Flacourtiaceae, Lauraceae, Fabaceae, Papilionaceae, Mimosaceae, Meliaceae, Melastomataceae, Myrsinaceae, Myrtaceae, Moraceae, Rubiaceae, Rutaceae, Sapindaceae and Sterculiaceae. To the South, about 250m there are houses with orchards of fruit tree species (e.g. guava, *Psidium guajava* L. mango, *Mangifera indica* L. hog plum, *Spondias purpurea* L., barbados cherry, *Malpighia emarginata* DC., orange, *Citrus sinensis* Osbeck and peach, *Prunus persica* (L.) (Uchoa et al., 2023). The monthly thermal variation was 18-28 °C and accumulated precipitation in the period was 1,200 mm. The study area has a history of land use that includes conversion of pasture to organic cultivation ten years ago, with the strategic maintenance of ecological corridors that integrate the orchard with fragments of native vegetation, offering alternative resources for the entomofauna of floral visitors throughout the year.

2.2. Data collection method

The observations and collections were carried out from February to November 2018, during the flowering periods of *B. orellana*. The captures of floral visitors occurred in the first 20 minutes of each hour, between 6:00 am and 6:20 pm, during ten non-consecutive days in 20 different annatto plants, with an average time of one minute per plant, until completing the 20 minutes, by two persons, working collaboratively and concurrently for about 1 min on each flower. All the plants were evaluated when it was in full bloom. In the remaining 40 minutes of observation, the behavior of floral visitors in an indeterminate number of flowers of different plants was evaluated, totaling 130 hours between collections and observations.

Floral visiting bees that collect pollen, are generally, classified into two guilds: pollinators and resource stealers. Pollinators are that floral visitors able to touch specific regions of their bodies in the reproductive structures of the flower when collecting floral resources (pollen), transferring it from the anthers to the floral stigma of *B. orellana*. On the other hand, floral resource stealers are the species of floral visitors that do not bump specific regions of their bodies in both reproductive organs (anthers and stigmas) during pollen collection, and consequently this behavioral pattern makes pollination unfeasible (Inouye, 1980; Richardson and Irwin, 2015).

Floral visitors were collected with entomological nylon nets and placed in a killing jar containing ethyl

acetate. The samples were separated by date and time of collection, placed individually in paper bags, and stored in a refrigerator in the *Laboratório de Insetos Frugívoros (LIF)*, *Faculdade de Ciências Biológicas e Ambientais (FCBA)*, *Universidade Federal da Grande Dourados (UFGD)*, Dourados, Mato Grosso do Sul state (MS), Brazil. The material remained under refrigeration until the specimens were mounted on entomological pins, labeled and incorporated into the Entomological Collection of the *Museu da Biodiversidade (MuBio)*, FCBA-UFGD, Dourados-MS, Brazil.

2.3. Annatto biology and flower morphology

Bixa orellana is native from Amazon and Atlantic Forest biomes (Brazil), but it is not endemic, with a wide geographic distribution in South and Central America. The flowering season is mainly from September to March, and can provide two or three annual harvests in the Central-West of Brazil, depending on the rainfall regime. Harvesting takes place approximately 30–45 days after pollination and flower fall. Each flower has an average, 420 stamens ($n = 12$ flowers evaluated), with poricidal dehiscent anthers that offer pollen in abundance, but no nectar inside. According to Queiroga et al. (2021), *B. orellana* has a compound flower with diameter between 3 and 5 cm; hermaphrodite, actinomorphic and grouped in panicles (10 to 15 cm long) at the ends of the branches; with five large, rounded and free petals. In pre-flowering, it's imbricated and has five greenish-brown sepals. Stamens with free filaments at the base, two thecae opening at the apex by two slits; uniformly colored fillets: white, yellow or violet. Flower can vary in color, as well as the capsules (green, red or yellow). Ovary

elevated by a gynophore, with a superior seder appearance, with seminal rudiments (10 to 60 capsules); two to four carpels fused into a locule; two parental placentas with several ovules, possibly with false septa, pluriovulated. Slender style, ending in a bilobate stigma.

2.4. Enhance in seed production by pollination

To analyze the increase in the production of seed in *B. orellana* by pollinators, 240 floral buds of five different plants were randomly marked. These floral buds were divided into six treatments, each containing 40 flower buds in the pre-anthesis phase.

2.5. Treatments (T1 to T3, November 2018)

A total 120 flower buds that were simultaneously marked, directly on annatto trees in the orchard, as described: T1 = Control: Evaluation of 40 flower buds, the base their branches were marked by light green colored tape and left in the orchard without any other type of handling, with free access to the wind and all animals in the external environment. T2 = Natural self-pollination: Each one floral bud ($n = 40$) was dressed in bags of organza tissue (mesh smaller than 1 mm) and marked by purple ribbon. T3 = Anemophilous pollination: Each one floral bud ($n = 40$) was inserted in bags of tulle tissue (mesh around 1 mm) and marked by orange ribbon. In the T3 treatment, to assess the occurrence or not of anemophilous pollination, flower buds were isolated with tulle-type tissue bags (see Figure 1).



Figure 1. Bagging of annatto flowers (*Bixa orellana*, Bixaceae) on the field (A = unity and B = set of flowers on a tree) pollination test to evaluate floral visitors/pollinators in an orchard under the organic cultivation system (November 2018).

