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„The pollination system of the common horse chestnut *Aesculus hippocastanum* (Hippocastanaceae) "

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Summary

Die gewöhnliche Rosskastanie *Aesculus hippocastanum* (Hippocastanaceae) hat die Eigenschaft, dass sich die Saftmale der Blüten während der Anthese von gelb, über orange zu rot verfärben. Es wird vermutet, dass die Rosskastanie ihren Bestäubern damit signalisiert, welche Blüten frisch, mit Nektar und daher attraktiv und welche Blüten alt, ohne Belohnung und demnach unattraktiv für diese sind. In der Literatur wird dieses Phänomen rein auf den Farbwechsel der Blütensaftmale zurückgeführt. Jedoch wurde in früheren Studien angemerkt, dass sich mit der Farbänderung der Saftmale auch der Duft verändert, welcher von der Blüte emittiert wird. Bisher wurde dies hauptsächlich durch das Riechen an der Blüte festgestellt und kaum chemisch analysiert und bewiesen.
In dieser Studie wurde zum ersten Mal an einer größeren Stichprobe untersucht, ob sich der olfaktorische Reiz tatsächlich mit der Umfärbung des optischen Reizes ändert. Hierzu wurden an Einzelblüten parallel Duft-, Farb- und Nektarmessungen durchgeführt. Es konnte festgestellt werden, dass sich der Blütenduft mit dem Farbwechsel der Blüten verändert. Sieben unterschiedliche Duftstoffe konnten als korrelierend mit den einzelnen Farbphasen der Blüte identifiziert werden: 1-Pyrrolin, 2-Methoxy-4-Vinylphenol, Indol, Methylisovalerat, unk1043, unk1185 und unk1873. Mit Hilfe eines bienenspezifischen Farbraums konnte gezeigt werden, dass die unterschiedlichen Färbungen der Rosskastanien-Blüte von *Bombus terrestris*, als Beispiel für einen natürlichen Bestäuber von *A. hippocastanum*, höchstwahrscheinlich wahrgenommen und auch unterschieden werden können, da sich das Reflektionsspektrum der Saftmale sowohl im Kontrast als auch in der Farbe unterscheidet. Außerdem konnte gezeigt werden, dass der Besuch der jungen Blüten mit gelbem Saftmal für die Bestäuber der gewöhnlichen Rosskastanie am lohnendsten ist, da in dieser Phase der Hauptteil des Nektars produziert wird.
Auf Grund der Ergebnisse in dieser Studie kann angenommen werden, dass die Blütenfarbe und möglicherweise auch der Duft als ehrliches Signal der gewöhnlichen Rosskastanie für ihre Bestäuber dienen. Dies sollte jedoch auch noch durch Verhaltensexperimente bestätigt werden.

Abstract

A characteristic of the common horse chestnut *Aesculus hippocastanum* (Hippocastanaceae) is a color change of their floral nectar guides during anthesis from yellow *via* orange to red. It is assumed that *A. hippocastanum* signals its pollinators, which flowers are young, contain nectar, and are attractive and which flowers are old, without any reward, and therefore unattractive for them. For the most part, this phenomenon is treated in the literature only concerning the color change of the nectar guides. In a few studies, though, it has been noted that the emitted floral scent does change in parallel to the color change of the flowers. However, this was observed primarily by taking a smell at the flowers and has not yet been tested using chemical analyses.
This study investigated a possible correlated change of visual and olfactory cues on a large sample of flowers for the first time using a combination of photo spectrometric and analytical chemical (gas chromatography/mass spectrometry) methods. The reflectance spectra measurements of the colored nectar guides were analyzed using a bee specific color vision model of *Bombus terrestris*, one of the natural pollinators of *A. hippocastanum*. In addition, the amount of nectar and its sugar concentration was measured for flowers at the different anthetic floral color stages.
It was shown that a floral scent change does occur and correlates with the color change of the flowers. Seven different scent compounds, 1-pyrroline, 2-methoxy-4-vinylphenol, indole, methyl isovalerate, unk1043, unk1185, and unk1873, which are correlating with the different color phases, could be identified. It could be demonstrated that the bumblebees likely perceive and distinguish between the coloration of the common horse chestnut´s flowers as the light reflectance spectra of the nectar guides differ in contrast and coloration. Furthermore, it could be demonstrated that the early, yellow-colored flowers are the most rewarding ones for the pollinators as it is in this phase, when most nectar is produced.
The results of this study indicate that the floral color and potentially even the scent serve as an honest signal for the pollinators of *Aesculus hippocastanum*. It must be noted that this needs to be tested also by behavioral experiments.

Introduction

Most angiosperms are insect-pollinated and attract the attention of their pollinators by olfactory and visual flower signals, such as odor, shape, and color (Chittka and Raine, 2006; Ollerton et al., 2011). Thus, these flower traits serve as communication channels between the flowers and their pollinating insects and are essential for long and short-distance attraction (Dötterl and Vereecken, 2010). Contrasting colors on flowers can further specify this communication, for example, by guiding the pollinators to the nectar reward in the form of so-called ´nectar guides´ or ´Saftmale´ after Sprengel (1793) and Tegrovsky and Pany (2019). The function of nectar guides was a much-discussed topic in the early literature before it was known that bees can perceive colors. Sprengel´s (1793) hypothesis that the colored spots on the flower lead the pollinators to the nectar was first rejected by Hess (1910), whose experiments falsely suggested that bees are color-blind and therefore cannot use the nectar guides as visual orientation. Later, it could be demonstrated that bees can be attracted by colored, visual stimuli such as nectar guides and even perceive different wavelengths of light (Knoll, 1922; Frisch, 1950), which supported Sprengel´s original hypothesis. A floral color change during anthesis is widespread in angiosperms and occurs in at least 78 families (Weiss and Lamont, 1997; Brito et al., 2015). Such a color change is often seen as an honest signal for pollinators signaling that there is no more nectar to collect as the flower has already been pollinated (e.g., in sky lupine *Lupinus nanus* or red sage *Lantana camara*; Schaefer et al., 2004). The color change of the complete flower, including stamens, style, and petals, e.g., in *Tibouchina pulchra*, changing color from white to pink during anthesis (Brito et al., 2015), is rarer than a color change of single parts of the flower, like nectar guides (Weiss, 1991). Whether this particular plant behavior is an actively induced color change by the plant itself, a result of a pH change after pollination, an effect of natural floral senescence, or the combination of these possible explanations is not clarified yet. However, Weiss (1991) could demonstrate that such a color change is of advantage both for plant reproduction and for the foraging success of the pollinators.

Volatile organic compounds (VOCs), lipophilic liquids with high vapor pressure and low molecular weight, occur in all plant parts such as leaves, seeds, stems, and flowers (Muhlemann et al., 2014). It is assumed that floral VOCs protect the plant against herbivores and pathogens but also are important in the interaction with flower-visiting insects to ensure pollination and reproduction, whereas the VOCs of all other plant parts are used for defense only (Muhlemann et al., 2014; Lawson et al., 2017). Karl von Frisch (1950) showed with his experiments that bees can perceive floral scents and associate them with a reward. Many factors can influence the floral scent composition, which is why variations occur among populations, across environmental gradients, with floral gender and even with floral color (Odell et al., 1999; Majetic et al., 2008; Ashman, 2009; Majetic et al., 2009; Burkle and Runyon, 2017).

The flowers of *Aesculus hippocastanum* L. contain primarily white petals and change the color of their nectar guides from yellow *via* orange to red during its few days of anthesis (Sprengel, 1793; Focke, 1889). After observing that only the flowers with yellow nectar guides produce the rewarding nectar (Focke, 1889; Kugler 1936), it could additionally be demonstrated that pollinators of *A. hippocastanum*, e.g., *Bombus terrestris* L., prefer to visit the flowers with the yellow nectar guides and can learn to avoid the flowers with the red ones (Kugler, 1936; Vogel, 1950; Lex, 1954). However, in all former studies, the floral color change has only been described by the human color perception and therefore classified in yellow, orange, and red nectar guides. In this study, reflectance spectra measurements of *Aesculus hippocastanum* flowers were done for the first time to test with a visual model system of bumblebees whether the pollinators can perceive and distinguish the different colored spots.

Other earlier studies on *A. hippocastanum* also identified a scent change correlating with the color change of the nectar guides (Lex, 1954). This specific phenomenon has only been described poorly in the literature and was mainly based on anecdotal evidence from smelling the flowers. It has only been investigated recently in greater detail, using chemical analysis (Stern, 2020). Based on a relatively small sample size, Stern (2020) could show that flowers with a yellow nectar guide differ in floral scent composition from flowers with a red nectar guide. The aims of this study are (1) to test for a correlated change of scent and color of the flowers of *Aesculus hippocastanum* based on a larger sample size, (2) to investigate nectar production during the three flowering stages to confirm that only yellow-colored flowers are rewarding and (3) to test whether pollinators (*Bombus terrestris)* can potentially perceive and distinguish between the colored nectar guides.

Materials and Methods

Studied Species and floral biology

Aesculus hippocastanum (Hippocastanaceae), the common horse chestnut, has its origin on the Balkan Peninsula (Avtzis, 2007) and is a common tree in parks, alleys, and even palace gardens because of its dense leaf canopy and attractive inflorescences (Lack, 2002). Its flowers are visited and pollinated by different bee species. The flowers are presented on big, candle-like, upright inflorescences (see Fig15, Appendix) formed by smaller sub-inflorescences of four to six flowers, called cincinni (Kugler, 1936). Each inflorescence is composed of functionally male, hermaphrodite, and female flowers, which differ in the functionality of stamens and pistil, respectively. Because of the temporal staggering of the flowers' anthesis, an inflorescence of *A. hippocastanum* is continuously flowering for two to four weeks, containing several anthetic flowers in different color stages simultaneously (Kugler, 1936). Female flowers are scarce, and even hermaphrodite flowers are not as frequent as functionally male flowers. Thus, all investigated non-male flowers in this study which were used for the sexual comparison were functional hermaphrodites.

Study Site and Sampling Strategy

This study was carried out during the flowering period of the *A. hippocastanum* from the 4th to the 29th of May in 2021 in the "Kastanien Allee" in the garden of Schloss Schönbrunn (48°11′N,°16°19′E; Fig16, Appendix) in Vienna, Austria. Bagging was necessary to study individual flowers' floral scent, color, and nectar secretion while avoiding bias by visitation, pollination, and nectar depletion by flower-visiting insects. To achieve this, a total of 130 entire inflorescences were covered with fine-meshed, reusable fruit and vegetable bags before anthesis (see Fig17A, Appendix). In addition, 120 small organza bags were used to cover sub-inflorescences (see Fig17B, Appendix). All in all, inflorescences on 26 trees were bagged.

The "Österreichische Bundesgärten" already labelled the trees with metallic number tags, which was adopted for this study. All investigated inflorescences were on the south-, south-east-faced side of each tree to exclude a bias of different sunlight exposure. The scent samplings took place between 10AM and 1PM. During that daytime, the scent emission of the flowers should be the strongest (F. Etl 2021, personal communication). Measurements were only performed in dry weather conditions with a minimum ambient temperature of 15°C.

Floral Scent Collection and Analysis

To increase and simultaneously standardize floral scent emission for the measurements, flower pools of ten to twenty male and five to fifteen hermaphrodite flowers were collected from each tree for each nectar guide color. With tiny scissors, flowers having the same nectar guide color were cut from the inflorescence and subsequently placed in a customized (12 cm x 15 cm) polyethylene oven bag (Toppits, Germany) using tweezers to avoid contamination. Using the dynamic headspace method (Dötterl et al. 2005; Braunschmid and Dötterl 2020), the emitted floral scent was collected either for five minutes after two minutes of vaporization time or for fifteen minutes without having the two minutes of vaporization. The vaporization period was used to minimize the amount of injury-derived scents the flowers might emit after being removed from the inflorescence, e.g., green leaf volatiles (GLVs) or herbivore-induced plant volatiles (HIPVs) caused by mechanical damage (Halitschka et al., 2004; War et al., 2011; Ameye et al., 2017). The customized oven bags were closed for scent collection to enclose the floral scent. With a rotary vane vacuum pump (G12/01EB, Gardner Denver, Germany) with an airflow of 0.200 l/min, the floral volatiles were collected using adsorbent quartz glass tubes (25mm in length and 2mm inner diameter) containing 1.5mg of Carbotrap B (mesh 20–40, Supelco, Germany) and 1.5 mg of Tenax TA (mesh 60–80; Supelco, Germany), which was fixed using glass wool plugs (see Etl et al., 2017; Braunschmid and Dötterl 2020)(see Fig18, Appendix). As a negative control, three samples of collected scent from empty oven bags were used. Scent measurements on an "empty" inflorescence (n=1), with all flowers removed, and cut petioles (n=1) were used to identify possible injury derived scents and to exclude the volatiles not emitted by the flower alone. The measurements of the empty inflorescence and petioles without flowers used in this study were done by Stern (2020).

