Arthropod-Plant Interactions (2021) 15:517–534
https://doi.org/10.1007/s11829-021-09844-2

ORIGINAL PAPER

Comparison of floral traits in *Calibrachoa* cultivars and assessment of their impacts on attractiveness to flower-visiting insects

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Received: 23 December 2020 / Accepted: 1 June 2021 / Published online: 29 June 2021

Abstract

Ornamental plants are appreciated by humans for their colorfulness, beauty, abundant flowering and long blooming periods. Many ornamental plants can also constitute an additional foraging resource for flower-visiting insects. However, the ability of the popular ornamental plant *Calibrachoa* to support urban insect communities is not well documented. In this study, 20 different *Calibrachoa* cultivars were selected and tested in regard to their insect friendliness based on standardized observations (I) in flight tents using the large earth bumble bee *Bombus terrestris* as a model species and (II) in open field trials. To investigate what floral characteristics might constitute attractiveness to bumble bees, various floral traits were recorded and compared across all tested *Calibrachoa* cultivars. Over a two-year period, a total of 6,327 foraging bumble bees were recorded in the tent observations. In the open field observations, we counted 4,188 flower-visiting insects. Our results revealed that (I) all *Calibrachoa* cultivars were visited by insects for foraging, (II) the number of insect visitors varied significantly among the 20 tested cultivars and (III) the cultivars displayed different floral traits. For the morphometric floral traits and the aroma profiles of *Calibrachoa,* only the mean nectar quantity and a few identified compounds could be correlated with attractiveness to the model species *B. terrestris*. We also found that the petal color of the tested cultivars had a significant impact on the number of visitors. Therefore, *B. terrestris* clearly preferred red or blue *Calibrachoa* cultivars over those with other petal colors. However, as the cultivar preferences in the different insect groups differed, it is highly recommended to use various cultivars in urban plantings. Nevertheless, efforts must be made to explain what additional floral traits make *Calibrachoa* and other ornamental plants generally attractive to flower visitors. This information can then be used for breeding purposes to increase the insect friendliness of ornamental plants.

Keywords Ornamental plant · *Calibrachoa* cultivars · Floral traits · Flower-visiting insects attractiveness

Introduction

Urbanization, the expansion of urban and suburban areas, is increasing worldwide (United Nations 2014), resulting in a drastic and permanent change in landscape utilization (Seto et al. 2013). As a consequence, the deterioration of the quantity and quality of natural and seminatural habitats is increased, which dramatically challenges wildlife (McIntyre et al. 2001; McKinney 2006; Winfree et al. 2009; Potts et al. 2010; Ushimaru et al. 2014). Nevertheless, some recently published studies concluded that cities could constitute a valuable habitat, or a retreat, for many insect species (Bates et al. 2011; Hall et al. 2017; Baldock et al. 2019; Theodorou et al. 2020), and scientists repeatedly argued that to support local insect diversity, green urban areas can play a crucial role (Matteson et al. 2008; Gunnarsson and Federsel 2014;

Handling Editor: Isabel Alves dos Santos.

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Hülsmann et al. 2015; Banaszak-Cibicka et al. 2018; Egerer et al. 2020; Wenzel et al. 2020).

Green urban areas such as parks, community gardens, cemeteries and public or private yards belong to the most anthropogenically altered and managed landscapes, providing extensive areas that frequently contain large numbers of flowering plant species. In contrast to natural habitats, urban plant species diversity is generally increased because exotic plants are willingly introduced and planted in addition to naturally occurring plants (Hope et al. 2003; Knapp et al. 2012). Previous studies demonstrated that exotic plants could constitute a useful floral resource for many bee species when they provide nectar and/or pollen (Comba et al. 1999; Corbet et al. 2001). Furthermore, some other studies showed that exotic plants can be equally attractive to flower visitors as native plants (Harrison and Winfree 2015; Lowenstein et al. 2019), and a recently published study indicated that exotic plants might even be a substitute for some native plants when they become scarce (Staab et al. 2020). However, the attractiveness of exotic plants seems to be dependent on the variety itself, and in some cases, different varieties of one species might show enormous differences in their attractiveness to insect flower visitors (Garbuzov and Ratnieks 2015; Erickson et al. 2020).

Generalist flower visitors such as honey bees and bumble bees must choose from a wide range of flowering plants, and they are constantly faced with the decision of what flower(s) to visit for foraging (Chittka and Raine 2006). Many flowering plant species, on the other hand, are dependent on insect pollination, and to attract pollinators, these flowers usually display various so-called floral signals. Generally, floral signals consist of visual cues, e.g., flower color and flower shape, as well as olfactory cues (flower scent) (Raguso 2004). In combination, floral signals constitute a complex interplay and form a combined stimulus in bees (Rowe and Guilford 1999), leading to the decision of whether a specific flower is visited or not. Although the importance of flower morphology and flower scent in native flowering plants to bumble bees has been intensively discussed in many previous publications (e.g.Harder 1983; Kunze and Gumbert 2001; Ishii 2006; Kulahci et al. 2008), it is still unclear what floral signals drive pollinator choice in exotic or ornamental plants, and so far, no study has investigated the importance of floral signals in these plants on pollinator attractiveness.

As public awareness of the current biodiversity loss, especially in insects, rises, the demand for insect-friendly garden plants increases simultaneously. Many lists recommending 'bee- and butterfly friendly' plants have been published so far, but these recommendations are often based on personal opinions rather than on scientific data (Garbuzov and Ratnieks 2014a). This is especially true for ornamental plants. Additionally, the high number of plant breeding programs that continuously lead to the creation of new and innovated cultivars with enhanced plant characteristics (De 2017) but mostly unknown degrees of insect friendliness aggravates this situation. One particularly relevant example is *Calibrachoa* Cerv., also known as *Calibrachoa* Lave & Lex., a fairly new but very popular ornamental plant with increasing economic importance (Rode et al. 2010; Jędrzejuk et al. 2017). The genus *Calibrachoa* Cerv. is native to Southern America, mainly Brazil, and the 27 associated species of this genus are all known to be self-incompatible and therefore highly dependent on insect pollination (Stehmann and Semir 1997; Olmstead et al. 2008; Fregonezi et al. 2012, 2013). The ornamental plant *Calibrachoa* hybrida was first introduced to the Japanese market in approximately 1990, followed by markets in Europe and North America (Murakami et al. 2004). However, these cultivars originated from the hybridization of various unknown wild *Calibrachoa* Cerv. species; therefore, the actual species origin of these cultivars remains unclear (Kishimoto et al. 2019; Liu et al. 2020). At present, the ornamental plant *Calibrachoa* hybrida is appreciated by gardeners for its high abundance of flowers and long blooming period, which lasts from approximately May to November in Central Europe. Additionally, garden centers and nurseries offer a wide variety of cultivars in a large range of appealing flower colors, which makes *Calibrachoa* hybrida a long-flowering, colorful and attractive bedding and balcony plant (Murakami et al. 2004). However, knowledge of *Calibrachoa* hybrida in regard to its insect friendliness is scarce, and to date, no study has investigated whether, and to what extent, differences in attractiveness exist among cultivars of *Calibrachoa* hybrida.

In this study, we present data on the attractiveness of 20 cultivars of the ornamental garden plant *Calibrachoa* hybrida to flower-visiting insects. The floral visitation patterns of the insects were monitored at two testing sites in southern Germany over a 3-year period (2017–2019), and therefore, two different approaches were devised: (I) tests under controlled, seminatural conditions in flight tents using *Bombus terrestris* as a model species and (II) field trials analyzing the whole spectrum of insects foraging at our tested plants. With this setup, we tried to assess whether and to what extent the attractiveness differed within the tested cultivars. As a further objective of our study, we compared some floral traits of these cultivars, e.g., flower size, flower color and flower scent, that are assumed to attract flower-visiting insects. The aim was to identify floral traits that can be directly linked with insect friendliness in our *Calibrachoa* cultivars.

