Sociobiology
An international journal on social insects

RESEARCH ARTICLE - BEES

Foraging specificity of *Tetralonia* (*Thygatina*) *macroceps* (Hymenoptera: Apidae: Anthophorinae) on *Argyreia cuneata* (Convolvulaceae)

Amala Udayakumar, Raghavendra Anjanappa, Kesavan Subaharan, Timalapur M Shivalingaswamy

Division of Germplasm Conservation and Utilization, ICAR-National Bureau of Agricultural Insect Resources (NBAIR), Hebbal Bengaluru, Karnataka, India

Article History

Edited by
Evandro Nascimento Silva, UEFS, Brazil
Received 25 May 2022
Initial Acceptance 07 February 2023
Final Acceptance 17 April 2023
Publication date 29 June 2023

Keywords
Argyreia cuneata, electroantennogram, foraging specificity, *Tetralonia macroceps*, volatile organic compounds.

Corresponding author
Amala Udayakumar
Division of Germplasm Conservation and Utilization, ICAR-National Bureau of Agricultural Insect Resources (NBAIR)
HA Farm Post, PB No 2491, Hebbal Bengaluru 560024 Karnataka, India.
E-Mail: amala.uday@gmail.com

Abstract

Floral specificity is a behavior that evolved due to mutualistic interactions between the plant-pollinator community. Flowers advertise themselves using visual or chemical cues to attract pollinators and gain reproductive success through pollination. Pollinators forage for rewards such as nectar or pollen produced by the flowers. We found that an anthophorid bee, *Tetralonia macroceps,* foraged specifically on *Argyreia cuneata* flowers. No visitation was observed on the flowers of *A. nervosa* though both belong to Convolvulaceae. *T. macroceps* was the most abundant floral visitor (5.21 bees/flower/5 min) on *A. cuneata* and did not visit *A. nervosa*. Mass flowering and narrow tubular flower structure with easy access to pollen in *A. cuneata* were the traits that accounted for the foraging specificity of *T. macroceps*. The present study investigates the preference of *T. macroceps* for the flowers and floral extracts of *A. cuneata* and *A. nervosa*. The bee visited 10.16 flowers/5 min of *A. cuneata*. *T. macroceps* were highly attracted to the flowers of *A. cuneata*. No bees were attracted to *A. nervosa*. The floral abundance of *A. cuneata* was relatively higher compared to *A. nervosa*. Pollen analysis of foraging bees of *T. macroceps* revealed the selective preference towards the pollen of *A. cuneata*. The highest number of bees preferred the extract of *A. cuneata* (7.75) compared to *A. nervosa* (0.50) in the Y-olfactory maze. Floral extract of *A. cuneata* caused the highest neuronal electroantennogram (EAG) response (1.48 mV) than *A. nervosa* (0.36 mV). Our preliminary studies indicated the presence of specific volatile organic compounds (VOCs) nonacosane (13.26%), hexatriacontane (12.06%), and beta farnesene (6.19%) observed in *A. cuneata* were absent in congener *A. nervosa*.

Introduction

Pollination is a mutualistic process vital for the reproductive success of many species of angiosperms (Mayer et al., 2011). Although some plant species rely on wind or water to transfer pollen from one flower to the next, the vast majority (almost 90%) of plant species need the help of animals for this task (Ollerton et al., 2011). Pollination was also reported to be mediated by vertebrates, such as birds, bats (Nathan et al., 2009; Subramanya & Radhamani, 1993), small mammals, and invertebrates, including flies (Sajid & Saeed, 2010), beetles (George et al., 1989), butterflies, moths (Sheppard & Oliver, 2004) and bees (Klein et al., 2007). The major share of pollination in plants is through bees (73%), followed by flies (19%), bats (6.5%), wasps (5%), beetles (5%), birds (4%), and butterflies (4%) (Abrol, 2009). Flowering plants and insect pollinators can form examples of a mutualistic relationship (Grass et al., 2018) wherein the flowers advertise themselves using visual or chemical cues to attract pollinators and gain reproductive success through insect visitation and pollination (Leonard et al., 2014; Chittka & Raine, 2006). In return, pollinators benefit mainly from the

nectar and pollen the flowers produce (Bronstein et al., 2006; Douglas, 2008). Visual cues in terms of flower color trigger the behavior of insects (Renoult et al., 2014; Larue et al., 2016). Volatile cues emanating from the flower play a major role in driving the behavior of both diurnal and nocturnal pollinators and ensuring flower visitation and pollination (Fenster et al., 2004; Raguso & Willis, 2005; Riffell et al., 2008). The pollen grains were also reported to emanate volatile odors that trigger a specific behavioral response from the bees (Dobson et al., 1996).

An anthophorid bee, *Tetralonia* (*Thygatina*) *macroceps* Engel & Baker 2006 (Apidae: Anthophoridae), was noted selectively foraging on the purple flowers of *Argyreia cuneata* (purple morning glory) (Convolvulaceae). This species belongs to a small group of bees confined to peninsular India and Africa (South of the Sahara) and is currently considered a subgenus of *Tetralonia* (Michener, 2000). *Tetralonia* bees were reported to be the second most abundant genus of bees in cotton fields in Zambia and Burkina in Africa (Stein et al., 2017). The flower visitation of the bee *T. fraterna* in the flowers of *Abelmoschus esculentus* (Malvaceae), *Ipomoea prescaprae,* and *I. arborescens* (Convolvulaceae) in Africa was reported by Pauly (1984). The flower visitation by *T. fraterna* increased the proportion of ovules resulting in an increased number of seeds and longer fruits in okra at Maroua, Cameroon (Ela et al., 2012). *A. cuneata* and *A. nervosa* are perennial vines that flower from March to October (Shivalingaswamy et al., 2020). *A. cuneata* has an endemic distribution in peninsular India, whereas *A.nervosa* has a global distribution (Indo-Malayan, China, and Mauritius). Both plant species are used in the Indian system of medicine (Ayurveda) and folklore medicines. *T. macroceps* is a buzz pollinator. Investigating its foraging specificity will aid in conserving this interesting bee species and its most preferred plant species, having medicinal value in an ecosystem.

The floral volatiles might be one of the factors influencing the selective forage preference of *T. macroceps* only towards *A. cuneata* in addition to floral abundance and pollen preference. The profiling of volatile organic compounds in the flowers of *A. cuneata* will provide probable information for its attraction towards *T. macroceps* compared to *A. nervosa*. The floral scent and visual cues synergistically attract *Tetralonia* bees for pollination (Spaethe et al., 2007). Modifying the key volatile components of flowers would play a vital role in the rate of visitation by a specific pollinator resulting in an enhanced pollination rate and reproductive success of crop plants (Parachnowitsch et al., 2012).

The present study was conducted to understand the flower visitors and floral traits of *Argyreia* spp, the foraging behavior of the dominant flower visitor (*T. macroceps*), and its preference for floral scents through electrophysiological responses in the antennae. The holistic data on the foraging behavior, influence of floral abundance, and pollen preference supported by behavioral preference and profile of volatile organic compounds will allow an understanding of the factors driving the selective foraging of *T. macroceps* in *A. cuneata* compared to *A. nervosa*.

Materials and Methods

Study site

The study was carried out at the experimental farm of the ICAR-National Bureau of Agricultural Insect Resources (NBAIR) Bengaluru, Yelahanka Campus (13.096932N, 77.56 759E), which is located within an urban landscape of the south Indian state of Karnataka. There are two patches of pollinator gardens at the study site. The first patch of the approximately one-acre area contains over 50 species of plants belonging to diverse families (trees, shrubs, herbs, and climbers), including two major foraging plants, *Argyreia cuneata* and *A. nervosa*. The remaining area (approx. 16 acres) is covered with orchard blocks (mango, sapota, cherimoya, guava, pomegranate, fig, lemons.) and blocks meant for seasonal crops (finger millet, pigeon pea, horse gram, *Dolichos* bean, and wide range of vegetable crops).