Using gas chromatography coupled to mass spectrometry (GC/MS) (QP2010Ultra, Shimadzu Corporation, Japan) combined with a thermal desorption unit (TD-20, Shimadzu, Japan) provided with a ZB-5 fused silica column (5% phenyl polysiloxane; length 60mm, inner diameter 0.25mm, film thickness 0.25µm, Phenomenex, USA), the flower pools were analyzed and their scent compounds identified (see Braunschmid et al., 2017; Etl et al., 2017; Braunschmid and Dötterl 2020). The scent pool samples were run with a consistent helium (carrier gas) flow of 1.5ml/min and a split ratio of one to one. Analysis started with a GC oven temperature of 40°C, which was increased by 6°C per minute until reaching 250°C, which then was held for one minute. The MS interface operated at 260°C, while the ion source operated at 200°C. In EI mode, the mass spectra of the samples were taken at 70 eV from m/z 30 to 350 (see Braunschmid et al., 2017). Afterwards, each flower pool spectrogram obtained by GC/MS analysis was manually edited and processed using GCMSolution 4.11 (Shimadzu Corporation, Japan). Volatiles occurring in pool samples and also in the negative controls were removed. Scent compound identification was made using the NIST 11, Wiley 9, FFNSC 2, Essential Oils and Adams 2007 mass spectral databases (see Dötterl et al., 2005; Etl et al., 2016; Braunschmid and Dötterl 2020). If possible, final scent compound identification and confirmation were made by S. Dötterl (2021) by comparing the mass spectra and retention times with available authentic standards (on a stock collection of Stefan Dötterl, University of Salzburg; see Etl et al., 2017).

Using canonical analysis of principal coordinates (CAP), the scent compound variations for the nectar guide colors were analyzed. With the color as a factor for groups, S17 Bray-Curtis similarity for resemblance, standardized samples by total, square roots transformations, and using PERMANOVA with 9999 permutations, the correlation of color on the scent was calculated (Primer 7, Version 7.0.21, PRIMER-e). Subsequently, a multiple regression was performed to evaluate the decisive scent compounds for each nectar guide color, only taking the scent compounds into account with a correlation of 0.3 or higher. Further analyses were only performed on these correlating scent compounds. Using PERMANOVA with the function adonis from the vegan package in R 4.0.3 (R CORE, Team, 2020), other possible influencing factors (tree ID and sex) were calculated. With the Kruskal-Wallis rank-sum test, the differences of the relative amount of each scent compound per nectar guide color were calculated.

Floral Nectar Collection and Analysis

The same flower pools, which had already been used for the scent collection were also used for the floral nectar measurements. The nectar collection was performed on ten male and ten hermaphrodite flowers from each pool. With a 2µl capillary (Minicaps, Hirschmann, Germany) the nectar of each flower was extracted (see Fig19, Appendix). To ensure that the total amount of nectar from a single flower could be collected, all petals were removed to access the nectar disc. To calculate the amount of each flower´s nectar, the length of the absorbed nectar in the capillary was measured to the closest mm. Knowing the total length (3.2cm) and volume (2µl) of the capillary, the exact quantity of nectar per flower in µl could be determined. The nectar was transferred (with a capillary holder) onto an eclipse handheld refractometer (45-81 Brix 50 Low Volume and 45-82 Brix 80 Low Volume, Bellingham + Stanley, UK) to measure the sugar concentration given in Brix degree. To calculate the actual sugar content (µg) for each flower, the measured sugar/solution (Brix degree), which is expressed as weight/weight-concentration (wwc), had to be converted to the weight/volume-concentration (wvc). According to Cruden and Hermann (1983), the following formula for a non-water-solution was used to correct measured concentrations:

$$wvc = 0.0046wwc + 0.9946 \qquad \text{[eqn. 1]}$$

To obtain the actual amount of sugar for each flower, the calculated correction factor (wvc) had to be multiplied with the originally measured concentration. The result is then given in mass (µg) / volume (µl). Afterwards, the nectar volume (µl) needs to be multiplied to gain the actual amount of sugar in µg. Referring to Kearns and Inouye (1993), the optical activity of different sugars in nectar solutions correlates with their amount of energy and can be seen as sucrose equivalents.

To calculate whether the factor sex has an impact on the amount of nectar and sugar quantity, first statistical analyses were performed only on the tree IDs on which both, male and hermaphrodite flowers, could be observed during this study by using a PERMANOVA (Permutational Multivariate Analysis of

Variances Using Distance Matrices) based on the Euclidean distance with 9999 permutations. Subsequently, the Wilcoxon rank-sum test with p-value adjustment of Bonferroni was performed as post hoc tests. Afterward, the same statistical analyses for all trees from which samples could be obtained were performed for each influencing factor.

Floral Color Measurement and Analysis

Again, the same flowers that had already been used for the scent and the nectar collection were also used for the nectar guide color measurements. Thus, ten male and ten hermaphrodite flowers of each pool were examined. One of the two petals containing a yellow, orange, or red-colored nectar guide was stuck on a flat piece of wood (Fig20, Appendix). This piece of wood was wrapped with black adhesive tape upside down, and the sticking area faced upwards. Sticking petals to the adhesive tape guaranteed flat placement for the spectral measurements. Using a photo spectrometer, the reflectance spectra of the colored nectar guides were measured. In addition, one white petal per flower pool was measured. For light reflection analysis, a deuterium and halogen lamp (Mikropack, DH-2000-BAL, Ocean Optics Inc., Dunedin, FL, USA) was used to cover the complete spectrum from 200 to 800nm. With a USB2000 Spectrometer (Ocean insight, USA), the reflected light was analyzed using the software package SpectraSuite (Ocean insight, USA). The spectrometer was calibrated using a certified reflectance standard (WS-1-SL, Ocean insight, USA). This white standard reflects the same intensity for all wavelengths (>96% from 250-2000nm). For 0% reflection (black calibration), the light flux to the sensor was interrupted using the shutter of the light source and blocking all ambient light.

The sensor probe was fixed in a probe holder to avoid distance fluctuation between the samples and the probe itself (Ocean Optics). Measurements were performed at an angle of 45° with respect to the petal surface. To measure the reflection spectrum of the nectar guides of *A. hippocastanum*, the probe holder was placed precisely on the colored spot of the flower. Using visual inspection ensured that the light source illuminated only the colored area. Spectral measurements between 300 and 700nm were collected on an average of 5 scans, exported as a text file, then converted and analyzed in Microsoft Excel. In addition, to obtain a single spectrum for each nectar guide color, all spectral measurements were averaged for each color. To model the color perception by *Bombus terrestris*, the spectral sensitivity functions of bumblebee photoreceptors were applied following standard procedures to demonstrate the color locus using the visual hexagon model (Chittka, 1992; Streinzer et al., 2009). The hexagon model assumed gray as the background. To calculate the distance between the mean values (yellow – orange – red – white – gray) in hexagon units (hu), the formula for the Euclidean distance was used.

Single Flower Nectar Production during Anthesis

To investigate whether the flowers of *A. hippocastanum* only produces nectar in the initial yellow stage or all phases of anthesis, twenty bagged flowers (males = 18, hermaphrodites = 2) from twenty trees were examined without removing the flower from the inflorescence. Thus, twenty yellow flowers were randomly chosen on the first day of the investigation. The nectar was collected with a 2µl capillary without removing the petals or destroying the flower. The colors of each flower´s nectar guides were noted on the consecutive two to seven days, and the nectar was collected again using a capillary. This procedure was carried out each day until every flower dropped from the inflorescence (ca. 5 days).

Further Details on Statistical Analysis

Statistical analysis and graphic visualization were mainly made in R 4.0.3 (R CORE, Team, 2020). The relative amounts of each scent compound and the scent compound table were created in Microsoft Excel for Mac 16.30 (Microsoft 365, 2019). For each performed PERMANOVA, all NAs had to be removed. The confidence interval for each statistical test was set at 95%, and therefore p-values above 0.05 were considered insignificant. P-values were adjusted if necessary, using the Bonferroni adjustment.

Results

Both male and hermaphrodite flowers of four trees were examined with respect to floral scent emission, nectar quantity, nectar quality, and floral color. Additionally, only male flowers were studied from 11 additional trees, as shown in Fig1. The mean ambient temperature during sample collection was 18.32°C (sd = 3.60°C).

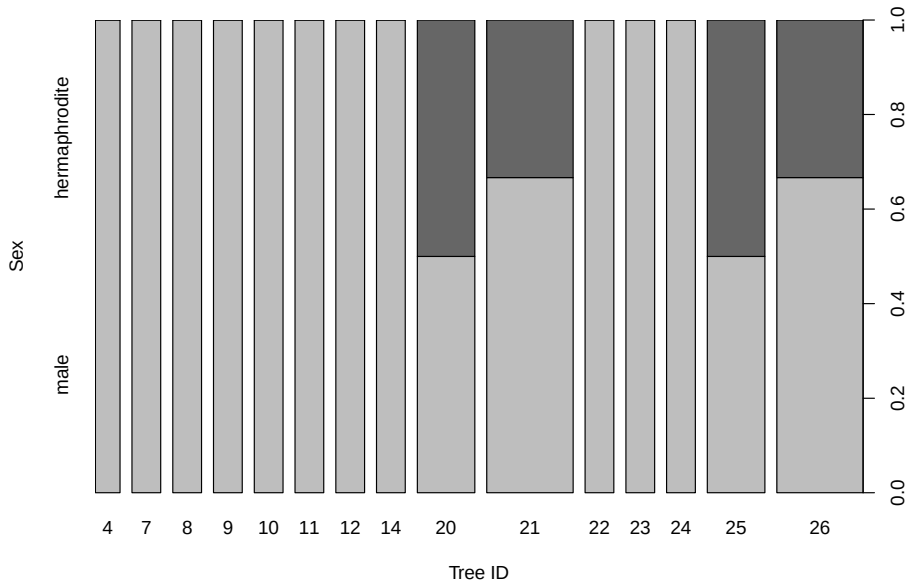

Figure 1; Overview of all tested tree IDs in this study. In light gray, the proportion (y-axis on the right) of the investigated male flowers per tree and the ratio of the investigated hermaphrodite flowers are shown in dark gray. 15 trees were examined during this experiment, 4 with hermaphrodite and male flowers, and 11 with male flowers only. The width of each column indicates the number of investigated flowers

Floral Scent

In total, 48 male flower pools with a mean of 18.52 flowers per pool (sd= 3.45) and 10 hermaphrodite flower pools with a mean value of 8.60 flowers per pool (sd= 3.57) were collected for scent analysis. The scent of 46 flower pools (36 male and 10 hermaphrodite) was collected for 5 minutes and 12 pools of male flowers only for 15 minutes.

A total of 63 different volatiles could be identified in the floral scent of *A. hippocastanum*. All scent compounds could be found in the entirety of all male, red-colored flower scent pools, while the scents of the male, orange-colored and yellow-colored flower pools each miss one scent compound, unk1525 was not present in the orange-colored and unkST1441 was not present in the yellow-colored flower pools. The hermaphrodite flowers had much fewer different volatile compounds in their floral scent. The highest number of compounds (45) could be detected in the orange scent, the second highest (39) in the red scent, and the lowest (37) in the yellow scent.