Materials & methods

Tested plant cultivars

Ornamental plants from the genus *Calibrachoa* Lave & Lex. (Wijsman 1990), hereafter referred to as the common name '*Calibrachoa',* were tested in this study. We chose these plants, as they (I) are available in a wide range of different petal colors, (II) have a long blooming period and (III) are commonly visited by insects for foraging. In Germany, *Calibrachoa* is known to be a nonnative (exotic) garden plant that grows annually. For this study, single flower *Calibrachoa* cultivars in five different petal color sets were selected: 'red', 'white', 'yellow', 'blue' and 'rave', which has radially symmetric patterns. Each color set consisted of four different genotypes, resulting in a total of 20 different *Calibrachoa* cultivars that were tested in this study. In Germany, these cultivars are currently widely available on sale and marketed in five series: MiniFamous® Neo, MiniFamous® Uno, MiniFamous® Piú, MiniFamous® and Rave® (Table 1).

In all three study years, the 20 *Calibrachoa* cultivars were supplied as cuttings by the plant breeding company Selecta One (Klemm & Sohn GmbH & Co. KG, Stuttgart, Germany, link to the company's website: https://www.selecta-one.com/en/home/), where they were derived from proprietary breeding lines. The subsequent cultivation of the cuttings was performed in a greenhouse at the State Horticultural College and Research Institute Heidelberg (Heidelberg, Germany). In mid-May, after cultivation, the plants were either planted outdoors in Heidelberg or transported to the State School for Horticulture in Stuttgart-Hohenheim (Stuttgart, Germany), where they were also planted outdoors.

Experimental setup, observations and hand net catching

This study was performed on the premises of the State Horticultural College and Research Institute Heidelberg (Heidelberg) and the State School for Horticulture in Stuttgart-Hohenheim (Stuttgart). The testing site in Heidelberg is located in a suburban area with a high degree of adjacent agricultural land with the following coordinates: 49°24′10.1"N 8°39′03.4"E. The testing site in Stuttgart is also located in a suburban area that is surrounded by mainly agricultural land. However, this location is situated inside a landscape conservation area, and the coordinates are as follows: 48°42′44.9"N 9°11′56.6"E. At both locations, three flight tents with the dimensions 4 m × 9 m × 3 m (width x length x height) were established and lined with landscape fabric (Mypex). Each flight tent was equipped with the 20 plant cultivars of *Calibrachoa*, which were planted in 30 L pots filled with soil (Einheitserde Primel & Viola (Patzer), medium texture with 2 kg Osmocote 5–6 M and 1 kg start fertilizer + iron chelate). Three individual plants of one cultivar were planted in one pot, and the 20 pots were arranged randomly within the flight tents. Automatic drip irrigation was connected to each pot to ensure adequate irrigation. When required, slug pellets (Bayer Mollustop®) and green lacewing larvae (Katz Biotech, *Chrysoperla carnea*) were applied to kill pests.

To determine the attractiveness of the 20 *Calibrachoa* cultivars to flower visitors, we performed two tests:

(I) Observations of bumble bee colonies in flight tents

Observations of the large earth bumble bee (*Bombus terrestris*) took place at both testing sites in Heidelberg and Stuttgart in 2017 and 2018. For this purpose, bumble bee colonies (Katz Biotech, *Bombus terrestris* mini colony or Sauter & Stepper, NATUPOL standard bumble bee colony *Bombus terrestris*) were purchased, and each flight tent was equipped with two bumble bee colonies. Each *Calibrachoa* cultivar was observed for 2 min, and the number of flower-visiting bumble bees was recorded. We only counted the visits when workers came in contact with the reproductive parts of the flowers and/or when they were observed actively foraging on the floral products of our plant cultivars. All 20 cultivars in the three flight tents were tested this way, and the observations were repeated three times per day. The observations were conducted only on days with favorable weather, i.e., on days with a minimum peak daytime temperature of 20 °C, low wind and full to partial sun. In Stuttgart, in 2017, there were 13 observation days, and in 2018, there were 12 observation days. In Heidelberg, in 2017, there were 15 observation days, and in 2018, there were 20 observation days. Observations took place between 07:00 am and 03:00 pm.

(II) Open field

Observations of the open field test without gauze were conducted only in Stuttgart in 2017 and 2019. Again, the number of flower visitors that were foraging on pollen or nectar from *Calibrachoa* was recorded, but this time, we used the 'snap-shot' method to acquire the data. In this method, the plants were observed just briefly, with a maximal duration of a couple seconds. In this short period of time, the number of foraging insects was recorded. The recorded insects were broadly identified in the field and then classified into the following five groups: honey bees (*Apis mellifera* L.), bumble bees (*Bombus spp*.), other wild bees (non-*Apis* and non-*Bombus*), hoverflies (Diptera: Syrphidae) and other pollinating insects (flies, wasps, beetles, but-

Table 1 The 20 *Calibrachoa* cultivars included in this study and the respective series in which they are currently marketed in Germany

#1—'Neo Sangria' MiniFamous® Neo 	#2—'Neo Firestorm' MiniFamous® Neo 	#3—'Uno Red' MiniFamous® Uno 	#4—'Piú Red' MiniFamous® Piú 
#5—'Neo White '12' MiniFamous® Neo 	#6—'Piú White' MiniFamous® Piú 	#7—'Uno White' MiniFamous® Uno 	#8—'Uno White + Yellow Eye' MiniFamous® Uno 
#9—'Neo True Yellow' MiniFamous® Neo 	#10—'Piú Yellow' MiniFamous® Piú 	#11—'Uno Yellow' MiniFamous® Uno 	#12—'Yellow 457' N/A 

Table 1 (continued)

#13—'Neo Blue' MiniFamous® Neo	#14—'Neo Royal Blue '16' MiniFamous® Neo	#15—'Dark Blue' MiniFamous®	#16—'Uno Blue' MiniFamous® Uno
			
#17—Rave 'Blue' Rave®	#18—Rave 'Cherry' Rave®	#19—Rave 'Peach' Rave®	#20—Rave 'Violet' Rave®
			

All cultivars were derived from the selecta one (Klemm & Sohn GmbH & Co. KG) proprietary breeding lines

> terflies and moths). All 20 cultivars in the three 'flight tents' were observed in this way, and the observations were again repeated three times per day. The observations were also performed only under favorable weather conditions and took place between 09:30 am and 04:45 pm. In 2017, there were 11 observation days, and in 2019, there were 9 observation days.

Additionally, foraging bumble bees and other wild bees were caught with a hand net (Bioform hand net, diameter 50 cm, mesh size 1 mm, Nürnberg, Germany) in three catching rounds on the mornings of July 10th, August 2nd and August 23rd in 2019. Within two-minute periods, all nectar or pollen foraging bumble bees and other wild bees on *Calibrachoa* were caught and killed with ethyl acetate. The captured bees were stored in the freezer until the final preparation and determination of the species.

Petal color analyses

The spectral reflectance of the petals of all 20 *Calibrachoa* cultivars was recorded from 300 to 700 nm with an Ocean Optics Jaz Spectrometer (Ocean Optics Inc., Dunedin, FL, USA). The light source was an Ocean Optics deuterium-halogen lamp that shone light over a fiber reflection probe (QR-400–7-UV–VIS, core diameter 400 µm, length 400 mm). The light reached the petal surface at an angle of 45°. A Zeiss MS 20 white ceramic plate and an open black film canister were used as black and white standards. For the measurements, only the flowering plants from the testing site in Stuttgart were used. Five flowers of each cultivar from all three tents were randomly picked and measured. All measurements were performed in 2019, except for the cultivar 'Yellow 457', as its spectral reflectance was already recorded in 2017.

Floral morphometric measurements

At both testing sites in Heidelberg and Stuttgart in 2017, the flower diameters (horizontally and vertically) and corolla diameters (horizontally and vertically) of 30 randomly picked flowers in all tested cultivars were measured with the help of a custom ruler, and the corolla depths of these flowers were further assessed with a tire tread gauge. In addition, at the testing site in Heidelberg, the lengths of the five stamens in 30 flowers per cultivar were recorded. For this measurement, the flowers were carefully cut in half with a sharp knife, and the lengths of the five stamens were measured with a ruler (Supplementary Information Fig. S1). To determine the nectar quantity per cultivar, we collected nectar samples at the testing site in Stuttgart in 2018. The samples (30 flowers randomly picked from each cultivar across all three tents) were collected between 10:00 am and 04:00 pm using 1–5 µL microcapillary tubes (Hirschmann Laborgeräte, ringcaps®, Eberstadt, Germany). The tubes were weighed on a precision scale before and after sampling, and the difference revealed the nectar quantity of the investigated flowers.