Foraging behavior of bees in *Argyreia cuneata* and *A. nervosa*

A. cuneata and *A. nervosa* were planted in 2012 in the pollinator garden. These plants were bushy creepers with over 100 flowers in bloom daily. Five plants of each species were taken for observation. From each plant, twenty-five flowers were considered for recording the observations regarding the number of flower visitors on *A. cuneata* and *A. nervosa* under open field conditions during the peak blooming period (June to August) for two consecutive years, 2016 and 2017. The observations on common flower visitors, number of bees/flower/5 min, number of flowers visited by dominant flower visitor, and time spent by the bee per flower were recorded visually by two observers from 7 am till 6 pm. Twenty-five fully opened flowers were marked and observed for the activity of flower visitors in *A. cuneata* and *A. nervosa* periodically, as given above. The common flower visitors collected in the study site were studied before they were taxonomically identified using keys and deposited in the National Insect Museum of ICAR-NBAIR, Bengaluru. The number of bees/flower/5 min was recorded. The number of flowers visited by the dominant flower visitor/5 min and the time spent by bee/flower were recorded. The bee's body diameter (width) was measured using a Metric flat-scale ruler. The inner diameter of the flowers of *A. cuneata* and *A. nervosa* was measured (Shaara, 2014). The different crops in bloom (crops and weeds) were observed daily for any foraging of *T. macroceps*.

Floral abundance in *Argyreia* spp.

The floral abundance was estimated by recording the total number of open flowers in the five vines by multiplying the mean number of open flowers per vine by the mean number of flower buds per individual vine (Stang et al., 2006). The floral abundance and the number of floral visitors in *A. cuneata* and *A. nervosa* were recorded for ten days.

The average floral abundance and the number of floral visitors in both *A. cuneata* and *A. nervosa* were worked out.

Pollen profile analysis of the bee, *Tetralonia macroceps*

Pollen analysis by acetolysis

Five reference slides were prepared from each sample of reference plants using the acetolysis method of Erdtman (1960). Slides were prepared using 5 µL of pollen pellet solution from the acetolysis process, and glycerin jelly was used as a mounting medium. Then, the slides were examined using a compound microscope set at 400x total magnification.

Analysis of pollen collected by *T. macroceps*

Twenty foraging bees of *T. macroceps* in the flowers of *A. cuneata* were hand-collected using collection tubes and kept in a freezer for 5 minutes. The pollen found in the hindlegs and body hairs of the anesthetized female bee was collected using a camel hair brush in an Eppendorf tube. The pollen analysis was done using the acetolysis method. The percentage of each pollen type was analyzed by counting 200 grains along two transects across the slide at a magnification of 400x. Pollen types representing less than 5% of the total count were excluded and considered contamination. For pollen analysis, 200 pollen grains per sample were counted, and the relative frequencies (percentages) of each pollen type were established by following the method proposed by Zander (1935). The identity of the pollen grains carried by the bee was compared with the pollen library of various plants on the campus. The results were analyzed in terms of pollen type dominance as D = dominant pollen (> 45%), A = accessory pollen (15% - 45%), and I = isolated pollen (3% - 15%).

Behavioral bioassays

Flight experiments to study the preference of *T. macroceps* for flowers of *A. cuneata* and *A. nervosa*

The flower preference (free-choice) of *T. macroceps* was studied in meshed cages (30x30x30 cm). A bunch of flowers of *A. cuneata* and *A. nervosa* was provided to the bees. Cut ends of the floral branch were placed in a vial with water to maintain the flower turgidity during the studies. Ten female bees were collected from the field 30 min before the start of the experiment. The bees were starved for 30 min before the beginning of the experiment with water satiation in a glass tube (100 cm^3), and the setup was placed at room temperature (25 ± 2 °C). The bees were released into the meshed cage one at a time to study their preference for landing. The time taken by the bee to land and the handling time by bees in flowers were recorded (Vibina & Subaharan, 2019).

Y-tube experiments to study the preference of *T. macroceps* for floral volatiles

The scent preference of *T. macroceps* towards the floral volatiles of *A. cuneata* was studied in a Y-tube olfactometer set up with an airflow of 0.5ml/min in the laboratory (Fig 1). Following different odor sources were compared for their preference,

- Flower of *A. cuneata* and Flower of *A. nervosa*
- Dichloromethane (DCM) alone and DCM extract of *A. cuneata*
- DCM alone and DCM extract of *A. nervosa*
- DCM extract of *A. cuneata* and DCM extract of *A. nervosa*

Flowers of *A. cuneata* and *A. nervosa* were placed one flower per arm in the two arms of the Y-tube to study the odor preference of the *T. macroceps*. About 1 µl of Dichloromethane (DCM) alone and DCM extract of *A. cuneata*, Dichloromethane (DCM) alone and DCM extract of *A. nervosa* and blank solvent (DCM) and DCM extract of respective flowers (*A. cuneata* and *A. nervosa*) were kept in the two arms of Y - tube to study the odor preference of *T. macroceps*. Ten female bees were collected carefully from *A.cuneata* by closing the flower as soon as the bee entered the flower for foraging. Ten bees were released one by one, and the respective odor preferred by the bee for probing was observed. The arms were cleaned using ethyl alcohol and air-dried after each run. The experiment was repeated fifty times. One set of ten bees was used for only a single replication. The number of non-participating bees in the behavioral assay was also recorded (Vibina & Subaharan, 2019).

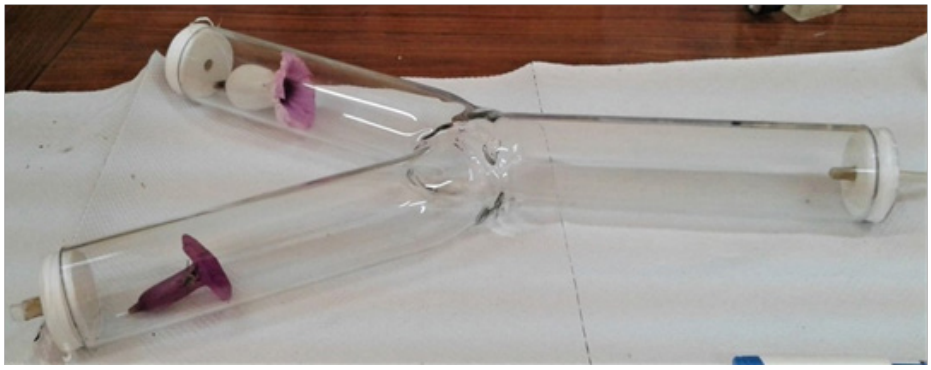

Fig 1. Y-tube olfactometer set up to study odor preference by *Tetralonia macroceps.*

Headspace sampling of volatiles from flowers of *Argyreia* spp.

The volatile compounds of *A. cuneata* and *A. nervosa* were collected by the dynamic headspace sampling technique for further analysis. The flowers were collected from the plants maintained at the pollinator garden of ICAR-NBAIR Yelahanka Campus and transferred to the 1L Erlenmeyer flask. Three replicates were maintained with three flowers per replicate. An air entrainment apparatus trapped the volatile organic compounds (VOCs) released from flowers. A gentle stream of air was pulled in through an activated charcoal cartridge at 30 ml/min, which was allowed to pass over the sample in a flask. This volatile saturated air was trapped in a glass tube containing absorbent Tenex 30 mg with glass wool on either side as stoppers. The VOCs were trapped for six hours, and the trapped volatiles in the adsorbent were eluted using HPLC-grade dichloromethane (400 µl) and condensed to 200 µl by passing a gentle stream of ultra-high pure nitrogen. The vials containing extracts were stored at -20^0 C until further analysis (Vibina & Subaharan, 2019).

Characterization of headspace volatiles from flowers of *Argyreia* spp.

One µl each of the condensed samples was injected into Gas chromatography (Agilent) GC- 7890A (G3440A) and MS-G3171A, and the column used was HP 5 MS Phenyl methyl siloxane capillary column (30 m × 250 µm i.d. × 0.25 µm film thickness, Agilent Technologies, USA. The temperature of the column and oven was maintained at 40ºC for 1 min and then gradually increased at the rate of 20ºC per minute to 280º C and held at 300º C for 10 min. The injector and column temperature were regulated at 250ºC, and the total run period was 23 min. The flow rate of Helium (Carrier gas) was maintained at 1 ml per min. An electron ionization (EI) mode with 70 eV ionization energy was used for the GC-MS identification. The components in EO were identified by retention time and mass fragmentation pattern using the NIST library (Vibina & Subaharan, 2019). Major constituents in the sample were verified by co-injecting the compounds. Tricosane (99% purity) and heneicosane (98% purity) sourced from Sigma Aldrich were co-injected.