Table 1; Identity and relative amounts (mean values in %) of floral scent compounds of yellow-, orange- and red-colored *Aesculus hippocastanum* flowers. tr = traces; if value was less than 0.05. The scent compounds (including unknowns: unk) are listed according to the gas chromatographic retention index (RI).

	Color	**yellow**		**orange**		**Red**	
	sex	♂	♀	♂	♀	♂	♀
	Sample size	17	3	15	3	16	4
	# of compounds	62	37	62	45	63	39
RI							
674	1-Pyrroline	4.0	-------	8.2	0.8	1.4	2.3
773	Methyl isovalerate	tr	-------	2.1	-------	0.6	0.5
881	2-Methyl-2-butenoic acid	0.1	0.1	0.2	0.2	0.0	-------
910	unk910	0.3	0.2	0.6	0.2	0.1	-------
943	unk943	0.2	-------	0.4	tr	0.2	-------
980	unk980	0.1	-------	0.2	0.4	0.1	-------

968	1-Heptanol	6.7	6.4	5.6	3.6	4.8	5.0
998	unk998	0.2	-------	0.2	-------	0.1	0.1
1021	unk1021	tr	-------	tr	tr	tr	-------
1035	unk1035	0.1	-------	0.4	0.4	0.1	-------
1043	unk1043	0.8	1.1	1.4	0.6	0.1	-------
1052	2,5-Dimethyl-4-hydroxy-3(2H)-furanone	0.5	tr	0.3	0.1	0.1	tr
1078	(Z)-Linalool oxide furanoid	1.8	7.7	0.8	1.0	2.8	2.1
1093	(E)-Linalool oxide furanoid	3.8	7.0	1.9	0.8	1.6	2.6
1119	2-Phenylethanol	3.0	0.2	0.4	0.2	0.5	0.2
1137	unk1137	0.2	tr	0.1	0.2	tr	-------
1145	Phenylacetonitrile	4.2	0.7	1.4	0.3	1.6	0.1
1165	unk1165	0.8	0.1	1.2	0.4	0.2	-------
1172	1-Acetyl-pyrrolidine	0.1	-------	0.3	-------	0.1	-------
1172	unk1172	0.5	0.9	0.1	0.3	tr	-------
1176	(E)-Linalool oxide pyranoid	0.5	0.6	0.3	0.2	0.2	0.3
1185	unk1185	3.9	3.4	1.4	1.2	2.5	3.8
1218	4-Vinylphenol	0.5	0.1	0.6	1.1	0.4	-------
1227	2-Aminobenzaldehyde	2.4	1.9	1.7	2.1	2.3	1.7
1304	Indole	36.9	46.6	46.3	55.3	54.9	60.7
1309	1-Nitro-2-phenylethane	0.3	-------	0.1	-------	0.3	------
1313	(Z)-Methyl cinnamate	0.1	tr	0.1	tr	tr	0.4
1323	2-Methoxy-4-vinylphenol	0.7	1.1	1.6	1.9	0.6	0.2
1328	unk1328	2.6	1.2	0.2	1.3	0.6	0.7
1355	Methyl anthranilate	0.2	-------	0.1	0.1	0.4	0.4
1365	α-Cubebene	0.4	0.1	0.1	tr	0.2	0.1
1375	unk1375	2.1	5.5	0.3	2.4	1.3	1.2
1377	unk1377	tr	-------	tr	-------	tr	-------
1388	unk1388	0.1	-------	0.1	-------	tr	-------
1394	(E)-Methyl cinnamate	0.7	0.6	0.3	0.2	0.3	0.4
1406	β-Bourbonene	1.2	0.7	0.6	0.2	0.9	2.0
1421	2-(Dimethylamino)benzaldehyde	6.8	9.0	4.2	9.5	6.8	9.2
1425	unk1425	3.4	0.7	8.9	5.7	4.6	2.0
1441	unkST1441	-------	-------	tr	-------	0.5	0.1
1452	(E)-Cinnamyl acetate	tr	2.3	0.2	1.8	0.1	-------
1452	unkST1452	0.2	tr	0.1	0.1	0.3	0.1
1478	γ-Muurolene	tr	-------	tr	-------	0.2	-------
1487	unk1487	0.5	-------	0.1	-------	0.8	tr
1505	unk1505	0.4	-------	0.1	-------	0.3	tr
1518	unk1519	0.1	tr	tr	tr	0.2	tr
1525	unk1525	tr	-------	-------	-------	tr	-------
1531	unk1531	0.2	-------	0.1	-------	tr	-------
1536	unkST1536	0.1	-------	tr	-------	0.1	tr
1542	unkST1542	0.2	tr	0.1	tr	0.1	tr

1543	unk1543	0.1	-------	0.1	tr	tr	-------
1545	unkST1545	tr	-------	0.1	-------	tr	-------
1549	unk1549	0.1	-------	0.1	-------	tr	-------
1550	unk1550	0.3	0.3	0.1	0.4	0.1	0.4
1567	Calacorene	0.2	-------	tr	tr	tr	tr
1588	unk1588	0.5	-------	0.1	0.1	tr	-------
1592	Vetivazulene	0.3	-------	0.1	-------	0.1	0.2
1619	unk1619	1.5	0.2	5.1	6.4	5.3	0.1
1623	Salvial-4(14)-en-1-one	0.3	tr	0.1	0.3	tr	0.1
1664	unk1664	0.4	0,6	0.1	0.1	tr	0.4
1832	unk1832	0.4	tr	0.1	0.1	0.1	0.9
1873	unk1873	3.6	0.3	0.6	-------	0.6	0.1
1965	unk1965	0.6	tr	0.2	tr	0.3	1.3

Probably due to a relatively small sample size of hermaphrodite flower pools compared to male ones, no significant correlation of the floral sex and the chemical composition of floral scents could be detected (PERMANOVA, p-value = 0.141). Because of that, male and hermaphrodite flowers were pooled for all further scent analyses.

Using PERMANOVA, a significant correlation of the floral color on the scent variations could be observed (p-value = 0.001). With the canonical analysis of principal coordinates (CAP; Fig 2), a scent compound variation of the different colored flowers of *A. hippocastanum* could be observed. It could clearly be shown that the floral scent changes during anthesis and correlates with the floral color change. With multiple regressions, seven scent compounds could be detected, which are correlating stronger than 0.3 with the different floral color stages. Whereby unk1043, unk1185 and unk1873 are correlating with the yellow-, 1-Pyrroline, 2-Methoxy-4-vinylphenol and unk1043 are correlating with the orange-, and Indole and Methyl isovalerate are correlating with the red-colored flower stage.

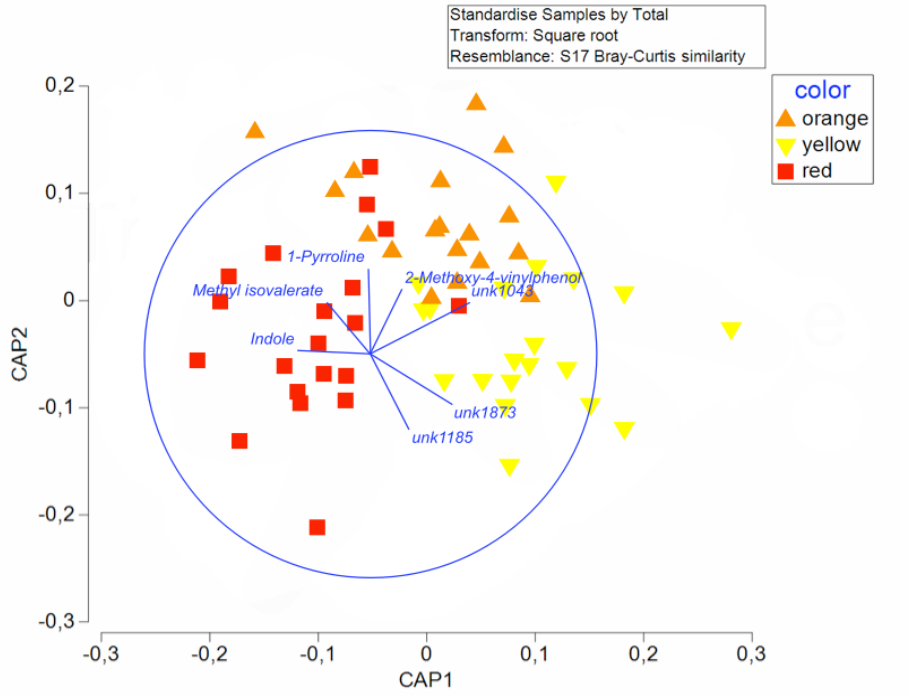

Figure 2; Canonical analysis of principal coordinates (CAP) based on standardized samples by total and square root transformed Bray-Curtis dissimilarity matrix of scent compounds in *Aesculus hippocastanum*. The vectors demonstrate the volatiles most correlating with floral scents containing different nectar guide colors.

Since no influence of the tree ID on the relative amount of the decisive scent compounds could be determined, each characteristic scent compound was compared only for the difference in the floral color stages.

As shown in Fig2 and Fig3, the volatiles correlating with the yellow scent are unk1185 and unk1873. Unk1043 is present the yellow and the orange floral scent (see Fig3A and Fig4B). For unk1043, a strongly significant difference between the relative amounts in the yellow and the red colored flowers, respectively, could be observed (p-value < 0.001). The relative amount of unk1873 significantly decreases with the color change, as shown in Fig3C. Thus, it differs between yellow- and orange-colored flowers (p-value = 0.014) and between the yellow and red ones (p-value = 0.013). Unk1185 does not show a significant difference in the relative amount between color stages, but a decreasing tendency is visible (Fig3B). Unk1185 and unk1873 occur in all investigated scent samples (yellow = 20; orange = 18; red = 20), whereas unk1043 could not be detected in one of the scent samples for each color (yellow = 19; orange = 17; red = 19).

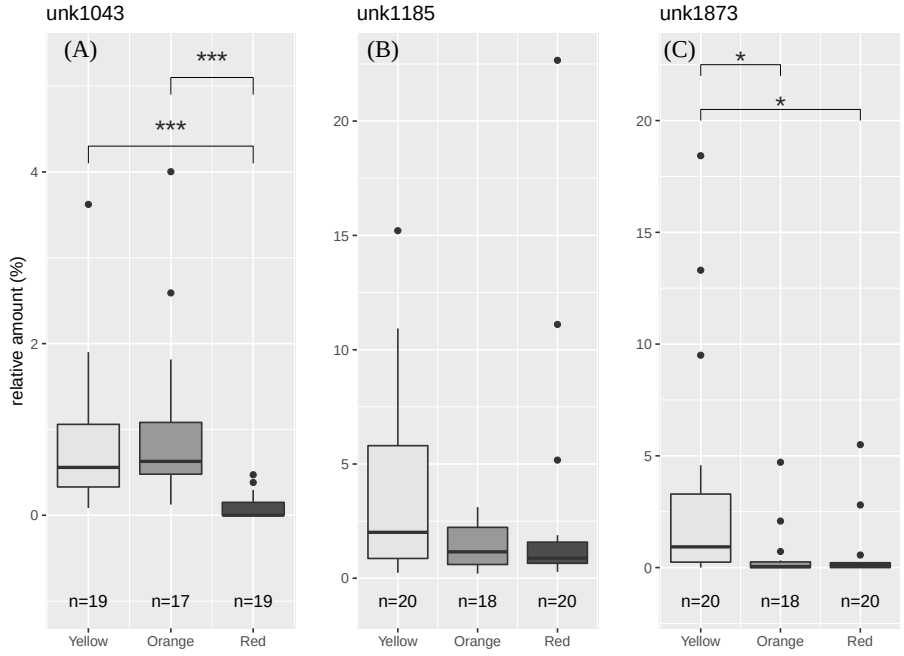

Figure 3; The relative amount of scent volatiles compared to the different color stages of the flowers of *Aesculus hippocastanum*. (A) Volatile unk1043; (B) Volatile unk1185; (C) Volatile unk1873. The numbers beneath each boxplot demonstrate the number of the volatile occurrences in all investigated samples.

