

The first record of conopid fly *Zodiomyia sumbaensis* as a parasitoid of a reed bee *Braunsapis mixta*

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Abstract

Bees play a crucial role in pollination, ensuring the reproduction of many flowering plants, but there is growing concern over the decline of bee populations worldwide. Female conopid flies are parasitoids that lay eggs in the bodies of aculeate Hymenoptera for example bees, and the resulting larvae develop as internal parasites and kill their host. While some aspects of conopid parasitism have been studied, there remains a lack of comprehensive understanding of the interactions between conopids and their host bees. The present study investigates the association between *Zodiomyia sumbaensis* Camras (Diptera Conopidae), and its host bee, *Braunsapis mixta* (Smith) (Hymenoptera Apidae) with a focus on the seasonal incidence of parasitism. Over a 24-months observation period, a total of 1067 puparia of *Z. sumbaensis* were collected in the bee nests of *B. mixta*. Percent parasitism and adult emergence rates of *Z. sumbaensis* were high during the monsoon season. The molecular characterization of *Z. sumbaensis* was carried out using the mitochondrial Cox-1 gene, providing valuable data for molecular identification. The study also reports the occurrence of hyper-parasitoid emergence holes in a few puparia. To the best of our knowledge, this is the first confirmed report of *Z. sumbaensis* as a parasitoid of *B. mixta* in India and elsewhere.

Key words: cashew, conopid, *Braunsapis mixta*, endoparasitoid, Apidae, *Zodiomyia*.

Introduction

Several anthropogenic factors have been associated with declining bee populations; parasites and pests are among the factors that can have a considerable impact on bee populations. Natural enemies of bees, which can occur in the nest brood and on free-flying adult bees may regulate the bee populations through top-down effects (Roulston and Goodell, 2011). The interactions of parasitoids with its host bee populations are poorly studied compared to other host insect taxa, such as Lepidoptera and Coleoptera (Malfi *et al.*, 2018). Recently, Vanitha *et al.* (2022) recorded adults of *Neochalcis breviceps* (Masi) (Hymenoptera Chalcididae) and *Diomorus indicus* Ahmad (Hymenoptera Torymidae) as larval parasitoids attacking *Braunsapis mixta* (Smith) (Hymenoptera Apidae). *B. mixta* a stem-nesting native bee, reported as an important pollinator of cashew plants (*Anacardium occidentale* L., Anacardiaceae) (Vanitha and Raviprasad, 2019) in India, and its nesting biology has been documented (Vanitha *et al.*, 2022). Up to 30 nest mates including nine females were found in a single nest. Unlike honey bees, limited information is available on the distribution, bioecology, foraging behaviour, and ecological impacts of natural enemies on several native bee populations.

In the present investigation, the interaction between *B. mixta* and a parasitoid, *Zodiomyia sumbaensis* Camras (Diptera Conopidae) is documented. The conopid flies lay their eggs on or in the bodies of other insects, mostly bees and wasps (Smith, 1966; Schmid-Hempel, 2001; Skevington *et al.*, 2010) and also on cockroaches and crickets (Freeman, 1966; Gibson and Skevington, 2013)

and parasitize them. Conopid flies comprise 863 species worldwide (Stuke, 2017). Female conopid flies typically use an in-flight attack system to assault bees (Stuke, 2017) and lay eggs. The larvae develop as internal parasitoids and eventually kill the host (Pouvreau, 1974; Muller *et al.*, 1996). The development of the larval parasitoid takes about 10-12 days after oviposition, during which the host is killed, and the parasite pupates *in-situ* (Smith, 1966; Pouvreau, 1974). In temperate species, the parasitoid hibernates where its host dies, and pupation takes place outside the nest (Smith and Cunningham-van Someren, 1970; Schmid-Hempel and Muller, 1991).

Z. sumbaensis belongs to the subfamily Zodioninae with *Zodiomyia* Camras as a monotypic genus. Within Zodioninae, only three genera are currently known: *Zodion* Latreille, *Parazodion* Krombein and *Zodiomyia*. *Z. sumbaensis* is widely distributed in the Oriental Region, with records from Sri Lanka, India, Malaysia, Indonesia, and Thailand (Stuke, 2019). *Physocephala* spp. (Conopidae) have been recorded on various bee genera, such as *Anthidium* F., *Anthophora* Latreille, *Apis* L., *Bombus* Latreille, *Centris* F., *Eucera* Scopoli, *Euglossa* Latreille, *Eulaema* Lepeletier, *Halictus* Latreille, *Megachile* Latreille, *Psithyrus* Lepeletier, and *Xylocopa* Latreille (Melo *et al.*, 2008; Santos *et al.*, 2008). Though, there is a mention of *B. mixta* as a host for conopid flies in Vanitha and Raviprasad (2021), the species identify of the conopid fly was not stated in it.