Electroantennography (EAG) for olfactory stimulation of *T. macroceps*

The responses of bees to olfactory stimuli were measured through Electroantennograms (EAG) by using an electroantennographic system (Syntech, Hilversum, The Netherlands) consisting of a dual-electrode probe for antenna fixation, a CS-05 stimulus controller, and an IDAC 232 box for data acquisition. A dual-electrode probe (EAG Combi-probe, Syntech) was used, and the proximal tip was connected to the recording electrode, and the scape was connected to the indifferent electrode. The antenna was fixed using Spectra 360 conductive gel (Parker, Orange, New Jersey) (Reinecke et al.,2005). The antenna was then flushed continuously with a stream of activated charcoal-filtered air to keep the air free of any volatiles. The headspace volatiles trapped from the flower was eluted using HPLC-grade dichloromethane (400 µl) and condensed to 200 µl by passing a gentle stream of ultra-high pure nitrogen. From this 200 µl, 10 µl was taken and used for EAG though the whole amount may not represent the total elute from a particular trapping period, qualitatively, it represents the composition as a higher volume of elute use in EAG will cause quick desensitization of the insect used. The trapped volatile compounds (10 µl) were applied on filter paper strips (Advantec 5C (110 mm) Japan, 3 cm length and 5mm diameter) and placed into the microtip pipettes (Tarson 100 – 1000 µl). This was connected to the stimulus controller by the Tygon rubber tube. After 10 sec, the solvent (HPLC-grade dichloromethane) was blown out with the first puff, and 60 sec later, the stimulus was puffed onto the antenna by injecting the vapor phase of the microtip pipette 15 mm upstream from the antenna in the continuous air stream (pulse time 0.5 sec, continuous flow 25ml/sec, pulse flow 21 ml/sec). The minimum delay between the stimulus puffs was 20 sec. Antennal responses to aliquots (extracts of *A. cuneata* and *A. nervosa*) were recorded with Ten new field-collected female bees as replicates (ten antennae). The EAG responses (mV, mean ± SD) were expressed as a summated response of neurons, sorted according to shape and amplitude emitted 1 sec after the onset of the stimulation (Vibina & Subaharan, 2019). A blank solvent (dichloromethane) served as a control in EAG studies.

Statistical analysis

The EAG response data of bee's antennae to volatiles from the headspace of flowers elute was pooled, the number of visitations made between the treatments and time differences was statistically analyzed by one-way analysis of variance (ANOVA), and the means were subjected to Tukey's test (SPSS software 12.0). Time spent by the bees in the respective arm containing the odor source was recorded.

Results

Foraging behavior of bees in *A. cuneata* and *A. nervosa*

The foraging activity of the bee, *T. macroceps,* was observed only from March to October, coinciding with the bloom of *A. cuneata* (Fig 2). Interestingly, the bee never preferred foraging on the flowers of congener, *Argyreia nervosa* (Hawaiian Baby Woodrose) (Fig 3). The flowers of *A. cuneata* were found to be visited by an array of bee visitors (bees/flower/5 min), such as *Tetralonia macroceps* (Total number of bees 240; mean 5.21 bees; range 0-8 bees), *Apis dorsata* (Total number 60; mean-1.30 bees; range 0-4 bees), *A. florea* (Total number 52; mean 1.13 bees; range 0-4 bees), *Ceratina hieroglyphica* (Total number 66; mean 1.43 bees; range 0-4 bees), *Amegilla zonata* (Total number 51; mean 1.10 bees; range 0-3 bees), *Xylocopa* sp. (Total

number 58; mean 1.03 bees; range 0-2 bees) and *Megachile* sp. (Total number 46; mean 0.83 bees; range 0-4 bees) (Fig 4). *T. macroceps* was the most abundant flower visitor (5.21 bees/flower/5min) on the flowers of *A. cuneata*. The average time spent by *T. macroceps* in the individual flower of *A. cuneata* was 1.53±0.63 sec. It was observed that the bees collected the pollen reward with the branched hairs of their abdomen and hind legs within this brief period spent in the flower. On average, *T. macroceps* visited 10.16 ± 1.60 (range 8-12 flowers) flowers of *A. cuneata* in five min. *A. nervosa* flowers were visited by only two species of bees *viz*., the leafcutter bee, *Lithurgus atratus,* and the large carpenter bee, *Xylocopa fenestrata* (Fig 5). The observations recorded on the entire set of flowering plants in the study site revealed that *T. macroceps* was never observed to forage on any other flowers. The number of *L. atratus* and *X. fenestrata* that visited the flowers of *A. nervosa* was 1.21 (Total number 37; range 1-3 flowers) and 1.80 (Total number 24; range 0 -3 flowers), respectively. The average time spent by *L. atratus* and *X. fenestrata* in the flower of *A. nervosa* was 2.26 and 1.33 sec, respectively. We recorded the body size in terms of the width of the *Tetralonia* bee (5mm) and compared the size of the flower openings of both *A. cuneata* (10 mm) and *A. nervosa* (15mm). The distance between the petal's tip and the anther cone's tip is relatively lesser in *A. cuneata* (13 mm) compared to *A. nervosa* (20 mm). The bees entering the flowers of both the species of *Argyreia* were observed to carry a copious amount of pollen on the branched hairs of their body parts.

Fig 2. Flower of *Argyreia cuneata* and *Tetralonia macroceps* entering the flower (Right).

Fig 3. Flower of *Argyreia nervosa* side and dorsal view.

