




Article

Response of Superoxide Dismutase (SOD) to Homogeneous and Heterogeneous Food Sources in Bumblebees (*Bombus terrestris*) and Honeybees (*Apis mellifera*)

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Abstract: The conception of “floral strips” is a strategy to provide more and different food sources for pollinators. The impact of “homogeneous” *Phacelia tanacetifolia* (“Phacelia”) and “heterogeneous” (flower mix) food sources on the enzyme activity of bumblebees (*Bombus terrestris*) and honeybees (*Apis mellifera*) under urban conditions has not been reported. Organisms responding to challenging environmental conditions are known to exhibit increases in oxidative stress parameters which in turn affect both physiological and metabolic parameters. A field study was conducted in Berlin-Dahlem, Germany, using the response of the “marker” enzyme superoxide dismutase (SOD) on food sources for assessment. SOD data is also shown from the wild bee *Megachile rotundata* (Fabricius 1787), obtained from three different locations in the federal state Brandenburg, Germany. The results demonstrate that the enzyme activity of SOD significantly increased in bumblebees visiting the flower mix compared to the Phacelia. The experimental approach had individual effects at the level of the species, bumblebees and honeybees, respectively. The activity of the biomarker SOD could be successfully used to assess the effects of the compositions of homogeneous and heterogeneous flower fields.

Keywords: *Apis mellifera*; *Bombus terrestris*; flower strips; *Megachile rotundata*; *Phacelia tanacetifolia*; superoxide dismutase; rural environment; urban environment



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1. Introduction

Bees (Hymenoptera: Apoidea) are the main group of pollinators, with approximately 20,000 species worldwide [1]. In Germany, more than 566 bee species with differences in their habitats, feeding habits, and nesting sources are known [2]. The decline of insects, including bees, is widely documented [3,4] and has been estimated at 9% decrease per decade [5]. Several factors could act as drivers of pollinator decline, e.g., land use changes, weather conditions, environmental pollution, but also diseases and pathogens [3,6]. These drivers affect the availability of pollen, nectar, and nesting sites and also pollinator health [3,7–9]. Several studies provide evidence that pollinator health, physiology, microbiota, reproductive capacity, and longevity are linked to the availability and diversity of floral sources [3,7,10,11].

Insects are exposed to diverse environmental stressors (agrochemicals, ultraviolet radiation, bacteria, and viruses), which are responded to by the production of “Reactive Oxygen Species” (ROS) [12]. ROS are free radicals, such as superoxide anion, hydroxyl, nitric oxide radical, and hydrogen peroxide. These metabolites are produced up to a certain level during physiological and metabolic processes, such as digestion, energy production, and

respiration, as well as at a higher level for detoxification and immune responses. Reactive species can be scavenged by several enzymatic and nonenzymatic detoxification systems [13]. In invertebrates' systems, ROS are produced by the response of plasma membrane nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, intracellular cytosolic xanthine oxidase, peroxisomal oxidases, endoplasmic reticular oxidases, and mitochondrial electron transport components [14], and references therein. Apart from this, the auto-oxidation of catecholamines, ubihydroquinone, hemoproteins, and flavin enzymes also generates ROS. However, in cases of hyperproduction of ROS or decreased antioxidative defense, oxidative stress develops [15]. In response to nutrient effects and/or stress, cells enter autophagy, which can lead to adaptation. Additionally, a number of small molecules also play a role in scavenging ROS, and some of these small molecules are plant-derived, whereas others such as carotenoids, α -tocopherol, ascorbic acid, and glutathione (GSH) can be synthesized by the insect itself.

Nutritional effects, such as the "availability" of pollen [16], result in a measurable increase in the antioxidative defense system. However, a distinction should be made here between the diversity of pollen and the amount of pollen. Thus, insects are continually subjected in different ways and intensities to oxidative stress from ROS. Superoxide dismutase (SOD, EC 1.15.1.1) is a key enzyme that plays a primary role in removing ROS, which has been identified in lepidopteran model insects (<https://metazoa.ensembl.org/> (accessed on 1 January 2023)) [12]. Three types of SOD protein have been reported in insects. SOD1 is a major cytoplasmic enzyme, SOD2 is a mitochondrial enzyme, and an extracellular, SOD3, has been identified in the hemolymph and molting fluid of insects. However, through a series of experiments, [12] four more unique and previously not-reported SODs were identified with different responses to physiology and different types of oxidative stress in *Bombyx mori*, the silk spinner, a lepidopteran insect.

