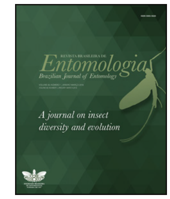
Revista Brasileira de Entomologia 68(1):e20230059, 2024

SOCIEDADE BRASILEIRA DE ENTOMOLOGIA
FUNDADA EM 1937

REVISTA BRASILEIRA DE
Entomologia
A Journal on Insect Diversity and Evolution



Living fruits of *Psychotria brachyceras* Müll. Arg. (Rubiaceae) as the main larval host of *Zygothrica orbitalis* (Sturtevant, 1916) (Diptera, Drosophilidae)

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ARTICLE INFO

Article history:
Received 26 July 2023
Accepted 29 January 2024
Available online 22 April 2024
Associate Editor: Sarah Oliveira

Keywords:
Drosophila suzukii
host plant association
larval breeding
oviposition site
trophic resource.

ABSTRACT

Zygothrica is a genus of Drosophilidae (Diptera) whose species utilize flowers and fungi for breeding sites, with records of fungi being used as courtship arena. Due to this habit, its representation in Drosophilidae surveys using banana-baited traps is generally low. However, *Zygothrica orbitalis* was well represented in a few samples with these traps. In this study, we report for the first time the breeding site of *Z. orbitalis* in living fruits of *Psychotria brachyceras* (Rubiaceae), noting that the use of living fruits is rare among Drosophilidae. The fructification of the plant occurs in the area of study from May to August, with previous collection records of the species in the Restinga (sandbank or strand) forest. Additionally, the emergence of some individuals of the invasive species *Drosophila suzukii* was observed, which highlights the necessity for continuous study of this plant to understand the dynamics between a native and an exotic species. Besides the ecological importance, our results are relevant for understanding the evolution of trophic resource use by the *Zygothrica* genus.

Introduction

Drosophilidae is a family of Diptera that is distributed worldwide and is primarily known for the genetic and evolutionary studies conducted with *Drosophila melanogaster* Meigen, 1830. Beyond genetics, however, Drosophilidae species have emerged as valuable scientific models in various biological fields. To date, there have been 4,692 species of Drosophilidae described, which are organized into 75 genera (Bächli, 2023). The genus *Drosophila* itself comprises 1,676 nominal species. There are 10 genera with species numbers ranging from 128 to 300, while the remaining genera contain fewer than 77 species each (Bächli, 2023).

Zygothrica Wiedemann, 1830a, with its 128 species predominantly found in the Neotropical Region (Bächli, 2023), is essentially mycophagous or anthophilic (Grimaldi, 1987; Gautério et al., 2020). Grimaldi (1987) notes that flowers, particularly large and fleshy ones, serve as the primary larval food resource for *Zygothrica* and some species utilize fungi as a courtship arena. More recently, Valer et al. (2016) recorded the emergence of different species of *Zygothrica* from fructification bodies of fungi. Given their feeding habits, *Zygothrica* species are only occasionally collected using standard banana-baited traps with fermented fruits, a methodology that captures preferentially species attracted by decaying fruits (Gottschalk et al., 2008).

As expected, *Zygothrica orbitalis* (Sturtevant, 1916) has been sporadically observed in surveys in various regions of Brazil. Most studies with banana-baited traps that have reported the presence of *Z. orbitalis* managed to collect only a few dozen individuals, even though tens or hundreds of thousands of Drosophilidae were sampled (De Toni et al., 2007; Gottschalk et al., 2007; Schmitz et al., 2007; Döge et al., 2007, 2008; Bizzo et al., 2010; Poppe et al., 2014; Coutinho-Silva et al., 2017; Duarte et al., 2018). Furthermore, with studies that specifically targeted Drosophilidae associated with fungi, *Z. orbitalis* was seldom captured: one specimen was collected visiting *Auricularia auricula-judae* (Bulliard) Quélet, but there are no records of *Z. orbitalis* emerging from fungi, in a study carried out by Valer et al. (2016) with 26 fungi species (Table S1).

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https://doi.org/10.1590/1806-9665-RBENT-2023-0059

We previously hypothesized that *Z. orbitalis* might be anthophilic like other species within its genus, but articles that report emergence of Drosophilidae from flowers in Brazil did not record the species, even though other *Zygothrica* species were (Santos and Vilela, 2005; Schmitz and Valente, 2019; Cordeiro et al., 2020). Santos and Vilela (2005) described Drosophilidae species associated with fallen and living flowers of Solanaceae from nine species; Schmitz and Valente (2019) investigated flowers from 125 plant species across 18 botanical families widely distributed in Brazil; and Cordeiro et al. (2020) investigated 82 plant species from 33 botanical families in Southern Brazil (Table S2).