Literature on *Z. sumbaensis* includes a short original description with morphological details by Gibson and Skevington (2013), faunistic data by Camras (1966) and Gibson and Skevington (2013), and a recent record with

images by Stuke (2019) but with no information on host insects or its biology. This study reports the association of *Z. sumbaensis* with *B. mixta* and provides information on its seasonal incidence.

Materials and methods

Study site and sample collection

The artificial bee nests developed for *B. mixta* (Vanitha and Raviprasad, 2021) consisted of thin bamboo canes having a node in each, and the small wooden blocks with drilled dead-end tunnels (holes) kept on ant - well stands (figure 1) are maintained at the bee conservation park of Indian Council of Agricultural Research - Directorate of Cashew Research (ICAR-DCR), Puttur, Karnataka, India. The nests were observed for incidence of parasitoids from October 2021 till September 2023. The study site is a hilly area situated between the west coast and the Western Ghats of India, located at 12.77°N and 75.22°E, with an average altitude of 87 m. The temperature in the area varies between 16.0 °C (minimum) and 40.0 °C (maximum). The relative humidity ranges from 43 to 98%, with levels typically above 90% from June to November months. In general, the region experiences heavy rainfall

during the southwest monsoon between June and September, with a mean annual rainfall of 3970 mm. The rainfall received during the study period was also typical of this rainfall pattern (figure 6).

Periodical cleaning of the bee nests was attended to get rid of spider webs and bee debris in the nests. Approximately, a total of 1650 nests were available for the occupation of bees, and we were sure that at any given time, at least 50% of the nests were always occupied by the bees. We are uncertain of the actual number of bees present in a nest at a given time, as it requires split opening of the nests, a destructive sampling. The presence of a guarding bee at its nest entrance made it easy to make a count on the occupied nests.

During the period of 2020-21, inspections of the trap nests revealed the presence of brown-coloured puparia outside the nest entrances (figure 2a), along with dead *B. mixta* bees. These puparia were likely ejected outside the nest by the adult *B. mixta* during its regular nest cleaning activities. Upon careful observation of intact trap nests, it was noted that some of the nests of *B. mixta* having narrow entrances were blocked by the puparia (figure 2b), which the bees were unable to push outside. A few puparia seen intact inside the abdomen of the dead *B. mixta* bees (with its lateral segments opened) confirmed them



Figure 1. Artificial nests for *B. mixta* (insert: natural nest of *B. mixta* in a dried cashew stick, and a foraging bee moving towards its nest entrance).

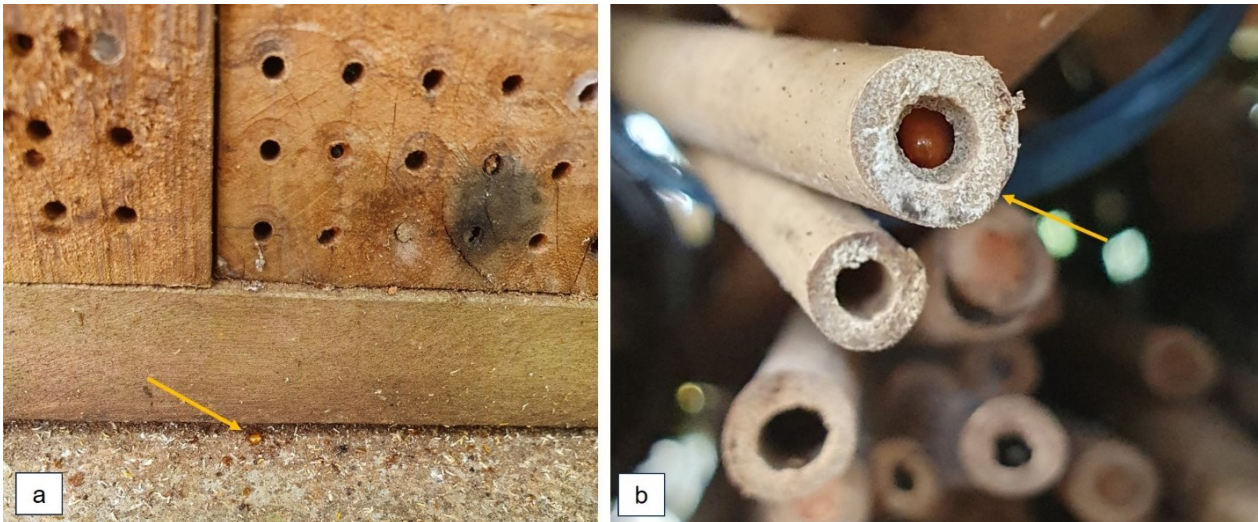


Figure 2. Puparium of *Z. sumbaensis*. **a)** outside the artificial bee nest (arrow), **b)** at nest entrance (arrow).