As already mentioned, and as shown in Fig4B, unk1043 also correlating with the orange color stage and a high significant difference could be observed regarding the relative amount between the orange and the red colored phase as well (p-value < 0.001). Additionally, two volatiles are correlating with the orange-colored flower scent, 1-pyrroline and 2-methoxy-4-vinylphenol. Considering the relative scent amount both are significantly different to the yellow- (1-pyrroline, p-value = 0.023; 2-methoxy-4-vinylphenol, p-value = 0.044) and to the red-colored flower scent (1-pyrroline, p-value = 0.008; 2-methoxy-4-vinylphenol, p-value = 0.004) of *Aesculus hippocastanum*, as seen in Fig4A and Fig4C. Both, 1-pyrroline and 2-methoxy-4-vinylphenol, occur in all investigated scent samples (yellow = 20; orange = 18; red = 20), whereas unk1043 could not be detected in one scent sample for each color (yellow = 19; orange = 17; red = 19).

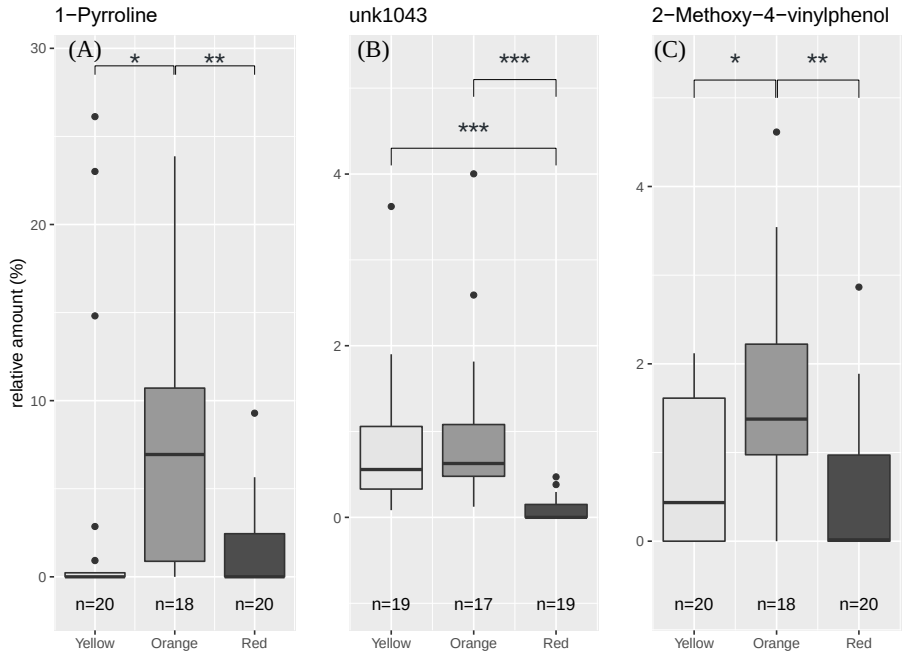

Figure 4; The relative amount of scent volatiles compared to the different color stages of the flowers of *Aesculus hippocastanum*. (A) Volatile 1-pyrroline; (B) Volatile unk1043; (C) Volatile 2-methoxy-4-vinylpenol. The numbers beneath each boxplot demonstrate the number of the volatile occurrences in all investigated samples.

Compared to the yellow- and orange-colored floral scents, only two characteristic volatiles could be determined for the flowers with the red nectar guides, methyl isovalerate and indole (see Fig2 and Fig5). Both floral scent compounds do not show a significant difference compared to the relative amounts of the yellow and orange scent, but as shown in Fig5, a strong tendency for an increase of both volatiles in the red-colored floral scent (methyl isovalerate, p-value= 0.051; indole, p-value = 0.054) can be observed. Both scent compounds could be determined in all scent pool samples (yellow = 20; orange = 18; red = 20).

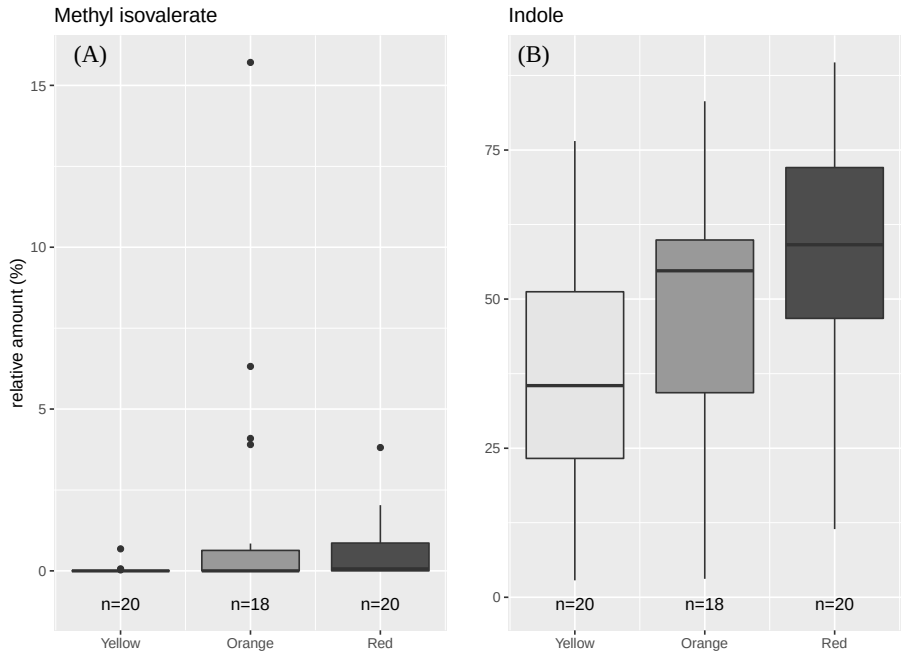

Figure 5; The relative amount of scent volatiles compared to the different color stages of the flowers of *Aesculus hippocastanum*. (A) The volatile methyl isovalerate; (B) and the volatile indole. The numbers beneath each boxplot demonstrate the number of the volatile occurrences in all investigated samples.

Floral Nectar Volume and Sugar Quantity

In total, 55 flower pools could be obtained from 15 trees to investigate the nectar quantity and quality, which was measured in µl nectar and µg sugar per flower. In summary 534 flowers (mean = 9.71 flowers/pool; mean = 1.098µl, sd = 0.808µl) could be gathered for this study, from which 191 flowers with a mean of 0.842µl (sd = 0.581µl) had a yellow, 149 an orange with a mean of 1.160µl (sd = 0.795µl) and 194 with a mean of 1.302µl (sd = 0.935µl) a red nectar guide color. On 4 of the 15 trees the rewarding flower system of both, hermaphrodite and male flowers were investigated (see Fig1).

From the four trees, from which male and hermaphrodite flowers could be gathered, 216 flowers (male = 137; hermaphrodite = 79) with a mean of 1.137µl (sd = 0.749µl) were used for the nectar analysis. Altogether, 80 yellow-colored (51 male and 29 hermaphrodites, mean = 0.873µl, sd = 0.630µl), 56 orange colored (40 male and 16 hermaphrodites, mean = 1.376µl, sd = 0.899µl) and 80 red colored flowers (46 male and 34 hermaphrodites, mean = 1.233µl, sd = 0.669µl) are the base of the first investigations, regarding sex as possible factor.

Performing a Shapiro-Wilk normality test for nectar volume (p-value < 0.001) and sugar content (p-value < 0.001) indicates that the samples are significantly different from a normal distribution. Thus, non-parametric statistical analyses were performed. With a non-parametric ANOVA (Permutational Multivariate Analysis of Variances using distance matrices = PERMANOVA), the influence of the factor sex on the nectar volume and the sugar content was calculated. With a p-value of 0.043, a statistically significant impact of the flower's sex could be determined, and as shown in Fig6, the hermaphrodite flowers have a substantial higher amount of nectar and sugar as a rewarding system (Wilcoxon rank-sum test; p-value for volume = 0.009; p-value for sugar content = 0.005). Thus, further sugar quantity and quality analyses were performed separately for male and hermaphrodite flowers.

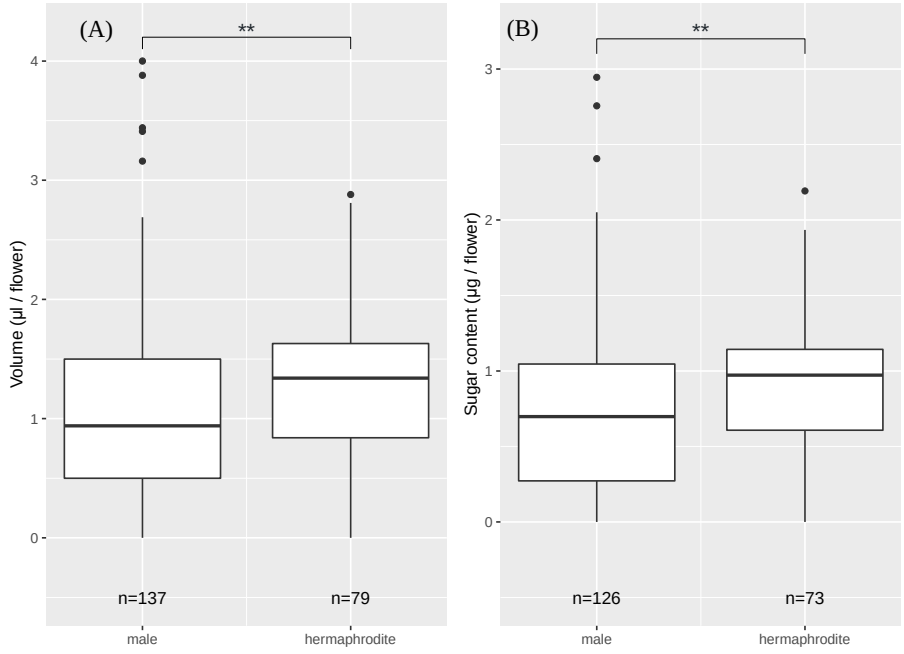

Figure 6; Comparison of the amount of nectar (µl per flower) (A) and quantity of sugar (µg per flower) (B) for the investigated male and hermaphrodite flowers of *Aesculus hippocastanum* trees, from which both flower sexes could be obtained. 37.23% of male flowers had a yellow, 29.20% an orange and 33.58% a red nectar guide. With regard to hermaphrodite flowers 36.71% had a yellow, 20.25% an orange and 43.04% a red nectar guide color. The dots above the boxplots present measure outliers. The "n" beneath each boxplot indicates the number of samples. The lines connecting the plots demonstrate the significant difference measured with the Wilcoxon-Rank-Sum test. * < 0.05; ** < 0.01; *** < 0.001

Altogether, 45 male flower pools with a mean of 10.11 flowers per pool (sd = 1.32), and therefore a total of 455 male flowers were investigated on the quantity of nectar per nectar guide color. The yellow flowers (n = 162) had a mean volume of 0.77µl (sd = 0.55µl), the orange flowers (n = 133) showed a mean volume of 1.16µl (sd = 0.79µl) and from the red ones (n = 160) a mean volume of 1.29µl (sd = 0.99µl) could be collected. For testing whether samples are normally distributed, a Shapiro-Wilk normality test for nectar volume (p-value < 0.001) and sugar content (p-value < 0.001) was conducted and it indicates that the samples are different from a normal distribution.