In contrast, three studies conducted in Brazil reported a significantly higher number of *Z. orbitalis* individuals. Santa-Brígida et al. (2019) collected 140 individuals flying over the fructification bodies of fungi in the Caxiuanã National Forest in the Amazon Biome. Monteiro et al. (2014) collected 1,749 individuals using banana-baited traps in a high-altitude forest in the Atlantic Forest Biome. Finally, Mendes et al. (2017) sampled 307 specimens with banana-baited traps in a Restinga (sandbank or strand) forest in the Pampa Biome.

Considering the records of *Z. orbitalis* obtained by Mendes et al. (2017), as well as the database of Drosophilidae assemblages in the Restinga forest in Southern Brazil using traps (Valer et al., 2013; Mendes et al., 2017) or associated resources (Valer et al., 2016; Mendes et al., 2019; Cordeiro et al., 2020), we focused on native fruits in this region as a potential trophic resource. The present study aims to describe the utilization of living fruits of *Psychotria brachyceras* Müll. Arg. (Rubiaceae) by *Z. orbitalis* and discuss the ecological implications of this atypical association.

Methodology

Host Plant and Study Area

The Horto Botânico Irmão Teodoro Luis (HBITL) is an approximately 23-hectares Federal Conservation Unit of Restinga forest within the Pampa Biome, located in Southern Brazil (31°48'54"S, 52°25'48"W). *Psychotria brachyceras* is an abundant and widely dispersed understory shrub in this area, characterized by its small purple fruits and white flowers (Figure 1). The plant is endemic to Brazil, encompassing both