Flower scent analyses

The flower scents of the *Calibrachoa* cultivars were collected by enclosing two flowers with an approximate weight of 0.5 g in headspace vials (20 mL); flowers were kept frozen until used for the analyses. The compounds from the flower scents were identified by using headspace solid-phase microextraction (HS-SPME) combined with a gas chromatography system equipped with a mass spectrometry detector (GC–MS). Each plant cultivar was sampled with three replications such that 60 flower samples were analyzed.

For HS-SPME, a carboxene/polydimethylsiloxane/divinylbenzene (CAR/PDMS/DVB) fiber (30/50 µm and 1 cm fiber length) (Supelco, Steinheim, Germany) was used. The samples were incubated for 0.5 min at 30 °C, followed by headspace extraction for 5 min at the same temperature. Afterwards, the analytes were directly desorbed in the split/splitless inlet at 250 °C using a GC – MS SPME liner for 1 min. After desorption, the fiber was cleaned under the conditions recommended by the manufacturer.

The GC–MS analyses were performed with a 7890B GC and 5977B MS (both Agilent Technologies, Waldbronn, Germany), an autosampler MPS robot and an olfactometry detection port ODP 3 (both Gerstel, Mülheim an der Ruhr, Germany). A polar Agilent J&W DB-WAXms column (30 m length × 0.25 mm inner diameter × 0.25 µm film thickness) (Agilent Technologies, Waldbronn, Germany) was used for separation of the flower scent compounds. Helium (5.0, Westfalen AG, Weißenhorn, Germany) was used as a carrier gas at a constant flow rate of 1.62 mL/min. The GC oven temperature was held at 40 °C for 3 min and then ramped at 5 °C/min to 240 °C. The 240 °C step was held for 10 min. The following MS parameters were applied: MS mode, scan; scan range, *m/z* 40–330; electron ionization energy, 70 eV; source temperature 230 °C; and quadrupole temperature 150 °C.

The data were analyzed using Agilent Mass Hunter B07.06 Gerstel Maestro (Gerstel, Mülheim an der Ruhr, Germany). The compounds of the flower scents were identified by their retention indices and a comparison of the mass spectra to those of authentic standards and data published in the literature, i.e., pherobase.com, webbook.nist.com and pubchem.ncbi.nlm.nih.gov.

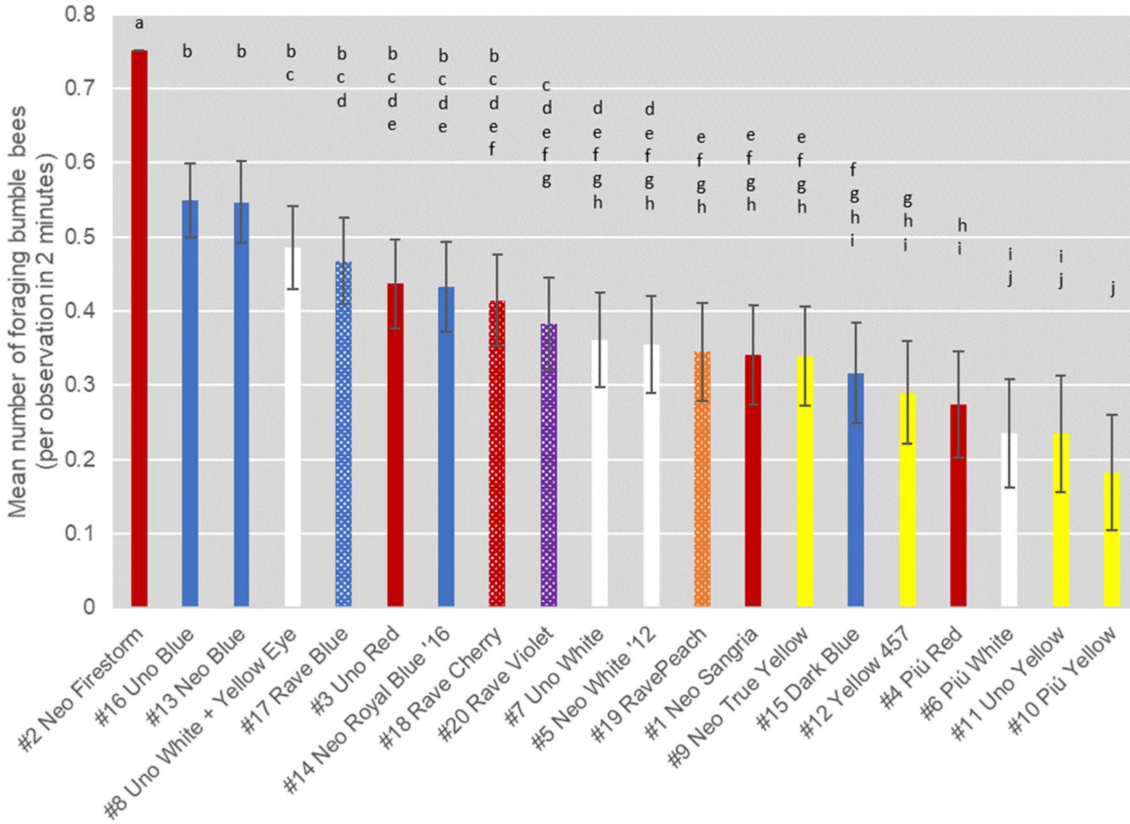

Fig. 1 Average number of foraging bumble bees (*B. terrestris*) and the standard deviation per *Calibrachoa* cultivar, which were controlled for any environmental and methodological impacts. The colors of the bars indicate the petal color of the respective cultivar. The letters above the bars represent significant differences based on the least squares means test, where cultivars sharing the same letter are not significantly different from each other ($P > 0.05$)

Statistical analyses

To make a scientifically objective statement regarding the attractiveness of the 20 tested *Calibrachoa* cultivars to bumble bees based on the flight tent observations, the number of bumble bee visits was controlled for any methodological and environmental impacts. Therefore, the data were analyzed using an ANOVA-type generalized linear model (GLM) based on the restricted maximum likelihood (REML) method using R 3.6.2 (R Core Team 2019) and the package ‘lme4’ (Bates et al. 2015). Based on the residual and Q-Q-plot, a Poisson distribution using the natural log-transformation was chosen for the GLM, which is quite typical for count data. The varietal effect was separated from the environmental effect of the year, repetition, repetition nested within the tent and location. For the least squares means test, the package ‘emmeans’ (Lenth 2020) was used. The obtained lsmeans values for the cultivar effect were then transformed back to the original scale. Tukey-adjusted *P*-values for the multiple pairwise comparisons among cultivars were calculated and visualized via a compact letter display. We proceeded similarly for the count data of the flower visitors in the open field observations. However, due to legibility reasons, we waived the graphical presentation of the letters in the figure.

Based on the spectral reflectance, the color locus of each cultivar was determined in the color hexagon according to Chittka (1992). Therefore, the statistical analyses of the spectral reflectance data and the graphical presentation on the color hexagon were performed in R 3.6.2 (R Core Team 2019) with the package ‘pavo’ (Maia et al. 2019). As we used the bumble bee *B. terrestris* as our model species in this study, the sensitivity maxima needed to be adjusted to fit its trichromatic system. The following values were taken as the maxima for the UV, blue and green receptors: 328 nm, 428 nm and 536 nm, respectively (Peitsch et al. 1992).

The GLM was further used to evaluate the importance of the bee-perceived petal color on the attractiveness to bumble bees. The varietal effect was completely replaced by the petal color effect to avoid confounding between the cultivar and color. Again, the least squares means test from the package ‘emmeans’ (Lenth 2020) was applied to the petal color effect data. We tested the effects of the series and the nectar quantities in a similar manner.

