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The impact of sex and reproductive status on the preference for floral bouquets in the hawkmoth, *Manduca sexta*.

Bachelor thesis

to obtain the degree of a

Bachelor of Science Biology

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List of Abbreviations

DCM	Dichloromethane
GC	Gas chromatography
GC-MS	Gas chromatography – mass spectrometry
MPI - CE	Max-Planck- Institute for Chemical Ecology
RH	relative humidity

Abstract

Manduca sexta is a nocturnal hawkmoth in the southwestern North America, which feeds mainly on *Agave palmeri* and *Datura wrightii*, which can also be used as an oviposition site. Olfactory signals, such as a strong scent, help *M. sexta* to locate host plants. While males can select potential sources of nectar primarily for their quality, as their own feeding is paramount, in females this focus shifts from feeding to oviposition after mating. In this thesis we want to investigate if the sex and reproductive status have an impact on the preference for floral bouquets. For this examination we tested the attraction of *A. palmeri* and *D. wrightii* to males, virgin females and mated females with a two-choice experiment (floral bouquets *versus* solvent) in a wind tunnel. Naïve moths who have no experience with odours responded to the floral bouquets with proboscis, tarsal or abdominal contact on the filter papers. We analysed the first contact, the number of moths contacting the filter papers and the net duration of these contacts. In all groups for both floral bouquets the first contact was shown on the floral-scented filter paper, which provides evidence for odour-guided behaviour in the hawkmoth *M. sexta*. The ratio of moths contacting the scented filter paper with the proboscis, was higher for the floral bouquet of *D. wrightii* than for *A. palmeri*, in all groups. However, the net duration of proboscis contact did not indicate any preference within the groups for any of the floral bouquets. Oviposition behaviour, which was only observed for mated females and one virgin female, was restricted for the floral bouquet of *D. wrightii*.

Zusammenfassung

Der im Süden Nordamerikas beheimatete, nachtaktive Schwärmer *Manduca sexta* ist nektarivor und ernährt sich hauptsächlich vom Nektar der *Agave palmeri* und *Datura wrightii*, welche zusätzlich noch als Eiablagepflanze genutzt werden kann. Olfaktorische Signale, wie zum Beispiel ein starker Blütenduft, helfen *M. sexta* diese potentiellen Wirtspflanzen zu lokalisieren. Männchen wählen potentielle Nektarquellen hauptsächlich nach ihrer Qualität aus, da deren eigene Ernährung im Vordergrund steht. Bei Weibchen wird der Fokus der eigenen Ernährung nach der Paarung auf die Auswahl einer geeigneten Wirtspflanze für die Eiablage verschoben. Im Rahmen dieser Bachelorarbeit soll untersucht werden, ob sich das Geschlecht und der Fortpflanzungsstatus der Motten auf die Präferenz von Wirtspflanzen und deren Blütendüften auswirkt. Im Windtunnel wird die Attraktivität der Blütendüfte von *A. palmeri* und *D. wrightii* für Männchen, jungfräuliche Weibchen und verpaarte Weibchen getestet. Dafür werden je einer der untersuchten Blütendüfte und das Lösungsmittel auf jeweils ein Filterpapier pipettiert. Naive Motten, die noch keine Erfahrung mit Düften haben, reagierten mit Proboscis-, Tarsal- oder Abdominal-Kontakt am Filterpapier auf die dargebotenen Blütendüfte bzw. das Lösungsmittel. Das gezeigte Verhalten am Filterpapier wurde bezüglich der Art des ersten Kontaktes, der Anzahl an Kontakten und der Dauer der Kontakte untersucht. Sowohl Männchen, jungfräuliche Weibchen als auch verpaarte Weibchen zeigten ihren ersten Kontakt am Filterpapier mit Blütenduft, wodurch geruchsgeleitetes Verhalten belegt werden kann. In allen Gruppen war die Anzahl der Kontakte am Filterpapier mit dem Blütenduft von *D. wrightii* höher als dem von *A. palmeri*. Bei der Nettodauer der Kontakte ließen sich jedoch keine Unterschiede bzw. keine Präferenz zwischen den Gruppen und Blütendüften feststellen. Eiablage-Verhalten konnte nur bei verpaarten Weibchen und einem jungfräulichen Weibchen beobachtet werden und ist auf den Duft von *D. wrightii* beschränkt.

1. Introduction

The study of interactions between animals and their environment is essential to clarify ecological issues and the importance of behaviours. First investigations have shown that Lepidoptera, more specifically moths, show different behaviours towards various odours and floral bouquets. Of great importance are olfactory information's, that can help moths to find possible nectar sources and oviposition sites. Interesting is the question of whether sexual preferences towards floral bouquets exist and whether these differences are reflected in the olfactory system. For mated females the role of olfactory information is very important as they must find a good host plant for their eggs. Therefore, it is very interesting to find out, for example, if mated females have specialized receptors to find good oviposition sites (Reisenman and Riffell, 2015). Preliminary results indicate that certain neurons are involved in female-specific behaviours such as oviposition (King et al., 2000; Reisenman et al., 2004). However, before this question can be resolved, behavioural tests are needed to find out if different plant preferences can be found in males, virgin females and mated females.

1.1 *Manduca sexta* as model organism

Manduca sexta is a nocturnal insect of the family Sphingidae and is distributed from South America to North America (Madden et al., 1945). Animals of both sexes have six yellow stripes on the abdomen and brown pattern wings with an expansion from 76 to 127 mm (Madden et al., 1945). Male *M. sexta* are sexually dimorphic, having larger antennas and a smaller body. Due to the short life cycle (30 – 50 days) and easily accessible and relatively large nervous system, *M. sexta* is a common model organism. *M. sexta* is a specialized nectarivorous insect that feeds almost exclusively on plants from the family Solanaceae (Madden et al., 1945; Yamamoto and Fraenkel, 1960). In the search for potential nectar sources, moths shows evidence for odour guided behaviour (zig-zag route), which divides in two phases (Faegri and Van Der Pijl, 2013). Moths downwind of a flower approach that flower, and reach the maximum odour concentration at the flower and may overpass it, at which point they turn back exhibiting a looping search pattern (Faegri and Van Der Pijl, 2013). In this way, they approach a potential source of nectar, but only land if the scent and visual stimuli match (Ramaswamy, 1988). Once they have identified a floral host, *M. sexta* can slow above the flower and hover to align its proboscis while feeding.

M. sexta prefers white, tubular, night-blooming flowers with a high odour emission (Dobson, 2006; Raguso and Willis, 2002; Sparks, 1970). Southwestern North America is home to at least 20 plants that meet these criteria and in addition have a special flower shape for long-tongued nocturnal hawkmoths as pollinators (Grant, 1983; Raguso and Willis, 2002). Pollen-load analysis revealed that *M. sexta* moths of both sexes mainly feed on *Agave palmeri* and *Datura wrightii* (Alarcon et al., 2008). During most of the summer, *M. sexta* feeds on the typically "bat-adapted" *A. palmeri*, and in the peak bloom of *D. wrightii*, hawkmoths transition to this classic hawkmoth pollinated plant (Riffell et al., 2008a).