The available information indicates that the use of "flower strips" has mitigated the negative impacts on bees and other pollinators caused by the scarcity and diversity of floral sources in agricultural landscapes, including urban areas [17]. Flower strips are areas that should provide food sources with mixtures of wild and cultivated plants exclusively for insects [18]. These flower strips are not subject to commercial use. Despite the success of flower strips to enhance the abundance and diversity of pollinators [11,19], the knowledge of how bees respond in physiological terms to a diverse availability of food sources on flower strips is limited. Agri-Environment Schemes (AES) [20] are being implemented in agricultural systems to stop the decline of pollinators. Scientific assessments of these schemes are still lacking, and only a few studies have examined the extent to which insect pollinators use the floral enhancements that are a part of AES and on which floral components they feed (i.e., pollen and/or nectar). To our knowledge, no field study in an urban area has been reported to date on the impact of "homogeneous" food supply (*Phacelia*) compared to "heterogeneous" food supply (flower mix) on the volume activity ("activity") of the "marker" enzyme SOD in bumblebees and honeybees. It is generally assumed that the quality of the environment in rural areas is higher than in urban ones; this fact was the motivation for setting up trap nests under "natural conditions" in the federal state Brandenburg. SOD data from the wild bee *Megachile rotundata* (Fabricius 1787), obtained from three different locations in the federal state Brandenburg, Germany, are shown in this study.

2. Materials and Methods

2.1. Construction of Trap Nests ("Insect Nesting Aids"/"Insect Hotels") and Places of Installation in Federal State Brandenburg, Germany

For identification, wild bees are caught with so-called "trap nests". The trap nests were constructed according to the methodology of [21]. They were inexpensive, made from natural materials, and can be easily placed in different habitats. A 30 cm long, 10 cm diameter PVC pipe forms the outer casing. Nest tubes were made of reeds (*Phragmites*) with a diameter of 4–10 mm and a length of 15 cm. The trap nests were protected from

predators by protective grilles over the entrance and were placed on wooden posts 1.5 m above the ground (Figure 1).

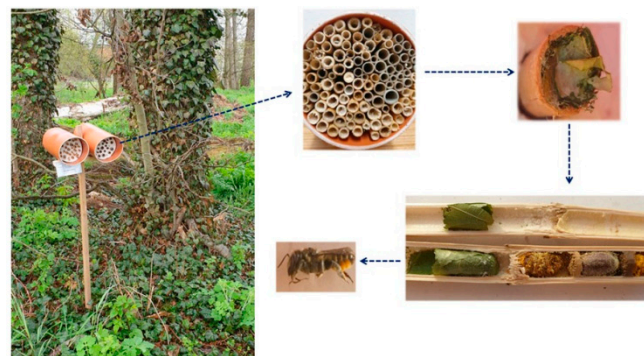


Figure 1. Example of the construction and installation of the trap nests in the federal state of Brandenburg, Germany.

2.2. Treatment before and after Overwintering of Trap Nests from Different Sites in the Federal State of Brandenburg, Germany

After deinstallation, the complete trap nests from the three locations in Brandenburg (Table 1) were stored in a cold chamber at 6 °C for overwintering until March 2021. In April 2021, the nest tubes were stored at constant temperatures of 24 °C and 60% relative humidity in an incubator (Memmert GmbH & Co. KG, Germany) until the wild bees hatched (emerged between 14 and 28 days). Until hatching, the nest tubes were controlled daily and the adult wild bees, corresponding to *M. rotundata* (Fabricius 1787), were in 1.5 mL microcentrifuge tubes (Eppendorf®, Hamburg, Germany) snap frozen in liquid nitrogen, and stored at −80 °C until processing and analysis (3.5). All handlings were in accordance with the “Code of Honor for Entomological Fieldwork” of the Federal Committee of Experts (BFA) Entomology at NABU-Naturschutzbund Deutschland e.V.

Table 1. Information on the trap nests installed at three locations in the federal state of Brandenburg, Germany.