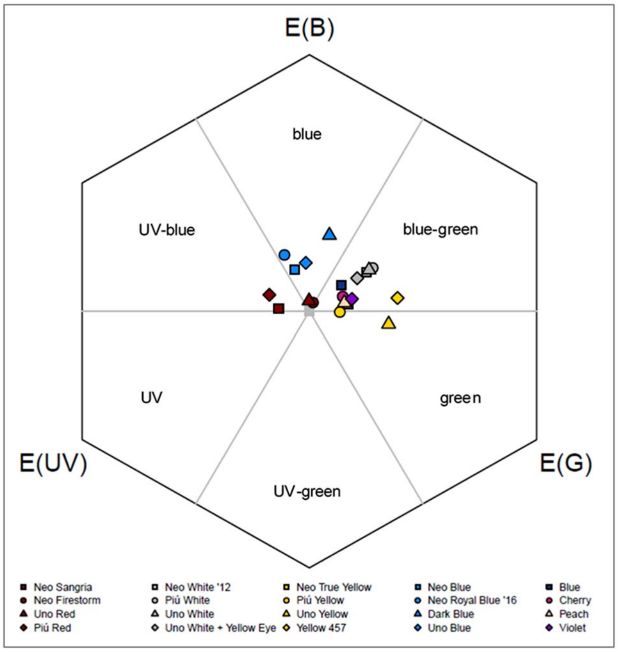

Fig. 2 The petal color loci of the 20 *Calibrachoa* cultivars compared in the color hexagon used by Chittka (1992), whereby '*B*' blue, '*U*' UV and '*G*' green

The floral morphometric measurements (horizontal/vertical flower diameter, horizontal/vertical corolla diameter, corolla depth and stamen lengths) were correlated with the count data of the response variable (number of bumble bee visits) in R 3.6.2 (R Core Team 2019) using Spearman's rank-order correlation (Spearman 1906). The importance of the methodological and environmental impact factors on the expression of the floral morphometric parameters was analyzed with ANOVA using a slightly modified model: the year effect was dropped since the floral morphometric measures were only carried out in 2017.

Visualization of the nonmetric multidimensional scaling (NMDS) of the single flower scent components depending on the pollinator attractiveness was performed with R 3.6.2 (R Core Team 2019) and the 'vegan' package (Oksanen et al. 2019). The raw data were the quantitative concentrations of the flower scent substances (peak area in units). The data were square-root transformed prior to the analysis, and the Bray–Curtis similarity index was used to measure the similarities/dissimilarities of the data. The stress value indicates the level of reliability of the two-dimensional plot whereby a stress equal to or below 0.1 is considered a fair representation.

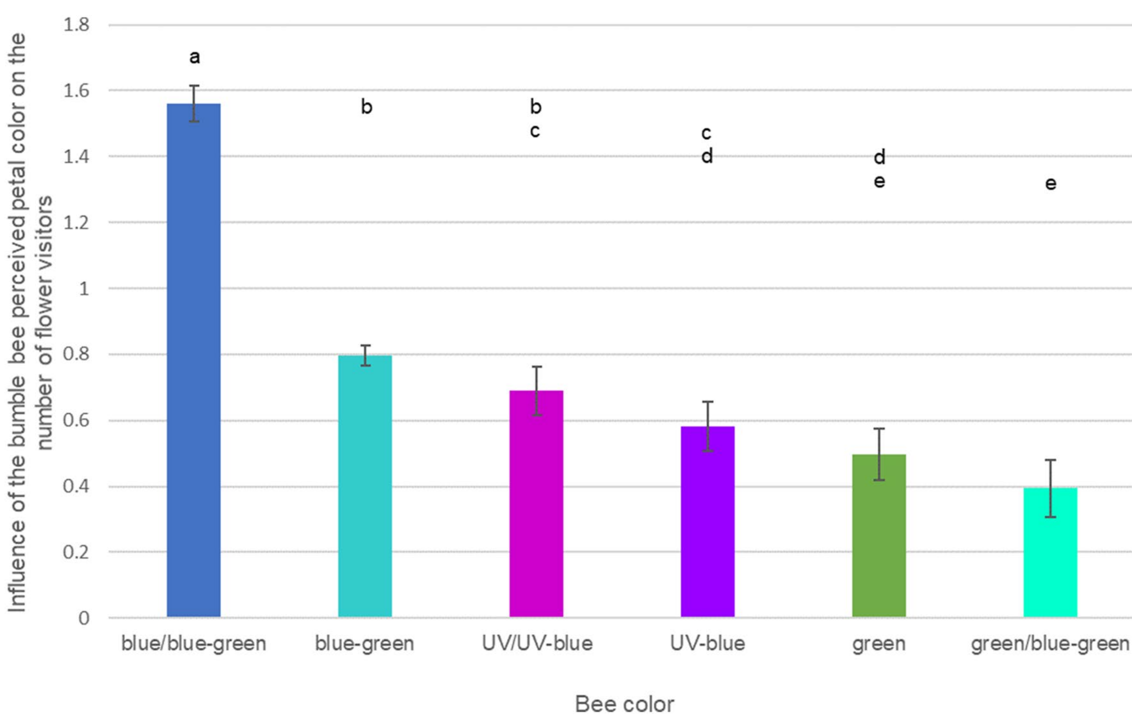

Fig. 3 Influence of the bumble bee-perceived petal color of the tested *Calibrachoa* cultivars on the number of *B. terrestris* flower visitors and the standard deviation. The letters above the bars represent significant differences based on the least squares means test, where bee-perceived colors sharing the same letter are not significantly different from each other ($P > 0.05$)

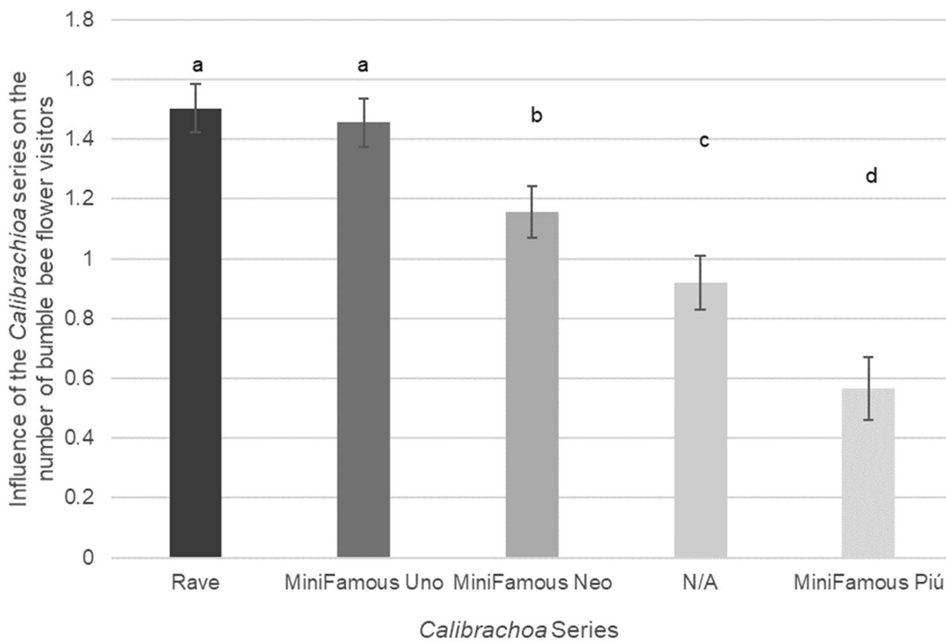

Fig. 4 Influence of the *Calibrachoa* series of the tested cultivars on the number of *B. terrestris* flower visitors and the standard deviation. The letters above the bars represent significant differences based on the least squares means test, where the series sharing the same letter are not significantly different from each other ($P > 0.05$)

To investigate whether the petal color and floral aroma profiles interact, we used ANOSIM and SIMPER (PAST 4.03). Data were compared using one-way analysis of similarity (ANOSIM, similarity index = Bray–Curtis, maximum permutations = 9,999) to determine if differences in the floral scent occurred among cultivars with the same petal color (as viewed by bees). The floral scents were also quantified through one-way similarity percentages (SIMPER, similarity index = Bray–Curtis) to identify similarities or dissimilarities among the same-colored cultivars.

Results

Variation in bumble bee attractiveness among the *Calibrachoa* cultivars

In total, 6,327 bumble bees were recorded foraging at the *Calibrachoa* plants across the two-year observation period in Heidelberg and Stuttgart. The number of *B. terrestris* visitors varied highly among the *Calibrachoa* cultivars. Of all tested cultivars, 'Neo Firestorm' was the most attractive cultivar, with 0.75 flower visitors on average in two minutes, followed by 'Uno Blue' and 'Neo Blue', with a mean of 0.55 flower visitors. The least attractive cultivar was 'Piú Yellow', with 0.18 flower visitors. Cultivar was a highly significant factor for attractiveness to the bumble bee *B. terrestris* (Wald test: $Df = 19$, Wald Statistic = 662.61, $P < 0.001$). The results of the least squares means test showed that the cultivar 'Neo Firestorm' was significantly different in the number of visitors compared with all other cultivars (Fig. 1).