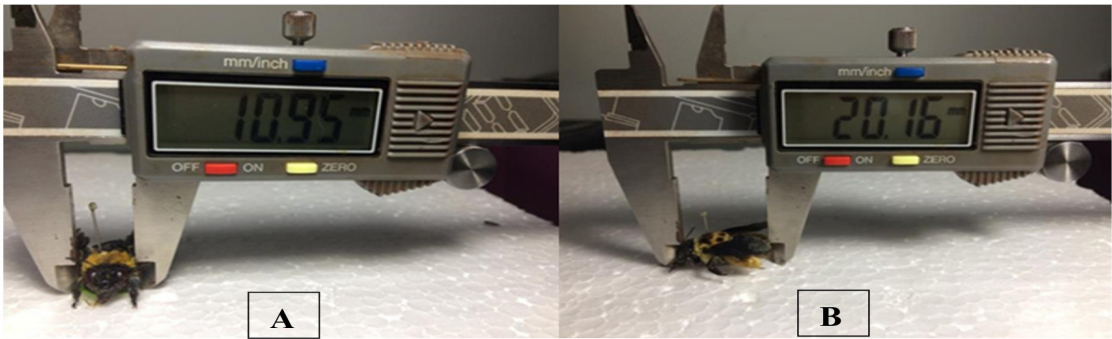


Figure 2. Digital caliper used to the measurements: (A) body width, (B) body length and Intertegular distance (ITD), not shown, of bees pollinating the flowers of *Bixa orellana* (Bixaceae).

2.6. Treatments (T4 to T6)

Four bee guilds were established with base in two different kind of measures (Body size-BS and Intertegular distance-ITD). These metrics were taken in individuals of different bee species flower visitors in *B. orellana*. We establish four bee guild, but only three bee guilds were used in this set of treatments (T3 to T6), as follow: T4 = Large body size (LBS); T5 = Medium body size (MBS) and T6 = Small body sizes (SBS). The treatments T4, T5, and T6 tested the pollination rate in *B. orellana* flowers by these bee guilds, established according to the two measures taken on their bodies. For the readings of data on the measures, a 150 mm 6 stainless steel MTX[®] digital caliper was used (Figure 2).

The Intertegular span (ITS) or Intertegular distance (ITD) is a measure considered coherent that directly correlates with the body size in Apoidea species (Cane, 1987; Cariveau et al., 2016; De Luca et al., 2019). Specimens from floral visitors were sorting according to their body size. For this purpose, body length = body size (BS) and the intertegular distance (ITD) of the specimens in each species were measured and used as a predictor of their body size for categorization and formation of guilds. Four categories were established: large body size: (ITD) > 4.0 mm, total body length > 14.0 mm; medium to large body size: ITD between 2.7 and 3.9 mm, body length between 10.2 and 13.9 mm; medium-small body size: ITD between 1.1 and 2.6 mm, body length between 6.3 and 10.1 mm; small body size: ITD < 1.0 mm, body length shorter than 6.2 mm. These categories were based on the classification of Roubik (1989); Viana and Kleinert (2005). Sampled bees were measured with a digital caliper and categorized into four guilds: (small body size, small to median body size, median body size and large body size), according to the measurement values (body size and intertegular distance) of the specimens (Figures 3, 4). In treatments T4, T5, and T6, the pollination rates by tree bee guilds (large, medium, and small body length [=size]) were evaluated, respectively.

To ensure the arrival of a bee as the first pollinator, flower buds were closed with tulle bags individually in the day before the flower opening, and bags were removed in the next day, minutes before the foraging time of a bee. The observations were determined by the limit of the observer's visual field, and the foraging time was noted,

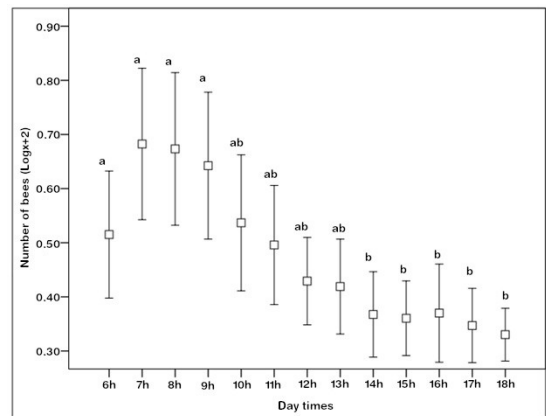


Figure 3. Abundance of bees collected with entomological net between 6 am and 6 pm, on *Bixa orellana* (Bixaceae) flowers, during 10 weekly collections in an orchard under organic cultivation system (Estância Biocenose, Fazenda Coqueiro, Dourados, Mato Grosso do Sul state, Brazil (February 10 and April 14, 2018). Kruskal-Wallis = $\{P(x>X^2); (p<0.001) = (\chi^2 = 91.95; gl = 12)\}$. Comparison of the number of bees by the bilateral Dunn-Bonferroni test ($p<0.05$), with e qual letters not significantly different.

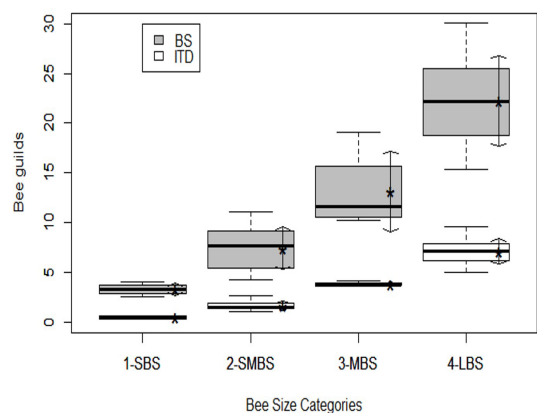


Figure 4. Guild of bee species (Hymenoptera: Apoidea) floral visitors of *Bixa orellana* (Bixaceae), based on two measurements: bee size (BS) and ITD (intertegular distance) of bee species. **Legend:** SBS = small body size; SMBS = small to medium body size; MBS = medium body size, and LBS = large body size ($P < 0.05$).

never exceeding 9:30 am. Before floral anthesis, tulle bags were removed for the free visitation by bees. After the visit, the category of bee guild was recorded, the flower was isolated again with the tulle bags to prevent new contacts and, after 48 hours, the protection was removed again for free floral development.

