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„The learning process of naïve bumblebees
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Aesculus hippocastanum”

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Summary

Viele Pflanzenarten nutzen unterschiedliche Modalitäten, um potenzielle Bestäuber anzulocken. Ihr Display kann diverse Informationen bereitstellen, wie zum Beispiel die eigene Fitness sowie die angebotene Belohnung. Diese Signale können in unterschiedlichster Weise von den blütenbesuchenden Insekten wahrgenommen werden. In bisherigen Studien sind visuelle und olfaktorische Reize die meistdiskutierte Art der Anlockung. Die gemeine Rosskastanie (*Aesculus hippocastanum*) ist zum Beispiel eine Art, welche ihre „Saftmale“, während der Anthese von gelb über orange zu rot verfärbt und zeitgleich mit dem Farbwechsel auch den Blütenduft ändert. In diesem Kontext konnte in bisherigen Studien beobachtet werden, dass Bienen hauptsächlich jene Blüten mit gelben Saftmalen anfliegen, während Blüten mit orangenem oder rotem Saftmal nicht oder nur selten besucht werden. Ob dieses Verhalten allein durch die Farbe oder auch durch den unterschiedlichen Blütenduft ausgelöst wird und wie diese Blütenmerkmale von den Bestäubern gelernt werden, wurde bisher noch nicht geklärt. Die vorliegende Studie analysierte anhand einer bekannten Hummelart (*Bombus terrestris*), welche ein wichtiger Bestäuber der gemeinen Rosskastanie ist, inwiefern sich blütennaive Individuen von einem für sie noch nie wahrgenommenen Duft oder einer noch nie gesehenen Farbe lenken lassen und ob sie lernen können, die verschiedenen Düfte und Farben zu unterscheiden.

Durch Verhaltensexperimente mit künstlichen Blüten und Duftextrakten, welche die echten Blüten von *A. hippocastanum* imitierten, konnte der Lernprozess der Hummel nachgestellt werden. Während eines Trainings in einer Flugarena wurde ausgewählten Sammlerinnen eine Auswahl an Kunstblüten geboten, welche entweder mit einem Duft- oder Farbstimulus präsentiert wurden. Der zu trainierende Reiz wurde mit einer Zuckerlösung belohnt. In einem anschließenden nicht-belohnenden zwei-Wahl-Test konnte beobachtet werden, ob der zuvor trainierte Stimulus gelernt wurde. Die Ergebnisse zeigen, dass sowohl der gelbe als auch der rote Duftreiz unabhängig voneinander gelernt werden konnten, jedoch nicht voneinander unterschieden wurden. Interessant ist auch, dass der Farbreiz schneller mit einer Belohnung assoziiert wurde als der angebotene Duftstimulus.

Abstract

Many plant species use different modalities to attract potential pollinators. Their display can provide information about their fitness or the offered reward. Thus, flower-visiting insects can perceive signals in different ways. In recent studies, the most frequently discussed stimuli were the attraction of pollinators by visual and olfactory cues. For instance, the common horse chestnut (*Aesculus hippocastanum*) is a plant species that changes the color of its nectar guides ("Saftmale") from yellow over orange to red during anthesis. In addition, the color change is correlated with a scent change. Previous studies observed that bees are more attracted to the yellow than to the orange and the red nectar guides, and, therefore, the flowers with yellow nectar guides are visited more often. Whether it is mainly the colors or the associated scents that trigger this behavior and how pollinators can learn flower characteristics could not be clarified yet. This study examined how flower naïve bumblebees (*Bombus terrestris*), a well-known pollinator of *A. hippocastanum*, are influenced by the color and scent stimuli and how they learn them.

Behavioral experiments with artificial flowers and scent extracts of the common horse chestnut flowers were used to explore the learning process of naïve bumblebees. During a training period in a flight arena, foragers were offered a selection of artificial flowers, presented with either a scent or color stimulus. The stimulus to be trained was rewarded with a sugar solution. In a subsequent non-rewarding two-choice test, it was possible to observe whether the previously conditioned stimulus was associated with a reward. The results show that both the yellow and red scent stimuli were learned independently, but the tested bumblebees could not distinguish between the associated scents. Interestingly, the color stimulus was learned more quickly than the scent stimulus.

Introduction

The first investigations regarding the foraging behavior of animals go back to the 1960s (reviewed in Pyke et al., 1997). They are related to the theory of optimal foraging, which attempts to explain how the correct decision for food (e.g., what food type to eat) and food-searching is made (Pyke et al., 1977). Considering this theory, social groups or individuals prefer food sources with the highest net profit absorption. To attract pollinators for reproduction, most plants use single or multiple modalities (Hasson, 1994) with metabolically costly stimuli, e.g., visual, olfactory, and acoustic signals (Raguso, 2004; Clarke et al., 2013; von Helversen & von Helversen, 1999; Lawson et al., 2017; Chittka & Raine, 2006) to advertise their reward (or to deceive their pollinators). This variety of floral cues, regarding their color, scent, shape, and size, is used by insects to find resources (Clarke et al., 2013) and those cues are even used to differentiate between rewarding and unrewarding flowers (Dyer & Chittka, 2004; Frisch, 1914; Menzel & Backhaus, 1991).

Each environment offers plenty of odors based on chemical compounds, which organisms with a sensory ability can perceive (Raguso, 2009). Flowers can produce volatile organic compounds (VOCs) and release them into the environment to defend themselves from herbivores and interact with their pollinators (Lawson et al., 2017; Muhlemann et al., 2014). Insects use these olfactory signals for food-searching, mate-detection, and related communication (Wright & Schiestl, 2009). The insects' antennae are equipped with various olfactory sensory neurons housed in different types of olfactory sensilla to recognize and differentiate between chemical compounds (Suchet et al., 2011; Chapman & Chapman, 1998; Chittka & Raine, 2006). For instance, Suchet et al. (2011) observed two subspecies of *Antirrhinum majus* L. (*A. majus* ssp. *striatum* and *A. majus* ssp. *pseudomajus*), which, besides color, are also distinct in their floral scents. At an antenna level, naïve bumblebees (*Bombus*) differentiated between the two floral scents and showed a higher antennal response to the scent with the higher amount of three specific volatile benzenoids. Their results showed that the emitted VOCs of these two subspecies innately influence the bumblebees' foraging choice behavior (Suchet et al., 2011).

Furthermore, it is argued that insects had to operate through a wide variety of viewing conditions depending on which habitat type they occupy, e.g., water, desert, forest, or even caves (Briscoe & Chittka, 2001). These environmental types exhibit different light intensities and vary in their spectral compositions (Endler, 1993; Lythgoe, 1972). Bumblebees, for example, can be found in many different habitats (Briscoe & Chittka, 2001) from Scandinavian alpine grasslands to Norway's northern coast, e.g., *Bombus alpinus* (L., 1758), or in Mediterranean habitats like Turkey or west Iran, e.g., *Bombus argillaceus* (Scopoli, 1763)

(Rasmont et al., 2015) and a few species even in tropical lowland forests (Endler, 1993). The ambient light, the light intensity, and color signals differ distinctly among those habitats (Briscoe & Chittka, 2001).

Sprengel (1793) was one of the first scientists who addressed the issue of floral color spots and introduced the term "nectar guides" ("Saftmale"). Later, Karl von Frisch (1950) observed that the nectar guides direct visitors to the nectar and that bees can perceive different wavelengths of light. Years later, another study confirmed the perception of varying nectar guides by pollinators (Knoll, 1992). In addition, it is assumed that these colored spots are essential and necessary for short distant attraction (Tregrovsky & Pany, 2019). According to Giurfa et al. (1995), UV-blue and green are the innate color preferences of bees. Bees have trichromatic color vision based on three photoreceptors (UV, green, blue) with different specific spectral sensitivity functions (Briscoe & Chittka, 2001). Generally, the green photoreceptor is most sensitive around 540nm, the blue photoreceptor around 440nm and the UV receptor around 350nm (Menzel & Backhaus, 1991; Peitsch et al., 1992; Briscoe & Chittka, 2001). Aside from a chromatic channel that processes the input of all three receptor types, bees are known to utilize an achromatic channel that receives input from the green-receptor alone and is used, e.g., in edge detection (Briscoe & Chittka, 2001; Dyer & Chittka, 2004; Menzel & Müller, 1996). All three photoreceptors are essential for flower recognition (Menzel & Müller, 1996; Diel, 2015).

In previous studies, the perception and detection of flowers by pollinators by both visual and olfactory impulses have been investigated (Dobson, 1987; Dötterl et al., 2011; Burger et al., 2010; Dötterl & Vereecken, 2010). Much attention has been devoted to the preference for floral color or scent by experienced and inexperienced specialized bees in a recent study (Dötterl, 2011). It could be shown that more experienced bees exhibit a stronger color preference, while the scent primarily attracts inexperienced bees. In summary, plenty of studies have discussed whether the flowers' scent or color is more important for the attraction of pollinators.

The common horse chestnut, *Aesculus hippocastanum* L., has large complex candle-shaped inflorescences, consisting of several partial inflorescences, including numerous individual flowers (Kugler, 1936). The flowers are monosymmetric and horizontally oriented and each has five large white petals (Eckehart, 2017). The upper two petals possess a single-colored nectar guide each. The nectar guides change their color (as perceived by a human observer) from yellow via orange to red during anthesis (Kugler, 1936; Sprengel, 1793). Previous studies observed that bees more frequently visit the flowers with yellow nectar guides than flowers with red ones (Kugler, 1936; Vogel, 1950; Lex, 1954; Stern, 2020). Furthermore, it was shown that the color change correlates with a difference in the scent composition (Sauer, 2022). Whether the behavior of preferentially and repeatedly visiting the yellow flowers is innate or learned and whether it is triggered by the color alone or by the different floral scents has not been thoroughly investigated and is currently poorly understood.

Honeybees (*Apis*), bumblebees, and other bee species are representatives of the superfamily Apoidea in the insect order Hymenoptera. They are among the most important pollinators in Europe (Rasmont et al., 2015). Generally, oligolectic bee species collect pollen from one type of plant or a group of closely related plants to feed their larvae (Michener, 2007). Therefore, it

is essential that bees can distinguish between, e.g., a hostplant and other plant species that co-occur (Dötterl, 2011). In contrast, polylectic bees (e.g., bumblebees) collect from different plant species (Vanderplanck et al., 2014). Concerning this matter, bumblebees must rely on sophisticated learning and memory skills to find essential food resources in their complex surroundings (Stanley et al., 2015). These skills allow bumblebees to navigate with a low energy input and to share the information with their nestmates (Stanley et al., 2015). The latter is not fully understood since signaling in bumblebees does not work through fluid transmission as in honeybees (Molet et al., 2009). It is assumed that a pheromone in combination with a floral scent released into the nectar pot results in enhanced scent learning performance (Molet et al., 2009).

Bombus terrestris (Latreille, 1802) is not only a well-known pollinator of *A. hippocastanum* (Kugler, 1936) but also used as an important model system for learning and memory research as well for sensory perception (Riveros & Gronenberg, 2009). This study aimed to examine whether flower naïve *B. terrestris* can learn the floral scent and color of *A. hippocastanum* flowers and whether the scent or the color (or their combination) is more decisive for choosing the different flowering stages. Due to the short flowering time of *A. hippocastanum*, about two weeks, it was impossible to conduct all experiments with real flowers. Therefore, scent extracts were produced and used for the scent experiments in this study. The scent extracts were compared with the scent measurements of the natural flowers (Sauer, 2022) to determine whether this method could also be used for future studies. Artificial flowers were used for the color experiments, and the color was compared with the measurements of natural flowers (Sauer, 2022).

Specifically, I asked (1) whether the colors of the artificial flowers are comparable with the natural colors of the *A. hippocastanum* flowers, (2) whether the scent extracts are comparable with the natural scent of *A. hippocastanum* flowers, (3) which nectar guide color and floral scent is preferred by flower naïve *B. terrestris*, (4) whether they can learn to associate the floral scents with a sucrose reward, (5) whether they can distinguish between scents and colors of different flowering stages, and (6) whether it is the scent or the color signals of the different flowering stages that are learnt faster by *B. terrestris*.

Material and Methods

Study Site and Studied Species

This study was carried out in a building of the "Österreichische Bundesgärten," located near Schloss Schönbrunn in Vienna. Two bumblebee colonies (*Bombus terrestris*, Koppert) were used (size at the beginning: $n_{\text{one}} = 80$ to 100 individuals; $n_{\text{two}} = \text{ca. } 120$ individuals). The first colony was set up on the 5th of May 2021 and used for the experiments between the 11th of May and 24th of June 2021. The second colony was set up on the 1st of July 2021, and experiments were carried out between the 3rd of July and the 5th of August 2021.

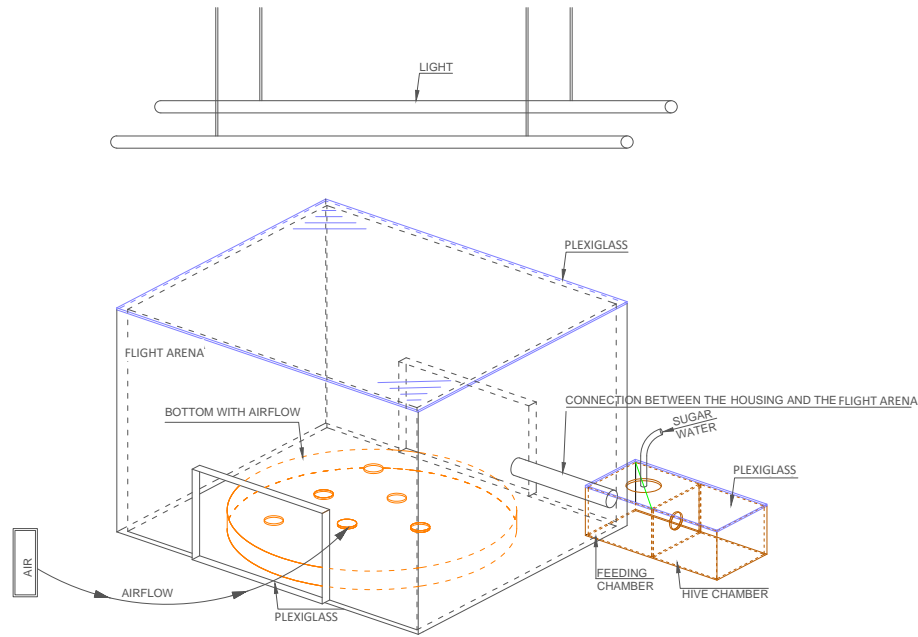


Figure 1; Experimental setup. Housing box (A) is divided into the hive- and feeding chamber. Flight arena (B) connected with housing box through a plexiglass tube. Plexiglass covers the top of both boxes and the doors on the sides—a Styrofoam disc with airflow support on the bottom of the flight arena and two non-flickering lights above. (Illustration designed by Ankica Žilić with AutoCAD)

Bumblebee Housing

The flower naïve bumblebee colonies were housed in a 415 x 195 x 130mm wooden box, divided into two equal chambers, one containing the hive and one used as a feeding chamber (see Fig. 1A). A hole (25mm in diameter) between the chambers allowed the bumblebees to move back and forth. The bottom was covered with cat litter (Clever), cleaned frequently, and completely changed every two weeks. Frozen pollen was provisioned into the hive daily, and a 25% sucrose solution was available for bees between experiments in the feeding chamber.