Differences in petal colors and impacts on bumble bee visitors

The comparison of the petal color reflectance in the *Calibrachoa* cultivars showed that the color loci of the investigated cultivars formed a gradient across the UV-blue, blue and blue–green sections in the color hexagon (Fig. 2). Furthermore, it can be seen that the majority of the color loci in the five color sets clustered as follows: the color set 'blue' appeared in the section bee-blue, 'white' and 'rave' appeared in the section bee blue–green, 'yellow' appeared in the transition between bee blue–green and bee-green, and 'red' appeared in the transition between UV-blue, bee-blue and bee blue/blue–green. Within each color set, the color loci distances of some cultivars were extremely small (< 0.1), and some of the cultivars were closely located to the center of the color hexagon (distance less than 0.1 hexagon distance) (Supplementary Information Table S1).

Statistical analyses linking the bumble bee-perceived petal color with the attractiveness of the *Calibrachoa* cultivars showed that petal color had a significant impact on the number of *B. terrestris* visitors (Wald test: $Df = 6$, Wald Statistic = 493.15, $P < 0.001$). The cultivars appearing blue/blue–green showed the most positive impact, followed by the blue–green, UV/UV-blue and UV-blue cultivars (Fig. 3). In contrast, the green color and the combination of green/blue–green showed less influence on the number of flower visitors. The results of the pairwise comparisons indicated that the blue/blue–green color was significantly different from the others.

Further statistical analysis showed that in addition to bee-perceived petal color, the series of investigated cultivars also

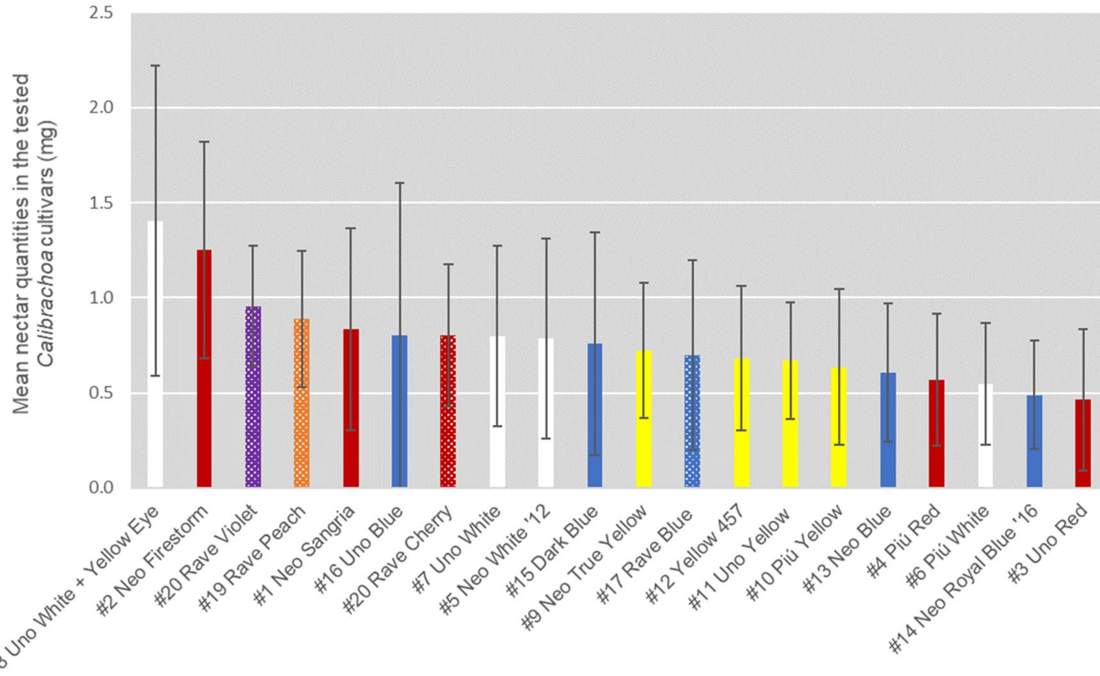

Fig. 5 Mean amount of nectar and standard deviations in the 20 investigated *Calibrachoa* cultivars. The colors of the bars indicate the petal color of the respective cultivar

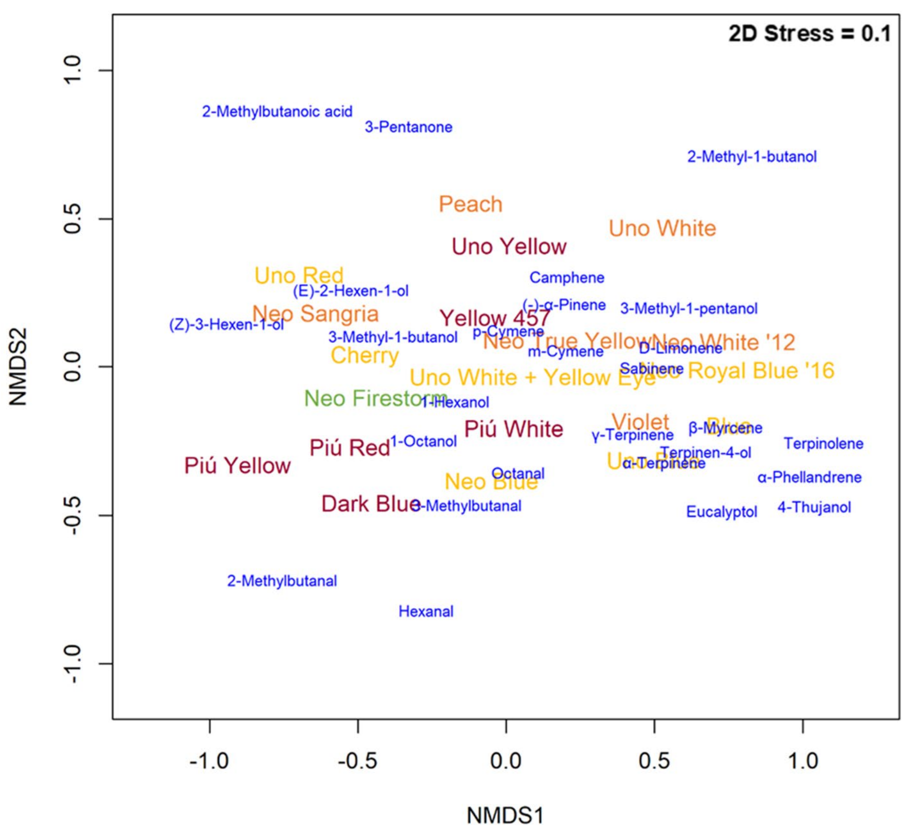

Fig. 6 Nonmetric multidimensional scaling (NMDS) of the aroma profiles of the 20 tested *Calibrachoa* cultivars classified into four groups 'highly attractive' (green), 'moderately attractive' (yellow), 'slightly attractive' (orange) and 'very slightly attractive' (red). This graphic representation is based on the quantitative analysis of the 27 flower scent compounds. The spatial proximity of some individual compounds to the highly attractive class indicates that these compounds might be relevant for the attractiveness of the floral scent to *B. terrestris*