2.7. Annatto seed production by pollination

To evaluate the differences in annatto seed increase or not by visiting bee species, capsules per branch and seed per capsule were counted. Annatto inflorescence clusters ($n = 20$) in pre-anthesis state were protected by voile fabric bags during the previous night. When the flowers opened, we observed which size of bee was the first to visit the flower, when the protection with the bag occurred and marking with ribbons of different colors to distinguish each treatment. The treatments: self-pollination and anemophilous pollination, flower was protected by the bags until it fell. On the other hand, the treatment control was totally exposed to natural conditions (without bag). The flowers of *B. orellana* fall on the same day that open, so they were kept protected until fruit formation. After maturation, the fruits were counted, recording how many fruits (capsules) per treatment, as well as the number of seeds per capsule. The seeds were separated and counted using a flat brush (Condor N02-474), placed on aluminum foil, dehydrated in a drying oven (FANEM-70x60, 3000 °C) at a constant temperature of 45 °C for 48 h and weighed on an analytical balance (BEL- 0.0001 g, M214AIH) in order to obtain the average dry weight.

2.8. Identification of the annatto's floral visitor bee species

The bee species were identified by Dr. Eduardo A. B. Almeida from *Departamento de Biologia, Faculdade de Filosofia Ciências e Letras (FFCLRP), Universidade de São Paulo (USP), Ribeirão Preto, SP, Brazil*, using dichotomous key and comparison with already identified bee species from Entomological Collection at that institution.

2.9. Data on population analysis

For analysis of the bee community populations, the following parameters were estimated based on Uramoto et al. (2005): Species Richness (S): total number of species observed in the community; Frequency: proportion of individuals of a species in relation to the total number of individuals in the sample; Constancy: percentage of samples in which a particular species was present. Classification of species regarding their constancy: (1) constant species (w) present in more than 50% of the samples, (2) accessory species (z) present in 25-50% of samples, (3) accidental species (y) present in less than 25% of samples. Dominant species: a species was considered dominant when its frequency was higher than $1/S$, where S = total number of species in the community. Diversity Index (Shannon-Weaner). Confidence Interval of H ($P = 0.05$), Equitability index, predominant species and the variance was determined by the Tukey test at 5% of probability. For comparison between the abundance of bees in different day times was applied Kruskal-Wallis test = $\{P(x > X_2); (p < 0.001) = (\chi^2 = 91.95; gl = 12)\}$, and the

bilateral Dunn-Bonferroni test ($p < 0.05$) for the number of sampled bees. In the evaluation of annatto pollination efficiency, the mean $\pm$ standard deviation was obtained, using ANOVA, followed by the Tukey test ($p < 0.05$).

2.10. Analysis on the bee guilds

To analyze whether the bee guilds (SBS = small body size; SMBS = small to medium body size; MBS = medium body size and LBS = large body size) of the flower-visiting species upon *Bixa orellana*, established based on body size measurements differ significantly among them, analysis of variance (ANOVA) was applied. To this end, the assumptions for performing the analysis of variance (ANOVA) were first verified. Considering that the homogeneity of variance was not met (p -value < 0.05), a non-parametric approach was chosen (Sheskin, 2011).

3. Results

A total of 910 individuals and 36 different species of four bee families (Hymenoptera: Apoidea) were collected: Apidae (25 species); Andrenidae (1 species), Colletidae (2 species) and Halictidae (8 species), during 130 hours of observations and collecting. The most abundant species were: *Apis mellifera* (182), *Exomalopsis analis* (153), *Tetragona elongata* (136), and *Epicharis flava* (89). Two species were the most frequent: *Apis mellifera* and *Tetragona elongata* (Table 1).

Four bee species were predominant, being the most abundant and frequent, in descending order: *Apis mellifera* (182 individual), *Exomalopsis analis* (153), *Tetragona elongata* (136) and *Epicharis flava* (89), being *E. flava* (large body size) one of the most efficient pollinators. Nonetheless, *A. mellifera* (median body size), was the most abundant, and *T. elongata* (medium to small body size), the third most abundant species, are resource stealers in *B. orellana* flowers, not pollinators. The bee species: *Exomalopsis auropilosa*, *Exomalopsis cf. analis*, *Exomalopsis fulvofaciata*, *Augochloropsis crassigena*, *Augochloropsis wallacei*, *Augochloropsis cf. melanochaeta*, *Augochloropsis cf. smithiana*, *Augochloropsis electra*, *Augochloropsis sp.*, *Geotrigona mombuca*, *Pseudaugochlora graminea* and *Hylaeus sp.* were classified as medium (10mm to 13mm) to small body size (< 7 mm). These bees shake anthers individually, by vibratory pollination behavior (Mesquita, 2008). This means that they can carry out some pollination in *B. orellana* flowers, but not so efficient as the bee species of the genera *Bombus*, *Centris*, *Epicharis*, *Oxaea* and *Xylocopa*, categorized as larger body size and heavy body masses (Table 2).

Behavioral differences between classes of visitors may explain the difference in the fruiting production by *B. orellana* found in this research. Annatto flowers visited by large-bodied bees are more productive, generating higher seed amount and with heavy mass by capsule (= fruit). In order for the pollen be released from the *B. orellana* flowers, it must be heavily shaken ("buzzed") by the bees. The larger the bee's body size, the greater the release of pollen grains, consequently increasing the chance of fruit production, generating a greater number of seeds and heavier fruits. Herein, the frequent occurrence of

Table 1. Diversity of bees (Hymenoptera: Apoidea) in annatto flowers (*Bixa orellana*, Bixaceae) and respective foraging times in an orchard under organic cultivation system. For each time recorded, the sum of 10 collections was considered, with 20 minutes of collection for each hour: Estância Biocenoze, Dourados, Mato Grosso do Sul state, Brazil (February 10 to April 14, 2018).