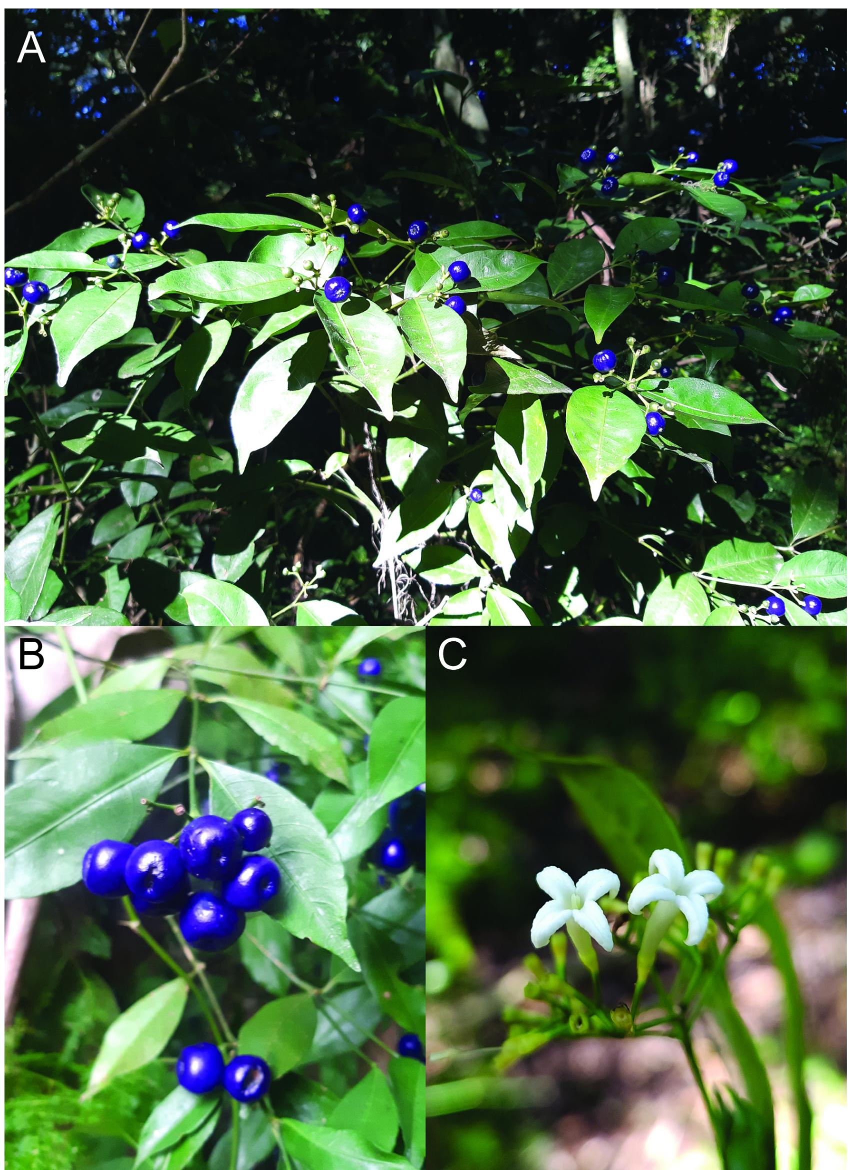

Figure 1 *Psychotria brachyceras* at the Horto Botânico Irmão Teodoro Luis (HBITL), located in the municipality of Capão do Leão, state of Rio Grande do Sul, Brazil. (A) Shrub of *P. brachyceras* bearing fruits; (B) Close-up of the ripe fruits; (C) Overall view of the flowers.

tropical and subtropical areas. It is distributed across Atlantic Forest and rainforests (Taylor and Gomes, 2015). Fewer records have been documented in the Amazon, Caatinga and Cerrado Biomes (EOL, 2023; GBIF, 2023; SIBBR, 2023). At HBITL, we observed fructification in February, with ripening starting from May to August.

Sampling

Ripe fruits of *P. brachyceras* were collected from the plant on April 14th, July 2nd, and July 6th, 2021. The ripe fruits were recognized by their intense violet color (Figure 1). After collection, the fruits were kept in three different ways for Drosophilidae emergence:

1) April 14th: Ten fruits were collected from each of 20 different sites within HBITL, with each site at least 10 meters apart from one another. Each fruit was placed individually in an autoclaved test tube containing one previously frozen fruit of *P. brachyceras* and non-hydrated Formula 4-24® Instant *Drosophila* Medium (Carolina Biological). The fruits were frozen to eliminate previous colonization. This procedure involved a total of 200 fruits.

2) July 02nd: Seven vials contained a variable number of ripe fruits (149, 207, 207, 212, 239, 249 and 322 fruits), taken from the plant, totaling 1,585 fruits. These fruits were kept in autoclaved sand. The number of fruits was obtained counting the seeds after the emergence of insects.

3) July 06th: Similar to the procedure described previously in method 1, but the vials contained only the autoclaved and non-hydrated Formula 4-24® Instant *Drosophila* Medium (Carolina Biological), without frozen fruits of *P. brachyceras*. This change in methodology was made following the observation that *Z. orbitalis* completes its development inside a single fruit. This procedure involved a total of 200 fruits.

The inclusion of nutritional supplementation (additional fruits and/or Formula 4-24® Instant *Drosophila* Medium) was conducted to optimize adult insect emergence by reducing larval competition (Grimaldi and Jaenike, 1984; Marco Silva Gottschalk, unpublished data). As the biology of *Z. orbitalis* was unknown and seemed to be very specific, we augment the trophic resource with more *P. brachyceras* fruits and/or a culture medium commonly used for breeding a large number of *Drosophila* species.

Additional pilot tests utilized autoclaved fruits or hydrated 4-24® Instant *Drosophila* Medium. However, these methods were discontinued as the autoclaved fruits fell apart, and hydrating the 4-24® Instant *Drosophila* Medium resulted in increased mold growth. Regarding the second methodology, it is one of the most commonly used procedures to study the fauna of fruit-emerging Drosophilidae, typically yielding higher richness and abundance of Drosophilidae. This method was employed to confirm the prevalence of *Z. orbitalis* in the studied plant species.

Following the emergence of the flies, the fruits were dissected, and the medium was stirred to locate insects, puparia, and larvae, which could indicate fruit colonization.

Taxonomic Identification

The Drosophilidae were identified based on their external morphology (Burla, 1956; Bächli et al., 2004; Vilela and Mori, 2014). The terminalia of some *Z. orbitalis* male were dissected following the procedures of Bächli et al. (2004) to confirm species identification according to Burla (1956). When the spiracles were intact, the puparium was also utilized for Drosophilidae identification. Voucher specimens were deposited in Museu de Ciências Naturais Carlos Ritter, UFPel, with the codes DRO-00537 to DRO-00551. Other insects were identified at the family taxa (Rafael et al., 2012).