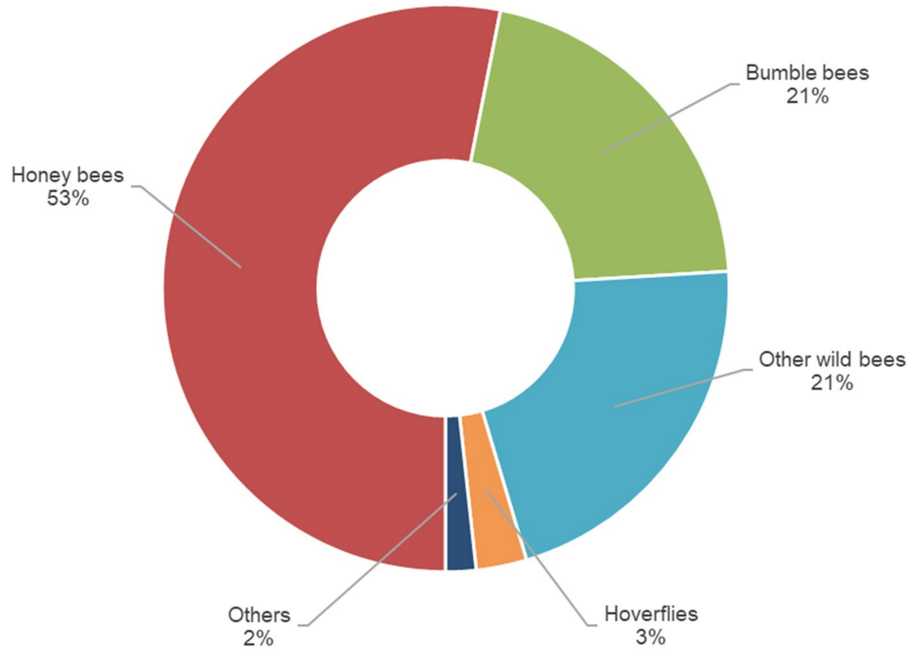

Fig. 7 Relative abundance of the different insect groups on the *Calibrachoa* cultivars in 2017 and 2019 in Stuttgart

had a highly significant impact on bumble bee attractiveness (Wald test: $Df = 5$, Wald Statistic = 292.50, $P < 0.001$). While the three series Rave®, MiniFamous® Uno and MiniFamous® Neo had a positive effect on the number of *B. terrestris* flower visitors, the series MiniFamous® Piú and the cultivar without a denomination negatively influenced the number of bumble bee visitors (Fig. 4). The pairwise comparisons showed that all combinations, except for Rave® and MiniFamous® Uno, were significantly different.

Floral morphometric parameters in the *Calibrachoa* cultivars

The mean floral morphometric parameters were highly variable among the 20 tested *Calibrachoa* cultivars (Supplementary Information Table S2). However, overall, the correlations of the floral morphometric parameters with the number of *B. terrestris* visitors were very low, with a correlation coefficient ranging only from 0.002 to 0.008. In addition, we found that the expression of the floral morphometric parameters not only depended on the cultivar but also to a substantial degree on external factors such as the location or the tent number. Regarding flower diameter, corolla diameter and corolla depth, these external factors explained between 45.9% and 60.1% of flower expression. In contrast, the lengths of the five stamens were mainly dependent on the *Calibrachoa* cultivar itself. This cultivar dependency ranged from 66.3% to 73.5%; finally, the external factors explained only 1/3 of the variation in the length of the stamen.

The mean nectar quantities varied highly within the 20 investigated cultivars (Fig. 5). In the cultivar 'Uno White + Yellow Eye', the highest amount of 1.41 mg was recorded, followed by 'Neo Firestorm', with a mean amount of 1.25 mg. In 'Neo Royal Blue '16' and 'Uno Red', we detected the lowest amounts of nectar, at 0.49 and 0.46 mg, respectively. The statistical analyses indicated that the mean nectar amount was a highly significant impact factor on bumble bee attractiveness (Wald test: $Df = 1$, Wald Statistic = 170.95, $P < 0.001$). However, the standard deviations in the sampled flowers were high, suggesting a high spread of nectar amounts in the flower samples.

Floral aroma profiles of the *Calibrachoa* cultivars and impact on bumble bee attractiveness

We identified 27 compounds in the floral aroma profiles of the *Calibrachoa* cultivars that belong to seven different chemical groups: monoterpenes (12 components), alcohols (7), aldehydes (4), alkanols (1), carboxylic acids (1), ketones (1) and menthadiene (1). Concerning the different *Calibrachoa* cultivars, qualitative and quantitative differences were apparent. The only compound that occurred in all cultivars was 3-methyl-1-butanol. Two substances, *p*- and *m*-cymene, occurred in 19 cultivars. The scarcest substance was 2-methylbutanoic acid, which was found only in the cultivar 'Uno Red'. The compounds were also present in varying concentrations in the tested cultivars. (-)-α-Pinene, sabinene, β-myrcene, α-terpinene, 3-methyl-1-butanol, γ-terpinene, *m*- and *p*-cymene and terpinen-4-ol were found at high concentrations in our samples. 2-Methylbutanal, 3-methylbutanal, 3-pentanone, camphene, hexanal, α-phellandrene, *D*-limonene, 2-methyl-1-butanol, eucalyptol, terpinolene,

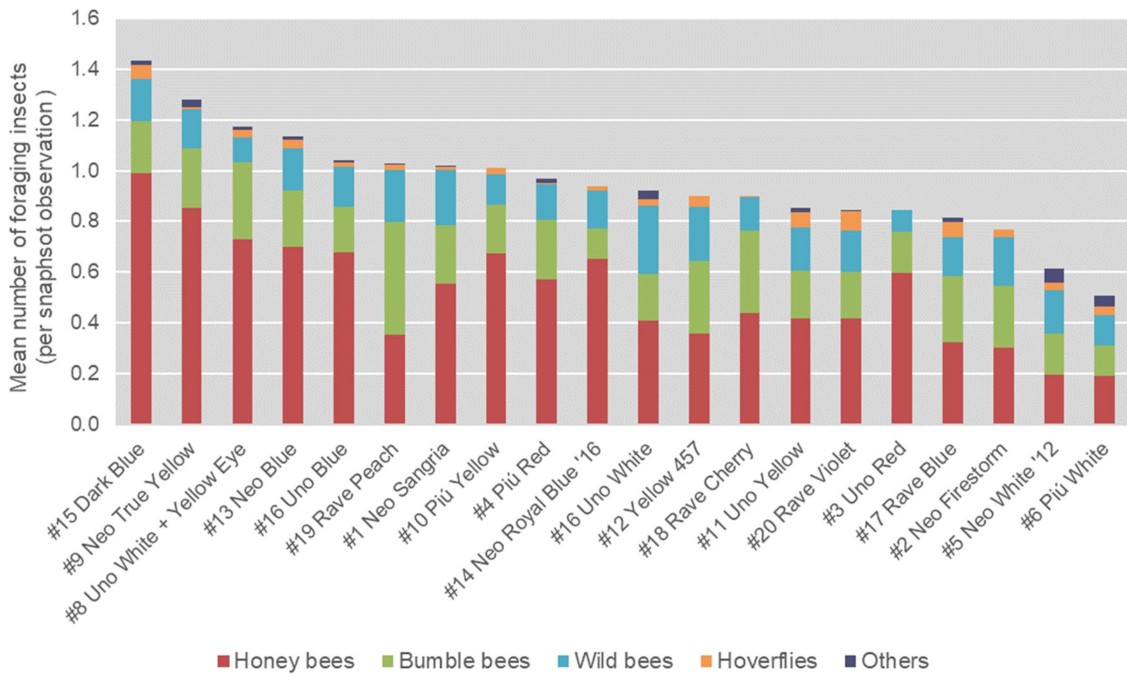

Fig. 8 Average number of foraging insects on the 20 different *Calibrachoa* cultivars. The mean values were controlled for any environmentals and methodological impacts

octanal, 3-methyl-1-pentanol, 1-hexanol, (*Z*)-3-hexen-1-ol, (*E*)-2-hexen-1-ol, 4-thujanol, 1-octanol and 2-methylbutanoic acid were detected in small concentrations or only in trace amounts (Supplementary Information Table S3).

NMDS based on the quantitative analysis of the aroma profiles of the 20 different *Calibrachoa* cultivars is displayed in Fig. 6. The figure shows that only a few individual substances in the floral aroma profile were directly associated with attractiveness to *B. terrestris*. Specifically, 7 compounds could be linked with highly or moderately attractive cultivars: 1-hexanol, octanal, α-terpinene, 4-terpinen-4-ol, β-myrcene, sabinene and (*E*)-2-hexen-1-ol. Typical compounds in *Calibrachoa* cultivars with very little attractiveness to bumble bees were camphene, (-)-α-pinene and 3-methylbutanal. Some compounds could not be linked with either particularly high or low attractiveness: 2-methylbutanoic acid, 3-pentanone, 2-methyl-1-butanol, 2-methylbutanal and hexanal.