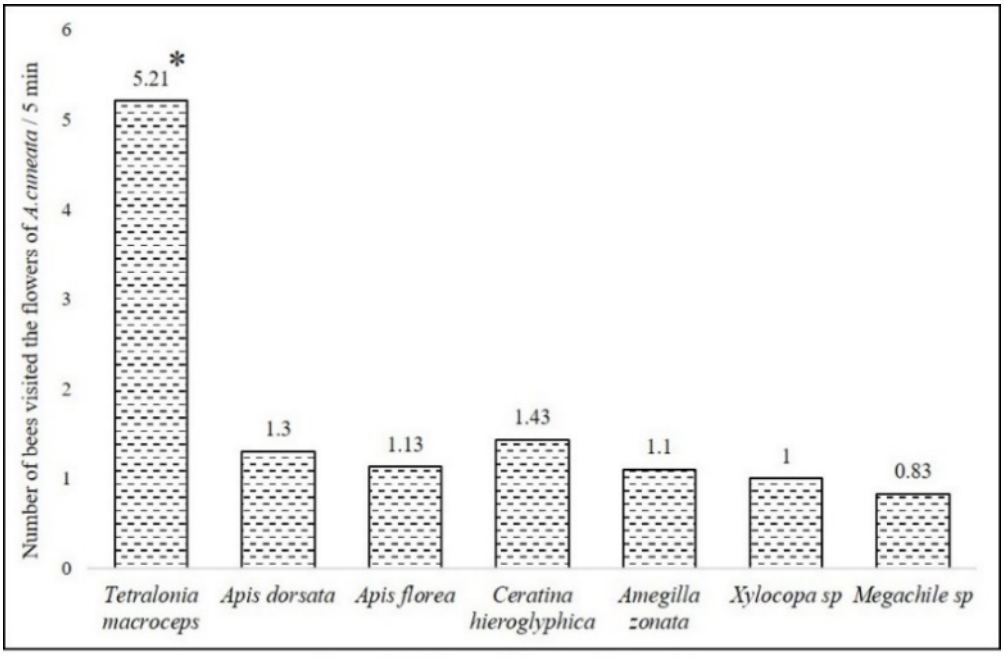

Fig 4. Flower visitors of *Argyreia cuneata.*

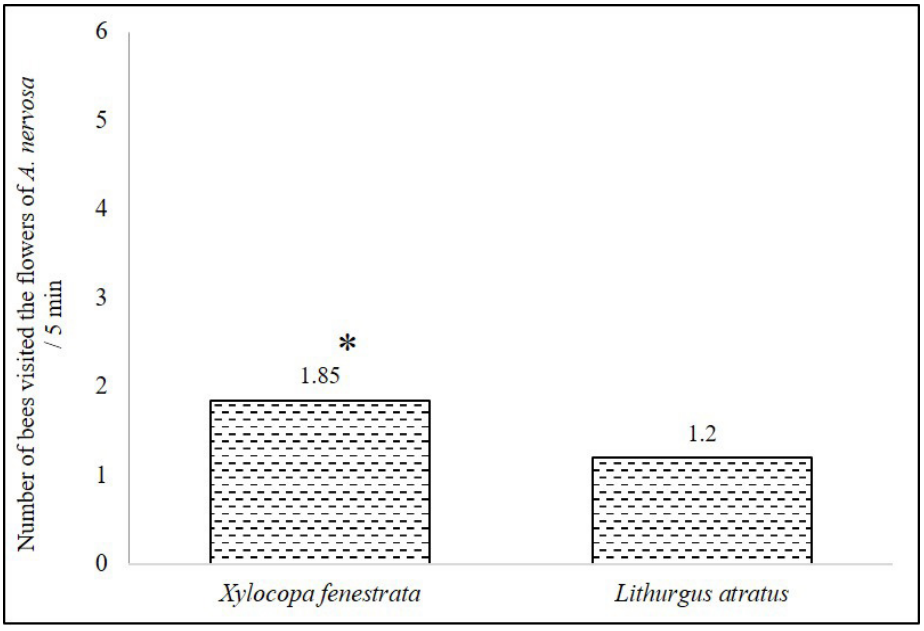

Fig 5. Flower visitors of *Argyreia nervosa.*

Influence of floral abundance on flower visitors

The total number of flowers available for floral visitors in *A. cuneata* across the ten flowering days ranged from 240 to 1073, with an average floral visitor of 5.00 ± 0.94 bees/day (total number = 55). Whereas the number of flowers available for flower visitors in *A. nervosa* across ten flowering days ranged from 64-989 (average of 275.60 ± 27.66) with an average floral visitor of 0.80 ± 0.63 bees/day (total number 8) (Fig 10, 11).

Pollen preference of *T. macroceps*

The pollen analysis of the foraging bees of *T. macroceps* revealed the presence of the pollen grains of *A. cuneata* alone. No other pollen grains were observed in the pollen load carried by the foragers of *T. macroceps*. Since the nests of *T. macroceps* remained untraceable by the authors, the brood pollen analysis was not carried out.

Behavioral bioassay

Flower preference of *T. macroceps* towards the flowers of *A. cuneata* and *A. nervosa*

In the meshed cages study, the bee took 30 min on average to approach the flower of *A. cuneata* and spent an average of 5.17 minutes on the flower. *T. macroceps,* after getting oriented towards the flower of *A. cuneata,* entered and rested inside the flower for a few seconds. There was a significant difference in the preference of bees towards the flowers of *A. cuneata* and *A. nervosa* in the Y-tube olfactometer assays (F value = 47.80; P < 0.0001) (Fig 6). On average, 6.20 bees moved toward the flowers of *A. cuneata*. None of the bees preferred to move towards the flower of *A. nervosa.* The non-participating bees in the Y-tube assay were 3.80.

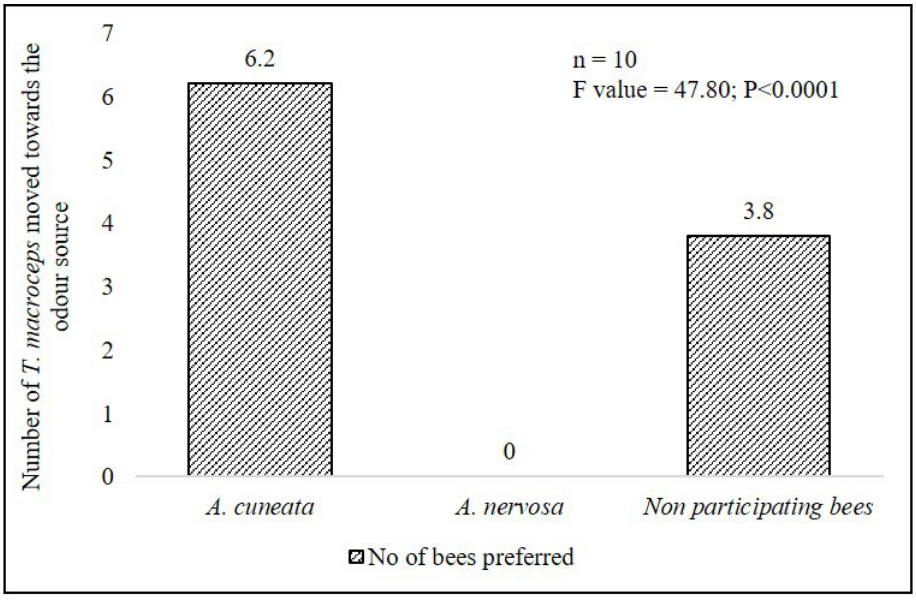

Fig 6. Preference of *Tetralonia macroceps* towards the flowers of *Argyreia* spp.

Preference of *T. macroceps* to floral volatiles of *A. cuneata* and *A. nervosa*

The preference of bees towards the dichloromethane (DCM) extracts of *A. cuneata* and solvent alone was also studied using a Y-tube olfactometer. The number of bees those preferred DCM extracts of *A. cuneata* was the highest (6.25) compared to the DCM (1.25) (Fig 7A) (F value = 14.77; P < 0.0001). The number of non-participating bees was 2.50. The lowest number of bees (0.75) preferred DCM extracts of *A. nervosa* and non-participating bees were 9.25 (Fig 7B) (F value = 35.70; P < 0.0001). The bees attracted to *A. cuneata* were found to land over the flower, and a few were observed to enter inside the flower. Moreover, in the choice studies using the solvent extracts (DCM) of both the flowers, *T. macroceps* highly preferred the DCM extract of *A. cuneata* (7.75 bees) compared to *A. nervosa* (0.50 bees) (Fig 8) (F value = 44.66; P < 0.0001). The results indicated the role of floral scent in attracting *T. macroceps* towards *A. cuneata*.

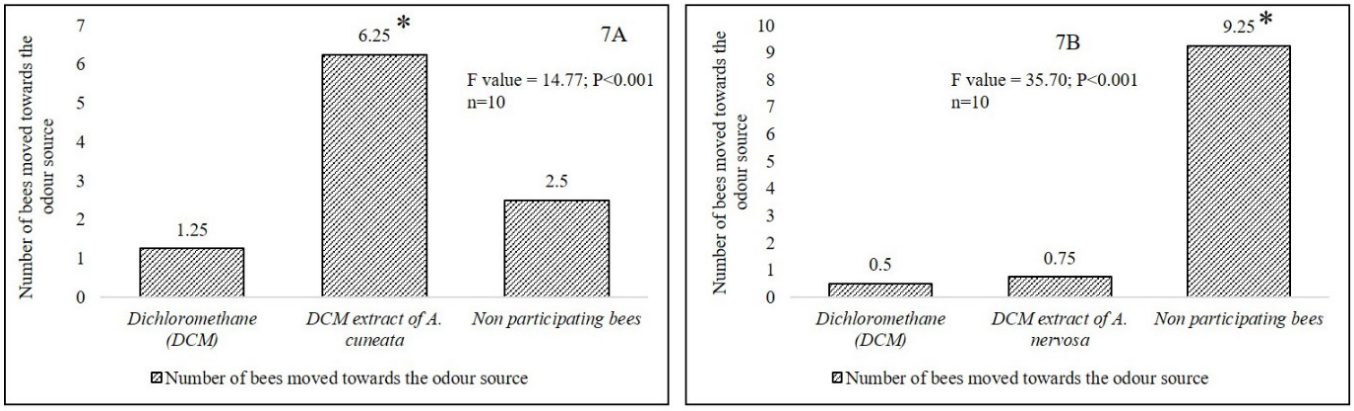

Fig 7. Preference of *Tetralonia macroceps* towards the DCM extracts of *A. cuneata* (7A) and *A. nervosa* (7B) in Y-tube olfactometer.