The plant identification was conducted based on general morphology and floral characteristics. A voucher specimen of the plant is deposited in the HerbárioPel, UFPel, with the code MBM00254514.

Analysis of fruit colonization

An Excel table was organized to catalog each fruit, whether isolated or grouped in vials, along with the taxa and number of emerging insects.

The rate of fruit colonization for isolated fruits was calculated by dividing the number of fruits with Drosophilidae emergence by the total number of fruits. In some cases, the puparium was counted as “Drosophilidae colonization” without the species identification. The rate of fruit colonization was also calculated separately for methods 1 and 3, as well as for each vial used in method 2. The larvae were not counted for colonization because they could not be identified.

The incidence of each species was determined by dividing the number of colonized fruits by the total number of fruits with Drosophilidae emergence.

Image capture and processing methods

The plants were photographed in the field using a Samsung Galaxy S10e SM-G970F cell phone camera.

The adults and fruit with puparia were photographed at several depths of focus using a Zeiss Discovery V.20 photomicroscope. Also, the male terminalia structures of *Z. orbitalis*, prepared according Frech-Telles et al. (2023), were photographed at several depths of focus using a Canon EOS REBEL T3 camera coupled to an Olympus BX51 microscope with a 20x objective lens. To indicate the scale bar on the pictures, an objective micrometer was also photographed at the same magnification. All the pictures were saved at the maximum resolution in JPEG format. The pictures taken at different depths of focus were stacked into an all-in-focus composite image by the rendering method “C” (pyramid) with the minimal smoothing setting of the Helicon Focus 7.7.5 Pro lifetime software (www.heliconsoft.com).

All figures boards were created in Adobe Photoshop CS6 v. 13.0 64x and the photos of terminalia were edited to remove the background.

Results

From the 400 isolated fruits (methods 1 and 3), pupae or adult Drosophilidae were observed from 75 fruits (19%). Among these, 87% were colonized by *Z. orbitalis* (Figure 2), while about 9% were colonized by *D. suzukii* (Matsumura, 1931) (Figure 3). For the remaining colonized fruits, the determination of the species identity was not possible. From two fruits, we observed the emergence of two *Z. orbitalis* individuals, and from one fruit we observed the emergence of four *D. suzukii* individuals. In all other cases, the emergence of only one fly per fruit was observed.

The emergence of flies from grouped fruits (method 2) was either equivalent to or greater in number than the emergence from isolated fruits (Table 1). We also observed two males and one female belonging to an undetermined species of the genus *Cladochaeta* Coquillett, 1900, seven microhymenopterans parasitoids, and one neuropteran larva. If we assume that typically only one fly emerges from each fruit, the colonization rate for the samples collected on June 2nd can vary between 0.18 and 0.45. However, this estimation might be on the higher side, as instances were noted where a single fruit housed multiple larvae. Additionally, a higher mortality rate should be taken into account for samples in which the fruits were isolated in tubes.