1.2 *Agave palmeri* – Composition of the floral bouquet and interaction with *M. sexta*

Agave palmeri (Agavaceae) is spread on upper mountains and slopes of hills in North America (Alarcon et al., 2008; Bronstein et al., 2009). During varying degrees of intensity blooming periods from June to September several flowers are formed, which remain open for up to 6 days (Scott, 2004; Slauson, 2000). Bright-yellow flowers, united at the receptacle into a tube (Cronquist and Garden, 1972), stand at 2 – 5 m height above the ground with several umbels and open flowers at one time (Alarcon et al., 2008; Alarcon et al., 2010). The strong rotten fruit odour mostly attracts bats, but also hawkmoths (Alarcon et al., 2008; Slauson, 2000). *M. sexta* is an occasional pollinator for *A. palmeri* (Howell and Roth, 1981), as this species is used exclusively as a nectar source (Alarcon et al., 2008; Riffell et al., 2008a). *A. palmeri* provides 616 µl of hexose- rich (12%) nectar, which sustain moths for longer durations as *D. wrightii* (Riffell et al., 2008a).

The floral bouquet of *A. palmeri* includes more than 60 compounds (Riffell et al., 2009b), most of which belong to the group of aliphatic esters (Riffell and Alarcon, 2013). Previous experiments have shown that the scent of *A. palmeri* can be mimicked from a mixture of butyl butyrate, myrcene, ethyltiglate, ethyl sorbate, benzaldehyde and propyl valerate (Riffell et al., 2009b). The most abundant ester in this floral bouquet is ethyl sorbate (Raguso, 2004), which alone elicits feeding behaviour in naïve hawkmoths (Bisch-Knaden et al., 2018). Therefore, ethyl sorbate is considered as main component in our experiment and composes the basis for following quantifications.

1.3 *Datura wrightii* – Composition of the floral bouquet and interaction with *M. sexta*

Datura wrightii (Solanaceae) is patchily spread throughout the low land areas in North America (Alarcon et al., 2008; Bronstein et al., 2009; Grant and Grant, 1979). During extended blooming periods from spring to autumn (Elle and Hare, 2000; Raguso et al., 2003), 50 – 100 white trumpet shaped flowers are produced each night (Baker, 1961; Raguso et al., 2003). This special flower shape is very well adapted to the mouthparts of the most frequent nocturnal visitor *M. sexta* (Alarcon et al., 2008; Raguso et al., 2003). Due to the sucrose-rich (22% of 56 µl nectar) and energetic nectar (Raguso et al., 2003; Riffell et al., 2008a), *D. wrightii* is a very attractive forage plant for *M. sexta*, but also serves for oviposition and as host plant for its larvae (Mechaber and Hildebrand, 2000; Mira and Bernays, 2002; Spathe et al., 2013). *D. wrightii* is a self-compatible plant (Elle and Hare, 2000), yet better fruits can arise if the plant was cross-pollinated (Raguso et al., 2003). Therefore, this insect-plant interaction is often described as non-obligatory mutualistic pollinator-herbivore association (Bronstein et al., 2009).

D. wrightii flowers produce a floral bouquet composed of more than 60 individual odorants, yet the scent can still differ within species (Raguso et al., 2003). Most odorants come from the groups of terpenoids and aromatics (Riffell et al., 2008a). This floral bouquet is effectively attractive to naïve *M. sexta* and elicits feeding behaviour (Desai and Raguso, 2001). It has already been shown that the complete floral bouquet of *D. wrightii* can be imitated by the three substances (±) linalool, benzaldehyde and benzyl alcohol and also eliciting feeding behaviour in naïve *M. sexta* moths of both sexes (Riffell et al., 2009a). Further experiments have shown that benzyl alcohol alone is sufficient to eliciting both proboscis and tarsal contact with a curled abdomen in naïve hawkmoths (Bisch-Knaden et al., 2018). Based on these findings, benzyl alcohol is considered as a main component in our experiments and composes the basis for following quantifications.

1.4 Aim of this thesis and preliminary thoughts

We seek to determine if sex or reproductive status of *M. sexta* determine their preference for differently scented *A. palmeri* or *D. wrightii* flowers, parsing both feeding and reproductive uses.

We hypothesize that regardless of sex or mated status, *M. sexta* will try to feed from both floral bouquets (Madden et al., 1945). Males might be expected to choose their nectar source only based on the quality of that source according to nectar volume (Alarcon et al., 2010), but other considerations such as which plants are frequented by egg laying females could influence their decisions (Alarcon et al., 2010; Reisenman et al., 2009). Further they are not expected to show sexual behaviour (Alarcon et al., 2010). It has already been shown that males feed longer and more frequently from *D. wrightii*, but also learn to use *A. palmeri* as a potential nectar source (Alarcon et al., 2010; Riffell et al., 2008a; Riffell et al., 2009a). Virgin females are also expected to choose their nectar sources based on the quality of that source. Research results have already shown that females feed longer and more frequently on *A. palmeri* (Reisenman et al., 2009), but laboratory naïve moths prefer *D. wrightii* over *A. palmeri* (Alarcon et al., 2010). Mated females may be expected to prefer *D. wrightii* to *A. palmeri,* as they are able to oviposit on former (Spathe et al., 2013). Findings in other Lepidopteran species showed that mated females change their preference from feeding to oviposition after mating (Saveer et al., 2012). Nevertheless, it could be shown that feeding increase the rate of oviposition wherefore mated females should try to feed from both *A. palmeri* and *D. wrightii* (Madden et al., 1945).

We want to analyse three different behaviours of the moths in response to these two different plants to determine if they serve different ecological niches for females *versus* male *M. sexta*: proboscis contact indicative of successful feeding, tarsi contact with a straight abdomen indicating chemosensory discrimination and tarsi contact with a curled abdomen indicating oviposition behaviour.

2. Materials and Methods

2.1 Breeding and handling of the study species *M. sexta*

M. sexta larvae were reared in the laboratory on an artificial diet (Grosse-Wilde et al., 2011). Wandering fifth instar larvae were separated individually and kept in a climate chamber (25 °C, 70 % relative humidity [RH]) until one week before hatching. Pupae and adults were kept in sex-separated climate chambers under a reversed light cycle (8 hr dark, 16 hr light). On the third day after hatching all moths were tested in the wind tunnel (Mechaber et al., 2002). They were starved, virgin and had no experience with plant volatiles. Moths were mated in a mesh cage (76 cm x 42 cm x 42 cm) containing two males for every female. Females were two days old, while males in the mating cage were older than three days and were used exclusively for mating assays. Before the mating assay was performed, the male abdomen was dipped in pink fluorescent powder. Mating success was confirmed when the females had a pink circle around their genital organs the next day.

2.2 Collecting odour

Since only the floral bouquet of *A. palmeri* and *D. wrightii* should be tested, first the odour must be collected. The floral bouquet of *A. palmeri* was collected in 08/2017 by Dr. Bisch-Knaden in Arizona. *D. wrightii* plants were bred in the greenhouse (23-25 °C, 50-70 % RH, Philips Sun-T Agro 400 W Na vapor bulbs, 350-500 µmol/m^2/s photosynthetic photon flux at plant level) of the Max-Planck-Institute for Chemical Ecology (MPI - CE) under a reversed light cycle (8hr dark, 16hr light). Therefore, the odour collecting could take place on-site.