Site	Geographic Coordinates (φ , λ)	Beeline * from Berlin-Dahlem	Installation Date	Deinstallation Date
Alt Madlitz, Briesen	52.385613, 14.291637	~65 km, south east	15 May 2020	17 October 2020
Groß Schönebeck, Schorfheide	52.898738, 13.550597	~54 km, north east	26 June 2020	24 October 2020
Schenkendöbern, Forst	51.793551, 14.573279	~104 km, south east	12 June 2020	10 October 2020

* calculation by <https://www.luftlinie.org> (accessed 1 February 2023; latitude φ , longitude λ).

2.3. Experimental Design for Different Food Sources for Bees

Two food source plots for bees (each ~600 m²) were established in 2021 at the experimental fields of the Free University Berlin (FU) and the Albrecht Daniel Thaer-Institute (ADTI at the Humboldt University of Berlin, HU) (52.47° N, 13.30° E, h = 51 m a.s.l.) under Berlin’s urban landscape, close to forest remnants and green areas. In April (23 April 2021), the plot with *Phacelia tanacetifolia* (Phacelia), cultivar “Beehappy” (“homogeneous food resource”) with a seed density of 12 kg/ha was planted on the area of FU (Figure 2), and the peak of flowering was observed during June. In May (11 May 2021), the plot with the flower mix “Veitshöchheimer Bienenweide” (“heterogeneous food resource”), obtained from Feldsaaten Freudenberger GmbH & Co. KG, 47800 Krefeld, with a seed

density of 12 kg/ha was planted at the area of ADTI (Figure 2), and the peak of flowering was observed during July. Both months correspond to the “food gap” for bees. The Veitshöchheimer Bienenweide is a bee pasture, consisting of flowering annual and perennial wild and cultivated species (69% herbs; 22% legumes; and 9% of a mixture of buckwheat (*Fagopyrum*), mallow (*Malva*), and sunflower (*Helianthus*)). Annual species, such as the marigold (*Tagetes*) and the sunflower flower in the year of sowing. The mullein (*Verbascum thapsus*) blooms in the second year, as well as perennial species such as meadow sage (*Salvia pratensis*) and clover (*Trifolium* L.). The more detailed composition, especially for the herbs, is shown at www.freudenberger.net. Surrounding both plots, crops of rapeseed, wheat, potatoes, and aromatic herbs were found. The two plots were about 1 km apart (Figure 2).



Figure 2. Schematic depiction of Phacelia plot at Free University Berlin (FU) and the flower mix plot at Albrecht Daniel Thaer-Institute (ADTI, HU) (<https://www.google.de/intl/de/earth/index.html>, accessed on 6 March 2023).

2.4. Bee Collection of *Bombus terrestris* and *Apis mellifera* from Phacelia and Flower Mix Plots

The sampling of bees was conducted following the “Code of Honor for Entomological Fieldwork” of the Federal Committee of Experts (BFA) Entomology at NABU-Naturschutzbund Deutschland e.V. Individuals were collected at midmorning during sunny days using sweep nets, on two sampling days separated by one week in June (5 individuals from *B. terrestris* and 5 individuals from *A. mellifera* collected at each sampling day) from Phacelia, and the same was done in July from the flower mix. Taxonomic identification was done a priori on site, considering that the most abundant bumblebee at the study site was *B. terrestris*. Both species were confirmed using the head and thorax of each individual using taxonomic keys. Subsequently, individuals were immediately introduced into 1.5 mL microcentrifuge tubes (Eppendorf®) and shock frozen in liquid nitrogen during transportation to the laboratory where they were finally stored at -80°C until further processing (Figure 3) and analysis (2.5).

2.5. Measuring Superoxide Dismutase (SOD) Activity

SOD activity was measured by LABOKLIN, 97688 Bad Kissingen (<https://laboklin.de> (accessed on 1 January 2022)), using a Superoxide Dismutase Assay Kit (Cayman Chemical, Ann Arbor, MI, USA, product no. 706002 [16]). The assay measures the three types of SOD (Cu/Zn, Mn, and FeSOD). Ten μL of the supernatant was diluted 1:500 with the supplied sample buffer, and the remainder of the assay was conducted following the manufacturer’s instructions. SOD in the bee samples was compared to a standard curve of SOD (provided by the manufacturer) and absorbance was read at 450 nm. SOD activity was expressed as units per mL.