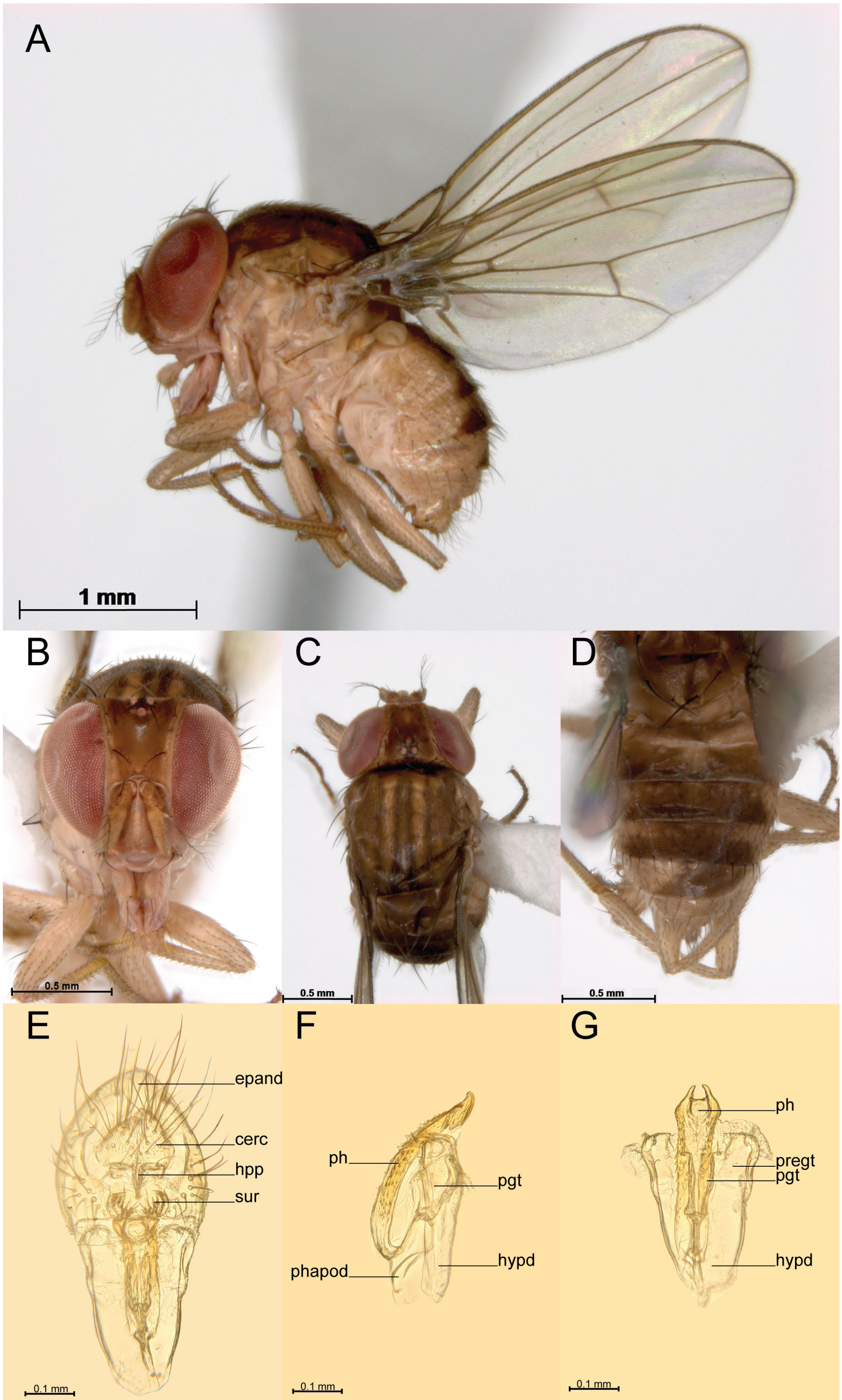

Figure 2 Male of *Zygothrica orbitalis* collected emerging from a fruit of *Psychotria brachyceras*. (A) Lateral view; (B) Frontal view of head; (C) Dorsal view of head and thorax; (D) Dorsal view of abdomen; (E) Posteroventral view of articulated periphallic and phallic organs; (F) Lateral view of hypandrium, phallus and associated sclerites; (G) Ventral view of hypandrium, phallus and associated sclerites. Abbreviations: cerc,cercus; epand, epandrium; hpp, hypoproctal plate; hypd, hypandrium; pgt, postgonite; ph, phallus; phapod, phallapodeme; pregt, pregonite; sur, surstylus.