With aid of an oven bag (Figure 1 No. 6; Toppits, Cofresco Frischhalteprodukte GmbH & Co. KG) the opened *D. wrightii* flower was carefully bagged. The experimental setup includes flow meter (Key Instruments, flow rate 0.1 l/min till 1 l/min, Serie FR2000) to pump and suck compressed air into the oven bag. The flow meter for pumping compressed air was set to a flow rate of 0.4 l/min (Figure 1 No.1). Trough two thin Teflon tubes (Figure 1 No. 2 & 4; Jenpneumatik TFL 4 x 2 mm), connected by a charcoal filter (Figure 1 No. 3; self-made in the MPI – CE workshop), compressed air was fed into the oven bag at the lower end. With aid of a Velcro fastener (Figure 1 No. 5) the oven bag was closed near the receptacle. A glass wool filter

(volatilecollectiontrap.com, VCT-1/4-3-POR-Q25MG) was inserted into a Peak (Jenpneumatik, self-made in the MPI – CE workshop) and positioned directly over the open flower. The fragrant headspace air was drawn through the filter via a thick silicone tube (Figure 1 No. 8; Sang-A Pneumatic CO, LTD, Size 6x4). Therefore, the pulling air flow meter was set to a flow rate of 0.3 l/min (Figure 1 No. 9). The upper end of the oven bag was closed with a Velcro fastener (Figure 1 No. 7). At around 11 am the odour collecting began and continued for 6 h. Trapped volatiles were eluted with 400 µl Dichloromethane (DCM; Carl Roth GmbH + Co.KG, CAS: 75-09-2) and stored in Vials (Macherey-Nagel GmbH & Co.KG, 1,5 ml) at -20 °C until analysis. Overall, the scent was collected from nine different flowers
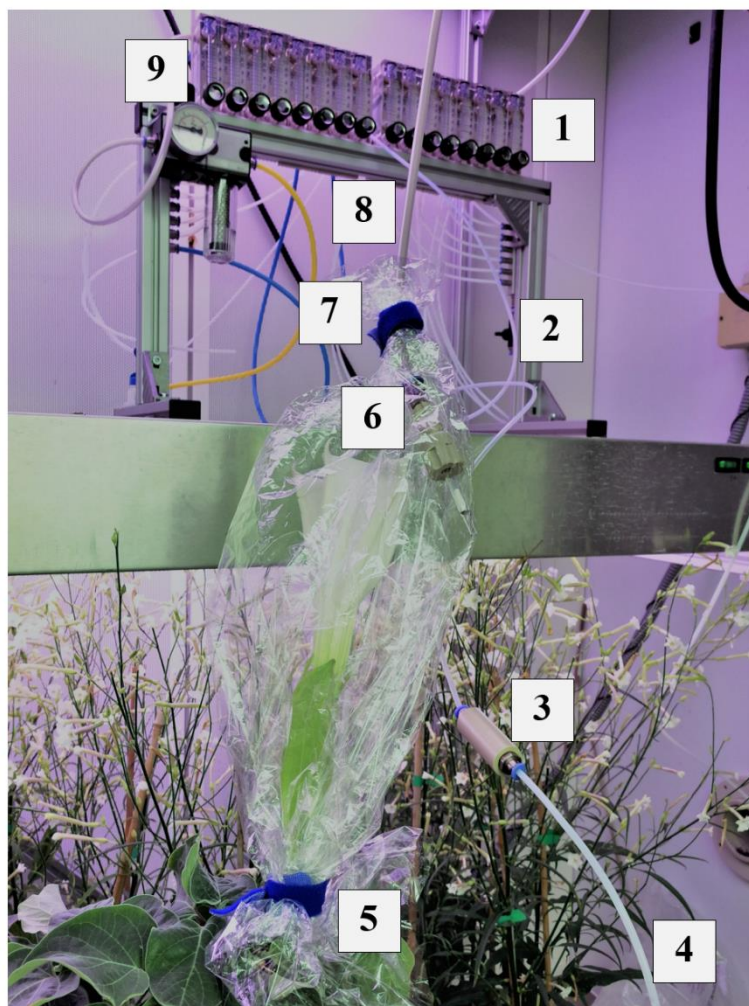

Figure 1: Experimental setup for the odour collecting of *D. wrightii*

2.3 Gas chromatography – mass spectrometry (GC – MS) analysis of the collected odours

The volatile samples were analysed using an Agilent 7890A gas chromatograph (Agilent Technologies) running in splitless mode. The GC was connected to an Agilent 5975C mass spectrometer (electron impact mode, 70 eV, ion source: 230 °C, quadrupole: 150 °C, mass scan range: 33 – 350 u; Agilent Technologies) with a polar column (HP-INNOWAX, 30 m length, 0.25 mm ID, 0.25 µm film thickness; J & W Scientific) under constant helium flow (1.1 ml/min). For the analysis 1 µl of each odour sample was used and DCM served as solvent. The initial oven temperature was 40 °C for 3 min, followed by an increasingly heating gradient of 5 °C/min up to 260 °C which was held for 15 min. The peaks of the Chromatogram, were identified by using the NIST MS Search 2.2 spectral library (Agilent Technologies, Santa Clara, CA, US) and verified with standards of the MPI – CE spectral library.

2.4 Quantification of ethyl sorbate and benzyl alcohol in the floral odour of *A. palmeri* and *D. wrightii*

The abundance of the main compound emitted by flowers of *A. palmeri* and *D. wrightii* was analysed using ethyl sorbate (Sigma-Aldrich Chemie GmbH, CAS: 100-51-6) and benzyl alcohol (Sigma-Aldrich Chemie GmbH, CAS: 2396-84-1). We tested these compounds at 6 of a $1:10^2$ concentrations in decadic steps ranging from 1 ng to 100 ng to create a calibration curve (Excel 2016, Microsoft Corporation, Redmond, WA, US). Using this calibration curve, we were able to determine, the concentration of both compounds in our odour collection. In previous wind tunnel experiments (Bisch-Knaden et al., 2018), 6 µl of a $1:10^2$ dilution of these compounds were shown to be attractive feeding cues. However, as the resources of the *A. palmeri* odour were limited, we used a 10-fold lower concentration of both flower bouquets and tested a sample volume that was equivalent to a concentration of the respective main compound in 6 µl of a $1:10^3$ dilution (*A. palmeri* 5.7 µg ethyl sorbate, *D. wrightii* 6.24 µg benzyl alcohol).

2.5 Experimental procedure in the wind tunnel

The floral bouquet of *A. palmeri* and *D. wrightii* was tested for its impact on the sex and reproductive status in the hawkmoth *M. sexta*. In a two-choice experiment, moths could choose between *A. palmeri*- or *D. wrightii*-scented filter papers *versus* the solvent alone. For this purpose, three experimental groups of moths were formed (n = 162 moths in total; n = 27 moths per group and floral bouquet): i) males, ii) virgin females and iii) mated females.