Flight and Test Arena

The housing box and the flight arena (950 x 815 x 600mm, Fig. 1) were connected by a plexiglass tube (20mm in diameter). Gates in the tube could be manipulated to manually control the access of individual bumblebees to the flight arena and the feeding chamber, respectively. The flight arena was topped with a UV-transmitting plexiglass and two fluorescent lamps (1x Sylvania Reptistar F30W; 1x Osram Biolux L30W/965) with an electronic ballast (>1kHz flicker-frequency) were placed above to ensure uniform non-flickering illumination. Experimental elements could be manipulated through two plexiglass doors at the sides of the flight arena. During experiments 1 and 2, a movable Styrofoam disc (710mm in diameter; 50mm height) was used. The Styrofoam contained six cutout circles (29mm in diameter), wrapped with aluminum foil, including a hole (5mm in diameter) in the center (see Fig. 2A). For the necessary airflow supply (mean= 0.167l/min; sd.= 0.065l/min) produced by an air pump, hoses (4mm in diameter) were run along the underside of the disc (see Fig. 2B). All bottom elements (floor and Styrofoam disc) were covered with gray cardboard (Canson 122 Gris Flanelle, Mi-Teintes). After each experiment, the connecting tube was blocked, and the flight arena and the aluminum foil were cleaned with alcohol (80% ethanol).

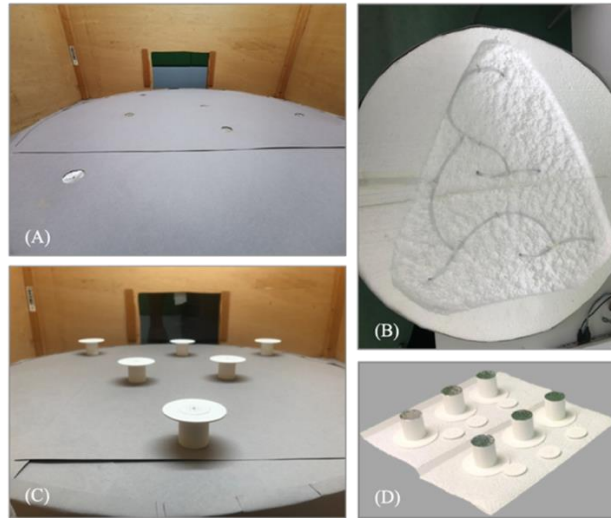


Figure 2; Styrofoam disc with airflow. (A) Styrofoam with six cutout circles wrapped with aluminum foil. (B) Integrated hoses on the underside of the Styrofoam were connected to an air pump. (C) Six artificial flowers were placed on six cutouts of the Styrofoam disc. (D) The bottom of the cylinder is closed with aluminum foil.

Artificial Flowers

All artificial flowers for this experiment were printed with a 3D printer (Ender-2, Creality). These white artificial flowers (used material: polylactic acid; PLA175W07, Velleman, Belgium) contained a cylinder (35mm height, 29mm in diameter) as a foothold, which simultaneously served as a scent chamber. The top of the cylinder had a disc (60mm in diameter) with an opening in the middle (29mm in diameter). The cylinder was closed with aluminum foil on the bottom side to prevent scent contamination on the floor (see Fig. 2D). Furthermore, the aluminum foil had five small holes to ensure sufficient airflow to support the volatiles emission of the artificial flowers. A second disc, containing 12 small holes (for airflow supply) and a cavity in the center (3mm in diameter), was placed on the first disc's opening for each experiment. During experiment 3, the white cavity of the second disc was replaced with yellow-colored (PLA; PLA175Y07, Velleman, Belgium) and red-colored dots (PLA; PLA175R07, Velleman, Belgium) to mimic the nectar guides of the natural flowers (Fig. 3). After each experimental run (see below), the artificial flowers were cleaned with alcohol (80% ethanol) to remove bumblebees' pheromones (Stout & Goulson, 2001) and other residues (e.g., scent residues).

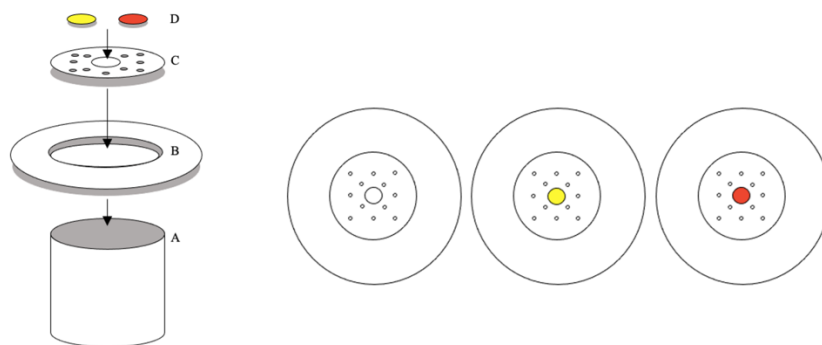


Figure 3; (Left) All parts of an 3D printed artificial flower. (A) Cylinder as a foothold and scent chamber, (B) first disc with an open center, (C) second disc with 12 small holes to enable airflow to pass to the top and a cavity in the center to add a 30% sugar water solution, (D) the cavity in the center simultaneously serves as a nectar guide color (yellow and red cavity). (Right) Schematic top view of artificial flowers.

Color Measurement

The spectral reflection of the yellow and red nectar guides and the contrast to the white petals of the natural flowers of *A. hippocastanum* (see measurement in Sauer, 2022) were compared with the spectral reflection of the artificial flowers. Therefore, the white artificial flowers, the yellow- and the red-colored dots were measured using a USB-2000 spectrometer (Ocean Optics, Dunedin, FL, USA). The USB-2000 spectrometer was calibrated against a white standard (Diffuse Reflectance Standard WS-1, Ocean Optics). For the measurement, a Deuterium/Halogen (DH-2 Bal, Ocean Optics B.V, Duiven, Nederland) light source was used, which covers a spectral range of <300 to >700nm. All spectral measurements were saved as a text file. To analyze the data, the text file was converted into an excel-file (Microsoft Excel 365; version 16.45), and mean reflectance spectra were calculated for each flower color.

To model the visual perception of the flowers by flower-visiting bumblebees, the color hexagon color space was used (Chittka, 1992). Photoreceptors were assumed to have adapted to the gray background of the flight arena. The Euclidean distance was used to calculate hexagon units (hu) between the natural and artificial flower values.

Flower Scent Extracts

At the beginning of this experiment, scent extracts from different flowering stages of male *A. hippocastanum* flowers had to be produced, because the flowering period lasted only two weeks. The purpose was to determine whether this method could be used for future studies and, therefore, to be independent of the flowering period. Therefore, male flowers with yellow and red nectar guides were collected. A pool of ten flowers of each stage was extracted in 3ml solvent (pentane, SIGMA-ALDRICH, anhydrous $\geq 99\%$; methanol, Honeywell, gradient grade $\geq 99,9\%$). For this purpose, the flowers were placed in an essence glass vessel (5ml volume) with tweezers and swiveled for one minute. Afterward, the flowers were removed. Most volatile organic compounds (VOCs) were thus washed from the flower surface into the solvent. The produced floral scent extracts were used for experiments 1–3 (see below). Seven extracts with pentane and six with methanol of the natural red flowers, nine with pentane and three with methanol of the natural yellow flowers, were made. In total, 25 scent extracts were used ($n_{\text{red}}= 13$; $n_{\text{yellow}}= 12$).

For statistical analyses, bias by the two used solvents had to be excluded. With the Wilcoxon rank-sum test the first five approaches (see below) for the first run (FR) with pentane were compared to the first five approaches with methanol for both the yellow and red flower extracts. The same test was conducted with the five choices in the test trials (TT).

Scent Measurement

The components of artificial flowers-scents had to be examined to investigate whether the produced scent extracts resemble the natural floral scent. Therefore, the dynamic headspace method was used (Dötterl et al., 2005). The equivalent amount of one flower from each flower extract was placed into the artificial flower and supplied with airflow (see experiments 1 and 2). With a polyethylene oven bag (Toppits, Germany), the artificial flower was enclosed from top to the base, where the bag was tightly constricted. The volatile substances could diffuse through the small holes on the upper plate and be trapped in the oven bag. With a rotary vane

vacuum pump (Gardner Denver, Germany; airflow 0.2l/min) connected to a quartz glass tube (25mm length; 2mm in diameter; see Dötterl et al., 2005; Etl et al., 2016), filled with 1.5mg each of the adsorbents Carbotrap B (mesh 20–40, Supelco, Germany) and Tenax TA (mesh 60–80; Supelco, Germany), the scent was collected for five minutes. Samples of pure methanol, pure pentane, a clean paper sheet, and the air space of the flight arena were collected with the same method and acted as negative control samples. Captured volatiles were analyzed with a GC/MS (gas chromatography; mass spectrometry; QP2010Ultra, Shimadzu Corporation, Japan) with helium as carrier gas with a flow of 1.5ml/min in a split ratio of 1:1. The initial GC oven temperature was 40°C, which was increased by 6°C per minute until it finally reached 250°C. The mass spectra of the scent extracts were then taken in EI mode at 70eV and 30–350m/z. With GCMSolution 4.11 (Shimadzu Corporation, Japan), individual scent spectrograms were edited manually. Based on the mass spectral databases (NIST 11, Wiley 9, FFNSC 2, Essential oils and Adams 2007), the scent components were analyzed (see Dötterl et al., 2005; Etl et al., 2016). Finally, the identified compounds' mass spectra and retention times were compared with the scent analysis of natural *A. hippocastanum* flowers (Sauer, 2022).

The focus of the comparison with Sauer (2022) was on the analyzed scent components, which are decisive for the respective color phases of *A. hippocastanum* flowers. The differences in the absolute amount of natural floral emitted scent and scent extract compounds were examined using the Kruskal-Wallis rank-sum test. Afterward, the Wilcoxon rank-sum test was used as a posthoc test to determine significant differences between groups. The graphic representation was plotted in Primer 7 (Version 7.0.21, PRIMER-e). Scent variations were divided into four color-scent groups (AFY= artificial flowers yellow-scented, AFR= artificial flowers red-scented, NFY= natural flowers yellow-scented, NFR= natural flowers red-scented). Canonical analysis of principal coordinates (CAP) was used with S17 Bray-Curtis similarity, square-root transformation to obtain standardized samples by total, and a PERMANOVA to evaluate the influence of these color-scent groups on the scent. Using multiple regression, a correlation of 0.3 or higher was used to decide which volatiles correlate with the scent groups.

Experimental Setup

At the beginning of this study, a few experiments were performed with natural flowers of *A. hippocastanum* (see Appendix “First setup”) to arrive at the following methodology. Tests performed took place between 8 AM and 6 PM, to test the hypotheses that “a preference for the yellow color and scent is present” and that “bumblebees can learn each scent independently and distinguish between the scents and the colors”. Furthermore, at each experimental start and end, the room temperature was measured with a thermometer.

Each experiment was divided into three stages (pre-training, training, test trial).

Pre-training: During pre-training, the bumblebees were primed to the experimental setup and trained to collect the sugar reward in the center of the artificial flowers. In experiment 3, therefore, five to eight white artificial flowers were randomly placed in the flight arena. In experiments 1 and 2, three to six artificial flowers, including the Styrofoam disc, were placed. A 30% (30g sugar in 100ml distilled water) sugar water solution (sucrose solution) was added to the central cavity of the artificial flower.

During pre-training, all bumblebees could freely enter the flight arena. Individuals which made several approaches (landings) to the artificial flowers (henceforth called “foragers”) and collected the sugar water were marked on the thorax with numbered non-toxic opalite plates.

Training: During the training stage, only a single forager was allowed to enter the flight arena at any given time. During this period, the “correct” choices (e.g., yellow scent; see below) were rewarded with 30 μ l of a 30% sucrose solution while the “incorrect” options (e.g., scentless) only contained 30 μ l of distilled water. Collected rewards were replaced immediately after a forager left the artificial flower. Each foraging bout (termed “run”) of a tested bumblebee started with the collection of a reward after leaving the colony and ended with the return to the hive. After every run, the arrangement of the artificial flowers was changed randomly to ensure that the location was not remembered.

The training ended with a minimum of 84 approaches. After the minimum was reached, the bumblebee was allowed to finish the run before the experiment/training was finished. Therefore, the total number of approaches is different for each tested individual.

Test trial: The test trial was carried out with two artificial flowers (positive vs. negative stimuli). During this test, both stimuli were filled with distilled water. The first five approaches to stimuli were counted.

Experiment 0: Learning Red Scent with Natural Flowers

Four experiments could be performed with natural red flowers (one flower in the scent chamber) instead of scent extracts with the same setup as in experiments 1 and 2 (see below). The Wilcoxon rank-sum test was used to determine whether there are differences in the frequency of choosing the correct decision relating to the natural red flowers and the red scent extracts. The Wilcoxon rank-sum test compared the first five approaches of the first run with red scent extracts with pentane, methanol against the natural red flowers. The same test was conducted with the five choices in the test trials.