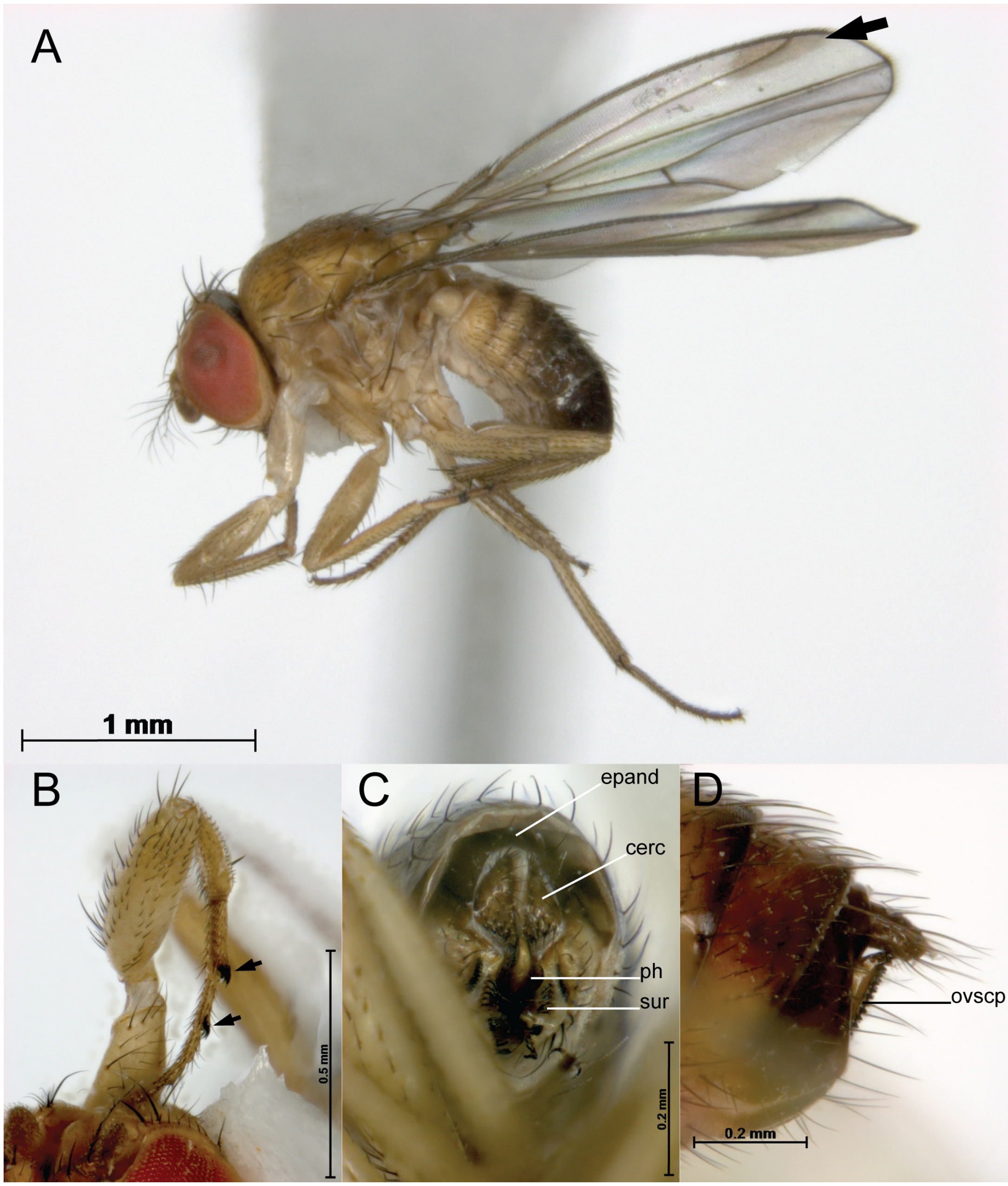

Figure 3 *Drosophila suzukii* collected emerging from a fruit of *Psychotria brachyceras*. (A) Male in lateral view (arrow indicating the dark spot at wing apex, at the intersection of veins R_{2+3} and C, typical of males of the species); (B) Detail of sex combs in foretarsus (arrows); (C) Posteroventral view of male periphallic organs; (D) Lateral view of the apex of abdomen of a female, showing the serrated oviscapt. Abbreviations: cerc, cercus; epand, epandrium; ovscp, oviscapt; ph, phallus; sur, surstylus.

Table 1
Use of *Psychotria brachyceras* fruits by Drosophilidae. Drosophilidae emerged from 200 fruits stored isolated in vials on April 14th and July 6th (*) and from multiple fruits per vial on July 2nd, 2021. The rate at which Drosophilidae colonize the fruits is indicated in parentheses.

Fruits	*Z. orbitalis*	*D. suzukii*	Total of Drosophilidae and rate of fruit colonization
200*	43	3	46 (0.23)
200*	22	4	31 (0.15)
			Including five unidentified Drosophilidae pupae, which lost the spiracles branches
149	64	0	64 (0.43)
207	51	2	53 (0.26)
207	42	0	42 (0.20)
212	41	2	43 (0.20)
239	101	6	107 (0.45)
249	45	1	46 (0.18)
322	113	8	121 (0.38)
Total= 1985	**522**	**26**	**548 + 5 pupae/ fruits (0.28)**

The fruits colonized by Drosophilidae became dry, and the seeds could be easily detached, whereas the fruits without fly colonization retained their pulp even months after collection. Some pupae were observed inside the fruits (Figure 4A), and by isolating the fruits, we were able to identify the puparium of *Z. orbitalis* (Figure 4B) due to its distinctive anterior spiracles with radially arranged branches resembling thin spines, while the anterior spiracles of *D. suzukii* have fewer radially arranged branches.