Floral Visitors Species	06-	07-	08-	09-	10-	11-	12-	13-	14-	15-	16-	17-	18-	Total
	06:20	07:20	08:20	09:20	10:20	11:20	12:20	13:20	14:20	15:20	16:20	17:20	18:20	
APIDAE														
<i>Apis mellifera</i> Linnaeus, 1758	6	14	19	8	16	20	15	16	14	10	22	14	8	182
<i>Bombus (Fervidobombus) morio</i> (Swederus, 1787)	13	15	9	2	1	—	—	—	—	—	—	—	—	40
<i>Centris (Aphemisia) cf. mocsaryi</i> Friese, 1899	—	2	1	1	—	—	2	—	—	—	—	—	—	6
<i>Centris (Metacentris) collaris</i> Lepeletier, 1841	—	1	—	2	—	—	1	—	—	—	—	—	—	4
<i>Centris (Ptilotopus) sponso</i> Smith, 1854	7	10	4	9	6	4	1	1	—	—	—	—	—	42
<i>Centris (Trachina) fuscata</i> Lepeletier, 1841	—	—	—	1	—	—	—	—	—	—	—	—	—	1
<i>Epicharis (Epicharana) flava</i> Friese, 1900	24	19	14	18	3	7	1	2	1	—	—	—	—	89
<i>Epicharis (Hoplepicharis) affinis</i> Smith, 1874	2	4	4	3	—	2	—	1	—	—	—	—	—	16
<i>Epicharis (Triepicharis) analis</i> Lepeletier, 1841	—	—	1	—	—	—	—	—	—	—	—	—	—	1
<i>Euglossa</i> sp.	—	—	1	—	—	—	—	—	—	—	—	—	—	1
<i>Eulaema (Apeulaema) nigrita</i> Lepeletier, 1841	—	—	1	—	—	—	—	—	—	—	—	—	—	1
<i>Exomalopsis (Exomalopsis) auropilosa</i> Spinola, 1853	—	—	2	—	—	—	1	—	—	—	—	—	—	3
<i>Exomalopsis (Exomalopsis) cf. analis</i> Spinola, 1853	9	33	44	34	19	9	4	1	—	—	—	—	—	153
<i>Exomalopsis (Exomalopsis) fulvofasciata</i> Smith, 1879	—	7	4	3	—	1	—	1	—	1	—	—	—	17
<i>Geotrigona mombuca</i> (Smith, 1863)	—	—	1	—	1	—	—	—	—	—	—	—	—	2
<i>Melipona (Melipona) quadrifasciata</i> Lepeletier, 1836	—	1	1	1	—	—	—	—	—	—	—	—	—	3
<i>Plebeia</i> sp.	—	—	—	1	3	2	—	1	—	—	—	—	—	7
<i>Scaura latitarsis</i> (Friese, 1900)	—	—	1	2	6	2	4	1	2	1	—	—	—	19
<i>Tetragona elongata</i> (Lepeletier & Serville 1828)	1	13	14	28	17	12	6	13	9	7	11	4	1	136
<i>Tetragonisca fiebrigi</i> (Schwarz, 1938)	—	—	1	—	4	4	1	—	1	—	—	—	—	11
<i>Thygater (Thygater) analis</i> (Lepeletier, 1841)	—	1	—	—	—	—	—	—	—	—	—	—	—	1
<i>Trigona hyalinata</i> (Lepeletier, 1836)	1	1	2	3	4	—	2	1	—	1	—	—	—	15
<i>Trigona spinipes</i> (Fabricius, 1793)	—	1	2	1	—	—	2	1	1	3	3	—	1	15
<i>Xylocopa (Neoxylocopa) frontalis</i> (Olivier, 1789)	—	1	9	10	2	—	—	—	—	—	—	—	—	22
<i>Xylocopa (Neoxylocopa) suspecta</i> Moure & Camargo, 1988	4	5	6	2	—	—	—	—	—	—	—	—	—	17

Table 1. Continued....

Floral Visitors Species	06-	07-	08-	09-	10-	11-	12-	13-	14-	15-	16-	17-	18-	Total
	06:20	07:20	08:20	09:20	10:20	11:20	12:20	13:20	14:20	15:20	16:20	17:20	18:20	
ANDRENIDAE														
<i>Oxaea flavescens</i> Klug, 1807	4	11	7	3	2	1	—	—	—	—	1	—	—	29
COLLETIDAE														
<i>Hylaeus</i> sp.	—	—	—	2	1	1	—	—	—	—	—	—	—	4
<i>Ptiloglossa</i> cf. <i>aenigmatica</i> Moure, 1945	4	2	—	1	—	—	—	—	—	—	—	—	—	7
HALICTIDAE														
<i>Augochloropsis</i> (<i>Augochloropsis</i>) <i>crasigena</i> Moure, 1943	1	5	12	9	11	3	1	—	—	—	—	—	—	42
<i>Augochloropsis</i> (<i>Augochloropsis</i>) <i>wallacei</i> (Cockerell, 1900)	—	—	2	2	—	2	—	—	—	—	—	—	—	6
<i>Augochloropsis</i> (<i>Paraugochloropsis</i>) cf. <i>melanochaeta</i> Moure, 1950	—	3	—	1	—	—	—	—	—	—	—	—	—	4
<i>Augochloropsis</i> (<i>Paraugochloropsis</i>) cf. <i>smithiana</i> (Cockerell, 1900)	—	—	1	—	1	—	—	—	—	—	—	—	—	2
<i>Augochloropsis</i> (<i>Paraugochloropsis</i>) <i>electra</i> (Smith, 1853)	1	6	—	1	—	—	—	—	—	—	—	—	—	8
<i>Augochloropsis</i> (<i>Paraugochloropsis</i>) sp.	—	1	—	—	—	—	—	—	—	—	—	—	—	1
<i>Pseudaugochlora graminea</i> (Fabricius, 1804)	—	—	—	—	—	—	1	1	—	—	—	—	—	2
<i>Temnosoma laevigatum</i> Smith, 1879	—	—	—	—	—	—	—	1	—	—	—	—	—	1
Total	77	156	163	148	97	70	42	41	28	23	37	18	10	910

Table 2. Analysis of the bee community (Hymenoptera: Apoidea) foraging on flowers of the *Bixa orellana* (Bixaceae) under organic cultivation system, Estância Biocenose, Fazenda Coqueiro, Dourados, Mato Grosso do Sul state, Brazil (February to April 2018).