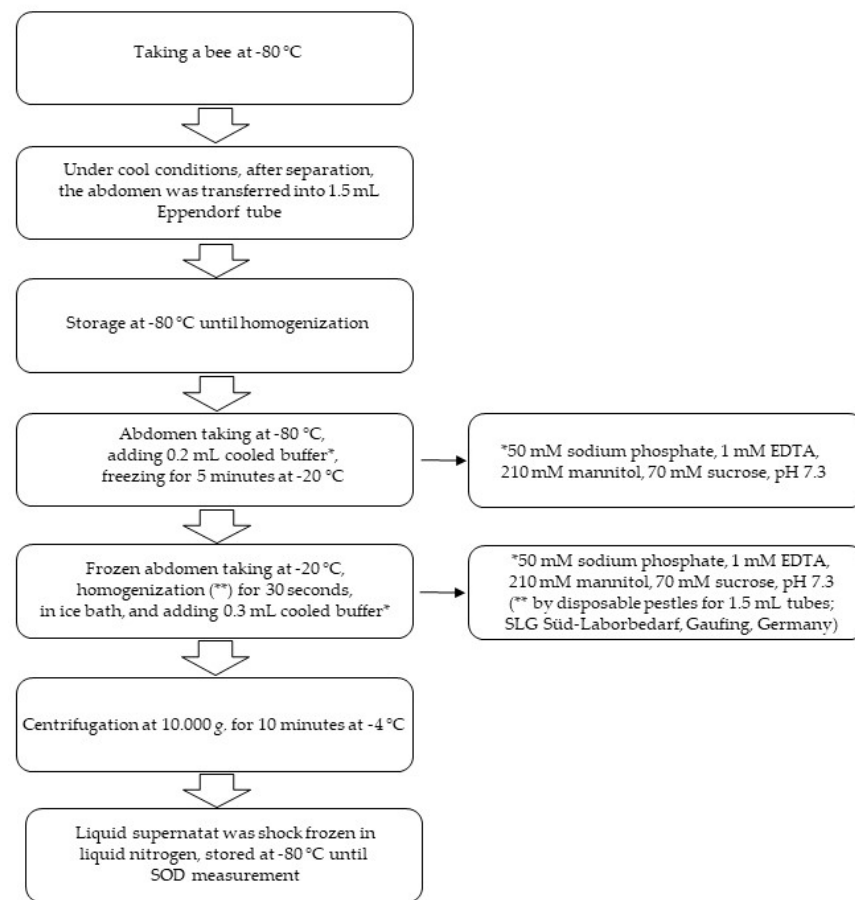


Figure 3. Schematic flow diagram for processing *Bombus terrestris* and *Apis mellifera* before superoxide dismutase (SOD) measurement by LABOKLIN.

2.6. Statistical Analysis

Standard statistical analyses (mean, standard error (SE), multiple comparison of means) was performed using IBM SPSS V29, using the one-way ANOVA with the Tukey HSD-test (Table 2), and the two-sided test, testing whether the means are different at $p = 0.05$ (Table 3).

Table 2. Superoxide dismutase (SOD) activity (U/mL) in hatched wild bee *Megachile rotundata* (2022) from different installation sites in the federal state Brandenburg (2021).

Site SOD Activity (U/mL)	Alt Madlitz, Briesen	Groß Schönebeck, Schorfheide	Schenkendöbern, Forst	Mean of 3 Sites ± SE
	37.96	32.25	25.59	
	18.23	9.83	31.14	
	26.82	12.31	41.04	
	22.75	27.88	15.83	
	18.57	34.77	29.48	
		28.63		
		40.50		
		36.19		
		42.47		
		28.77		
Mean ± SE	24.87 ^a ± 1.81	29.36 ^a ± 3.42	28.62 ^a ± 4.09	28.05 ± 2.13

Similar letters indicate nonsignificant differences (ANOVA, Tukey-HSD test, $p = 0.05$).

Table 3. Superoxide dismutase (SOD) activity (U/mL) in *Bombus terrestris* and *Apis mellifera* collected from Phacelia in June 2021 and from flower mix in July 2021 in Berlin-Dahlem.