Experiment 1: Learning Red and Yellow Scents Independently

The extract equivalent of one flower was pipetted onto a piece of print paper (40 x 20mm; hp Everyday) and placed in the scent chamber of the artificial flower. Three of the six flowers were equipped with scent and the sucrose reward, and three of them as blank with the solvent and water as “reward” (see Fig. 2C). The third amount of the used scent extract was refilled after each fourth run to ensure continuous evaporation of volatiles. In the test trial the print paper was replaced by a fresh paper sheet.

To test whether the bumblebees learn to associate the red and yellow scent, respectively, with the sucrose reward, the first five decisions in the first training run (assumed to reflect the innate preference) were compared against the five decisions of the test trial using a Wilcoxon rank-sum test. With a binary logistic regression model, the influence of the number of approaches on the probability of making the correct choice was calculated. All analyses were performed in R 4.0.3 (R Core Team; 2020). Every treatment and test trial were filmed with a video camera (Sony HDR-CX405) fixed on a tripod and reviewed on a computer afterwards, to ensure that all approaches were correctly noted during the experiment.

Experiment 2: Learning Yellow vs. Red Scent

Based on the same procedure as the independent scent experiments, bumblebees were tested for whether they can learn to discriminate between the yellow and red scent. Therefore, three artificial flowers with the yellow scent and a sucrose reward were presented against three with red scent and water. Only extracts made with the same solvent were tested against each other to exclude any influence of the solvent type. In addition, a possible innate scent preference was tested using the first five decisions of each bumblebee's first run. Thus, the proportion for yellow and red decisions was compared using the Wilcoxon rank-sum test. All other statistical analyses were performed as in experiment 1.

Experiment 3: Learning Yellow vs. Red Color

The color experiment was conducted without the Styrofoam disc at the bottom and airflow. Five rewarding yellow-colored flowers and five non-rewarding red-colored flowers (Fig. 3) were randomly placed in the flight arena. The first five choices made by the naïve bees during the first run were considered innate preferences. Therefore, the innate proportion for yellow and red color decisions was compared with the approaches of a non-rewarded test trial using a Wilcoxon rank-sum test. All other statistical tests were conducted as in experiments 1 and 2.

Results

Color Measurement

To compare the spectral reflectance of artificial ($n_{\text{yellow}}=10$; $n_{\text{red}}=10$; $n_{\text{white}}=20$) and natural ($n_{\text{yellow}}=191$; $n_{\text{red}}=193$; $n_{\text{white}}=54$; measured by Sauer, 2022) flowers the mean reflectance curves were calculated (Fig. 4). There are small differences between the reflectance of artificial (AF) and natural (NF) flowers (Y= yellow; R= red; W= white). Exemplifying, in the region of the blue light wavelength (400nm-500nm), the natural flowers contain a small peak of up to 15% reflectance, but the AF did not reflect in this region.

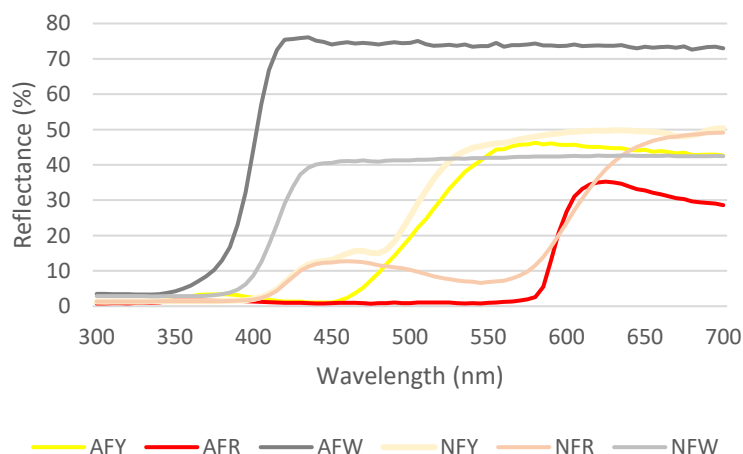


Figure 4; Mean spectral reflectance curves of measured artificial (AF) and natural (NF; Sauer, 2022) flowers. The x-axis indicates the wavelength spectrum from 300nm to 700nm (UV= 300-400nm; blue= 400-500nm; green=500-600nm; red= 600-700nm). The y-axis demonstrates the reflectance intensity (0%-100%). Y= yellow; R= red; W= white.

The color hexagon model of *Bombus terrestris* is shown in Fig. 5. To estimate the color perception of bumblebees, the hexagon distance between nectar guides of the artificial and natural flowers was calculated. In addition, the contrast between color loci and the background (center= bottom of the flight arena= gray cardboard= colorless for bumblebees) was calculated. Those distances are presented in Table 1.

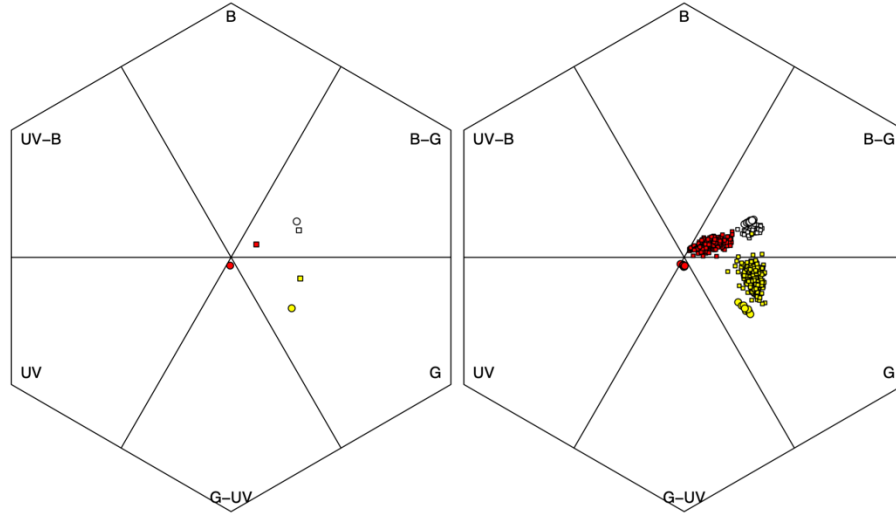


Figure 2; Color hexagon model of *Bombus terrestris*. (Left) Mean color loci for artificial (circle) and natural flowers (square; Sauer, 2022). (Right) Individual measurements for artificial (circles) and natural (squares) flowers. Lines divide the hexagon into arbitrary color sectors (B blue, B-G blue-green, G green, G-UV green-UV, UV ultraviolet, UV-B UV-blue).

The comparison of the artificial flowers showed that the bumblebees should be able to distinguish all offered visual stimuli. The artificial yellow and the white show the largest differences among all visual stimuli in the experimental setup (e.g., $AFY/AFW = 0.342hu$; $AFW/AFR = 0.313hu$). The smallest differences are between the red-colored dot and the background (background/ $AFR = 0.033hu$). The AFW showed the highest similarity to the NFW . Overall, enough color contrast between white and background and between white and the nectar guides in natural and artificial flowers is present (Table 1). Although there is a large variation among the natural spectra, some artificial colors occupy similar loci in the color space ($AFY/NFY = 0.122hu$; $AFR/NFR = 0.132hu$; $AFW/NFW = 0.036hu$).

Table 1; Euclidean distances between mean color loci in the hexagon color space (given in hexagon units (hu)). (NF= natural flower; AF= artificial flower; Y= yellow; R= red; W= white; background= grey cardboard of the flight arena). The background presents the center of the hexagon model (see Fig. 15).

	<i>NFY</i>	<i>NFR</i>	<i>NFW</i>	<i>AFY</i>	<i>AFR</i>	<i>AFW</i>
<i>NFR</i>	0.218					
<i>NFW</i>	0.190	0.176				
<i>AFY</i>	0.122	0.287	0.308			
<i>AFR</i>	0.279	0.132	0.303	0.294		
<i>AFW</i>	0.225	0.182	0.036	0.342	0.313	
<i>Background</i>	0.283	0.111	0.286	0.311	0.033	0.293

Scent Measurement

A total of 28 compounds which were also found in yellow-phase flowers of *A. hippocastanum* and 24 compounds which were also found in red-phase flowers, were detected in the scent extracts (Table 2). Sauer (2022), on the other hand, identified far more components in those natural flowers (yellow scent= 61; red scent= 63). In this regard, 45% of the original yellow scent compounds and only 38% of the red scent compounds could be extracted by the solvents.

Sauer (2022) detected a total of three unknown substances (unk1043, unk1185, unk1873) characteristic for the yellow flower scent, and two (methyl isovalerate; indole) for the red flower scent. Compared to these latter results, four out of five important compounds responsible for the difference between the two color-phases could be detected in the scent extracts in this study. All missing and not extracted substances in the scent extracts compared to the measurements of Sauer (2022) are presented in the Appendix (Table 4).

Table 2; The mean value of the relative amount (%) of analyzed yellow and red flower-extract compounds of *A. hippocastanum* are listed. Sample size= number of used scent extracts. Number of compounds= total of detected compounds in both scent groups. RI= Kovats retention index. tr(trace)= values less than 0.05. Occurrence= number of samples in which the compound was detected. Unk= unknown compound. Thick marked compounds= indicative compounds for the yellow (unk1043, unk1185, unk1873) and red floral scent (methyl isovalerate, indole).

scent extracts		yellow	red		
sample size		12	13		
number of compounds		28	24		
RI	name			occurrence Y	occurrence R
881	2-Methyl-2-butenic acid	0.544	1.161	5	4
943	unk943	1.203	---	6	0
968	1-Heptanol	4.076	3.343	6	13
1043	unk1043	13.347	4.786	11	13
1052	2,5-Dimethyl-4-hydroxy-3(2H)-furanone	1.657	1.105	4	4
1078	(Z)-Linalool oxide furanoid	4.183	6.114	10	9
1093	(E)-Linalool oxide furanoid	10.860	11.679	6	7
1119	2-Phenylethanol	5.114	1,829	12	10
1176	(E)-Linalool oxide pyranoid	0.806	1.013	5	5
1185	unk1185	37.217	49.751	10	13
1227	2-Aminobenzaldehyd	tr	2.374	1	7
1304	Indole	7.467	8.468	6	10
1313	(Z)-Methyl cinnamate	---	0.216	0	4
1328	unk1328	tr	---	1	0
1365	α -Cubebene	0.755	0.057	2	1
1375	unk1375	---	tr	0	1
1394	(E)-Methyl cinnamate	0.377	0.355	2	3

1406	β -Bourbonene	1.078	1.636	4	8
1425	unk1425	0.465	---	3	0
1441	unkST1441	1.645	0.135	3	4
1452	(E)-Cinnamyl acetate	tr	5.126	1	3
1452	unkST1452	0.293	---	2	0
1487	unk1487	2.521	0.132	5	2
1505	unkST1505	0.303	0.220	3	2
1518	unk1518	0.696	0.081	4	2
1525	unk1525	---	tr	0	1
1536	unkST1536	0.093	0.103	3	1
1542	unkST1542	tr	---	2	0
1545	unkST1545	1.638	0.052	3	1
1550	unk1550	---	tr	0	1
1567	Calacorene	0.371	tr	2	1
1588	unk1588	---	tr	0	2
1619	unk1619	---	tr	0	1
1623	Salvial-4(14)-en-1-one	0.396	0.086	4	3
1664	unk1664	0.148	---	1	0
1832	unk1832	0.121	0.051	2	1
1873	unk1873	0.299	---	2	0
1965	unk1965	2.277	tr	5	1

There are some apparent differences when comparing the absolute amount (in ng) of the responsible yellow scent compounds of *A. hippocastanum* flowers between the experimental scent extracts (AFY, AFR) and the natural floral scents (NFY, NFR). All essential substances accountable for the yellow scent are present in the scent extracts. Unk1043 and unk1185 occur in a similar amount compared to the yellow flowers (Fig. 6A, B). In addition, unk1043 occurs in an almost equal amount as in the natural red flowers, but a noticeable dissimilarity for unk1185 ($\chi^2 = 7.83$; $p < 0.05$; Fig. 6B) could be observed. A surprisingly higher quantity causes the dissimilarity in the red scent extracts. The yellow and red scent extracts significantly differ in the substance unk1873 compared with the natural flowers ($\chi^2 = 33.15$; $p < 0.001$; Fig. 6C). No unk1873 could be detected in the red scent extracts but a little in the yellow extract (Fig. 6C).

The red flower extracts exhibited one out of two accountable volatiles for the red-phase scent of *A. hippocastanum* flowers (Fig. 7). Methyl isovalerate could not be detected in both extracts, which in comparison was present in the analysis of the red natural flowers (Fig. 7A). Therefore, there is a difference between those scented solvents and the natural floral scent ($\chi^2 = 17.06$; $p < 0.001$). The quantity of indole differs in the artificial yellow and red scent from natural flowers ($\chi^2 = 33.15$; $p < 0.001$; Fig. 7B). The solvent extracted a much smaller amount of indole compared to the quantity in natural flowers.