as parasitoids of *B. mixta*. The puparia found outside the nests were collected at varying time intervals of 4 to 8 days using a fine camel hairbrush, individually transferred into glass test tubes, and taken to the laboratory (N = 1067). The length and width of puparia were measured using a digital caliper (Mitutoyo make) (N = 35). The emerged conopid flies were collected and preserved in 70% ethanol. A set of 171 flies were examined for sex ratio. The specimens were identified as *Z. sumbaensis* following the redescription by Stuke (2019). Voucher specimens are deposited at ICAR-National Bureau of Agricultural Insect Resources, Bengaluru, India.

Molecular characterization

The specimens of *Z. sumbaensis* were subjected for molecular characterization using the mitochondrial cytochrome c oxidase I (mtCOI) gene. Genomic DNA was extracted from the adult individuals using a modified CTAB method (Thube *et al.*, 2022). PCR reactions were performed to amplify partial COI gene sequences with a 25 µl reaction volume, comprising 2.0 µl of genomic DNA (100 ng), 1.0 µl each of the forward (LCO1490 5'-GGTCAACAAATCATAAAGATATTGG-3') and reverse (HCO2198 5'-TAAACTTCAGGGTGACCAAAAATCA-3') primers (Folmer *et al.*, 1994) (10 pmol/ml), 1.0 µl of 10 mM dNTPs, 4.0 µl of 10X PCR buffer containing MgCl₂, 1.0 µl of DyNAzyme II DNA Polymerase (2 U/µl), and 15.0 µl of molecular biology-grade water (Thermo Fisher Scientific, USA). Thermocycling (Bio-Rad T100) consisted of an initial denaturation at 94 °C for 3 minutes, followed by 30 cycles of denaturation at 94 °C for 20 seconds, annealing at 50 °C for 30 seconds, extension at 72 °C for 30 seconds, and a final extension at 72 °C for 5 minutes (Thube *et al.*, 2018). The amplified product was evaluated with electrophoresis using a 1.0% agarose gel (Sambrook and Russell, 2001). Polymerase chain reaction (PCR) purification (Bioline Meridian Bioscience, Bioline GmbH, Luckenwalde, Germany) was performed, and the purified products were subjected to Sanger sequencing (Agrigenome Pvt. Ltd., "SmartCity Kochi", Infopark Road, Kakkanad, Kerala, India).

Seasonal incidence and emergence pattern of conopid flies

Regular inspections were conducted in the trap nests for the presence of conopid puparia for two years from October 2021 till September 2023. A minimum of five collections was done every month, and the data were pooled for monthly observations. The dead bees found outside the trap nest floor surface which were intact but not parasitized were also collected between January 2023 and September 2023 to understand the extent of natural mortality of the bees (N = 525) and the male to female ratio. In the laboratory, the puparia were kept at room temperature (24-30 °C) and observed daily for the emergence of adult conopid flies. The puparia were examined using Nikon stereo zoom microscope (SMZ 745T model) attached with 5 MP microscope camera (UC501S model) using VIMAGE 2016 software to check for adult emergence, any signs of parasitism, or pupal dryness. The time interval between the collection of puparia and the emergence of the parasitoids was recorded to understand the pupal development period, but the exact date of parasitism was not known.

Data analysis

Descriptive statistics was done in XL Miner Analysis ToolPak in excel and the mean values are expressed as Mean ± SD throughout the text. Based on the number of non-emerged puparia from the total collection, the percentage of adult emergence was calculated. But the proportion of parasitism could not be determined as the total number of bees present in each trap nest could not be recorded without destruction of nests. As the study site is in a high rainfall zone area, we assessed the emergence rate of adults in relation to rainfall received per month and the number of rainy days using correlation and regression analyses. The rainfall data has been recorded on-site from the institutional meteorological observatory, but the data on temperature and relative humidity was not fully available for the observation period.