Species Food Source	<i>B. terrestris</i>		<i>A. mellifera</i>	
	Phacelia	Flower Mix	Phacelia	Flower Mix
	33.32	116.30	31.85	27.88
	22.61	47.97	36.19	34.77
	19.77	112.35	35.82	42.16
	9.72	94.30	42.63	41.41
	19.53	85.41	36.19	27.79
	20.75	56.55	29.58	22.75
	23.12	78.89	48.63	29.22
	23.44	73.98	39.08	21.71
	17.27	103.45	29.48	130.97
	21.71	112.81	22.40	140.55
Mean	21.12 ^{bB}	88.20 ^{aA}	35.19 ^{aA}	51.92 ^{aB}
SE	1.86	7.58	2.34	14.15

Different small letter indicates significant difference (two-tailed *t*-test, $p = 0.05$) of species between Phacelia or flower mix; different capitals indicate significant difference (two-tailed *t*-test, $p = 0.05$) of Phacelia or flower mix between species, *B. terrestris* and *A. mellifera*, respectively.

3. Results and Discussion

3.1. SOD Activity in Hatched Wild Bee *Megachile rotundata* from Different Sites in the Federal State Brandenburg

It is well known that cities have various problems related to emissions they put into the environment and the so-called “carbon footprint”. This can affect the quality of the environment to varying degrees. It can be generally assumed that the quality of the environment in rural areas is higher than in urban ones; this fact was the motivation for setting up trap nests under “natural conditions” in the federal state Brandenburg. From trap nests in Brandenburg, the species *M. rotundata* was dominant (~90%). This “alfalfa-leaf-cutting bee” is distributed in Southern and Central Europe. The preferred habitat includes inland dunes, sand and clay pits, flood dams in contact with alluvial forests, south-facing and structurally rich forest edges, and also parks and gardens in urban areas [22]. Flowers and leaves, e.g., of Pelargonium, Hortensia, Rosa, Euphorbia, Reseda, Syringa, and Vitis are used as food sources and nesting materials. In the United States and Canada, *M. rotundata* is the main pollinator of alfalfa (*Medicago sativa* L.) for seed production [23].

The SOD activity in hatched bees of *M. rotundata* (Table 2)—without any contact to flowers—was on average (\pm SE) 24.87 (\pm 1.81), 29.36 (\pm 3.42), and 28.62 (\pm 4.09) U/mL for the three sites, Alt Madlitz, Groß Schönebeck, and Schenkendöbern. For this species, there are no statistical differences in the SOD activity between the three rural sites. Therefore, the mean value of the pooled SOD activity ($n = 20$) for the three locations in Brandenburg was 28.05 (\pm 2.13) U/mL (Table 2). However, it should be considered that *M. rotundata* is a different species compared with *B. terrestris* and *A. mellifera*, because it is a solitary bee species, which does not build colonies or store honey.

3.2. Superoxide Dismutase (SOD) Volume Activity (U/mL) in *Bombus terrestris* and *Apis mellifera* Collected from Phacelia and from Flower Mix at Berlin-Dahlem

The SOD activity in *B. terrestris* (Table 3) from Phacelia was 21.12 (\pm 1.86) U/mL (June 2021) and significantly increased in the flower mix by the factor 4.2 to 88.20 (\pm 7.58) U/mL (July 2021). In *A. mellifera* the SOD activity (Table 1) from Phacelia was 35.19 (\pm 2.34) U/mL and 51.92 (\pm 14.15) U/mL from the flower mix, (collected June, July), respectively, and was statistically not different. In Phacelia, however, *A. mellifera* had a significantly ($p = 0.05$) higher SOD activity (by a factor of 1.7) than *B. terrestris*, 35.19 (\pm 2.34) and 21.12 (\pm 1.86) U/mL, respectively. In the flower mix, the SOD activity was higher (by a factor of 1.7) in *B. terrestris* ($p = 0.05$) compared to *A. mellifera*, 88.20 (\pm 7.58) and 51.92 (\pm 14.15) U/mL, respectively.