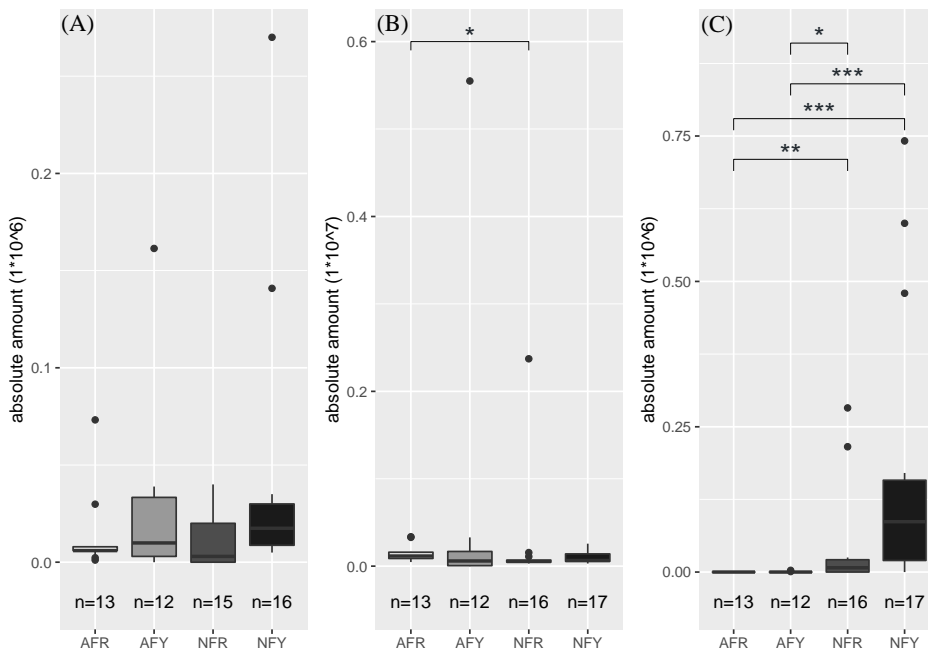


Figure 3; The absolute volatile amount (ng) of the yellow and red flowering stages of *A. hippocastanum* is compared among the scent extracts (AFR= artificial flowers red-scented, AFY= artificial flowers yellow-scented) and the natural flowers (NFR= natural flowers red scented, NFY= natural flowers yellow scented). (A) unk1043, (B) unk1185, (C) unk1873. Statistical analysis= Kruskal-Wallis rank-sum test; Posthoc= Wilcoxon rank-sum test; *<0.05, **<0.01, ***<0.001.

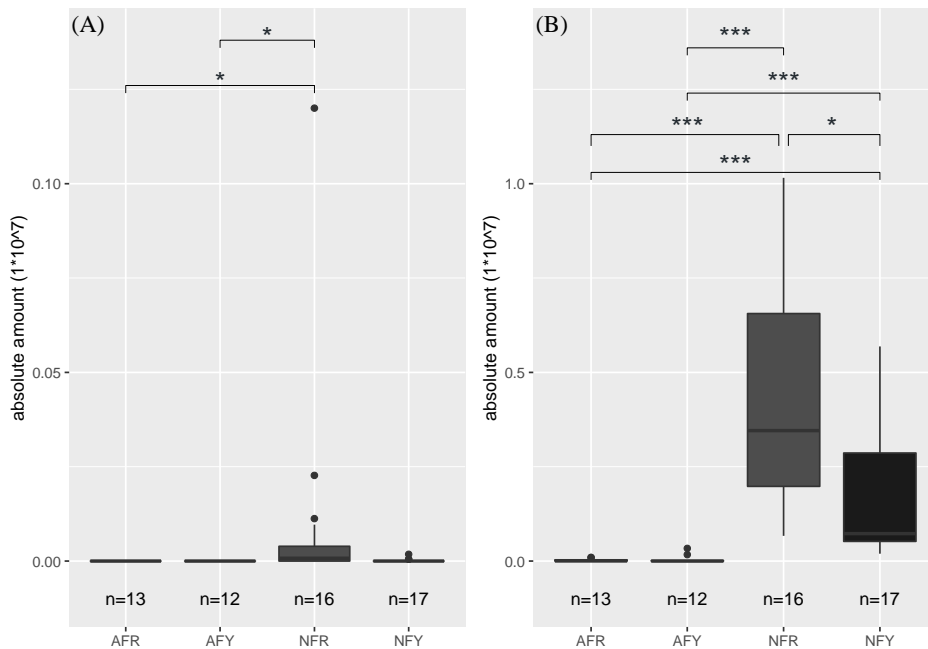


Figure 4; The absolute volatile amount (ng) of the yellow and red flowering stages of *A. hippocastanum* is compared among the scent extracts (AFR= artificial flowers red-scented, AFY= artificial flowers yellow-scented) and the natural flowers (NFR= natural flowers red scented, NFY= natural flowers yellow scented). (A) methyl isovalerate, (B) indole. Statistical analysis= Kruskal-Wallis rank-sum test; Posthoc= Wilcoxon rank-sum test; *<0.05, **<0.01, ***<0.001.

The disparity between natural scents and scent extracts is more pronounced than between extracts of yellow and red flowers (Fig. 8). The crucial compounds differ significantly in quantity between phases in the natural flowers but not significantly in the solvent extracts. It is noteworthy that indole is present in higher amounts in the red natural flowers than in the red extracts (Fig. 7B).

Furthermore, a higher unk1185 quantity in AFR than in NFR and the difference of the unk1873 amount between NFY and AFY could be observed. Overall, extracting all components (Table 4, Appendix) of yellow and red *A. hippocastanum* flowering phases did not work. The amount of the substances, which define the difference between the two-color phases are different from the natural flowers.

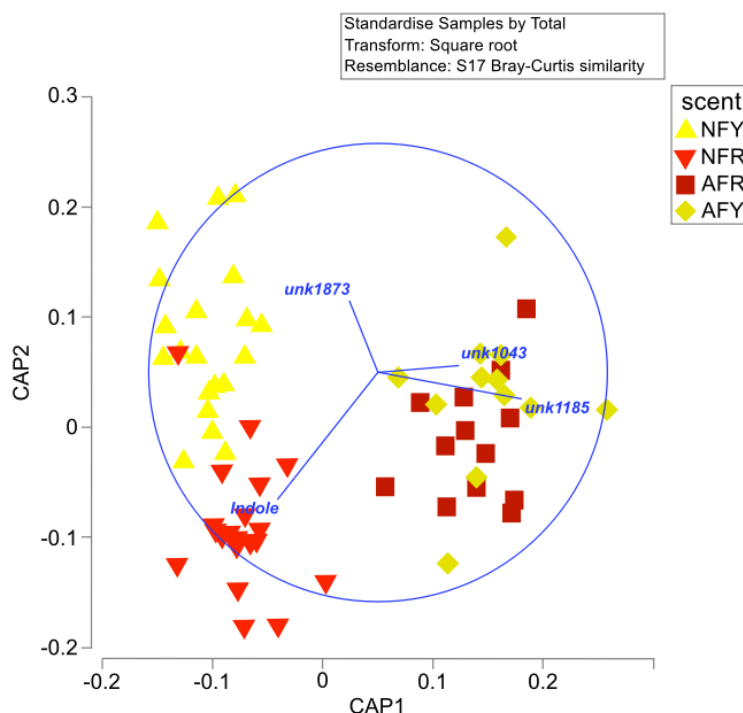


Figure 5; Canonical analysis of principal coordinates (CAP). Bray-Curtis similarity, square-root formatting to obtain standardized samples by total, and PERMANOVA were used. Vectors present the compound with the highest correlation to the scent extracts (square; AFR, AFY) and natural scent (triangle; NFR, NFY) of *A. hippocastanum* flowers containing yellow and red nectar guides and the difference between them.

Influence of Solvent on Decision

The Wilcoxon rank-sum test showed no significant differences between pentane and methanol extracts for each flower color (p-value: $FR_{\text{yellow}} = 0.376$; $TT_{\text{yellow}} = 0.758$; $FR_{\text{red}} = 0.376$; $TT_{\text{red}} = 0.453$; see Fig. 19 in Appendix).

Experiment 0

Pooling

The test showed no difference in the frequency voting of the red scents with methanol and pentane against the natural red floral scent (p-value: $FR = 0.724$; $TT = 0.405$; see Fig. 20 in Appendix). Hence, those four individuals were added to the red control group. Therefore, two solvent groups were pooled for further analysis (yellow control group $n = 9$; red control group $n = 14$).

Experiment 1

The mean temperature before and after each experiment was 23.0°C (sd.= 1.9°C, May to June) and 24.9°C (sd.= 0.9°C, July to August).

Yellow Control Group

About 75% (n= 9) of all marked bumblebee individuals (n= 12) participated successfully in this control group. It was not possible to force each bee to participate in all three experimental stages for this study. Some of them ceased foraging after one to three runs or died shortly afterward. However, nine scent-unexperienced were successfully trained to associate the yellow-scent phase of *A. hippocastanum* with a sucrose reward. Increasing preferences for the rewarding stimulus were already observed during the training phase. The choice probability for the scented stimulus started around 50% and rose to about 75% for the rewarded stimulus during the training period (Fig. 9; individual learning curves are given in Fig. 21, Appendix). The number of approaches significantly influenced the decision for the yellow-phase scent ($p < 0.001$).

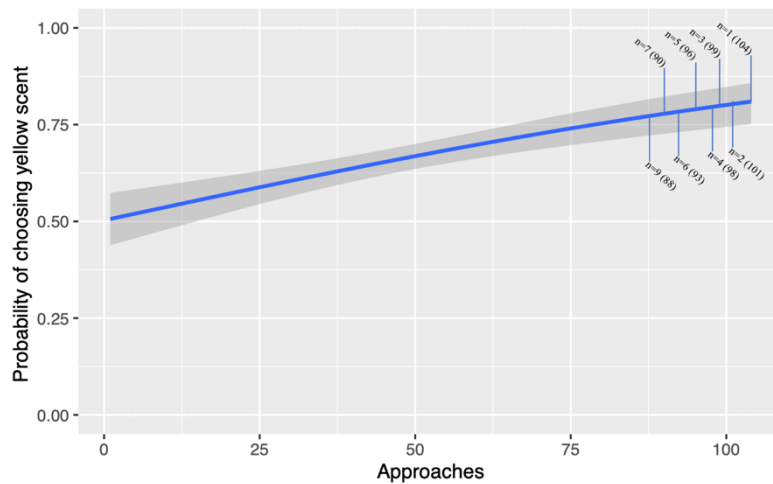


Figure 6; Learning performance on yellow-phase scent extracts of *A. hippocastanum* by *B. terrestris*. The influence of the number of approaches on the probability of choosing the yellow-phase scent of *A. hippocastanum* is demonstrated using binary logistic regression. The y-axis shows the likelihood of selecting the correct choice, and the x-axis the number of approaches during the training period. Note= since the training phase did not consist of the same number of approaches for each bee, the number of individuals is given for each approach number where it decreased from the initial number (n= 9).

Naiïve bumblebees, which have never experienced the yellow-phase scent of *A. hippocastanum* before, generally showed no innate preference when given a choice between yellow-scented or scentless artificial flowers (Fig. 10). After training with a minimum of 88 approaches, the tested bees chose significantly more scented artificial flowers in a non-rewarded test than their initial preference ($p < 0.01$). At an individual level, bumblebees showed a similar learning behavior and a higher preference for the rewarding stimulus after training ($FR_{\text{median}} = 0.20$, min.= 0, max.= 0.80; $TT_{\text{median}} = 1$, min.= 0.60, max.= 1; Fig. 10).

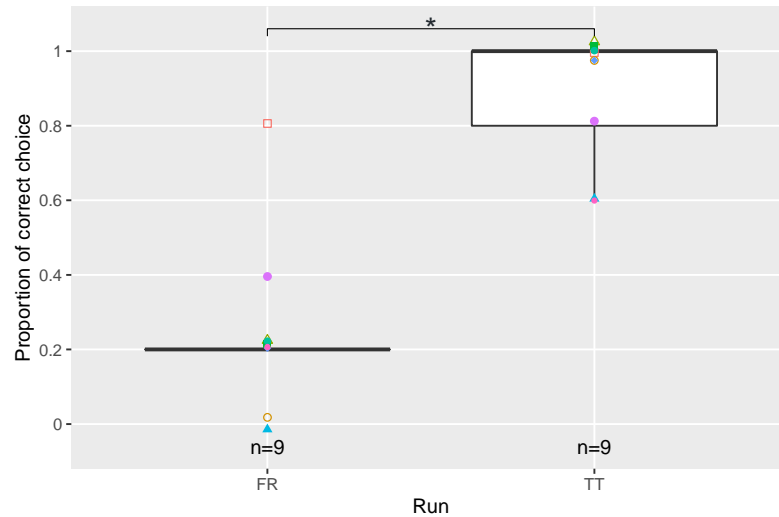


Figure 10; Learning of yellow-phase scent extracts by *B. terrestris*. The proportion of correct choices for the first five approaches in the first run (FR) was compared with the test-trial (TT) approaches. Colored symbols in FR and TT are vertically distributed to avoid overlapping and refer to the same bumblebee specimen and the line between them indicates the learning process of each bumblebee (n= 9). The thicker the line, the more individuals showed the same learning process. Statistical test= Wilcoxon rank-sum test; **<0.01.

Red Control Group

78% (n= 14) of all marked bumblebee individuals (n= 18) completed all experimental phases for this control group. Increasing preferences for the rewarding stimulus were already observed during the training phase, with an initial choice for the scented artificial flower around 50%, which rises to around 70% during the training phase (Fig. 11). A significant impact of the number of approaches (min.= 91) on the probability for choosing the rewarded stimulus indicates an influence on the decision for the red-scented phase of *A. hippocastanum* flowers ($p < 0.001$). Individual learning curves for all tested bumblebee individuals are demonstrated in the Appendix (Fig. 22).

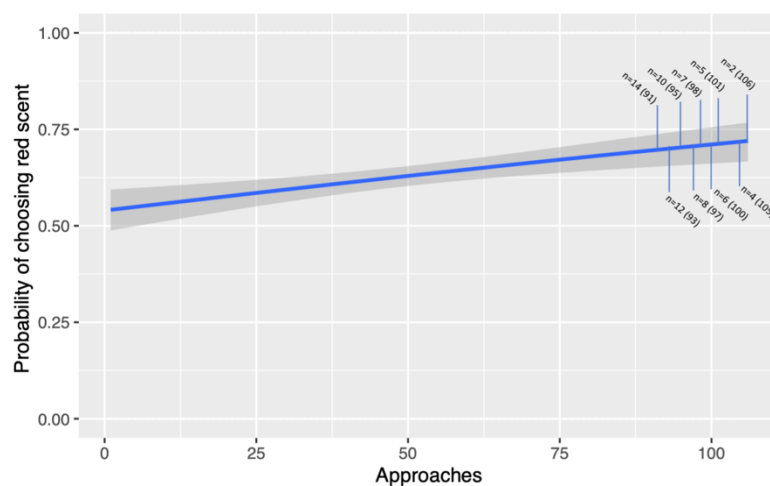


Figure 11; Learning period of red-phase scent extracts of *A. hippocastanum* by *B. terrestris*. The influence of the number of approaches on the probability of choosing the red-phase scent of *A. hippocastanum* is demonstrated using binary logistic regression. Y-axes shows the likelihood of selecting the right choice, and the x-axis represents the number of approaches during the training period. The closer the value of y is to one, the higher the probability for an individual making the correct choice. Note= since the training phase did not consist of the same number of approaches for each bee, the number of individuals is given for each approach number where it decreased from the initial (n= 14).