Results

Diagnostic features of puparia of *Z. sumbaensis*

A total of 1067 *Z. sumbaensis* puparia was collected outside the trap nests between October 2021 and September 2023. The puparia variable in sizes were observed along with dead adults of *B. mixta* outside the nest entrances (figure 2). Most of the puparia were found on the basal frame of the nest structure, while a few remained just outside the nest entrance without falling on the basal frame. These oval-shaped puparia displayed two prominent spiracles at the distal end. The posterior spiracles were convex apically, not forming a plate. Initially, the

freshly formed puparia were orange in colour with blackish spiracles, later turning orange-brown, and eventually appearing dark brown toward adult fly emergence (figure 3a). The length of the puparia ranged from 2.34 to 3.50 mm (mean = 2.92 ± 0.30 mm, N = 35) and the breadth varied from 1.77 to 2.67 mm (mean = 2.09 ± 0.28 mm, N = 35) (table 1). In the emerged puparia, excluding the dorsal flap seven segments were clearly visible. Puparia were easily visible inside the parasitized dead bees as the abdominal segments of bees were ventrally opened with puparia projecting out (figure 3b). The observations indicated that only one puparium could successfully develop and pupate within a bee.



Figure 3. Puparia of *Z. sumbaensis*. **a)** puparia of conopid flies, **b)** pupa inside bee abdomen, **c)** final larval instar of *Z. sumbaensis* dissected out of *B. mixta*, **d)** emergence of *Z. sumbaensis* adult.

Table 1. Morphometrics of *Z. sumbaensis* puparium, adult emergence, and sex ratio.

Details	Mean \pm SD	Range (Min-Max)
Puparium length (mm) (N = 35)	2.92 ± 0.30	2.34 - 3.50
Puparium width (mm) (N = 35)	2.09 ± 0.28	1.82 - 2.67
Number of puparia collected/month	63.18 ± 32.18	10 - 163
Days to adult fly emergence from the day of collection (days) (N = 171)	14.14 ± 8.56	1 - 29
Adult emergence over months (%)	39.23 ± 24.81	9.09 - 88.00
Sex ratio of adult flies emerged (N = 171)		1.45

Among the puparia found outside the nests which were transferred by the nest mates of the bees, 94.5% were devoid of any bee body parts on them, while the rest had parts of bee abdominal portions attached to it. Further examination revealed that all the parasitized bees were female. Out of the 525 intact dead bees (unparasitized) collected outside the trap nests between January 2023 and September 2023, 94.09% were female bees, suggesting a dominance of females in *B. mixta* nest population compared to its males. Logistic regression analysis indicated an insignificant relationship between the size of conopid pupae (length and breadth) and the adult fly emergence ($\chi^2 = 3.68$, $P = 0.15$). Similarly, there was no significant relationship between size of pupae and emergence of female and male flies ($P = 0.99$ and 0.14 , respectively).

On February 11, 2021, when an immobilized female bee collected outside the nest was dissected out under laboratory conditions, a fully grown third instar conopid larva was observed (figure 3c). The fly larva occupied the entire abdominal hemocoel of the bee, with its head turned towards the thorax and the spiracles turned towards the last abdominal segments of bee, indicating the final larval instar. It measured 3.3 mm in length and 2.5 mm in breadth, appeared whitish with two black posterior spiracles. It turned into a whitish puparium within a day when kept separately in a Petri plate. The puparium was orange in colour on the first day and gradually darkened. After completing the pupal stage, the adult *Z. sumbaensis* emerged after 23 days by creating an incision on the top of the cranial portion of the puparium (figure 3d).

Diagnostic features of adult *Z. sumbaensis*

The adult flies were small, blackish brown, with hyaline wings and white patterns on their legs (figure 4). They are characterised by the presence of elongated pedicel; short arista, only about as long as the first flagellomere; hind femora thickened, especially in females; female flies have a prominent theca, which is a typically forward-pointing thumb-like protuberance. Molecular characterization of

adult flies was carried out using the mitochondrial Cox-1 gene. The obtained sequences were aligned using BioEdit (Biological sequence alignment editor - Tom Hall, <http://www.mbio.ncsu.edu/BioEdit/bioedit.html>) and blasted in the NCBI (<http://www.ncbi.nlm.nih.gov/>) as well as the BOLD database (<http://www.boldsystems.org/>). Sequence similarity was analysed with the available sequences, and the sequences were deposited in NCBI. The GenBank accession number obtained was ON040904. Among the successfully emerged conopid flies, female flies were found to be dominant (59.65%), and the male to female ratio is 1:1.45.