At least one hour before the experiment, individual moths were transferred in mesh cages (13 x 14 cm) to an acclimatization chamber (25 °C; 70 % RH; 0.3 lux). Two acrylic glass poles (40 cm high) were put upright and 40 cm apart from each other at the upwind end of the wind tunnel (Figure 2; Plexiglas: L x H x W: 2.5 m x 0.9 m x 0.9 m; 70 % RH; 0.3 lux; wind speed 40 cm/s). Firstly, an individual moth was put on a platform (40 cm high) on the downwind end of the wind tunnel. The diluted odorant and the solvent DCM were each pipetted on a round filter paper (diameter: 3.7 cm) with 10 µl mineral oil (Carl Roth GmbH + Co.KG, CAS: 8042-47-5) on top. The filter papers were prepared in the same room as the wind tunnel to keep the odour as strong as possible. The specific volume of the odour is depending on the concentration of ethyl sorbat in *A. palmeri* and benzyl alcohol in *D. wrightii*. When the filter papers were clipped onto the acrylic poles the mesh cage was opened. Some moths flew right away, and others took up to two minutes with wing fanning. The moths could fly for three minutes inside wind tunnel. During this time, their behaviour at the target was filmed (Sony Handycam DCR-SR35E, night shot mode). The duration and type of contact of each moth was analysed. After each trial the filter papers were renewed, and the position of the odour was changed frequently. All moths were only used once and after the experiment they were all killed by putting them into the freezer.
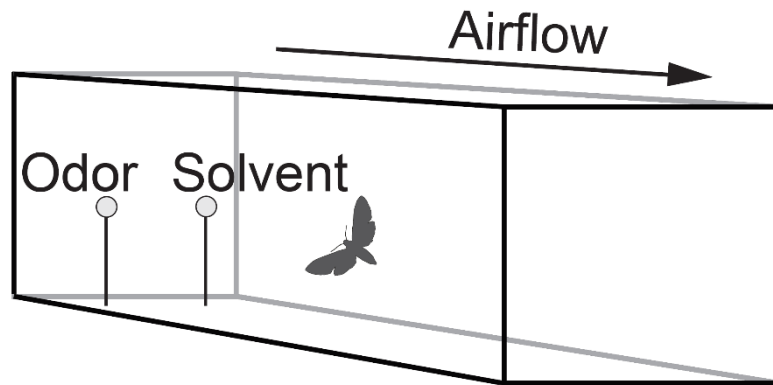
Figure 2: Simplified representation of the two-choice experiment in the wind tunnel (Bisch-Knaden et al., 2018).

During all behaviours, the moths should continue hovering, as they sit down and stop hovering it is not counted as a desired contact. If the moth extends its proboscis in flight and tries to feed from the filter paper, this behaviour is considered as proboscis contact. If the moth clings to the filter paper in flight with the tarsi and has a straight abdomen, this behaviour is considered as tarsal contact. Oviposition or tarsi contact with a curled abdomen is a behaviour for egg deposition in lepidopteran females (L. Eaton, 1986). Therefore, gravid females contact a potential oviposition-site with their tarsi and the last two abdominal segments are extruded to permit egg placement (L. Eaton, 1986; Sparks, 1973; Yamamoto et al., 1969).

2.6 Statistical Analysis

The evaluation of the videos from the wind tunnel experiment was accomplished with VLC Media Player (VideoLAN, Paris, France) and Excel 2016 (Microsoft Corporation, Redmond, WA, US). The subsequent evaluation of the raw data was done with IBM SPSS Statistics 20 (IBM, Armonk, NY, US). For the two-way ANOVA and the Fisher´s exact test, PAST (Paleontological Statistics, http://folk.uio.no/ohammer/past/) and GraphPad InStant (GRAPHPAD SOFTWARE, LLC, La Jolla, CA, US; Version 13) were used at a significance level of $\alpha = 0.05$ ($p < 0.05$ significant; $p > 0.05$ not significant).

3. Results

3.1 Floral bouquet of *A. palmeri* and *D. wrightii*
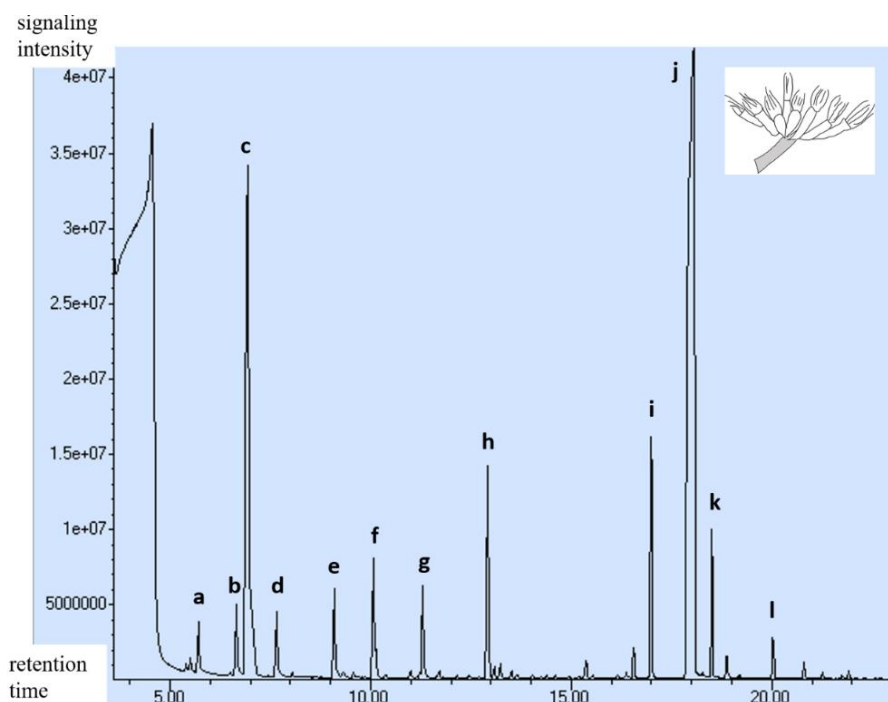
Figure 3: Chromatogram of the floral bouquet of *A. palmeri*. The main components are marked alphabetically: (a) α - Pinene, (b) (+) Camphene, (c) Ethyl isovalerate, (d) β - Pinene, (e) Propyl isovalerate, (f) Isobutyl isovalerate, (g) Ethyltiglate, (h) Isoamyl isovalerate, (i) & (k) 2, 4 - Hexadienoic acid ethyl ester, (j) Ethyl sorbate, (l) Propyl sorbate.
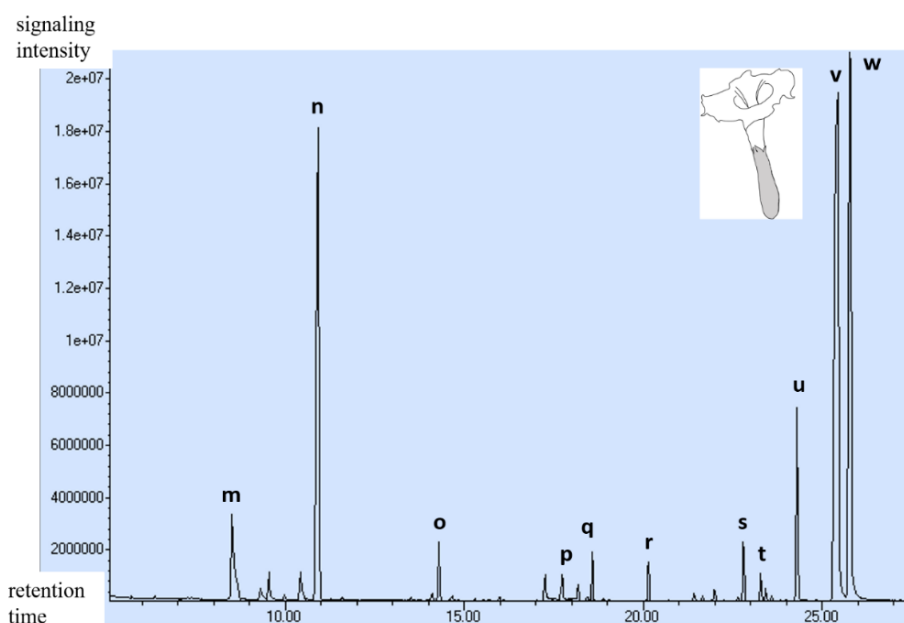
Figure 4: Chromatogram of the floral bouquet of *D. wrightii*. The main components are marked alphabetically: (m) β – Myrcene, (n) β – (Z) – Ocimene, (o) (Z) – 3 – Hexen – 1 – ol, (p) Benzaldehyde, (q) Linalool, (r) Methyl benzoate, (s) α – Citral (t) α – Farnesene, (u) Nerol, (v) Geraniol, (w) Benzyl alcohol.