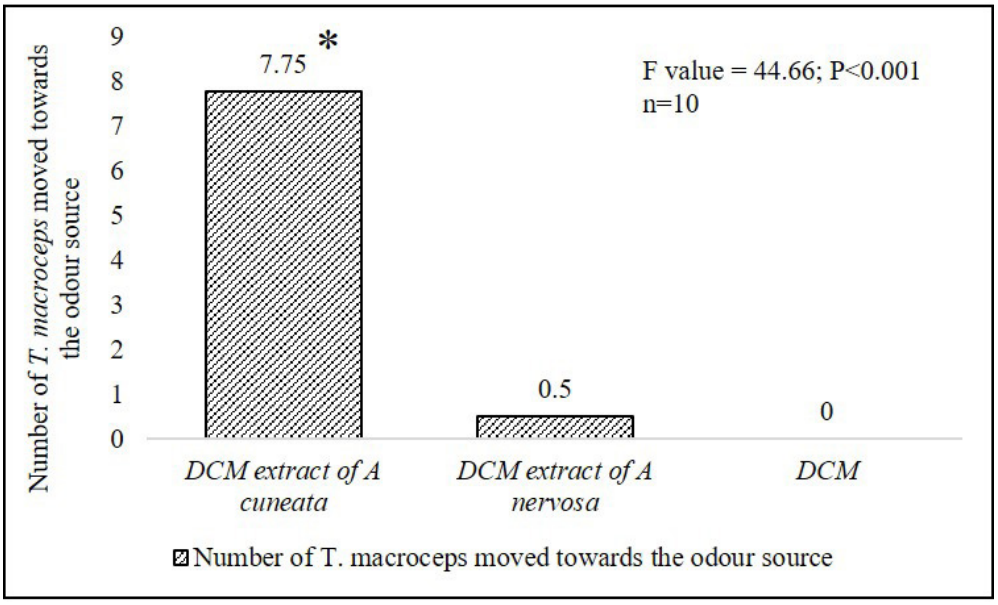

Fig 8. Preference of *Tetralonia macroceps* towards the odour source of two floral extracts of *Argyreia* spp. in Y-tube olfactometer.

Chemical characterization of *Argyreia* spp. by GC-MS analysis

Since the foraging plant specificity of the bee, *T. macroceps,* was noticed in *A. cuneata*, studies were conducted to understand the volatile scent profile of *Argyreia* spp. towards the bee. The compounds were identified, and the chemical formula and molecular weight are presented in Table 1. The percentage of the composition was reported for individual flowers based on the relative peak percent area of the chromatogram. The major compounds in *A. cuneata,* according to percent composition, were Nonacosane, 3-methyl (13.26%), Hexatriacontane (12.06%), and Tricosane (9.43%). Pentacosane (5.69%) 2-Methyltetracosane (1.810%), and i-Propyl 16-methylheptadecanoate (1.527%) were observed to be major compounds by composition in *A. nervosa*.

Table 1. Comparison of different volatile organic compounds (VOC) and their composition in *Argyreia* spp.

Component	Chemical formulae	Retention Index	*A. cuneata*	Percentage composition in *A. cuneata*	*A. nervosa*	Percentage composition in *A. nervosa*
Caryophyllene	$C_{15}H_{24}$	1420	*	1.005	-	-
Trans bergamontene	$C_{15}H_{24}$	1432	*	2.38	-	-
Beta farnesene	$C_{15}H_{26}$	1454	*	6.19	-	-
Alpha farnesene	$C_{15}H_{24}$	1505	*	0.871	-	-
Quinoline, 6-methyl-2-phenyl-	$C_{16}H_{13}N$	NA	*	5.02	-	-
Heneicosane	$C_{21}H_{44}$	2101	*	2.092	-	-
Docosane	$C_{22}H_{46}$	2198	-	-	*	0.557
i-Propyl 16-methyl-heptadecanoate	$C_{21}H_{42}O_2$	NA	-	-	*	1.527
Tricosane	$C_{23}H_{48}$	2303	*	9.43	-	
2-Methyltetracosane	$C_{25}H_{52}$	2442	-	-	*	1.810
Pentacosane	$C_{25}H_{52}$	2496	-	-	*	5.69
Heptacosane	$C_{27}H_{56}$	2705	-	-	*	1.45
9-Octyldocosane	$C_{30}H_{62}$	2939	*	4.06	-	-
Nonacosane, 3-methyl-	$C_{30}H_{62}$	2970	*	13.26	-	-
Untriacontane	$C_{31}H_{64}$	3105	*	6.08	-	-
Hexatriacontane	$C_{36}H_{74}$	3598	*	12.06	-	-

*Compounds present
'NA' – Identification based on mass spectrum only.

Electroantennograph (EAG) response of *T. macroceps* to *Argyreia* spp.

The EAG response (mV, mean ± SD) of *T. macroceps* against headspace extract of the floral volatiles to *A. cuneata* and *A. nervosa* was assessed. There was a significant difference in the electroantennogram response of *T. macroceps* towards the floral extracts of *A. cuneata* and *A. nervosa* (F value = 130.89; $P < 0.0001$). The extracts of *A. cuneata* cause the highest neuronal response to the bee antennae. The mean EAG response in control (DCM) was 0.16mV (Fig 9). Floral extract of *A. cuneata* caused the highest mean antennal response (1.48 mV), significantly higher than the floral volatiles of *A. nervosa*. The bee antennal response to *A. nervosa* extracts was 0.36 mV, significantly lower than *A.cuneata*. This indicated that the bee has antennal receptors for the floral volatiles of *A. cuneata*.

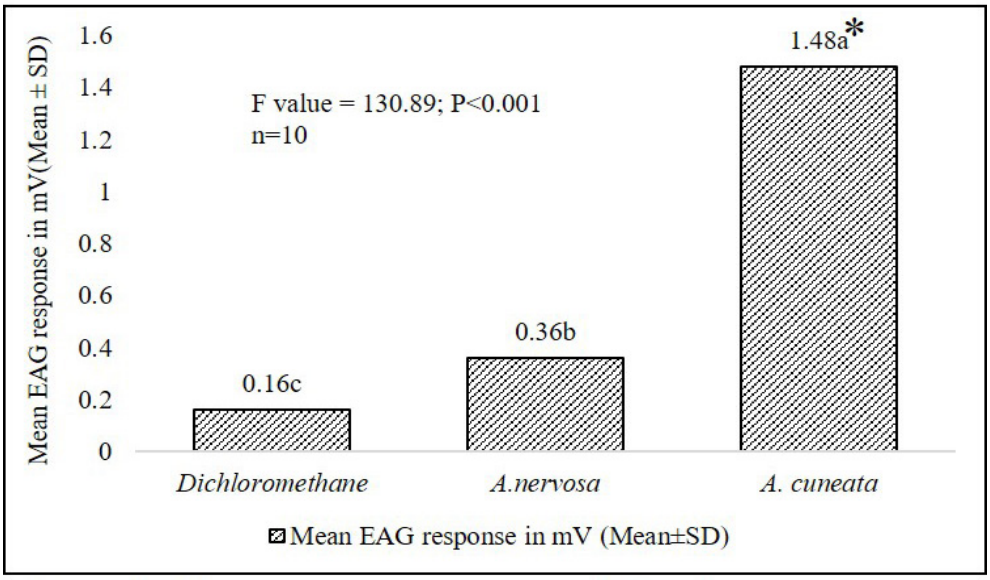

Fig 9. Electroantennogram response of *Tetralonia macroceps* to *Argyreia* spp.

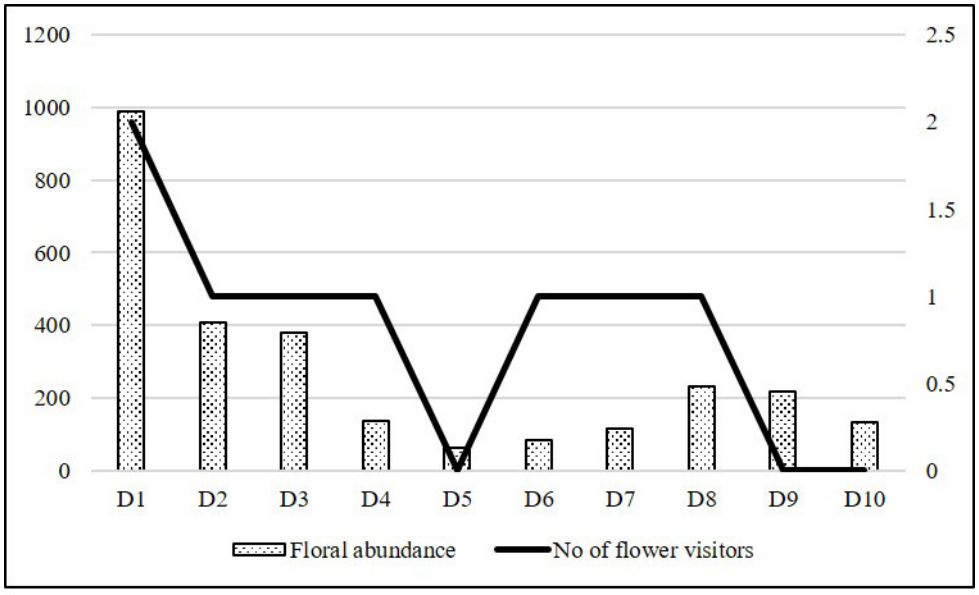

Fig 10. Floral abundance vs flower visitors in *A. nervosa*.