Seasonal incidence and adult emergence of *Z. sumbaensis*

The number of puparia collected per month was less than 50 in trap nests from November 2021 till April 2022 (figure 5). More than 70 puparia were collected every month from May 2022 till August 2022, which coincided with the rainy season of the study region (figures 5, 6). During the month of January 2023 alone, a total of 162 puparia were collected, and the numbers gradually declined during the subsequent months. The number of rainy days per month had a non-significant relationship with the number of puparia collected in that month. There was a significant influence of number of rainy days per month on the emergence of *Z. sumbaensis* ($N = 24$, $r = 0.44$, $P = 0.05$). Number of rainy days in the corresponding month showed significant relationships with the collection of puparia ($F = 0.05$, $P = 0.0007$), and the emergence of *Z. sumbaensis* adults ($F = 3.4$, $P = 0.04$). The number of rainy days in the previous month showed non-significant relationships with the collection of puparia ($F = 0.0005$, $P = 0.98$), and the emergence of *Z. sumbaensis* adults ($F = 2.41$, $P = 0.13$). Similarly, the amount of rainfall received per month had non-significant relationship with the number of puparia collected and the number of adult fly emergence in that particular month ($P \geq 0.05$).

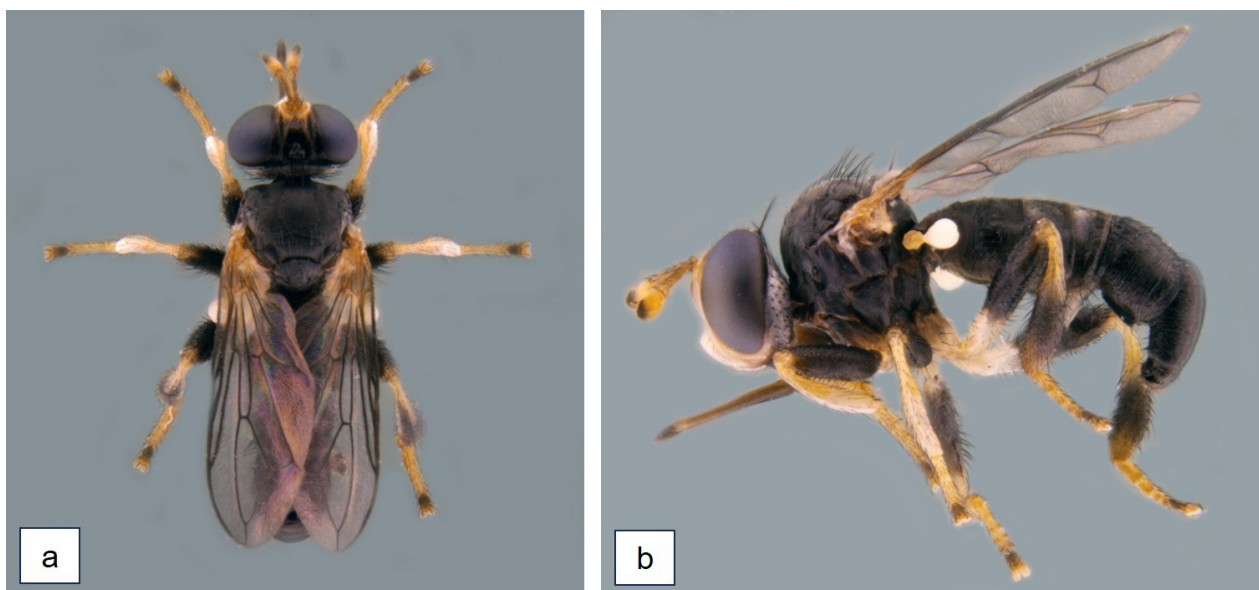


Figure 4. *Z. sumbaensis* adult. **a)** dorsal view and **b)** lateral view.