A strongly significant correlation of the amount of nectar and sugar content of male flowers could be calculated for the factors tree ID (PERMANOVA, p-value < 0.001) and nectar guide color (PERMANOVA, p-value < 0.001). This indicates a high variation of the nectar volume and sugar quantity between the investigated trees (as seen in Fig7). Six tree groups could be determined using the Kruskal-Wallis test for the amount of nectar. As shown in Fig7A, group A containing five tree IDs (ID10, ID24, ID4, ID25 and ID9), group B seven (ID24, ID4, ID25, ID9, ID21, ID26 and ID22), group C eight (ID4, ID25, ID9, ID21, ID26, ID22, ID7 and ID12), group D seven (ID21, ID26, ID22, ID7, ID12, ID8 and ID14), group E also eight (ID26, ID22, ID7, ID12, ID8, ID14, ID20 and ID23) and group F containing four tree IDs (ID14, ID20, ID23 and ID11). As demonstrated in Fig7B, seven different groups of tree IDs could be determined for the floral sugar content. Five in group A: ID10, ID24, ID4, ID25, and ID9. Also, five in group B: ID24, ID4, ID25, ID9, and ID21. Six in group C: ID4, ID25, ID9, ID21, ID22 and ID7. Six in group D: ID25, ID9, ID21, ID22, ID7 and ID12. Another six in group E: ID21, ID22, ID7, ID12, ID8, and ID26. Eight trees in group F: ID22, ID7, ID12, ID8, ID26, ID14, ID23, and ID20. And finally, four trees in group G: ID14, ID23, ID20, and ID11.

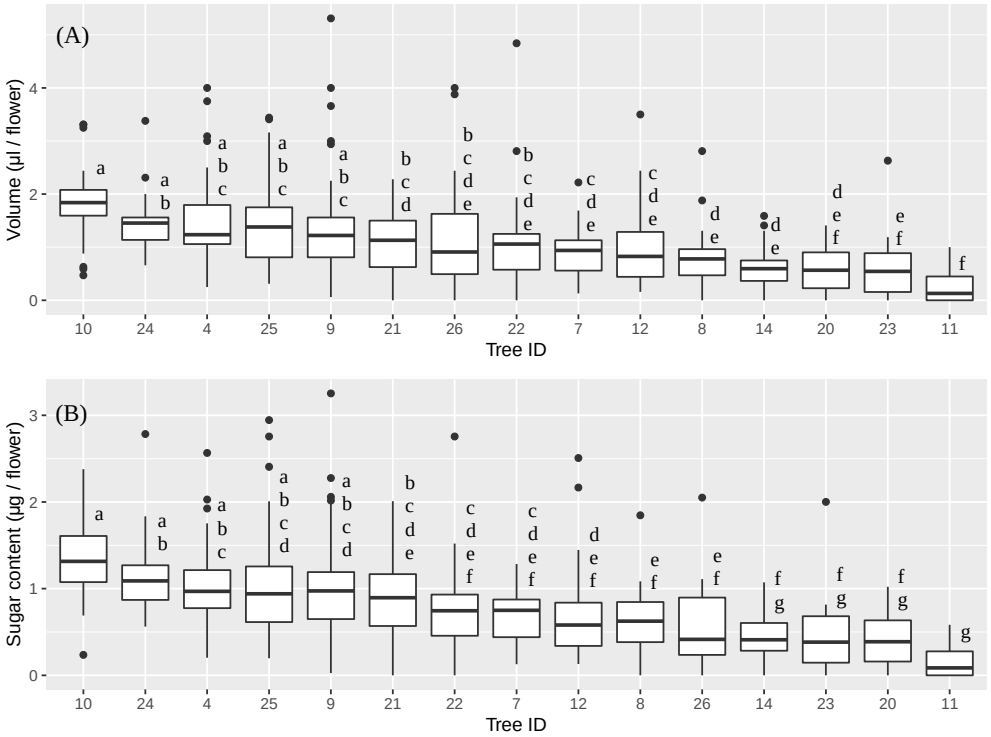

Figure 7; Variation of the investigated trees on (A) the amount of nectar in µl per flower and (B) the sugar content in µg per flower of male flowers. The calculated statistical groups using the Kruskal-Wallis test are marked with letters.

In addition to the influence of the tree ID, the flowers with different floral nectar guide colors also differ in the amount and quality of their nectar. The Kruskal-Wallis rank sum test showed a clear significance between the nectar amount of the three-color groups (p-value < 0.001). With pairwise comparisons using Wilcoxon rank-sum test with p-value as post hoc analysis, a significance regarding the floral nectar measurements could be shown in the amount of nectar between the yellow and orange (p-value < 0.001)

as well as between the yellow and red flower stage (p-value < 0.001). No difference could be observed in the nectar volume of the orange and the red colored flowers (p-value = 1), as seen in Figure 8A.

A total of 435 male flowers were examined concerning sugar content in µg per flower. As in the volume observations, the yellow-colored flowers (n = 158) had a lowest sugar content of all groups with a mean of 0.54µg (sd = 0.37µg). The orange flowers (n = 131) showed a sugar quantity with a mean value of 0.88µg (sd = 0.57µg), similar to the flowers with the red nectar guides with a mean of 0.91µg (sd = 0.65µg). A highly significant difference (p-value < 0.001) between the three groups could be shown using the Kruskal-Wallis rank-sum test. The same result as in volume analysis could be demonstrated for the sugar content, calculated with the pairwise comparisons using Wilcoxon rank-sum test with as post hoc analysis. Thus, a highly significant difference could be observed between the amount of sugar of the yellow and the orange-colored flowers (p-value < 0.001) and between the yellow and the red ones (p-value < 0.001). Furthermore, no statistically significant difference in the floral sugar quantity could be measured between the orange and the red flower phase (p-value = 1), as seen in Figure 8B.

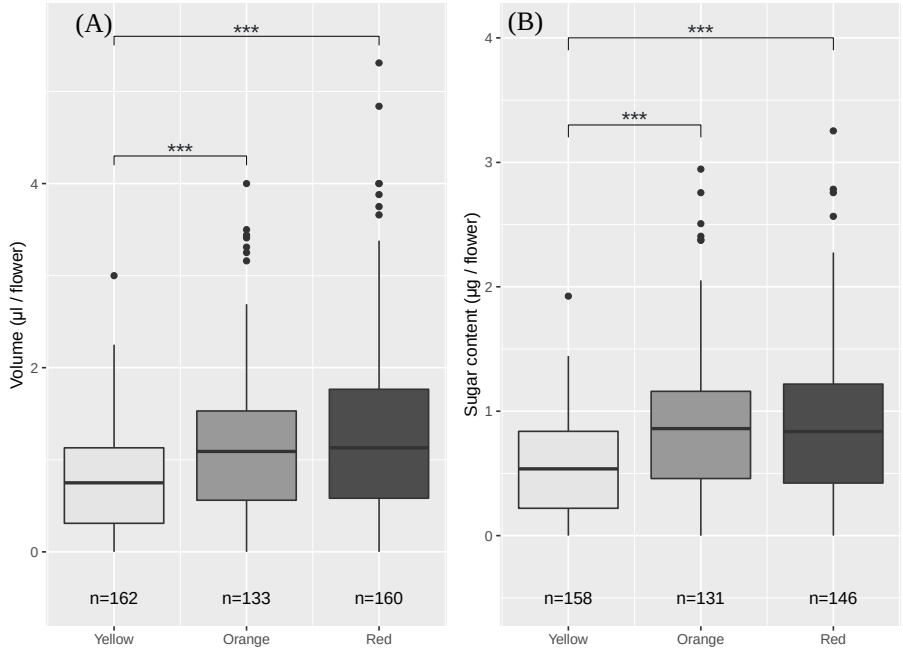

Figure 8; The nectar volume (A) in µl per flower and the nectar sugar content (B) in µg per flower are displayed for the three nectar guide colors yellow, orange and red of male *Aesculus hippocastanum* flowers. The dots above the boxplots present measure outliers. The "n" beneath each boxplot indicates the number of samples. The lines connecting the plots demonstrate the significant difference measured with the Wilcoxon-Rank-Sum test.
* < 0.05; ** < 0.01; *** < 0.001

For hermaphrodite flower nectar measurements, ten flower pools with a mean of 7.90 flowers per pool (sd = 2.42), and therefore a total of 79 flowers were investigated. The flowers having a yellow nectar guide (n = 29) showed a mean nectar quantity of 1.22µl (sd = 0.64µl). A mean nectar amount of 1.17µl (sd = 0.85µl) for the orange-colored (n = 16) and a mean of 1.34µl (sd = 0.59µl) for the red ones (n = 34) could be measured. Despite the samples not showing a difference to a normal distribution (Shapiro-Wilk normality test; p-value for volume = 0.140; p-value for sugar content = 0.217), the non-parametric PERMANOVA was performed to estimate an influence of the tree ID and color stage of the flowers on the nectar quality and quantity. Both factors did not show any influence on the floral rewarding system for hermaphrodite flowers (tree ID: p-value = 0.214; floral color: p-value = 0.213), as seen in Fig9 and Fig10.

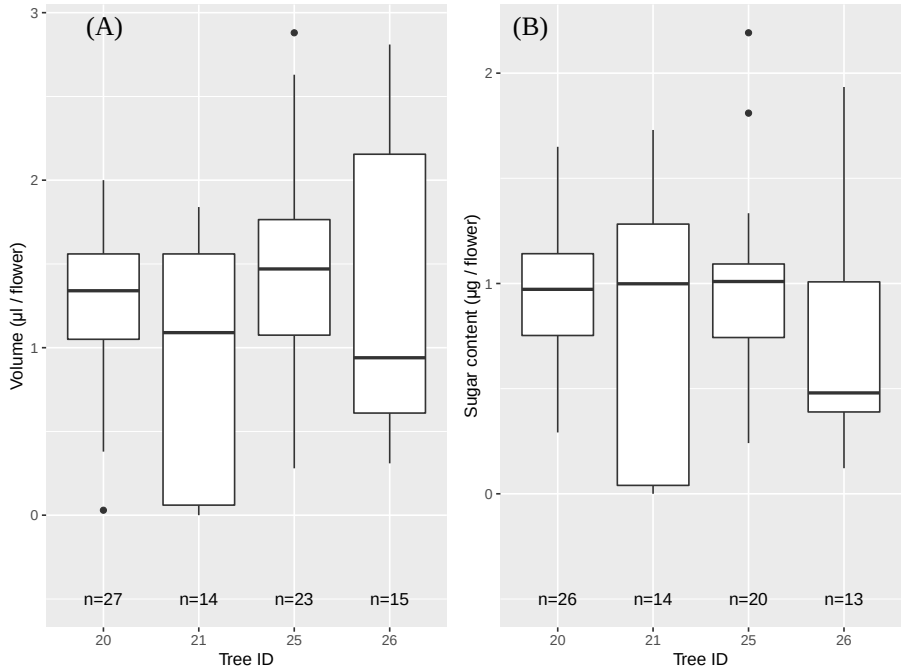

Figure 9; Variation of the investigated trees on (A) the amount of nectar in µl per flower and (B) the sugar content in µg per flower of hermaphrodite flowers. Referring to Kruskal-Wallis test all four trees are part of the same statistical group.

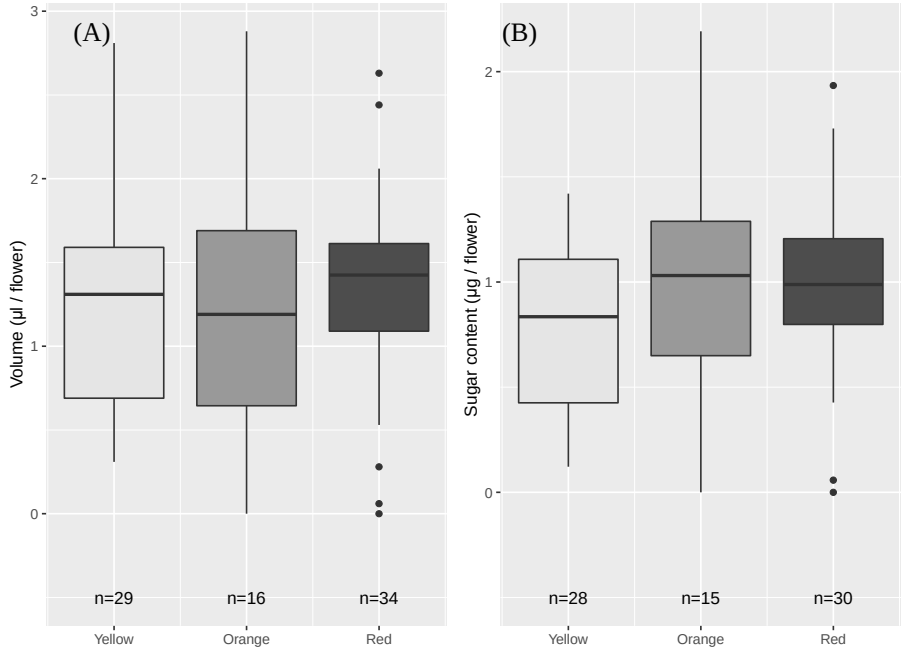

Figure 10; The nectar volume (A) in µl per flower and the nectars sugar content (B) in µg per flower are displayed for the three nectar guide colors yellow, orange and red of hermaphrodite *Aesculus hippocastanum* flowers. The dots above the boxplots present measure outliers. The "n" beneath each boxplot indicates the number of samples. No significant differences could be found.