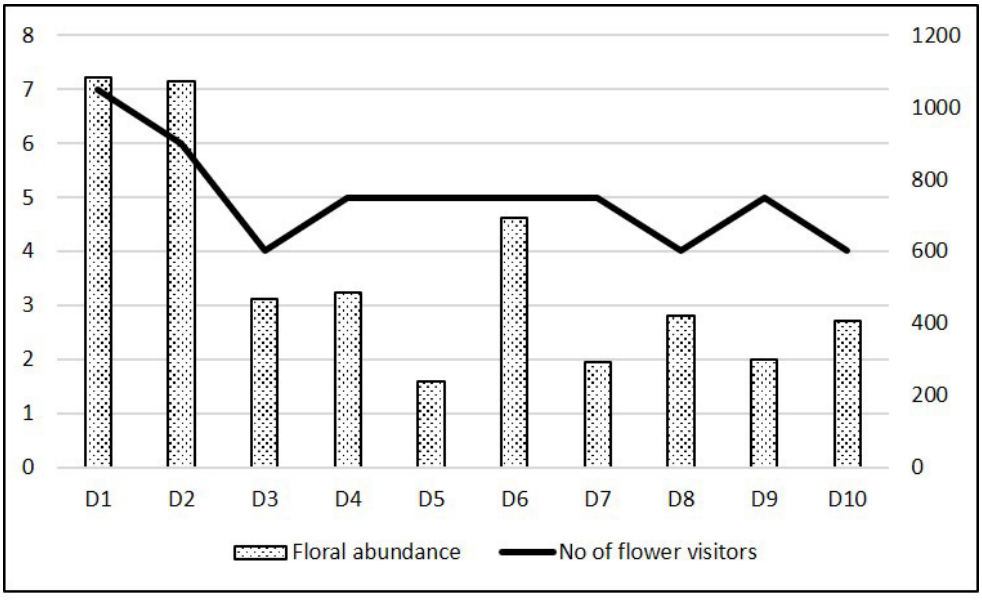

Fig 11. Floral abundance vs flower visitors in *A. cuneata*.

Discussion

Foraging behavior of bees in *A. cuneata* and *A. nervosa*

The flowers of *A. cuneata* were visited by seven different species of bees with a relatively higher abundance of *T. macroceps*. These bees forage the flowers of *A. cuneata* with a buzzing sound. On the contrary, *A. nervosa* flowers were visited only by two species of bees (*X. fenestrata* and *L. atratus*). The flowering of *A. cuneata* commences in mid-March and lasts till the end of October, and the active foraging of *T. macroceps* could be observed during this period (Shivalingaswamy et al., 2020). Our observations over two years confirmed the foraging plant specificity of this bee species towards *A. cuneata.* Such specificity was never observed on any other plant in the study site. The marked foraging preference of *Tetralonia* towards the pollen from the flowers of *Argyeria populifolia* belonging to Convolvulaceae was reported by Karunaratne et al. (2005).

The body size of the bee and tubular flower morphology permitting easier entry and moving around space inside the flower of *A. cuneata* and exit might be some of the reasons for the floral specificity of *T. macroceps* towards *A. cuneata*. The flower of *A. nervosa* is slightly bigger, with a relatively larger inner diameter than *A. cuneata*. The morphological fit of the bee with the sexual organs of the flower depends upon the traits of the flower (Stout, 2000; Kuriya et al., 2015). Tubular flowers with a narrow inner diameter of the flower that enables rapid movement inside the flowers with easier access to the pollen grains might be the reason for the foraging specificity of *T. macroceps* to

A. cuneata compared to *A. nervosa*. *T. fraterna* as a flower visitor of *Ipomoea prescaprae* and *I. arborescens* in Africa belonging to the family Convolvulaceae with similar tubular flower morphology was reported by Pauly (1984). Moreover, the flowers of *A. nervosa*, upon anthesis, were observed to be visited by the carpenter bee, *X. fenestrata,* which collects the pollen from the flower in branched hairs all over its body. The floral traits are important in determining pollination, influencing pollinator visitation and efficiency (Armbruster et al., 2013). In the current study, *T. macroceps* visited more flowers of *A. cuneata* in a five min period. In *A. cuneata*, the opening of multiple flowers occurred in a single flower cluster, unlike in *A. nervosa,* wherein the opening of single flowers was commonly observed. Multiple flower openings in *A. cuneata* might have attracted the bees as they were observed to move swiftly from one flower to another in the same cluster to collect the pollen rewards (Giurfa et al., 1996). Bees prefer to visit more flowers per plant to collect the rewards. *T. macroceps* was the first flower visitor on the flowers of *A. cuneata* in large numbers soon after the onset of anthesis. The bees were observed to collect the copious pollen reward in their branched hairs all over the body during foraging hours. The other floral visitors viz., *Apis dorsata*, *A. florea*, *C. hieroglyphica*, *Amegilla zonata*, *Xylocopa* sp, and *Megachile* sp appeared to be 'chance / incidental visitors' of the flowers of *A. cuneata,* making the floral visitation after the complete depletion of pollen reward by *T. macroceps*.

Floral abundance vs. Flower visitation in *A. cuneata* and *A. nervosa* and Pollen preference studies

Increased floral abundance was recorded in *A. cuneata,* with more floral visitors than in *A. nervosa*. The abundance of flowers providing the reward greatly influences bees' number of flower visits (Possingham, 1992; Dreisig,1995). Investigations on foraging bees of *T. macroceps* revealed the sole preference towards the pollen of *A. cuneata*. However, this data needs further investigation in the brood pollen analysis. Since the nesting habits of *T. macroceps* are non-traceable, this can be a future line of work for the authors.

Pollen analysis

Floral specificity and odor preference of *T. macroceps*

Our studies confirm that *T. macroceps* is a specific forager of *A.cuneata* flowers. There are no reports on foraging flora and nesting of *T. macroceps* from India or elsewhere. In the Y-tube assays, *T. macroceps* preferred the flower and floral extract of *A. cuneata* in choice studies. The foraging bees communicate with their conspecifics about the food source through the chemical information that they have experienced (Mas et al., 2020). The olfactory cues in floral constancy play a major role compared to visual cues as influenced by the presence of specific compounds in unique ratios in the flowers that attract the bumblebees (Kunze & Gumbert, 2001). The foraging bees were observed to handle the flowers of *A. cuneata* for a brief period collecting the pollen reward on its body hairs. The pollen of *A. cuneata* is easily accessible to the bee, which could also be a reason for its specificity to the flower. Pollen-collecting behavior as an attributing factor for specificity was reported in the passionflower bee *Anthemurgus passiflorae,* which was reported to be specific to *Passiflora lutea* L (Neff & Rozen, 1995).