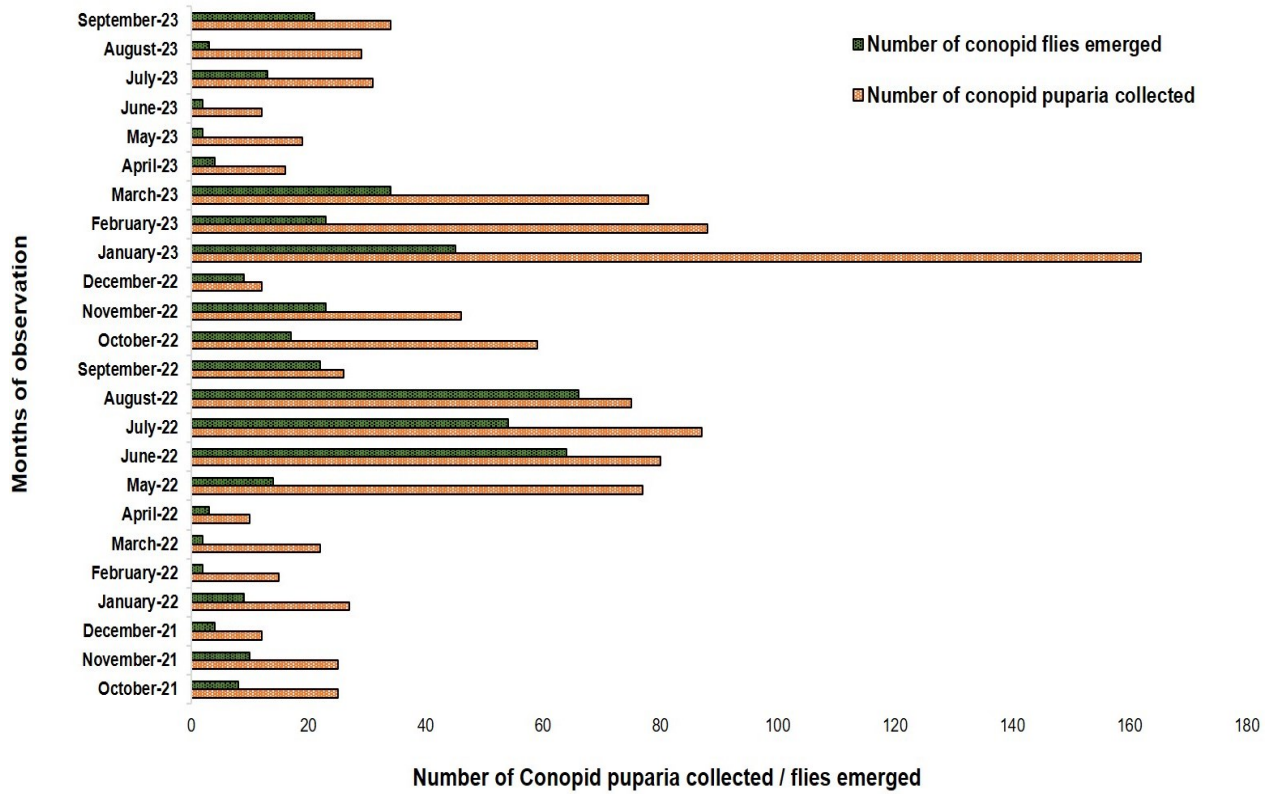


Figure 5. Month-wise number of conopid puparia collected and number of adult conopid flies emerged.