Bumblebees trained to associate a sucrose reward with the scent of red-phase flowers of *A. hippocastanum* showed no initial preference for scented artificial flowers and such that were only provisioned with solvent (Fig. 12). After training, bees chose significantly more scented artificial flowers in a non-rewarded test than their initial preference ($p < 0.05$). At an individual level, 9 out of 14 bees showed a higher preference for the rewarding stimulus after training ($FR_{\text{median}} = 0.50$, $\text{min.} = 0$, $\text{max.} = 0.80$; $TT_{\text{median}} = 0.60$, $\text{min.} = 0.60$, $\text{max.} = 1$; Fig. 12).

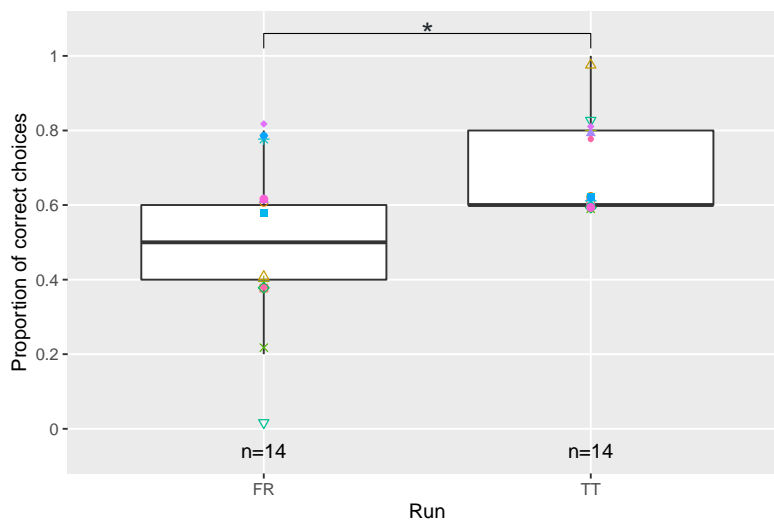


Figure 12; Learning of red-phase scent extracts by *B. terrestris*. The proportion of correct choices for the first five approaches in the first run (FR) was compared with the test-trial (TT) approaches. The lines between each colored symbol indicate the learning process of each tested bumblebee. The thicker the line, the more individuals showed the same learning process. Colored symbols in FR and TT are vertically distributed to avoid overlapping and refer to the same bumblebee specimen ($n = 14$). Statistical test= Wilcoxon rank-sum test; $* < 0.05$.

Experiment 2

Yellow vs. Red Scent

Nine scent-unexperienced foragers of were presented with two different scent phases of *A. hippocastanum* flowers with yellow scent as a rewarded stimulus and red as non-rewarded. Every marked individual ($n = 10$) participated in all three experimental stages of this study. An initial choice for the scented stimulus starts at around 60% for the yellow scent and rises during the training phase to about 70% (Fig. 13). An impact of the number of approaches ($\text{min.} = 90$) on the rewarded stimulus indicates an influence on the decision for the yellow-scented phase of *A. hippocastanum* ($p < 0.05$) during the training phase (Fig. 8). Individual learning curves for all tested bumblebee individuals are demonstrated in Fig. 23 (Appendix).

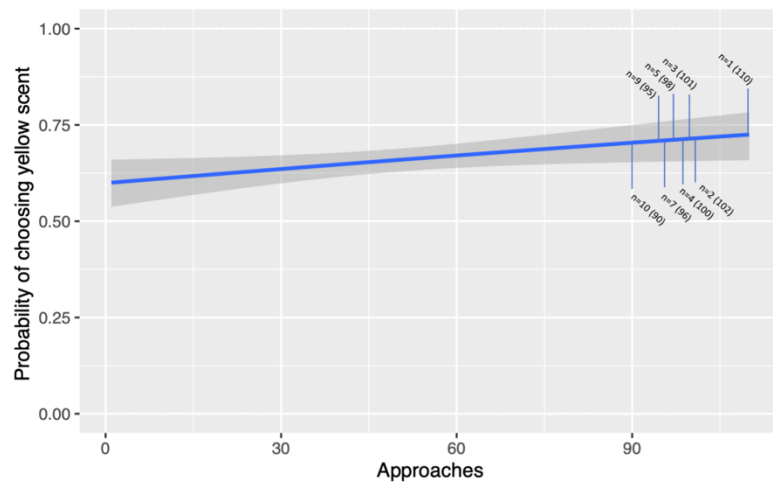


Figure 13; Learning period of yellow-phase vs. red-phase scent extracts of *A. hippocastanum* by *B. terrestris*. The influence of the number of approaches on the probability of choosing the yellow-phase scent of *A. hippocastanum* is demonstrated using binary logistic regression. Y-axes show the likelihood of selecting the correct choice, and the x-axis is the number of approaches during the training period. Note= since the training phase did not consist of the same number of approaches for each bee, the number of individuals is given for each approach number where it decreased from the initial (n= 10).

B. terrestris trained to associate a sucrose reward with the yellow-phase scent and to differentiate it from the red-phase scent showed an initial preference for the white, yellow-scented artificial flowers ($p < 0.05$; $\text{Yellow}_{\text{median}} = 0.60$, $\text{min.} = 0.20$, $\text{max.} = 1$; $\text{Red}_{\text{median}} = 0.40$, $\text{min.} = 0$, $\text{max.} = 0.80$; Fig. 15). After training with a minimum of 90 approaches, the tested bees showed no significance for choosing more yellow-scented artificial flowers in a non-rewarded test than their initial preference ($p > 0.05$; Fig. 14). A higher preference for the rewarded stimulus after training was shown in five out of ten bees ($\text{FR}_{\text{median}} = \text{TT}_{\text{median}} = 0.60$, $\text{min.} = 0.20$, $\text{max.} = 1$).

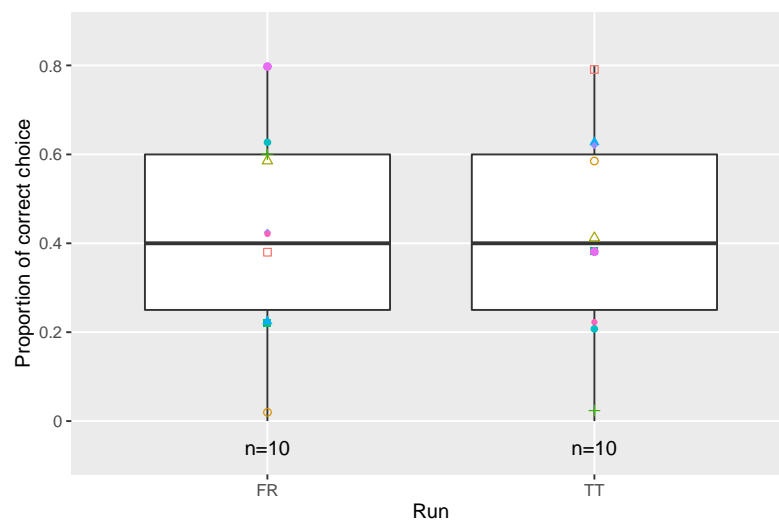


Figure 14; Learning of yellow-phase scent extracts against red-phase scent extracts by *B. terrestris*. The proportion of correct choices for the first five approaches in the first run (FR) was compared with the test-trial (TT) approaches. The lines between each boxplot indicate the learning process of each bumblebee. The thicker the line, the more individuals showed the same learning process. Colored symbols in FR and TT are vertically distributed to avoid overlapping and refer to the same bumblebee specimen (n=10). Statistical test= Wilcoxon rank-sum test.

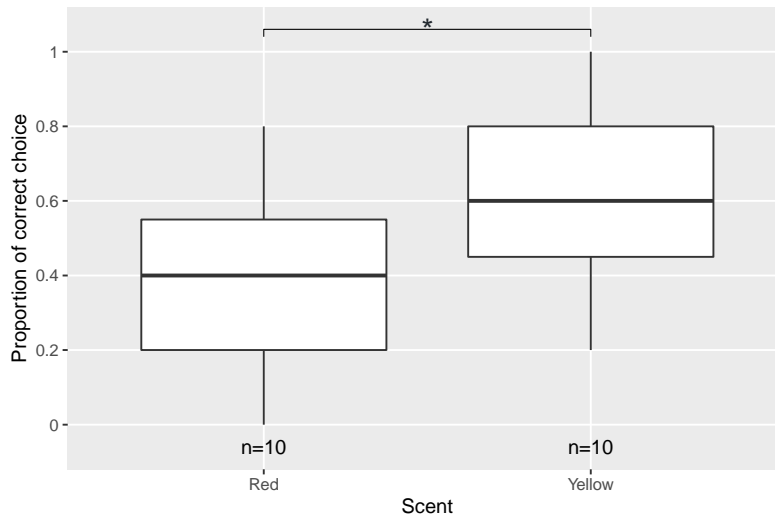


Figure 15; Innate scent extract preference of *B. terrestris*. The choice frequency for the first five approaches of the first run from experiment 2. Y-axes present the proportion of choices, and the x-axes are the rewarded yellow-phase and the non-rewarded red-phase scent of *A. hippocastanum*. n= 10. Statistical test= Wilcoxon rank-sum test; *<0.05.

Experiment 3

Yellow vs. Red Color

Every marked forager (n= 5) participated in all three experimental stages in this group. A fast-increasing preference for the rewarding stimulus was already observed during the training phase. An initial choice for the yellow-colored artificial flower starts at around 40%. With an increase of approaches, the value of y is closer to 100%, which indicates a clear impact of the number of approaches on the correct choice ($p < 0.001$; Fig. 16). After only 20 approaches, the frequency of making the correct decision increases by over 75% and already reaches its maximum after 60 approaches In the Appendix (Fig. 24), every learning curve of each tested bumblebee individual is demonstrated.

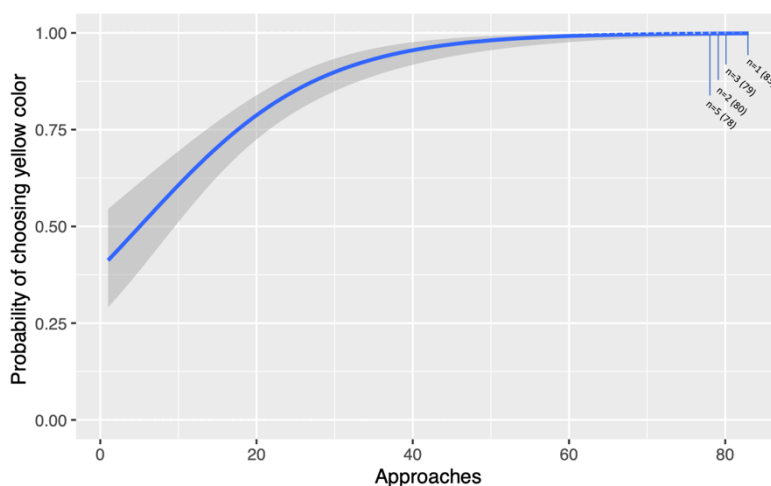


Figure 16; Learning period of the yellow-phase against the red-phase color of *A. hippocastanum* by *B. terrestris*. The influence of the number of approaches on the probability of choosing the yellow-phase color of *A. hippocastanum* is demonstrated using binary logistic regression. The y-axis shows the likelihood of selecting the right choice, and the x-axis is the number of approaches during the training period. The closer the value of y is to one, the higher the probability for an individual making the correct choice. Note= since the training phase did not consist of the same number of approaches for each bee, the number of individuals is given for each approach number where it decreased from the initial (n= 5).

After the tested bumblebees' training success toward the yellow-colored reward, four out of five bees choose more yellow-colored artificial flowers in a non-rewarded test than their initial preference, but overall, no significant preference for the yellow color could be observed (p -value= 0.07; $FR_{\text{median}}= 0.60$, $\text{min.}= 0.20$, $\text{max.}= 1$; $TT_{\text{median}}= 1$, $\text{min.}= 0.80$, $\text{max.}= 1$; Fig. 17). Bumblebees were trained to associate a sucrose reward with the yellow-phase color and differentiate it from the red-phase color and no innate preference for both colored artificial flowers were observed ($\text{Yellow}_{\text{median}}= 0.60$, $\text{min.}= 0.20$, $\text{max.}= 1$; $\text{Red}_{\text{median}}= 0.40$, $\text{min.}= 0$, $\text{max.}= 0.80$; Fig. 18).

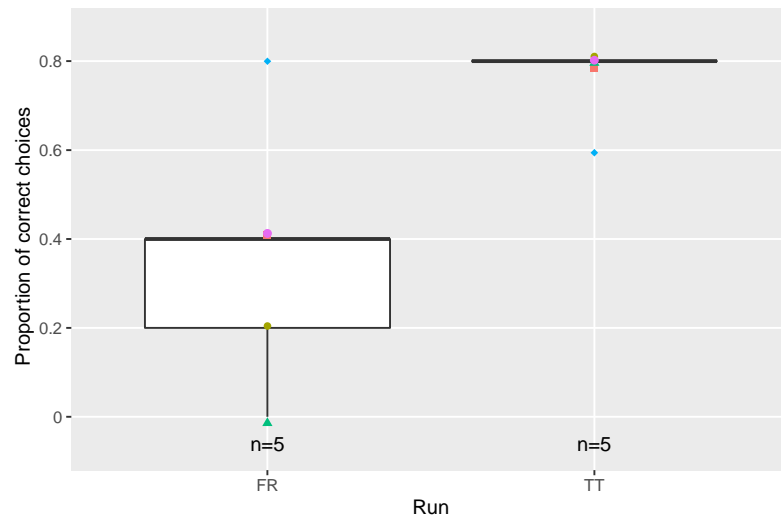


Figure 17; Learning of yellow-phase color against red-phase color by *B. terrestris*. The proportion of correct choices for the first five approaches in the first run (FR) was compared with the test-trial (TT) approaches. Colored symbols in FR and TT are vertically distributed to avoid overlapping and refer to the same bumblebee specimen and the lines between them indicate the learning process of each bumblebee ($n= 5$). The thicker the line, the more individuals showed the same learning process. Statistical test= Wilcoxon rank-sum test.