The boxplots of the nectar volume and the amount of containing sugar for each tree can be seen in the Appendix of this study (see Figure 21 (male) and Figure 22 (hermaphrodite), Appendix).

Floral Color Analysis

The exact same number of flowers used for the floral nectar measurements (n_{total}= 534, n_{yellow}= 191, n_{orange}= 149, n_{red}= 194) was used for the single petals' reflectance spectra. Fig11 shows the mean reflectance curves of each nectar guide color and the white petals (1/pool; n = 55). Due to the visual sensitivity range of bumblebees (300 – 600nm), Fig11 demonstrates that all four colors reflect wavelengths perceivable for bumblebees but do not stimulate the UV-receptor (300-400nm), as also shown in Figure 12 (see Fig23, Appendix, for curves with standard deviation). The white petal absorbs in the UV-range and reflects about 40% of the incident light throughout the range from 400 to 700nm. The yellow, orange and red nectar guides absorb in the UV- range and reflect light at about 425nm with 15% intensity, from which on the nectar guides reflect and absorb different wavelengths. Thus, the color reflectance spectra of nectar guide colors do clearly differ among each other as demonstrated in Fig11.

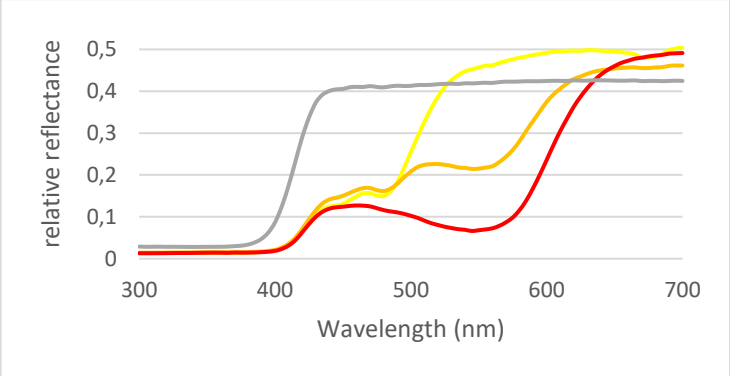

Figure 11; Mean reflectance spectra of each nectar guide color and the white petal of *Aesculus hippocastanum*. On the x-axis the wavelength from 300 to 700nm is demonstrated, and on the y-axis, the relative reflectance. The gray line illustrates the mean value for the white petal reflectance, the yellow line for the yellow, the orange for the orange, and the red for the red nectar guide reflectance curve.

In Fig12, the visual hexagon models of *Bombus terrestris* as a representative species of the pollinators of *A. hippocastanum* are plotted. The center of the hexagon represents the color gray, which is colorless for bumblebees. The hexagon edges express the visual receptors of the bumblebee (B = blue, G = green, and UV = ultraviolet). In Fig12A the mean values of all color measurements in this study (Fig12B) are plotted. The further a single locus is located from the center of the hexagon, the higher the color contrast. In addition, the further the loci of different nectar guides are spaced from each other, the higher the difference of the colors and thus the better the bumblebees can distinguish between those colors (Chittka, 1992). Therefore, the yellow nectar guide color and the white petals have the highest contrast for their pollinators (Table 2).

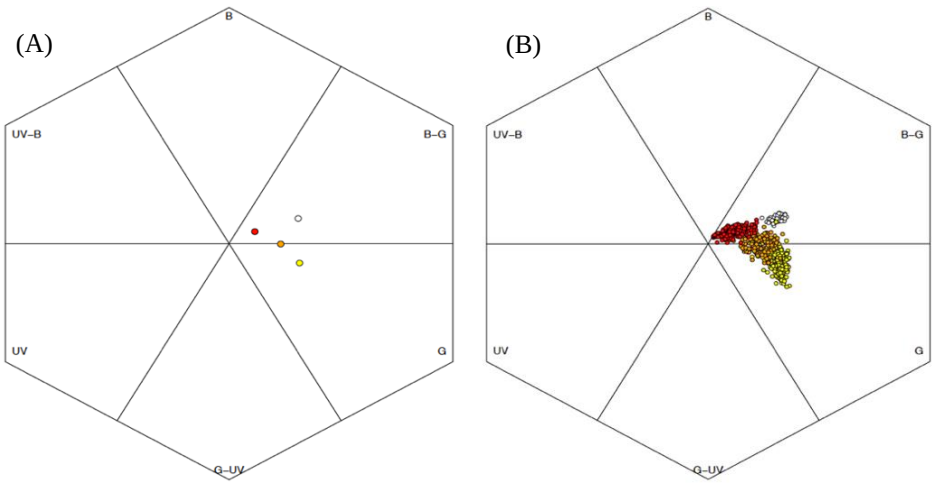

Figure 12; The color hexagon model of *Bombus terrestris*. The three visual receptors of *B. terrestris* are demonstrated (B = blue; G = green; UV = ultraviolet), and the center represents the color gray, which is colorless for *B. terrestris*. The further the loci are located from the center, the higher is the color contrast. (A) shows the mean color measurements for yellow, orange, red, and white. (B) shows all measurements performed for yellow, orange, red, and white nectar guides in this study.

It could be determined that the highest difference of 0.286hu can be seen between the color of the white petal and the center of the hexagon. The second and third highest difference is between gray and the yellow nectar guide reflectance (0.283hu) and between yellow and red (0.217hu). The red nectar guide color is the closest dot to the center of the hexagon but still perceived- and distinguishable for bumblebees with 0.111hu. The slightest difference regarding the measured color reflectance is between the yellow and the orange nectar guides (0.108hu) (see Fig12A & Table 2).

Table 2; All calculated hexagon units between the mean color measurements (gray-white; gray-yellow; gray-orange; gray-red; white-yellow; white-orange; white-red; yellow-orange; yellow-red; orange-red) of the color hexagon model (Figure 3A).

	White	Yellow	Orange	Red
Gray	0.286	0.283	0.198	0.111
White		0.189	0.128	0.176
Yellow			0.108	0.217
Orange				0.112

Floral Nectar Production during Anthesis

The nectar measurements on the same flowers on consecutive days showed, that the nectar production of a single flower mainly takes place on the first day of anthesis (see Fig13). It clearly can be seen that the nectar volume (µl) is the highest on day one and that it is not replenished by the plant after the nectar has been removed. This result indicates that there will hardly be a reward for upcoming flower visitors after a pollinator visits a flower.

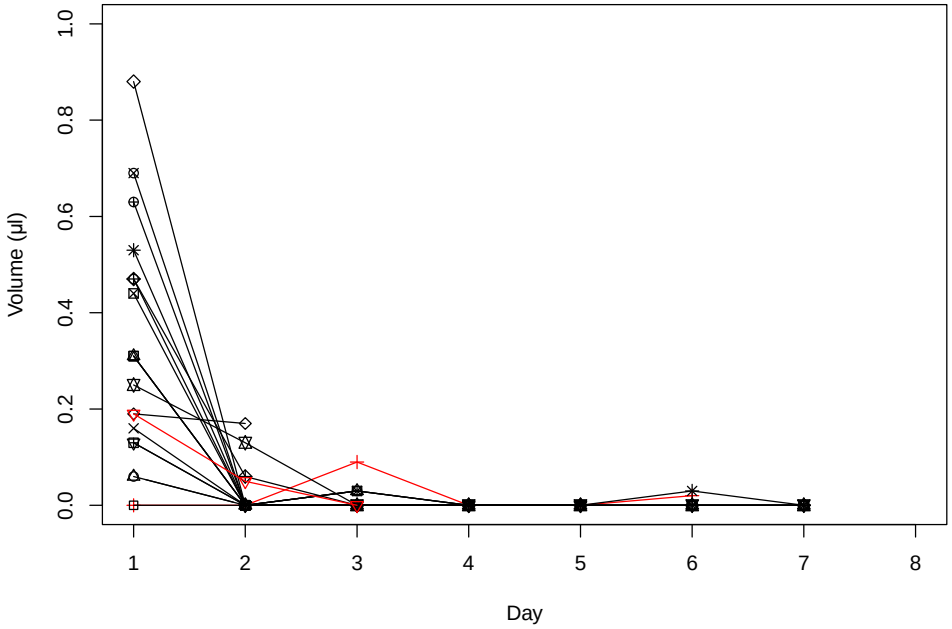

Figure 13; The nectar production in µl of single flowers of *Aesculus hippocastanum* during their anthesis. The black lines indicate the male flowers (n = 18), and the red lines the hermaphrodite (n = 2) observed flowers. Not all flowers showed an anthesis duration of eight days. The end of the anthesis is demonstrated by the end of the line in the graph.

The proportion of the nectar guides color during the single flower observation is shown in Figure 14. The number of observed flowers decreases over time, indicating the inflorescences dropping flowers. It is shown that on the first day, 100% of the investigated flowers contained a yellow nectar guide color; on day two, only 50% had the yellow-colored spot, and the other 50% already changed to orange. On day three, the first red-colored nectar guides occurred (47.37%) in the same number as orange-colored flowers. On day three, only one out of 19 flowers (5.26%) was still in the yellow flower phase. On day

four, most of the nectar guides turned red (n = 16; 94.12%), and only a single flower (5.88%) still had an orange nectar guide. From day five to seven, all flowers contained a red-colored spot, and the number of flowers decreased rapidly from 14 on day five to 8 (day six) to 7 on day seven. On the last observation day (day eight), no flowers were left to investigate the amount of produced nectar, and anthesis was over in all estimated flowers.

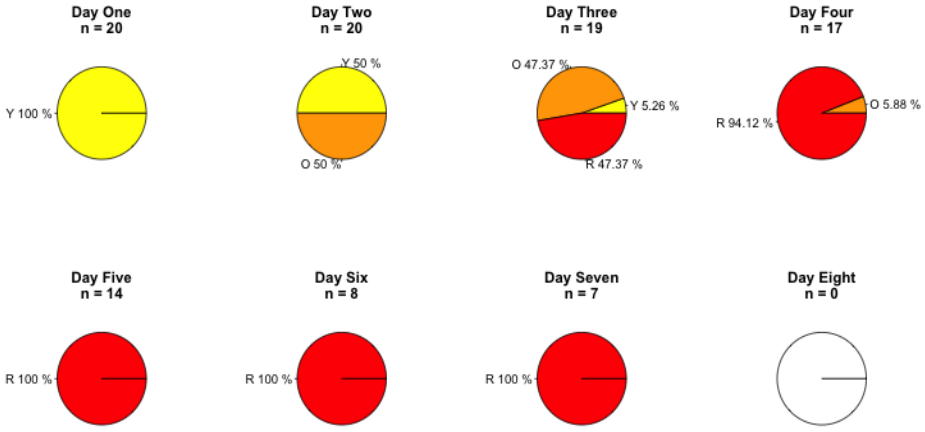

Figure 14; Proportion of each nectar guide color during the single flower observations. Each pie represents one day. The number of observed flowers is given in n. The proportion of the nectar guide colors is given as color inside the pie and in percentage (%) aside from the pie. On the eighth day, no flowers were left to observe. Y = yellow; O = orange; R = red.