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The chromatograms of the GC – MS show the signal intensity plotted against the retention time of the different chemical components of the floral bouquet of *A. palmeri* (Figure 3) and *D. wrightii* (Figure 4).

The floral bouquets of *A. palmeri* (Figure 3) and *D. wrightii* (Figure 4) differ in their chemical composition. The floral bouquet of *A. palmeri* consists mainly of different esters (c, e – l) and a few terpenes (a, b, d, Figure 3). Ethyl sorbate, the main component for the quantification, has a retention time at 18 min. Various terpenes (m, n, q, s -v), a few aromatics (p, r, w) and alcohols (o) compose the floral bouquet of *D. wrightii* (Figure 4). The main component for the quantification, benzyl alcohol, has a retention time at 25.7 min.

3.2 Two-choice behavioural assay in the wind tunnel

To investigate if there is a difference in the preference of these two floral bouquets in the hawkmoth *M. sexta*, we performed two-choice assays in the wind tunnel. Moths were tested with a filter paper scented with a diluted flower extract, and a filter paper with the solvent alone. We analysed three parameters of the moth´s behaviour: i) the first contact each moth made with one of the filter papers, ii) the number of moths that contacted the scented filter paper, and iii) the duration of contacts at scented *versus* unscented filter papers. Contacts were done with the proboscis, with tarsi and a straight abdomen, or with tarsi and a curled abdomen, and were analysed separately. It was also examined whether males, virgin females, or mated females behaved differently.

Table 1: First contact of *M. sexta* with a filter paper in the wind tunnel. The sample size is given underneath the table.

type of contact	Agave palmeri						Datura wrightii					
	males*		virgin females*		mated females*		males*		virgin females*		mated females*	
	odour	solvent	odour	solvent	odour	solvent	odour	solvent	odour	solvent	odour	solvent
proboscis	7	2	8	1	2	-	16	2	16	1	11	1
tarsi	2	-	2	1	4	1	1	5	3	1	4	2
abdomen curling	-	-	-	-	-	-	-	-	1	-	4	-

*n = 27

In the two-choice experiment with *D. wrightii*-scented filter paper *versus* unscented filter paper, moths generally first contacted the scented site (Table 1). Within all groups, proboscis contacts occurred more often than tarsal contacts. The first (and only) tarsal contacts with abdomen curling, however, were performed by 1 virgin female and 4 mated females at the *D. wrightii*-scented filter paper.

In the experiments with *A. palmeri*-scented filter papers *versus* unscented filter papers, each type of contact was again shown more often for the first time at the scented site (Table 1). Within the group of male's tarsal contact with a straight abdomen, however, occurred more frequently on the solvent site. As in the *D. wrightii*-experiment, proboscis contacts occurred more often than tarsal contacts. None of the moths curled its abdomen at one of the filter papers.

3.3 Number of moths contacting the scented filter paper

Figure 5: Percentage of moths with proboscis contact at the *A. palmeri*- and *D. wrightii*-scented filter papers. If moths had no contact at all or exclusively contact with the solvent filter papers it is rated as no contact.