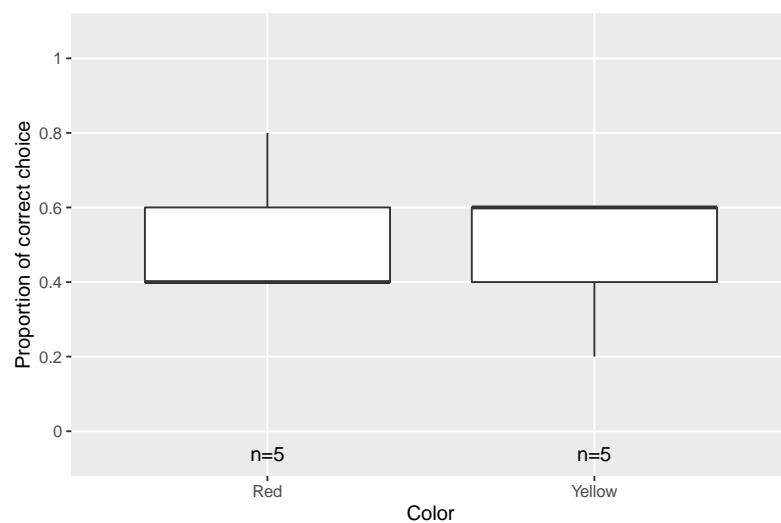


Figure 18; Innate color preference of *B. terrestris*. The choice frequency for the first five approaches of the first run from experiment 3. Y-axes present the proportion of choices, and the x-axes are the rewarded yellow-phase and the non-rewarded red-phase color of *A. hippocastanum*. $n= 5$. Statistical test= Wilcoxon rank-sum test.

Discussion

This study investigated the learning ability of naïve bumblebees to associate a scent or color stimulus with a reward and demonstrates a learning success with respect to the scents and colors of *A. hippocastanum* flowers. The results show clearly that naïve *B. terrestris* can learn to associate floral scent extracts with a sucrose reward. These results correspond with Wright & Schiestl's (2009) argument that Hymenoptera provide some of the best examples of holometabolous insects' learning capabilities. Therefore, they argued that olfactory cues could be associated with food and that activities such as feeding are guided by the olfactory sense. For most animals, associative learning is used to adapt to the constantly changing environment and reliance on (changing) olfactory cues shows that the ability to learn is required (Wright & Schiestl, 2009).

Spectral measurements showed that the colors of the artificial flowers and the nectar guides of the natural flowers of *A. hippocastanum* (Fig. 4-5; Table 1) are comparable. The closer the dots in the hexagon, the more similar the colors are, the more challenging it is to distinguish them, and the higher the likelihood that artificial and natural colors are perceived as equal by the bumblebees. The synthetic red color is near the Hexagon center and, therefore, appears colorless to the bumblebees. Still, Garcia et al. (2017) determined that *B. terrestris* can distinguish colors with a success rate of 80% if the hexagon units (hu) are between 0.02 and 0.03 apart from each other and, therefore, bumblebees potentially can perceive the red color. Based on the comparison of the spectral distances, this study indicates that *B. terrestris* can perceive the colors of the artificial flowers. Thus, it can be assumed that artificial flowers can be used as an appropriate model for visual stimuli experiments.

Bumblebees learned to associate the yellow color with a reward during the training period and were able to distinguish yellow from red. In a non-rewarding test, learning was not statistically significant, likely due to the small sample size. Previous studies showed that bees often make wrong decisions and that a longer learning phase is necessary to be able to distinguish between similar colors than between different colors (Dyer & Chittka, 2003). However, it is only beneficial for bees if the floral colors can be distinguished from the background, and if this condition is not met, bees use other cues to find the flowers they want to visit, for example flower shape (Dyer & Chittka, 2003). Likewise, Giurfa et al. (1994) argued that bees also use the floral scent for establishing if, e.g., the color is not differentiable. Equally, white 3D printed rewarded and non-rewarded flowers were used in this study and differed only in their nectar guide color. Thus, white color, scent, and shape between offered flowers did not vary in the color experiment. On the one hand, this fact corresponds with the argument for short-distance attraction by the nectar guides (Tregrovsky & Pany, 2019) as it can be assumed that the bumblebees had to orient themselves close to the flowers to recognize the nectar guide color. On the other hand, the fact that the yellow color was learned relatively quickly without any floral scent stimuli or shape orientation and additionally being the most rewarding flower stage (Sauer, 2022) could indicate that the color is an honest signal and that the color of the nectar guides could therefore be of greater importance in the pollination strategy of *A. hippocastanum*.

The scent extracts did not show high similarity with the natural scent of *A. hippocastanum* flowers. The scent bouquet evaluation in this study showed that the extracts from

yellow vs. red flowers differ in the number of scent compounds compared with the measurements of the natural flowers. At least 45% of the yellow and 38% of the red scent compounds of *A. hippocastanum* flowers could be extracted compared to the measured compounds in Sauer (2022). Furthermore, all three scent compounds characteristic for the yellow (unk1873, unk1043, unk1185) and one (indole) out of two for the red scent phase (indole, methyl isovalerate) could be found in the volatile phase of the scent extracts (Table 2; Fig. 6-7). The absolute amount of unk1873 was significantly lower than in the natural yellow flowers. The other two substances are very similar to the natural yellow flowers in terms of their absolute quantity. In contrast to the natural red flowers, indole seemed to be poorly extracted. However, Burkle & Runyon (2017) argued that collecting and identifying VOCs has become easier with new technologies and modern techniques (e.g., the headspace sampling) offer an advanced picture of volatiles compared to older technologies (e.g., solvent extractions). In this study, the headspace method was not used to produce eluates for the scent experiments but to examine the extracts for their components. Wright & Smith (2004) assumed that the quality of a scent signal can affect the learning ability of a visiting pollinator. Considering this, it may be assumed that the scent extracts may have not exactly the same effect on the learning behavior of *B. terrestris* as the scent of natural flowers of *A. hippocastanum*. Furthermore, these results indicate that the use of scent extracts of the flowers of *A. hippocastanum* is not necessarily as straightforward as assumed and that future studies might either have to use natural flowers for their experiments or should consider different methods for scent extraction (e.g., eluate).

This study showed that bumblebees can perceive the scent extracts individually, and overall, a positive but not equally fast learning effect could be determined between the yellow and red scent control groups (Fig. 9-12). Interestingly, in the training period of the yellow scent control group, a mean of 75 approaches were sufficient to exceed already the 75% mark of choice frequencies for the right decision. Many more approaches were necessary to get close to 75% probability for choosing correctly in the red control group. When comparing with experiment 2 (yellow against red scent extract), the tested individuals never achieved 75% of the probability for choosing the correct choice even with a maximum of around 100 approaches. The better-extracted volatiles of the yellow scent phase could explain why all tested bumblebees were learning the yellow scent faster than the red.

Furthermore, this study shows that the learning success is faster in the color experiments than in the scent experiments. Bumblebees needed only a mean of 60 approaches to reach 100% probability for the correct decision during the training period (Fig. 16). The hypothesis that with a reward, olfactory signals can be learned faster than visual signals like in Wright & Schiestl (2009) and Menzel & Müller (1996) could not be confirmed in the present study. On the contrary, it appears that the color was learned faster. A reason for the slower increase in the likelihood of the correct choice during the training in the scent experiments could firstly be non-perfect scent extraction (see above) or the pre-training. During the latter, rewarded odorless artificial flowers were used to identify potential foragers. After the pre-training, the same artificial flowers were presented with an added/new scent during the training period. Thus, differences in the artificial flowers from unscented to scented were present. For example, Manning (1956) observed that foraging bumblebees avoid choosing dandelion (*Taraxacum officinale* L.) when adding a rose scent to them. Therefore, bees could also perceive changes in

the smell of flowers. In a different study, Wright & Schiestl (2009) showed that adding an aromatic compound to scentless flowers (which appears scentless to the human nose) such as borage and alkanet decreased landings by bees. However, they argued that bees could perceive even minor scent modifications. This aspect could have negatively affected the learning success because trained bumblebees were no longer rewarded with odorless artificial flowers but with scented.

Surprisingly, the tested bumblebees could not distinguish between the scent extracts of *A. hippocastanum* flowers, although each scent extract could be learned individually (Fig. 9-12). According to Wright & Smith (2004), bees can identify single compounds of a floral scent, even if the scent is very complex. However, at the beginning of the training period, bumblebees showed an innate preference for the yellow scent stage compared to red. Unlike natural flowers, the scent extract analysis showed no significant differences between the two scents (Fig. 14). This would explain why bumblebees could not successfully discriminate between the yellow and red scent extracts. Furthermore, it is noteworthy that indole is present in higher amounts in the natural red flowers than in the red extracts, indicating less success in extracting this compound. It could be assumed that indole has a more significant role in both flowering scent stages, and the differences in the amount of this compound in the scent extracts are responsible for *B. terrestris*' inability to distinguish between the yellow and red scent extract. This assumption would correspond with Martínez-Harms et al. (2018), who investigated different scent mixtures with different levels of indole and showed that honeybees can use indole to discriminate between different scents.

This study indicates a significant innate preference of *B. terrestris* for the yellow scent extract and a tendency to prefer the yellow color, but the sample size examined is too small to draw general conclusions. Assumptions, which relate to the innate scent or color preferences, may be made. For instance, Dötterl et al. (2019) argued that inexperienced naïve bumblebees are olfactory orientated and only with more experience, visual orientation is used more often. Similar observations were reported in the study by Dobson (1994), in which naïve bees were initially dependent on scent stimuli and only learned visually with more experience. Those studies support the assumption that the inexperience of bumblebees caused the scent preference in this study.

Regarding the lack of color preference at the beginning of the training period, it can be assumed that the color dots are too small to be seen from a distance and that bumblebees, therefore, must orient themselves by the white artificial flowers and then learn the color later. This likely agrees with the natural conditions as Focke (1889) hypothesized that the big white inflorescences of *A. hippocastanum* flowers are used for long-distance attraction. Likewise, Tregrovsky & Pany (2019) argued that the colored dots only serve short-distance attraction. Nevertheless, to answer whether the scent or the color of *A. hippocastanum* flowers is more important for the behavior of a bumblebee or whether the decision is triggered by the color alone could not be finally clarified in this study. Therefore, more experiments would be necessary.

Ideas for future studies: Finally, some suggestions for improvements for future studies on this topic. The methodology could be adapted in different ways. For example, experiments with actual flowers of *A. hippocastanum* (since the probability of offering the entire scent bouquet is higher) and better-extracted scent samples could be conducted. Alternatively, a punishment

for the non-rewarding choice could be added as an enhancement of the correct stimulus (Riveros & Gronenberg, 2009). As bees can learn high scent concentrations faster (Wright & Smith, 2004), it could also be considered to increase the amount of the offered scent in artificial flowers. Furthermore, the use of a second flight arena could be considered. During this study, the difficulty was to let only individual foragers into the experimental arena as many individuals also wanted to fly out. A second flight arena would allow to offer other individuals the opportunity for flight experiences and, therefore, prevent the experiment's traffic jam (seen in Gumbert, 2000). In this case, the experimental setup would be the same, except for two flight arenas instead of one, both separately connected to the hive chamber. The first arena would be for foragers and, therefore, for experimental runs, and the second for the entire colony (e.g., mating or flying). Complementing the setup with a second arena would allow better and faster regulation of foragers' bouts.

Conclusion

Bombus terrestris is a well-known pollinator of *A. hippocastanum*, whose nectar guide color and floral scent changes during anthesis. Behavioral experiments were conducted to test the influence of these floral traits on the bumblebee's pollinating behavior. The results show that the tested bumblebees likely use the nectar guides as an honest signal, whereas they cannot distinguish the floral scents. The scent extracts used in this study did not comprise all volatiles that occur in natural flowers. Thus, a more comprehensive understanding of the common horse chestnut's pollination systems requires future studies with natural flowers to determine whether also the scent serves as an honest signal.

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Appendix

First Setup/ Preliminary Experiments

The experimental design of the first setup was slightly different from the methodology that was finally used in this study. Preliminary experiments were conducted with natural flowers of *A. hippocastanum*. Ultimately, this phase lasted two weeks, as the flowers faded quickly. Considerations of the three steps (pre-training, training, test trial) were precisely the same as in experiments 1, 2, and 3 (but without airflow).

First Setup; Scent of Natural Flowers

Experiments were conducted with natural yellow and red flowers of *A. hippocastanum* to observe whether *B. terrestris* can associate the yellow flower scent with a reward (sucrose solution) and distinguish it from the red. Therefore, one yellow flower was placed in one of 5 roll edge glasses (Rollrandglas 30 x 19mm; volume= 3ml) and one red flower in one of five additional roll edge glasses. Each glass was covered with a white artificial flower and randomly re-distributed in the flight arena after each run. The yellow scented artificial flowers were rewarded with 30µl of a 30% sucrose solution and the red-scented flower with water as non-reward. After each fourth run, the flowers were replaced by a new one. For the test trial a new flower was placed in the edge glass again.

First Setup; Scent of Natural Flowers and Color of Artificial Flowers; Conflict

This part of the first setup aimed at identifying the most important stimulus for the bumblebee, i.e., whether the color or the scent of *A. hippocastanum* is more attractive. The training stage contained the yellow and red flowers as scent stimuli (see “First Setup; Scent of Natural Flowers”), the yellow and red color dots on the artificial flowers as a visual stimulus. Five yellow scented and colored artificial flowers were rewarded during training, and five red scented and colored non-rewarded. Bumblebees were trained to associate the yellow scented and colored flowers as a reward and tested whether they could do so in a non-rewarded

test trail. Furthermore, those two fragrances were exchanged in a second unrewarded test trial to create a conflict between color and scent and to be able to observe whether the visual or olfactory stimulus is more critical.