Discussion

Visual flower cues like nectar guides are suggested to be important for short-distance attraction of pollinators (Sprengel, 1793; Weiss, 1991). In this study, it could be observed that each nectar guide color and the white petals reflect light in the visual spectrum of *Bombus terrestris* (300-600nm; see Fig11). Due to the reflectance spectrum of the nectar guides and white petals, it can be assumed that the colored spots on the flowers and the petals of *Aesculus hippocastanum* appear colorful for bumblebees. Additionally, the observed color contrasts of the nectar guides with the adaptation background (grey) expressed in hexagon units (hu) are much higher than 0.02hu to 0.03hu (see Tab2), according to Garcia et al. (2017) to be the minimal needed difference for bumblebees to distinguish between different colors with a success rate of 80%. Thus, this study shows that bumblebees, which is one group of legitimate pollinators of *A. hippocastanum*, can perceive and distinguish the differently colored nectar guides. These findings are corroborated also in a behavioral study by Žilić (2022), who found that bumblebees can be trained on the different nectar guide colors of *A. hippocastanum* and associate them with a reward. Therefore, the hypothesis of a short-distance attraction function of nectar guides originally proposed by Sprengel (1793) can be supported.

The most essential activities of insects like mating and feeding rely upon olfactory signals, which therefore play a significant role in an insect's life (Wright & Schiestl, 2009). Because of that, Wright & Schiestl (2009) argued that pollinators can use the floral scent as a meaningful cue to distinguish between different plant species and can learn to associate floral scent with a reward (e.g., pollen or nectar). Additionally, it is argued that bees learn olfactory flower cues earlier and remember them longer than visual flower cues (Menzel, 1985; Wright and Schiestl, 2009). Especially naïve bees, which have never visited a flower before, are said to orient themselves in their environment only by olfactory cues first and learn to orient themselves by visual cues much later when they are more experienced using visual orientation points, like landmarks (Dobson and Bernays, 1994, Dötterl & Vereecken, 2010). On the other hand, Menzel and Müller (1996) found out that bees learn visual cues significantly faster than olfactory cues, which could be supported by a recent study using the floral traits of *A. hippocastanum*

(Žilić, 2022). In addition, Žilić (2022) could observe that the bees can associate the scent of yellow and red nectar guides with a reward but are not able to distinguish between the two. As floral scent extracts of *A. hippocastanum* flowers and artificial flowers were used, the scent bouquet was not fully comparable.

Insect pollinators can perceive and also distinguish many different scent compounds and mixtures, which can be measured using gas chromatography coupled to electroantennographic detection (GC/EAD) (Dötterl & Vereecken, 2010 and references therein). As in this study the main correlating volatiles for the yellow-colored *A. hippocastanum* flowers, unk1043, unk1185 and unk1873, are unknown scent compounds, no assumptions about the perception and the influence on the pollinator's behavior can be made. Besides unk1043, the volatiles 1-pyrroline and 2-methoxy-4-vinylphenol were the most characteristic compounds for the orange flower phase. Both scent compounds are known to attract pollinators and therefore can be perceived by insects (Chen et al., 2015; Nunes et al., 2016). Chen et al. (2015) observed that 1-pyrroline is the main compound of *Stemona japonica* (Blume) Miq. (Stemonaceae), mainly visited and pollinated by small dipterans, which were even attracted by a recreated synthetic floral scent. Even 1-pyrroline alone could attract many insects, but not the usually pollinating family. Nunes et al. (2016) investigated the orchid *Dichea pendula* (Aubl.) Cogn. (Orchidaceae), pollinated by orchid-bees, and determined 2-methoxy-4-vinylphenol as the main scent compound of the floral scent, which even attracted orchid-bees in behavioral bioassays using the pure synthetic compound. The most characteristic scent compounds for the red-colored flowers of *A. hippocastanum* in this study are indole and methyl isovalerate. The latter is hardly known as attractant but rather as an inhibitor against a bacterial infecting fungus (Gong et al., 2019) and nematode parasites in tomatoes (Ayaz et al., 2021). Indole is related to the scent of faeces and is also a known angiosperm volatile, which, inter alia, can be perceived by flies (Johnson et al., 2020) and honeybees (Martínez-Harms et al., 2018). Martínez-Harms et al. (2018) demonstrated that honeybees can discriminate different scent mixtures by their relative levels of indole alone. Taking this result into account, it can be assumed that bees and therefore even the pollinators of *A. hippocastanum* can distinguish the scent of the different colored flowers by indole alone, as the indole content varies across the three stages. For supporting this hypothesis behavioral experiments on bumblebees using different concentrations of indole – simulating the indole emission of *A. hippocastanum* flowers – need to be done.

It is assumed that floral scent in combination with visual flower cues increase the attractiveness of plants for its pollinators and also supports the pollinators' floral constancy in comparison to just an olfactory or visual stimulus alone (Wright & Schiestl, 2009 and references therein, Martínez-Harms et al., 2018). In this study, the hypothesis, and observations of Stern (2020) that the floral emitted scent change does correlate with the color change of the flower's nectar guides of *Aesculus hippocastanum*, could be further supported. Former studies assumed that mainly the color of the nectar guides is influencing pollinator behavior by signaling which flowers are worth to be visited and which are not (Vogel, 1950; Lex, 1954). By showing that the color change is closely associated with a change in floral scent in the flowers of *A. hippocastanum*, it is possible that the emitted scent may have an equal or higher impact in the short distance communication as bumblebees can detect scents from a close range and even avoid flowers after their scent has changed (Manning, 1956; Wright & Schiestl, 2009). This could stand for the possibility that young bees and bumblebees learn which flowers are rewarding and which are not by using the floral scents of the differently colored flowers. Over time, they adopt the color system for future flower visits (see Dobson and Bernays, 1994, Dötterl & Vereecken, 2010). While the exact function of the emitted scent could not be determined in this study, it provides a fundamental question for upcoming investigations and experiments. However, at least a color-supporting function by the floral scent seems very likely.

A highly significant difference regarding the amount of nectar and sugar quantity between the yellow and orange as well between the yellow and red colored flowers could be shown in the present study whereas the orange- and red-colored flowers showed more amount of nectar and sugar than the yellow-colored (see Figure 8). This result matches the hypothesis of this study and the observations of Focke (1889) and Kugler (1936), i.e., that the yellow flowers are the most rewarding and therefore most profitable flowers for the pollinators of *A. hippocastanum.* As the complete inflorescences were covered with fine mashed bags before the anthesis started to avoid nectar collection by pollinators, the flowers

in the red color stage possessed the complete amount of produced nectar. Would the flowers with orange and red nectar guides still produce nectar, a clear increase in the amount of nectar would be observable between the orange and the red flower phase. Thus, it is possible that the nectar from the yellow-colored flowers was collected in the middle of the yellow-colored stage, and the nectar production was not quite finished during the investigation time. Another possible explanation is that the nectar production does not stop abruptly but slowly decreases when the flower is already changing its nectar guide color. In both cases, the result would lead to a higher quantity of nectar in orange and red flowers. However, it is fact, that the nectar is not resorbed by the plant during aging and therefore is present even in old flowers, if not visited by any pollinators. This could indeed be helpful for pollinators after a bad weather period, when the insects were not able to visit any flowers (J. Schönenberger, 2022, personal communication). This result is also supported by the single flower investigations in this study, which showed that on the first day of anthesis nectar is present and after removing the nectar the flower does not replenish the nectar that the old flowers would still be worth visiting by the pollinators (see Fig. 13).

In general, it might be more advantageous for a plant to drop the old flowers, which cannot be fertilized anymore to save energy (Sprengel, 1793). Based on this claim, Focke (1889) came up with his hypothesis of a positive influence of a bigger inflorescence on the long distant attraction of pollinators. Therefore, dropping the old flowers would decrease the visual stimulus for the insects from a long distance. Even the scent emission by the inflorescence is stronger by having more scented flowers, which increases the long-distance attraction and the learning of insect pollinators (Wright & Schiestl, 2009 and references therein). In addition, if the floral color would not change during aging, potential pollinators would still visit also older flowers with a high chance of not getting any reward, which could negatively impact the flower constancy of the pollinators (Tegrovsky and Pany, 2019). A recent study observed the approaching behavior of bumblebees to artificial flowers containing the nectar guide colors of *A. hippocastanum* (Žilić, 2022). Žilić (2022) could not observe any color preference of the bumblebees first visits, which also indicates that the pollinators are attracted by the big white area of the inflorescence / artificial flower and afterwards need to learn to use the nectar guide colors as a short distance stimulus for finding the rewarding flowers.

Based on the observations from Stern (2020) that even un-pollinated flowers change their nectar guide colors at about the same time as pollinated flowers do, the color change in *A. hippocastanum* may occur due to natural senescence. As it has been reported that at least two biosynthetic pathways of floral color pigments and volatiles are strongly related (Ben Zvi et al., 2008; Martínez-Harms et al., 2018) the scent may change as well due to natural senescence. Ben Zvi et al. (2008) determined that benzenoids/phenylpropanoids volatiles as well as blue, red, and purple (anthocyanins) pigments are produced in the shikimate pathway, whereas Stange (2016) found out that homoterpenoids/apocarotenoids volatiles and yellow, orange, and red (carotenoids) pigments are derived from the 2-C-methyl-d-erythritol 4-phosphate pathway. However, according to Muhlemann et al. (2014), the majority of VOCs synthesis is still unknown and further research needs to be done. However, it could be demonstrated, that volatiles and pigments occur in specific combinations in the flowers of *Papaver nudicaule* L. (Papaveraceae) (Martínez-Harms et al., 2018) and even for *Aesculus hippocastanum* it could be observed that the colored nectar guides emit a more intensive odor than the white petal itself (Kugler, 1970). Yan et al. (2016) investigated that the scent of *Quisqualis indica* L. (Combretaceae) changes with the floral color change and concluded that this presumable is caused by the interlinked traits of color and scent and natural senescence. This could indicate that the color and scent change of the *A. hippocastanum* flowers is not an honest signal for its pollinators but a cue the bumblebees are able to learn and use for their advantage. However, the color change and maybe also the scent change is assumed to be of advantage for the flower and the pollinators, which supports the hypothesis that these flower traits may serve as an honest signal. To conclude whether the visual and olfactory flower traits of *A. hippocastanum* serve as honest signals needs to be examined in further behavioral studies.

Future Studies

Because of the possibility that the flowers were harmed during the nectar collection without cutting them off the inflorescences and therefore were influenced in the nectar production on the consecutive days, a natural nectar collection by bees could bring better results here. Thus, single flowers have to be marked before anthesis. These flowers should be observed to see when each flower starts anthesis. However, when the flower is attractive for its pollinators, it should not be covered but be observe until the first flower-visiting insect extracts the entire amount of nectar. This can be assumed by observing the insect's visiting duration on the fresh young flower. Due to that natural nectar collection, the flower will not be harmed and the nectar production after a first visit can be measured on consecutive days.

Also, it would be interesting to examine the fertilization success in different anthesis stages of female and hermaphrodite flowers and whether even flowers with red nectar guides can be fertilized if they had not been visited before. For such a study, three groups of flowers and, because the female flowers are rare, a considerable number of bagged inflorescences would be necessary. The first group will be the investigation group, in which female flowers will be pollinated by hand in different flower stages (yellow, orange, red). The second and the third group will serve as control groups. One group will not be pollinated by hand or by insects and, therefore, will be bagged before the start and until the end of anthesis. This group will serve as a control to prove that the *A. hippocastanum* cannot do self-pollination. The second and third group will not be covered with bags. After the flowering period, every developing fruit per inflorescence will be counted, and the ratio of fertilization success will be measured.

As already mentioned, behavioral experiments with the nectar guide colors and different floral scent phases need to be done to proof whether the colors and volatiles can be perceived and distinguished by the pollinators of *Aesculus hippocastanum* and to clarify, whether the flower traits serve as honest signals or not.

Regarding the scent, electro-physiological investigations of the pollinator's antennae would be a final proof, which volatiles the pollinators perceive and to which they react the most to. With following behavioral experiments, the crucial scent compounds of the different floral phases could be examined.