Bee species	Individuals	Size	Dominance	Abundance	Frequency	Constancy	Classification
<i>*Apis mellifera</i> Linnaeus, 1758	182	MBS	D	VA	F	W	RS
<i>Bombus (Fervidobombus) morio</i> (Swederus, 1787)	40	LBS	D	A	VF	Y	P
<i>Centris (Aphemisia) cf. mocsaryi</i> Friese, 1899	6	LBS	D	R	IF	Y	P
<i>Centris (Melacentris) collaris</i> Lepeletier, 1841	4	LBS	ND	R	IF	Z	P
<i>Centris (Ptilotopus) sponso</i> Smith, 1854	42	LBS	D	A	VF	W	P
<i>Centris (Trachina) fuscata</i> Lepeletier, 1841	1	LBS	ND	R	IF	Z	P
<i>*Epicharis (Epicharana) flava</i> Friese, 1900	89	LBS	D	VA	VF	W	P
<i>Epicharis (Hoplepicharis) affinis</i> Smith, 1874	16	LBS	D	C	F	Y	P
<i>Epicharis (Triepicharis) analis</i> Lepeletier, 1841	1	LBS	ND	R	IF	Z	P
<i>Euglossa</i> sp.	1	LBS	ND	R	IF	Z	P
<i>Eulaema (Apeulaema) nigrata</i> Lepeletier, 1841	1	LBS	ND	R	IF	Z	P
<i>Exomalopsis (Exomalopsis) auropilosa</i> Spinola, 1853	3	SBS	ND	R	IF	Z	P
<i>*Exomalopsis (Exomalopsis) cf. analis</i> Spinola, 1853	153	SBS	D	VA	VF	W	P
<i>Exomalopsis (Exomalopsis) fulvofasciata</i> Smith, 1879	17	SBS	D	C	F	Y	P
<i>Geotrigona mombuca</i> (Smith, 1863)	2	SMBS	ND	R	IF	Z	RS
<i>Melipona (Melipona) quadrifasciata</i> Lepeletier, 1836	3	MBS	ND	R	IF	Z	P
<i>Plebeia</i> sp.	7	SBS	D	D	IF	Y	RS
<i>Scaura latitarsis</i> (Friese, 1900)	19	SBS	D	C	F	W	RS
<i>*Tetragona elongata</i> (Lepeletier & Serville 1828)	136	SMBS	D	VA	VF	W	RS
<i>Tetragonisca fiebrigi</i> (Schwarz, 1938)	11	SBS	D	C	F	Y	RS
<i>Thygater (Thygater) analis</i> (Lepeletier, 1841)	1	SMBS	ND	R	IF	Z	P
<i>Trigona hyalinata</i> (Lepeletier, 1836)	15	SMBS	D	C	F	W	RS
<i>Trigona spinipes</i> (Fabricius, 1793)	15	SMBS	D	C	F	W	RS
<i>Xylocopa (Neoxylocopa) frontalis</i> (Olivier, 1789)	22	LBS	D	C	F	Y	P

Legend: **Bee body size:** SBS = small body size; SMBS = small to medium body size; MBS = medium body size and LBS = large body size; **Dominance:** ND - non-dominant; D - dominant; SD - super dominant; **Abundance:** R - rare; D - scattered; C - common; VA - very abundant; SA - super abundant; **Frequency:** IF - infrequent; F - frequent; VF - very frequent; Z - accidental Y - accessory W - constant. ***Predominant species** (indicators), and **Floral Visitors Classification:** P - pollinator; RS - resource stealer.

Table 2. Continued...

Bee species	Individuals	Size	Dominance	Abundance	Frequency	Constancy	Classification
<i>Xylocopa</i> (<i>Neoxylocopa</i>) <i>suspecta</i> Moure & Camargo, 1988	17	LBS	D	C	F	Y	P
<i>Oxaea flavescens</i> Klug, 1807	29	LBS	D	C	F	W	P
<i>Hylaeus</i> sp.	4	SBS	ND	R	IF	Z	RS
<i>Priloglossa</i> cf. <i>aenigmatica</i> Moure, 1945	7	SBS	D	D	IF	Z	P
<i>Augochloropsis</i> (<i>Augochloropsis</i>) <i>crassigena</i> Moure, 1943	42	SMBS	D	A	VF	W	RS
<i>Augochloropsis</i> (<i>Augochloropsis</i>) <i>wallacei</i> (Cockerell, 1900)	6	SBS	D	R	IF	Z	RS
<i>Augochloropsis</i> (<i>Paraugochloropsis</i>) cf. <i>melanochaeta</i> Moure, 1950	4	SBS	ND	R	IF	Z	RS
<i>Augochloropsis</i> (<i>Paraugochloropsis</i>) cf. <i>smithiana</i> (Cockerell, 1900)	2	SBS	ND	R	IF	Z	RS
<i>Augochloropsis</i> (<i>Paraugochloropsis</i>) <i>electra</i> (Smith, 1853)	8	SBS	D	D	IF	Z	RS
<i>Augochloropsis</i> (<i>Paraugochloropsis</i>) sp.	1	SBS	ND	R	IF	Z	RS
<i>Pseudaugochlora graminea</i> (Fabricius, 1804)	2	SMBS	ND	R	IF	Z	RS
<i>Temnosoma laevigatum</i> Smith, 1879	1	SMBS	ND	R	IF	Z	RS
<i>Xylocopa</i> (<i>Neoxylocopa</i>) <i>frontalis</i> (Olivier, 1789)	22	LBS	D	C	F	Y	P

Legend: Bee body size: SBS = small body size; SMBS = small to medium body size; MBS = medium body size and LBS = large body size; Dominance: ND - non-dominant; D - dominant; SD - super dominant; Abundance: R - rare; D - scattered; C - common; A - abundant; VA - very abundant; SA - super abundant; Frequency: IF - infrequent; F - frequent; VF - very frequent; Z - accidental Y - accessory W - constant. *Predominant species (indicators), and Floral Visitors Classification: P - pollinator; RS - resource stealer.

large body size (= heavy body mass) bee species, such as: *Bombus morio*, *Centris sponsa*, *Centris fuscata*, *Epicharis flava*, *Epicharis affinis*, *Epicharis analis*, *Xylocopa frontalis*, and *X. suspecta* are the mainly responsible for the pollination in *B. orellana*. These big body species make short visits in their foraging behavior, generally less than 1 minute (personal observation), and restricted to the first morning time (6-9h), as show in Table 1. This result is due to their large body size and heavy body mass, ability to fly quickly and ability to vibrate multiple anthers at once.