The ratio of moths that had proboscis contact with the *A. palmeri*-scented filter paper was lower in mated females (7 %) than both in males (33 %) and virgin females (33 %; p = 0.039, Fisher´s exact test, Figure 5). When tested with the *D. wrightii*-scented filter paper, the ratio of moths with proboscis contacts was similar in all three groups (44 – 63 %; p = 0.275, Fisher´s exact test, Figure 5). A comparison between the two floral bouquets reveals that mated females contacted the *D. wrightii*-scented filter paper more often than the *A. palmeri*-scented filter paper (p = 0.004, Fisher´s exact test). Males and virgin females show a trend in the same direction (p = 0.056, Fisher´s exact test).
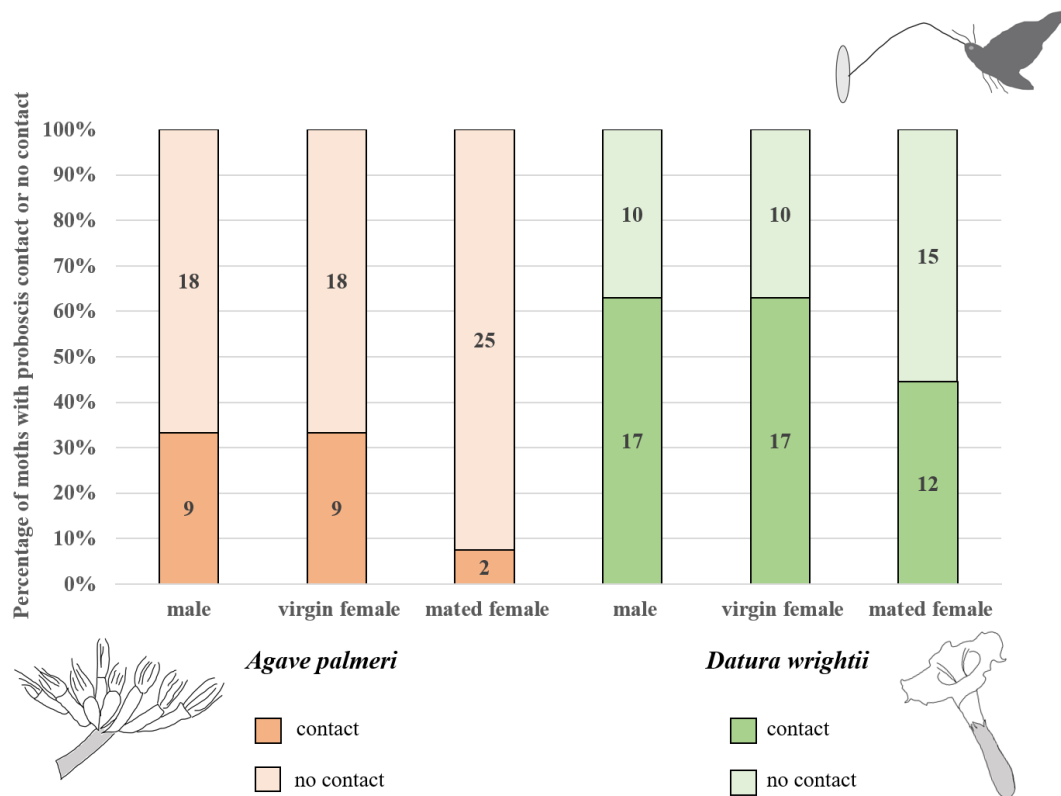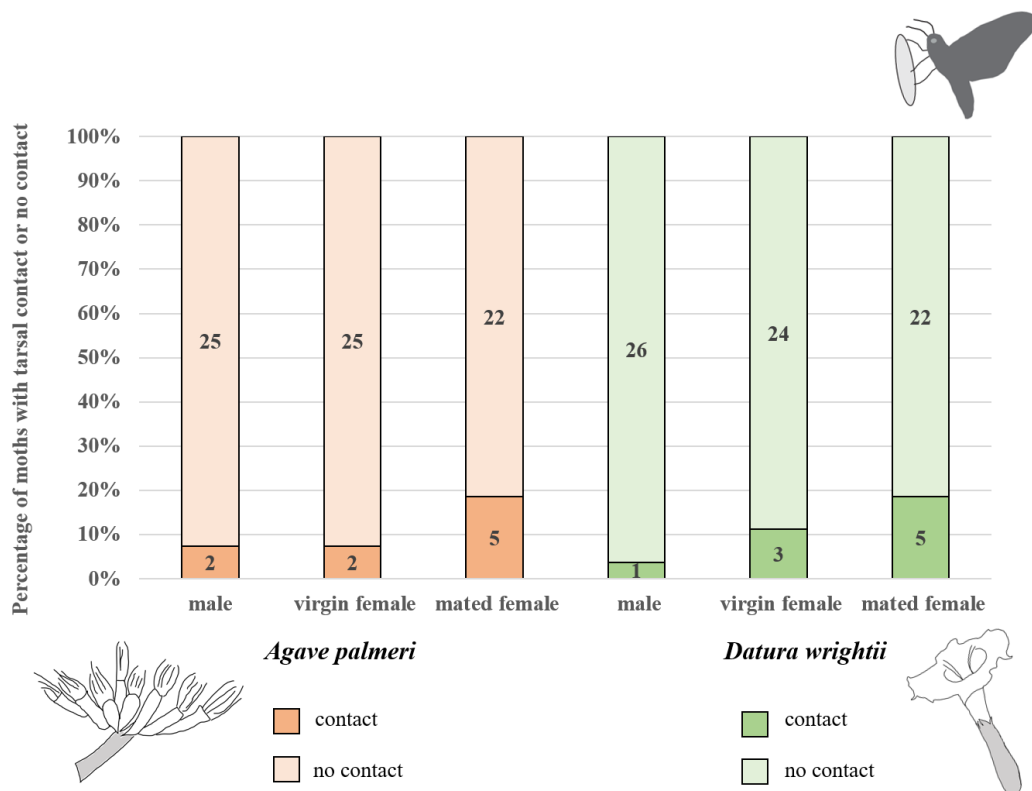
Figure 6: Percentage of moths with tarsal contact at the *A. palmeri*- or *D. wrightii*-scented filter papers. If moths had no contact at all or exclusively contact with the solvent filter papers it is rated as no contact.

The ratio of moths that showed tarsal contact with a straight abdomen at the scented filter papers was similarly low in all three groups, and for both tested floral bouquets (4 – 19 %, Figure 6).

Tarsal contacts with abdomen curling occurred only with the *D. wrightii*-scented filter paper (Table 1).

The ratio of moths that had proboscis contact with a filter paper scented with a diluted flower extract was lower for *A. palmeri* (25 % (20 / 81)) than for *D. wrightii* (57 %, (46 / 81), p < 0.0001, Fisher´s exact test, Figure 5). Tarsal contact with a straight abdomen at a filter paper scented with a diluted flower extract was the same for *A. palmeri* and *D. wrightii* (11 % (9 / 81), Figure 6).

3.4 Net duration of contacts with proboscis and tarsi

To investigate if sex and reproductive status influences the preference for floral bouquets in *M. sexta*, the net duration of proboscis (Figure 7) and tarsal (Figure 8) contacts was analysed.
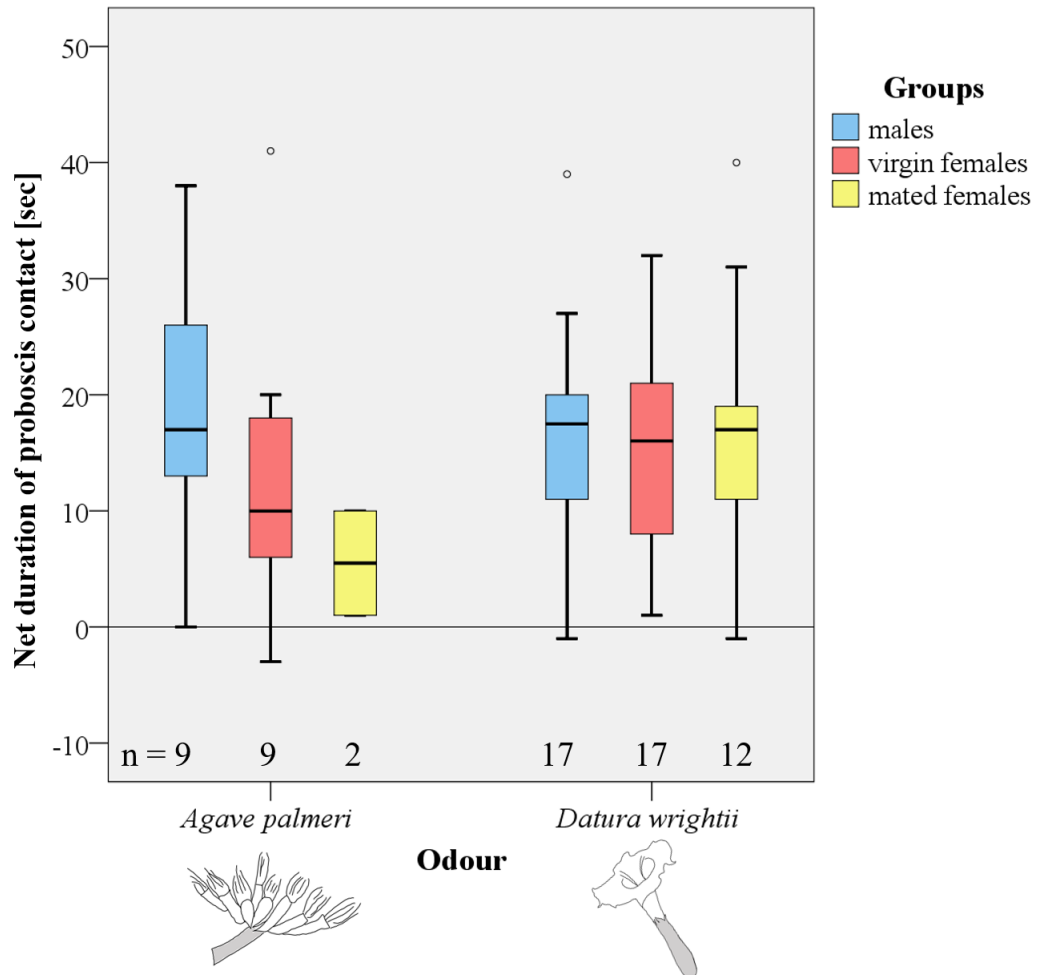
Figure 7: Net duration of proboscis contact at the *A. palmeri*- and *D. wrightii*-scented filter papers by the hawkmoth *M. sexta*. Net durations of contacts were calculated as contact time at the scented filter paper minus contact time at the unscented filter paper. Box plots represent the median net proboscis contact duration for each floral bouquet and group (vertical line in the box). Minimum and maximum values (whiskers) are shown together with 25th and 75th percentiles (left and right margins of the box). The sample size is given underneath each boxplot.