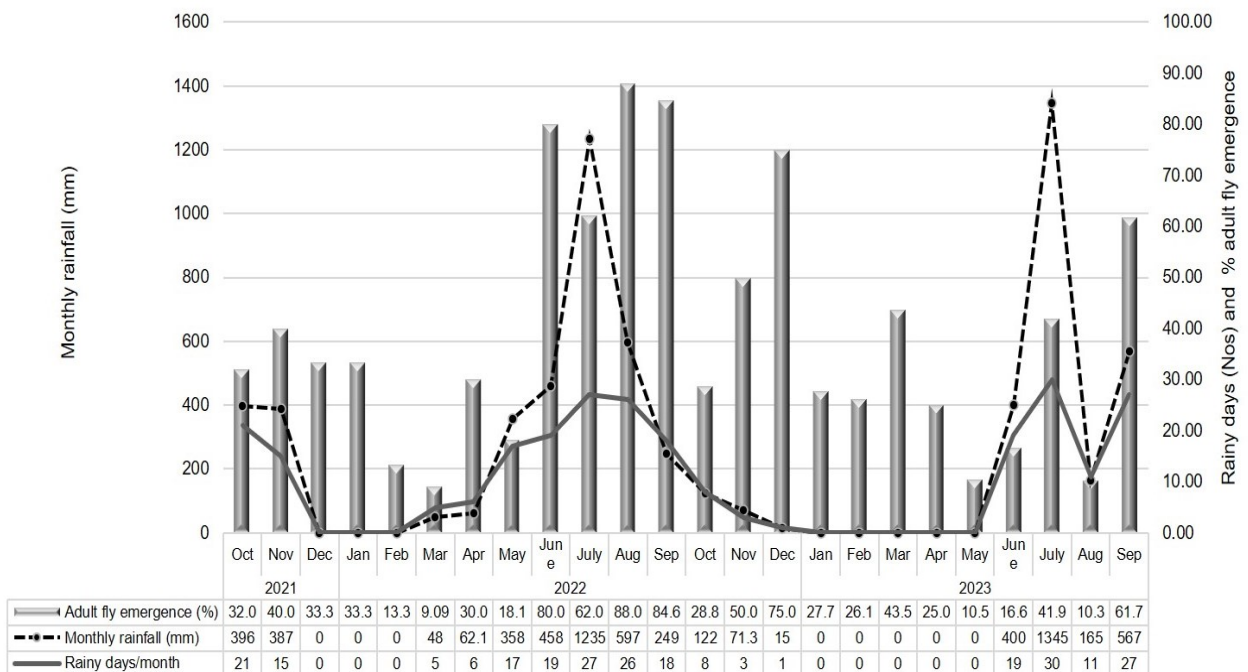


Figure 6. Month-wise percent adult fly emergence of *Z. sumbaensis*, rainfall and number of rainy days.

Out of 1067 puparia collected, 454 flies emerged successfully, indicating 39.23% adult emergence. The percentage of adult emergence was lowest (9.09%) during March 2022, but highest (88.00%) during August 2022. From October to May 2022, adult emergence was less than 40 %, except during December 2022 (figure 6). Out of the 99 non-emerged puparia examined under a microscope, 14 puparia had a hole without any contents inside, while others were dried up. These holes are indicative of emergence of hyperparasitoids, and further observations are needed to get more insights on its relationship.

The frequency distribution of the adult emergence rate of *Z. sumbaensis* over 24 months of observations showed that out of the total puparia collected every month, adult fly emergence was less than 50% in 17 months, while more than 75% adult emergence was observed in three months (June, August, and September 2022) (figure 7). The interval between the collection of *Z. sumbaensis* puparium and adult fly emergence ranged from 1 to 29 days during the observation period. Most of the flies emerged within 20 days of collection, with a mean of 14.14 ± 8.56 days.

Discussion

In this study, observations indicated that only one puparium of *Z. sumbaensis* can develop successfully inside *B. mixta* female. This is in line with Salt (1961) and Schmid-Hempel and Schmid-Hempel (1996), who indicated that due to intra and inter-specific competition in conopids, only one larva is able to develop successfully in a host. In case of larger bees like *Bombus* sp. and *Eulaema* sp., adult conopid fly emerged through the parasitized bee's body as the conopid puparia remained inside the bee abdomen (Freeman, 1966; Rasmussen, 2009), whereas in *B. mixta*, adult fly emergence is not through the parasitized bee's body as the nest mates have thrown out the intact puparia and the body parts of dead bees outside the nests.

With our observations, it is difficult to determine whether conopid flies can discriminate between already parasitized bee hosts and unparasitized ones. It was also hard to identify the parasitized live bees among the foraging bees that returned to the nest as they all appeared normal without any behavioural differences. This is in support of Muller (1988) and Heinrich and Heinrich (1983), who suggested that bees behave normally until the parasite larva has reached its last stage. The size of puparia of *Z. sumbaensis* is smaller compared to *Physocephala* spp. for example, the maximum length of puparia of *Z. sumbaensis* was 3.50 mm, while it was 10 mm in *Physocephala* sp. (Lucia *et al.*, 2010); 10.3 mm in *Physocephala wulpi* Camras (Stuke *et al.*, 2011) indicating species specific differences. Similar to *P. wulpi*, the puparia of *Z. sumbaensis* were oval with two prominent spiracles at the distal end with a convex apex (Stuke *et al.*, 2011).

The puparium of *Z. sumbaensis* had its head alignment towards the thorax of *B. mixta* and the spiracles towards the last abdominal segments, indicating the beginning of the fly pupation as reported by Abdalla *et al.* (2014). Similar to the findings of Malfi *et al.* (2014) in *Bombus* spp.,

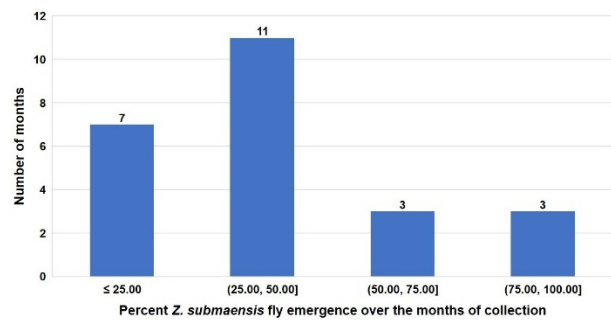


Figure 7. Frequency distribution of percent adult emergence of *Z. sumbaensis* over 24 months.