The diversity index (Shannon-Weaner) was $H = 2.63$, indicating that individuals are randomly collected from a large population. The Equitability Index was $E = 0.72$, assuming that 72% of the maximum theoretical diversity was obtained through the sampling carried out here. The analysis of seed yield data for *B. orellana* under the organic and non-irrigated cultivation system are summarized below (Table 3).

The bee foraging peak occurred between 6 am and 2 pm, decaying through the course of the day. Two classes of foraging bees were recognized based on their activity period of visiting annatto flower's: 1) from 6 to 9 am and, 2) from 2 to 6 pm (Figure 3).

In the analysis of bees to establish guilds based on their body sizes (BS) and Intertegular Distance (ITD), the Kruskal-Wallis test was used instead of ANOVA, and indicated a significant difference (p value < 0.05) between BS and ITD categories. For comparison between guilds, was applied the bilateral Dunn-Bonferroni test. Using this test, there was significant differences between the bee guilds: small body size (SBS) and medium body size (MBS); small body size (SBS) and large body size (LBS); small to medium body size (SMBS) and large body size (LBS), presenting a p -value < 0.05 in these comparisons. So, some of these body size categories are significantly different (Figure 4).

Additionally, we found that the body size (BS) and intertegular distance (ITD) of bee species, used to categorize their guilds, are positively correlated. The intertegular distance (ITD) or intertegular span (ITS) means: the shortest possible distance between the tegulae (right and left) in each individual of the bee species (Figure 5).

The most efficient bee species to the annatto flower's pollination, based in seed production were that with big body and big intertegular distances, such as: *Bombus (Fervidobombus) morio* (Swederus, 1787), *Centris (Ptilotopus) sponsa* (Smith, 1854), *Epicharis (Epicharana) flava* (Friese, 1900), *Oxaea flavescens* (Klug, 1807) and *Xylocopa (Neoxylocopa) frontalis* (Olivier, 1789) (Figure 6).

4. Discussion

Pollination is a process strongly based in mutualisms between animals and plants. The bees are the most prominent taxa of pollinators and the effectiveness of pollen grains transference to the flower stigma could be dependent to the pollinator's body size as well as to their behavior during interactions with flowers (Zych et al., 2013; Willmer and Finlayson, 2014). In specialized floral systems, such as the poricidal flowers, certain bee species

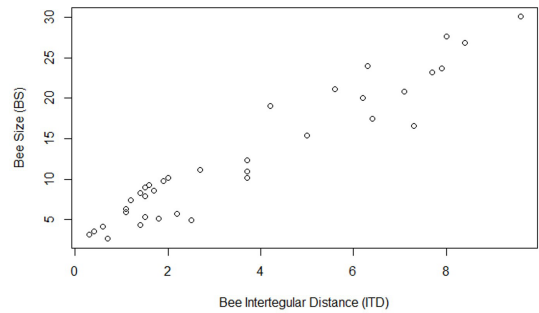


Figure 5. Relationship between two types of measurements performed on individuals of each specimen of the species belonging to the four bee guilds (Hymenoptera, Apoidea) floral visitors of *Bixa orellana* (Bixaceae). Treatments: body size (BS) and intertegular distance (ITD).

Legend: Pearson correlation coefficient = 0.9551437; $P < 0.05$; significant positive correlation, p -value $< 2.2e-16$.

exhibit behavior of vibrating, which is essential for pollen release and effective pollination (Delgado et al., 2023). These integrated characteristics highlight the importance of bees as pollinators and emphasize the complexity of plant-pollinator interactions.

Some specific morphologies and behaviors between plant species and floral visitors regulates which insects have the potential to pollinate certain plant species. In Apoidea, pollination efficiency, for many plant species, correlate to bee body size and vibratory behavior. Larger bees apply higher amplitude vibrations to the poricidal anthers, removing and transferring more pollen between flowers, which favors fruiting, even with a reduced time per visit (De Luca et al., 2019). This effect may be associated with the greater vibration force applied, since this force is proportional to the bee's body mass, according to the relationship $\text{force} = \text{mass} \times \text{acceleration}$ (Nunes-Silva et al., 2010; De Luca et al., 2019). On other hand, big bees are more likely to contact the stigma due to their size exceeding the spatial separation between the flower's sexual organs (Solís-Montero et al., 2015). Although they deposit more pollen per visit, large bees also remove large amounts, generating a cost for the plant (De Luca et al., 2019), but in the case of *B. orellana*, probably, this cost is compensated by the increase in the viable seed production.

Smaller bees may fail to reach the stigma of relatively larger flowers, such as *B. orellana* flowers (diameter 3 to 5 cm and grouped in panicles with 10 to 15 cm long) while foraging for floral resources. Even though, they transport less pollen and may have lower transfer efficiency, play an essential role in biodiversity and ecosystem stability, pollinating several plant species, including those of great agricultural and environmental importance. Limitations in pollen transfer can affect the reproductive success of plants and the natural regeneration of habitats, reinforcing the need to conserve these pollinators and their environments (Solís-Montero et al., 2015; Mesquita-Neto et al., 2021).

The pollen release rate in flowers with poricidal anthers (like annatto), depends directly on the amplitude

Table 3. Reproductive efficiency of *Bixa orellana* with flowers subjected to different methods of pollination in an orchard under the organic cultivation system, *Estância Biocenose, Fazenda Coqueiro, Dourados-MS, Brazil* (February 10 to April 14, 2018).