The ANOSIM analyses of the interaction between the bumble bee-perceived petal color and the floral scent of the cultivars showed that although we received an overall significant ratio of dissimilarities, it was only weakly positive ($R = 0.1034$, $P < 0.05$). In addition, only two significant ratios could be found in the pairwise comparisons, namely, the pairs green/blue–green and blue ($R = 0.39$, $P < 0.05$) and green/blue–green and blue–green ($R = 0.57$, $P < 0.05$) (Supplementary Information Tables S4, S5). In SIMPER, the overall dissimilarity of the floral scent depending on the cultivar, which was grouped based on petal color, amounted to 68.9%. The four substances (-)-α-pinene, sabinene, *p*-cymene and 3-methyl-1-butanol contributed almost 70% of this dissimilarity (data available upon request).

Other foraging insects on the *Calibrachoa* cultivars

A total of 4,188 insects foraging on *Calibrachoa* were counted in the two years of field observation in Stuttgart. The majority of the flower visitors were honey bees at 53% (2,224 counted individuals). The bumble bees and other wild bees were equally abundant at 21% (877 and 894 counted individuals, respectively). Hoverflies (3%, 121 individuals) and other pollinating insects (2%, 72 individuals) were rarely observed (Fig. 7).

Within the tested *Calibrachoa* cultivars, the average number of insect visitors to the flowers varied greatly (Fig. 8). In sum, the cultivar 'Dark Blue' was visited most frequently, with an average of 1.4 flower visitors per snapshot observation. The least visited cultivar was 'Piú White', with only 0.5 foraging insect visitors. The observed insect groups also showed differences in their preference regarding the cultivars. 'Dark Blue' was favored by honey bees, and 'Rave Peach' was favored by bumble bees. The other wild bees preferred 'Uno White', while hoverflies were mainly observed on 'Rave Violet' and the other insects on 'Neo White '12'. The statistical analyses showed that the *Calibrachoa* cultivar had a highly significant impact on the number of insect visitors across all groups (Wald test: $Df = 19$, Wald Statistic $= 282.41$, $P < 0.001$).

Hand-netted bumble bees and other wild bee species on *Calibrachoa* cultivars

During the three catching periods in Stuttgart, 182 foraging bees were captured with a hand net (Supplementary Information Fig. S2). The most frequent genus was *Lasioglossum* (95 individuals), followed by *Bombus* (57 individuals) and *Halictus* (28 individuals). Only one individual each was captured from the two genera *Anthidium* and *Andrena*. In total, 12 different wild bee species were captured, with *Lasioglossum morio* being the most frequent, with 28%. Except for *Lasioglossum glabriusculum,* which is currently classified as vulnerable in Germany (IUCN Red List, vulnerable), none of the caught wild bee species are currently listed on the IUCN Red List.

Discussion

Calibrachoa as a foraging resource for flower-visiting insects

In this multiyear study with more than 10,000 counted plant–insect interactions in tent and open field observations, we demonstrated that *Calibrachoa* constituted a suitable foraging resource for a high number of flower-visiting insects and that the occurrence of *Calibrachoa* increased the overall local food availability. During the observation intervals, we observed flower-visiting insects collecting both pollen and nectar, concluding that *Calibrachoa* served as an appropriate resource for both floral rewards. A previous study from our workgroup using detailed observation and melissopalynological methods confirmed that bees foraging on *Calibrachoa* indeed collected *Calibrachoa* pollen and nectar (Kretschmer 2017).

However, not all 20 *Calibrachoa* cultivars tested in this study seemed to be equally suitable, as some cultivars were visited considerably more often for foraging than others. This finding is in line with a comprehensive study performed by Garbuzov and Ratnieks (2015), where several hundred different aster cultivars were tested in regard to their flower visitor attractiveness. Their results clearly showed that the insect attractiveness to the main flower visitors – honey bees and hoverflies – varied greatly within the tested cultivars (Garbuzov and Ratnieks 2015). Such cultivar-related differences in the attractiveness of ornamental plants to insect visitors have also been described in previous studies on other ornamental plants, e.g., *Zinnia* (Yeargan and Colvin 2009; Erickson et al. 2020), *Lavandula* (Garbuzov and Ratnieks 2014b), *Dahlia* (Garbuzov et al. 2015), *Lantana*, *Lobularia*, *Pentas*, and *Tagetes* (Erickson et al. 2020). As a result, these studies (including the present study) impressively show how important it is to continuously test new breeds and already established ornamental plant cultivars to provide information on their insect friendliness. This information might then be communicated with plant breeding companies as well as end consumers. Thus, the promotion of insect-friendly ornamental cultivars and the subsequent preferred use of these cultivars in urban plantings can be accomplished. This strategy is urgently needed to increase the urban nutritional resources for flower-visiting insects and to support the successful conservation of local insect diversity.

Preferences of the bumble bee *Bombus terrestris* to *Calibrachoa* cultivars

The large earth bumble bee *B. terrestris* was attracted to the 20 different *Calibrachoa* cultivars observed in this study to different degrees, and the mean number of *B. terrestris* visitors was found to vary up to fourfold. This large difference in bumble bee visitor attractiveness among the *Calibrachoa* cultivars raises the question of what floral trait might be responsible for this variation. Interestingly, we found that the cultivar 'Neo Firestorm', which is characterized by its bright red petal color, was clearly preferred over the other cultivars. However, to date, only one study in ornamental plants has investigated some flower traits that are known to attract flower-visiting insects (Garbuzov and Ratnieks 2015). By using the British national collection of asters, scientists tried to correlate some floral traits with the number of counted flower visitors in 228 different aster cultivars, and in conclusion, only bloom intensity could be directly linked with attractiveness to honey bees and hoverflies. Additional floral parameters, such as flower color, disc floret area and total capitulum, did not significantly influence the attractiveness to flower visitors. In contrast, we clearly demonstrated in our study that petal color represented an important factor in the attractiveness of *Calibrachoa* cultivars to *B. terrestris*. Cultivars that appeared blueish-green or UV-blue to the bumble bees were considerably visited more often for foraging than cultivars that appeared solely green or with a high proportion of green. This finding is in line with some previous studies that also showed that UV and blue colors are particularly interesting for bumble bees in artificial and natural flowers (Gumbert 2000; Reverté 2016). However, the high preference of bumble bees for the red-flowered cultivars is rather uncommon. For a long time, the color red was assumed not to be perceived by bees due to the lack of a corresponding receptor (Peitsch et al. 1992); therefore, a possible reason for this lack of perception was considered to be the exclusion of bees from the relevant pollination systems (Raven 1972; Rodríguez-Gironés and Santamaría 2004). Previous studies contrastingly showed that despite their rarity, some red flowers might indeed be pollinated by bees (Chittka and Waser 1997; Martínez-Harms et al. 2010; Chen et al. 2020). This could be the result of an improved perception of the flowers, which is achieved either by a sufficient reflection of UV or UV-blue light or a sufficient achromatic contrast of the red flowers (Martínez-Harms et al. 2010; Chen et al. 2020). Both aspects might apply in our case to red-flowered *Calibrachoa* cultivars. However, due to contradictory results, conclusive evidence regarding the importance of petal colors in ornamental plants for flower visitors is still lacking. However, it has been suggested that in some genera and species, color can be used as a predictor for insect visitation, and it has been shown that petal color is indeed important for flower-visiting insects, e.g., in *Zinnia* ssp. and *Lobularia maritima*. However, this could not be verified in *Symphyotrichum* spp. (asters) and *Lantana camara* (Garbuzov and Ratnieks 2015; Erickson et al. 2020).

In addition to visual floral signals, many bee species additionally rely on olfactory signals, either in combination or as a single signal (Burger et al. 2010; Schäffler et al. 2015). In this study, in total, we identified 27 single floral scent compounds, with the majority of them belonging to the chemical groups of monoterpenes and alcohols. Both chemical groups are known to contain substances that occur as secondary metabolites in flowering plants and can have attractive functions in pollinators (Levin et al. 2001; Wink 2016; Cordeiro et al. 2016). Among the 20 *Calibrachoa* cultivars, we found the floral aroma profiles to be highly varying but without a discernible pattern, e.g., dependent on petal color. The statistical analyses on the interaction between the floral scent and petal color also indicated only a weak positive correlation. A further attempt to correlate the single floral scent compounds to attractiveness to bumble bees revealed that only 7 compounds contributed to cultivar attractiveness, while the remaining 20 compounds had little or no impact. As we obtained these results by statistical evaluations only, we highly recommend verifying these results in further laboratory bioassays to separately test the effects of olfactory and visual signals of *Calibrachoa* flowers on *B. terrestris* (see Burger et al. 2010; 2017).