Discussion

Although *Zygothrica* has been characterized as a mycophagous and anthophilic genus (Grimaldi, 1987), there have been no observations of *Z. orbitalis* utilizing fungi or flowers as breeding sites for their larvae (dos Santos and Vilela, 2005; Valer et al., 2016; Schmitz and Valente, 2019; Cordeiro et al., 2020). Capturing *Z. orbitalis* in traditional banana-baited traps is also rare, despite numerous studies using this methodology. However, in some instances, adults of this species have been captured in high numbers flying over the fungi (Grimaldi, 1987; Santa-Brígida et al., 2019), or in banana-baited traps (Monteiro et al., 2014; Mendes et al., 2017). The highest abundance of *Z. orbitalis* was recorded in the months of July and August in Amazon (Santa-Brígida et al., 2019), Atlantic Forest (Monteiro et al., 2014) and Pampa Biomes (Mendes et al., 2017). Also, Döge et al. (2007), who analyzed samples from Atlantic Forest areas, recorded *Z. orbitalis* primarily from April to September. The sampling of *Z. orbitalis* coincided with the ripening of *P. brachyceras* fruits in HBITL from May to August aligning with the described phenology of the plant. Its fructification period ranges from May to July (Taylor and Gomes, 2015). The sampling in the Amazon, Atlantic Forest, and Pampa Biomes is in concordance with the distribution of the plant in Brazil (EOL, 2023; GBIF, 2023; SIBBR, 2023). However, two issues remain unresolved: *P. brachyceras* has only been recorded in Brazil, unlike *Z. orbitalis,* which has also been recorded in Panama (Bächli, 2023). Furthemore, the type species was collected on Taboga Island, Panama, in 1907, and initially named *Drosophila orbitalis* (Sturtevant, 1916). This suggests that the species might be capable of utilizing other plant for larval breeding. Secondly, since the fruit is not available year-round, the persistence of *Z. orbitalis* could depend on the use of other plants, or the species might undergo diapause. In *D. melanogaster*, diapause is triggered by shorter day lengths, which block vitellogenesis (Anduaga et al., 2018). Unlike *D. melanogaster*, *Z. orbitalis* may respond to longer days or higher temperatures, but this requires further investigation.

Beyond the ecological importance of the association between *P. brachyceras* and *Z. orbitalis*, this relationship is essential for understanding the evolution of the genus *Zygothrica*. Grimaldi (1987) proposed a phylogenetic hypothesis for *Zygothrica* species using morphological traits, characterizing the genus as predominantly anthophilic, and associating traits such as heavily sclerotized, narrow, and elongated oviscapes with oviposition in flowers. He suggested that mycophagy in *Zygothrica* is a retention of a primitive trait. In Grimaldi's phylogenetic hypothesis, *Z. orbitalis* was placed in a separate group (Grimaldi, 1987), suggesting that it represents a distinct lineage. Gautério et al. (2020) presented a molecular phylogenetic hypothesis in which *Z. orbitalis* was found clade together with *Z. dispar* (Wiedemann, 1830b), *Z. mesopoeyi* Burla, 1956, *Z. vittimaculosa* Burla, 1956, *Z. vittinubila* Burla, 1956, *Z. vittipunctata* Burla, 1956, and *Z. zygopoeyi* Burla, 1956, most of which are anthophilic species. This suggests that *Z. orbitalis* may have either acquired a new habit within the group or developed it in parallel with the origin of anthophilic habits. Zhang et al. (2021) presented similar results to those of Gautério et al. (2020), but with different taxonomic sampling.

Notably, *Z. orbitalis* uses living fruits, which is an uncommon feature for frugivorous Drosophilidae, as they typically utilize ripe and fallen fruits. The exceptions are *D. suzukii* and potentially *D. subpulchrella* Takamori and Watabe in Takamori et al., 2006, which can pierce and oviposit in fruits with soft skin (Atallah et al., 2014). The ability of *Z. orbitalis* to pierce *P. brachyceras* fruits warrants further investigation. Lastly, the presence of *D. suzukii* in *P. brachyceras* fruits raises questions regarding competition between species, as this fruit is the only known breeding site for *Z. orbitalis*. *Drosophila suzukii*, an exotic species first recorded in South Brazil in 2013 (Deprá et al., 2014; Mendes et al., 2017), is considered a pest due to its ability to use non-rotten fruits for rearing larvae by piercing soft-skinned fruits. *Drosophila suzukii* is polyphagous (Poyet et al., 2015), a trait that contributes to the species' successful dispersion into different areas and complicates management strategies. Poyet et al. (2015) also described the ability of *D. suzukii* to utilize fruits from various plant families and to establish itself in natural ecosystems and gardens. Given that *Z. orbitalis* and *D. suzukii* did not emerge from the same fruit and that the number of *D. suzukii* emergence was lower, direct competition between the species seems