Treatments	Fruitification (n)	Seeds (n)	Weight (g)
Control (T1)	3.48 a	32.23 a	1.00 a
Self-pollination (T2)	0.51 bc	3.73 c	0.06 c
Anemophilous pollination (T3)	0.22 c	1.10 c	0.02 c
Bees of big body size (T4)	2.65 a	19.60 b	0.49 b
Bees of medium body size (T5)	1.55 b	6.55 c	0.20 c
Bees of small body size (T6)	1.02 bc	1.02 c	0.07 c

Legend: n: number, g: weight in grams; Data expressed as means ± standard deviation; ANOVA, followed by Tukey's test, P < 0.05; treatments with equal letters do not differ significantly.



Figure 6. The top five most efficient bee species (Hymenoptera: Apidae) to annatto flowers (*Bixa orellana*, Bixaceae) pollination and seed production in orchard under organic production system without irrigation, Dourados, Mato Grosso do Sul state, Brazil (February 10 and April 14, 2018).

of vibrations generated by the bees, which are influenced by the physical coupling between the bee and the flower, typically mediated by mouth or other body part touching the anther (Woodrow et al., 2024). In these flowers, pollen remains encapsulated within the anthers, and will be released, when specific bees perform buzz pollination (Buchmann and Hurley, 1978) or pollination by vibration. This structural adaptation of the anthers restricts pollen release, making plants highly dependent on female vibratory bees for reproduction (Vallejo-Marín, 2019). For pollen to be released from *B. orellana* flowers, it must be strongly shaken (“buzzed”) by bees (Rocha and Polatto, 2017). Larger-bodied bees release greater amounts of pollen grains (Nunes-Silva et al., 2010; Vallejo-Marín, 2019; De Luca et al., 2019), thereby increasing the likelihood of fruit production, leading to higher seed quantity and more heavy fruits (annatto capsules).

In this research, the high frequency and constancy of native bee species with large body size, such as: *Bombus morio*, *Centris sponsa*, *Centris fuscata*, *Epicharis flava*, *Epicharis affinis*, *Epicharis analis*, *Euglossa* sp., and *Eulaema nigrita* (Table 1), stood out. These bees play a significant role in *B. orellana* pollination due to their large size, heavy body mass, ability to fly quickly, and capability to vibrate multiple anthers simultaneously, as show herein.

The first bees to visit the flowers of *Bixa orellana* in the morning (06:00 am to 09:00 am) touch the anthers of the flowers and vibrate, creating a shrill noise, and causing the pollen grains to stick to their body bristles. These early bee visitors are the species with large (14mm to 20.16mm) or medium body size (10mm to 13mm). Bees of this guild visit several flowers in a short period: typically, less than one minute. In contrast, the guild of bees that forage on annatto flowers between 2:00 pm and 6:00 pm have distinct foraging behavior and morphological characteristics: visiting (collectors) have medium to small body size and do not touch the flower's reproductive structures directly, and their visiting/collecting time was considerably longer per flower (Table 2).

Research to control the access of pollinators to flowers and evaluate the outcome of different treatments on seed and fruit production is very important, because they allow to properly identifying key species in the pollination process (Klein et al., 2007). This is especially the case for plants of economic interest, such as the annatto tree. Bees are considered the most relevant group of pollinators (Shepherd et al., 2003), being predominant and the economically more important in the most geographic regions of the world (Kremen et al., 2007). In this research, during the first four collections, most of individuals of bees captured belonged to *Apis mellifera*, a trend not repeated during subsequent collections. The initial dominance of *A. mellifera* may be related to some external factors, such as the occurrence of two apiaries on the nearby (200 m to 300 m apart of the orchard), resulting in greater abundance of this exotic species, but mainly in the first four samplings, adding 81.32% of all captured individuals of the honey bee in this research. As pointed out by Kremen et al. (2007), *A. mellifera* is efficient to pollinate flowers of some crop, but inefficient to several other plant species. Several native and crop plant species depend on native bee species to

reproduce and to be productive, both in nature (native forests) and agro systems.

Annatto flowers do not have nectar but are extremely rich in pollen. This suggests that *A. mellifera*, in this case, behaved as a resource stealer. Minussi and Alves-Dos-Santos (2007), reported that *A. mellifera* is highly generalist, found in diverse kinds of habitats, including urban, agricultural or in natural forests, well preserved or in an advanced state of degradation areas. Bee species from the Andrenidae, Apidae (except *Apis*), Colletidae, Halictidae and Megachilidae families are known to carry out vibration pollination (Buchmann and Hurley, 1978; Harter et al., 2015; Cardinal et al., 2018). Pollination efficiency depends on other behavioral characteristics related to bee foraging, such as the frequency of visits to flowers (Hrncir et al., 2008; Nunes-Silva et al., 2010). This frequency to a particular plant species is correlated with the amount of pollen grains transferred to the flower stigma by insects and, consequently, higher fruit and seed productivity.

Herein, the control treatment (T1), which allowed free access to all pollination agents, beyond pollination by the wind (anemophilous) and self-pollination, presented the highest mean values of fruit formation (3.48 ± 0.42), number of seeds (32.23 ± 3.15) and seed weight (1.00 ± 0.12 g). This was expected, since in the absence of barriers and in the presence of abundant pollinating agents, the pollination process is maximum. In annatto there is a small rate of anemophilous pollination, as well as self-pollination, which were added to the work of the other pollinating agents in the field. Pollination by large bees (T4) showed the second highest efficiency in relation to free access to all pollinating agents, which in this paper are denominate Control. The treatments self-pollination (T2) and anemophilous pollination (T3) were ineffective, with means close to zero (see Table 3). The statistical overlap between the treatments T4, T5 and T6 (Table 3) suggests that additional factors, such as competition for resources or microclimatic variations in the field, may be influencing the results.