It is very likely that in addition to the already described floral signals, other floral characteristics might have contributed to the varying attractiveness of the *Calibrachoa* cultivars. Previously published literature demonstrated that the quality and quantity of pollen and nectar can drive floral choices in pollinators (Krömer et al. 2008; Di Pasquale et al. 2013; Somme et al. 2015). To a certain extent, this might also apply to *Calibrachoa* cultivars. Although we found the nectar quantity in the *Calibrachoa* flower samples to be highly variable, we obtained the first indications that the nectar quantity indeed impacted attractiveness. Therefore, whether nectar and pollen analyses can be performed and correlated with bee attractiveness in ornamental plants should be considered in more detail in further studies.

Based on these findings, we suggest classifying the tested *Calibrachoa* cultivars as 'attractive' (> 0.5 *B. terrestris* visitors) and 'less attractive' (< 0.5 *B. terrestris* visitors) cultivars according to their number of *B. terrestris* visitors. Thus, the three most attractive cultivars, 'Neo Firestorm', 'Uno Blue' and 'Neo Blue', might be recommended for urban plantings. As the remaining cultivars have proven to be a less suitable foraging resource for *B. terrestris*, we suggest using these cultivars in combination with other bumble bee-friendly plants, e.g., *Dahlia* x *hortensis*, *Lavandula angustifolia*, *Nepeta racemose*, *Stachys officinalis* and *Scabiosa columbaria* (Rollings and Goulson 2019; Marquardt et al. 2020).

Preferences of other flower-visiting insects

Previous studies have demonstrated that different insect groups show group-specific preferences for ornamental plant species and even cultivars (Garbuzov and Ratnieks 2014b; Erickson et al. 2020). Our comprehensive results on the preference of *B. terrestris* for *Calibrachoa* cultivars might therefore not be representative of other flower visitors, and our study should be repeated with other insect groups.

In the open field observations, we recorded high numbers of flower visitors collecting pollen and nectar on all 20 offered cultivars, indicating that *Calibrachoa* is very likely also contributing to the nutrition of a large spectrum of foraging insects. Bees represented the group with the largest number of individuals. Among this group, more than half of the observed individuals belonged to honey bees, which are considered 'supergeneralists' and are known to forage from a large range of native and nonnative flowering plants (Memmott and Waser 2002; Potts et al. 2010). The other half consisted of both bumble bees and other wild bees in equal shares. Our data indicate that bumble bees and generalist species of wild bees exclusively foraged on the offered *Calibrachoa* cultivars. This can be explained by the lack of *Calibrachoa*-associated bee species in Germany, which is consistent with other studies where mainly generalist bee species were found foraging on nonnative ornamental plants (Memmott and Waser 2002; Frankie et al. 2005; Geslin et al. 2013; Lowenstein et al. 2019; Erickson et al. 2020).

With this study, we highlighted that the use of the ornamental plant *Calibrachoa* increased the overall food availability for flower-visiting insects, at least for generalist bee species. Furthermore, due to the extended blooming period and the high abundance of flowers per plant, *Calibrachoa* has the potential to lengthen the flowering season and to provide a reliable and continuous foraging resource during periods of food shortage. For these reasons, *Calibrachoa* can certainly be advantageous for urban insect communities. However, the present study also illustrated large differences in the attractiveness of the tested *Calibrachoa* cultivars to different flower-visiting insect groups. To support a great diversity of insects, it is recommended to use I) cultivars that can be labeled "insect-friendly" through standardized tests and II) various cultivars that cover the specific preferences of different flower-visiting insect groups.

Methodological approaches

The floral aroma profile of *Calibrachoa* was analyzed for the first time in this study by using headspace solid-phase microextraction (HS-SPME) coupled with gas chromatography (GC–MS). We relied on this method because it has been proven to be suitable for aroma profile analyses and the identification of a broad range of floral odorants in plants (Bicchi et al. 2007; Rigling et al. 2019). Due to organizational reasons, however, we were obliged to freeze the flower samples prior to the floral scent analyses. Whether and how this might have affected the aroma profiles of our samples is currently a matter of speculation, as data regarding the effects of the freezing process on floral scent are still inconclusive (see Salvador et al. 2017; Culleré et al. 2013; but Xiao et al. 2015). Additionally, to date, no data of (unfrozen) *Calibrachoa* floral scents have been published, so a comparison of results is not possible. However, studies suggest that freezing is one of the most suitable preservation methods, and the observed decrease in volatile compounds in comparison with freshly analyzed material is still within an acceptable level (Salvador et al. 2017).

Interestingly, we found the expression of some floral morphometrics to be particularly dependent on the environmental context. This dependency, however, varied greatly within the examined floral morphometric parameters. While the lengths of the stamens were mainly cultivar-specific and less dependent on the environment, the expression of flower diameter, corolla diameter and corolla depth was highly dependent on the environmental context, such as the location and flight tent number. The strong impact of environmental factors on these morphometric characteristics is most likely the reason why we could not find a correlation of these characteristics with the attractiveness to insects.

The amount of nectar within a flower might also contribute to their attractiveness. Here, we found the nectar quantities of the flower samples to be highly variable, which might be traced back to the fact that we picked the flower samples randomly and independent of their age. However, unpublished observations indicate that nectar production in *Calibrachoa* depends not only on environmental factors such as daytime and weather conditions but also on the age of the flower (Dominik Losert, personal communication). To obtain reliable results, we suggest a standardized method to record nectar quantities in *Calibrachoa* cultivars, in which the age of the flower is recorded and considered in the evaluations.

The identification of distinct floral traits in *Calibrachoa* could be useful for breeding practices to increase insect friendliness in *Calibrachoa* cultivars. Unfortunately, this intention has become very challenging for two reasons. First, the expression of some floral morphometric characteristics depended on environmental factors. This makes selective breeding of insect-friendly cultivars on the basis of these characteristics almost impossible. Second, the investigation of the aroma profiles in *Calibrachoa* cultivars revealed only a few single compounds that may be related to insect friendliness. However, in contrast to the floral morphometric traits and the floral aroma profile, we obtained clear indications that the red and blue petal colors in the *Calibrachoa* cultivars promoted higher attractiveness to the model species *B. terrestris*. This information can be utilized and should be further pursued to generate *Calibrachoa* cultivars that are more attractive for flower-visiting insects.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11829-021-09844-2.

Acknowledgements We thank Wolfgang Henle, Michael Kurz and the whole team from the State School for Horticulture and Agriculture (SfG) in Stuttgart-Hohenheim for providing us free space to install the testing areas, including the three flight tents, on their premises. Special thanks go to Mathias Zäpfel and his trainees (State Horticultural College and Research Institute (LVG) Heidelberg) for the setup of the testing plants at both locations in Heidelberg and Stuttgart-Hohenheim in all three study years. Acquiring all the data would not have been possible without the help of Susanne Bonk (LVG), Marietheres Klinger (LVG) and Leni Stahl (SfG), so thank you for your support. MM owes particular thanks to Prof. Dr. Manfred Ayasse, Dr. Hannah Burger, Dr. Omer Nevo and the whole team from the Chemical Ecology workgroup at the Institute of Evolutionary Ecology and Conservation Genomics at Ulm University for permission to use the Jaz spectrometer and for their support while performing the spectral reflectance measurements. Finally, we are grateful for the helpful comments and suggestions from two anonymous reviewers who helped to improve the earlier version of the manuscript.

Author contributions DL, UR and PR conceived the research; MM, LAK and NW collected the data; LK, MM, KS and MR analyzed the data; and MM, LK and PR wrote the paper.

Funding Open Access funding enabled and organized by Projekt DEAL. The project was funded by the European Agricultural Fund for Rural Development: Europe investing in rural areas with the participation of the State of Baden-Württemberg.

Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

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