Volatile profile of *Argyreia* spp. and electroantennogram response of *T. macroceps*

The volatile profile and EAG for antenna response of *T. macroceps* revealed its attraction and preference towards *A. cuneata*. This attraction may be due to certain compounds, such as nonacosane, 3-methyl, and hexatriacontane, found in relatively higher concentrations in the floral extracts of *A. cuneata*. The attraction to *A. cuneata* might also be due to specific compounds like caryophyllene, beta farnesene, and alpha farnesene, which are absent in *A. nervosa*. Beta farnesene in *A. cuneata* was at a higher level (6.19%), which was absent in *A. nervosa*. This could be a source of attraction of *T. macroceps* bees to *A. cuneata* than *A. nervosa*. The marked innate preference of the newly emerged bee, *Chelostoma rapunculi* (Lepeletier,1841), towards one specific plant species, *Campanula trachelium,* over two other co-existing/ co-flowering species of *Campanula* sp is influenced by the presence of aliphatics, sesquiterpenes and spiroacetal group of volatiles accounting for its floral constancy (Milet-Pinheiro et al., 2015). The antennal response of *T. macroceps* to the volatile floral extract of *A. cuneata* was higher than *A. nervosa*. Terpene compounds like farnesene, myrcene, and *p*-cymene served as olfactory cues in the attraction of resin and flower-seeking bees (Leonhardt et al., 2010). Conditioned proboscis extension assays revealed a positive response for beta farnesene in 85% of the honeybees present in the floral volatiles of oilseed rape, *Brassica napus* (Blight et al., 1997). VOCs released from the flowers have been recorded as a prime factor in attracting pollinators (Theis & Raguso, 2005; Muhlemann et al., 2006). The olfactory receptors are fine-tuned to respond to the variation in the odor profile of their host (Lawson et al., 2012), and the ratio of volatile blends directly affects the insect's orientation (Salvagnin et al., 2018). The volatile profile of *A. cuneata* revealed the presence of caryophyllene (1.00%) and alpha farnesene (0.87%) in addition to major other compounds. Caryophyllene and alpha farnesene caused a positive behavioral response in *Neocorynura* aff. *centroamericana* (Damon & Roblero, 2007, Dotterl & Vereecken, 2010). Caryophyllene, a most widespread sesquiterpene floral volatile, was reported to be a major floral sent in almost 50% of angiosperm families and one of the 12 most common volatile compounds in floral scents playing an attractant role (Knudsen et al., 2006;

Hori & Namatame, 2013) in flowers. Pentacosane and 2-Methyltetracosane were found in relatively higher proportions in the flower extracts of *A. nervosa*. Though few hydrocarbons are effective as short-range cues, pentacosane is a major compound. There is little information on its role in causing insects' orientation, which could have been why *A. nervosa* not attracting *T. macroceps*. The relatively higher proportion of volatiles in *A. cuneata* (Nonacosane – 13.26%; Hexatriacontane – 12.06%) compared to *A. nervosa* might be a reason for the foraging specificity of *T. macroceps* towards *A. cuneata* compared to *A. nervosa*. The proportion of volatile compounds influencing the specific preference of *Melipona solani* and the bumblebee *Bombus impatiens* in the olfactory studies towards the floral extracts of *Solanum rostratum* was reported by Montero et al. (2018).

Conclusion

In the present study, the floral preference of solitary bee, *T.macroceps* to *A. cuneata* was evident through floral abundance, flower visitation, and pollen preference coupled with the volatile compounds attributed to the foraging specificity of *T. macroceps* only towards the plant, *A. cuneata*. Detailed further studies on the association of *Tetralonia* bees with different host plants may consolidate information on their oligolectic behavior. This bee species helps conserve a plant with pharmacological importance, *A. cuneata*. Hence, it is prudent to decipher the volatile cues to which the bees are attracted and use VOCs to attract them to crop ecosystems for enhanced pollination.

Acknowledgments

The authors are thankful to Dr. K.D. Prathapan, Professor, KAU, Thiruvananthapuram for confirmation of a specific bee encountered on *Argyreia cuneata* as *Tetralonia* (*Thygatina*) *macroceps.* We thank Dr Chandish R Ballal, Director, ICAR-NBAIR for her constant support and encouragement in our efforts in the conservation of pollinators.

Authors' Contributions

AU: investigation, formal analysis, writing-original draft, writing-review & editing.
RA: investigation, writing-review & editing.
KS: investigation, writing-review & editing.
TMS: conceptualization, writing-review & editing.

References

Abrol, D.P. (2009). Plant-pollinator interactions in the context of climate change - an endangered mutualism. Journal of Palynology, 45: 1-25.

Armbruster, S.A., Corbet, A.J., Vey, M., Shu-Juan, L. & Shuang-Quan, H. (2013). In the right place at the right time: *Parnassia* resolves the herkogamy dilemma by accurate repositioning of stamens and stigmas. Annals of Botany, 113: 97-103.

Blight, M.M., Metayer, M.L., Delegue, M.H.P., Pickett, J.A., Poll, F.P. & Wadhams, L.J. (1997). Identification of floral volatiles involved in recognition of oilseed rape flowers, *Brassica napus* by Honeybees, *Apis mellifera*. Journal of Chemical Ecology, 23: 1715-1727.

Bronstein, J.L., Alarcon, R. & Geber, M. (2006). The evolution of plant-insect mutualisms. New Phytologist, 172: 412-428. doi: 10.1111/j.1469-8137.2006.01864.x.

Chittka, L. & Raine, N.E. (2006). Recognition of flowers by pollinators. Current Opinions on Plant Biology, 9: 428-35. doi: 10.1016/j.pbi.2006.05.002.

Damon, A. & Roblero, P.S. (2007). A survey of pollination in remnant orchid populations in Soconusco, Chiapas. Tropical Ecology, 48: 1-14.

Dobson, H.E.M., Groth, I. & Bergstrom, G. (1996). Pollen advertisement: chemical contrasts between flower and pollen odors. American Journal of Botany, 83: 877-885.

Dötterl, S. & Vereecken, N.J. (2010). The chemical ecology and evolution of bee-flower interactions: A review and perspectives. Canadian Journal of Zoology, 88: 668-697. doi: 10.1139/Z10-031.

Douglas, A.E. (2008). Conflict, cheats and the persistence of symbioses. New Phytologist, 177: 849-858. doi: 10.1111/j.1469-8137.2007.02326.x.

Dreisig, H. 1995. Ideal free distributions of nectar foraging bumblebees. Oikos, 72: 161-172.

Ela, M.A., Ali, M., Fohouo, F.N.T. & Messi, J. (2012). The importance of a single floral visit of *Eucara macrognatha* and *Tetralonia fraterna* (Hymenoptera: Apidae) in the pollination and the yields of *Abelmoschus esculentus* in Maroua, Cameroon. African Journal of Agricultural Research, **7**: 2853-2857.

Engel, M.S. & Baker, D.B. (2006). A New Species of *Tetralonia* (*Thygatina*) from India, with Notes on the Oriental Fauna (Hymenoptera: Apidae). American Museum Novitates, 3527: 1-9. doi: 10.1206/0003-0082.

Erdtman, G. (1960). The Acetolysis method. A Revised Description. Svensk Botanisk Tidskrift, 54, 561-564.

Fenster, C.B., Armbruster W.S., Wilson, P., Dudash, M.R. & Thomson, J.D. (2004). Pollination syndromes and floral specialization. Annual Review of Ecology, Evolution, and Systematics, 35: 375-403. doi: 10.1146/annurev.ecolsys.34.011802.132347.

George, A.P., Nissen, R.J., Ironside, D.A. & Anderson, P. (1989). Effects of nitidulid beetles on pollination and fruit set of *Annona* sp. hybrids. Scientia Horticulturae, 39: 289-299.

Giurfa, M., Vorobyev, M., Kevan, P. & Menzel, R. (1996). Detection of coloured stimuli by honeybees: minimum visual angles and receptor-specific contrasts. Journal of Comparative Physiology A,178: 699-709. doi: 10.1007/BF00227381.

Grass, I., Bohle, V., Tscharntke, T. & Westphal, C. (2018). How plants' reproductive success is determined by the interplay of antagonists and mutualists. Ecosphere, 9: 2-15. doi: 10.1002/ecs2.2106.

Hori, M. & Namatame, M. (2013). Host plant volatiles responsible for the invasion of *Stenotus rubrovittatus* (Heteroptera: Miridae) into paddy fields. Journal of Applied Entomology, 137: 340-346.

Karunaratne, W.A.I.P., Edirisinghe, J.P. & Gunatilleke, C.V.S. (2005). Floral relationships of bees in selected areas of Sri Lanka. Ceylon Journal of Science (Biological Science), 34: 27-45.

Klein, A.M., Vaissière, B.E., Cane, J.H., Steffan-Dewenter, I., Cunningham, S.A., Kremen, C. & Tscharntke, T. (2007) Importance of pollinators in changing landscapes for world crops. Proceedings of Royal Society B, Biological Sciences, 274: 303-313. doi: 10.1098/rspb.2006.3721

Knudsen, J.T., Eriksson, R., Gershenzon, J. & Stihl, B. (2006). Diversity and distribution of floral scent. The Botanical Reviews, 72: 1-120.

Kunze, J. & Gumbert, A. (2001). The combined effect of color and odor on flower choice behavior of bumblebees in flower mimic systems. Behavioral Ecology, 12: 447-456.