It should be considered [20,24] that both *B. terrestris* and *A. mellifera*'s most flower visits were for collecting nectar. Only 22% of honeybee visits and 3% of bumblebee worker visits were for pollen collection. Nectar properties vary with environmental factors, pollinator visits, and microbial contamination [25]. Nectar is a viscous liquid, rich in glucose, fructose, and sucrose, that contains minerals and fragrances, free amino acids, proteins, inorganic ions, and secondary plant compounds (plant defense chemicals). The naturally occurring ratios of each sugar vary between plant species; bee-pollinated plants tend to be sucrose-dominant and glucose-poor, whereby sucrose-rich nectars are more common in protected, tubular flowers [26], and references therein, [25]. This behavior may lead to lower SOD activity values for the homogeneous stand of *Phacelia* in *A. mellifera*, compared to the flower mix.

A further explanation lies in the purple flower color of *Phacelia*, typical to the occurrence of anthocyanins. These are water-soluble plant pigments in the cell sap of almost all higher plants and they give flowers, fruits, and vegetables an intense red, violet, or blue color. Anthocyanins are among the most effective antioxidants, which primarily act as free-radical scavengers, but also absorb short-wave UV light and pass on radiant energy as heat.

Results on honeybees [15] kept in traditional, “natural accommodation” hives (“trmka hives”) without any human influence, compared to commercially managed standard hives, showed that both hive types resulted in marked differences of superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), and malondialdehyde (MDA). Only the SOD activity in the traditional hives was significantly higher compared to bees from commercial hives, where the CAT, GST, and MDA concentration was significantly increased in comparison to the traditional hives. It is known that SOD catalyzes the dismutation of the superoxide radical (O_2^-) into hydrogen peroxide (H_2O_2), the first-line defense against ROS; it was assumed, that significantly higher values of oxidative stress parameters (CAT, GST, and MDA) in commercial hives resulted from higher burdens of parasites.

Flower contact is inevitably linked to pollen contact. The crude protein (CP) content of pollen is reported in the wide range from 2.5% to 61%. The protein content of the pollen of blooming plants may influence the floral choice of pollinators [27]. In contrast to many wild bee species, which depend on certain flowers, the honeybee flies to everything that is in bloom, making it very flexible. For *A. mellifera* this may be the reason for the wider adaptation range of SOD activity when visiting homogeneous or heterogeneous flower plots, 35.19 (± 2.34) U/mL and 51.92 (± 14.15) U/mL, respectively (not significant, Table 3).

Pollen is required for a bee's own energy supply as well as for feeding larvae and young nurse bees. The CP content of pollen collected by *A. mellifera* is given in a range from 7 to 37% [28]. The nutritional benefits to honeybees of floral sources of pollen [29] can be divided into three categories: pollens with more than 25% CP (excellent quality; e.g., *Lupinus angustifolius* (lupines): 34%), 20–25% CP, (average quality; e.g., *Vicia sativa* (vetch): 24%), and lower than 20% (poor pollen quality; e.g., *Helianthus annuus* (sunflower): 13%).

Summarized, the reasons for the marked upregulation of the SOD activity for *B. terrestris* for the flower mix plot can be explained as follows: (a) Superoxide inactivates in the citric acid cycle the enzyme aconitase (EC 4.2.1.3) (catalyze isomerization of citrate to isocitrate via cis-aconitate); (b) For the inactivation of other ROS and RNS (Reactive Nitrogen Species); (c) And/or protection against diseases and so to maintaining health and fitness. The half-life of superoxide is less than one second in the mmol range and ~14 h in the nmol range. Superoxide dismutase is one of the few known catalytically perfect enzymes ($\sim 7 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$), because a catalytic reaction occurs at almost every encounter with the substrate and the reaction is only limited by the rate of diffusion [30]. The activity of the SOD, thus, represents the current metabolic status of *B. terrestris* and *A. mellifera*.

4. Conclusions

Our experimental approach—monofloral vs. polyfloral field plots combined with measurement of SOD activity—had individual effects at the level of the species, bumblebees

and honeybees, respectively. Therefore, the activity of the biomarker SOD could also be used to assess the effects of different compositions of mixed flower fields on other wild bees.

To improve the assessment of the benefit from different wild and cultivated plants for wild bees, the content and profile of anthocyanins, the total phenol content, and the antioxidant capacity of nectar should be analyzed. With such an approach, the spectrum of flower colors from white to yellow/orange and red to dark purple could be selected first, with the flower morphology being considered secondarily. This could improve the decision for plant selection for the composition of flower strips, based on the “quality” of nectar.

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Data Availability Statement: All data of this study available in the text.

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