The variations in fruit and seed production in the annatto can be explained in function of the morphology and the behavior of the species bees that visits their flowers. We verified that the efficiency in the pollination of *B. orellana* is related to bee vibration behavior while touch the stamens of the flower, as well as bee body size, as demonstrated in this research. The flowers visited by species of big bees are more productive, resulting in higher abundance of fruits and more heavy seeds in compare to flowers visited by bee species of medium and small body size (Table 3), corroborant with our previous hypothesis. The fruiting rate by self-pollination and anemophilous pollination in annatto in this research was very low (0.5 for self-pollination and 0.2 for anemophilous pollination), as shown (Table 3). These results corroborate with Umadevi et al. (2020) findings, who indicated that fruit formation in annatto can occur from self-pollination but is much lower in compare to xenogamy (= crossing pollination between different individuals of the same plant species). Self-pollination generated low levels of fructification in geitonogamy (pollination between flowers of the same

plant), due to protandry (male flowers ripen earlier than female ones). Herein, we found that the natural agitation by wind of *B. orellana* flowers releases pollen grains from the poricidal anthers to the stigma of other flowers of the annatto, but in very small quantities. In this case, *B. orellana* may represent a transition between anemophilous to entomophilous pollination syndromes, as there was fruit set in all tests performed.

The large bees are more efficient pollinators of annatto flowers than medium and smaller ones. Prior to this research there was no evidence that bees with big body size (or body mass) could be associated with pollination efficiency of annatto, *B. orellana*, generating larger fruits with a larger number of seeds and, consequently, greater weight (mass) of the final product (seeds) (Table 3). There was only one initial hypothesis in previous studies (Nunes-Silva et al., 2010) about this type of service provision by bees. However, we assume that the vibration force is proportional to the size and body mass. Then, the force is equal to the mass multiplied by the acceleration (Nunes-Silva et al., 2010); therefore, bees with large body size would be more effective in releasing pollen grains from the anthers and would more probable to transfer them to the stigma. Furthermore, large bees have a higher likely of simultaneously touching the male and female organs of the flower, ensuring greater efficiency in the pollination process (Willmer and Finlayson, 2014; Delgado et al., 2023). In orchards and in the nature, the conservation of these native bee species is essential for the maintenance of these environmental services. Given the worldwide importance of the annatto and its economic value to rural producers of food and raw materials for industry, it is essential to preserve these native bee species in the Neotropical Region, reconciling rural development with biodiversity conservation.

In this study, for test if floral visitor (including pollinator) could be classified as efficient or not, other criteria were used in addition to fruiting, such as the number and weight of seeds (Table 3). To *B. orellana* cultivation there is a need for pollinating agents, preferably large native bees, with vibration behavior of the anthers to achieve success in the production of fruits and seeds and, consequently, make the cultivation economically viable of the annatto tree. Annatto production is essential to provide natural dye for the food, beverage, pharmaceutical and cosmetic industries, and is urgent the need of conservation native bee species and their habitats for the sustainable use their environmental services, making annatto production viable for rural producers in the Neotropical Region.

To establish bee guilds in researches about floral visitors, an easy parameter is to take as base, body measures of the individual of those sampled species. For this purpose, it makes no difference whether someone measure the body length (BS) of individuals of the bee species or their intertegular distance (ITD), because there is a strong positive correlation between both measurements (Figures 4 and 5). Whom for the first time explored the intertegular distance in Apoidea was Sir James H. Cane from Auburn University at Alabama, USA. He found that that measure, named by him as intertegular span (ITS) = (Intertegular distance-ITD) correlates with the dry weights of several species of

solitary female bees. Here, we found a new relationship between the bee body length (= bee body size) and their intertegular distance (ITD).

In metrics to score bee size, the use of intertegular distance (ITD) maybe prioritized. Because, as a rule, it is not possible to take these measurements simultaneously with sampling of the floral visitor species in the field. On the other hand, dead bees shrink in length, especially their abdominal segments, which may telescope over others, making dead individuals smaller in compare to living conspecifics. Therefore, ITD should be a more reliable parameter. Bee thorax rings are generally more stable and probably better reflect the size relationship between living and dead conspecifics.

In conclusion, at least 36 species of Apoidea from four families (Apidae, Andrenidae, Colletidae and Halictidae) visits annatto flowers during the two main flowering seasons (March-April and November-December) in Dourados-MS, Brazil. It's possible to establish bee guilds based on two metrics: whatever either a matter of measuring body length (BS) or measuring the shortest distance between tegulae (ITD) of individuals of the same species, as these measurements are positively correlated. The production of fruit and seed in annatto by self-pollination and by the wind (anemophilous) is very low, being inviable this cultivation in the absence of pollinator agents. The big size bees (= big body mass) are most efficient on the pollination of the annatto flowers and increase fruit and seed production. The native bee of the genera: *Bombus*, *Xylocopa*, *Centris*, *Epicharis*, and *Oxaea*, are the best pollinators of annatto, "achiote" (Spanish) or "colorau", "urucum" (Portuguese), due to their ability to carry out pollination by vibratory movements that release pollen grains from the poricidal anthers. The species *Epicharis flava*, *Centris sponsa*, *Bombus morio*, *Oxaea flavescens*, *Xylocopa frontalis* and *X. suspecta*, are the main pollinators of annatto flowers, generally forage between 6 to 9h in the morning and spend less than 1 minute in *Bixa orellana* flowers. Entomophilous pollination is essential to making annatto production economically viable.

5. Research Needs

The next steps could be to evaluate: Whether the bee species pollinators of *Bixa orellana* has life cycles: semivoltine, univoltine, bivoltine or multivoltine. Whether the diversity and species composition of bees visiting annatto flowers varies in the two main different annual flowering seasons (March-April and November-December). If the use of artificial nests, distributed on the nearby or inside annatto orchards, would influence the abundance, diversity and in the maintenance of bee species pollinators of *Bixa orellana* in the Neotropical Region.

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Data Availability Statement

The entire data set that supports the results of this study was published in the article itself.

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