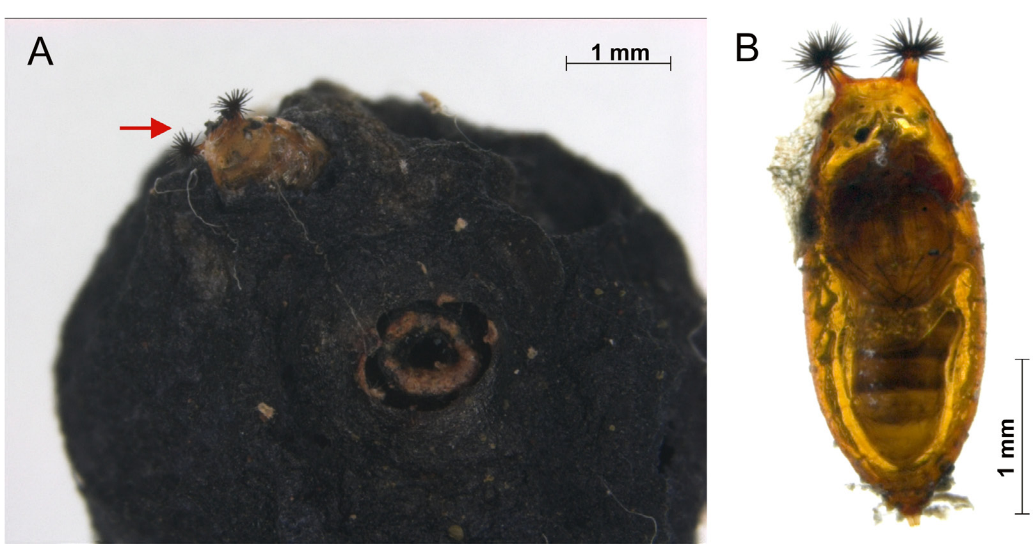

Figure 4 Unopened puparia of *Zygothrica orbitalis*. (A) Red arrow pointing to puparium in *Psychotria brachyceras* fruit; (B) Dorsal view.

unlikely. However, long-term monitoring is essential to understand the potential competitive interaction between these species.

This study revealed that the fruits of *P. brachyceras* are predominantly colonized by the Drosophilidae species *Z. orbitalis*, and occasionally by *D. suzukii*. The ability of *Z. orbitalis* to utilize living fruits for breeding suggests a specific ecological niche and an uncommon behavior among frugivorous Drosophilidae, while the presence of *D. suzukii*, an exotic species, raises questions about potential competitive and ecological impacts in the long term, emphasizing the need for ongoing monitoring and further research.

Acknowledgments

The authors are grateful to the anonymous reviewers for their valuable comments and to the area editor for their editorial guidance, both of which have greatly contributed to the improvement of this manuscript.

Funding

Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) supported this work (process 309186/2022-6).

Conflicts of interest

The authors declare no conflicts of interest.

Author contribution statement

MFM and MLB designed and performed the experiments, species identification and writing manuscript. MSG: supervision and Drosophilidae species identification. RL: plant species identification. MFM, MLB and MSG: Reviewing and Editing. All authors have read and agreed to the published version of the manuscript.

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Supplementary material

Supplementary material accompanies this paper.

Table S1 - Associations between fungi and Drosophildae specimens documented in studies conducted in Brazil. Fungi, refers to the taxonomical identification of fungi; Reference, indicates the bibliographical reference where the record is documented; Collection Methodology, specifies whether the adults were collected with a sweep net or entomological aspirator (Flying Over), or were collected upon emergence from mushrooms used as breeding sites (Emergence).

Table S2 - List of species of plants sampled in Brazil which had flowers examined by the Drosophilidae emergence. *Zygothrica orbitalis* were not recorded emerging from any sample.

This material is available as part of the online article from https://doi.org/10.1590/1806-9665-RBENT-2023-0059