The median net duration of proboscis contacts per moth was similar across all groups and the two floral bouquets (Table 2) and ranged from 6 sec (mated females at *A. palmeri*-scented filter paper) to 17.5 sec (males at *D. wrightii*-scented filter paper).

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Table 2: Two-way ANOVA estimating the effects of sex / reproductive status and floral bouquet on the net duration of proboscis contacts in two-choice experiments with *A. palmeri* and *D. wrightii versus* solvent.

| Factor (df)	Summed square	F	p
Group (2)	79,3	0,373	0,690
Floral bouquet (1)	48,32	0,455	0,503
Interaction (2)	234,4	1,103	0,338
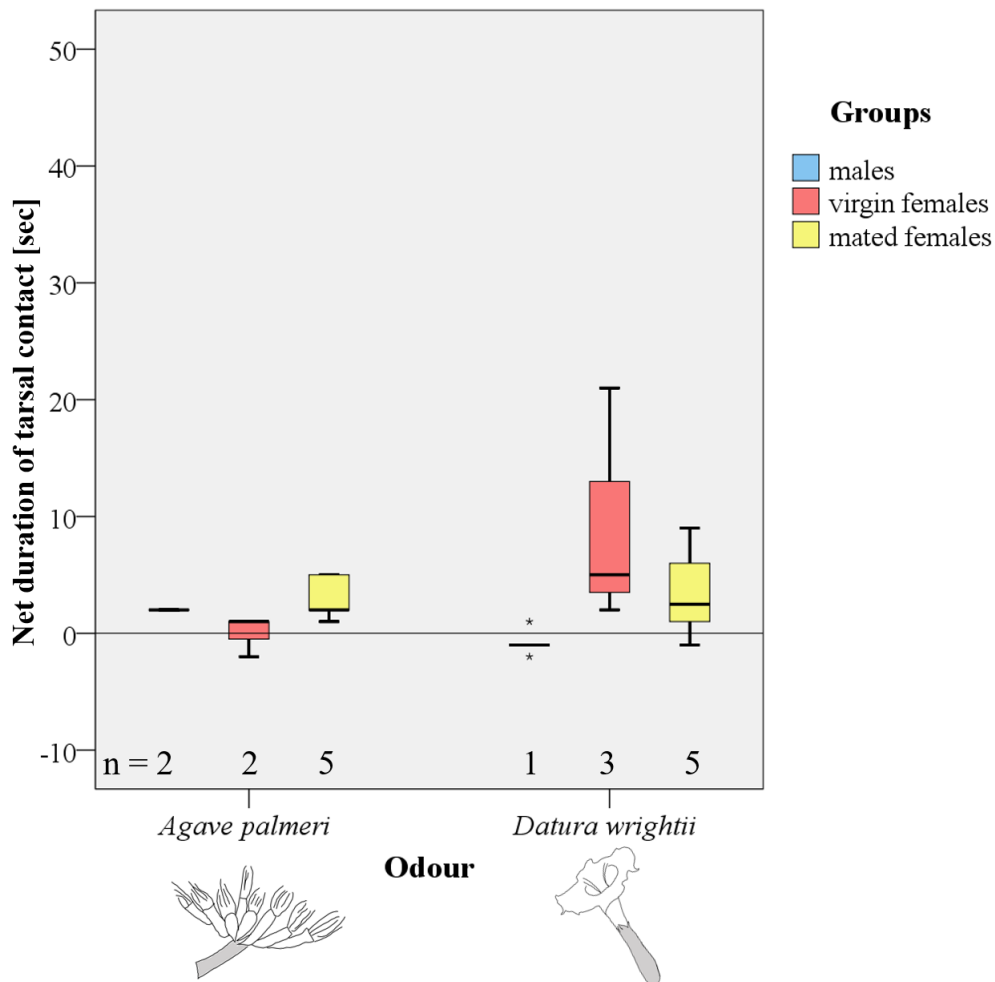
Figure 8: Net duration of tarsal contact at the *A. palmeri*- and *D. wrightii*-scented filter papers by the hawkmoth *M. sexta*. Net durations of contacts were calculated as contact time at the scented filter paper minus contact time at the unscented filter paper. The sample size is given underneath each boxplot.

The median net duration of tarsal contacts with a straight abdomen was highest for mated females at *D. wrightii*-scented filter papers (5 sec). However, as this behaviour was rarely observed (n = 1 – 5 per experiment, Figure 8), a statistical analysis was not possible. Tarsal contacts with a curled abdomen were only observed at *D. wrightii*-scented filter papers and lasted from 18 sec (n = 4 mated females) to 37 sec (n = 1 virgin females). Three mated females oviposited on the filter paper.

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4. Discussion

The fundamental question of this thesis is whether sex and reproductive status have an impact on the preference for floral bouquets in the hawkmoth *M. sexta*. We wanted to investigate if a difference in behaviour between males, virgin females and mated females in relation to the floral bouquets of *A. palmeri* and *D. wrightii* can be observed. All groups were tested in a two-choice experiment in the wind tunnel with a diluted floral extract on filter paper *versus* the solvent on filter paper.

4.1 Differences in the floral bouquet of *A. palmeri* and *D. wrightii*

Gas chromatographic analysis of the collected odours revealed different compositions of both floral bouquets (Figures 3 and 4, Riffell, Alarcon and Abrell 2008), which might lead to behavioural differences. The floral bouquet of *A. palmeri* consists mainly of different esters and some terpenes (Figure 3). Behavioural tests showed that aliphatic esters, such as ethyl sorbate, are attractive chemicals for *M. sexta*, and elicit feeding behaviour in naïve moths (Bisch-Knaden et al., 2018). These rare floral volatiles are not released by typical hawkmoth pollinated flowers like *D. wrightii* (Knudsen et al., 2006; Knudsen and Tollsten, 1993). The floral bouquet of *D. wrightii* mainly consists of terpenes (e.g. linalool) and a few aromatics (e.g. benzyl alcohol; Figure 4). Linalool and benzyl alcohol are important indicators for an attractive oviposition site (Bisch-Knaden et al., 2018; Reisenman et al., 2009), and are not present in the floral bouquet of *A. palmeri*.