First Setup; Control Groups

For the yellow flower control group, five yellow scented artificial flowers (one flower in each glass) and five empty scentless artificial flowers were randomly distributed in the arena. In contrast, the red flower control group included five flowers in each glass. Since it seemed as if the bumblebees did not perceive the flower scent through the artificial flowers well, an airflow supply had to be added finally. This part was conducted with a few natural red flowers. Ultimately, the natural flowers withered quickly, and therefore extracts had to be made to continue the experiments with airflow.

First Setup; Results

The addition of natural *A. hippocastanum* flowers to the artificial flowers in preliminary experiments did not indicate perception of the two scents by the bumblebees. Neither one nor five flowers per roll edge glass showed any influence on the decision (Table 3; experiments= SNF, CGR). The conflict experiment (combination visual and olfactory stimuli) showed a preference for the yellow color and red scent in a non-rewarding test trial. Although the innate tendency does not differ from that in a non-rewarding test (Table 3; experiment= SNF and CAF). The bumblebee's learning behavior with respect to the two combinations can be attributed to the visual stimulus when assuming that the bees did not perceive both scents in this setup. An improvement could already be observed during training with airflow supply. The influence of the number of approaches has a more significant impact on the decision (Table 3; experiment= CGRA) than those without airflow; therefore, all further scent experiments were conducted with airflow supply.

Table 3; Results of the preliminary experiments. For statistical analysis of SNF (scent experiment with adding natural flowers), SNF and CAF (combination experiment with adding natural flowers and colored artificial flowers), and all three control groups (CGY= control group yellow; CGR= control group red; CGRA= control group red; natural flowers with airflow). The first five decisions in the first run were compared against the five decisions of the test trial using a Wilcoxon rank-sum test. With a binary logistic regression model, the influence of the number of approaches on the probability of making the correct choice was calculated. In a non-rewarded conflict test, the number of approaches was compared using Wilcoxon rank-sum test (C= yellow color with red scent against red color with yellow scent).

experiment	setup	amount of marked <i>B. terrestris</i>	amount of participated <i>B. terrestris</i>	Wilcoxon rank-sum test (p-value)	binary logistic regression model (p-value)
SNF	first setup	8	5	0.465	0.159
SNF and CAF	first setup	6	5	0.269	*** 0.001
C	first setup	6	5	*0.019	
CGY	first setup	1	1	low sample size	low sample size
CGR	first setup	3	3	0.103	0.897
CGRA	experimental design	4	4	0.099	*** 0.001

*<0.05; **<0.01; ***<<0.001

Additional Graphs

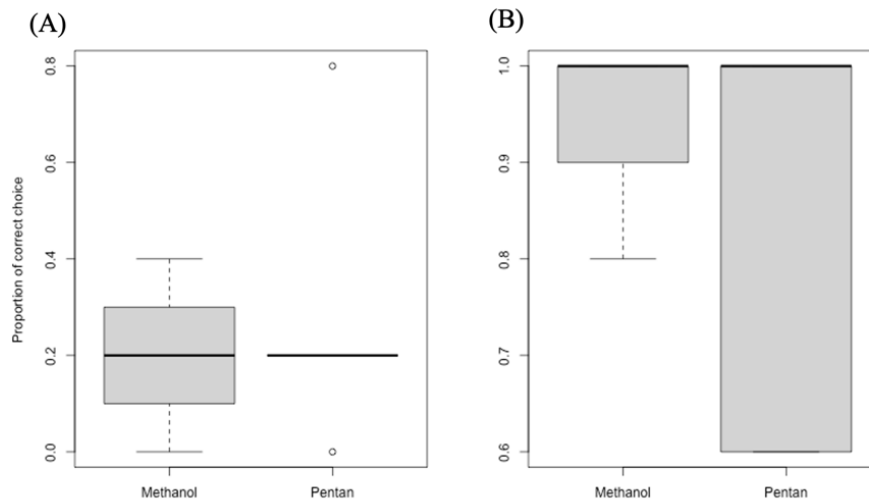


Figure 19; The proportion of correct choices were calculated. (A) Comparison between the first five approaches in the first run of yellow scent extracts with methanol and pentane. (B) Comparison between the first five approaches in the test trial. ($n_{\text{methanol}}=3$; $n_{\text{pentane}}=6$).

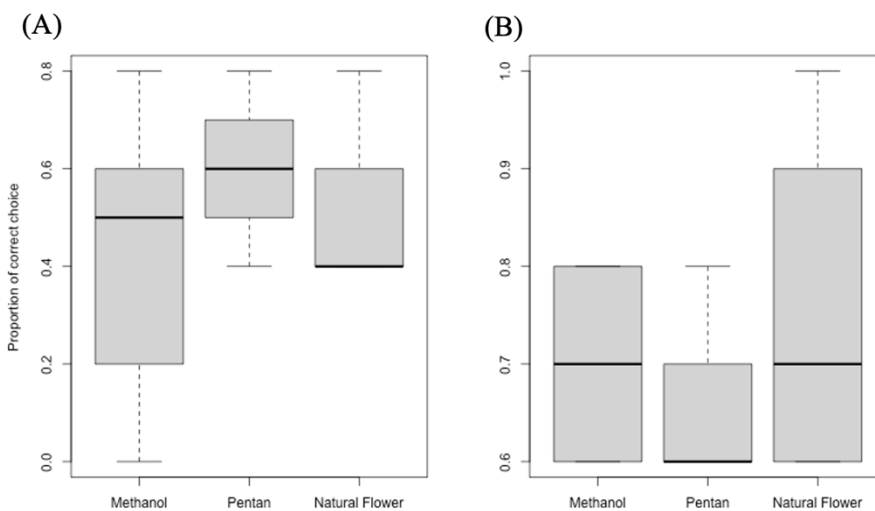


Figure 20; The proportion of correct choices were calculated. (A) Comparison between the first five approaches in the first run of red scent extracts with methanol and pentane against the natural flower scent. (B) Comparison between the first five approaches in the test-trial. ($n_{\text{methanol}}=6$; $n_{\text{pentane}}=4$; $n_{\text{natural flower}}=4$).

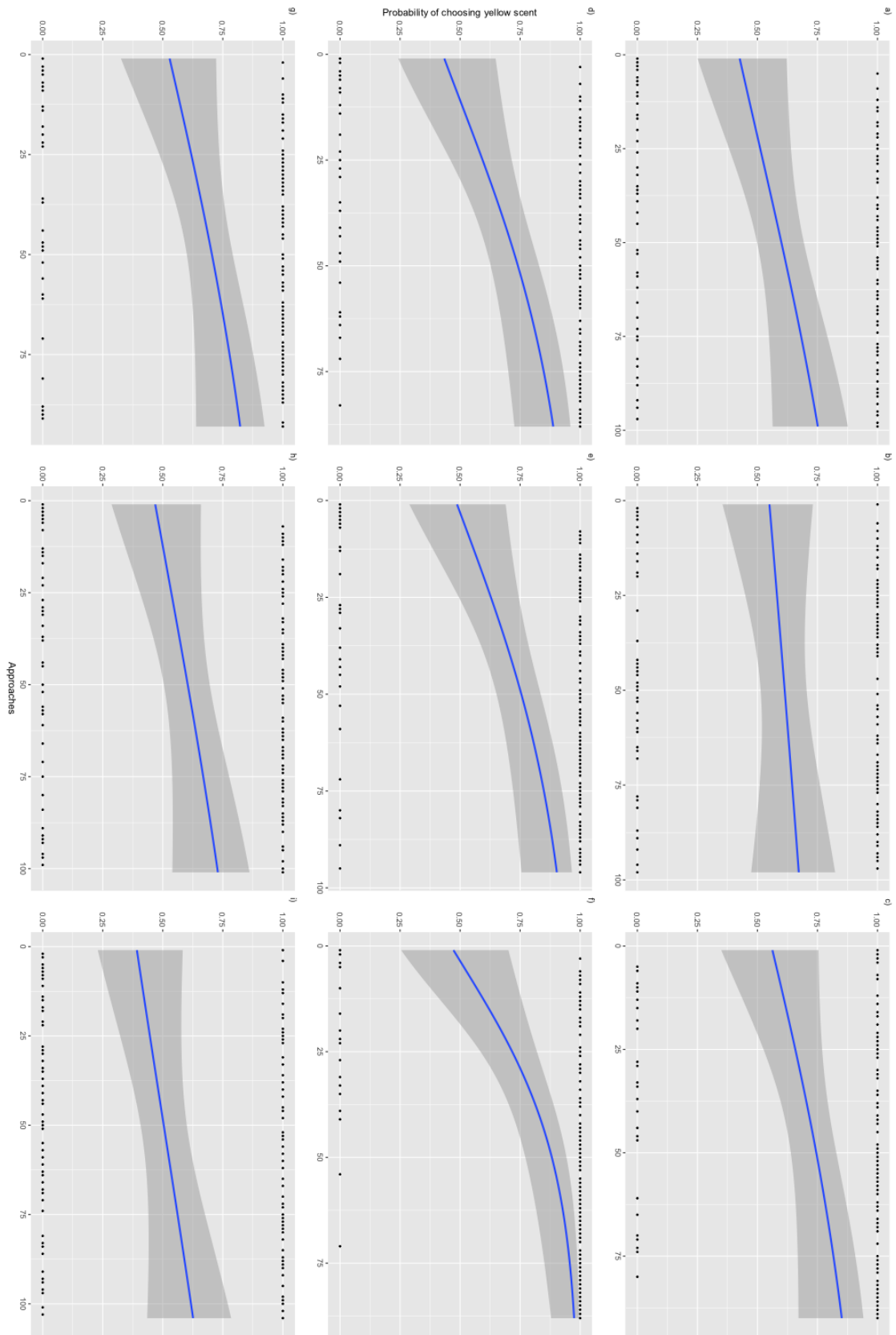


Figure 21; Learning period of yellow-phase scent extracts of *A. hippocastanum* by every tested *B. terrestris* (a-i). The influence of the number of approaches on the probability for choosing the yellow scent is shown.

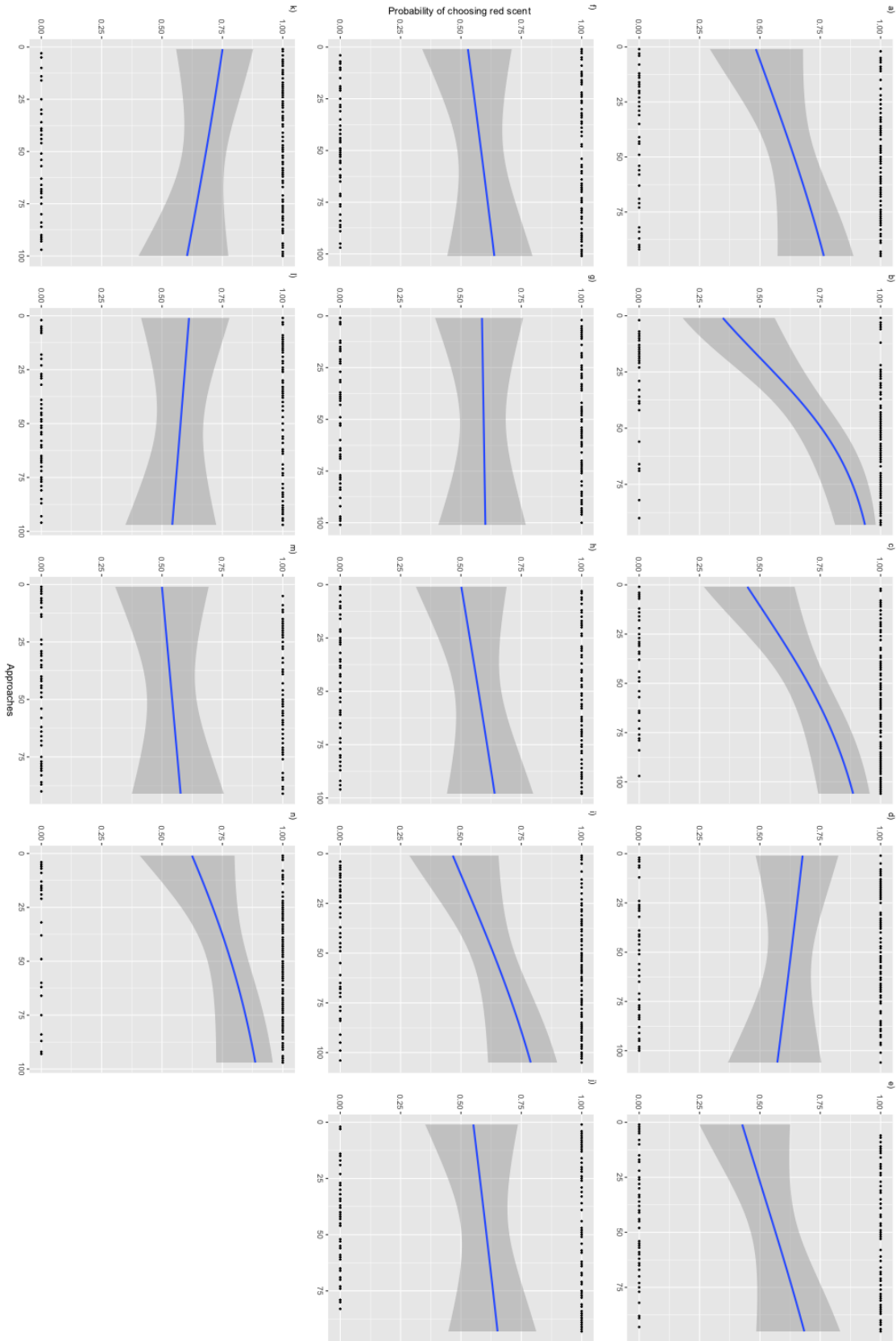


Figure 22; Learning period of red-phase scent extracts of *A. hippocastanum* by every tested *B. terrestris* (a-n). The influence of the number of approaches on the probability for choosing the red scent is shown.