the adult flies of *Z. sumbaensis* that emerged from bigger puparia were slightly bigger in size. The time taken for a freshly formed *Z. sumbaensis* puparium to develop as an adult was 23 days only, while it was longer (32 days) in *Physocephala* sp. (Schmid-Hempel and Schmid-Hempel, 1990). Like in *P. wulpi* (Stuke *et al.*, 2011), the emerged puparia of *Z. sumbaensis* had seven visible segments. Adult emergence pattern of *Z. sumbaensis* is found similar as recorded in *Physocephala rufipes* F. (Cumber, 1949), *Zodion cinereum* (F.), *Physocephala tibialis* (Say) where adult fly uses its ptilinum (ptilinal sac) to emerge from its puparium and utilizes its legs and mouthparts as levers to pry itself from the host's body (Polidori *et al.*, 2005; Gibson *et al.*, 2014). Similar to the observations made on *Centris analis* (F.) (Moure-Oliveira *et al.*, 2019), the parasitized bees remained inside the nest cavities itself until their death. In *Bombus* spp., self-burial behaviour has been reported when parasitized by *P. tibialis* (Malfi *et al.*, 2014). This self-burial behaviour appears remote in *B. mixta* as large number of the parasitized dead bees and the fly pupae were found at the trap nests itself.

If we consider that half of the total nests were occupied by the bees and a mean of 7.0 individuals were found per nest as reported by Vanitha *et al.* (2022), then a total of 5,775 individuals would have present in the trap nests at a given time. At a sex ratio of 1:1, one can expect 2,887 females would develop in them. As 1,067 puparia were collected over a period of two years a parasitism percentage of 36.97 has been noticed. However, we are not sure whether all the parasitized bees died in the trap nests; hence in-depth studies by dissecting the nests and the female bees found in the nest shall help to understand the extent of parasitism. Earlie, higher rate of 60 to 80% parasitism in free flying bumble bees by the Conopids were reported (Schmid-Hempel and Schmid-Hempel, 1990; Gillespie, 2010; Malfi *et al.*, 2014), which might be due to the low level of host defence against conopid parasitoids involving cellular defence reactions as reported by Salt (1968), Schmid-Hempel and Schmid-Hempel (1996; 1989).

Observations on the monthly collection of *Z. sumbaensis* puparia revealed that parasitism rate peaked earlier in the year 2022 compared to 2023. Though uncertain, this difference could be attributed to the unseasonal heavy rains observed during April-May 2022, whereas there was no rain during April-May 2023 and very less rain

during the late May 2023 in the study region. A similar type of variation in parasitism between years was observed with *Stylogaster neglecta* Williston at the University of Toronto at Mississauga, where the parasitism rate in late July 2018 was higher than the parasitism rates found in July 2017 (Etzler *et al.*, 2020). The percentage of adult flies successfully emerging from the puparia also varied between months. According to Malfi *et al.* (2019), survival of the conopids during its larval stage, was strongly influenced by the daily background mortality rate (BMR) (i.e. sources of death other than conopids) applied to the host population, where, at 11% as the BMR only 22.7-29.8% of conopids survived, but using 3% as BMR, 50.4-62% of conopids survived. Out of 99 non-emerged puparia observed, 14 were with a hole in each, possibly indicating the emergence hole of hyperparasitoids. In *Physocephala* sp., occurrence of *Pediobius williamsoni* Girault (Hymenoptera Eulophidae) as hyperparasitoids was reported (Burks, 1966), but we need to document the hyperparasitoid species occurring on *Z. sumbaensis*.

Conclusion

The study provides important insights into the association between *Z. sumbaensis*, a conopid fly, and its host bee, *B. mixta*. The research has documented variation in parasitism rates and adult fly emergence across different months. Furthermore, the presence of hyperparasitoid emergence holes in some non-emerged puparia warrants further investigation. The successful molecular characterization of *Z. sumbaensis* adds valuable data to aid in future molecular identification and ecological studies. Overall, the findings of this study contribute to our understanding of the dynamics between conopid flies and their host bees, shedding light on present parasitism rates. Further studies are required to understand the implications of Conopids on *B. mixta* population and to explore the means for conservation of this important native bee.

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The authors declare that they have no competing interests relevant to the content of this article.

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