Vapor pressure is defined as evaporation rate of a liquid (Speight, 2017) and can thus provide information about the volatility of a substance. The main compound of the floral bouquet of *A. palmeri*, ethyl sorbate, has a higher vapour pressure than benzyl alcohol, the main component of *D. wrightii* (0,418 mm / Hg at 25 °C *versus* 0.094 mm / Hg at 25 °C; ´Aromatic/Hydrocarbon/Inorganic Ingredients Catalog information´). Thus, the main component of *A. palmeri* evaporates more quickly than the one of *D. wrightii*. Investigations of whether the presence of odours is similar over the duration of the behavioural experiment (3 min) for both floral bouquets are necessary. In addition, the experiment could be performed with 10 times higher concentration of both main components of the floral bouquets. Perhaps then the attractiveness of the *A. palmeri* scent for *M. sexta* would increase.

4.2 Results of the behavioural assay in relation to both floral bouquets

Three parameters of the moth's behaviour were analysed: i) the first contact each moth made with one of the filter papers, ii) the number of moths that contacted the scented filter paper, and iii) the duration of contacts at scented *versus* unscented filter papers. In relation to both floral bouquets, moths showed three different types of behaviour: proboscis contact, tarsal contact with a straight abdomen and tarsal contact with a curled abdomen. The first contact each moth made with one of the filter papers was more frequently shown at the scented site for both floral bouquets (Table 1). As already shown in previous experiments, when the floral bouquet of *A. palmeri* or *D. wrightii versus* a solvent was tested, moths choose the odour (Riffell et al., 2008a). These results suggest odour-guided behaviour or odour-modulated flight behaviour as already assumed earlier (Willis and Arbas, 1991). Moths showed more proboscis contact than tarsal contact on the scented site (Table 1), which suggest a higher attempt of feeding behaviour.

The ratio of moths that contacted *A. palmeri*-scented filter paper with the proboscis was significant lower in mated females than in males and virgin females (Figure 5). In previous behavioural experiments, it has been observed that mated females feed longer than males on *A. palmeri* and thereby gain a considerable amount of energy (Alarcon et al., 2010). Post-mating nectar feeding doubles the amount of fertile eggs in females (Madden et al., 1945; Schmidt-Busser et al., 2011). Therefore, in the floral bouquet of *A. palmeri* might be especially attractive for mated females. For males and females however, *A. palmeri* is used as rich nectar source until *D. wrightii* has reached its full bloom (Alarcon et al., 2008; Bronstein et al., 2009; Riffell et al., 2008a). With the floral bouquet of *D. wrightii*, the ratio of moths that contacted the filter paper with the proboscis was similar in all three groups (Figure 5). Considering the ratio of moths having proboscis contact, a significant preference for *D. wrightii* over *A. palmeri* could be observed. Other results have shown that *M. sexta* is innately attracted to the floral bouquet of *D. wrightii* and particularly laboratory naïve moths prefer this odour (Alarcon et al., 2010; Riffell et al., 2008a; Riffell et al., 2008b). However, the net duration of proboscis contact was similar in all groups for both floral bouquets (Figure 7). Pollen load analysis of wild hawkmoths showed that males carried more pollen from *D. wrightii* than females and males feed longer on this plant than females (Alarcon et al., 2010) Females caught in their natural habitat, however, carried more

pollen from *A. palmeri* than males, probably because they feed longer on this plant than males (Alarcon et al., 2010). These results differ from ours, which may be explained by the already mentioned volatility of the *A. palmeri* odour. At Alarcon et al. (2010), they tested real flowers that could be more attractive than floral-scented filter papers in our experiment. Some scientist argue that moths need to learn to feed from nectar sources, such as *A. palmeri* (Riffell et al., 2008b).

There are different opinions among scientists about the interplay and importance of floral bouquets and visual signals. It could be shown that moths never extended their proboscis when a source of odour lacks visual display (Raguso and Willis, 2002). Therefore, the simultaneous presence of floral bouquets and visual display is important to evoke nectar feeding behaviour such as the proboscis extension reflex (Raguso et al., 2003; Raguso and Willis, 2005). Especially naïve moths preferred the display of olfactory and visual cues (Riffell and Alarcon, 2013). While olfactory and visual cues together increase the time, moths feed on *D. wrightii*, no synergetic effects have been reported for *A. palmeri* (Riffell and Alarcon, 2013). Previous publications have shown that the behaviour can be influenced by the flower shape, but more by the size than the shape itself (Kaczorowski et al., 2012). In this regard, it could be tested whether a flower imitation or a bigger filter paper in this experiment could trigger a higher moth response than with a round filter paper. Comparing the number of moths which contacted the filter with the proboscis, the filter paper from our experiment is just as attractive as the real flowers in Riffell and Alarcon (2013). That shows that the visual display in our experiment already been very attractive and that the floral bouquets are a more specific cue for *M. sexta* than visual display (Raguso et al., 2003).

Tarsal contact with a straight abdomen rarely occurred in all groups and both floral bouquets (Figure 6). Additionally, net duration of tarsal contact with a straight abdomen turned out to be quite low within all groups for both floral bouquets, but highest for mated females (Figure 8). Previous publications have shown that the choice of a suitable host plant involves tarsal contact of the female with the leaf surface before oviposition (Mechaber et al., 2002; Yamamoto et al., 1969). In future experiments one could try, whether changing the structure of the filter paper, and increasing the humidity would increase the number of moths that oviposit as texture and moisture are important for short-distance recognition of hostplants and increase the oviposition response (Faegri and Van Der Pijl, 2013; Sparks, 1973). However, many findings

suggest that olfactory cues are more important for mated females to accept a host plant (Spathe et al., 2013; Yamamoto and Fraenkel, 1960).

Mated females contacted the *D. wrightii*-scented filter paper with their tarsi more often than both, males and virgin females (Figure 6). Additionally, tarsal contact with a curled abdomen was only observed for the floral bouquet of *D. wrightii* within the groups of virgin females and mated females (Table 1). *M. sexta* prefers to oviposit on *D. wrightii* (Spathe et al., 2013), yet it has not been observed that mated females lay eggs on *A. palmeri* (Alarcon et al., 2008). As observed in our experiment, virgin females lay eggs when in contact with a suitable host plant. This has already been reported for *M. sexta* with the potential hostplant *Lycopersicon esculentum* (Mechaber et al., 2002) Research from other Lepidoptera suggest that olfactory coding and preference in moths change after mating (Saveer et al., 2012), which may explain the low interest of mated females in the floral bouquet of *A. palmeri*.

4.3 Summary and Outlook

The results show that the behaviour of moths in the wind tunnel was odour-guided as moths made their first contact with the scented filter paper. They further indicate that *M. sexta* preferred *D. wrightii* over *A. palmeri* for nectar feeding, because the ratio of moths contacting the scented filter paper with the proboscis was higher when tested with *D. wrightii* than with *A. palmeri*. Considering the net duration of proboscis contact, however, no different preferences could be observed. Tarsal contact with a straight abdomen was the same for all groups and both floral bouquets. Mated females, showed tarsal contact with a curled abdomen only for the floral bouquet of *D. wrightii*. Thus, only an influence on the preference of sex and reproductive status for mated females could be observed. Whether this preference of female moths also reflected on the neuronal level remain to be investigated.

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