Conclusion

Aesculus hippocastanum is a bee-pollinated species, presenting its flowers on big, candle-like, upright inflorescences. The color change of the floral nectar guides is correlating with a scent change emitted by the flowers and is linked to the floral nectar production. It can be assumed that this combination influences the behavior of the pollinators as well as reproductive success of the plant itself. The results of this study show that bumblebees are likely to perceive and distinguish the different colored nectar guides, but it was not examined whether the scent compounds can be perceived and distinguished as well. As it is not clear whether the color and scent change of the flowers is an honest signal by the plant or not further research is needed for a better understanding of the common horse chestnuts pollination system.

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Appendix

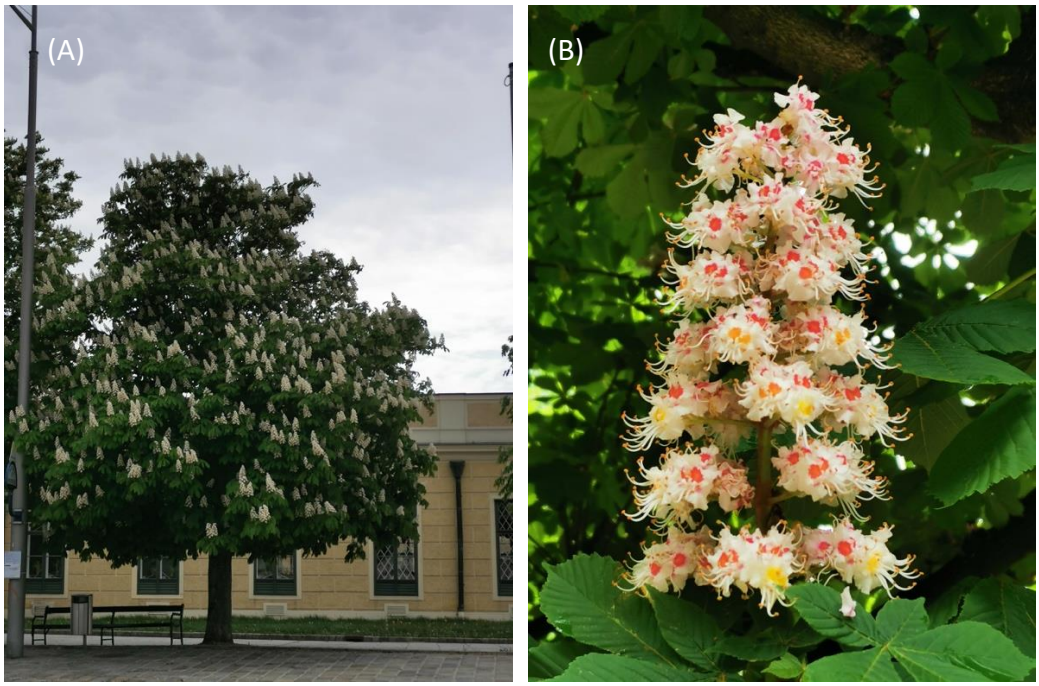

Figure 15; (A) The common horse chestnut, *Aesculus hippocastanum*, in full bloom in front of the entrance to the Schlossgarten Schönbrunn. (B) The candle-like inflorescence of *Aesculus hippocastanum* with its yellow, orange and red nectar guides.

Figure 16; The „Kastanien Allee“ in the Schlossgarten Schönbrunn from north to south. It contains four rows of *Aesculus hippocastanum* trees, each individually marked by the Österreichische Bundesgärten. Flower investigations were only done on the sun-exposed side of each tree (east = left side of the trees from this point of view).

Figure 17; (A) Fully bagged inflorescence with a reusable fruit and vegetable bag to prevent any bias caused by flower visiting insects. The green cord around the opening of the bag was used for closure and fixing the bag at the tree. (B) Small organza bag covering a sub-inflorescence, cincinnus, of *Aesculus hippocastanum*. One young flower in anthesis and three buds before anthesis are covered.

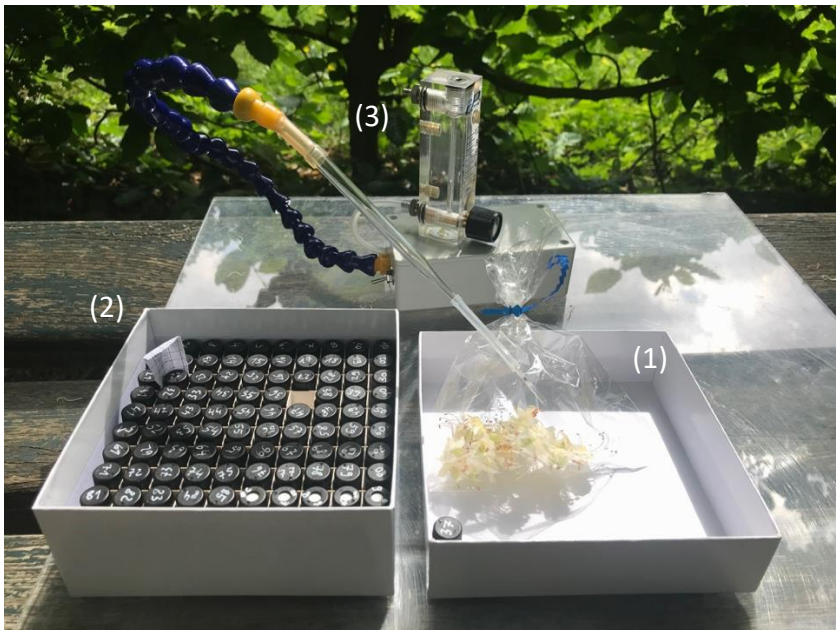

Figure 18; (1) Rotary vacuum pump, (2) scent traps (adsorbent quartz tubes) and (3) the scent pool of yellow colored *Aesculus hippocastanum* flowers in a customized oven bag.

Figure 19; Nectar collection with a 2µl capillary of *Aesculus hippocastanum* flower.

Figure 20; Petals containing a yellow, orange or red nectar guide stuck flat on a black adhesive tape wrapped upside down around a wood for photo spectrometric measurements.

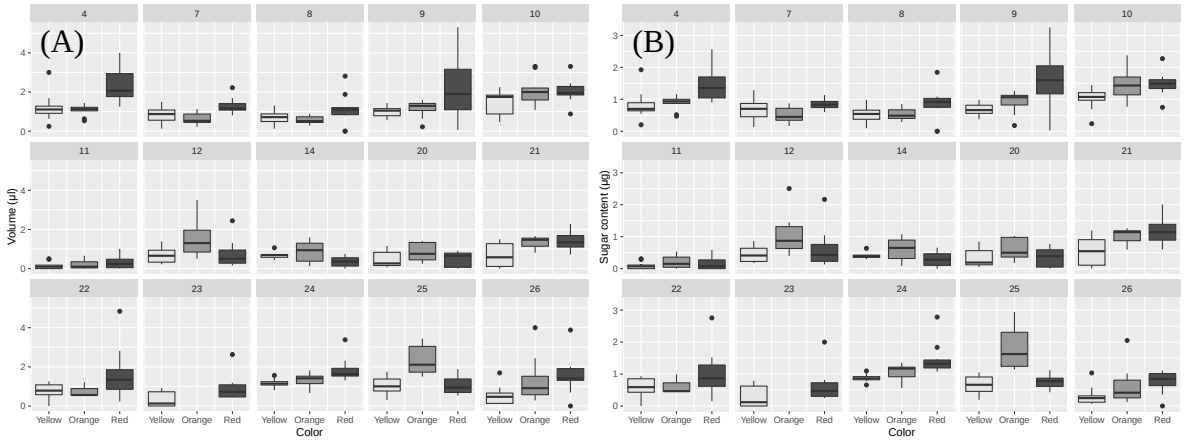

Figure 21; Nectar volume (A) and sugar content (B) for all investigated male flowers for each individual tree.

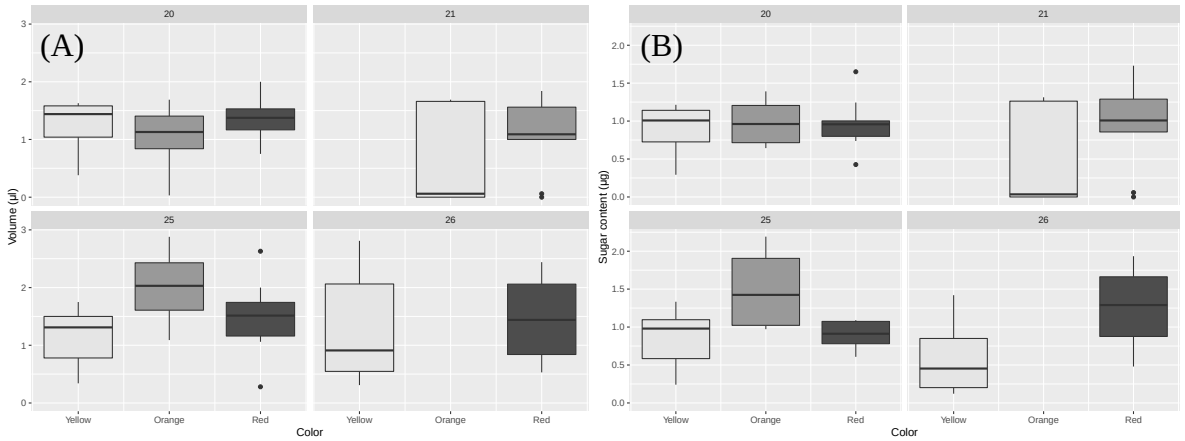

Figure 22; Nectar volume (A) and sugar content (B) for all investigated hermaphrodite flowers for each individual tree.

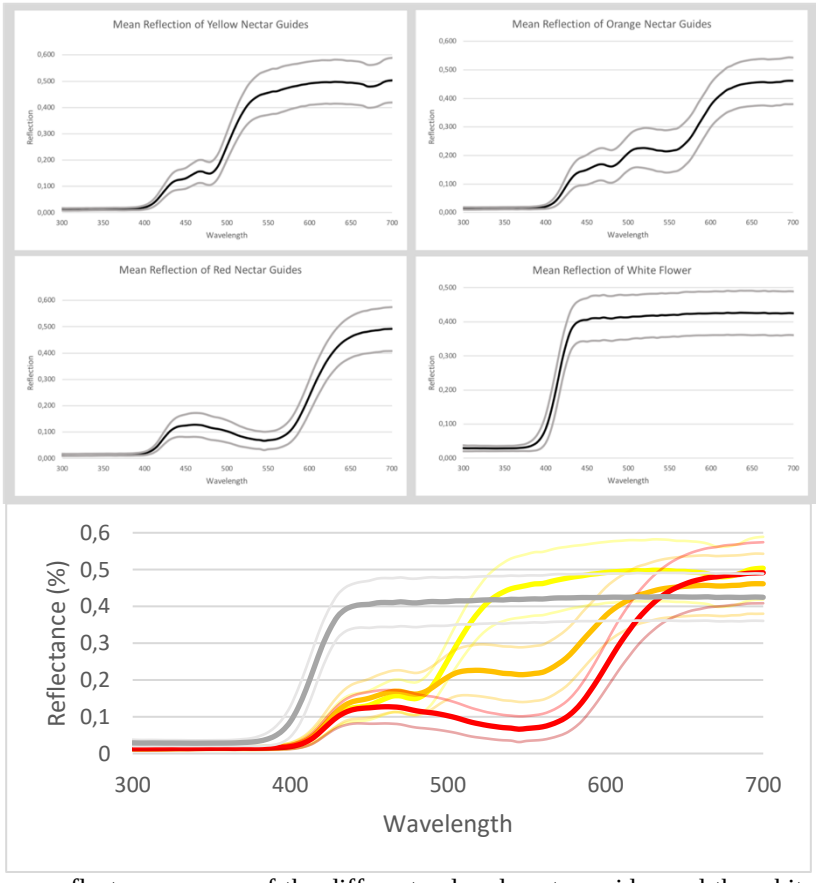

Figure 23; The mean reflectance curves of the different colored nectar guides and the white petal of Aesculus hippocastanum. (A) mean reflectance of the yellow nectar guides; (B) mean reflectance of the orange nectar guides; (C) mean reflectance of the red nectar guides; (D) mean reflectance of the white petals (E) all mean and standard deviation curves combined.