Kuriya, S., Hattori, M., Nagano, Y. & Itino, T. (2015). Altitudinal flower size variation correlates with local pollinator size in a bumblebee-pollinated herb, *Prunella vulgaris* L. (Lamiaceae). Journal of Evolutionary Biology, 28: 1761-1769.

Larue, A.A.C., Raguso, R.A. & Junker, R.R. (2016). Experimental manipulation of floral scent bouquets restructures flower-visitor interactions in the field. Journal of Animal Ecology, 85: 396-408.

Lawson, M.J., Craven, B.A., Paterson, E.G. & Settles, G.S. (2012). A computational study of odorant transport and deposition in the canine nasal cavity: implications for olfaction. Chemical Senses, 37: 553-566. doi: 10.1093/chemse/bjs039.

Leonard, A.S. & Masek, P. (2014). Multisensory integration of colors and scents: insights from bees and flowers. Journal of Comparative Physiology A, 200: 463-74. doi: 10.1007/s00359-014-0904-4.

Leonhardt, S.D., Zeilhofer, S., Bluthgen, N. & Schmitt, T. (2010). Stingless bees use terpenes as olfactory cues to find resin sources. Chemical Senses, 35: 603-611.

Mas, F., Horner, R., Brierley, S., Harper, A. & Suckling, D.M. (2020). The scent of individual foraging bees. Journal of Chemical Ecology, 46: 524-533. doi: 10.1007/s10886-020-01181-7.

Mayer, C., Adler, L., Armbruster, W. S., Dafni, A., Eardley, C., Huang, S.-Q., Kevan P.G., Ollerton, J., Packer, L., Ssymank, A., Stout, J.C. & Potts, S.G. (2011). Pollination ecology in the 21st century: key questions for future research. Joiurnal of Pollination Ecology, 3: 8-23. doi: 10.26786/1920-7603.

Michener, C.D., The bees of the world. Baltimore: Johns Hopkins University Press, 2000, xiv + [1] + 913 pp.

Milet-Pinheiro, P., Ayasse, M. & Dötterl, S. (2015). Visual and olfactory floral cues of *Campanula* (Campanulaceae) and their significance for host recognition by an oligolectic bee pollinator. PLoS One, 10: e0128577. doi: 10.1371/journal.pone.0128577.

Montero, L.S., Garcia, S.C., Rosas, D.A., Crisostomo, J.F.G., Polanco, M.V., Conesa, J.G. & Lopez, L.C. (2018). Pollinator preferences for floral volatiles emitted by dimorphic anthers of a buzz-pollinated herb. Journal of Chemical Ecology, 44: 1058-1067. doi: 10.1007/s10886-018-1014-5.

Muhlemann, J.K., Waelti, M.O., Widmer, A. & Schiestl, F.P. (2006). Post pollination changes in floral odor in *Silene latifolia*: adaptive mechanisms for seed-predator avoidance? Journal of Chemical Ecology, 32: 1855 1860. doi: 10.1007/s10886-006-9113-0.

Nathan, P.T., Karuppudurai, T., Raghuram, H. & Marimuthu, G. (2009). Bat foraging strategies and pollination of *Madhuca latifolia* (Sapotaceae) in Southern India. Acta Chiropterologica, 11: 435-441.

Neff, J.L. & Rozen, J.G. (1995). Foraging and nesting biology of the bee, *Anthemurgus passiflorae* (Hymenoptera: Apoidea), descriptions of its immature stages, and observations on its floral host (Passifloraceae). American Museum Novitates, 3138: 1-19.

Ollerton, J., Winfree, R. & Tarrant, S. (2011) How many flowering plants are pollinated by animals? Oikos, 120: 321-326. doi: 10.1111/j.1600-0706.2010.18644.x.

Parachnowitsch, A.L., Raguso, R.A. & Kessler, A. (2012). Phenotypic selection to increase floral scent emission, but not flower size or colour in bee-pollinated *Penstemon digitalis*. New Phytologist, 195: 667-675. doi: 10.1111/j.1469-8137.2012.04188.x.

Pauly, A. (1984). Contribution a l'etude desgenres afrotropicaux de Nomiinae. Revue de Zoologie Africaine, 98: 693-702.

Possingham, H. P. 1992. Habitat selection by 2 species of nectarivore/habitat quality isolines. Ecology, 73: 1903-1912.

Raguso, R.A. & Willis, M.A. (2005). Synergy between visual and olfactory cues in nectar feeding by wild hawkmoths, *Manduca sexta*. Animal Behavior, 69: 407-418. doi: 10.1016/j.anbehav.2004.04.015.

Reinecke, A., Ruther, J. & Hilker, M. (2005). Electrophysiological and behavioral responses of *Melolantha melolantha* to saturated

and unsaturated aliphatic alcohols. Entomologia Experimentalis et Applicata, 115: 33-40.

Renoult, J.P., Valido, A., Jordano, P. & Schaefer, H.M. (2014). Adaptation of flower and fruit colours to multiple, distinct mutualists. New Phytologist, 201: 678-686.

Riffell, J.A., Alarcon, R., Abrell, J., Davidowitz, G., Bronstein, J.L., Hildebrand, J.G. (2008). Behavioral consequences of innate preferences and olfactory learning in hawkmoth-flower interactions. Proceedings of the National Academy of Sciences USA, 105: 3404-3409.

Sajad, A. & Saeed, S. (2010). Floral host plant range of Syrphid flies (Syrphidae: Diptera) under natural conditions in Southern Punjab, Pakistan. Pakistan Journal of Botany, 42: 1187-1200.

Salvagnin, U., Malnoy, M., Thöming, G., Tasin, M., Carlin, S., Martens, S., Vrhovsek, U., Angeli, S. & Anfora, G. (2018). Adjusting the scent ratio: using genetically modified *Vitis vinifera* plants to manipulate European grapevine moth behavior. Plant Biotechnology Journal, 16: 264-271. doi: 10.1111/pbi.12767.

Shaara, A.H.F. (2014). The foraging behavior of honey bees, *Apis mellifera*: a review. Veterinarni Medicina, 59: 1-10.

Sheppard, C.A. & Oliver, R.A. (2004). Yucca moths and yucca plants: Discovery of the most wonderful case of fertilisation. American Entomologist, 50: 32-46. doi: 10.1093/ae/50.1.32

Shivalingaswamy, T.M., Amala, U., Gupta, A. & Raghavendra, A. (2020). Non-Apis bee diversity in an experimental pollinator garden in Bengaluru – a Silicon Valley of India. Sociobiology, 67: 593-598.

Spaethe, J., Moser, W.H. & Paulus, H.F. (2007). Increase of pollinator attraction by means of a visual signal in the sexually deceptive orchid, *Ophrys heldreichii* (Orchidaceae). Plant Systematics and Evolution, 264: 31-40. doi: 10.1007/s00606-006-0503-0.

Stang, M., Klinkhamer, P.G.L. & Meijden, E.V.D. (2006). Size constraints and flower abundance determine the number of interactions in a plant flower visitor web. Oikos, 112: 111-121.

Stein, K., Coulibaly, D., Stenchly, K., Goetze, D., Porembski, S., Lindner, A., Konaté, S. and Linsenmair, E.K. (2017). Bee pollination increases yield quantity and quality of cash crops in Burkina Faso, West Africa. Scientific Reports, 7: 17691 doi: 10.1038/s41598-017-17970-2.

Stout, J. (2000). Does size matter? Bumblebee behavior and the pollination of *Cytisus scoparius* L. (Fabaceae). Apidologie, 31: 129-139. doi: 10.1051/apido:2000111.

Subramanya, S. & Radhamani, T.R. (1993). Pollination by birds and bats. Current Science, 65: 201-209.

Theis, N. & Raguso, R.A. (2005). The effect of pollination on floral fragrance in thistles. Journal of Chemical Ecology, 31: 2581-2600. doi: 10.1007/s10886-005-7615-9.

Vibina, V. & Subaharan, K. (2019). Electrophysiological and behavioral response of red palm weevil, *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Dryophthoridae) to fermented coconut sap neera. Journal of Plantation Crops, 47: 82-89. doi: 10.25081/jpc.2019.v47.i2.5767.

Zander, E. (1935). Beitrage zur Herkunftsbestimmung bei Honig. I Reichsfachgruppe Imker, Berlin. II. Liedloff. Loth & Michaelis. Leipzig, p. 465.