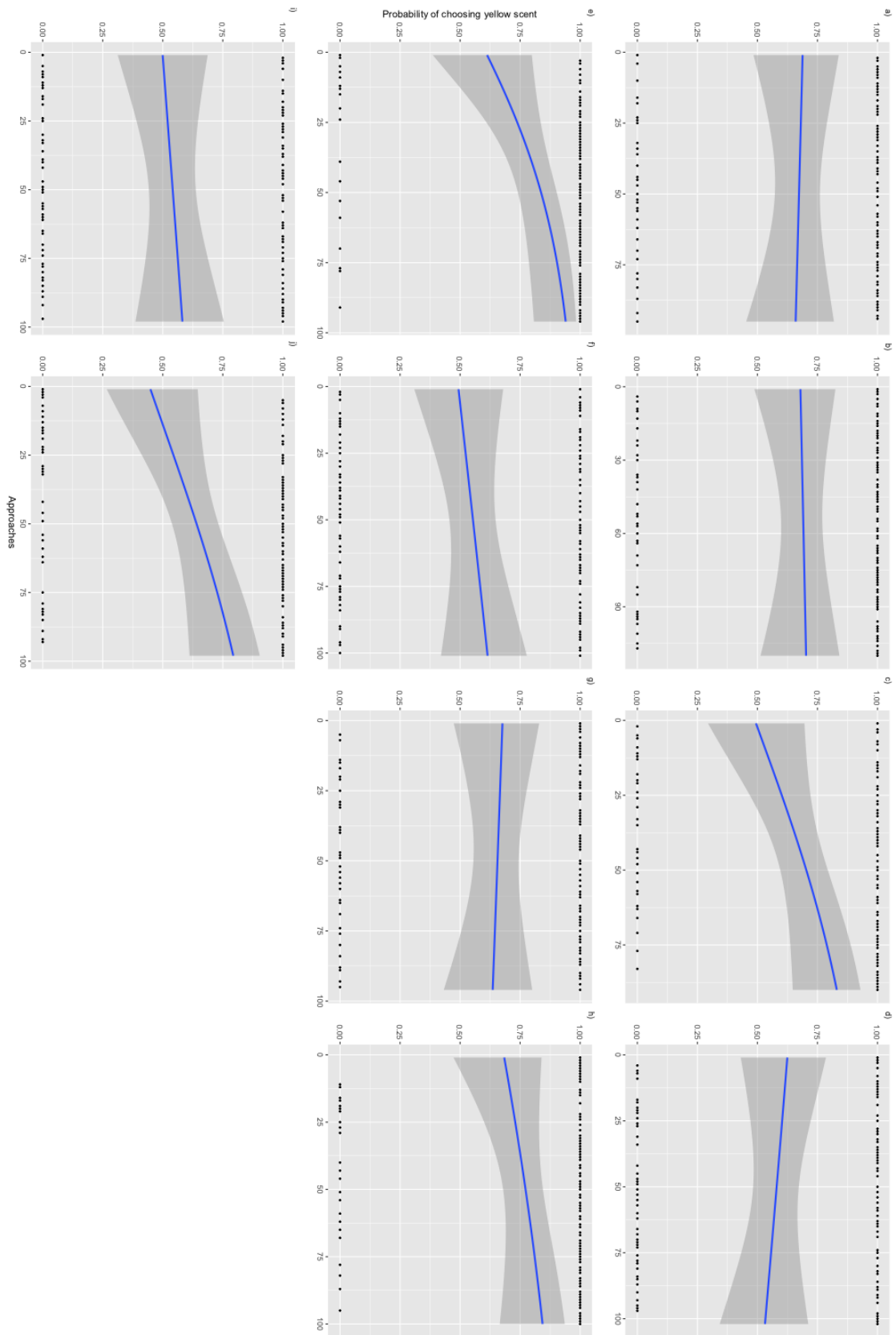


Figure 23; Learning period of yellow-phase scent extracts against red-phase scent extracts of *A. hippocastanum* by every tested *B. terrestris* (a-j). The influence of the number of approaches on the probability for choosing the yellow scent is shown.

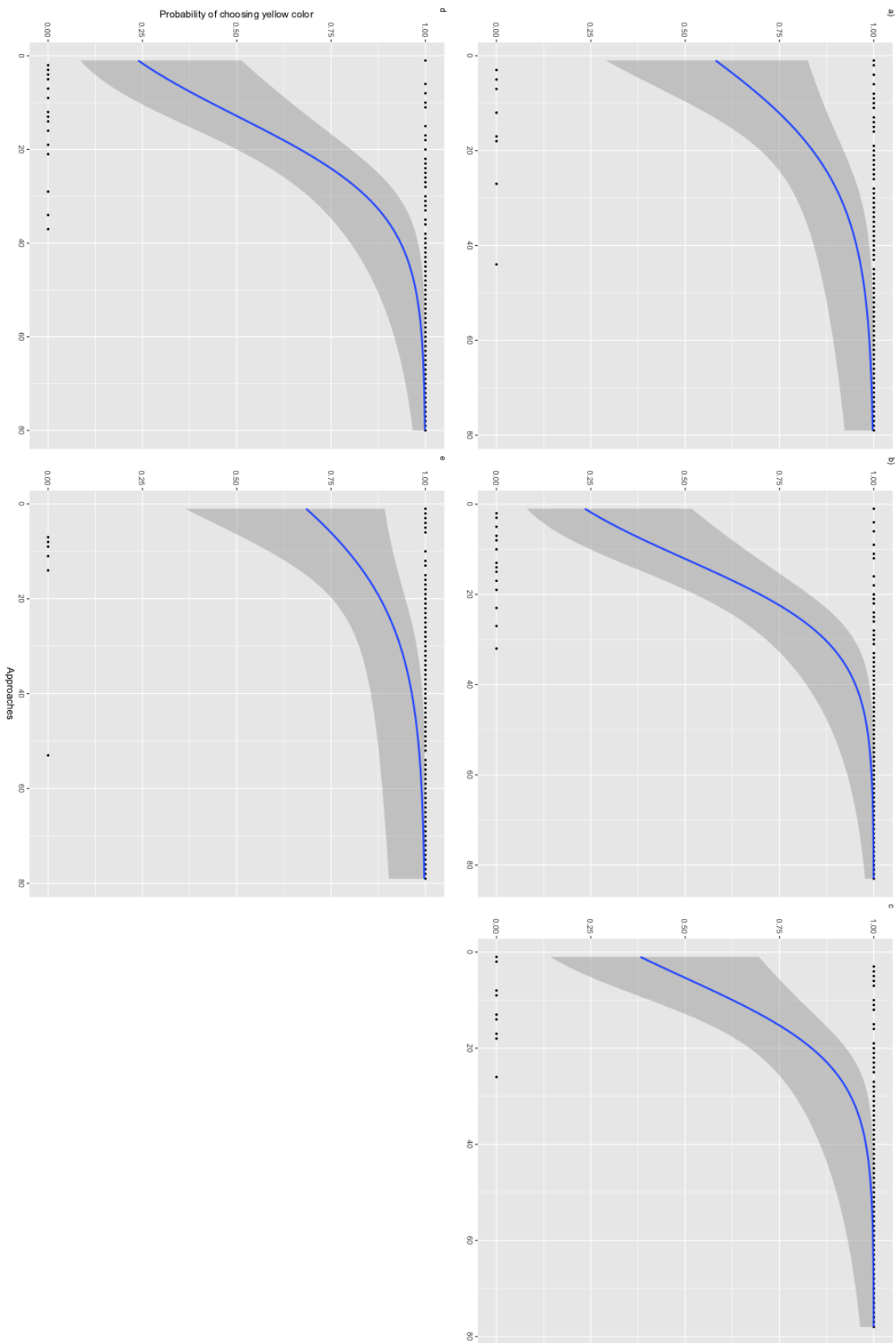


Figure 24; Learning period of the yellow phase against the red-phase color of *A. hippocastanum* by every tested *B. terrestris* (a-d). The influence of the number of approaches on the probability for choosing the yellow color is shown.

Scent Compounds

Table 4; Combined scent measurements of the natural *A. hippocastanum* flowers from Sauer 2022 (grey background) and the scent extract of *A. hippocastanum* flowers from Žilić 2022 (white background). The table shows which scent components were not extracted during the production of the extracts.

RI	Name	m/z	Yellow	Yellow Sd.	Red	Red Sd.
674	1-Pyrroline	41,69,42,68,39	3.984	8.297	1.402	2.174
773	Methyl isovalerate	74,57,59,85,101	0.046	0.16	0.648	1.028
881	2-Methyl-2-butenic acid	55,100,39,54,82	0.544	0.995	1.161	3.790
910	unk910	58,100,55,43,71	0.303	0.765	0.095	0.231
943	unk943	84,42,55,56,39	1.203	2.743	---	---
980	unk980	101,73,43,144,55	0.136	0.19	0.086	0.151
968	1-Heptanol	56,70,55,41,43	4.076	5.735	3.343	2.934
998	unk998	116,89,42,117,41	0.213	0.449	0.134	0.349
1021	unk1021	80,123,53,79,81	0.023	0.038	0.006	0.009
1035	unk1035	43, 114	0.099	0.291	0.142	0.275
1043	unk1043	99,44,98,42,41	13.347	14.508	4.786	5.605
1052	2,5-Dimethyl-4-hydroxy-3(2H)-furanone	43,128,57,85,45	1.657	3.159	1.105	2.103
1078	(Z)-Linalool oxide furanoid	59,94,93,68,111	4.183	7.265	6.114	7.982
1093	(E)-Linalool oxide furanoid	59,94,55,93,43	10.860	19.531	11.679	14.826
1119	2-Phenylethanol	91, 92, 122, 65	5.114	4.587	1.829	3.324
1137	unk1137	43,102,56,57,44	0.171	0.31	0.043	0.078
1145	Phenylacetonitrile	117, 90, 116, 89, 51	4.184	13.697	1.589	5.63
1165	unk1165	57,73,58,44,86	0.811	2.035	0.213	0.349
1172	1-Acetyl-pyrrolidine	43,70,113,85,55	0.075	0.3	0.053	0.207
1172	unk1172	84,94,114,68	0.452	0.851	0.011	0.037
1176	(E)-Linalool oxide pyranoid	68,94,59,67,43	0.806	1.285	1.013	2.193
1185	unk1185	57,97,68,109,82	37.217	34.139	49.751	21.102
1218	4-Vinylphenol	120,91,119,65,39	0.492	0.964	0.356	0.583
1227	2-Aminobenzaldehyd	93,94,121,66,65	0.008	0.025	2.374	3.123
1304	Indole *	117,90,89,63,58	7.467	15245	8.468	12.312
1309	1-Nitro-2-phenylethane	104,105,77,79,103	0.253	1.014	0.325	1.258
1313	(Z)-Methyl cinnamate	131,103,162,142,161	---	---	0.216	0.487
1323	2-Methoxy-4-vinylphenol	150,135,107,77,73	0.692	0.809	0.613	0.833
1328	unk1328	147,119,146,90,64	0.009	0.030	---	---
1355	Methyl anthranilate	119,151,92,120,65	0.229	0.77	0.352	0.678
1365	α -Cubebene	161,105,119,91,81	0.755	2.015	0.057	0.198

1375	unk1375	43,81,107,55,58	---	---	0.004	0.014
1377	unk1377	84,41,56,143	0.019	0.077	0.019	0.052
1388	unk1388	140,69,97,57,56	0.071	0.166	0.025	0.064
1394	(E)-Methyl cinnamate	131,103,162,77,161	0.377	1.238	0.355	0.652
1406	β -Bourbonene	81,123,80,161,91	1.078	2.115	1.636	3.045
1421	2-(Dimethylamino)benzaldehyde	93,121,66,65,149	6.835	6.573	6.801	6.199
1425	unk1425	57,73,86,58,44	0.465	0.880	---	---
1441	unkST1441	161,120,105,79,81	1.645	4.528	0.135	0.236
1452	(E)-Cinnamyl acetate	115,43,133,134,92	0.010	0.035	5.126	13.597
1452	unkST1452	161,105,91,119,81	0.293	0.713	---	---
1478	γ -Muuroloene	161,81,91,93,105	0.004	0.015	0.166	0.643
1487	unk1487	161,105,162,204,119	0.465	0.863	0.817	3.057
1487	unk1487	161,105,162,204,119	2.521	4.821	0.132	0.446
1505	unkST1505	161,105,119,91,133	0.303	0.803	0.220	0.641
1518	unk1518	161,105,91,204,119	0.696	1.136	0.081	0.205
1525	unk1525	161,81,120,91,105	---	---	0.003	0.009
1531	unk1531	84,112,155, 70,43	0.177	0.573	0.038	0.083
1536	unkST1536	161,105,91,119,204	0.093	0.253	0.103	0.358
1542	unkST1542	161,105,134,119,204	0.023	0.057	---	---
1543	unk1543	100,70,43,113,41	0.058	0.19	0.036	0.082
1545	unkST1545	159,160,202,131,144	0.022	0.06	0.024	0.094
1549	unk1549	111,177,83,55,110	0.126	0.298	0.007	0.014
1545	unkST1545	159,160,202,131,144	1.638	3.111	0.052	0.182
1550	unk1550	102,174,76,51,50	---	---	0.042	0.145
1567	Calacorene	157,142,200,141,156	0.371	0.839	0.019	0.065
1588	unk1588	184,80,57,43,157	0.000	---	0.024	0.064
1592	Vetivazulene	170,155,198,156,183	0.268	0.968	0.09	0.128
1619	unk1619	87,73,85,74,43	---	---	0.007	0.024
1623	Salvial-4(14)-en-1-one	123,81,43,124,93	0.396	0.812	0.086	0.176
1664	unk1664	117,160,83,43,91	0.148	0.491	---	---
1832	unk1832	154,70,71,125,41	0.121	0.293	0.051	0.175
1873	unk1873	57,84,127,43,56	0.299	0.762	---	---
1965	unk1965	70,194,166,94,138	2.277	7.023	0.031	0.106